THE EFFECTS OF ENVIRONMENTAL CONDITIONS ON QUORUM SENSING AND COMMUNITY INTERACTIONS IN CORAL-ASSOCIATED BACTERIA

by

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Abstract

The coral holobiont contains diverse communities of bacteria that play a role in the maintenance of coral ecosystems, however little is known about the structure and conservation of the host-bacterial relationship. Declines in coral ecosystems have been partly attributed to outbreaks of disease in tropical and sub-tropical regions, which have been linked to increasing temperatures. Bacteria are thought to play a role in some of these diseases, however little is understood about the mechanisms behind disease progression or the series of events involved in the shifts of coral-associated bacteria from conserved, potentially beneficial communities to those including potential pathogens. Investigations into a cold-water gorgonian coral, *Eunicella verrucosa*, have shown similar bacterial communities to those present in tropical and sub-tropical regions, with high proportions of *Spongiobacter* and *Endozoicomonas* genera, suggesting an important role for these associates in the coral holobiont irrespective of location or the presence of zooxanthellae. A shift in bacterial community with disease was also shown, with suggestions that sedimentation and depth may affect the extent of bacterial community alteration.

With the increasing knowledge that bacteria exhibit elaborate systems of intercellular communication (quorum sensing; QS) to allow a population response and to control the expression of genes for pathogenesis, antibiotic production and biofilm formation, the present study showed the presence, stability and species-specific nature of *N*-acyl-homoserine lactones (AHLs; most prevalent type of QS) *in situ* in a number of coral species. This finding and a high proportion of coral-associated bacteria found producing AHLs suggests an important role for QS in the coral holobiont. Further, AHL signals have been shown to break down in *Stylophora pistillata* kept at 30 °C, which coincided with a drop in bacterial numbers and a changing bacterial community which included more quorum quenching (QQ; AHL-degrading) bacteria. Temperature was shown to affect AHL-QS in a strain-dependent manner in *E. verrucosa* isolates, suggesting that the decline seen in *S. pistillata* is not primarily an effect of temperature. Further experiments with three species of soft coral (*Sinularia* sp., *Discosoma* sp. and a gorgonian) showed no such decline in AHLs at 30 °C and instead show a coral-specific response to temperature, including the ability of coral extracts to inhibit putative pathogens. A decline in the ability of crude coral extract to degrade AHLs in the

Discosoma sp. and the high QQ activity in crude extract from all three species suggests a role for QQ in the coral holobiont, confirmed by the high percentage of QQ found in coral-associated bacterial isolates; again suggesting a role in the maintenance of bacterial communities. Further investigations attempted to link QS and QQ to antagonism and susceptibility in coral associated bacteria; however these results were inconclusive. The thesis concludes that priority should be given to further research of QS and QQ in the coral holobiont, which will reveal important knowledge that may lead to future mitigation of some forms of coral disease.

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CHAPTER 5: THE EFFECTS OF INCREASED TEMPERATURE ON BACTERIAL COMMUNITY DYNAMICS, QUORUM SENSING AND ANTAGONISTIC INTERACTIONS IN THREE SPECIES OF CORAL (*Discosoma* sp., *Sinularia* sp. and a gorgonian)

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Bacteria and other micro-organisms are ubiquitous in the marine environment. They are taxonomically diverse, biologically active and colonise all marine habitats, from the deep oceans, to shallow estuaries. There is growing appreciation for the fundamental dependence that all biomes have upon their microbial constituents, which holds true for marine ecosystems and particularly for coral reef ecosystems (Azam & Worden, 2004). Bacteria are the most diverse and numerous organisms known to associate with reef-building corals (Rohwer et al., 2002); however to date little is known about the structure and maintenance of the host-bacterial relationship (Kelman et al., 1998; Knowlton & Rohwer, 2003).

Declines in coral ecosystems have been attributed to a number of natural and anthropogenic factors, including tourism, ocean acidification, eutrophication and disease. Incidences of coral diseases are thought to have increased (Richardson, 1998) with the appearance of new, previously un-described diseases (Richardson et al., 2001) and the re-emergence of more virulent forms of known diseases (Hayes & Goreau, 1998). Bacteria are thought to play a role in some of these diseases; however little is understood about the mechanisms behind disease progression (Ainsworth et al., 2007) or the series of events involved in the shift in coral-associated bacterial communities, for example, from mutualism to pathogenesis (Thurber et al., 2009). Increases in disease prevalence have also been attributed to environmental conditions associated with global climate change (Barber et al., 2001; Harvell et al., 1999) and the disturbance of the bacterial communities associated with corals (Bourne et al., 2007; Gochfeld & Aeby, 2008; Munn, 2011; Pantos et al., 2003; Rosenberg & Loya, 2004).

A picture of the normal microbial flora associated with healthy corals is beginning to form (Ainsworth et al., 2010). However, little progress has been made in understanding how these communities are maintained or in investigating coral-bacterial and bacterial-bacterial

interactions at the cellular and molecular levels (Weis et al., 2008). Understanding the dynamics of disease outbreaks and resistance are essential when predicting the epidemiological modes of these diseases, as is understanding the evolutionary and ecological dynamics of mutualisms, which depends on knowing what organisms are involved and how they perform as partners (Knowlton & Rohwer, 2003).

Scientists are increasingly recognising that bacteria do not always exist as individual cells, but are colonial organisms that exploit elaborate systems of intercellular communication to facilitate their adaptation to changing environmental conditions (Whitehead et al., 2001). This cell-to-cell communication, known as quorum sensing (QS), is widespread in bacteria (Lerat & Moran, 2004) and has been shown to be important in population expression of genes for pathogenesis, symbiosis, biofilm formation, antibiotic production and expression of virulence factors – including in bacteria phylogenetically similar to those known to infect corals (Davies et al., 1998; Dunny & Winans, 1999; Milton, 2006; Stoodley et al., 2002). Due to the large and diverse bacterial community associated with corals, QS may play an important role in the maintenance of coral species-specific bacterial communities but may also allow pathogens to invade under adverse environmental conditions. However, to date little research has shed light on the presence of QS in coral-associated bacterial communities or the potential for the use of these signals to maintain natural populations.

This literature review aims to (a) give a comprehensive insight into the knowledge we hold with respect to the coral 'holobiont' and its bacterial communities; (b) summarise the increasing threats to these systems; and (c) rationalise research investigating coral-microbial partners and their QS systems.

1.2 Ecology and evolution of the coral 'holobiont'

All corals fall into the Anthozoa, a subphylum of the Cnidaria; a large and ecologically important phylum, including about 9400 species (Wood, 2005) with great diversity (see Figure 1.1 for groupings within the Anthozoa). Corals form the structural and biological framework of some of the most diverse, productive and economically important marine ecosystems in the world (Lesser et al., 2007), providing the structure to reefs and supporting a huge diversity of organisms (Kooperman et al., 2007). They are a source of livelihood and food for at least 100 million people worldwide, supporting major industries (fishing and tourism), playing a key role in stabilising coastlines (Lesser, 2004) and are a potential source of drugs for the pharmaceutical industry. Corals are also highly diverse and widespread in cold water areas such as high-latitude and deep-sea regions of the oceans (Cairns & Chapman, 2001; Hall-Spencer et al., 2007); the seas around Britain and Ireland supporting over 70 species of anthozoans, including 12 species of coral (Wood 2005). Scleractinian and gorgonian corals can form dense stands in these regions, increasing the structural complexity of habitats and contributing significantly to biodiversity, just as on tropical coral reefs (Roberts et al., 2006).

Tropical reef building corals are often composed of mutualistic partnerships or symbioses, between the cnidarian hosts, their photosynthetic dinoflagellate endosymbionts (zooxanthellae) and a complex community of microbes (Weis et al., 2008), including bacteria, archaea, viruses and fungi. As such the 'coral holobiont' was coined (Margulis, 1993), used first to refer to the symbiosis between the coral and the zooxanthellae, and more recently to refer to the coral animal, zooxanthellae and associated microbiota (e.g. Wegley et al., 2004). These partnerships form the trophic and structural foundations of the coral reef ecosystem, developing through a massive and long-term build-up of calcareous skeletons, secreted by modular cnidarians, on which other organisms may grow. Zooxanthellae and some bacteria form stable and species-specific associations with corals, while other associations are less specific (Wegley et al., 2007). Our understanding of these associations has been transformed in recent decades and is now of

more than academic interest, as corals become increasingly threatened by factors that could destabilise these associations. For the purpose of this project this review will focus on the coral host, bacterial associates and briefly discuss symbiosis with zooxanthellae.
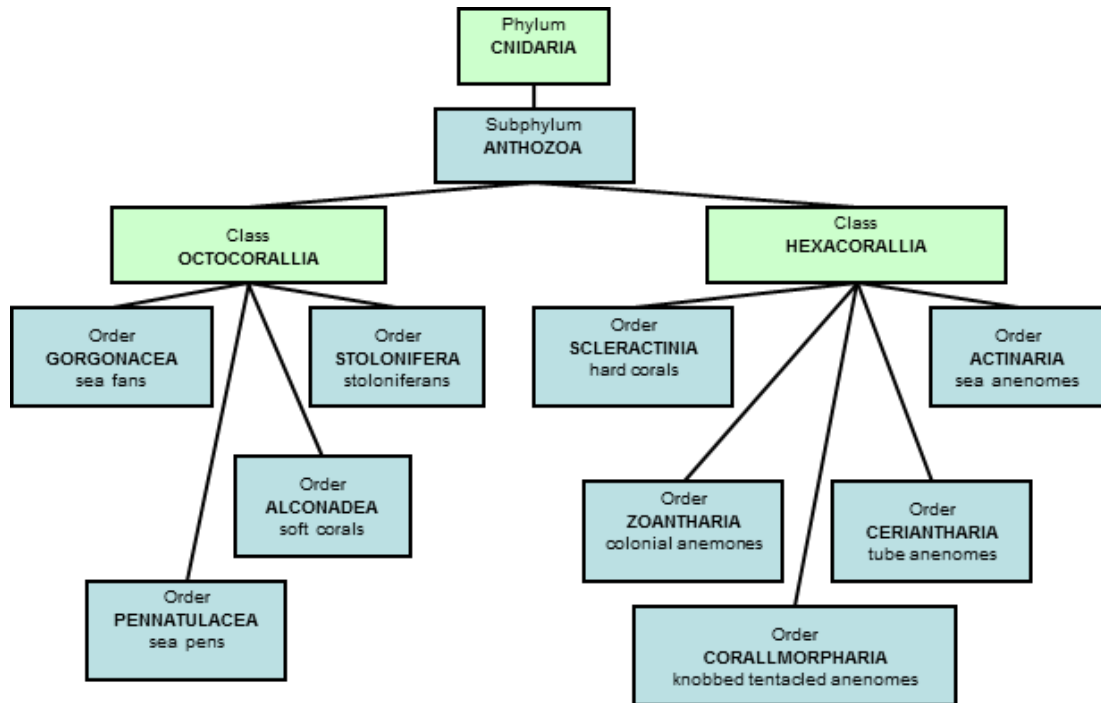
Figure 1.1 Groupings within the Anthozoa (adapted from Wood, 2005).

1.2.1 The Host

All anthozoans are made up of polyps, which have a similar basic structure – however they can live solitarily (e.g. sea anemones and soft corals) or form colonies (e.g. sea fans and tropical hard corals). The polyp has a hollow cylindrical blind-ended sac, a mouth surrounded by a ring of hollow retractable tentacles and is connected to the gastric cavity by the pharynx. The internal gastric cavity is divided by partitions, called mesenteries, which connect at the edge to the pharynx, the free edges forming mesenterial filaments. However, colony formation differs between groups (Mullen et al., 2004).
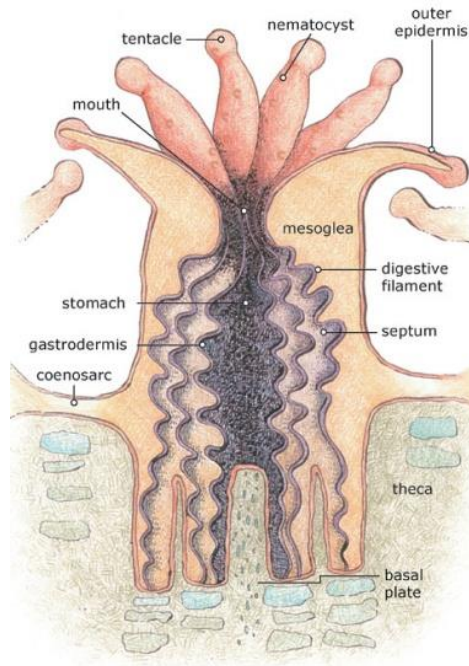
Figure 1.2 Anatomy of a coral polyp (from http://www.solcomhouse.com/coralreef.htm)

The surface of living coral is covered by a muco-polysaccharide material that provides a matrix for bacterial colonization and allows the establishment of a natural bacterial community (Kelman et al., 1998), with the numbers and diversity of microbes exceeding that found in the surrounding water (Ducklow & Mitchell, 1979; Herndl & Velimirov, 1986; Paul et al., 1986; Ritchie & Smith, 1995). This surface mucus-rich microlayer (SML) is highly productive and

can extend a few centimetres above the surface of the coral. In this layer, physical and chemical changes are rapid and dynamic (Kuhl et al., 1995; Rosenberg & Loya, 2004), which may affect the diversity of microorganisms found there. Specialised mucus cells present in the coral epidermis secrete the mucus containing polymers that form a highly hydrated viscoelastic polymeric gel (Meikle et al., 1988); however much of the fixed carbon that makes up the SML originates from the zooxanthellae (Patton et al., 1977). The coral pathogen *V. shiloi* has been found to adhere to a beta-D-galactopyranoside-containing binding site in the mucus, which interestingly were shown to be absent in corals lacking zooxanthellae, further linking production of mucus to zooxanthellae (Banin et al., 2001). The SML sustains high bacterial growth (Herndl & Velimirov, 1986) that presumably results from the degradation of mucus constituents and co-secreted lipids (Ducklow & Mitchell, 1979), however the mucus also cleans the coral surface and aids in the capture of food (Ducklow & Mitchell, 1979; Lewis & Price, 1975). The physical structure of this environment has not been studied in detail; however it is likely to be as complex as the seagrass rhizosphere, which is stratified, with respect to nitrogen fixing bacteria, from anaerobic to aerobic environments (Ritchie & Smith, 2004). Further, the chemical composition of the mucus varies both qualitatively and quantitatively by species but also with environmental factors such as water motion, irradiation and availability of nutrients (Ritchie & Smith, 1995; Brown & Bythell, 2005). It has therefore been suggested that environmental conditions coupled with coral physiological state determine the microbial community associated with the coral mucus and, in turn, changes in microbial community structure may affect coral physiology too (Kooperman et al., 2007).

1.2.2 Defence mechanisms

In common with all animals, anthozoans need to protect themselves against the lethal or debilitating consequences of microbial or parasitic invasion, especially due to their large surface area to tissue ratios. They have a suite of highly efficient defence mechanisms to rid themselves of sediment, settling organisms and potential pathogens, including biochemical properties of the

mucus, phagocytic cells that can engulf and destroy microorganisms and antimicrobial chemical defences (Cooney et al., 2002; Mullen et al., 2004; Sutherland et al., 2004; Ritchie 2006; Ward et al., 2007). However, there is comparatively little known about the mechanisms employed by corals to resist pathogens (Sutherland et al., 2004; Mullen et al., 2004) or the consequences of environmental change on these (Lesser et al., 2007).

Coral mucus has been proposed to enhance resistance by providing a physical barrier, aiding in the removal of microbes via ingestion by the coral host, sloughing to avoid colonization by invasive microbes (Ducklow & Mitchell, 1979) and acting as a medium for secreted allelochemicals with antimicrobial properties (Brown & Bythell, 2005). In scleractinians, the mucociliary system of the epidermis plays an important role in host immunity (Warr & Cohen, 1991) with mucous secretory cells usually abundant in the epidermis releasing copious quantities of mucus (Bigger & Hildemann, 1982), whereas gorgonians generally contain far fewer of such cells (Mullen et al., 2004).

Like other invertebrates, cnidarian polyps also possess innate immunity, which is non-specific and will not alter with subsequent exposure. Phagocytosis in invertebrates is extremely important in defence and in the Cnidaria this is accomplished by wandering amoebocytes within the mesoglea (Young, 1974; Patterson & Landolt, 1979) that take part in wound healing and tissue reorganisation (Chapman, 1974; Bigger & Hildemann, 1982). High concentrations of amoebocytes have been found in *E. verrucosa* coenchyme (Hall-Spencer et al., 2007) and other gorgonian species (Olano & Bigger, 2000) and generally tend to occur in dense clusters, forming a layer beneath the epidermis or are present on the surface (Mullen et al., 2004), acting as a first line of defence. Hutton and Smith (1996) have shown *Actinia equina* amoebocytes to be actively phagocytic *in vitro,* producing oxygen radicals that are thought to be involved in intracellular killing of phagocytosed material. Amoebocytes in the Scleractinia are few and scattered but contain acidophilic granules that have been considered to be lysosomes or

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peroxisomes (Olano & Bigger, 2000); however, the density and size of these cells vary by species and by colony.

The production of chemical defences that deter both fungal and bacterial pathogens have been studied in some detail for a number of model anthozoans. One example is the immune response of the gorgonian, *Gorgonia ventalina* and other closely related species to the fungal pathogen *Aspergillus sydowii* (Couch et al., 2008). *A. sydowii* infections result in the encapsulation of advancing hyphe through a dense layer of aragonite (Bentis et al., 2000) with the primary line of defence appearing to be a cellular response facilitated by wandering amoebocytes (Ellner et al., 2007). This gorgonian species is known to produce antifungal lipid metabolites (Kim et al., 2000a; Kim et al., 2000b), chitinases (Kramer & Muthukrishnan, 1997; Douglas et al., 2007), melanin (Petes et al., 2003; Mullen et al., 2004; Mydlarz et al., 2008) and oxidative enzymes such as peroxidases (Mydlarz & Harvell, 2007) as defensive mechanisms. The antibacterial enzyme lysozyme is also found in phagocytic cells and is responsible for breaking down the peptidoglycan walls in bacteria (Salton, 1952). The presence of melanin deposits and phenoloxidase (PO) activity, both commonly associated with innate immunity in invertebrates, have also been confirmed in Scleractinian and Alcyonacea corals from the Great Barrier Reef (Palmer et al., 2008; Palmer et al., 2010).

Many soft-bodied marine invertebrates are thought to produce secondary metabolites that exhibit antibacterial and antifungal activities (Harder et al., 2003). These have been shown in sponges (McCaffrey & Edean 1985), ascidians (Wahl et al., 1994), gorgonian octocorals (Kim, 1994; Jensen et al., 1996) and soft corals (Slattery et al., 1995; Kelman et al., 1998). Scleractinian corals also produce chemical defences, although these are less well studied (Gochfeld & Aeby, 2008). Studies have found that coral extracts deter predatory fish (Gochfeld, 1997) and affect bacterial growth in a number of Australian (Gunthorpe & Cameron, 1990; Koh, 1997) and Caribbean (Gochfeld et al., 2006) corals. Further, Kelman et al. (1998) found

extracts from the Red Sea soft coral, *Pareythropodium fulvum* to exhibit antimicrobial activity against several co-occurring and potentially pathogenic marine bacteria, with high activity being found particularly against a *Vibrio* sp. isolated from necrotic coral tissue. Gochfeld and Aeby (2008) have also shown high levels of antibacterial chemical defence in three species of Hawaiian reef corals against known coral pathogens *V. coralliilyticus* and *Serratia marcescens*; activity varying by coral colony, population and species but also being highly selective against certain bacterial strains rather than broad spectrum in nature. Geffen and Rosenberg (2005) added to this by showing that mild mechanical stress on *Pocillopora damicornis* caused the release of potent broad spectrum bactericidal material that killed the pathogen *V. coralliilyticus*, suggesting that environmental stress may trigger defence.

Studies have suggested that these defences may be more active against foreign bacteria and specific in their action (Gochfeld & Aeby, 2008; Harder et al., 2003). Gochfeld and Aeby (2008) observed no antimicrobial activity against coral-associated bacterial strains isolated from the coral tissue or its mucoid surface, compared to known pathogens. *Dendronephthya* sp. extracts have also been shown to inhibit growth of benthic bacterial isolates, affecting their ability to attach to agar, more successfully than coral-associated isolates (Harder et al., 2003). Inhibition of attachment was also found in compounds produced by *P. damicornis* which allow bacteria to remain intact and motile but lose their ability to form colonies. This suggests that *P. damicornis* combats microbial fouling by producing compounds that inhibit bacterial growth or attachment. This is similar to the strategy of the red alga, *Delisea pulchra* which produces furanone-type compounds that interfere with QS signalling in potential fouling bacteria (Hentzer et al., 2002). Such results have prompted questions concerning the ability of the coral host to select for beneficial bacterial associates, how they achieve this, what role these bacterial associates play and what effect environmental stress has on this process.

Importantly, to date scientists have used whole tissue or crude aqueous extracts with little attempt to determine the mode of action of such compounds or to address the mechanisms for their production or identify the tissue responsible (Gochfeld & Aeby, 2008). Using whole tissue extracts essentially measures the immune response of the whole 'holobiont' and thus variations in immunity may be derived from the coral, its endosymbiotc algae, its microbial community or a combination of all three (Couch et al., 2008). Numerous natural products from marine invertebrates show striking structural similarities to metabolites of microbial origin, suggesting that microorganisms are the source of these metabolites or are intricately involved in their biosynthesis (Anand et al., 2006). Symbiotic marine bacteria have already been shown to be responsible for the production of secondary metabolites that were previously thought to be derived from the host (Elyakov et al., 1991; Mikki et al., 1996) and levels of enzymes such as peroxidases, superoxide dismutase and exochitinase have been measured in various invertebrates, algae and bacteria (Hawkridge et al., 2000; Wong et al., 2004; Mydlarz & Harvell, 2007). Many marine algae and bacteria contain a suite of antimicrobial compounds (Couch et al., 2008) and some anti-fungal properties (Gil-Turnes et al., 1989). However, Ritchie (2006) has shown that UV-irradiated mucus from healthy *A. palmata* inhibited the growth of potentially invasive microbes by up to 10-fold, with activity against Gram-positive and Gram-negative bacteria as well as potentially invasive microbes. With increasing threats to coral ecosystems, it is increasingly important to determine the true source and production of these compounds to aid in our understanding of coral disease resistance and how they are affected by the rapidly changing environment.

1.2.3 Symbiotic zooxanthellae

The best known symbiosis occurring within many corals is the host's relationship with photosynthetic dinoflagellates of the genus *Symbiodinium* ("zooxanthellae") (Knowlton & Rohwer, 2003). Zooxanthellae are of major importance for the nutrition and physiology of corals, especially in oligotrophic tropical waters (Fallowski et al., 1984; Leutenegger et al.,

2007), releasing enough photosynthetically fixed carbon to meet the host's respiratory demands and assisting in the assimilation and conservation of nitrogen (Santos et al., 2004). This yield of fixed carbon and oxygen are major components of both animal and bacterial respiration (Rosenberg et al., 2009). As most of the carbon in the coral mucus is thought to come from stimulation and excretion of compounds and the uncoupling of the balanced growth of the zooxanthellae (Falkowski et al., 1993) some scientists believe that zooxanthellae may contribute to specific properties of the coral mucus which may influence bacterial populations (Banin et al., 2001). Scientists have also proposed that stressed corals can alter their densities of dinoflagellate symbionts (i.e. bleach) and regain a new mixture of symbiotic algae that are more adapted to the stressful conditions, known as the 'Adaptive Bleaching Hypothesis' (Buddemeier & Fautin, 1993). However, this hypothesis has been debated. It also remains unclear as to whether changing algal partners is sufficiently rapid or effective enough for corals to survive the challenge of temperature stress (Glynn et al., 2001).

1.2.4 Bacterial and Archaeal associates

Non-eukaryotic microbes play important ecological roles in all ecosystems, but it is only in the past few years that scientists have begun to realise the potential roles of bacteria in normal and perturbed coral physiological states (Ainsworth et al., 2006). Studies have demonstrated that bacterial communities associated with corals are different from the surrounding water and sediment communities (Frias-Lopez et al, 2002; Harder et al, 2003) and can be species specific (Ritchie & Smith, 1997; Rohwer et al., 2001, 2002; Littman et al., 2009). For example, similar bacterial communities have been found in *Montastraea franksi* coral reefs up to 10 km apart (Rohwer et al., 2001) whereas different coral species living in close proximity have distinct bacterial communities (Rohwer et al., 2002). They may also be spatially specific on the coral surface; with specific ribotypes associated with only the growing tips of *Porites furcata* (Rohwer et al., 2002).

Recent studies have begun to look at differences between coral 'residents' or putative symbionts, potential 'visitors' or transient bacteria and possible 'pathogens' (e.g. Ritchie, 2006) and have found specific bacterial groups associated with diverse groups of corals (Cooney et al., 2002; Rohwer et al., 2002; Bourne & Munn, 2005). Coral-associated bacteria have also been found to be extremely diverse in terms of richness and abundance, with ~1000 ribotypes documented in culture-independent analyses (Rohwer et al., 2001, 2002; Cooney et al., 2002; Frias-Lopez et al., 2002). Coral-associated Archaea are also known to differ from those in the water column (Wegley et al., 2007), however to date little attention has been given to studies on the diversity and roles of Archaea in the coral holobiont and they do not appear to be associated with specific corals (Rosenberg & Loya, 2004; Wegley et al., 2007).

Many studies have found members of the Gammaproteobacteria to be abundant on corals and have been identified in acroporids (Ritchie, 2006; Ritchie & Smith, 2004; Rohwer et al., 2002; Ainsworth et al., 2006) and in *Porites* species (Wegley et a., 2007). Further, associations have been found with specific Gammaproteobacteria, including ribotype PA1, found in over 50 samples of *P. astreoides* and some *P. compressa* samples (Wegley et al., 2007). *P. furcata* has also been found to harbor a specific member of the Gammaproteobacteria closely related to PA1 (Rohwer et al., 2002), suggesting this group may associate with *Porites* spp. generally. The majority of sequences retrieved from tissue slurry libraries from *Pocillopora damicornis* are also affiliated with this class (Bourne & Munn, 2005) as were sequences from the Antarctic soft coral, *Alcyonium antarcticum* (Webster & Bourne, 2007). Culture-dependent studies have also shown members of this class to produce antibiotics that limit the growth of other bacteria alongside considerable resistance to other antibiotics (Long & Azam, 2001), suggesting a potential role in coral defense. However, members of the *Vibrio*-genus, also members of the Gammaproteobacteria, are also known pathogens of corals (e.g. Ben-Haim et al., 2003). Within the Gammaproteobacteria, of particular interest are members of the genus *Spongiobacter*, which have been found to dominate sequences from clone libraries in pre- and post-bleached corals (Bourne et al., 2007) and within clone libraries from three other *Acropora* species, accounting

for between 17 and 35% of clones (Littman et al., 2009). Being such a dominant member of many coral-associated microbial communities and retrieved from both sponges and corals, *Spongiobacter* spp. may play a key role in holobiont function and supports the hypothesis that these animals may shape their microbial partners for either nutritional or protective benefits.

Members of other groups, such as an Alphaproteobacteria related to *Silicibacter lacuscaerulensis* have also been shown to be ubiquitous in healthy corals of several species, with a possible role in nitrogen fixation (Wilkinson & Fay, 1979; Shashar et al., 1994; Kühl et al., 1995; Rohwer et al., 2001, 2002). Pantos and colleagues (2003) also found dominance of this subclass in *Montastrea annularis* samples, with molecular studies indicating that this group dominates marine systems (Dang & Lovell, 2002). In contrast, the 'mucusy' coral *Diploria strigosa* is more commonly associated with the Bacteriodetes, possibly because this group is more adept at breaking down complex polysaccharides (Rohwer et al., 2002).

Conserved phyla of bacteria also seem to exist between coral species, with a metagenomic analysis of *Porites astreoides* finding the most prominent groups to be the Proteobacteria (68%), Firmicutes (10%), Cyanobacteria (7%) and Actinobacteria (6%) (Wegley et al., 2007). Rohwer et al. (2002) found the same four phyla as the most dominant groups in a further *Porites* sp. study, suggesting it may be fruitful to look to the general properties of some of these phyla in future hypothesis building. Littman and colleagues (2009) also found *Roseobacter* and *Stenotrophomonas*-affiliated sequences in libraries associated with three different *Acropora* species at two different sites, making these two groups possible candidates for investigation as players in coral nutrient cycling or the production of antimicrobials. The marine *Roseobacter* clade contains several genera of marine bacteria with heterogeneous physiological properties including sulfite reduction (Sorokin, 1995), metabolism of dimethysulfoniopropionate (DMSP) (Zubkov et al., 2001, 2002), production of toxins (Hold et al., 2001) and aerobic anoxygenic photosynthesis (Algaeir et al., 2003). As such the *Roseobacter* genus is suspected to have

important functional roles within the coral holobiont, including the ability to produce secondary metabolites (Gram et al., 2002), and to produce antagonistic activity against other marine bacteria and algae (Brinkhoff et al., 2004; Rao et al., 2005).

To date, the majority of studies investigating coral-associated bacterial communities have focused on corals found in warm water environments and known to suffer from prolific outbreaks of disease. However, there is little information on bacterial isolates associated with cold-water corals available. It would prove interesting to compare tropical-zooxanthellae containing corals with azooxanthellae corals and both with cold-water zooxanthallae and azooxanthallae relations.

Care needs to be taken when assessing coral-associated bacteria however. Littman et al. (2009) found that while the same bacterial ribotypes were found within all the corals investigated, dominant members of the community differed between locations on seemingly healthy corals. In their study one of the sites had some land-derived pollutants discharging onto the reef, while the other site was uninhabited (Littman et al., 2009), showing the importance of establishing all variables in an assessment of 'healthy' or 'natural' coral-associated microbial communities. Johnston & Rohwer (2007) have also recently failed to find an adherent microbial community on the surface of the reef-building coral *Porites compressa* and a few *in situ* hybridization studies have shown that bacteria were rare and appeared as isolated cells within the tissues of healthy corals (Ainsworth et al., 2006; Ainsworth & Hoegh-Guldberg 2009). To date, sampling protocols have involved either whole animal maceration after washing with sterile seawater or syringe-aspiration of coral surface material, with the exception of a few recent studies (e.g. Sweet et al., 2011). As such, contamination from seawater is possible as is disruption of the physical organisation of bacteria. As coral mucus also traps particles and microbes that pass by in the water column (Wild et al., 2004), attempts at understanding the importance of coral-associated microbial communities may be misleading due to the fact that coral mucus is

indiscriminate. Studies have shown that although the numbers of bacteria present in the mucus and tissue are similar (Bourne & Munn, 2005; Koren & Rosenberg, 2006), the species found are very different (Bourne & Munn, 2005; Koren & Rosenberg, 2006; Sweet et al., 2011), hence researchers need to be aware of these differences when surmising roles of coral-associated bacterial isolates.

1.2.5 Potential roles for coral-associated bacteria

Authors have noted that, as yet, there is no clear picture of the ecological roles of the diverse microbial associates with their non-perturbed coral hosts (Rowher et al., 2001; Knowlton & Rohwer, 2003) or many details of the structural arrangement of microbial communities and their interactions with coral tissue (Ainsworth et al., 2006; Ainsworth & Hoegh-Guldberg 2009). Unravelling the nature of the associations between the coral host and bacterial associates is difficult due to the biochemical capabilities of bacteria being so diverse in comparison with dinoflagellates (Knowlton & Rohwer, 2003). However, mechanisms are likely to include specific interactions, signal transduction pathways that allow certain species to evade the host's innate immune system and ecological competition between species for the host niche. Culture and culture-independent methods have now provided details of the diversity and biogeography of coral-associated microbes and as such, future investigations need to concentrate on their metabolic contributions to the function of the coral holobiont and the methods by which they protect the host and maintain stable communities, information of which is still relatively limited (Wegely et al., 2007).

Heterotrophic bacteria have long been recognised as an important source of carbon and nitrogen cycling in coral reefs (Sorokin, 1973). It is reasonable to assume that some coral bacteria will benefit the coral holobiont by fixing nitrogen (Reshef et al., 2006), as it is a process carried out exclusively by prokaryotes (Reshef et al., 2006) and oligotrophic waters in which many corals flourish contain little useable sources of nitrogen. A number of bacteria known to fix both

carbon and nitrogen have been observed in coral-associated communities (Wilkinson & Fay, 1979; Shashar et al., 1994; Cooney et al., 2002; Rohwer et al., 2002; Lema et al., 2012). Nitrogen fixation has been found in coral-associated Cyanobacteria (Lesser et al., 2004) and Archaea (Siboni et al. 2008). Coral-associated bacteria have also been found to degrade dimethylsulfoniopropionate (DMSP), including *Spongiobacter* spp. (Raina et al., 2009), already mentioned in this review. Bacteria also possibly play a key role in the catabolism of material captured by the host. Cellulose, chitin and other complex polymers are highly abundant organic compounds in the sea and are often only degraded by bacteria – which would allow corals to obtain energy from a complex mixture of polymers (Kushmaro & Kramarsky-Winter 2004) by using the bacteria as a 8food source (Sorokin, 1973; Sebens, 1998). Bacteria are much better than corals at assimilating nutrients at low concentrations; as such it is possible that bacteria may scavenge for limiting nutrients (e.g. iron and vitamins) that are then harvested by the coral (Knowlton & Rowher, 2003), a hypothesis supported by observations that corals ingest their own mucus (Coles & Strathmann, 1973). Further, during the day coral tissue and their boundary layers are highly aerobic (due to the production of oxygen by algae) but at night the tissues and mucus may become anaerobic, suggesting that many coral microbes may carry out anaerobic metabolism, producing products which may serve as nutrients for corals during the day (Siboni et al., 2008). Increasingly scientists are recognising that microbes often interact as a consortium to, for example, ensure effective utilisation of nutrients (Munn, 2004) when single organisms cannot carry out particular metabolic transformations alone. This behaviour could be cooperative and coordinated or emergent and opportunistic. Foster (2005) has suggested a model that shows cooperation between similar bacteria to secure a niche because they share like genes and possibly modes of communication. However, irrespective of this, due to the organic compounds produced by one group of bacteria often being substrate for another in such physically close microbial populations, it is possible that a change in one population will directly influence co-inhabiting species and possibly the entire coral holobiont.

Another function of communities found associating with corals may be to protect the holobiont from pathogens (Klaus et al., 2007), by means of intraspecific competition (Rohwer et al., 2002; Reshef et al., 2006; Ritchie et al., 2006; Rosenberg et al., 2007) and/ secretion of antibiotic substances (Koh 1997; Klaus et al., 2007). Evidence from human and other animal studies shows resident microbial communities to contribute to resisting bacterial infection (Silva et al., 2004). In corals the domination of specific groups, such as the genus *Spongiobacter* in pre- and post-disease corals (Bourne et al., 2007) and their association with a number of different species of anthozoans suggests either the importance of such groups in maintaining a balanced holobiont or that the coral is producing something that these bacteria can utilise in an oligotrophic setting.

A range of studies has shown a large proportion of coral-associated bacteria to produce antibiotics (Kelman et al., 2006; Ritchie et al., 2006; Nissimov et al., 2009; Rypien et al., 2010). One important pioneer study was the demonstration of a novel mucus-mediated selection of bacterial symbionts in *Acropora palmata,* which revealed 20% of cultured bacteria from *A. palmata* to display antibiotic activity against one or more ecologically relevant tester strains (Ritchie, 2006). This implies a microbial contribution to the antibacterial activity described for coral mucus (Ritchie, 2006). Anand and colleagues (2006) showed 21% of 75 bacterial strains isolated from four species of coral native to the Indian Ocean produced antibiotics with activities ranging from broad spectrum to species specific against *Bacillus subtilis*, *Escherichia coli*, *Vibrio parahaemolyticus*, *V. harveyi* and a fungal pathogen *Candida albicans*. Vibrio species are a rich source of biologically active bacterial metabolites, with 19 compounds being reported in marine strains, including sponge-derived isolates (Kobayashi et al., 1994; Wagner-Döbler et al., 2002). Marine *Pseudomonas* species, very common in sea-water, have also been found to produce bioactive compounds such as Micacocidin A, B, C and C-14, a cyclic dipeptide (chitinase inhibitor) (Kobayashi et al., 1998; Dobler et al., 2002; Zheng et al., 2005), and terrestrial *Bacillus* sp. are also recognised as a wide source of antimicrobial agents (Gebhardt et al., 2002). All these groups are represented in sponge and coral tissue.

There is also evidence that coral extracts are more capable of inhibiting the growth of bacterial isolates from the benthos, where only half of coral-associated bacteria are inhibited (e.g. *Dendronephthya* sp.) indicating tolerance and possible symbiosis between some of the isolates and the coral host (Harder et al., 2003). Studies also indicate that some epibiotic bacteria evoke an inhibitory effect on growth and attachment of co-occurring bacterial species competing for the same niche (Boyd et al., 1999; Thakur et al., 2000), which suggests a need for sensing other bacterial populations. The production of antibiotics in the marine bacterium *Streptomyces tenjimariensis* is induced by the presence of at least 12 marine bacterial isolates, which induced levels inhibiting the growth of potential competitors (Slattery et al., 2001). Importantly, the mechanism by which *Streptomyces tenjimariensis* senses competitors and up-regulates antibiotic production is currently unclear, but recent studies of QS circuits in bacteria suggest that this phenomenon may be relatively widespread, and may be mediated by cell to cell signalling and response (Bachofen & Schenk, 1998; Gray, 1997).

1.3 Disease outbreaks in corals world-wide

Although there has been some controversy over the increase in marine diseases, which is often correlated with global climate change (Munn, 2011), after normalisation for the increase in research on marine diseases, Ward and Lafferty (2004) suggest that there has still been a clear increase in coral disease (between 1970 and 2001). Coral diseases were first studied intensively in the Caribbean, where successive outbreaks have contributed to significant losses to major reef-building species and resulted in major ecosystem changes. Disease is now emerging as a problem for reefs throughout the Indo-Pacific (Gochfeld & Aeby 2008) – exemplified by the 22- to 150-fold increase in White Syndrome over 5 years on the Great Barrier Reef (Willis et al., 2004). Diseases have been described in all the major reef systems of the world and are even emerging in cold-water systems (Hall-Spencer et al., 2007). If, as predicted, the effects of global

warming continue to escalate, it is vital that we develop a better understanding of the role of microbes in the health of corals (Munn, 2011).

Recent investigations into coral diseases have shown that observation based approaches used in the past are insufficient to characterise coral diseases (Richardson et al., 2001). While a disease is defined as a process resulting in tissue damage or alteration of physiological function, producing visible symptoms (Stedman, 2005), the mechanistic variations of individual diseases require detailed approaches in order to understand each disease process (Richardson et al., 2001). Disease causation (i.e. aetiology) may be attributed to pathogens, environmental stressors, or a combination of both (Sutherland et al., 2004). Biotic diseases, caused by pathogenic microorganisms, may be associated with environmental stressors that can hinder the response of the host, promote growth or virulence of pathogens, trigger the pathogenic process or increase the rate of disease transmission. Abiotic diseases result from both natural and human-induced environmental conditions, which may be exacerbated by secondary opportunistic infections (Peters, 1997).

A number of studies have implicated microbes as aetiological agents of disease in corals (Peters et al., 1983; Kushmaro et al., 1996; Kushmaro et al., 1998; Banin et al., 2001; Ben-Haim & Rosenberg, 2002; Denner et al., 2003; Richardson & Kuta, 2003), however many of these are now contested as 'primary' pathogens. For example, white plague type II disease, first described from an epizootic in the Florida Keys, was originally thought to be caused by *Aurantimonas coralicida*, an Alphaproteobacterium (Richardson, 1998). White plague II has now spread throughout the wider Caribbean and has expanded its host range, affecting the largest host range of around 40 species of scleractinian corals (Remily & Richardson, 2006). A more recent study using microarray analysis of *Montastrea faveolata*, showed all the same disease signs of white plague type II, but with not a single sequence of the suspected pathogen in any of the samples (Sunagawa et al., 2009). This suggests either an opportunistic infection by bacteria or that this

disease is caused by another pathogen entirely. However, this disease has been shown to have some degree of temperature dependence, with optimal growth of the pathogen between 30°C and 35°C (Remily & Richardson, 2006) suggesting that this is an opportunistic infection that follows environmental stress.

Since the 19th century it has been generally accepted that in order to prove the causation of disease by a biotic agent, Koch's postulates must be fulfilled (Koch, 1882), stating that the putative pathogen must be found in every diseased individual, isolated and grown in pure culture and that the disease must be induced in experimental organisms by transferring the pathogen from culture. This has been the basis for determining microbial agents as the cause of coral disease. However, in some cases this method proves limiting, such as where disease is caused by a consortium of microorganisms (Sutherland et al., 2004) or where pathogens are opportunistic and non-specific bacteria that exploit the compromised health of the coral after exposure to environmental stressors, as suggested by Lesser et al. (2007). It is therefore extremely important to look to the modification of the natural bacterial community when assessing coral diseases, as well as seeking a single microbial cause, as we have been trained to do by the legacy of Koch, Pasteur and other founders of pathogenic microbiology. In addition, in infection experiments to prove Koch's postulates for a particular pathogen, sample sizes are sometimes small and the inoculum of questionable ecological significance (Lesser et al., 2007). Particular diseases are also often assigned a group of limited characteristics without details of underlying cellular and structural characteristics, which make it impossible to conclude that the same disease has been produced in the laboratory (Lesser et al., 2007).

1.3.1 Association of *Vibrio* sp. with coral disease and the effects of temperature

Of the nine coral infectious diseases whose pathogens have been identified and characterised using Koch's postulates, six of these are thought to be caused by species of the family Vibrionaecae (Kushmaro et al., 2001; Ben-Haim et al., 2003; Bally & Garrabou, 2007; Sussman

et al., 2008). This adds to many previously described vibrio infections of shrimps, clams and fish (Sussman et al., 2009). A number of other studies have found significant correlations between signs of disease in corals and elevated abundance of *Vibrio* spp. in tropical and sub-tropical corals (Cervino et al., 2004; Gil-Agudelo et al., 2006; Bourne et al., 2007; Thompson et al., 2005; Sussman et al., 2008), as well as in *Eunicella verrucosa* in the English Channel (Hall-Spencer et al., 2007). Specifically, *Vibrio shiloi* and *V. coralliilyticus* have been shown to cause bleaching and necrosis in *Oculina patagonica* in the Mediterranean (Kushmaro et al., 1997) and *Pocillopora damicornis* in the Indian Ocean and the Red Sea, respectively (Ben-Haim et al., 2003). However, there has been ample controversy over the role of some of these pathogens, exemplified by the work by Rosenberg and colleagues on the infection of *O. patagonica* by *V. shiloi* (Kushmaro et al., 1998; Banin et al., 2001; Rosenberg & Falkovitz, 2004) and the proposal of the "coral probiotic hypothesis" to explain the absence of *V. shiloi* from bleached tissue. This hypothesis suggests that a changing microbial consortium will allow adaptation of the coral host over time (Reshef et al., 2006; Rosenberg et al., 2007, 2009). This hypothesis, as well as the role of *V. shiloi* in bleaching has been criticized for disregarding the evidence that the break-down in the symbiosis between the zooxanthellae and the coral host is responsible for bleaching (Leggat et al., 2007) and demonstrates the need for further research into the role of bacterial symbionts and pathogens in the coral holobiont.

Vibrios are Gram-negative, usually motile rods, which are mesophilic and chemoorganotrophic and have a facultatively fermentative metabolism (Thompson et al., 2005). They play a role in nutrient regeneration in the marine environment by taking up dissolved organic matter, producing essential poly-unsaturated fatty acids and degrading chitin (Milton, 2006). They are prolific producers of antimicrobials as well as the most resistant, suggesting an important role in structuring and maintaining bacterial communities within marine environments. Many vibrios also specialise in multiple host attachment and detachment (Tamplin et al., 1990; Kirn et al., 2005), suggesting a broad scope for potential infection of corals by vibrios.

In the case of *Oculina patagonica, Vibrio splendidus* was the most abundant bacterial species found in summer and winter (Koren & Rosenberg, 2006) and shifts in equilibria between *Pseudomonas* sp. and *Vibrio* sp. in healthy corals to *Vibrio* sp. dominance have been seen when corals are bleached (Ritchie & Smith, 1995, 2004). *Vibrio shiloi* and *Enterovibrio* sp., similar to species associated with coral bleaching in the Mediterranean, have been found as 'visitor' microbes in 'mucus selective' experiments in *Acropora* (Ritchie, 2006), with an increase in their abundance to the predominant groups with the loss of antibiotic properties of coral mucus with increasing temperature (28-30°C), suggesting that they are capable of opportunistic infection when the conditions are suitable.

Studies of *V. shiloi* in the *O. patagonica* model have shown *V. shiloi* to become more virulent at higher water temperatures and chemotactic to coral mucus (Toren et al., 1998; Banin et al., 2000); penetrating the coral epidermis (Banin et al., 2000), differentiating into a VBNC state (viable but non-culturable) and multiplying intracellularly reaching over 10^8 bacteria per cm^3 coral tissue (Kushmaro et al., 1998; Israely et al., 2001; Banin, 2003). Upon multiplication, *V. shiloi* produces a proline rich peptide toxin, which inhibits *Symbiodinium* photosystems (Banin et al., 2001) and superoxide dismutase, important in antioxidant defence. With adhesion, production of the toxin and expression of superoxide dismutase all being temperature dependent reactions, *V. shiloi* has been shown to only infect and survive in the summer - elevated seawater temperatures have also been shown to be a major factor in *Vibrio cholera* pandemics (Colwell, 1996).

Production of the putative virulence factor superoxide dismutase (SOD) also show significant increases at 28°C in *V. coralliilyticus* and *V. harveyi*, in contrast to cultures at 16°C (Munn et al., 2008) which is in accordance with the increased levels of protease associated with virulence observed at higher temperatures by Ben-Haim et al. (2003). The same was found for catalase

activity in both strains (Munn et al., 2008). Several cases of protease associated virulence in vibrios have been found (Milton et al., 1992; Lee et al., 2006). The *V. coralliilyticus* protease shows maximum activity at pH 8-11 and temperatures in the range of 30-40°C and is a suspected metalloproteinase (Ben-Haim et al., 2003). Vibrios isolated from diseased *E. verrucosa* tissues are also potent producers of proteolytic and cytolytic compounds and caused extensive tissue damage to the gorgonian (Hall-Spencer et al., 2007). Further, links have also been demonstrated between white syndrome disease signs in Indo-Pacific coral and the presence of *Vibrio* spp. possessing a zinc-metalloprotease gene (Sussman et al., 2009). Protein homologues of this gene have been identified as key virulence factors of vibrio pathogens in fish, shrimp, and molluscs – which act to digest mucin and other connective tissue components (Sussman et al., 2009). Sussmann et al. (2009) showed that the expression of zinc-metalloprotease by White Syndrome coral pathogens was density dependent, with the greatest proteolytic activity measured at the end of the logarithmic phase, where bacterial cell density in cultures reached 1×10^9 cells ml^{-1}, indicating a possible role for QS systems. More integrated knowledge about bacterial virulence, temperature and QS is important for greater understanding of the progression of diseased lesions and the change of natural communities and could also to lead to possible future developments of technologies for early warning and possible prevention of bacterial-induced coral disease (Ben-Haim & Rosenberg, 2004).

1.3.2 A changing microbial consortium

Although bacterial community profiles appear to reflect phylogenetic relationships among coral species, these community profiles have been shown to change when corals become diseased (Rosenberg & Loya, 2004). The mechanisms and processes for these changes are not yet clear but several explanations have been posited, including compromise of the natural bacterial community allowing pathogens to invade, components of natural populations becoming pathogenic; and penetrate host barriers (Gochfeld & Aeby, 2008), all of which may be caused or exacerbated by environmental factors. Research has shown that shifts in the microbial

community can occur in the whole coral colony even when just a small part of the colony shows signs of disease (Pantos et al., 2003) and that, in the case of bleaching, the shift occurs prior to both visual signs and decreases in zooxanthellae (Bourne et al., 2007). This suggests that initial stages of imbalance in the coral holobiont may involve the breakdown of natural bacterial communities and that this occurs as a whole colony response, suggesting a role for some sort of communication between bacteria or between bacteria and host.

Bacterial diversity has been found to increase in bleached and diseased samples when compared directly to healthy specimens (Bythell et al., 2002; Cooney et al., 2002; Frias-Lopez et al., 2002; Pantos et al., 2003; Bourne et al., 2007; Sussman et al., 2008). In a white plague-like disease of *Favia favus* culturable bacteria at the site of infection increased more than a thousand-fold (Barash et al., 2005), even though only a small proportion was identified as the causative agent, *Thallosomonas loyarana* (Thompson et al., 2006). This also suggests the breakdown of a balanced community and the control of bacterial populations, allowing invasion of pathogens and secondary opportunists. In black band disease (BBD) this is seen with the appearance of Delta- and Epsilon-proteobacteria and the loss of Gamma- and Beta-proteobacteria (Cooney et al., 2002), which is likely to change the physiological function of these communities, affecting coral health. Further, striking similarity (36% of sequences) can be seen between populations associated with BBD and a white plague- like disease affecting *Monstastrea annularis* (Cooney et al., 2002; Frias-Lopez et al., 2002; Pantos et al., 2003), suggesting the development of a specific community around a unique microenvironment, when compared to 4% similarity between healthy samples. Similarities included a similar Alphaproteobacterium ribotype, closely related to the causative agent of juvenile oyster disease (Cooney et al., 2002; Pantos et al., 2003) and ribotypes implicated in other diseases; including toxin producing species from the Rhodobacter group, which are associated with paralytic shell fish poisoning (Hold et al., 2001), which again suggests opportunistic pathogenesis.

Ritchie (2006) has demonstrated a loss of antibiotic activity from the coral mucus of *A. palmata* during a prolonged bleaching period, possibly allowing transient and potentially pathogenic microogranisms to invade. It has also been suggested that the variation in coral response to stressors may be mediated by species-specific dynamics of the symbiont community (Rohwer & Kelley, 2004) and that qualitative changes in coral mucus, which occur during bleaching (Lasker et al., 1984), may change bacterial populations due to a change in available carbon sources (Rosenberg & Loya, 2004). As already mentioned, due to the organic compounds produced by one group of bacteria often being substrate for another, it is possible that a change in one population will directly influence co-inhabiting species too. However, it is also possible that resident bacteria may just be outcompeted by certain bacteria that thrive under warmer conditions, such as members of the genus *Vibrio* (Lipp et al., 2002; Rosenberg & Ben-Haim, 2002; Thompson et al., 2005). Foster's model (2005) however, shows cooperation between similar bacteria to secure a niche because they share like genes and possibly modes of communication, which Ritchie (2006) suggests is broken down by vibrios competing for space and reducing the relatedness among beneficial surface bacteria sharing similar gene products (such as feeding enzymes and virulence factors). It may also be that communication systems themselves are affected by environmental stress, allowing others to invade. These ideas need further investigation if we are to understand the true dynamics of these communities and how they breakdown with environmental stress and disease.

1.4 Could quorum sensing be controlling coral-associated bacterial communities?

Quorum-sensing (QS) or cell-to-cell signalling is a form of microbial chemical communication that relies on the production, release and detection of chemically diverse, small signal molecules into the extracellular environment and their subsequent concentration-dependent detection, resulting in the regulation of genes on a population level (Fuqua et al., 1994; Swift et al., 2001).

At the beginning of this project QS was yet to be studied in relation to coral-associated bacteria and is still unexplored as a potential mechanism of control in *in situ* coral niches.

QS is present in many bacterial species and even though the actual chemical signals, relay mechanisms and target genes controlled by these systems vary, they all allow bacteria to alter their behaviour on a population-wide scale in response to increasing signal concentrations and/or microbial numbers (Waters & Bassler, 2005). Given the dense bacterial communities associated with marine eukaryotes, such as sponges (Taylor et al., 2004), macroalgae (Jensen et al., 1996), ascidians (Wahl, 1995) and corals (Ducklow & Mitchell, 1979; Shashar et al., 1994; Richie & Smith, 1995; 1997; Rohwer et al., 2001; Frias-Lopez et al., 2002; Reshef et al., 2006; Guppy & Bythell, 2006; Koren & Rosenberg, 2006) and the density dependent nature of bacterial signalling it is likely that bacterial communities associated with corals and other marine organisms contain species with QS regulated behaviour. Cell-to-cell communication allows a population of bacteria to coordinate gene expression and therefore their behaviour as a group (Miller et al., 2002). QS has been shown to be important in pathogenesis, symbiosis, dispersal, DNA transfer, colonization, biofilm formation, expression of virulence factors, production of antibiotics and other extracellular enzymes in many bacteria (Davies et al., 1998; Stoodley et al., 2002; Dunny & Winans, 1999; Swift et al., 2001; Milton, 2006). For coral associated bacteria, QS signalling could allow bacterial symbionts to maintain their own populations and defend against pathogens, or alternatively, allow for pathogens to gain a 'foothold' in adverse environmental conditions. If coral-associated bacteria confer protection to the host via QS circuits and the subsequent production of antimicrobial compounds or enzymes, it is important to understand the mechanisms of up-regulation and control, especially in relation to the changing environment and with the increasing incidence of coral disease.

1.4.1 Quorum sensing: a brief summary

To date, the frequency of QS in the marine environment is poorly documented (Case et al., 2008); however several distinct families of QS signal molecules, along with their cognate

synthase gene(s) and associated signal transduction apparatus have now been described. Gram-negative bacteria employ N-acylhomoserine lactones (AHLs), 2-alkyl-4-quinolones (AQs), γ-butyrolactones, furanones, long-chain fatty acid derivatives, fatty acid methyl esters, peptides, the 4,5-dihydroxy-2,3-pentandione (DPD) derivatives collectively referred to as autoinducer-2 (AI-2) (Atkinson & Williams, 2009) and autoinducer 3 (AI-3), an α-hydroxyketone (Higgins et al., 2007). Gram positive bacteria generally communicate using modified oligopeptides such as autoinducing peptides (AIPs) made by *Staphylococcus* (Williams, 2007) with 'two-component' type membrane-bound sensor histidine kinases as receptors (Waters & Bassler, 2005). Members of the actinomycetes such as *Streptomycetes* have been shown to use γ butyrolactone molecules for QS; these molecules are structurally similar to AHLs (Takano, 2006). Gram positive bacteria have also been found to share the (AI-2)/ LuxS QS system (Winzer et al., 2002; Winzer & Williams, 2003) with Gram negative bacteria. It has been suggested that any number of extracellular bacterial metabolites have the potential to function as signal molecules (Yim et al., 2006); however it is important to differentiate between a true signal molecule involved in cell-to-cell communication and other metabolites, namely:

1. accumulation in the extracellular environment under certain physiological conditions/ response to environmental change/ specific growth phase;

2. recognition by a specific cell surface/ cytoplasmic bacterial receptor;

3. induction by a cellular response that extends beyond physiological changes required to metabolise/ detoxify a molecule (Winzer et al., 2002; Atkinson & Williams, 2009)

QS mechanisms in Gram-positive and Gram-negative bacteria are now being studied extensively, with potential as new and promising targets for reducing bacterial infections in humans, animals and plants (Czajkowski & Jafra, 2009). The first and most intensively investigated of these are the N-Acyl homoserine lactones (Williams, 2007).

1.4.2 The N-Acyl homoserine lactones (AHLs) in Gram negative bacteria

Nealson and Hastings (1970) described the first AHL system in the bioluminescent bacterium *Vibrio fischer*i. AHL-based QS is now known to be broadly distributed among Proteobacteria, however the *V. fischeri* system has remained an important model, particularly through studies of the symbiotic relationship between *V. fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes*. This system involves the production of AHLs that accumulate with increasing bacterial numbers within the light organs of *E. scolopes*. At threshold concentrations of signal, the expression of genes required for bioluminescence is induced (Waters & Bassler, 2005). Central to the AHL family of quorum sensing genes are the pair *luxI* and *luxR* and their homologues, which respectively code for AHL synthase, Lux-I (over 100 of which currently appear in the bacterial genome databases) (Atkinson & Williams; 2009) and the membrane bound receptor LuxR which also acts as a transcription factor (Rice et al., 1999). *luxI* is transcribed at a low basal rate, which allows for a constant low rate of production of the AHL signal, but as the population density increases, there is sufficient AHL to bind to LuxR receptor. The LuxR-AHL complex induces the expression of *luxI*, which creates a positive feed-back loop, flooding the environment with the signal and causing the entire population to switch into 'QS-mode' and produce light (Waters & Bassler, 2005). This autoinduction circuit is critical, allowing for rapid increase in signal production and induction of light production by all members of the population (Rice et al., 1999). The AHLs themselves are composed of a five membered homoserine lactone ring with varied amide linked acyl side-chains (Figure 1.3) that vary in length, oxidation state, saturation level of the carbon chain and the presence of substituents (Chhabra et al., 2005). The N-acyl moieties of naturally occurring AHLs identified to date range from four to 18 carbons in length and may be saturated or unsaturated (Chhabra et al., 2005).
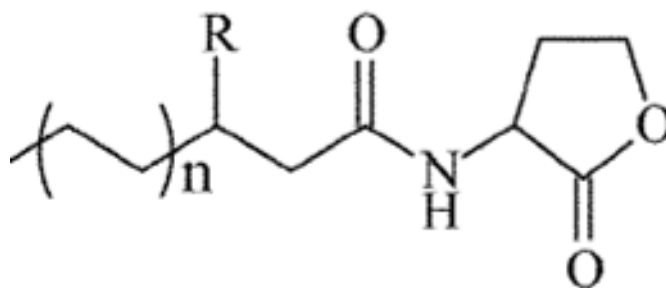
Figure 1.3 Example of an N-acyl homoserine lactone (AHL) molecule.

In complex microbial communities it is likely that multiple AHL signals are present, however evidence shows that each species can distinguish, measure and respond to its own accumulation of signal molecule, due to the specificity between the LuxR proteins and their cognate AHL signals. This specificity is derived from the diverse set of fatty acyl side chains of varying length, backbone saturation and side-chain substitutions incorporated into the AHL signals (Waters & Bassler, 2005). Bacteria of the same species can also produce distinct AHLs in a strain-dependent manner (Czajkowski & Jafra, 2009). However, many transcriptional activator proteins accept AHLs with longer or shorter chain lengths than the cognate molecule (Winson et al., 1998) and so it is likely that some cross-communication between distinct sympatric populations of bacteria can occur.

1.4.3 AHLs in marine bacteria

AHL-producing bacteria have been isolated from 'marine snow' (Gram et al., 2002), marine sponges (Taylor et al., 2004; Mohamed et al., 2008); marine water column eukaryotic algae (Wagner-Döbler et al., 2005) and marine sub-tidal biofilms (Huang et al., 2007; 2009; Tait et al., 2009). Recently, AHL-producing bacteria have also been isolated from corals (Golberg et al., 2011). Taylor and colleagues (2004) first reported AHL production in a sponge-associated *Vibrio* sp. (tentatively *V. campbellii*) and Mohamed and colleagues (2007) described the diversity of QS signal production in sponge-associated Proteobacteria. Thirteen of 23 isolates

from *Mycale laxissima* and five of 25 isolates from *Ircinia strobilina* were found to produce AHLs. However, signals were also detected in two of eight strains from the surrounding water column and types and amounts of AHLs produced varied considerably from strain to strain (Mohamed et al. 2008), leaving doubt as to the importance of QS in some marine strains in the natural environment. Of the AHL positive strains in this study, 67% were Alphaproteobacteria (predominantly *Roseobacter* lineage) and 67% were Gammaproteobacteria (containing isolates closely related to species of *Vibrio*, *Thalassomonas* and *Spongiobacter*). Interestingly, Taylor et al. (2004) also isolated an alphaproteobacterium positive for AHL production in the Australian sponge *Cymbastela concentrica*, which falls into the dominant *Roseobacter* clade AHL$^+$ microbes isolated by Mohamed et al. (2008). Such results beg the question – does AHL-based signalling in such sponge-associated bacteria control functions that have significance to both sponge-microbe interactions and the structure of the bacterial community? Such a question also needs to be given priority in the study of the coral holobiont.

Although the majority of work on AHLs in the marine environment has been carried out in the laboratory, the recent work by Huang and colleagues (2009) established similar pattern of AHL production in biofilms from coastal marine environments to those seen in the laboratory. AHL-producing *Pseudoalteromonas* sp., members of the Rhodobacteraceae, Alphaproteobacteria and Bacteroidetes were identified, however dominant AHLs changed over time, suggesting dynamic shifts in phenotypic control or the succession of new AHL-producing bacterial community. They showed a decrease in diversity of AHL signals over time and an increase in signal from those still being produced – suggesting a decrease in bacterial diversity and an increase in dominance of certain bacteria in the biofilm community (Huang et al., 2009). It has been reported that some *Vibrio* sp., a genus with members known to act as coral pathogens, employ biofilm formation to survive in the marine environment, with QS playing an important role in this process (Bartlett & Azam, 2005; Milton, 2006). Huang et al. (2009) showed AHL-producing vibrios to be dominant in the first day of biofilm formation, but not subsequently, suggesting that vibrios are one of the pioneer groups in natural biofilm communities but are then

superseded by other bacteria which may outcompete vibrio succession under normal conditions, possibly as a result of bacterial cross-talk between other members of the community.

1.4.4 Bacterial cross-talk and symbiosis

Initial research on QS focused on the roles that signalling systems may have in individual populations. However, the discovery that different Gram-negative bacteria could make similar/ identical signal molecules prompted the idea that these signals may be exploited as 'cross-talk' mechanisms between organisms sharing the same environment; relating interspecies information between symbionts and competitors (Hardman et al., 1998; Xavier & Bassler 2003; Sperandio et al., 2003). The demonstration by Cervino and colleagues (2004) that four *Vibrio* sp. together produce signs of disease in the coral *Montastraea* sp. more rapidly suggests that these four species act in consortium which may require some form of inter-species communication. It has been proposed that while AHL QS systems are primarily intra-specific systems, the AI-2 autoinducer signalling system acts as an interspecific communication system, to detect or respond to conditions sensed by other bacteria in the same environment (Bassler, 2002; Chen et al., 2002). Indeed, supernatants from numerous *Vibrio* spp. have been shown to induce the *V. harveyi* system (Bassler et al., 1997). Further, McDougald and colleagues (2003) have found AI-2-like signalling in *V. vulnficus* and *V. angustum* (inducing starvation adaptation and stress resistance) to be competitively inhibited by a signal antagonist. This indicates, at least in these species of *Vibrio*, that AI-2-like signalling systems may function as an interspecies communication system, capable of 'cross-talk' and regulation of environmentally relevant phenotypes. Investigations into AI-2 cross-talk between *V. harveyi* and *E. coli* have found that when co-cultured, *V. harveyi* produced only 18% of the bioluminescence it produced in pure culture (Xavier & Bassler, 2003), a result of the internalization and degradation of AI-2 by *E. coli*.

In the laboratory, AHLs have also been shown to mediate the interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm co-cultures (An et al., 2006). Another elegant study investigated unidirectional signalling between *P. aeruginosa* and *Burkholderia cepacia*, both opportunistic human pathogens that cause chronic infections in cystic fibrosis patients. During chronic co-infection, the two bacteria co-exist within a biofilm. Given that both use the same chemical language, it is not surprising that there is cross-communication between the two of them. Using GFP-based biosensors, Reidle and colleagues (2001) were able to show that *B. cepacia* responded to the AHLs produced by *P. aeruginosa*, but not *vice versa*. This cross-communication may also enhance the virulence of the consortium. Earlier work in which spent culture supernatant of *P. aeruginosa* was added to growing cultures of *B. cepacia* found increased production of siderophores, lipase and protease in the latter species (McKenney et al., 1995), suggesting something secreted by *P. aeruginosa* enhances phenotypes in *B. cepacia*. Cross-talk can also take place between Gram-positive and Gram-negative bacteria. One example is the coexistence of *P. aeruginosa* and *S. aureus* in the lungs of cystic fibrosis sufferers, where AHLs from *P.aeruginosa* can influence the expression of virulence determinants in *S. aureus* (Qazi et al., 2006) and 2-alkyl-4-quinolones (AQs) induce the formation of small colony variants and increased resistance to antibiotics (Hoffman et al., 2006).

LuxI and LuxR homologs have only been found in Proteobacteria, Cyanobacteria (Sharif et al., 2008) and recently the Bacteroidetes (Huang et al., 2008; Romero et al., 2011), although many bacteria are also known to have extra LuxR-type proteins or incomplete QS circuits. For example, in *Rhizobium leguminosarum bv. viciae,* BisR, an extra LuxR-type protein allows induction of conjugal transfer only when a quorum of 3O-C14-HSL-producing bacteria is established in the vicinity (Danino et al., 2003). In *P. aeruginosa*, the unpaired QscR negatively regulates QS systems and is more sensitive to AHL signals not produced by *P. aeruginosa*, suggesting that this protein may function by responding to AHLs produced by co-inhabiting microbes (Ledgham et al., 2003; Lee et al., 2006). The presence of additional LuxR homologs

and other incomplete systems suggest a possible selective pressure to maintain these homologues for sensing and responding to QS signals produced by competing bacteria (Case et al., 2008). This is not surprising given that bacteria rarely live in monoculture and rather in complex communities, however this hints at a level of social sophistication previously not thought to exist among bacteria. If different bacterial species can exchange QS signals in natural environments, this gives them the potential to stimulate important behaviours in neighbours and possibly establish functional mixed communities (Bauer & Robinson, 2002; Williams, 2007), co-ordinating community behaviour (Lewenza et al., 1999; Valle et al., 2004; Qazi et al., 2006; Decho et al. 2009). Of relevance to this thesis, this could include aiding in the maintenance of specific coral-associated communities and protecting the coral host from pathogens. Bearing this in mind, especially when considering the prevalence of coral disease, it is imperative to understand the potential role of coral-associated bacteria in maintaining their own communities under normal and perturbed environmental conditions by investigating the role of quorum sensing in these communities.

1.4.5 Impacts of climate change on the coral holobiont and virulence of pathogens

There seems to be a general consensus that the increase in disease emergence seen in the last decade may be a result of recent changes to the marine environment caused by anthropogenic factors such as pollution and global climate change (Munn, 2004). Corals have evolved under relatively stable and uniform environmental conditions and are very sensitive to natural or man-made physical and chemical changes in water conditions (Sheppard, 1982), which are becoming more evident on highly impacted and rapidly changing reefs (Ainsworth et al., 2007).

One of the main factors affecting coral reefs is thought to be increasing sea surface temperature. With record breaking temperatures occurring more frequently (NOAA, 2011) and predictions of an increase of average global sea surface temperature ranging from 1.8 to 4.0ºC in the 21st century (IPCC, 2007) it is vital that we better understand the role of microorganisms in coral

health and disease and develop better diagnostic tools to monitor the emergence and development of disease. Bacterial communities associated with corals have been shown to be dynamic in response to environmental changes (Ritchie & Smith, 1995, 2004; Pantos et al., 2003; Barash et al., 2005; Koren & Rosenberg, 2006); including water temperature (Ben-Haim et al., 2003; Cervino et al., 2004; Rosenberg & Falkovitz, 2004; Bruno et al., 2007) and correlated with shifts in coral microbial associations that have led to disease outbreaks. However, the mechanisms and significance of these shifts in relation to coral health and disease progression is presently relatively unknown (Littman et al., 2009). Factors which adversely affect the health of corals will also affect the conditions of other reef dwellers, due to the interactions occurring within this complex ecosystem (Peters, 1984). To understand how these changes will affect the coral holobiont, it is necessary to understand the roles of microbes and their responses to such changes (Wegley et al., 2007) by carrying out comprehensive studies that emphasize investigating factors responsible for increased susceptibility of corals to opportunistic infections by microbes (Lesser et al., 2007). Climate change is affecting coral reefs in at least two fundamental ways; the first is through increasing sea surface temperatures; the other is through ocean acidification, arising from increasing concentrations of atmospheric CO_2 entering the ocean (Weis et al., 2008).

Bacterial bleaching by *V. shiloi* and *V. coralliilyticus* is thought to involve temperature controlled virulence factors (Banin et al., 2001; Ben-Haim et al., 2003; Rosenberg, 2004). Similarly, pathogens isolated from gorgonian aspergillosis and black band disease have been shown, in the laboratory, to have growth optimas at elevated temperatures (Alker et al., 2001; Richardson & Kuta, 2003). Certain authors also speculate that the temperature-induced change in the microbial community prior to bleaching could result in a decrease in antibiotics secreted by symbiotic microorganisms, thereby causing coral to become more susceptible to bacterial infection (Rosenberg et al., 2009; Bourne et al., 2008), however the mechanisms for such changes are not yet understood. Rypien and colleagues (2010) have recently shown a decrease in the ability of coral-associated bacteria to deter other bacteria with increased temperatures.

With increases in temperature causing such impacts on coral-associated microbes, it will only become more important to understand the mechanisms behind these changes in bacterial communities, increasing virulence, decreasing antibiotic production and susceptibility to antagonism. A key question is therefore the extent to which such changes can be explained by the phenomenon of QS.

Increased atmospheric concentrations of carbon dioxide are also producing a rapid change in the pH of seawater (Royal Society, 2005). Very few studies have addressed the effects of decreased pH and increased carbon dioxide on marine bacteria. Preliminary experiments have found rapid declines in bacterial production with decreases in pH over 96 hours (Coffin et al., 2004), changes in pH having the potential to reduce metabolic rates (Seibel & Walsh, 2001) and significantly alter microbial community structure (Nold & Zwart, 1998). Further, the impact of reduced pH is more pronounced when bacteria are grown at warmer temperatures (Coffin et al., 2004). We have very little understanding to date of the long-term effects of CO_2 on bacterial communities, however we do know that AHLs are unstable at relatively high pH, with short-acyl chains being most affected (Yates et al., 2002). In terms of the coral host, there is a pH change during transmission from the external seawater environment (pH 8.0) to the surface mucus layer (pH 5.8) (Phillips, 1963), possibly making AHLs more stable when located within the SML. Thus, a pH-mediated virulence mechanism could have a profound effect on coral pathogen virulence and the infection process. Denner et al. (2003) found that *Aurantimonas coralicida*, the putative pathogen associated with white plague II, produces and excretes NH_3, which has been postulated to be a mechanism for disease pathogenesis as an inhibitor of photosynthesis. However, Remily and Richardson (2006) also suggest that NH_3 may contribute to raising the pH of the surface mucus layer, promoting faster growth of the pathogen. Decho and colleagues (2009) have also found AHLs extracted from microbial mats to contain a surprising diversity of AHLs and abundance changes that may be related to pH. Diel cycling within the mat environment appeared to significantly reduce the relative abundances of certain AHLs, through alkaline pH lactonolysis.

Temperature and pH interactions have been found to influence growth and metabolism in many bacteria (Cheng & Chen, 1998) and to influence the degree of both virulence and infectivity (Mayo & Noike, 1996). Preliminary evidence has shown that with increased temperature, *V. harveyi* reduces production of signal molecules and that this is possibly linked to turnover of AHLs by this strain (Tait et al., 2010). The possibility that this AHL-inhibitory activity may also affect signalling within the coral community, allowing *Vibrio* sp. to gain a foothold, is intriguing. AHL signal molecules are known to undergo hydrolysis in a pH-, temperature and acyl-chain length-dependent manner (Yates et al., 2002). In seawater of pH 8.2 the degradation of AHL molecules was temperature-dependent, indicating that AHLs produced in seawater have short half-lives (Tait et al., 2005). Further, molecules with longer N-acyl chains diffused more slowly from an agarose matrix than those with shorter chains (Tait et al., 2005). Thus, at lower pH (6.8), AHL signal molecules are relatively stable whereas in seawater, typically pH 8.2, AHLs are very unstable and this hydrolysis increases with increasing temperature (Tait et al., 2005). In another synergistic study of *A. coralicida*, a distinctive synergistic relationship between pH and temperature was found; increasing the temperature from 25 to 35°C expanded the pH tolerance of the pathogen from a minimum of 6.0 down to 5.0 (Remily & Richardson, 2006). Elevated temperature allowed *A. coralicida* to colonise the low pH environment of the mucus layer as a potential initial stage of infection. Thus, AHL stability and virulence may be affected by both temperature and pH, and there may be a trade-off between the two, potentially causing fluctuations in microbial community structure – with the potential to affect a range of environmental niches and oceanic processes. Therefore, using the coral 'holobiont' as a model system, this research will take us closer to understanding how microbial populations and processes may change in future oceans. Further, insights into the mechanisms behind host-microbial interactions due to microbial competition and communication may enhance our ability to predict how the coral holobiont will cope with climate change.

1.5 Aims and objectives

The aims of this thesis were to (1) explore the presence of conserved bacterial communities in a cold-water coral to compare to tropical counter-parts; (2) investigate the presence of quorum sensing in the Cnidaria; and (3) examine the impacts of temperature on quorum sensing, with particular emphasis on the responses of bacterial communities, such that results can be discussed in terms of the likely impacts of increasing sea-surface temperatures on marine bacterial communities.

- Chapter 2 investigated the hypothesis that the cold-water coral, *Eunicella verrucosa*, has conserved bacterial communities associated with its tissues and that these communities evolve to include a potential coral pathogen with the proliferation of tissue necrosis. Bacterial communities associated with apparently healthy colonies of *E. verrucosa* were assessed over three sites in Southwest England and compared to colonies with signs of tissue necrosis, with a hypothesis that bacterial community structure and diversity is conserved across sites and is different in healthy and diseased colonies of *E. verrucosa*.

- Chapter 3 used thin-layer chromatography with the *Agrobacterium tumefaciens* (NTL4) *Acyl*-homoserine lactone (AHL) biosensor to assess the presence, temporal and spatial stability, and quantity of AHLs in cnidarian extracts. *Anemonia viridis* was employed to investigate variation in AHL signalling *in situ* in rock pools over a tidal cycle, with the hypothesis that AHL-signalling can be detected in cnidarian extracts.

- Chapter 4 examined the hypothesis that increasing ocean temperatures will affect bacterial quorum sensing in the scleractinian coral *Stylophora pistillata* and that this occurs prior to changing bacterial communities.

- Chapter 5 repeated the investigation in chapter 4 with three corals of varying phylogeny (a gorgonian coral, *Discosoma* sp. and *Sinularia* sp.), scrutinized the effect of increasing temperatures on the detection of AHLs and AHL-degradation (quorum quenching) and attempted to link these changes to bacterial communities and the prevention of pathogen colonisation. The hypothesis being that increased temperatures will have an impact on the detection of AHL signals and AHL-degradation in extracts of a Gorgonian coral, *Discosoma* sp. and *Sinularia* sp..

- Chapter 6 explored the presence of AHL-QS and –QQ in a range of coral-associated bacteria and the effect of temperature on QS, QQ and bacterial antagonism towards other coral-associated bacteria by *Eunicella verrucosa* bacterial isolates with the hypothesis that temperature will affect bacterial interactions.

- Chapter 7 sought to synthesise the major findings from the experiments and observations carried out within this study, in order to augment our current understanding of the impacts of increased temperatures on coral-associated bacterial community structure; and to identify key knowledge gaps for future study.

CHAPTER 2

DISTURBANCE TO CONSERVED BACTERIAL COMMUNITIES IN THE COLD WATER GORGONIAN CORAL *EUNICELLA VERRUCOSA*

2.1 Introduction

Recent marine disease epizootics have reduced the abundance of a variety of endangered, commercially valuable and habitat-forming species (Harvell et al., 1999), including a number of corals from tropical and sub-tropical systems; where an increasing number of coral diseases are being described (Bourne et al., 2009). There is strong evidence that conserved microbial communities associated with corals (as discussed in the general introduction) have a beneficial effect, conferring resistance to the host by producing anti-bacterial agents (Shnit-Orland & Kushmaro, 2009; Nissimov et al., 2009), which are compromised during disease (Ritchie et al., 2006). Increased host susceptibility (Lesser et al., 2007) and increased pathogenicity of coral-associated microbes (Rosenberg & Ben-Haim, 2002; Bruno et al., 2007) have both been proposed to be driving incidences of disease. In addition, disturbance to the fragile relationships between the coral host and bacterial communities have been linked to a variety of environmental factors, including thermal abnormalities (Harvell et al., 2002; Rosenberg & Ben-Haim, 2002), increased nutrients (Bruno et al., 2003) and sedimentation (Voss & Richardson, 2006). With an increasingly changing marine environment, it is therefore essential to understand the progression of disease and its link to environmental stress by documenting the alteration of coral bacterial communities to provide accurate diagnosis of coral disease for researchers and ecosystem managers (Ainsworth et al., 2007).

Recently, disease outbreaks have been noted in gorgonian corals (Order: Alcyonacea) in temperate waters of the north-west Mediterranean (Cerrano et al., 2000; Martin et al., 2002) and in SW England (Hall-Spencer et al., 2007). Mass mortality by tissue necrosis has been observed for several species and although the cause of this tissue loss has not been clearly defined, opportunistic pathogenic bacteria have been implicated in a number of gorgonians (Harvell et al., 2001; Cerrano et al., 2000; Martin et al., 2002). Using culture-based methods Hall-Spencer et al. (2007) found an increase in the diversity of culturable bacteria from healthy to diseased *Eunicella verrucosa* tissue, many of which closely matched to *Vibrio splendidus*. Members of

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the family Vibrionaceae are associated with disease in other coral species, including infections correlated with temperature stress (Kushmaro et al., 1996, 1997; Ben-Haim & Rosenberg, 2002; Ben-Haim et al., 2003). Hall-Spencer et al. (2007) also demonstrated that these vibrio isolates induced tissue necrosis at 20°C but not at 15°C in the laboratory, suggesting a possible link between *E. verrucosa* disease and temperature.

Details of the microbial communities thought to associate with non-perturbed gorgonian coral tissue and how these change with the progression of disease are scant, with no comprehensive molecular analysis of these shallow cold-water coral bacterial associates. Given a greater knowledge of these communities we may further our understanding of multispecies mutualism, the effect of environmental conditions on these associates and aid our identification of species that may play a key role in maintaining coral health. Further, documenting shifts in microbiota, if they occur prior to signs of visible stress, may also allow the use of microbiology as bio-indicators of both environmental change and disease (Pantos et al., 2003; Bourne & Munn et al., 2005). Using molecular based methods, the aim of this study was to investigate microbial communities associated with the pink sea fan, *E. verrucosa,* a cold-water gorgonian coral on the international 'red list' of threatened species and known to be important in the functional ecology of the benthic environment in which it is found (Hall-Spencer et al., 2007). Colonies were monitored for disease at three sites off the SW coast of England, during 2008, and healthy and diseased colonies were sampled in June and September of 2008. Sedimentation, temperature and irradiance were also recorded.

2.2 Materials and Methods

2.2.1 Field observation and sample collection

This study was conducted at three sites off the SW coast of England from June 7th to September 16th, 2008. Three sites were chosen for differences in depth and substratum (see Figure 2.1 for

an image of site 1; Table 2.1 for site summary). Two sediment traps (English et al., 1997) were deployed at each site for 12 days in June and September 2008 and seawater temperature and irradiance were measured at each site every 15 min throughout the monitoring period using HOBO® data loggers (Onset, Massachusetts, USA). On June 7th, *E. verrucosa* colonies at each site were evaluated for visual signs of necrotic tissue, epibiont cover and fouling to determine colony health. Six healthy colonies and two diseased colonies (depending on presence) of similar size from each site were tagged. Evaluation of visual condition of colonies was repeated on September 16th. Recruitment of *E. verrucosa* was assessed using abundance data from 2007 and 2008 surveys.

On the June 7th and September 16th, 4 cm branches of each tagged colony were sub-sampled. Branches were placed in plastic bags underwater and immediately taken to the surface where they were washed three times in 0.2 µm filtered, sterile phosphate buffered saline (PBS; pH 8.0) to remove loosely attached microbes. The branches were then placed aseptically in RNA Later (QIAGEN, Hilden, Germany) and kept on ice before transporting back to the laboratory and storing at –80°C until analysis.

Figure 2.1 *Eunicella verrucosa* colonies at site 1, SW England. Image by Fred Wobus.

Twenty litres of water was collected from monitoring site L4 (50° 15.00'N, 4° 13.02'W), filtered (1.6 µm GF/A filter; Whatman) and then applied directly to a Sterivex filter (0.22 µm; Millipore) to assess the bacterial community present in the water column at the time of sampling. Following filtration, each Sterivex was pumped dry, frozen in liquid nitrogen and stored at −80°C until analysis.

2.2.2 DNA extraction and purification

DNA was extracted using the DNeasy Blood and Tissue Extraction Kit (QIAGEN) according to the manufacturer's protocol for animal tissues, with an extension of the 56°C incubation to overnight. To extract DNA from water column samples, the methodology of Neufeld et al. (2007) was used. Following the extraction, total nucleic acids were eluted in 200 µl of nuclease-free water and total DNA was quantified by spectrophotometry (Nanodrop 1000, Thermo Scientific) and diluted to 20 µg µl^{-1}.

2.2.3 PCR amplification and Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA gene

A nested PCR approach was used to amplify 16S rRNA for DGGE. DNA was amplified in a 25 µl reaction using 0.2 µmoll^{-1} of primers 9bfm and 1512uR (5' GAGTTTGATYHTGGCTCAG-3' and 5' ACGGHTACCTTGTTACGACTT-3', respectively) (Mühling et al., 2008), 2 mmoll^{-1} MgCl$_2$, 0.2 mmoll^{-1} of each dNTP, 1x PCR buffer (Promega, Southampton, UK), 0.25 U of Go *Taq* Flexi (Promega) and 0.4 ng of DNA using the following conditions: 1 cycle at 96°C for 4 min; 35 cycles at 96°C for 1 min, 53°C for 1 min and 72°C for 1 min and one final extension 1 cycle at 72°C for 5 min. Each PCR reaction was conducted in triplicate. Following successful amplification, PCR products were diluted 1:10 with dH$_2$O and primers 341f (5'-CCTACGGGAGGCAGCAG-3') with a 40-bp GC clamp and 907r (5'-

CCGTCAATTCMTTTGAGTTT-'3) were used to amplify a 566-bp section of the 16S rRNA gene (Mühling et al., 2008). The reaction mixture contained 0.5 µmoll^{-1} of each primer, 0.15 mmoll^{-1} of each dNTP, 1.5 mmoll^{-1} MgCl$_2$, 1x PCR buffer and 0.15 U of Go *Taq* Flexi (Promega) in a total volume of 60 µl. The amount of diluted DNA added to each reaction depended on band brightness from agarose gels from the first PCR round (from 0.1 µl to 2 µl of diluted PCR product was added). Temperature cycling for PCR amplification was 1 cycle at 94°C for 5 min; 22 cycles at 94°C for 1 min, 65°C for 1 min (decreasing by 0.5°C every cycle) and 72°C for 1 min, 15 cycles at 55°C and one final extension at 72°C for 7 min. PCR products were pooled, cleaned using the QIAquick PCR purification kit (QIAGEN) and total DNA was quantified by spectrophotometry (Nanodrop). DGGE was performed using the DCode^{TM} System (BioRad) with 800 ng of cleaned PCR products on an 8% polyacrylamide gel with urea and formamide as denaturants (30–60% gradient), at 60°C and a constant voltage of 60V for 18 h. Subsequently, gels were stained with SYBR Green 1 (Molecular Probes, Eugene, OR, USA) for 30 min and then washed with deionised water for 30 min. Samples were run on gels twice to check for reproducible sample patterns.

2.2.4 Clone library construction, sequencing and phylogenetic analysis

PCR products amplified using primers 9bfm and 1512uR (as above) were pooled by site, month and health status, (*e.g.* five replicates from site 1 healthy colonies, in June), cleaned by gel extraction (Wizard® SV Gel and PCR Clean-Up System; Promega), quantified and diluted to 20 µg µl^{-1}. Clone libraries were constructed for water and coral tissue using a pGEM®-T easy cloning kit (Promega) and *E.coli* JM109 (Promega) competent cells, using the manufacturer's protocol. Clones containing inserts were verified using the vector primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5' –CAGGAAACAGCTATGAC-3'). Temperature cycling for PCR amplification was 1 cycle at 94°C for 3 min; 30 cycles at 95°C for 30 s, 59°C for 30 s and 72°C for 30 s and one final extension at 72°C for 5 min. Forty clones were randomly selected for each coral library and each water library, and directly sequenced using a

BigDye Terminator v3.1 cycle sequencing kit (ABI) and the M13F primer for the sequencing reaction. Sequences were analysed on an ABI3100 automated sequencer. Generally, only one strand of the DNA fragment was sequenced, proving sufficient bases for taxonomic identification. The 16S rRNA gene sequences were compared to sequences stored in GenBank (NCBI database) using the BLAST algorithm to identify bacteria and archaea associated with *E. verrucosa* and aligned using CLUSTALW in MEGA 4 (Molecular Evolutionary Genetics Analysis; Tamura et al., 2007), with the closest match to sequences from BLAST searches.

2.2.5 Presence of zooxanthellae

PCRs were performed using dinoflagellate specific primers symITSFP (5'-CTCAGCTCTGGACGTTGYGTTGG-3') and symITSRP (5'-TATCGCRCTTCRCTGCGCCCT-3') as described by van Oppen et al. (2001) were used to amplify zooxanthellae ITS1 region from coral tissue DNA samples.

2.2.6 Statistics

GelCompar (Applied Maths) was used to identify DGGE bands within the bacterial profiles and construct a binary matrix based on presence and absence of aligned bands. Levene's test for equal variances of environmental variables between sites was performed in Minitab 6.0 (Minitab) as were ANOVAs and Tukey tests to test for differences between sites. In Primer-E6 v6 (Clarke & Warwick, 2001) a Bray-Curtis similarity matrix of DGGE bands (presence/ absence data) and of clone libraries (standardised by total, square root transformed) with nonmetric multidimensional scaling (MDS) enabled visualisation of similarities between samples. Hierarchical clustering (group average) with SIMPROF tests tested for structure in the subset of data corresponding to each branch of a dendogram (1000 permutations, significance level 5%). To assess variability as a measure of disturbance to coral bacterial communities the Multivariate Dispersion (MvDISP) was performed on DGGE and clone library data.

PERMANOVA analysis established the importance of site, month and health in determining bacterial community composition for DGGE data (Primer-E v6). The Shannon-Weaver (H') test investigated diversity, Pielou's (J') test investigated evenness and average taxonomic distinctness (Δ+) took phylogenetic relatedness of different classes into account for clone library data.

2.3 Results

2.3.1 Environmental perturbations

Table 2.1 summarises environmental variables by site. In June, seawater temperature at site 3 was significantly higher (ANOVA $F=12.68$, $p<0.001$) and in September, site 1 had a significantly lower temperature (ANOVA $F=7$, $p<0.01$) than other sites. Light intensity (lux) was significantly lower at site 1 ($\sqrt{}$-transformed data; ANOVA $F=25.76$, $p<0.001$). Sediment trap analysis showed site 3 to have a significantly higher sediment load in June and September (ANOVA $F=20.70$, $p<0.005$; $F=10.63$, $p<0.01$), compared to sites 1 and 2.

Table 2.1 Site descriptions

	Mean value ±SD (where appropriate)					
Site	**Site 1**		**Site 2**		**Site 3**	
Month	June	September	June	September	June	September
Latitude	03˚58.116′W	-	04°07.261′W	-	04˚08.881′W	-
Longitude	50˚17.102′N	-	50°18.168′N	-	50˚20.021′N	-
Depth (m)	24 – 27	-	15 - 25	-	6 - 9	-
Sediment type	Medium-fine grain	-	Coarse grain	-	Fine grain	-
Substratum type	Wreck	-	Rocky reef	-	Artificial reef	-
Average Temperature (°C)	14.81 ± 0.44	16.72 ± 0.05	14.97 ± 0.41	16.68 ± 0.10	15.58 ±0.37	16.83 ± 0.08
Temperature (max-min) (°C)	13.85-15.47	16.62-16.81	14.04-16.81	16.52-17.00	14.61-17.48	16.52-17.57
Average Light (lux)	119.4	-	547.4	-	475.5	-
Sediment (mg cm^{-2}d^{-1})	5.84 ± 2.08	2.31 ± 0.64	4.34 ± 0.57	3.08 ± 0.48	12.36 ± 1.81	16.12 ± 7.1

2.3.2 Coral recruitment and fouling

Visual signs of disease in *E. verrucosa* were evident in September at all three sites; however, the numbers of colonies affected were much smaller compared to previous years (Hall-Spencer et al., 2007). Only one colony – found at site 1, showed signs of disease during the June survey. Affected colonies had patches of necrotic tissue that were soft and white with areas of exposed black gorgonian skeleton, where the tissue was sloughed, allowing colonisation of epibionts. Healthy gorgonians typically had tough, orange-pink coenenchyme. Figure 2.2 shows a visually healthy *E. verrucosa* colony (A) and a colony with extreme cover of fouling organisms (B).

This level of fouling was not experienced by the diseased colonies sampled in this study. In a two-way ANOVA with post-hoc tests of the effects of site and season on *E. verrucosa* abundance and degree of fouling (data not shown), site 1 had significantly higher recruitment and significantly lower fouling than sites 2 and 3 ($F=30.985$, $p<0.001$; $F=23.087$, $p<0.001$).
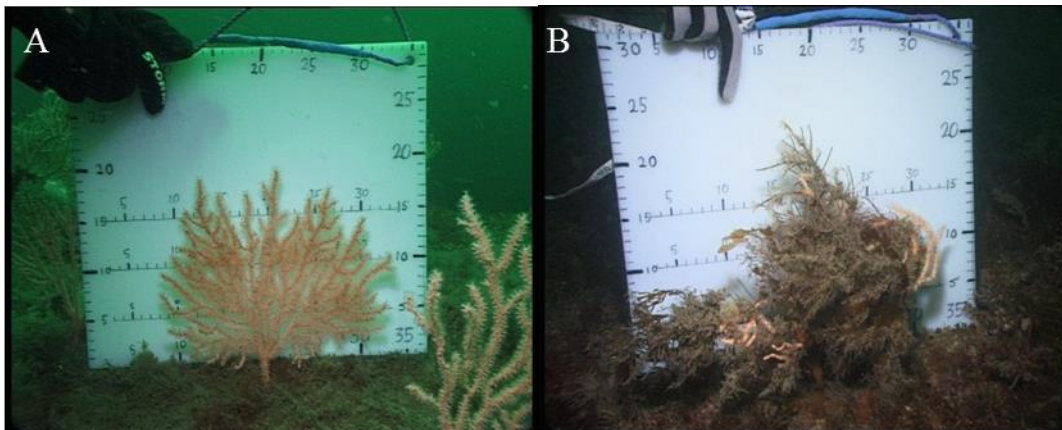
Figure 2.2 Visually healthy *E. verrucosa* colony (A) and an example of an *E. verrucosa* colony covered with fouling organisms (B) at site 2. In this study there were no fouling organisms present on diseased colonies from which samples were taken.

2.3.3 Microbial community changes: DGGE analysis of bacterial 16S rRNA genes

Thirty-seven *E. verrucosa* branches and two water samples were collected for analysis of their associated microbiota. While the high stress of the 2-dimensional Multidimensional scaling (MDS) plot does not emphasise the distinction between water and coral samples, SIMPROF groupings show these bacterial communities to be distinct (Figure 2.3). In June, DGGE fingerprints from colonies at sites 1 and 2 grouped tightly on the MDS and showed heterogeneity with SIMPROF tests (Figure 2.3). In contrast, the bacterial profiles from colonies at site 3 shared similar community structure to that found in coral samples from sites 1 and 2 in September (all but one sample from site 3 in June, one from site 2 in September and three from site 1 in September clustered together in the SIMPROF tests). From June to September, the bacterial profiles of colonies at site 1 and 2 shifted and clustered separately to the same colonies

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sampled in June. In September, bacterial profiles at site 3 were mostly heterogenous but clustered separately from other sites in September, with one colony profile clustering with diseased samples. Interestingly, this colony showed no visual signs of disease. Diseased samples from all three sites, whether in June or September, also grouped closely on the MDS and clustered in the SIMPROF tests, away from healthy colonies, suggesting the presence of a distinct bacterial community (Figure 2.3). While June samples (Sites 1 and 2) and diseased samples showed tight clustering on the MDS, September samples were more dispersed. MvDISP analysis also highlighted the more dispersed nature of September samples (site 1: 1.095, 2: 1.146, 3: 1.464) compared to their June counterparts (site 1: 0.354, 2: 0.885, 3: 1.056).
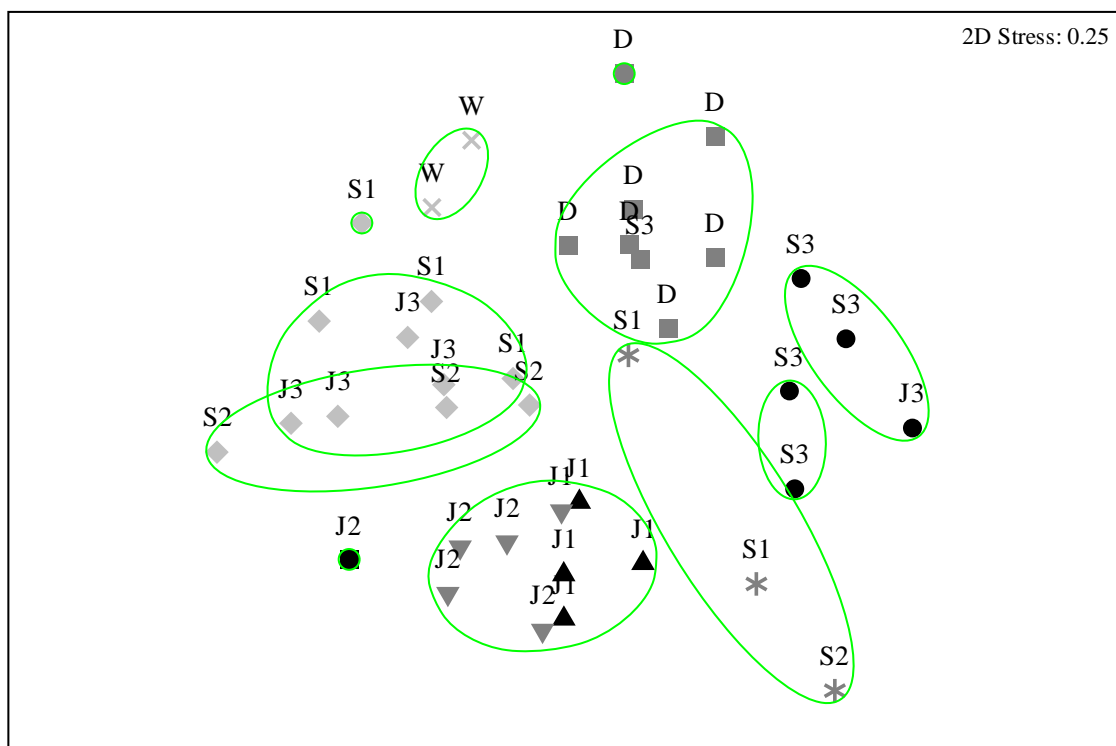
Figure 2.3 Multidimensional scaling (MDS) plot of microbial community DGGE bacterial profiles in *E. verrucosa* colonies based on a Bray-Curtis resemblance matrix of presence and absence data. Labels represent June (J) and September (S) sites (1-3) for healthy samples. Diseased (D) samples and Water (W) samples are also represented. Symbols represent SIMPROF test results, showing structure in bacterial communities between samples and clustering represents similarity between samples (50%).

In an analysis of DGGE data, PERMANOVA analysis (factors: site, month and health) showed health of colonies to be the determining factor in bacterial community composition (Pseudo-F = 2.86, P<0.01). However, significant interactions were found between site x health and site x month (Pseudo-F=1.87, P>0.05; Pseudo-F=2.83, P<0.001), indicating the complex nature of this data set. Without diseased samples complicating the analysis, significant differences in community structure were also found between site (F=2.59, P<0.001) and month (F=4.84, P<0.001) in a two-factor ANOVA. In pairwise tests, differences between sites 1 and 3 and sites 2 and 3 were responsible for overall site differences seen.

2.3.4 Microbial community composition: clone library analysis

MvDISP based on Genus and Class level phylogeny confirmed DGGE results and highlighted that June samples were less dispersed than September samples, indicating higher disturbance in September. Calculated diversity indices based on NCBI top hits for species data, including Shannon-Weaver index (H') and Pielou's evenness values (J') (Table 2.2) highlighted the increase in diversity at each site from June to September and peaking in diseased tissue samples. J' showed June libraries (and site 1 September) to be more dominated by few species, whereas diseased colonies, water samples and September site 3 colonies showed more of an even distribution of bacterial classes within their communities (Table 2.2), suggesting that no one group takes over when the coral suffers from disease. Average taxonomic distinctness ($\Delta+$) confirmed high diversity and unrelatedness of water samples. It also confirmed taxonomic distinctness to be lowest in June samples and highest in September diseased samples, indicating that no single ribotype thrives under disease conditions (Table 2.2).

Table 2.2 Diversity indices for class level clone library analysis of bacteria associated with water samples, healthy coral colonies in June (J) and September (S) and diseased (D) colonies at three sites.

	Water		Site 1				Site 2			Site 3		
	J	S	J	S	J (D)	S (D)	J	S	S (D)	J	S	S (D)
Total number of clones	40	40	40	40	40	40	40	40	40	40	40	40
Shannon-Weaver diversity (H')	2.2	2.5	0.4	0.7	1.3	1.4	0.7	1.1	2.1	0.8	2.3	2.2
Pielou's evenness (J')	0.77	0.89	0.31	0.47	0.71	0.64	0.37	0.65	0.79	0.43	0.90	0.79
Average Taxonomic Distinctness ($\Delta+$)	77.4	77.2	66.7	73.8	71.6	75.2	71.0	72.2	75.2	70.2	77.7	75.7

Water libraries were dominated by Alphaproteobacteria and the Bacteroidetes groups, but also contained a range of other bacterial groups (Table 2.3). Within these groups, no one sequence on the NCBI database was dominant, with the majority of clones representing distinct NCBI hits. In contrast, healthy coral tissues were dominated by Gammaproteobacteria, representing 80-90% of clones in June. In September this dominance varied, with 80%, 67.5% and 20% of clones at sites 1, 2 and 3 respectively (Table 2.3). In September, Alphaproteobacteria increased at sites 2 and 3 and the Bacteroidetes and Cyanobacteria at sites 1 and 3, in addition to the Firmicutes at site 2 and the Planctomycetes and the Deltaproteobacteria at site 3.

Analysis of clone libraries from visually diseased colonies revealed dominance of clones affiliated to the Gammaproteobacteria (site 1, June 58% and September 60%; site 2, 43%; site 3, 28%); although this represented a drop in number compared to healthy libraries, with the

exception of site 3 in September. In diseased samples the Alphaproteobacteria (site 3; 30%), the

Bacteroidetes (site 1, 23%) and the Verrucomicrobia (site 2; 13%) also dominated. However,

site 2 also had many affiliations to the Alphaproteobacteria and the Bacteroidetes. Further,

clones affiliated most closely with the Betaproteobacteria, Deltaproteobacteria, Planctomycetes,

the Cyanobacteria and the Chloroflexi appeared in diseased libraries and were not present in

their healthy counterparts. See appendix 1 for phylogenetic trees of forward sequences.

Table 2.3 Diversity of microbial groups found in clone library analysis of coral branches and
water in June (J) and September (S) and diseased (D) samples.

Bacterial Group	Water		Site 1				Site 2			Site 3		
	J	S	J	S	J(D)	S(D)	J	S	S(D)	J	S	S(D)
Gammaproteobacteria	4	7	36	32	23	24	34	27	17	32	8	11
Alphaproteobacteria	17	5	1		6	4	1	4	3	4	5	12
Flavobacteria	3	8			4	5					2	2
Sphingobacteria				1					1			
Bacteroidetes	7	1				4			5			1
Betaproteobacteria	1		2						3	1	1	2
Deltaproteobacteria									1		1	2
Cyanobacteria	3	7		1					2	2	7	3
Verrucomicrobia	1	6							5			1
Actinobacteria		1										
Firmicute								1	1			
Planctomycetes		2			1						5	
Chloroflexi												2
Bacteria	4	3	1	6	6	3	5	8	2	1	11	4

The similarities and patterns between clone libraries can be visualised in Figure 2.4 which

shows June (J) and September (S) clone libraries to the left of the plots, with an exception of the

September site 3 clone library, which is situated closer to diseased (D) and water libraries (to

the right of the plots), demonstrating a shift in community structure. While there is no pattern to the distribution of many bacterial classes over the MDS plots, such as for the Betaproteobacteria, patterns of change can be seen in the Gammaproteobacteria, Alphaproteobacteria and the Flavobacteria (Figure 2.4).

Figure 2.4 Multidimensional scaling (MDS) bubble plots based on class level clone library data showing relative abundance of bacterial classes at each of the three sites (1,2 and 3), in June (J) and September (S) and in diseased (D) samples. Water clone libraries in June (J) and September (S) are also shown. Diameter of circles (1, 2, 3, 4) represent the relative number of Gammaproteobacteria (40, 28, 16, 4), Betaproteobacteria (3, 2.1, 1.2, 0.3), Alphaproteobacteria (20, 14, 8, 2) and Flavobacteria (8, 5.6, 3.2, 0.8) represented in the clone libraries.
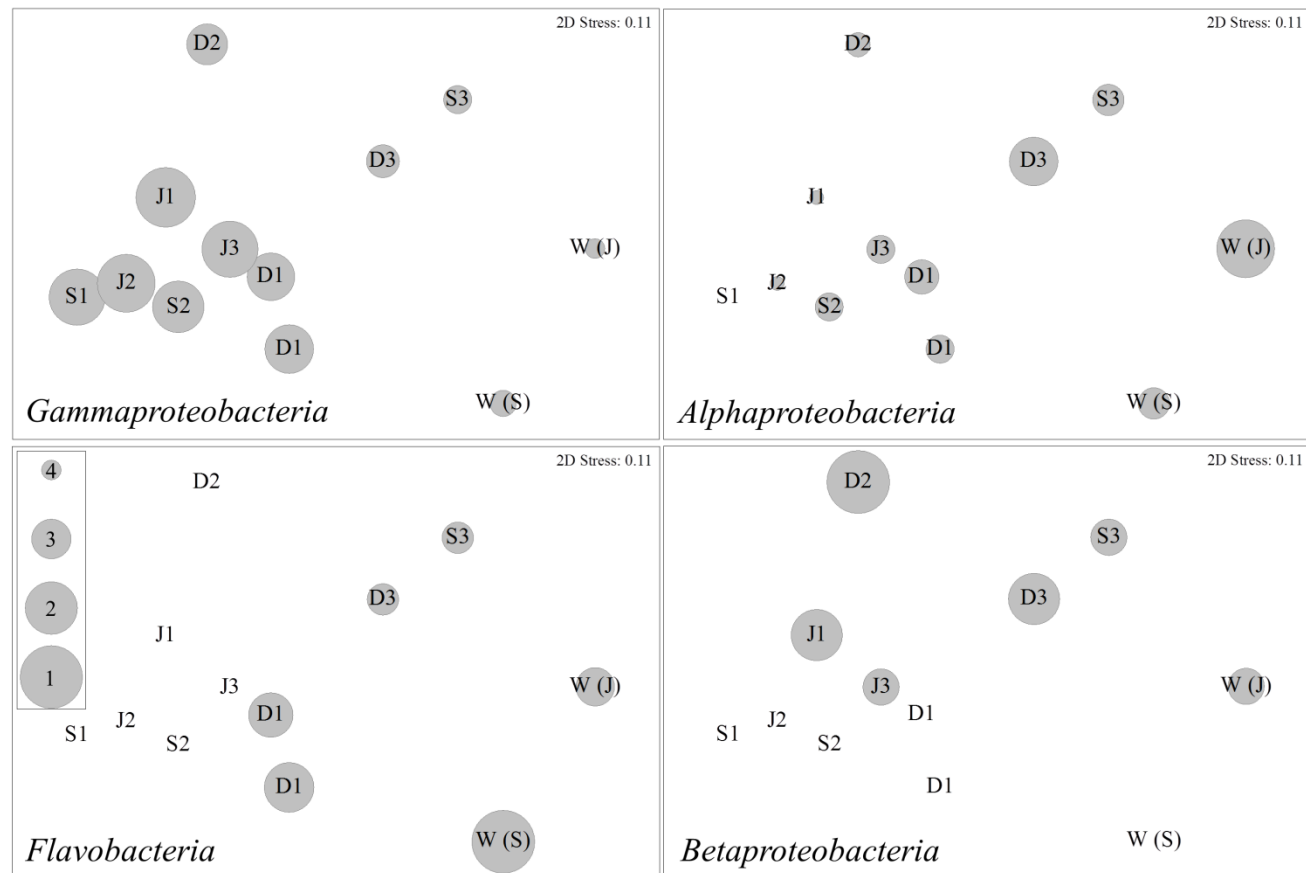
Clone libraries from diseased coral tissue did not show an increase in any one particular species/hit when the community changed, however clone libraries from healthy coral tissue had a large proportion of Gammaproteobacteria clones that affiliated with *Spongiobacter* sp. (FJ457274) and *Endozoicomonas montiporae* (FJ347758) in the NCBI database (Table 2.4). None of the clones from the water samples matched these sequences in the NCBI database. Interestingly, there were differences between the number of clones affiliated to these two species found in clone libraries from coral tissue sampled in June and from diseased coral tissue, by site, with total numbers originally lower at site 3 (Table 2.4). A number of other clones grouped with sequences retrieved from previous studies of microbial diversity associated with healthy corals. These included the Gammaproteobacteria *Spongiobacter nickelotolerans* (AB205011), *Endozoicomonas elysicola* (AB196667), uncultured *Spongiobacter* (EF657844) and *Spongiobacter* sp. (FJ889678). Other members of the Gammaproteobacteria, such as the vibrios were only represented by one clone from diseased coral tissue from site 1 in June. Sequences that most closely affiliated with *Delftia* sp. were found in three of the clone libraries (Site 3, September and diseased; Site 2 diseased) and were also found in *Acropora millepora* from the Great Barrier Reef (Bourne et al. 2007). Similarly, sequences matching *Achromobacter* sp. were found in *E. verrucosa* as well as in *A. millepora* and *Montastraea franksi* tissue.

Table 2.4 Most common affiliations in the NCBI database for clones from *E. verrucosa.*

Closest Relative	*Spongiobacter* sp.		*Endozoicomonas* sp.		
Accession number	FJ572274		FJ347758		
	Similarity (%)	% of clone library	Similarity (%)	% of clone library	Total %
Library					
Site 1					
June	≥ 96	30.0	≥ 97	50.0	80.0
September	≥ 96	27.5	≥ 97	45.0	72.5
Diseased (J)	≥ 96	17.5	≥ 97	22.5	40.0
Diseased (S)	≥ 96	22.5	≥ 97	30.0	52.5
Site 2					
June	≥ 95	52.5	≥ 97	10.0	62.5
September	≥ 96	22.5	≥ 97	15.0	37.5
Diseased (S)	≥ 95	17.5	≥ 97	10.0	27.5
Site 3					
June	≥ 96	15.0	≥ 97	22.5	37.5
September	≥ 96	2.5	≥ 97	7.5	10.0
Diseased (S)	-	0	≥ 97	2.5	2.5

2.3.5 Presence of zooxanthellae

The presence of *Spongiobacter* prompted us to investigate whether *E. verrucosa* tissue contained zooxanthellae since it has been suggested that *Spongiobacter* spp. may break down the high levels of DMSP produced by endosymbiotic dinoflagellates in coral tissue (Raina et al., 2009) and no previous study has confirmed the lack of zooxanthellae in *E. verrucosa*. No sequences were amplified from the tissue of *E. verrucosa* using dinoflagellate specific primers symITSFP and symITSRP (van Oppen et al., 2001) for the zooxanthellae ITS1 (data not shown).

2.4 Discussion

2.4.1 Conserved bacterial communities inhabiting the gorgonian coral *E. verrucosa*

This study confirms that bacterial communities associate with *E. verrucosa* and supports the hypothesis that corals have microbial consortia that differ from the surrounding environment (Rohwer et al., 2001; Cooney et al., 2002; Frias-Lopez et al., 2002; Rohwer et al., 2002; Bourne & Munn, 2005). Previous studies have shown that coral-associated bacteria can be species-specific (Rohwer et al., 2002) and also spatially specific, with similar bacterial communities inhabiting different species of closely related coral in the same location, but differing between location (Littman et al., 2009). DGGE analysis in this study provides evidence of conserved bacterial communities associating with *E. verrucosa* colonies at the same site and demonstrates conservation of these communities between sites (1 and 2). While communities at site 3 do not cluster as closely with sites 1 and 2, similar bacterial communities exist between replicate colonies at site 3. This suggests that although bacterial populations associating with *E. verrucosa* are conserved, there may be environmental factors that cause bacterial populations to differ to some degree by site.

Phylogenetic analysis of clone libraries further confirms these patterns, displaying distinct differences in sequence affiliation from water samples, dominated by Alphaproteobacteria and Bacteroidetes*,* to healthy *E. verrucosa* libraries, which were dominated by Gammaproteobacteria. Many studies of coral tissue have found Gammaproteobacteria to be abundant (Bourne & Munn, 2005; Ritchie, 2006; Ritchie & Smith, 2004; Rohwer et al., 2002; Ainsworth et al., 2006; Webster & Bourne, 2006; Wegley et al., 2007). In culture-based studies, Gammaproteobacterial dominance is thought to be related to their ability to produce antibiotics that limit the growth of other bacteria, in addition to their resistance to antibiotics (Long & Azam, 2001). Such results pose interesting questions, including how such microbial communities are maintained, to what extent microbial mutualisms exist within coral tissue and what functions these may play in coral health (Knowlton & Rohwer, 2003).

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Of these Gammaproteobacteria, a high abundance of clones from clone libraries associated with healthy coral tissue affiliated with species from the *Endozoicomonas* and *Spongiobacter* genera. *Endozoicomonas montiporae* is a member of the Oceanospirillales and was originally isolated from *Montipora aequituberculata*, an encrusting pore coral (Yang et al., 2010). Sequences affiliated with *Endozoicomonas* species are dominant in DGGE bands from the sea anemone *Metridium senile* (Schuett et al., 2007), were found associating with the ascidian *Cystodytes dellechiajei* (Martinez-Garcia et al., 2007), the marine sponge *Halichondria okadai,* the nudibranch *Elysia ornata* (Schuett et al., 2007) and the coral *Pocillopora damicornis* (Kurahashi & Yokoto, 2006). This Gram-negative, strictly aerobic, rod-formed bacterium represents a novel genus and is closely related to the genera *Oceanospirillum*, *Microbulbifer* and *Marinobacter*, of which almost all species are marine isolates. *Spongiobacter* sequences have previously been retrieved from sponges, the colonial ascidian *C. dellechiajei* (Martinez-Garcia et al., 2007) and found to dominate clone libraries in pre- and post-disease corals, e.g. in *Acropora millipora* from the Great Barrier Reef (Bourne et al., 2007) and three other *Acropora* species (Littman et al., 2009). The dominance of these two groups in many marine invertebrates and in *E. verrucosa* clone libraries lead to two possible conclusions. This could suggest that these bacteria are important members of the microbial community in this gorgonian coral and that they play a key role in the function of the holobiont, supporting the hypothesis that these animals shape their microbial partners for nutritional or protective benefits. Alternatively, it could suggest that these bacteria have substrate specificity and take advantage of the niche that this gorgonian coral and other marine invertebrates provide. Raina et al. (2009) has suggested that *Spongiobacter* sp. may play a role in degrading DMSP, produced by symbiotic algae, or zooxanthellae, which are known to associate with a number of corals. This prompted us to look for the presence of zooxanthellae in *E. verrucosa*, with no positive result. Different hypotheses could explain the role of the *Spongiobacter* sp.. For example, it has been suggested that specific invertebrate-microbial associations play a major role in coral health by protecting the host from potentially invasive microbes (Klaus et al., 2007), competing for nutrients and niche allocation (Koh, 1997; Klaus et al., 2007) and producing antibiotics (Ritchie, 2006). For example, Ritchie (2006) demonstrated that bacteria within the mucus of healthy corals inhibit the growth of other

bacteria 10-fold. The high representation of these two groups in healthy coral tissue could indicate that they are involved in excluding invading microorganisms as suggested for *Spongiobacter* sp. by Bourne et al. (2007).

2.4.2 Spatial differences to bacterial diversity

A number of factors have been shown to correlate with changing bacterial diversity associated with corals, including increased nutrients (Bruno et al., 2003) and temperature; which have been shown to disrupt bacterial populations and increase severity of disease in a number of corals (Harvell et al., 2007; Ward et al., 2007). Voss & Richardson (2006) have also observed increased sedimentation rates at sites of disease when compared to healthy sites, indicating that sediments may play a role in coral infections. In environments where corals are found, several factors may contribute to these differences. For this study three sites that differed in depth, and thus temperature and light intensity, as well as in substratum and sediment accumulation, were chosen with the aim of understanding if bacterial populations associated with *E. verrucosa* were stable and conserved. While these variables are insufficient to capture the extent of variation between the three sites, they may give clues as to some of the environmental factors at work. While sites 1 and 2 had similar bacterial communities, site 3 communities were less similar to sites 1 and 2, most diverse (H') and show higher multivariate disturbance.

Site 3 had a significantly higher temperature in June and had significantly higher sediment loads in both months, when compared to the other two sites. Site 3, at 6–9m is also the shallowest site. Black band disease has been shown to be more prevalent at shallow sites (Kuta & Richardson, 2002) while mean sediment rates for reefs not subject to stresses from human activities are <1 to ca 10mg cm^{-2}d^{-1} (Rogers et al., 1990), which is indeed lower than sedimentation seen at site 3. However, to date there have been no comprehensive studies of the effect of increased sedimentation (e.g. from dredging) on the function of coral bacterial communities and as one of a potentially large number of stressors, it is difficult to differentiate the response of bacterial

communities to sedimentation (Rogers et al., 1990). However, sediment particles can smother reef organisms, decrease recruitment (Harvell et al., 2007) and potentially act as vectors for coral disease (Voss & Richardson, 2006), possibly altering the balance between the coral and its resident microbiota.

2.4.3 Temporal and health related changes to bacterial diversity

This study demonstrated a shift in the bacterial community associating with healthy to diseased *E. verrucosa* colonies, with an increase in bacterial diversity and increased taxonomic distinctness in September and more so in diseased samples, suggesting temporal stress to these communities. It also demonstrated that no one group was responsible for this shift and instead showed bacterial changes to differ by site. Previous studies have demonstrated shifts and increasing diversity in the microbial populations from healthy to bleached or diseased corals (Bythell et al., 2002; Cooney et al., 2002; Frias-Lopez et al., 2002; Pantos et al., 2003; Gil-Agudelo et al., 2006; Bourne et al., 2007; Sussman et al., 2008). Studies into specific coral diseases have also shown the appearance of certain bacterial groups with the onset of disease, for example in Black Band Disease Delta- and Epsilonproteobacteria appear and Gamma- and Betaproteobacteria are lost (Cooney et al., 2002). This has been hypothesised to change the physiological function of these communities, affecting coral health. Striking similarity (36% of sequences) can be seen between communities associated with BBD and a white plague- like disease affecting *Monstastrea annularis* (Cooney et al., 2002; Frias-Lopez et al., 2002; Pantos et al., 2003), suggesting the development of a specific community around a unique microenvironment. Bourne et al. (2007) also showed an increase in the retrieval of *Vibrio*-related sequences associated with *A. millepora* colonies during bleaching. However, there have been no such studies carried out on shallow, cold-water species.

Diseased *E. verrucosa* tissue was still dominated by similar groups within the Gammaproteobacteria; however the second most abundant class of bacteria differed by site.

Affiliations to bacterial classes not present in healthy coral tissue also appeared in the diseased libraries, potentially altering the function of this bacterial community. *Endozoicomonas*- and *Spongiobacter*-affiliated sequences seen in the healthy libraries were reduced in clone libraries from visually diseased samples, suggesting a breakdown in the natural community. Bourne et al. (2007) witnessed a similar event during bleaching of *A. millepora*, with a decrease in *Spongiobacter*-affiliated clones from 41% in pre-bleached corals to 3% during bleaching, suggesting vulnerability of these groups to environmental stress, unbalancing the coral-microbial community. In comparison to the culture-based study by Hall-Spencer et al. (2007), where increases in the numbers of bacteria observed in diseased tissue were most closely matched to *Vibrio splendidus*, only one *Vibrio*-affiliated clone was present in any of the clone libraries, found in a diseased colony at site 1 in June. This could be due to the relative time period at which sampling occurred in the progression of disease in each study. However, the observed differences may also be due to the culture-dependent nature of the Hall-Spencer et al. (2007) study compared to this culture-independent analysis. This highlights the need for employing a range of techniques when studying microbial diversity in environmental samples (Donachie et al., 2007). Alternatively, it may suggest that the 'invading' bacteria seen solely or in higher abundance in diseased samples are opportunistic pathogens as suggested by Hall-Spencer et al. (2007) and aren't always present when disease symptoms occur. Opportunistic pathogens infect compromised or previously stressed individuals, as opposed to primary pathogens that cause disease in an uncompromised host. However, this study also provides evidence that bacterial communities change prior to visual signs of disease. This indicates that the coral may already be diseased and visual signs have yet to appear or that the conserved bacterial communities are compromised first, which leads to disease. Casadavell and Pirofski (1999) proposed the damage-response continuum to describe microbial pathogenesis, in which damage/ disease can be mediated by a pathogen or the host. For most infectious diseases, the nature and extent of damage depends on the immune status of the host (Casadavell & Pirofski, 1999). In the context of the coral holobiont, environmental conditions may put stress on natural bacterial communities, which have been suggested to have a role in the coral immune system (Ritchie, 2006; Shnit-Orland et al., 2009; Nissimov et al., 2009). In turn, this may have

consequences for coral homeostasis by allowing opportunistic pathogens to invade, creating a stress continuum which results in disease. This hypothesis needs to be investigated further to gain a deeper understanding of the progression of coral disease.

In conclusion, this study has revealed new evidenced that *Eunicella verrucosa*, has conserved bacterial communities associated with its tissues, members of which closely match genera found in a number of tropical corals. This study has provoked further questions concerning the role of these specific associates in this azooxanthellate coral holobiont, where previous studies have suggested functions relating to the breakdown of zooxanthellae products for the *Spongiobacter* genera. Further, it has strengthened the case for further research into assessing the role of key players in maintaining coral health, such as the *Spongiobacter* and *Endozoicomonas* genera and how they are affected by environmental stress. Although there is a range of studies that have revealed the microbiota of healthy corals, this is the first molecular investigation into the microbiota of a shallow, cold-water coral. This study suggests that no particular pathogen is associated with *E. verrucosa* tissue necrosis, that the changing microbiota may occur prior to visual signs of disease and that sedimentation and depth may affect the extent of bacterial community alteration; all of which are new and interesting insights for the future protection of this protected species in the UK.

CHAPTER 3

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DIVERSITY AND TEMPORAL STABILITY OF ACYL-HOMOSERINE LACTONE SIGNALLING IN *IN SITU* CNIDARIAN-ASSOCIATED BACTERIA

3.1 Introduction

Quorum sensing (QS), or cell to cell communication via the production of extracellular signal molecules, allows bacteria to monitor the environment for other bacteria and to alter behaviour on a population-wide scale in response to changes in the number/ species present in a community (Waters & Bassler, 2005). Phenotypes allowing for pathogenesis, symbiosis, biofilm formation, antibiotic production and expression of virulence factors are known to be under quorum sensing control (Davies et al., 1998; Dunny & Winans, 1999; Milton, 2006; Stoodley et al., 2002), many of which may be important in maintaining cnidarian-associated bacterial communities. However, as yet no research has established the importance or even presence of these signalling systems in cnidarians.

While much laboratory-based information exists on quorum sensing, comparatively little is known about how quorum sensing operates in the complex setting of the natural environment (Horswill et al., 2007) and how it affects microbial community parameters such as diversity and function (Valle et al., 2004). Outside the confines of laboratory cultures and under rapidly changing natural conditions, signal molecules are potentially susceptible to degradation or alteration once outside the cell (Decho et al., 2010). Decho et al. (2009) investigated temporal stability of AHLs in marine microbial mats and found a daily shift in AHL production, with an abundance of longer chain molecules present at night, which decreased during the day (Decho et al., 2009) due to alkaline lactonolysis at the higher day time pHs (Yates et al., 2002). Fluctuations in the geochemical and photochemical conditions that characterize natural environments present substantial challenges to even the short-term persistence of intact signals and efficient utilization of QS (Decho et al., 2010) which presents a question as to the importance of signalling in the natural environment.

The best characterised QS signalling system involves N-acyl-homoserine lactones (AHLs). These signals were initially described in a small number of proteobacterial species (Fuqua & Greenberg, 2002; Williams et al., 2007) and have recently been reported in the Cyanobacteria *Gloeothece* PCC6909 (Sharif et al., 2008) and members of the Bacteroidetes (Huang et al., 2008; Romero et al., 2010). AHLs have also been suggested to have an important role in eukaryotic-prokaryotic interactions in the marine environment (*e.g.* algae (Tait et al., 2005; 2009; Weinberger et al., 2007), invertebrate larvae (Dobretsov *et* al., 2008; Huang et al., 2007)), for example, in mediating the settlement of eukaryotic zoospores on bacterial biofilms (Joint et al., 2002; Tait et al., 2005), which suggests AHL production may play a role in the ecology of higher organisms.

AHLs are only active as QS signals in their intact, lactone based form. The lactone moiety of AHLs is highly susceptible to rapid, base-catalysed abiotic degradation (Yates et al., 2002; Tait et al., 2005) and AHLs are very short-lived in aqueous media with pH levels, in the range of seawater (>pH6; Yates et al., 2002). AHLs are also degraded enzymatically in natural systems (Delalande et al., 2005; Wang & Leadbetter, 2005), a process termed quorum quenching. The limited density of bacteria encountered in the open ocean and low chemical stability of AHLs in seawater has led to the suggestion that AHL-mediated QS activity may be concentrated in specific microhabitats (Hmelo & Van Mooy, 2009), such as marine biofilms (Huang et al., 2007) and potentially the coral mucus layer. However to date there is little evidence that QS is active *in situ* in bacteria associated with higher marine organisms or, like many coral-associated bacterial communities, are cnidarian species specific or spatial and temporally stable.

The aim of this study was to investigate the presence, diversity and stability of AHLs in various cnidarians. To gain greater understanding of the temporal role and stability of AHLs *in situ*, AHL signalling in the Snakelocks anemone, *Anemonia viridi*s was studied in intertidal pools over a 16 hour period. Signalling patterns were recorded alongside determinations of the

presence and activity of bacteria using denaturing gel electrophoresis of the bacterial 16S rRNA sequence from DNA and RNA extractions and compared to the changing pool chemistry recorded over the section of the diurnal cycle. AHLs were detected using the *Agrobacterium tumefaciens* NTL4 (pCF218)(pCF372) (Fuqua & Winans, 1996) AHL bioreporter and thin layer chromatography. This biosensor is an NT1 derivative which does not produce its own AHLs and carries a traG::lacZ reportor fusion. The reporter gene is induced when its transcription activator, TraR detects a cognate exogenous AHL and resulting in the production of β-galactosidase enzyme, detected by the use of X-gal as a substrate, which gives a sensitive colorimetric response to a wide range of AHLs (Steindler & Venturi, 2007).

3.2 Methods

3.2.1 Cnidarian sampling

Replicate samples were collected from a number of cnidarian species (see Table 3.1) including samples kept in a stable, long-term aquarium setting and samples collected directly from their natural environment. For colonies needing fragmentation, sections were removed from colonies aseptically and all samples were immediately washed three times in 0.2 μm filtered, sterile phosphate buffered saline (PBS; pH 6.5). Samples were either frozen in liquid nitrogen and stored at −80°C, stored on ice in the dark until analysis (no more than 30 min) or analysis (below) was carried out immediately (see Table 3.1). Additionally, to assess where AHL-signalling was concentrated in the tissue of the sea anemone *A. viridis*, tentacles were collected alongside whole individuals.

Five rock pools of varying size and position on the shore, containing approximately 20 *A. viridis* (brown variety; Figure 3.1A), were tagged with small floating buoys at Mount Batten, a rocky intertidal location in Southwest England on September 10th, 2011 (Figure 3.1B and 3.1C). One (whole) anemone was collected with aseptic technique from each of the pools at low tide

(08:00) on the same day. A further anemone was collected from each pool every three hours until 23:00. All anemones were approximately the same size. All samples were immediately washed three times in 0.2 μm filtered, sterile phosphate buffered saline (PBS; pH 6.5) to remove loosely attached microbes, frozen in liquid nitrogen and stored at -80°C until analysis. Salinity, temperature, pH, dissolved oxygen (YSI 63 meter), light and access of the pool to the main body of water were recorded at each time point for each pool. The dimensions of each pool were also recorded.

3.2.2 *A. viridis* DNA and RNA extraction

Samples were briefly removed from -80°C. On ice, two cuttings of tentacles (~ 0.03 g each) were taken from each sample under aseptic conditions using sterile, RNase treated equipment. Samples for DNA extraction were defrosted on ice while samples for RNA were immediately extracted using the RNeasy extraction kit (QIAGEN) according to the manufacturer's protocol for total extraction of RNA from animal tissues, except that samples were first placed in 1 ml RTL buffer (from kit) with 10 μl 2-mercaptoethanol and homogenised using a needle homogeniser for 1 min. Homogenate was then drawn into a sterile syringe and expelled five times before centrifuging at 18,000 xg for 3 min and following the remainder of the protocol. Samples were eluted in 60μl RNase-free water and stored at -80°C. DNA was extracted and diluted as in 2.2.2.

3.2.3 Reverse transcription of *A. viridis* RNA

RNA was quantified by spectrophotometry (Nanodrop 1000, Thermo Scientific) and diluted to 39 ng μl^{-1} in RNase free water. cDNA was synthesised using 0.1 μl of diluted RNA using the Quantitect Reverse Transcription kit (QIAGEN) with the provided blend of oligo-dT and random primers.
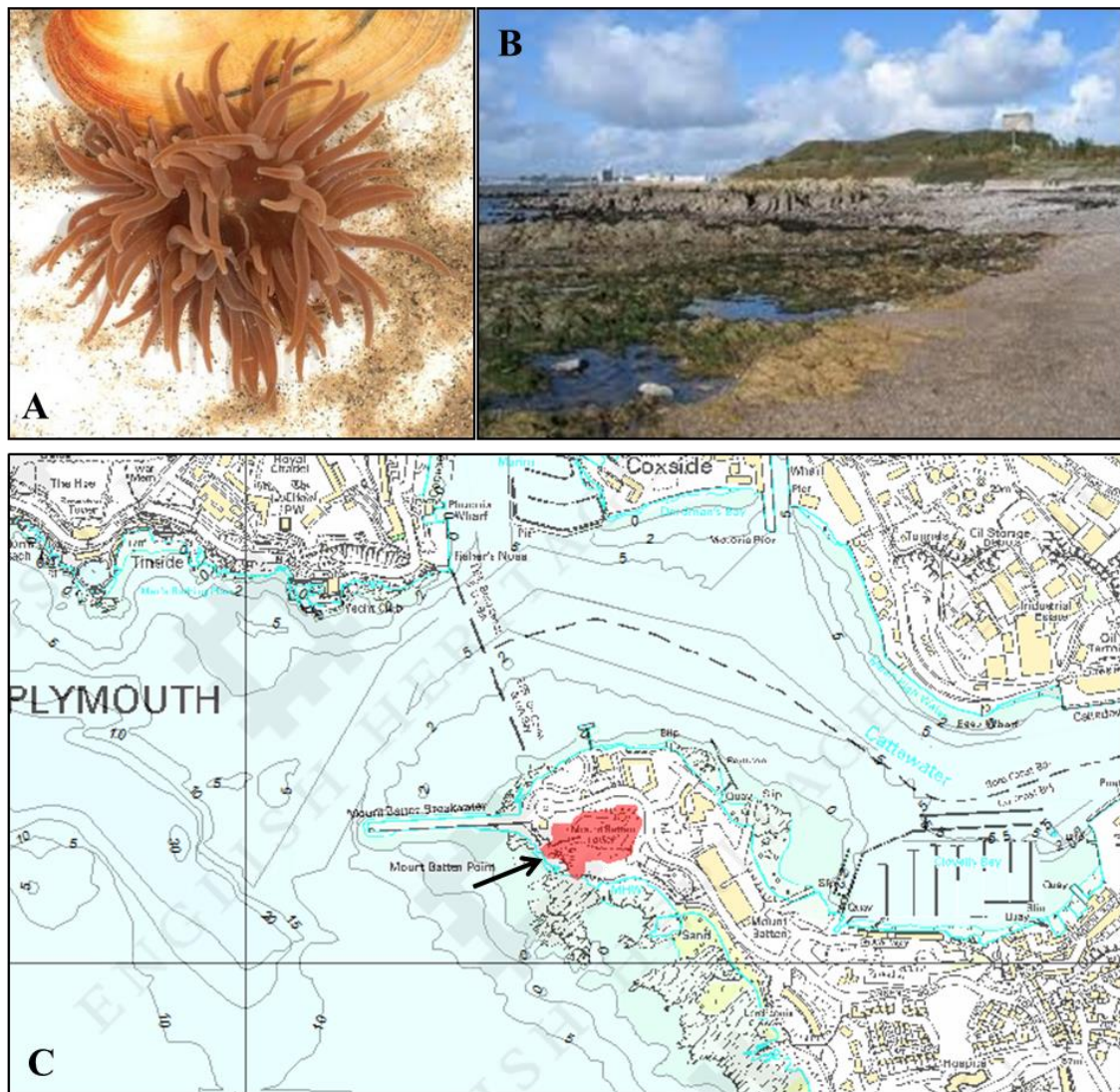
Figure 3.1 Picture of the sea anemone *Anemonia viridis* found at Mount Batten (A), location of Mount Batten beach (C) and Mount Batten beach, Plymouth, UK (B).

3.2.4 *A. viridis* **PCR amplification and Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA**

A nested PCR approach was used to amplify 16S rRNA for DGGE, using the methodology described in 2.2.3, except that DGGE was performed using the INGENYphorU^{TM} System (Ingeny, Netherlands) with 15 µl PCR products and a denaturant gradient of 20–60%. Subsequently, gels were stained with ethidium bromide (50 µg ml^{-1}) for 1 h and then washed with deionised water for 30 min. This process was repeated twice to check for reproducible results.

Bands of interest were cut from the DGGE gel using aseptic technique, placed in 50 μl sterile dH$_2$O, vortexed and incubated over night at 4°C. 1 μl was then re-amplified in a PCR reaction using primers 341f (5'-CCTACGGGAGGCAGCAG-3') and 907r (5'-CCGTCAATTCMTTTGAGTTT-'3) using the methodology in 2.2.3. Positive products were sequenced using the 341f primer and an ABI 3730 XL (LGC Genomics, Germany). Only one strand of the DNA fragment was sequenced and sequences analysed as in 2.2.4.

3.2.5 Sample homogenisation and *A. viridis* protein analysis

All cnidarian samples were placed in 2 ml sterile PBS (pH 6.5) and were homogenised until no visible tissue remained. Once homogenised, 100 μl of *A. viridis* samples was placed in a microcentrifuge tube and kept at 4°C in the dark until analysis. Total protein was measured using Bradford quick start dye reagent in flat bottom multi-well plates using the manufacturer's protocol (Bio-Rad). Protein values were taken in triplicate, with a blank of sterile PBS. Bovine serum albumin (BSA) was used to create a standard curve, achieving an R^2 of 0.98 before using this to determine protein content of samples.

3.2.6 Dichloromethane extraction of AHLs

Dichloromethane (DCM, 10 ml) was added to the remainder of the cnidarian samples, which were shaken vigorously for 1 min and centrifuged at 4,000 xg for 5 min. DCM extract (7.5 ml, bottom layer) was removed and placed in a clean Falcon tube. This process was repeated three times and the resulting extract was left to dry in a fume hood. Dried extracts were re-suspended in 500 μl DCM, vortexed and removed to a 1.5 ml microcentrifuge tube. Extracts were left to dry and stored at -20°C.

3.2.7 Visualisation of AHLs from cnidarian tissue

Dried extracts were re-suspended in 50 µl acetonitrile, vortexed and applied to RP18 F_{254} thin-layer chromatography (TLC) plates (20 cm x 20 cm; VWR International). For *A. viridis* samples, the amount of sample applied was normalised to 2.8 mg total protein. A mobile phase of 60% (v/v) methanol was used to separate extracts. TLC plates were overlaid with the biosensor *Agrobacterium tumefaciens* NTL4 (pCF218)(pCF372) (Fuqua & Winans, 1996), following the methodology of Mohamed et al. (2007). The detection limit for this biosensor varies for each AHL, however it is sensitive to all 3-oxo-acyl-homoserine lactones, C6-14 -acyl-homoserines lactones and C6-10 -3-hydroxyl-acyl-homoserine lactones (Steindler & Venturi, 2006). Following incubation at 30 °C overnight, the plates were examined for the presence on blue spots, indicative of AHL production. N-acyl-homoserine lactone synthetic standards were used as markers; using 5 µl of stock containing 5 µM *N*-oxohexanoyl-L-homoserine lactone (OC6), 0.2 µM *N*-oxohexanoyl-L-homoserine lactone (OC8) and 0.5 mM *N*-oxodecanoyl-L-homoserine lactone (OC10). Area-density analysis of spots on ImagePro Plus software determined the amount and detectable presence of various putative AHLs.

3.2.8 LC/MS identification of acyl-homoserine lactones from cnidarian tissue

AHLs were identified by liquid-chromatography mass-spectrometry (LC/MS) as described before (Laue et al., 2000; Shaw et al., 1997) using enhanced product trap experiments (EPI) triggered by precursor ion scanning between the m/z range 150-500 and in particular for the fragment ion m/z 102 which is characteristic for the homoserine lactone ring moiety. The EPI spectra (m/z range 80-400) containing a fragment ion at m/z 102 were compared for the retention time and spectral properties to a series of corresponding synthetic AHL standards, including 3-OH, 3-Oxo and unsubstituted AHLs with even chain lengths from C4-C14. This analysis was carried out at the University of Nottingham by Nigel Halliday and colleagues.

3.2.9 Statistical analysis

GelCompar (Applied Maths) was used to identify DGGE bands within the bacterial profiles for *A. viridis* samples and construct a binary matrix based on presence and absence of aligned bands. In Primer-E v6 (Clarke & Warwick, 2001) a Bray-Curtis similarity matrix of DGGE bands (presence/ absence data) was performed. Hierarchical clustering (group average) with SIMPROF tests tested for structure in the subset of DGGE data corresponding to each branch of a dendogram (1000 permutations, significance level 5%). Nonmetric multidimensional scaling (MDS) enabled visualisation of similarities between data from DNA and RNA-extracts (with hierarchical clustering overlaid). PERMANOVA analysis was used to relate differences in signals to environmental factors.

3.3 Results

3.3.1 Presence of putative AHLs in cnidarian samples

The presence of autoinduction signals in dichloromethane (DCM) extracts from various corals and anemones were shown using thin-layer chromatography (TLC) bioassays employing the sensitive bacterial reporter strain *A. tumefaciens* NTL4 (pCF218)(pCF372) for screening AHL molecules (Fuqua & Winans, 1996). Representative results of AHL reporter strain bioassays are shown in Figure 3.2. This reaveled that detectable accumulations of a diverse range of putative AHLs of various chain lengths were extracted from various natural and aquarium kept cnidarians.

Ten of the 13 cnidarian species tested (77%) induced blue colouration of the *A. tumefacians* biosensor. Examples can be seen in Figure 3.2. Autoinduction activities were detected in fragments as small as 0.016 g, suggesting high concentrations of these extracellular signals in the tissues. No colouration was induced in the negative controls (phosphate buffered saline) whereas colouration was induced by synthetic AHLs on all plates. Presence of AHLs and the

number of AHLs with different chain lengths detectable by the *A. tumefaciens* biosensor are summarised in Table 3.1.
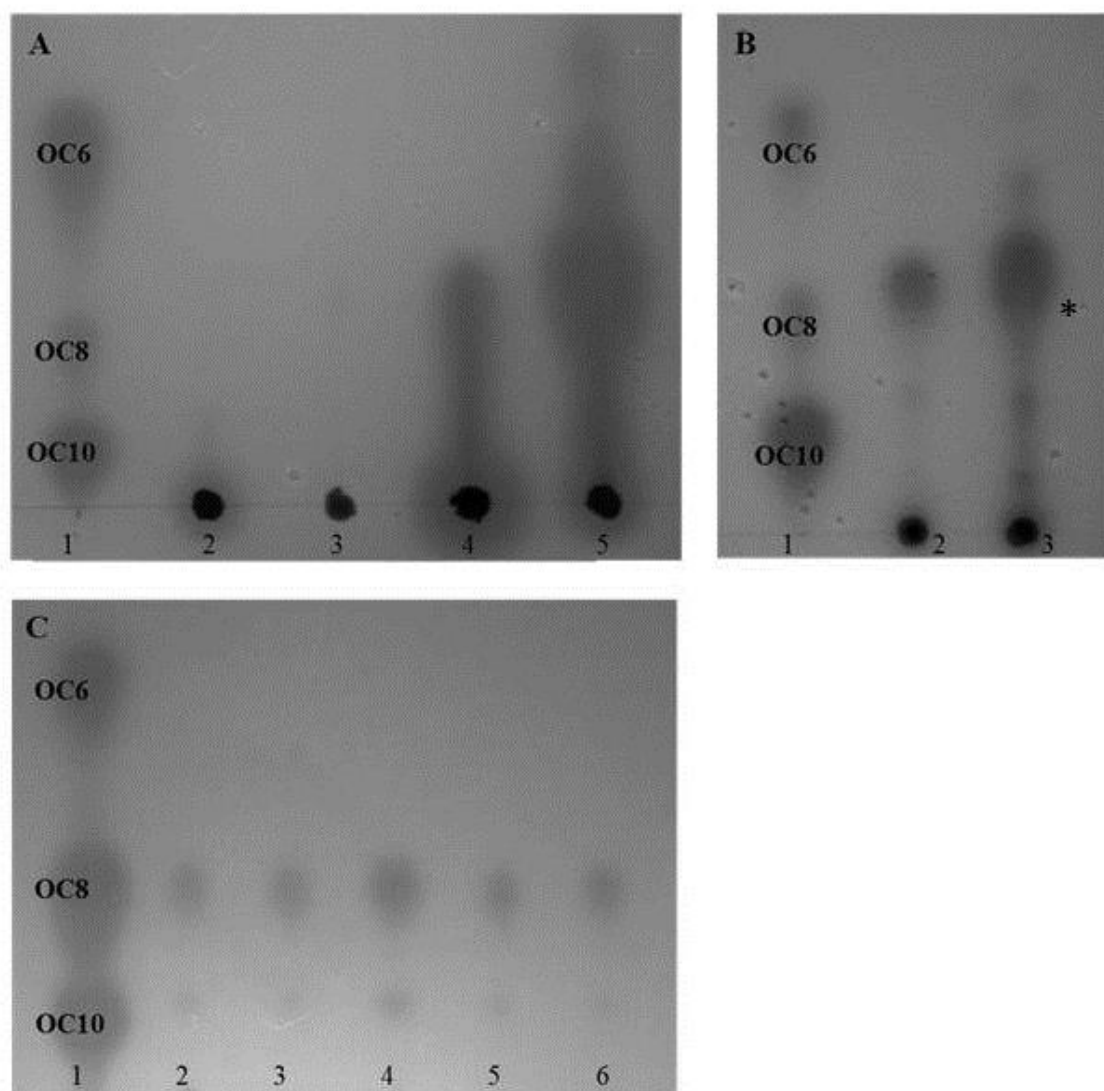
Figure 3.2 Reverse phase thin-layer chromatography profiling of AHLs extracted from Cnidrians. 5 µl of *N*-acyl-homoserine lactone synthetic standards were used as markers: 0.5 µM N-oxohexanoyl-L-homoserine lactone (OC6), 0.2 µM N-oxohexanoyl-L-homoserine lactone (OC8) and 0.5 mM of N-oxohexanoyl-L-homoserine lactone (OC10) (Lane 1). Images show profiles from *Discosoma* sp. (A2), Mushroom coral (A3), *Sinularia* sp. (A4), *Aptasia* sp. (A5); *A. viridis* (green variety) tentacles (0.6g; B2) and whole individual (17g; B3); and five

fragments of *Stylophora pistillata* (frozen, C2-4; immediately analysed, C5-6). Star represents AHL with tail shape.

3.3.2 Repeatability and species specificity of AHL profiles

The majority of the spots seen on TLC plates had similar migration rates to the mid- and long-chain synthetic AHL standards (N-oxohexanoyl-L-homoserine lactone (OC8) and N-oxodecanoyl-L-homoserine lactone (OC10)) with the presence of a spot that migrated alongside OC8 synthetic standard found in the majority of samples that tested positive on the TLC plates. In contrast, shorter-chain putative AHLs were only detected in Actinaria; which seems unrelated to fragment weight (Table 3.1). TLC analysis also revealed spots with tail shapes e.g. in *Sinularia* sp. (Figure 3.2 B3, represented by *), which could indicate the presence of a 3-oxo-substitution on the AHL compound (Golberg et al., 2011).

Extracts showed a differing degree of diversity of putative AHL signals, varying from 1−6 different chain lengths detected depending on the origin (Table 3.1). Strongest signals and greater diversity of putative signals were detected in the Alcyonacea and the Actinaria. In the Actinaria, or sea anemones, analysis of whole individual (Figure 3.2B 2: 4.45−17.1 g) versus tentacles (Figure 3.2B 3: 0.6-0.97 g) showed signal to be concentrated in the tentacles when compared by weight (n=3).

AHL profiles from fragments of the same species also showed good reproducibility. Even when extracted from individuals on different days (*A. viridis,* n=*3* and *S. pistillata,* n=3) the same TLC profiles were found. Freezing had no effect on the detectability of AHL signals (Figure 3.2C) which will prove important in future work.

Table 3.1 Summary of cnidarian species tested for presence of putative AHLs with TLC. Table summarises sample weight, methods used, including when tissue homogenisation was carried out after collection (on frozen tissue (F), immediate analysis (< 2 min; I) or delayed analysis (< 30 min; D)) and whether the samples were from the aquarium (A) or the natural environment (NE), detection of signal, number of putative AHLs detected (based on spot number), estimated chain length of putative AHLs (short (S), C2-C6; medium (M), C6-C10; long (L), C10-14; estimated by their position relative to standards) and whether the AHL profile was repeatable. For *Sinularia* sp. and *Aptasia* sp., where TLC plates were smeared, only distinguishable AHLs have been counted. Aquarium samples were collected from Plymouth Marine Laboratory, UK (A1), the National Marine Aquarium, Plymouth, UK (A2). Samples from the natural environment were collected from South West England (NE1) and the Red Sea, Eilat, Israel (NE2). * denotes fragments that contained high percentages of skeletal material.

	Species	Sample wet weight (g)	Method	AHL signal detected	AHLs detected	Chain length	Replicated
Gorgonacea (seafans)	*Eunicella verrucosa*	2.0 *	I (A1)	Yes	2	M, L	No replicates
	Gorgonian sp.	2.4 - 6.0 *	D (A2)	No	-	-	Yes
Alcyonacea (soft corals)	*Discosoma* sp. (1)	0.1 - 0.79	D (A2)	Yes	2	M, L	Yes
	Discosoma sp. (2)	3.2	D (A2)	Yes	2	L	No replicates
	Sinularia sp.	8.0	D (A2)	Yes	2	M, L	No replicates
	Mushroom coral	4.5	D (A2)	No	-	-	No replicates
Scleractinia (hard corals)	*Balonphylia regia*	5.1 *	F (NE1)	Yes	1	L	Replicates combined
	Pocillopora sp.	0.085 - 0.73	I (NE2)	No	-	-	Yes
	Favia sp.	0.016 - 0.088	I (NE2)	Yes	1	M	Yes
	Acropora eurystoma	0.21 - 0.29	I (NE2)	Yes	2	M	No
	Stylophora pistillata	Standardised by protein content	I, F (NE2)	Yes	2	M, L	Yes
Actinaria (sea anemones)	*Anemonia viridis*	0.6 - 17.0	I, F (A2, NE1)	Yes	1-6	S, M, L	Yes
	Aptasia sp.	1.6	D (A2)	Yes	4	S, M, L	No replicates

3.3.3 Temporal and spatial stability of AHLs in *A.viridis* found in rock pools over a tidal cycle

To further investigate spatial and temporal stability of signalling *A. viridis* was chosen due to the strength of signal detected in previous analysis and its availability locally. Presence of putative AHLs in whole *A. viridis* was assessed in five rock pools of varying size and substratum and, consequently, water chemistry. Samples were taken every three hours over 16 h to include a tidal cycle and periods in light and dark (08:00–23:00). Pools ranged from 0.33 m to 4 m in length (Table 3.2) and pool chemistry changed with time (Figure 3.3). Due to position, pools 3, 4 and 5 were subjected to longer periods without connection to the main body of water at low- and mid-tide. These pools also contained higher dissolved oxygen (DO; mg/L) at 11:00 compared to pools 1 and 2. DO was highest at 11:00 for all pools and dropped significantly by 23:00. While salinity varied little in the pools over the tidal cycle except for pool 1 (decreasing to 12 ppt at low tide), pH varied greatly over the tidal cycle with the most extreme example seen in pool 1, varying from pH 7.01 (08:00; low tide) to pH 8.45 (17:00; after high tide). Temperature varied from an average of 14.5°C to 22.1°C in just 4 h in rock pools, although this variability was similar in all four pools.

Table 3.2 Description of rock pools from which *A. viridis* samples were removed.

	Pool				
	1	2	3	4	5
Position on shore	Low	Low	Mid	Mid	Mid
Size of pool (m)	4 x 3	4 x 1	0.3 x 0.3	3 x 0.5	1 x 4
Substratum	Sediment	Sediment	Rock	Rock	Rock
Anemones in pool	20	20	20	20	20
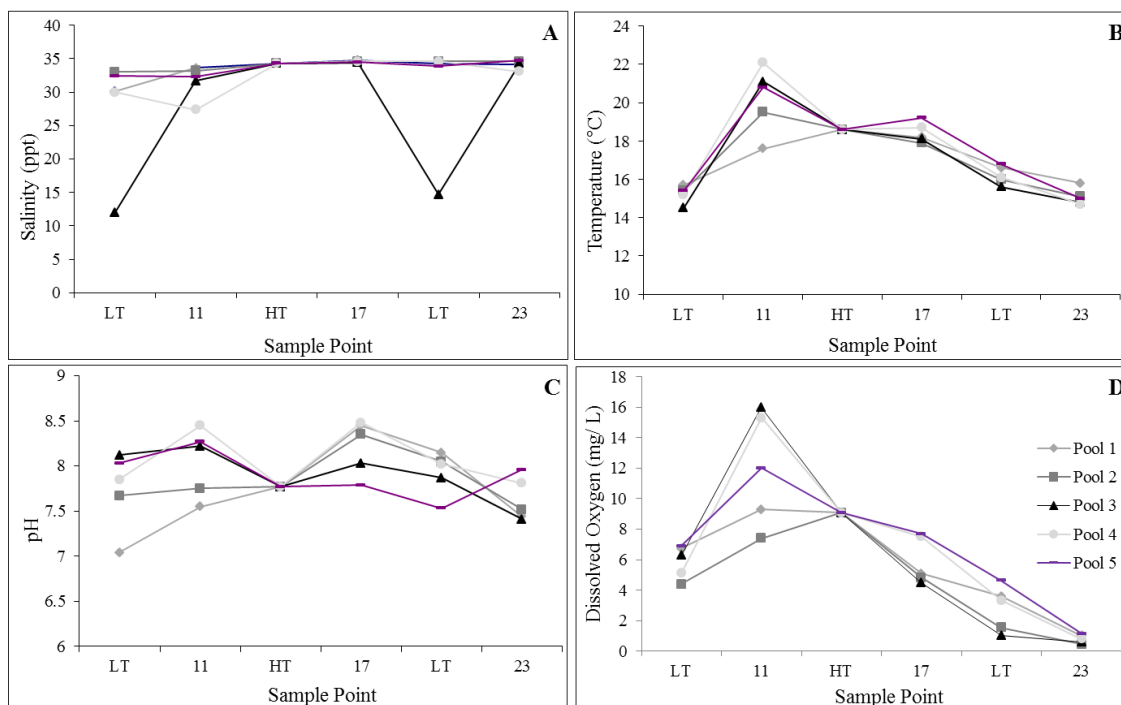
Figure 3.3 Changing salinity (ppt; A), temperature (°C; B), pH (C) and dissolved oxygen (mg/L; D) over the 16 hour section of the diurnal sample during which *A. viridis* were removed from rock pools. Sampling points correspond to exact times *A. viridis* samples were taken. Values at high tide (HT) were averaged from three sets of values along the shore. Numbers on x-axis correspond to time or low tide (LT) and high tide (HT) which were sampled at 08:00 (LT), 20:00 (LT) and 14:00 (HT). Dawn was at 06:45 and dusk was at 19:45.

Dichloromethane extracts from individual *A. viridis* taken immediately from the field and frozen in liquid nitrogen were also found to induce the bacterial reporter strain *A. tumefaciens* NTL4 (pCF218)(pCF372) biosensor, using TLC to separate putative AHLs (Figure 3.4). Despite variability in pool chemistry, profiles of detectable AHLs remained relatively stable in anemones from different pools. Four different spots were detected, determined by their migration with a mobile phase of 60% methanol on TLC plates, suggesting the presence of a variety of signalling by bacteria associated with *A. viridis*. Slight variability in the resting place of some spots is likely to be the result of slight changes in migration of different extracts possibly due to the presence of other compounds, however it may also signify the presence of a modified form of AHL. For the purpose of this analysis, the former was assumed. All four spots migrated at similar rates or below the longer-chain synthetic AHL standards (N-oxohexanoyl-L-

homoserine lactone (OC8) and N-oxodecanoyl-L-homoserine lactone (OC10) found to the left of the plate (Figure 3.4). However, not all spots were found in each sample (Figure 3.5).

Detectable AHL profiles remained relatively stable in all samples analysed. However, some variation was observed over the monitoring period, quantified in a crude analysis of spot area using density analysis with Image ProPlus software (Figure 3.5), made possible as *A. tumefaciens* responds to AHLs in a concentration dependent manner. Spot 1, which ran just above AHL standard *N*-oxohexanoyl-L-homoserine lactone (OC8) on TLC plates was found in all samples tested. However, in all pools there was an upward trend from 08:00 to 20:00 when signals peaked in all pools except pool 5. Although not present in all samples and less abundant, spot 3 shows a similar trend. At 23:00 spot 3 either remained at similar levels to those found at 20:00 or were slightly depressed, while still higher than other time points. However at 23:00, spot 1 dropped back to values seen elsewhere. Spots 2 and 4 showed no obvious trends. Spot 2 was more abundant in pools 3, 4 and 5. Spot 4 was also found in all samples. Samples from pool 5 showed similar patterns to all the other pools; however, spots were smaller and depressed at 20:00 in comparison to other pools.
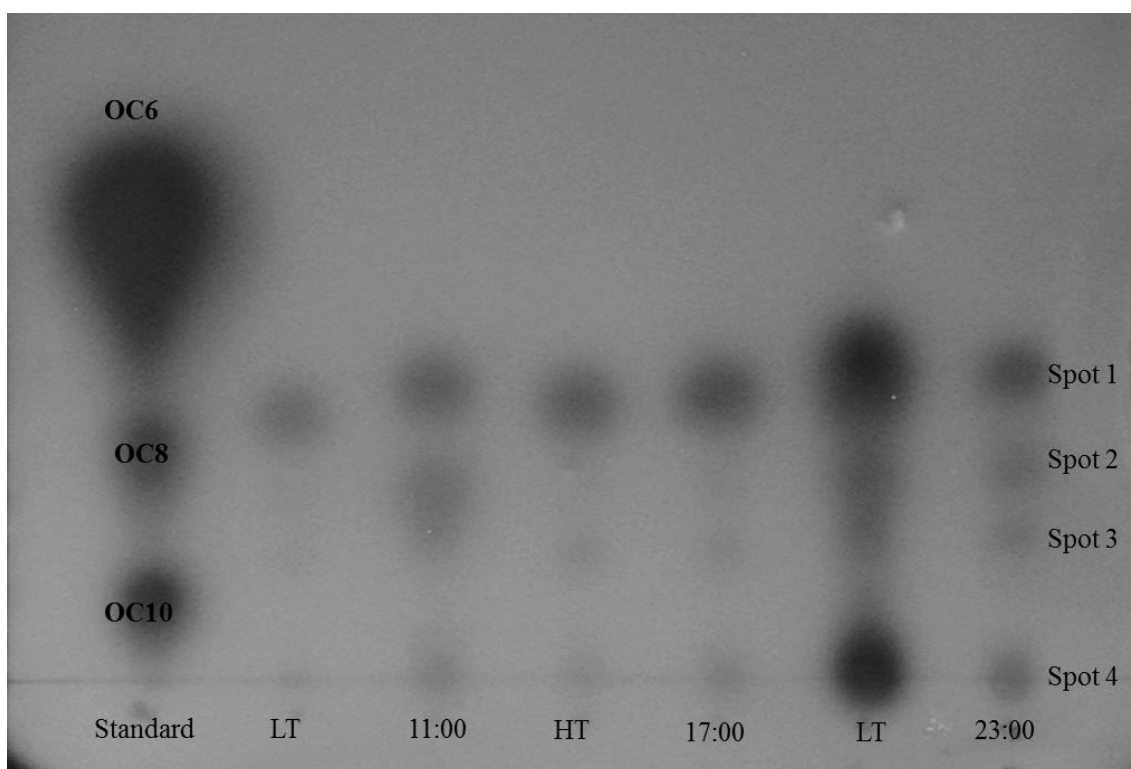
Figure 3.4 Reverse thin-layer chromatography profiling of AHLs extracted from *Anemonia viridis*. Standard lane shows 5 μl of AHL synthetic standard made up of 5 μM N-oxohexanoyl-L-homoserine lactone (OC6), 0.2 μM N-oxohexanoyl-L-homoserine lactone (OC8) and 0.5 mM of N-oxodecanoyl-L-homoserine lactone (OC10); and dichloromethane extract corresponding to 2.8 mg total protein from *A. viridis* in pool 4 at 08:00 (LT), 11:00, 14:00 (HT); 17:00, 20:00 (LT) and 23:00.

The image analysis method used is a crude analysis of putative AHLs on biosensor treated TLC plates. Values given (Figure 3.5) correspond to AHL detected from 2.8mg *A. viridis* protein analysed by density and area of blue colouration induced by samples and standardised to the area of induction of the OC8 standard. Although this method is not precise enough to allow accurate quantification of AHLs, it is accurate enough to give some indication of relative abundance.
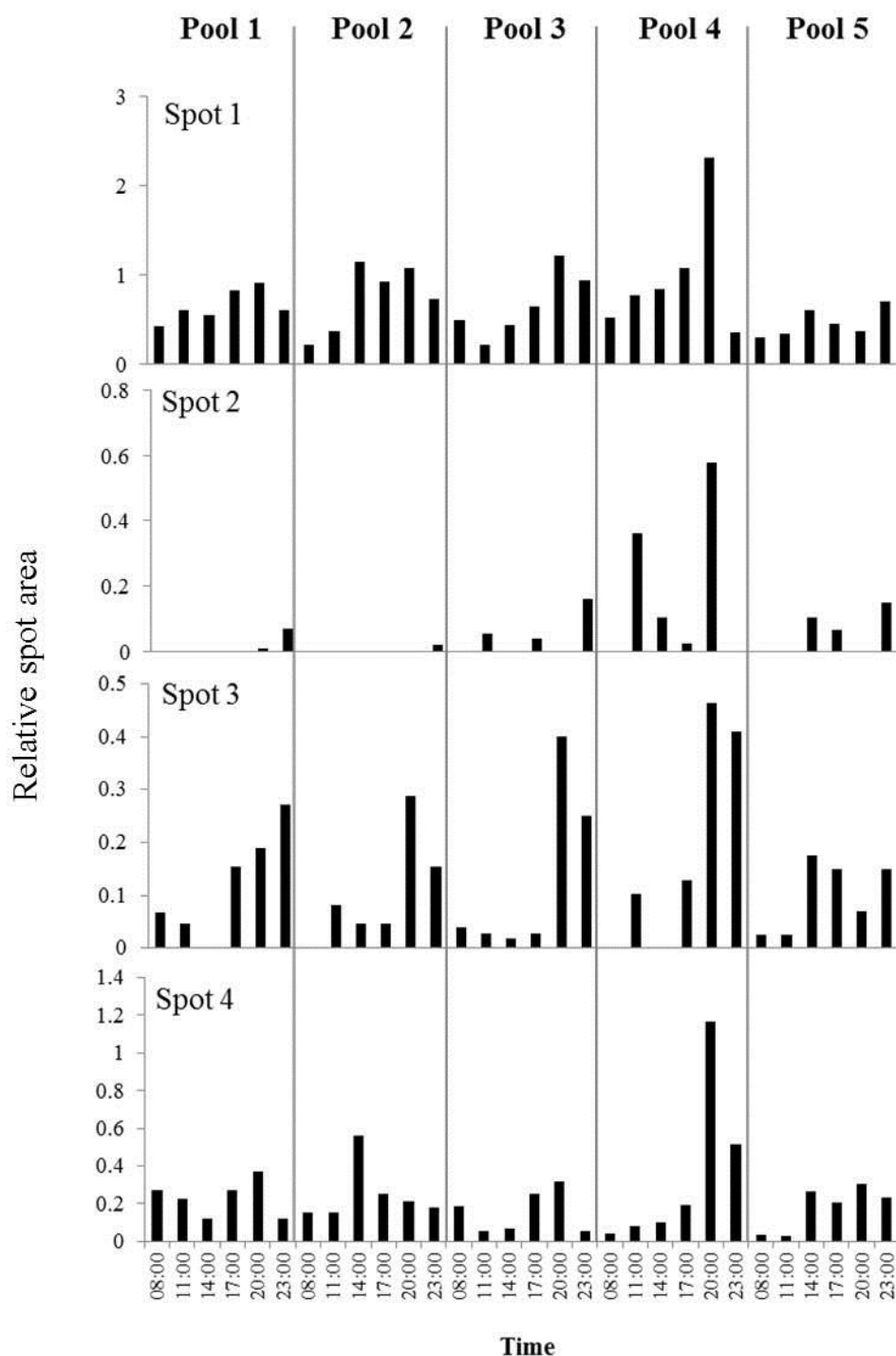
Figure 3.5 Analysis of spots from *A. viridis* extracts from reverse thin-layer chromatography plates based on area and density analysis using Image Pro Plus. Values correspond to relative area that *A. tumefaciens* bioreportor was induced by 2.8 mg *A. viridis* protein.

Figure 3.6 shows a multidimensional scaling (MDS) analysis of the AHL TLC profiles, except for one sample (pool 4; 20:00) which was omitted from the analysis due to its concentrated signal, to allow visualisation of other differences. Hierarchical clustering and SIMPROF analysis shows statistically significant evidence of genuine clusters in samples based on AHL profiles, separating samples taken at 08:00 and 11:00 from samples taken at 20:00 and 23:00. This can be visualised in Figure 3.6 where samples from 08:00 and 11:00 can be seen to the right of the plot clustering together at a distance of 0.48, while the majority of samples from 17:00–23:00 can be seen clustered to the left, suggesting some differences in AHL profiles as a result of time of day. Samples at 14:00 (high tide) were the most variable (1.39) in a Multivariate dispersion (MvDISP) analysis and are scattered over the MDS plot (Figure 3.6). A further MvDISP analysis looking at variability of signals within pools showed pool 1 to have the least variable signals over the experimental time period (0.67); with pool 4 having the most varied signals (1.46).

PERMANOVA analysis of AHL profiles with time point and pool as fixed terms showed significant differences between time points (Pseudo-F=2.98, p<0.005) but not between pools (Type 1 model). Covariates (normalised environmental factors) were added to the model one by one to assess the importance in determining AHL profiles. However, none of the environmental factors recorded had significant interactions with the model, suggesting that time of day is the main factor contributing to AHL signalling in *A. viridis*.
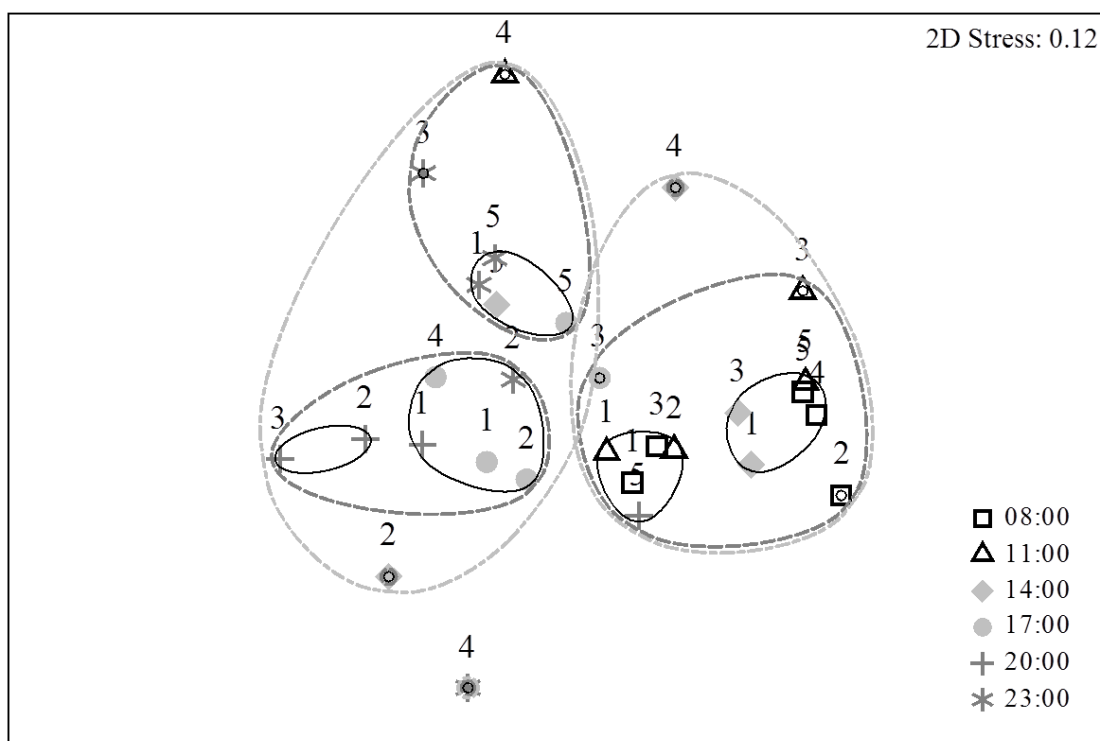
Figure 3.6 MDS comparing AHL profiles from TLC plates based on a Euclidean distance resemblance matrix of square root transformed abundance data. Labels represent pool number (1 − 5) and symbols represent time (key on graph). Samples are clustered at 0.24 (solid line), 0.36 (dashed dark grey line) and 0.48 (dashed light grey line) distances based on a hierarchical clustering analysis.

3.3.4 LC/MS Results

Subsequent purification of DCM extracts with LC/MS revealed the presence of a number of putative AHLs in *A. viridis* extracts (Figure 3.7A-F). Results confirm the presence of a number of AHLs including C6-AHL, C8-AHL, 3-OH-C8-AHL, 3-OH-C10-AHL and potentially 3-oxo-C10-AHL. The 3-oxo-C10-AHL has a lactone ring (size 102.2) and acyl-side chain the same molecular weight as C10-HSL but runs off the column more slowly compared to the standard. Due to the presence of the lactone ring (*m/z* 102) and because these samples activated AHL bioreporters, these molecules are most likely AHLs, but perhaps with an novel acyl side chain.
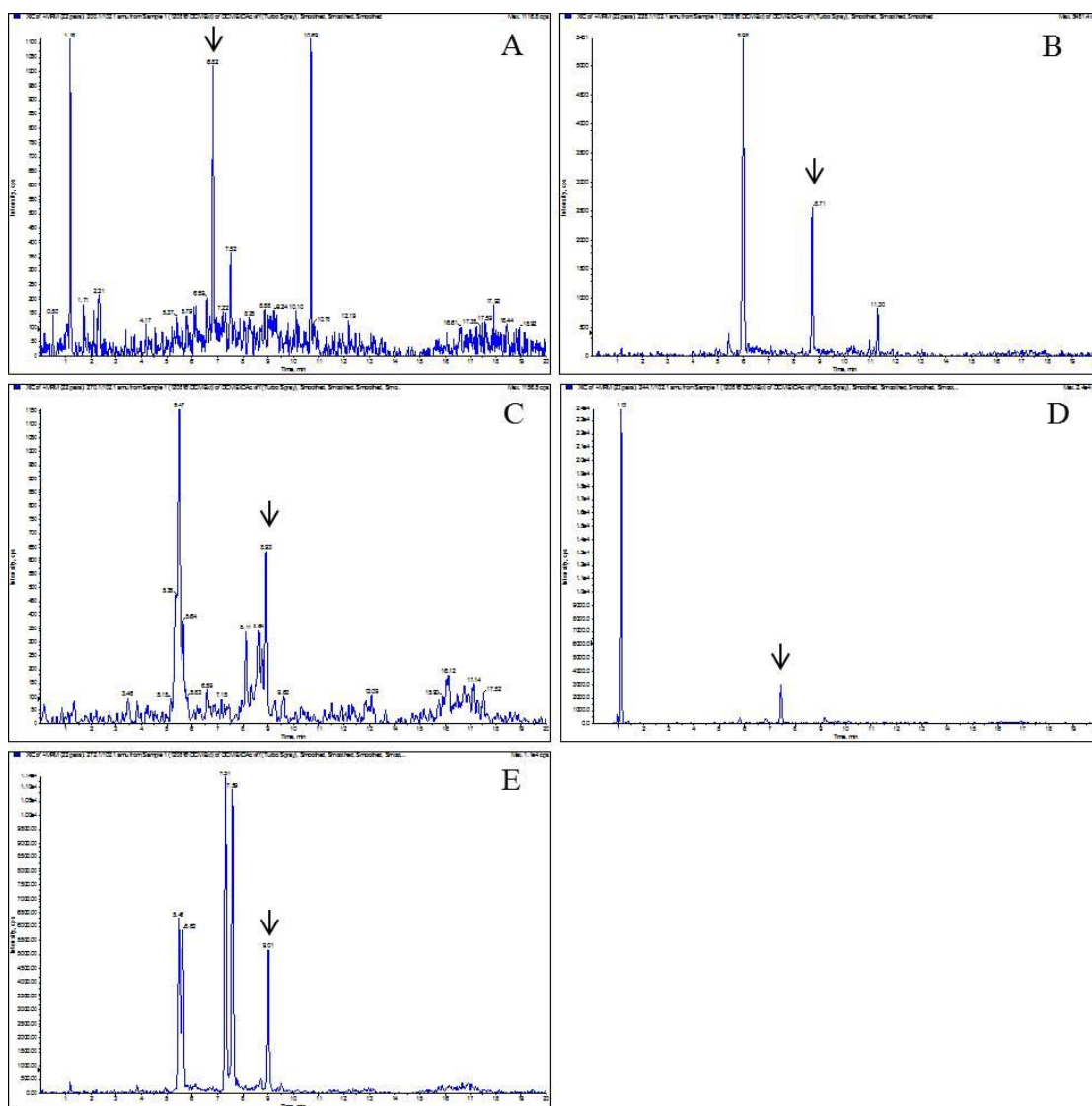
Figure 3.7 Mass spectra (LC/MS) of a representative *A. viridis* extract containing a number of peaks of putative AHLs (A-E). These peaks (labelled with an arrow) matched peaks from synthetic AHL standards C6-AHL (A), C8-AHL (B), 3-Oxo-C10-AHL (C), 3-OH-C8-AHL (D) and 2-OH-C10-AHL (E) and were not present in the control sample (data not shown).

3.3.5 Relating putative AHLs to bacterial community structure in *A. viridis*

PCR-DGGE profiles of 16S rRNA genes isolated from *A. viridis* DNA extracts are shown in Figure 3.8. Cluster profiles based on Bray-Curtis resemblance matrix of presence/ absence data with SIMPROF test to detect statistically similar clusters of samples showed all samples to cluster in the same group. This suggests that a similar total bacterial community occurs in all

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samples. Interestingly, however, no one band in the DGGE analysis was found throughout all the samples (Figure 3.8). However, a number of bands were present in more than 10 samples, including three that were identified to their closest match in the NCBI database (see Table 3.3). A number of other unsequenced bands were also found in more than 10 of the individuals, labelled in Figure 3.8 in accordance with their position on the gel (323.95, 392.00, 399.85, 412.02 and 422.05).

Interestingly, cluster analysis of PCR-DGGE profiles of 16S rRNA isolated from *A. viridis* RNA extracts (Figure 3.9) revealed a different story (Figure 3.10; Bray-Curtis resemblance matrix of presence/ absence data with SIMPROF test). SIMPROF analysis displays some differences between samples (Figure 3.10), with samples clustered into three statistically significant groups. Samples from 23:00 (pool 2-5) had significantly different DGGE profiles to other samples. Using 16S rRNA amplifications (from RNA extracts) as a proxy for active bacterial cells, this suggests a change in bacterial activity at 23:00. Samples from 08:00 and 11:00 can be seen in the far-right group (Figure 3.10) except for some samples from pool 1 and 2, which correspond to pools with sediment substratum and access to main body of water at mid-tide. 20:00 samples cluster in the middle group but are joined by an assortment of samples from other times and pools. Multivariate Dispersion analysis (MvDISP) shows samples at high tide (14:00) to be most dispersed (1.257) in terms of the active bacterial community associated with anemones, which matches dispersion seen in signal profiles at high tide. However samples from 20:00 and 23:00 are almost as discrete (1.103 and 1.174 respectively). In an MvDISP analysis of pool, samples from pools 1 and 2 are the most dispersed throughout the experimental time period.
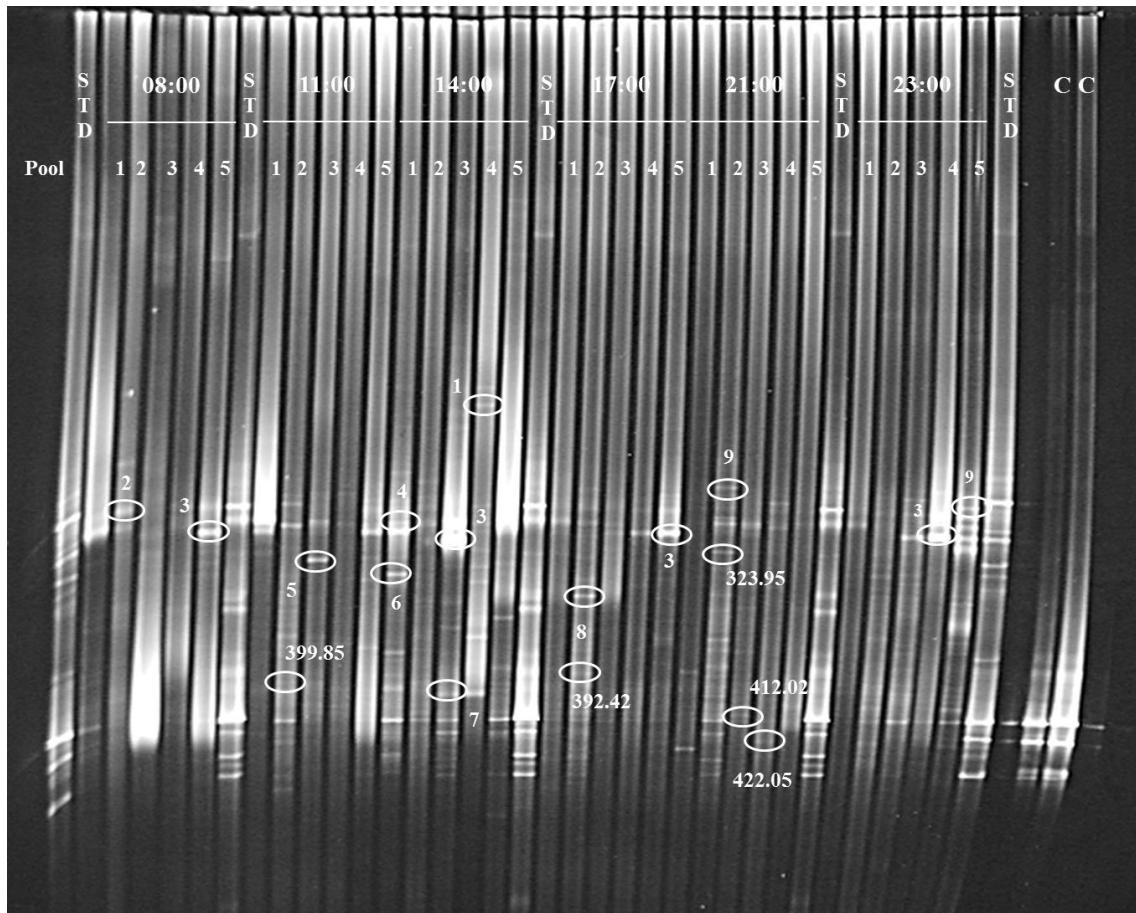
Figure 3.8 Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments extracted from *A. viridis* DNA samples. Labels at the top of the image relate to sampling time, with lanes corresponding to pool 1-5 under each time. Standards (STD) are RNA samples to allow for comparison between gels. Controls (C) can be seen to the right. Numbers (1-9) correspond to bands successfully excised, re-amplified, sequenced and run through the NCBI database to identify prominent bands. See table 3.3 for identities. Other prominent bands appearing in >10 samples are also circled with their reference position labelled (323.95, 392.00, 399.85, 412.02 and 422.05).
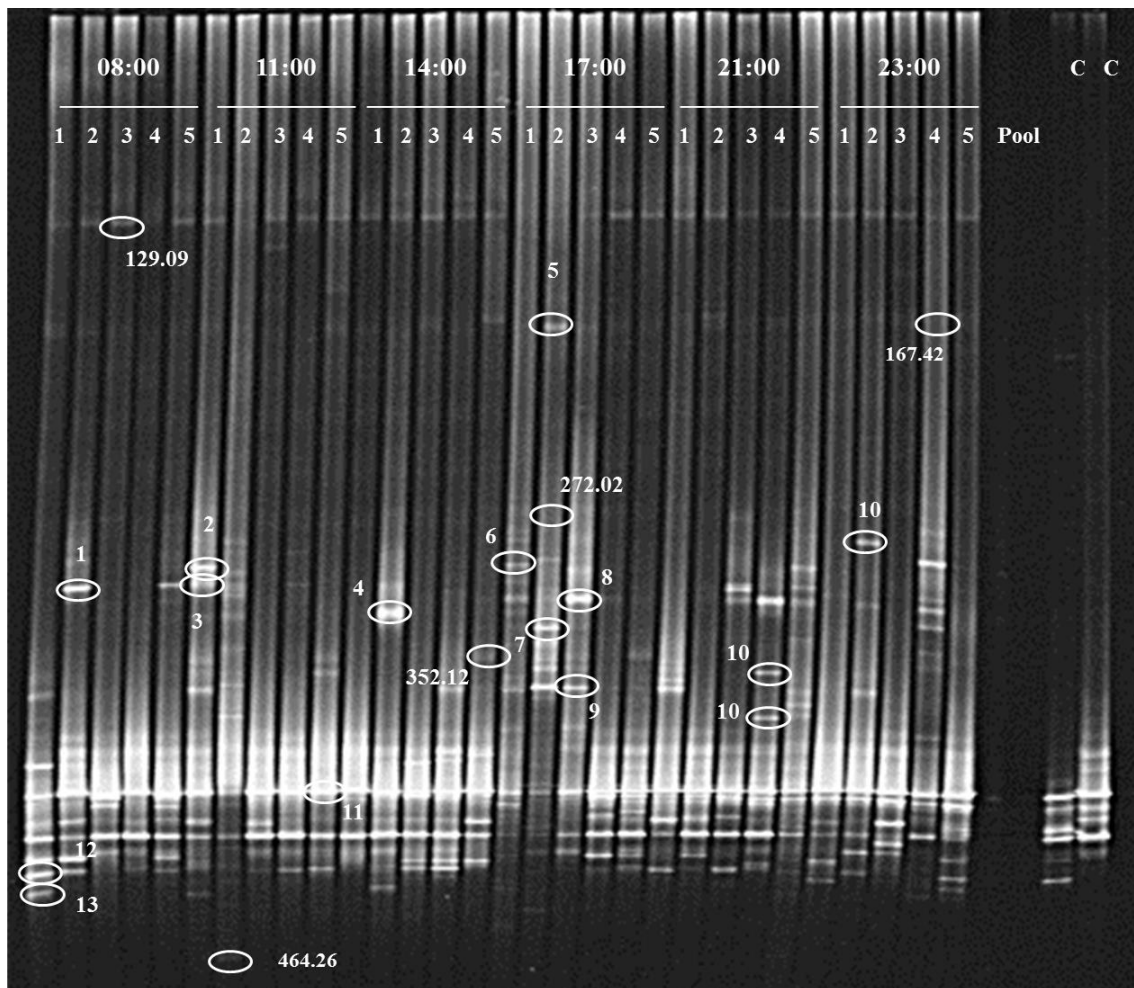
Figure 3.9 Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments extracted from *A. viridis* RNA samples. Labels at the top of the image relate to sampling time, with lanes corresponding to pool 1-5 under each time. Controls (C) can be seen to the right. Circles and numbers (1-14) correspond to bands successfully excised, re-amplified, sequenced and run through the NCBI database to identify prominent bands. See table 3.3 for identities. Other prominent bands appearing frequently are also circled with their reference position labelled (129.09, 167.42, 272.02, 352.12 and 464.26).
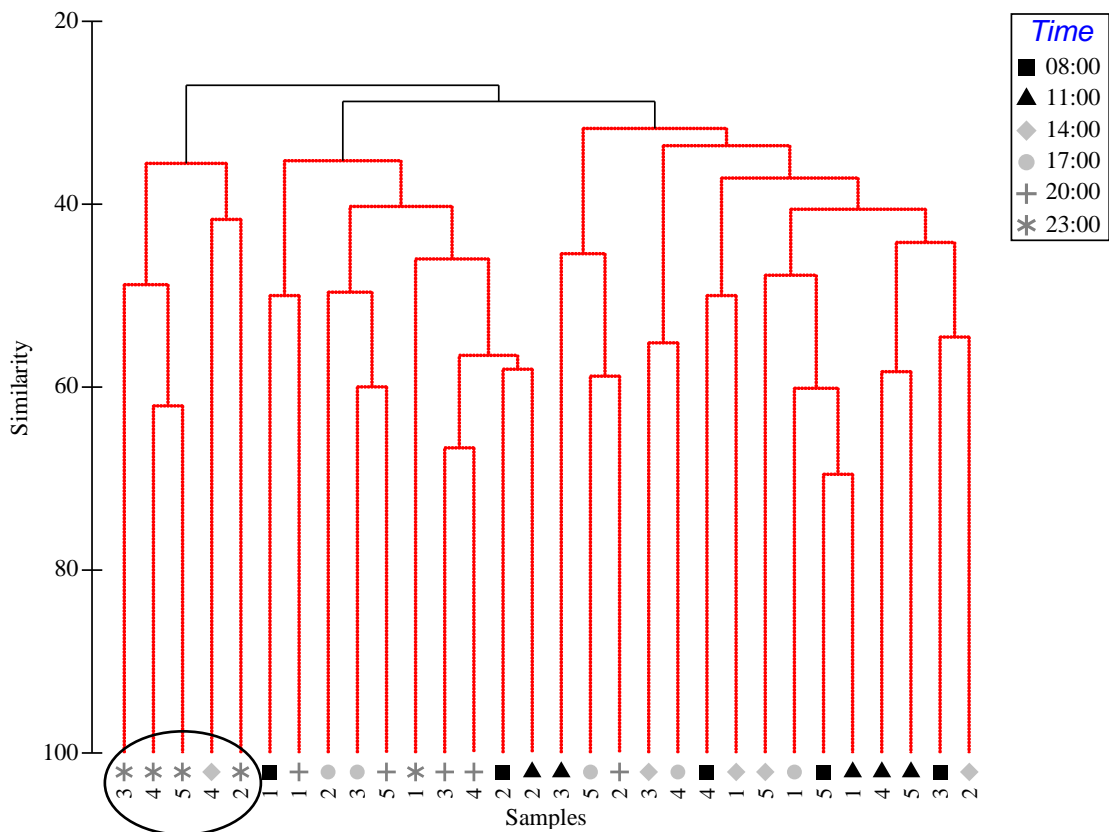
Figure 3.10 Cluster analysis of DGGE band profiles of 16S rRNA gene fragments extracted from *A. viridis* RNA samples. Cluster analysis is based on Bray-Curtis resemblance matrix of presence/ absence data using group average hierarchical clustering. Red lines denote SIMPROF results showing where statically similar clusters occur.

A number of bands were present in over 10 RNA samples, including bands that we have identified to their closest match in NCBI. See Table 3.3 for details. Unidentified bands 129.94, 169.67, 272.70, 352.40 and 464.26 were also found in more than 10 of the samples (Figure 3.9). In a SIMPER analysis, a number of DGGE bands were responsible for differences seen between RNA extracts taken at 23:00 and the rest of the samples, however no one band contributed more than 6%. Bands included band 167.42 which contributed to 15.74% similarity in samples taken at 23:00 and contributed to dissimilarity at all time points and band 237.88 which contributes 15.74% to similarity at 23:00 and to dissimilarity with 11:00, 14:00 and 21:00 samples. A number of other bands contribute to differences observed, being present at other time point but not at 23:00, including uncultured bacteria originally isolated from the coral *Montastraea faveolata* (GU118683). However, while there are some patterns in the activity of *A. viridis*

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bacteria over time, other bacteria, such as the cultured Gammaproteobacteria (GQ347793) are present in many of the samples irrespective of time or pool, which make any trends hard to establish (see Table 3.3 for BLAST information).

MDS analysis of DGGE banding profiles from DNA and RNA amplifications of the 16S rRNA sequences from *A. viridis* samples (Figure 3.11) establishes that different bacterial community profiles are seen when investigating DNA and RNA extracts. Hierarchical cluster analysis with a SIMPROF test shows bacterial RNA and DNA to belong to two distinct communities based on DGGE bands, with almost all RNA samples clustering at 40% (Figure 3.11). To ensure this difference was not based on gel differences or alignment difficulties, RNA standards used in the DNA extract DGGE (Figure 3.8) have been plotted on the MDS (Figure 3.11). Although these standards are not perfectly in line with those on the RNA gel, suggesting some gel effect, they do cluster more closely (at 40%) with RNA samples and lie close to original samples and not DNA samples on the MDS (Figure 3.11), signifying a limited gel effect.

Table 3.3 Most common affiliations in the NCBI database for bands successfully excised, re-amplified and sequenced from 16S rRNA PCR-DGGE of DNA and RNA extracts of *A. viridis*.

Origin	Band number	Frequency in samples	Most common affiliation	Accession number	Sequence similarity (%)
DNA	1	1/30	uncultured Fusobacteria	JQ579948	98
DNA	2	17/30	uncultured bacterium	GU362941	100
DNA	3	14/30	uncultured coral bacterium	GU118683	96
DNA	4	18/30	uncultured Flavobacteriales	HQ692014	100
DNA	5	5/30	uncultured Bacteroidetes	AY847473	96
DNA	6	8/30	*Maribacter* sp.	CP002157	100
DNA	7	1/30	uncultured Fusobacteria	JQ579948	98
DNA	8	9/30	uncultured Bacteroidetes	JQ579971	100
DNA	9	7/30	*Geobacillus* sp.	KC551260	99
RNA	1	11/30	uncultured Cyanobacterim	FJ53106	99
RNA	2	9/30	*Geobacillus* sp.	KC551260	99
RNA	3	8/30	uncultured Mollicute	EF137402	87
RNA	4	14/30	uncultured coral bacterium	GU118683	96
RNA	5	10/30	*Saprospira* sp.	AB088635	94
RNA	6	10/30	uncultured Sphingobacterium	EF664090	86
RNA	7	6/30	Flexibacter litoralis	AB681019	99
RNA	8	11/30	uncultured coral bacterium	GU118683	96
RNA	9	13/30	uncultured Gammaproteobacteria	GQ347793	97
RNA	10	9/30	*Geobacillus* sp.	KC551260	99
RNA	11	30/30	*Delftia* sp.	JF274926	100
RNA	12	10/30	*Geobacillus thermoleovorans*	NR07491	99
RNA	13	6/30	uncultured Cyanobacterium	FJ53106	99
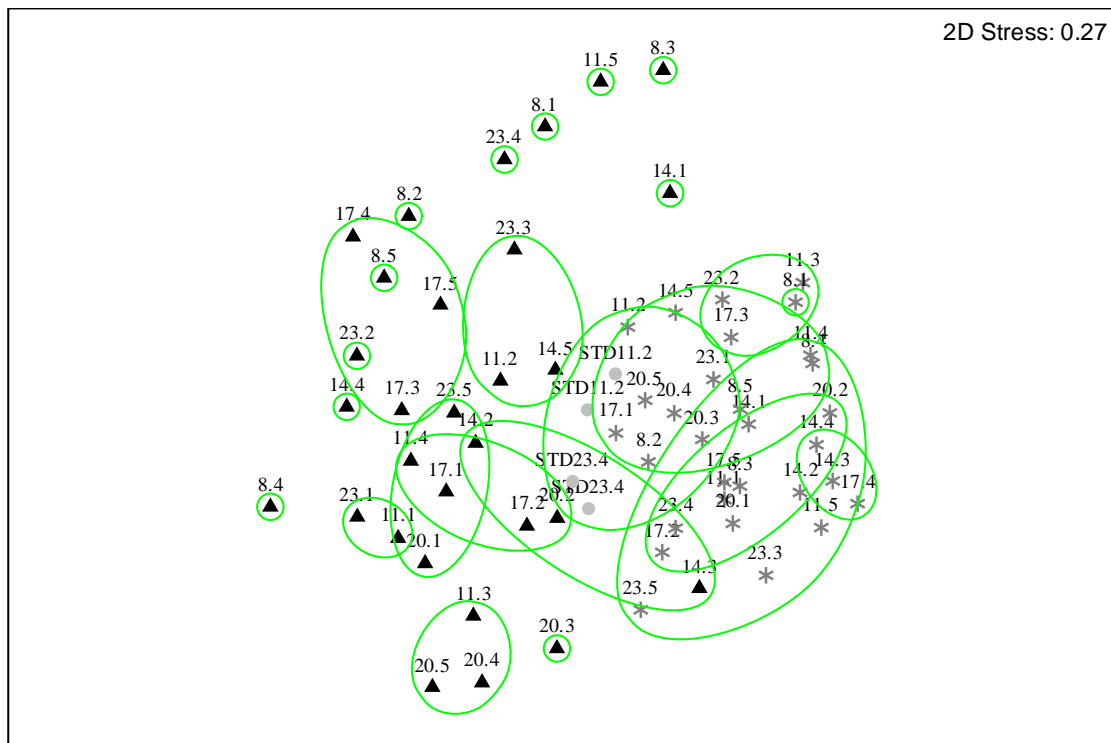
Figure 3.11 MDS comparing banding profiles of 16S rRNA sequences amplified from DNA (triangles) and RNA (stars) based on a Bray-Curtis resemblance matrix of presence and absence data. Labels represent time and pool number. RNA standards on DNA gel to check gel alignment correspond to RNA sample 11.2 and 23.4 (circles). Clusters denote similarity between samples at 40 % based on a hierarchical clustering analysis.

3.4 Discussion

Quorum sensing (QS) allows bacteria to alter their behaviour on a population-wide scale in response to changes in number and or species present in the community (Waters & Bassler, 2005), which could have profound effects on the host microbial ecosystem. It has been proposed that bacteria might use this intercellular communication to dominate in a natural niche or as a weapon to compete for survival (Waters & Bassler, 2005; Defoirdt et al., 2008) however many questions remain about how quorum sensing operates in natural environments.

3.4.1 Presence and estimated quantity of putative AHLs in cnidarians

This study has established the presence of putative AHL signal molecules in a range of cnidarian species. Results show 10 out of 13 of the cnidarian species tested to induce blue colouration of *A. tumefaciens* NTL4. The lack of presence of detectable signals in the remaining three species may be due to the restricted amount of soft tissue available and the detection limits of the biosensor as opposed to no signalling occurring in these species. LC/MS data confirms the presence of a number of AHLs in *A. viridis* extracts, one of which was tested against the 3-Oxo-C10-AHL standard, confirming the presence of a lactone ring and an acyl side chain with the same molecular weight as the 3-Oxo-C10-AHL standard. However, different retention times of the molecules and the different fragmentation patterns suggests that this may be an AHL not previously identified, perhaps having a novel acyl side chain. This AHL may be produced by a coral-associated bacterium, or alternatively these molecules may have been modified once excreted, either by the action of other bacteria or by the coral host.

AHL-producing bacteria have been isolated from a number of marine sources including marine snow (Gram et al., 2002), sponges (Taylor et al., 2004; Mohammed et al., 2008), water column eukaryotic algae (Wagner-Döbler et al., 2005), tidal (Tait *et al*., 2005) and subtidal biofilms (Huang et al., 2007) and now also from corals (Golberg et al., 2011). In natural environments the addition of AHL-QS blockers and AHLs have also been found to change bacterial community composition and function, including in marine biofilms (Dobretsov et al., 2007). However, the presence of signal molecules has been observed in a limited number of natural systems (Decho et al., 2009; Hmelo et al., 2009; McLean et al., 1997).

In the laboratory, QS has been found to regulate genes and phenotypes allowing for pathogenesis, symbiosis, biofilm formation, antibiotic production and expression of virulence factors (Davies et al., 1998; Dunny & Winans, 1999; Milton, 2006; Stoodley et al., 2002) as well as mediating interactions between bacteria and their eukaryotic hosts (algae: Teplitski et

al., 2003; Tait et al., 2005, 2009; Weinberger et al., 2007; plants: Teplitski et al., 2004; Klein et al., 2009; invertebrate larvae: Dobretsov et al., 2007; Huang et al., 2007; fungi: Hogan et al., 2004). For example, some plants 'listen' to bacterial QS signals, such as the model legume *Medicago truncatula* which detects nanomolar concentrations of QS signals and responds globally with significant changes in a range of proteins (Mathesius et al., 2003). All of these factors could prove important in maintaining bacterial populations associated with cnidarian species. The realisation that high diversities of bacteria associate with marine invertebrates (Bourne & Munn, 2005; Ducklow & Mitchell, 1979; Webster et al., 2007), the spatially and temporally stable nature of these communities seen in corals (Harder et al., 2003, Knowlton & Rohwer, 2003) and the vertical transmission of bacteria from parent to offspring in the coral *Porites astreoides* (Sharp et al., 2011) suggest that these communities play important roles in these systems, which may be facilitated by QS. However, detection of signals with the biosensor used does not necessarily relate to quantity of AHL needed to induce phenotypic change in bacterial populations. Kaplan & Greenberg (1985) suggest that QS systems in Proteobacteria are generally not induced until AHL concentrations reach at least 10 nM. Although only a crude image analysis was possible, facilitated by comparing area-density of spots induced by cnidarian extracts on TLC plates running at the same rate as the OC8 standard (1 µM; Image ProPlus), concentrations can be estimated. It is estimated that the assays detected a range of 4–43 µM AHLs from Actinaria, 5–13 µM for Alcyonacea tested and 0.07–0.2 µM for Scleractinia tested. Previous studies have found addition of 2 µM to induce changes in bacterial community and function in activated sludge (Valle et al., 2004) and 1–100 nM to stimulate defences in *M. truncatula*, modulating primary and secondary metabolism (Mathesius et al., 2003). This suggests that AHL signals in cnidarians are found in concentrations that are able to induce phenotypic change in bacterial populations and possibly the cnidarian host.

Accurate measurements of quorum sensing signals are also likely to be complicated by inherent microspatial variability in microbial abundance and microbial processes that are likely to occur in coral tissue. Such variability in dense marine environments presents obstacles in estimating

the relevant (to QS) concentrations of AHLs experienced by cells under natural conditions (Decho et al., 2010). While complex and diverse coral-bacterial associations have been proposed to occur in several regions including within the coral tissue layers, endolithic layer of skeleton and also in the coral surface mucus layers (Ritchie & Smith, 1997; Brown & Bythell, 2005; Ritchie, 2006; Ainsworth et al., 2006), more detailed studies of coral tissue layers have found bacteria to be limited to bacterial aggregates within the gastrodermis (Ainsworth & Hoegh-Guldberg, 2009; Peters et al., 1983; Peters, 1984; Rohwer et al., 2002). AHL molecules are likely to accumulate to higher concentrations within small 'clusters' of bacteria, when compared to areas having lower cell densities (Decho et al., 2008), therefore suggesting that QS would be a possible mechanism for communication within these tight clusters of bacteria. Further, fully induced proteobacteria synthesise AHLs at a rate of 1 amol cell^{-1} h^{-1} (Wang & Leadbetter, 2005). Therefore in a steady state a minimum of 1.2×10^9 cells l^{-1} would be required. Previous studies of coral-associated bacteria using DAPI cell counts have identified bacteria associated with coral mucus at densities of 5.3×10^8 l^{-1} to 1.8×10^9 l^{-1} (Garren et al., 2010) and 1.6×10^7 cm^{-3} in healthy coral tissue (Luna et al., 2007), suggesting that bacteria are present in high enough quantities in cnidarian tissue to facilitate the use of quorum sensing as a mechanism for inducing phenotypic change.

3.4.2 Diversity and stability of detectable putative AHLs

Thin-layer chromatography (TLC) of cnidarian extracts detected a wide-range of putative AHLs with varying chain length, which were reproducible between fragments from the same species, suggesting the importance of AHL signalling in these environments. Some extracts also revealed spots with tail shapes which could indicate the presence of a 3-oxo-substitution on the AHL molecule (Golberg et al., 2011), which will change the functional and taxonomic specificity of the AHL compared to one with the same migration rate without a tail (Hmelo et al., 2009). Extracts showed a differing degree of diversity of AHL signals detected throughout the samples, which do not seem to relate to amount of wet tissue used and may instead relate to

bacterial diversity and/or the number of phenotypes being controlled within the coral holobiont. The presence of different AHL molecules could be the result of a single bacterial species or functional group but is more likely to be an accumulation of an array of interactions within and across functional groups allowing for rapid responses to a range of environmental changes experienced within these ecosystems. Strongest signals and greater diversity of putative signals were detected in the Alcyonacea and the Actinaria which may relate to the number of bacteria found within these tissues or the importance of AHL-QS in these species.

Short-chain AHLs were only detected in the Actinaria, while the majority of the spots seen on the TLC plates ran at similar rates to the longer-chain synthetic AHL standards (N-oxohexanoyl-L-homoserine lactone (OC8) and N-oxodecanoyl-L-homoserine lactone (OC10)) suggesting a dominance of long-chain signals associated with the cnidarian species tested. Short chain AHLs are known to degrade more rapidly in seawater (Hmelo et al., 2009; Yates et al., 2002) and thus longer chain AHLs may be better optimised to function in a seawater medium. This is consistent with numerous reports that AHL-producing marine bacteria often produce long-chain rather than short-chain AHLs (Schaefer et al., 2002; Wagner-Döbler et al., 2005). However, it may be possible that these short-chain signals are effective in the environment over shorter distances (Hmelo et al., 2009) or are buffered from seawater chemistry by their invertebrate host, only to be broken down more rapidly once the animals are removed from their natural environment, thus preventing detection.

While this study has focused on AHL signalling, it is likely that other forms of signal molecules are also used within these bacterial communities, such as the 2-alkyl-4-quinolones (AQs) – which have recently been detected within marine communities (Twigg, *unpublished*) and a marine *Alteromonas* sp. (Long et al., 2003), diketopiperazines in halophilic archaeon (Tommonaro et al., 2012), γ-butyrolactones, furanones and autoinducer-2 (AI2-), used by Gram negative bacteria (Atkinson & Williams, 2009) and modified oligopeptides, such as

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autoinducing peptides (AIPs) used by Gram positive bacteria (Williams, 2007). Identification of these molecules would necessitate a larger study using a range of other biosensors, where biosensors are available, but would be an interesting next step to allow greater comprehension of how QS signals regulate bacterial function.

3.4.3 Temporal stability of putative AHLs in *A.viridis*

Investigations into the temporal stability of AHLs produced *in situ* in *A. viridis* revealed remarkable similarity between AHL profiles over the 16 h observational period, suggesting the presence of a specific bacterial community and a particular, stable role for these AHL signals that do not simply relate to adapting to changes in the environmental conditions seen during the diurnal cycle in these rock pools. The production of stable signals may inhibit sub-communities of bacteria (Golberg et al., 2011) or may be responsible for defence against invasive microbes by promoting genes for the production of antimicrobials. The production of antimicrobials active against planktonic bacteria, coral pathogens and biomedically important microbes by bacteria has been shown in a number of corals (Kelman et al., 2006; Ritchie, 2006; Nissimov et al., 2009; Shnit-Orland et al., 2009), as well as in other invertebrates such as sponges (Thakur et al., 2004) and ascidians (Karthikeyan et al., 2009).

The stability of the putative AHLs, despite massive fluctuations in rock pool chemistry (especially with pH changes from 7.0 – 8.5) is surprising considering previous studies concerning microbial mats, which found a daily shift in AHL production, from an abundance of longer chain molecules (C8 and C10) at night, which decrease during the day (Decho et al., 2009) due to alkaline lactonlysis at higher pHs (Yates et al., 2002). Researchers have suggested this may allow bacteria to utilise diel pH periodicity to alternate their day/night induction of specific sets of genes (Decho et al., 2009). The lack of this effect suggests that *A. viridis* may control internal pH, allowing long-chain AHL molecules to remain active in periods of high external pH. However, while signals remain relatively stable, this study finds some variability in

the detection of AHLs to relate to time of day, with an increase to some signals at night, as seen in the study by Decho et al. (2009). This correlates to dark conditions, a more anoxic environment and slight increases in pH, which may be a result of changing bacterial function or metabolic activity associated with bacterial communities throughout the day.

3.4.4 Relating AHL stability in *A. viridis* to DNA and RNA bacterial communities and specific bacterial sequences

The majority of studies to date looking at bacterial communities associated with marine invertebrates have either used DNA extracts to assess diversity or looked at phenotypes of specific isolates. To gain a broader picture of bacterial diversity this study has assessed the bacterial community with both DNA and RNA extracts of *A. viridis* samples and found significant differences in bacterial communities between the two methods. While DNA shows what is abundant, looking directly at the ribosomes (RNA) is thought to show which bacterial cells are active. Critically though using rRNA to determine the active bacteria relies on more RNA being present in active bacterial cells. It is known for example that ammonia-oxidising bacteria retain appreciable cellular concentrations of rRNA even during idle periods (Morgenroth et al., 2000) and that smaller cells are likely to have lower rRNA than larger cells, even though they could be metabolically more active (Kamke et al., 2010). However, it is widely accepted that in most bacteria rRNA levels are correlated with cellular growth rate and activity (Delong et al., 1989; Poulsen et al., 1993) and previous studies have used this approach to investigate the activity of marine bacteria, for example in marine plankton, sediment and gas hydrate communities (Moeseneder et al., 2001, 2005; Mills et al., 2005; Gentile et al., 2006; Rodriguez-Blanco et al., 2009).

In this study, no one band in DGGE community profiles based on 16S rRNA amplification from DNA or RNA extracts was present in every sample, which leads to the conclusion that the AHL profiles obtained from *A. viridis* samples are the result of a consortium of bacteria which

contribute to the overall AHL pool. Results of a recent study suggest the same, with 30% of coral bacterial isolates found to produce AHL molecules; suggesting AHL signalling is widespread in coral bacteria (Golberg et al., 2011). Differences seen between RNA and DNA extracts suggests that there may be differences between abundant bacteria and metabolically active bacteria in *A. viridis* extracts and that even with the high diversity of bacteria present and varying randomly throughout samples, there may be a more conserved group of active bacteria present. Further, variability of both the detected AHL signals and the bacterial communities (as assessed by RNA extracts) in rock pools was greatest at high tide suggesting a correlation between the 'active' components of the community and the intensity of AHL signals, which warrants further investigation.

DGGE profiles of the 16S rRNA sequences from RNA extracts of *A. viridis* show the presence of a number of bands that have been identified as belonging to groups of known quorum sensing bacteria. One band, relating most closely to an uncultured bacterium originally isolated from the coral *Montastraea faveolata* (GU118683) and found in 19 out of 30 of the samples, has a secondary affiliation with a *Reichenbachiella* sp. (AB540004; 93% similarity), which is a member of the Bacteroidetes. This group, although not as well studied has been found to produce a number of AHL molecules (Huang et al., 2008; Romero et al., 2010) including C14-HSL, C8-HSL, 3-oxo-C4-HSL and 3-oxo-C6-HSL from four isolates in sub-tidal biofilms (Huang et al., 2008). Another band, identified as an uncultured Cyanobacteria found in 17 out of 30 anemones also belongs to a group recently found to include QS bacteria. Studies of the epilithic colonial cyanobacterium *Gloeothece* PCC6909 have found production of *N*-octanoyl homoserine lactone (C8-AHL) (Sharif et al., 2008), which is the first time AHL signaling has been identified in this group. Two clades of bacteria consistently detected in *Porites astreoides*, a *Roseobacter* clade and a *Marinobacter* sp. (Sharp et al., 2011) also belong to groups of known QS bacteria. Finally, a band identified as an uncultured member of the Gammaproteobacteria, found in 13 out of 30 samples, is part of the best studied and largest groups of known AHL producers. In a recent study of cultivated coral-associated bacteria, AHL producers were found

to frequently cluster in the Proteobacteria and of these 90% were affiliated with the Gammaproteobacteria (Golberg et al., 2011). Ainsworth & Hoegh-Guldberg (2009) showed bacterial aggregates in both *Acropora aspera* and *Stylophora pistillata* from the Great Barrier Reef to be made up of only one morphotype, identified as members of the Gammaproteobacteria and they suggested that this group may prove to be a universal symbiont group associated with corals. In this study, the signal associated with *A. viridis* was found to be concentrated in the tentacles. A recent investigation using light and scanning electron microscopy to look at bacterial distribution within the anemone *Metridium senile* showed bacterial aggregates living in the tentacles. Sequence analysis of these aggregates revealed three subgroups of Proteobacteria including the gammaprotobacterium *Endozoicomonas elysicola* (98%) (Schuett et al., 2007). This group is found in a number of marine invertebrates (Martinez-Garcia et al., 2007; Schuett et al., 2007; Kurahashi & Yokoto, 2006; Chapter 2) and as such is worthy of further investigation concerning their ability to use quorum sensing to regulate behaviour in cnidarian associated bacterial communities.

In conclusion, this study has shown AHL-signalling to occur *in situ* in the coral holobiont for the first time, corroborating the few reports that QS may play an important role *in situ* marine bacterial communities (Decho et al., 2009; Hmelo et al., 2009; McLean et al., 1997). The presence of spatially and temporally conserved AHL-signalling profiles in cnidarian extracts suggests that AHL-signalling is at least one of the mechanisms by which coral-associated bacteria may maintain their communities and that AHL-producing bacteria may have an important role in the coral holobiont. The species specific nature of these AHL profiles links to the species specific nature of coral-associated bacteria (Ritchie & Smith, 1997; Rohwer et al., 2001, 2002; Littman et al., 2009) and further indicates that the coral host or at least the particular niches it provides potentially determines the composition of the natural bacterial community and the functions they carry out.

CHAPTER 4

TEMPERATURE MEDIATED DISRUPTION TO BACTERIAL COMMUNITIES AND THEIR QUORUM SENSING SIGNAL MOLECULES WITHIN THE CORAL *STYLOPHORA PISTILLATA*

4.1 Introduction

Disturbance to the fragile relationships between the coral host and bacterial communities have been linked to increasing temperatures associated with global climate change (Barber et al., 2001; Harvell et al., 1999, 2002; Rosenberg & Ben-Haim, 2002). Understanding the dynamics of disease outbreaks and the resistance of certain species as well as the ecological dynamics of mutualisms within the coral holobiont will be essential to help predict and mitigate against coral disease in the future. However, this depends on knowing what organisms are involved, how they perform as partners (Knowlton & Rohwer, 2003) and how temperature affects these relationships.

It is likely that many microbial symbionts have strict temperature thresholds, which when exceeded could result in a breakdown of symbiosis followed by reduced host fitness (Webster & Hill, 2007). Increased temperatures may also cause a shift from symbiotic to pathogenic function for some microbial species (Webster et al., 2008). However, this information is limited despite the critical contribution of microbes to marine biomass, diversity and ecosystem function (Webster et al., 2010).

Numerous studies have shown coral-associated bacterial communities to shift with environmental stress and disease (Littman et al., 2009; Ainsworth et al., 2009; Bourne et al., 2008), which has the potential to alter the fragile balance and roles within these communities. These communities could be altered as a result of a change in quorum sensing (QS) signalling due to evidence that QS molecules are degraded more rapidly at higher temperatures (Yates et al., 2002) and evidence that temperature affects the production of signal molecules in a number of marine *Vibrio* spp. (Tait et al, 2010). Kimes et al. (2012) has also presented data showing the up regulation of numerous quorum sensing proteins in *Vibrio corallilyticus*, a known coral pathogen, with increased temperatures. However, all these studies have been carried out in pure

cultures, not assessing quorum sensing under natural conditions. During shifts in bacterial communities increases in quorum quenching (QQ) bacteria; bacteria with mechanisms that inactivate QS, by either enzymatic inactivation or the production of inhibitors/ antagonists of signal reception (Dong et al., 2001, 2007), could also cause a change in bacterial signalling and disrupt the roles of bacterial associates in the coral holobiont. Increasing sea water temperatures could therefore significantly impact marine bacteria and their roles in the marine environment (Webster & Hill, 2007) – including by altering the ability of microbes to produce signalling compounds or by causing a shift to bacterial communities (Webster et al., 2010).

Marine bacteria play an important role in the coral holobiont by providing defence against invading microbes (Ritchie, 2006; Nissimov et al., 2009; Shnit-Orland et al., 2009), maintaining bacterial community structure (Bourne et al., 2009; Reshef et al., 2006), and cycling nutrients; therefore, a shift in community structure or altered ability to produce/ use signalling molecules could have serious implications for coral health and maintenance. The aim of this study was to investigate the effect of temperature on acyl-homoserine lactones (AHLs) in the scleractinian coral *Stylophora pistillata*. Total bacterial numbers and bacterial community structure were also assessed to establish bacterial dynamics over the experiment.

4.2 Methods

4.2.1 Coral collection and maintenance

The corals used in this study were collected by SCUBA from the coral nursery at the Interuniversity Institute of Marine Science in Eilat (IUI 29°30'N; 34°55'E, Gulf of Aqaba), Red Sea on January 25, 2010. Four colonies of *S. pistillata* were collected at 5 m depth and fragmented into approximately 8 cm long sections (n = 50), taking care to minimise the area of exposed tissue. Fragments were fixed to coded plastic mounts with underwater cement and epoxy resin (Figure 1C) and immediately placed in running seawater for 1 h to remove excess

cement and resin. After 1 h, fragments were then transferred to shaded outdoor tanks (Figure 1B) and an open-flow-through system providing seawater from the same location at which coral fragments originated (temperature 23°C, salinity 3.9%, pH 8.2). Corals were maintained with a natural photoperiod, with light intensity reaching an average of 597 μmol m^{-2}s^{-1}. Corals were acclimatised for two weeks until full recovery of the tissue, during which time corals were fed twice weekly with *Artemia salina* nauplii.

Following the acclimation period fragments were distributed between 9 tanks (10 L), three for each temperature treatment (Figure 4.1A & B), situated in one of three 1000 L holding tanks, to act as a water bath to stabilise the temperature (Figure 4.1B; 1-3). The distribution of fragments was carried out using a random number table. Fragments were acclimatised to experimental temperatures of 26°C (average summer temperature) and 30°C (acute stress corresponding to peak summer temperatures) over 7 days and maintained under these conditions for 7 further days, with natural diurnal fluctuations. The remaining fragments were kept at an ambient temperature of 23°C (average winter temperature). To maintain a constant temperature in the nine tanks, water from the Red Sea was continuously supplied to each tank at a rate of 150 ml min^{-1}, with one 100W adjustable heating rod in each experimental tank and one 100W and one 50W heating rod maintaining the temperature of the water baths. Each 10 L tank was fitted with a water pump (Eheim) to provide continuous water movement (Figure 4.1A) and temperature was measured every 30 min in the middle tank of each treatment using a HOBO© recorder.

At each experimental time point, samples were chosen using a random number table. When tanks reached experimental temperatures five fragments were removed for analysis from the ambient treatment (T0). Further fragments were removed at 48 hours after experimental temperatures were reached (48 h; n=5 for each treatment) and after 7 days (7 d; n=5 for each treatment). Five further fragments were collected from the coral nursery (where experimental

fragments were collected) on the last day of sampling to compare the experimental analysis to wild coral fragments.
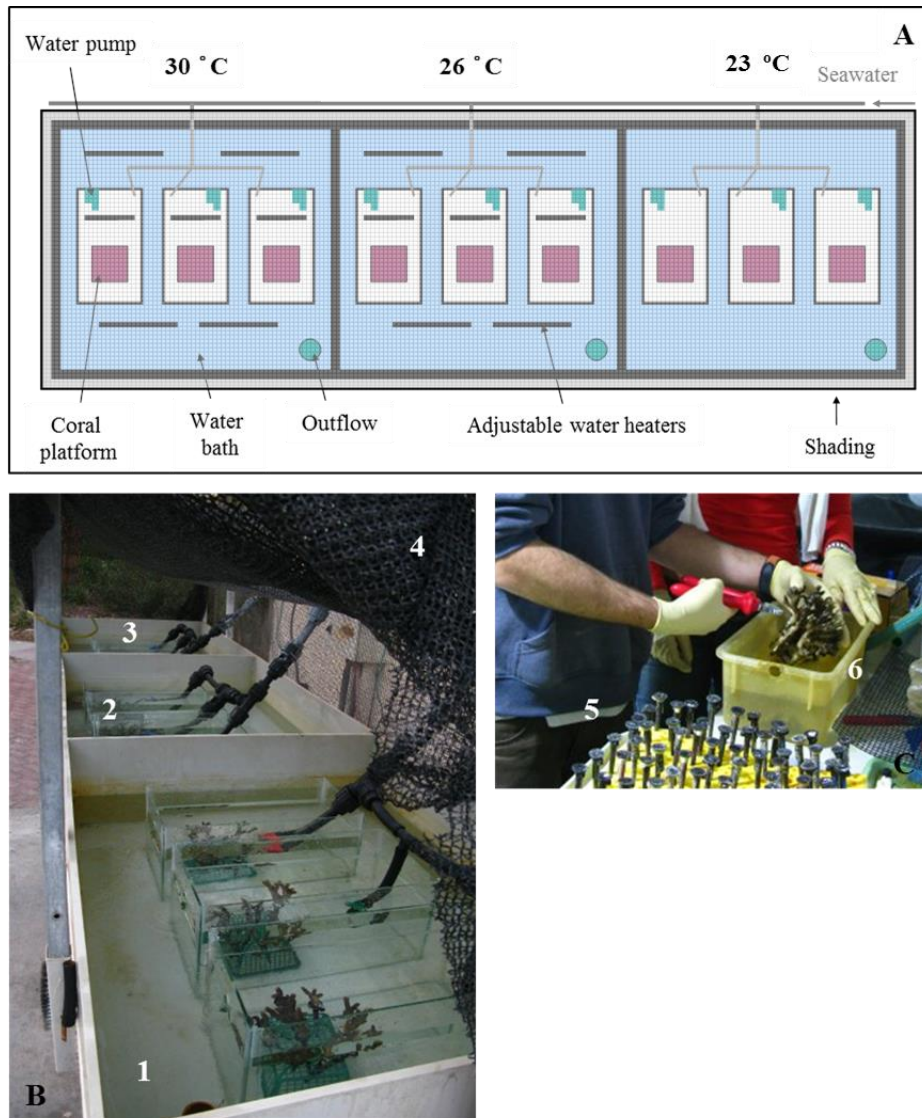
Figure 4.1 Diagram of experimental setup (A), facility at Interuniversity Institute of Eilat, Israel (B) and fragmentation of *S. pistillata* (C). Image B shows water baths at 23°C (1), 26°C (2) and 30°C (3) with three experimental tanks within each water bath containing *S. pistillata* fragments. Shading is provided by a heavy net mesh (4). Image C shows plastic mounts (5) and one of four parent colonies of *S. pistillata* (6).

4.2.2 Coral tissue removal and processing

Fragments were removed from experimental tanks and maintained at the same temperature until analysis (<30 min). Fragments were washed three times in sterile phosphate buffered saline (PBS; pH 6.5) and the tip of a branch of each fragment (~1.0 cm^3) was removed aseptically for analysis of bacterial DNA. DNA was extracted and diluted as in 2.2.2 and stored at –20°C. The remainder of the tissue was then removed from the coral skeleton using an airbrush, mounted with sterile PBS. Total volume of tissue sample and PBS was recorded and kept on ice at all times while the skeleton was air dried for surface area analysis. Coral tissue and PBS were homogenised until no tissue was visible. Homogenate (1 ml) was removed and placed in 3% filter sterilised formaldehyde for DAPI measurements. The remaining sample was then centrifuged at 2,000 xg, for 5 min at 4°C and the supernatant was removed to a clean 50 ml falcon tube; 100 µl was removed for host protein measurements. All sub-samples were stored in the dark at 4°C until analysis. The remaining pellet was re-suspended in 2 ml sterile PBS, homogenised and centrifuged at 2,000 xg for 5 min at 4°C. The supernatant was removed and pooled with the previous supernatant. The remaining pellet was then re-suspended in 1 ml sterile PBS, homogenised and centrifuged again, then re-suspended in 1 ml PBS; 100 µl was removed for zooxanthellae counts. The remaining sample was centrifuged as above and the supernatant was removed fully. Acetone (90%, 1 ml, 4°C) was added to the sample, vortexed and incubated at 4°C for 24 h for chlorophyll analysis.

4.2.3 Dichloromethane extraction of acyl-homoserine lactones (AHLs)

Dichloromethane (DCM; 10 ml) extraction was carried out as in 3.2.6 and stored at

-20°C.

4.2.4 Normalisation of fragments for quantification of other parameters: coral surface area and host protein content

After air-drying, surface area of coral fragments was measured using a standard paraffin wax technique to standardise zooxanthellae data. Coral fragments were weighed while a vat of wax was heated in a water bath to 60°C. Once at temperature, each fragment was dipped in the liquid wax (using tweezers on the base) for 4 s and quickly removed. Care was taken to remove drips and not cover the base of the fragments in wax. Once dry, fragments were weighed again and the difference was calculated. A linear equation used previously to determine surface area of corals (Relative Surface Area = 27.579 * weight difference (g) + 0.858) and determined by objects of known surface area and weight (Fine, *pers. comm.*), was used to calculate surface area of the coral fragments.

To standardise all other measurements host protein was measured using Bradford quick start dye reagent as in 3.2.5. Bovine serum albumin (BSA) was used to create a standard curve, attaining an R^2 of 0.97 before using to determine protein concentrations of coral fragments.

4.2.5 Visualisation of AHLs from coral tissue

Dried extracts were re-suspended in 50 µl acetonitrile, vortexed and applied to RP18 F_{254} thin-layer chromatography (TLC) plates (20x20 cm; VWR International). The amount of sample applied was normalised to 5.0 mg host protein and the assay was carried out and analysed as described in 3.2.7.

4.2.6 DAPI staining and image analysis

Tissue extracts (1 ml diluted to 1/100 with PBS) were mixed with 30 µl 4',6-diamidino-2-phenylindole stain (DAPI, in ultrapure water) and 10 µl Triton-X100 (0.1%) to break up

aggregates. Samples were then vortexed for 30 s and incubated in the dark with agitation for 10 min. Samples were vortexed again and 40 μl of sample was pipetted onto a clean microscope slide and covered with a cover slip (11 cm^2).

Stained samples were visualised on a fluorescence microscope (excitation 340–380 nm; emission 435–480 nm; x1000 magnification). Thirty images were taken per sample and analysed using ImagePro Plus imaging software (this analysis was carried out for T0 and 7d samples only, due to time constraints).

4.2.7 Zooxanthellae counts and chlorophyll quantification

Samples for zooxanthellae counts were vortexed and diluted 1:10 with sterile PBS. Zooxanthellae were counted in four corner squares of a Neubauer haemocytometer for each sample. This process was repeated twice for each sample, giving 8 counts (this analysis was carried out for T0 and 7d samples only due to time constraints).

For chlorophyll analysis, samples were removed the fridge, kept in the dark, vortexed for 30 s and diluted 1:10 with acetone (90%). Optical density of samples were recorded in a glass cuvette and spectrophotometer at wavelengths 630 nm, 664 nm and 750 nm, using acetone (90%) as a blank. Chlorophyll (μg) was calculated using the following formula: Chlorophyll (μg) = 11.43 * (OD(664) – OD(750)) – 0.4 * (OD(630) – OD(750)) (Fine, *pers. comm*).

4.2.8 PCR amplification and denaturing gradient gel electrophoresis (DGGE) analysis

A nested PCR approach was used to amplify 16S rRNA genes for DGGE using the methodology described in 2.2.3. PCR products were all checked for similar intensity of bands

on agarose gels and DGGE was performed using the Ingeny[TM] System (BioRad) and carried out as described in 3.2.4.

4.2.9 Amplification and 454-pyrosequencing of 16S rDNA V4/V5 region

PCR amplification of the 16S rDNA hypervariable region V4/V5 was performed with primer pools containing two forward and three reverse primers as described by Huse et al. (2010), giving a 408bp amplicon. Each sample was amplified using one of 12 primer pools, each pool being identifiable by a unique decamer multiplex identifier (MID) tag sequence to enable sample multiplexing. MID sequences were taken from the Roche Technical Bulletin (TCB No. 005-2009) *"Using Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry – Extended MID Set"* (April 2009). See appendix 2 for primer sequences, MID tags and pool/sample identification. Amplicon libraries were prepared using 20 ng μl^{-1} DNA stored at – 20°C. PCR was carried out using 0.4 μM of each primer, 1x PCR buffer (Promega, Southampton, UK), 0.15 mM of each dNTP, 3.0 mM $MgCl_2$ and 0.6 U of Go *Taq* Flexi (Promega) in a total volume of 60 µl using the following conditions: 1 cycle at 94°C for 3 min; 35 cycles at 94°C for 30 s, 57°C for 45 s and 69°C for 1 min and one final extension cycle at 69°C for 5 min. Each PCR reaction was conducted in triplicate. PCR products were run on 2% agarose gels and the correct band extracted using aseptic technique (due to the presence of multiple bands). Amplicon preparation was carried out in triplicate, and purified first by gel extraction using the Wizard® SV Gel and PCR Clean-Up System (Promega) and then using the Agencourt AMPure XP Purification System (Beckman Coulter, Bromley, UK) according to section 3.2.2 of the Roche Amplicon Library Preparation Method Manual for the GS FLX Titanium Series (October 2009). Following elution in 10µl of molecular biology grade water, the DNA concentration for each product was confirmed using the PicoGreen assay against standard DNA curves with $R^2 \geq 0.99$ (Invitrogen) in a Tecan infinite M200 microplate reader with i-Control 1.5 software (Tecan Group Ltd, Männedorf, Switzerland) set to an excitation

wavelength of 480 nm and emission wavelength of 520 nm. Samples were then stored at -20°C, prior to sequencing.

DNA libraries were prepared for sequencing using the Roche emPCR Method Manual – Lib-L MV for the GS FLX Titanium Series (October 2009; rev. Jan. 2010). This medium-volume protocol allows for 8 separate sequencing regions per PicoTitre Plate (PTP). Briefly, each tube contained a mix of equal concentrations of 12 DNA samples, each amplified using a different primer pool (1 to 12) (appendix 2). Each primer pool contained an equimolar amount of each forward and reverse primer, each labelled with the same tag sequence; thereby, using 12 differently-labelled primer pools, each sequencing region on the PTP can accommodate a multiplex of 12 pooled DNA samples. To prepare DNA beads for sequencing, the Roche Sequencing Method Manual for the GS FLX Titanium Series (October 2009) was used. The GS FLX Titanium sequencer was then set to run for 200 cycles (approximately 350-450bp read lengths), using the setting for amplicon image processing only.

Data were processed by Karen Tait at Plymouth Marine Laboratory using the in-built Roche image processing software, which removes background noise from the image and performs signal processing. Sequence data were de-multiplexed and denoised using AmpliconNoise (Quince et al., 2011), and OTUs and taxonomy assigned using the Quantitative Insights into Molecular Ecology (QIIME) pipeline (http://qiime.org). OTUs were then picked and compared at 95, 97 and 99% similarity and taxonomy assigned by BLAST analysis against a Silva database (www.arb-silva.de).

4.2.10 Statistical analysis

Minitab 6.0 was used to perform normality tests; Levene's test for equal variances, Kruskal-Wallis test for non-parametric light data and ANOVAs and Tukey tests to investigate differences in bacterial and zooxanthellae numbers, AHL signals and chlorophyll content

between treatments. GelCompar (Applied Maths) was used to identify DGGE bands within the bacterial profiles and construct a binary matrix based on presence and absence of aligned bands. Using Primer-E6 v6 (Clarke & Warwick, 2001) a Bray-Curtis similarity matrix of DGGE bands (presence/ absence data) and of 454-data (standardised by total, square root transformed) were constructed. Hierarchical clustering (group average) with SIMPROF tests tested for structure in the subset of DGGE data corresponding to each branch of a dendogram (1000 permutations, significance level 5%) and Nonmetric multidimensional scaling (MDS) enabled visualisation of similarities between treatments in 454-data (with hierarchical clustering overlaid). The Shannon-Weaver (H') test investigated diversity and Pielou's (J') test investigated evenness for 454- data.

4.3 Results

4.3.1 Experimental conditions

Over the experimental period the average temperatures for the three treatments were 22.75°C (23°C), 25.50°C (26°C) and 29.53°C (30°C). The water baths kept temperatures relatively stable over the 7 day experiment, however fluctuations over the day and night cycle can be seen in Figure 4.2. Fluctuations over 24 h cycles average around 2.7°C for the 23°C treatment and 2.0°C for both 26°C and 30°C treatments. While the average temperature in 23°C and 26°C was relatively stable with a fluctuation of 0.34°C and 0.56°C respectively over the experiment, the average temperature changed by 1.51°C in the 30°C treatment over the experimental time period, increasing from a daily average of 28.78 °C to 30.22 °C on day 1 and 7 respectively. Light data recorded by HOBO© instruments were unreliable due to the angle at which they were situated in the tanks. However, light was recorded prior to the experimental periods over all tanks to assess the similarity between them (data not shown), showing light peaking at 597 μmol m^{-2}s^{-1} on average with no significant difference between tanks (Kruskal-Wallis non-parametric test) and at similar levels to those experienced in the coral nursery (M. Fine pers. comm.)

B		23 °C	26 °C	30 °C
Average (whole experiment)		22.75	25.50	29.48
Day				
1	Average	22.68	25.49	28.78
	Max	24.64	26.39	29.95
	Min	22.05	24.85	28.26
2	Average	22.62	25.29	29.12
	Max	24.64	27.37	30.76
	Min	21.95	24.74	28.56
3	Average	22.72	25.75	29.32
	Max	24.45	27.47	30.56
	Min	22.05	24.64	28.85
4	Average	22.54	25.19	29.17
	Max	24.45	26.68	30.46
	Min	21.86	24.64	28.66
5	Average	22.65	25.43	29.68
	Max	24.64	26.78	31.47
	Min	21.66	24.64	28.75
6	Average	22.88	25.58	30.29
	Max	24.64	26.59	31.27
	Min	22.05	25.03	29.45
7	Average	22.72	25.45	29.92
	Max	25.32	26.59	31.27
	Min	22.53	25.32	29.35

Figure 4.2 Temperature data for experimental period as recorded by HOBO© situated in the middle tank for each temperature. Profiles show fluctuations in temperature, taken every 30 min over experimental period, with sampling points highlighted (A). * denotes where HOBO © lost power. Table shows average temperatures (over experiment and by day) as well as maximum and minimum values recorded in 24 h (B).

4.3.2 Detection of putative AHLs in *Stylophora pistillata*

Thin-layer chromatography (TLC) of *S. pistillata* DCM extracts detected a number of putative AHLs with varying chain-length (Figure 4.3). Three spots were detected within the samples tested, two of which had similar migration rates to the longer-chain synthetic AHL standards (*N*-oxohexanoyl-L-homoserine lactone (OC8) and *N*-oxodecanoyl-L-homoserine lactone (OC10) and one that migrated between OC6 and OC8. This spot, spot 1, was only present in nine of the 40 samples (Figure 4.3). In contrast, spots 2 and 3 (Figure 4.3) were detected in 40 and 37 of the samples, respectively, showing relatively stable production of AHLs by bacterial communities associated with *S. pistillata* and suggesting a potentially important role for them in the coral holobiont.

4.3.3 Temperature effects on putative AHLs in *Stylophora pistillata*

Although putative AHLs were detected in the majority of samples from all temperatures, the quantity of AHL detected was quite variable between individual fragments in the same treatment, as can be seen by the error bars in Figure 4.4. Quantities of AHLs detected also differed markedly in samples from the 30°C temperature treatment after 7 d, when compared with other samples (Figure 4.4). Spot 1 was only detected in 23°C treatments (T0, four fragments; 48 h, one fragment; 7 d, one fragment and two wild fragments) and in one fragment from the 26°C treatment after 7 d exposure.
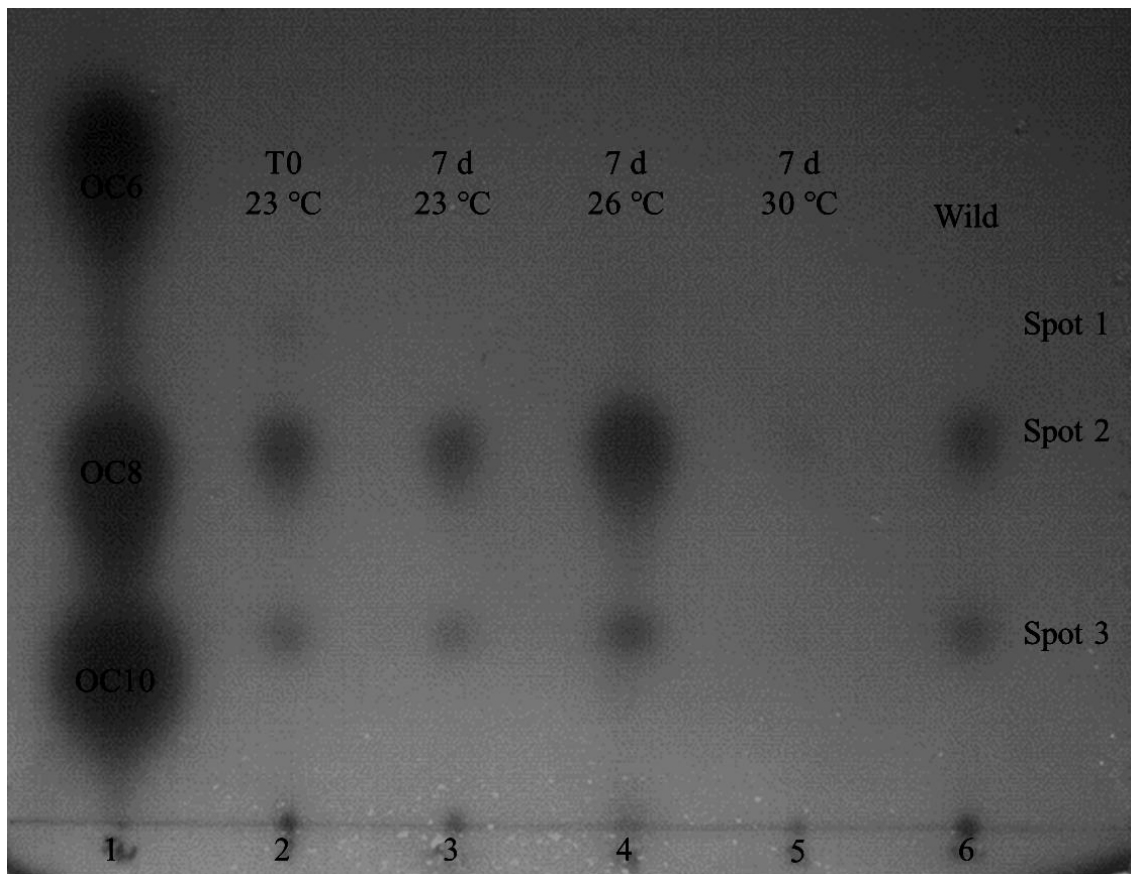
Figure 4.3 Reverse thin-layer chromatography profiling of AHLs extracted from *Stylophora pistillata*. From left to right columns show 5 µl of AHL synthetic standard made up of 5 µM *N*-oxohexanoyl-L-homoserine lactone (OC6), 0.2 µM *N*-oxohexanoyl-L-homoserine lactone (OC8) and 0.5 mM of *N*-oxodecanoyl-L-homoserine lactone (OC10) (1); and dichloromethane extract corresponding to 5.0 mg host protein from *S. pistillata* at the beginning of the experiment (2), after 7 days at 23°C (3), 26°C (4), 30°C (5) and in a wild sample (6). Spots are labelled for further analysis.

Spot 2 was found consistently in all samples but was most abundant in wild samples with an average of 0.214ng AHL/mg host protein being produced. In experimental samples kept at ambient temperature (23°C) this dropped to an average of 0.146 ng AHL/mg host protein. At 26°C, AHL production recovered to values more consistent with wild samples (0.195 ng AHL/mg host protein). However, all these values were relatively stable when compared to average values of AHL detected in fragments kept at 30°C. After 7 d, the amounts detected were severely reduced to values of 0.020 ng AHL/mg host protein; however after 48 h at 30°C spot 2 was detected in similar quantities to ambient temperature treatments. An ANOVA

confirmed differences seen (F=2.34 P<0.05); however, due to variability in production of AHL signals, Tukey tests only found significant differences between the samples at 30°C after 7 d when compared to samples taken from 26°C at all time-points and wild samples. This analysis however, is based on the area of blue colouration of the *A. tumefacians* biosensor induced by *S. pistillata* extracts on TLC plates, using area analysis in Image Pro Plus. Values given (Figure 4.4) correspond to AHL detected from 5.0 mg *S. pistillata* host protein compared to area of blue colouration induced by OC8 synthetic standard at concentration of 2.5 ng. This method is not considered to be precise enough to allow accurate quantification of AHLs and therefore this analysis can only estimate relative abundance and approximate quantities.

Spot 3 shows a similar pattern to spot 2. Average signal is highest in wild samples with an average area of 0.10 units. This dropped to an average of 0.054 units at ambient temperatures in experimental conditions, and further to 0.045 units at 26°C and 0.029 units at 30°C. Samples taken after 7 d again dramatically reduced, to an average of 0.012 units. While quantities of spot 2 can be estimated as it runs alongside the OC8 standard, spot 3 may represent very different quantities, hence using relative area instead of estimating quantities.
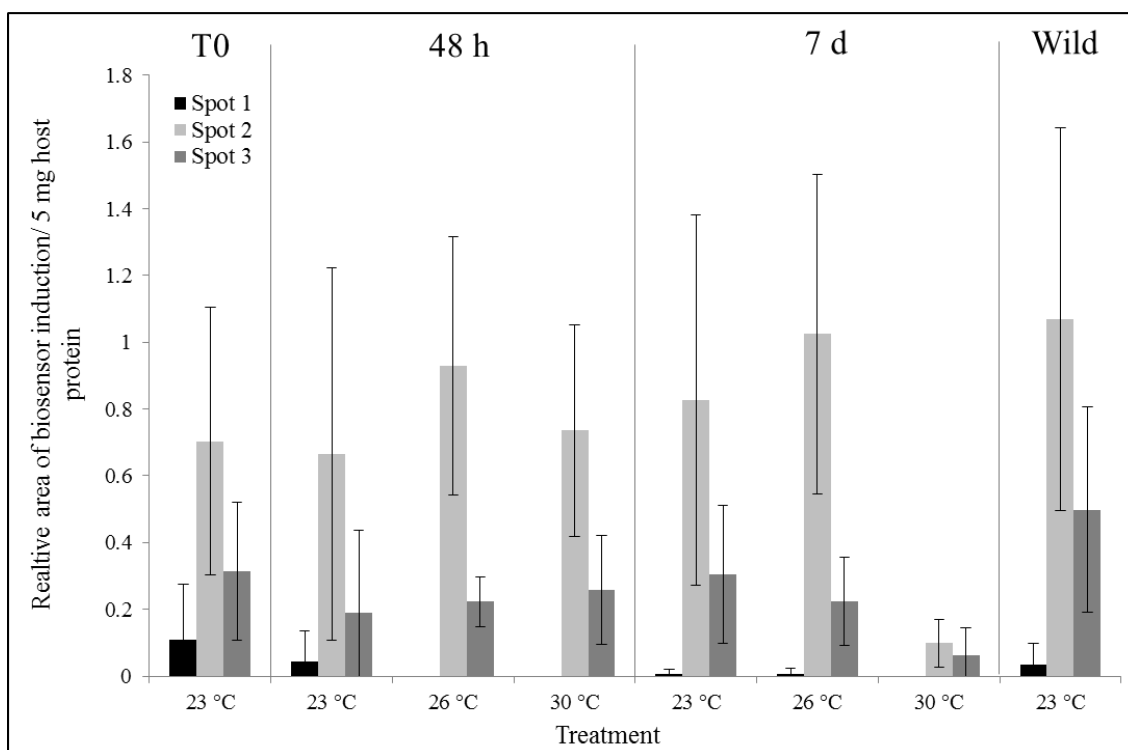
Figure 4.4 Analysis of reverse thin-layer chromatography using area analysis of spots from *S. pistillata* extracts (Image Pro Plus). Values correspond to relative area of biosensor *A. tumefaciens* NTL4 induced by 5.0mg *S. pistillata* host protein on TLC plates. Error bars represent standard deviation.

4.3.4 Bacterial numbers as assessed by DAPI staining

To establish a comparison between signalling profiles and bacterial community dynamics, a number of analyses were carried out, including DAPI staining of bacterial cells to establish the numbers of bacteria present in samples. DAPI staining of sub-samples from *S. pistillata* fragments provided relative numbers of bacteria found in each fragment (Figure 4.5). However, due to time constraints and equipment failure, only analysis of T0, 7 d and wild samples were achieved.

Bacterial counts were reduced in samples from 26°C and 30°C treatments after 7 d (Figure 4.5). ANOVA confirms a difference between samples ($\sqrt{\ }$ -transformed data; F=3.24; p<0.05); however Tukey tests do not show significant differences between individual treatments.

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Number of bacteria associated with *S. pistillata* tissue range from 8.5×10^9 to 5.1×10^{10} total numbers in fragments, corresponding to 2.0×10^6 to 1.0×10^7 in 5.0 μg host protein.
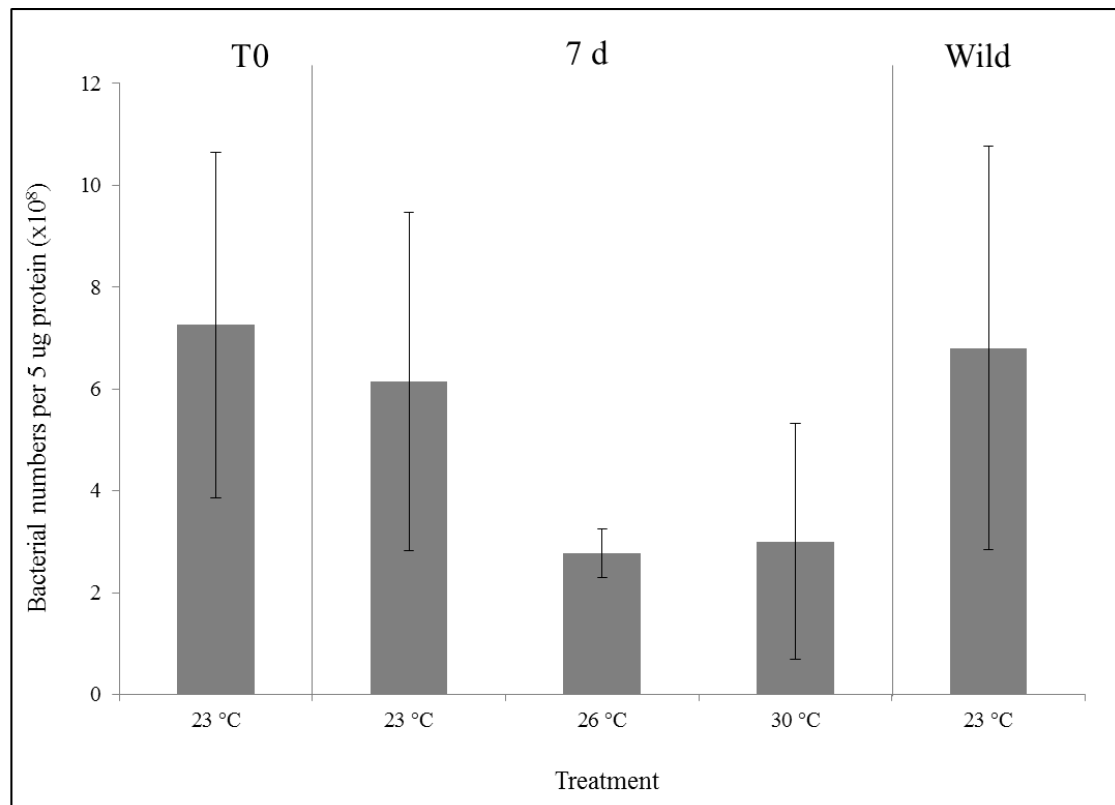
Figure 4.5 Average DAPI counts (n=5) from *S. pistillata* tissue corresponding to 5μg of host protein for T0, 7 days and wild samples. Error bars represent standard deviation.

4.3.5 Bacterial community profiles using PCR of bacterial 16S rRNA genes and Denaturing gradient gel electrophoresis (DGGE)

Hierarchical clustering of bacterial community structure based on DGGE banding patterns (Figure 4.6) shows no obvious grouping/ change in community structure between treatments (Figure 4.7). Although not found as having a distinct community structure in a SIMPROF analysis, T0 samples cluster together (Figure 4.7), not showing similarity between them – but showing dissimilarity to all other samples.
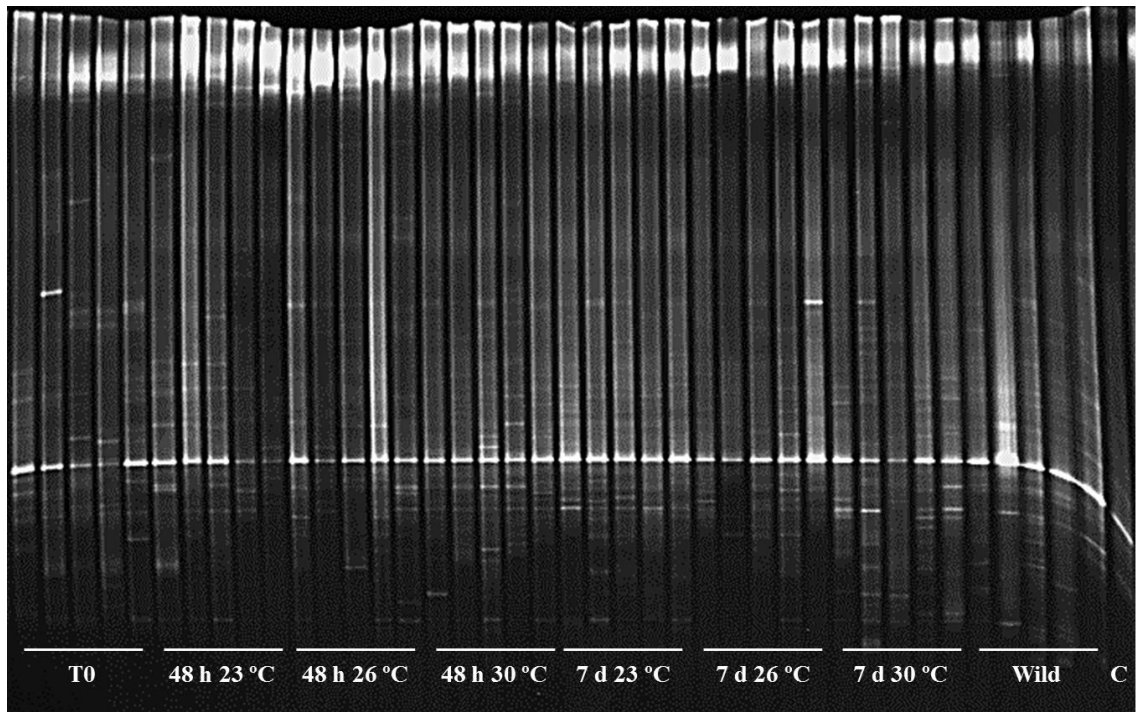
Figure 4.6 Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA gene fragments extracted from *S. pistillata* samples and control (C).
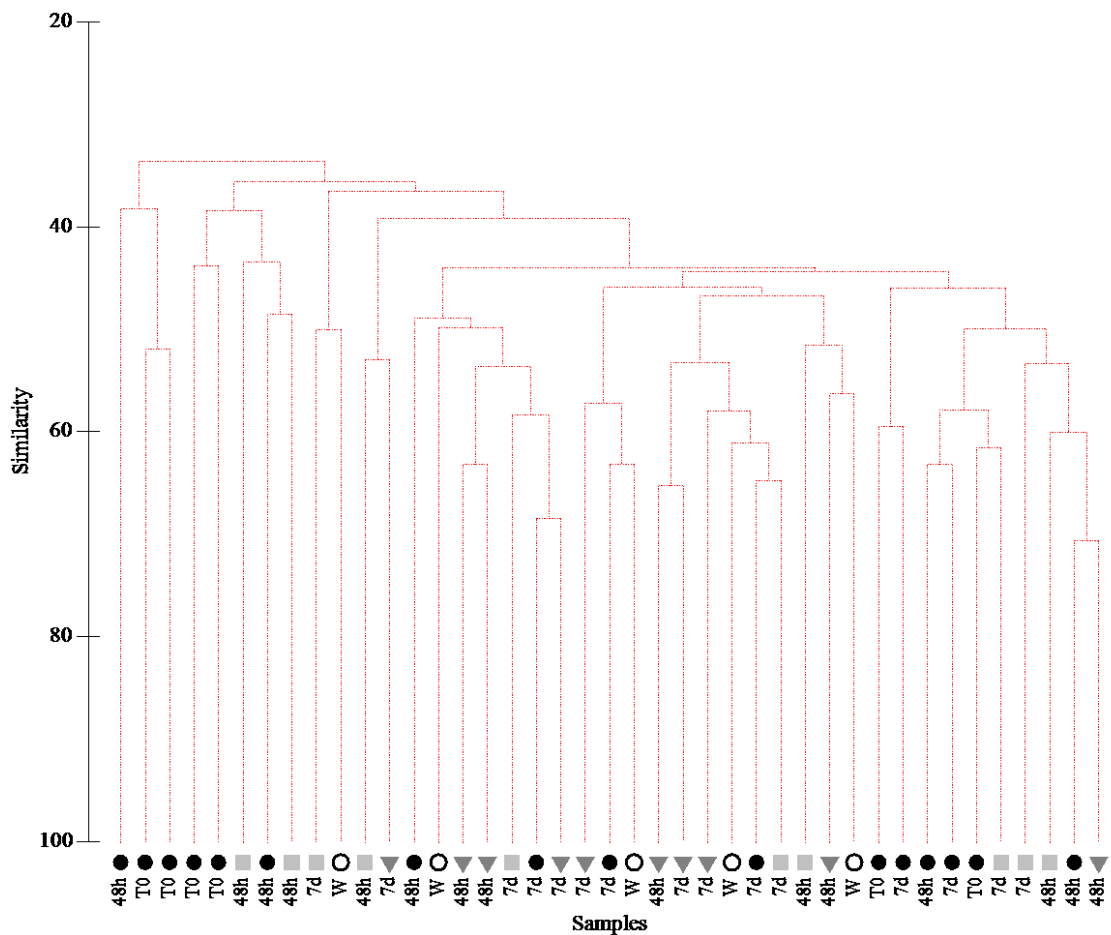
Figure 4.7 Cluster analysis of DGGE data based on a Bray-Curtis resemblance matrix using presence and absence data. Black circles represent samples at 23°C, squares represent 26°C, triangles represent 30°C and clear circles represent wild samples. Red lines represent SIMPROF test results, showing no difference in the bacterial community between samples.

4.3.6 Bacterial community analysis using 454-pyrosequencing of bacterial 16S rRNA genes

Detailed analysis of bacterial communities associating with *S. pistillata* using 454-pyrosequencing revealed greater differences than DGGE data. Picking operational taxonomic units (OTUs) was carried out at 95, 97 and 99% similarity to investigate the best cut off for assessing coral bacterial communities. However, Kruskal-Wallis tests show no significant differences between the number of OTUs or the percentage of singleton OTUs (OTUs with only one sequence associated with it per sample) in these data sets. As result the 99% cut off data was used in further analyses. Details of OTU data can be seen in Table 4.1.

Multidimensional scaling based on a Bray-Curtis similarity matrix of standardised (by total) and √-transformed data, shows the distribution of samples in multivariate space based on these 97 sequences per sample with Family level phylogeny (Figure 4.8). This plot shows Wild and T0 samples to the bottom right of the plot (except one), 30°C samples to the bottom left (except one) and most other samples clustering in the middle with groups clustering at 40% similarity (Figure 4.8). This suggests that the bacterial community changes in 30°C samples but that there may also be an experimental effect on bacterial community composition, with experimental ambient temperatures clustering away from T0 and Wild samples.

Table 4.1 Details of 454-pyrosequencing operational taxonomic units picked at 99% similarity and resampled for 97 sequences per sample.

Timepoint	T0					48 hours									7 days									Wild				
Temperature	23 °C					23 °C		26 °C				30 °C			23 °C			26 °C			30 °C			23 °C				
Sample	1	1B	2	3	4	25	28	16	17	18	19	20	23	29	39	40	41	45	46	48	50	51	53	1	2	3	4	5
Original Sequences	4472	6559	6680	7522	6259	5277	3291	3460	944	4663	6131	4338	11303	326	3992	97	6680	6789	15390	948	2993	1549	297	3634	750	4668	2048	6829
no. OTU's (99 %)	1482	1687	2468	1837	1068	1189	347	626	130	1074	657	737	371	77	649	56	727	1143	1024	259	629	403	181	1238	86	1496	765	1852
no. singletons	1044	1067	1515	997	805	712	220	381	81	629	362	397	201	44	321	37	454	577	446	181	415	266	140	769	65	1000	552	1050
no. singletons (%)	70.4	63.2	61.4	54.3	75.4	59.9	63.4	60.9	62.3	58.6	55.1	53.9	54.2	57.1	49.5	66.1	62.4	50.5	43.6	69.9	66.0	66.0	77.3	62.1	75.6	66.8	72.2	56.7
Resampled - data refined to 97 sequences per sample																												
no OTU's (99 %)	59	54	86	64	44	55	26	48	23	59	32	59	28	33	55	56	40	63	39	50	56	59	69	80	22	67	59	79
no. singletons	52	48	79	54	36	50	19	41	15	52	26	47	16	20	41	37	29	51	31	39	41	45	59	73	13	59	49	70
no. singletons (%)	88.1	88.9	91.9	84.4	81.8	90.9	73.1	85.4	65.2	88.1	81.3	79.7	57.1	60.6	74.5	66.1	72.5	81.0	79.5	78.0	73.2	76.3	85.5	91.3	59.1	88.1	83.1	88.6
J'	0.97	0.97	0.91	0.97	0.94	0.97	0.98	0.97	0.92	0.96	0.91	0.93	0.98	0.97	0.95	0.98	0.95	0.98	0.94	0.97	0.97	0.98	0.97	0.98	0.87	0.98	0.96	0.99
H' loge	3.51	3.43	2.67	3.66	3.12	3.53	3.83	3.47	2.55	3.56	2.69	2.79	3.78	3.46	3.20	3.57	3.21	3.70	3.10	3.46	3.54	3.78	3.61	3.76	2.35	3.85	3.48	3.98
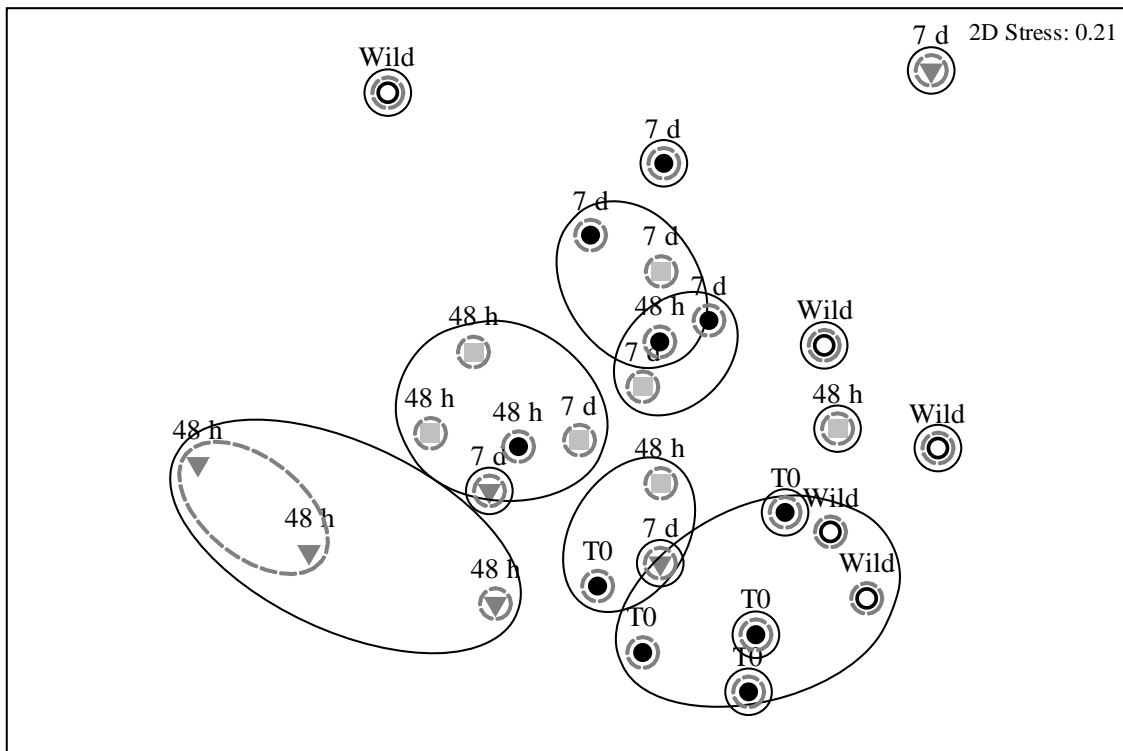
Figure 4.8 MDS of 454-pyrosequencing data. Based on a Bray-Curtis resemblance matrix using family level analysis for 97 sequences per sample with OTUs picked at 99% similarity. Data are standardised by total and square root transformed. Labels represent time points and symbols represent 23°C samples (black circles), 26°C samples (squares), 30°C samples (triangles) and wild samples (clear circles). Clustering is based on 40% (solid line) and 60% (dotted line) similarity.

ANOSIM tests at Family level phylogeny for all ambient temperature samples confirmed this, showing a significant difference between time-points (Global-R=0.417; p<0.005). Pairwise tests showed significant differences between samples taken at T0 and from the wild with those taken after 7 d (p<0.05). However, significant differences were also found between temperature treatments (Global-R=0.234; p<0.005), with pairwise tests showing significant differences between samples taken from the 30°C treatment and all other samples (p<0.05) and between the 26°C treatment and wild samples (p<0.005). ANOSIM analysis at Class and Phylum phylogenetic distinctions confirmed time-point differences (between T0 and 48 h/7 d at Class level and additional differences between 48 h and 7 d samples at Phylum level) but did not confirm temperature changes – with no significant difference between treatments found. This

suggests that temperature affected the presence of specific OTUs but that these changes remained within the same higher groupings of bacteria e.g the Proteobacteria.

At Family level, Multivariate Dispersion (MvDisp) analysis shows 30°C samples taken after 48h to be the least dispersed (0.133) and 30°C samples taken after 7 d to be the most dispersed (1.667). All other treatments lie within these two values with T0 23°C samples, wild samples and 48h 26°C samples (1.17, 1.075 and 1.108, respectively) more dispersed than 23°C samples collected after 48 h and 7 d as well as 26°C after 7 d (0.6, 0.55 and 0.75, respectively). This suggests the succession of a specific bacterial group in all fragments taken after 48 h from the 30°C treatment and potentially the breakdown of a specific community after 7 d, with different communities found in different samples. Also interesting is the effect of time on 23°C samples, with less dispersed bacterial communities existing after 48 h and 7 d compared to T0 and wild samples, which suggests an experimental effect, possibly signifying the loss of a number of natural OTUs. However, analysis of diversity showed no significant differences between evenness (J') or diversity (H') at Family or Phylum level between samples.

4.3.7 Changing bacterial communities: experimental and temperature effect

A more detailed analysis of bacterial groups in all of the treatments using 454-analysis shows Phylum level changes to bacterial community structure (Figure 4.8). The Proteobacteria were the largest group found in the 454-libraries, making up between 29.6 and 67.5% of sequences. Samples from T0 and 30°C were the only samples that had an average of less than 50% of their communities made up by Proteobacteria. Of the Proteobacteria, the most abundant class is the Gammaproteobacteria, making up between 23% (30°C) and 38% (26°C) of the total bacterial community, with an average of 37% of the total bacterial community in both 23°C and wild samples.

The Planctomycetes were also found in high numbers in Wild and T0 samples contributing an average of 16.3 and 15.5%, respectively; however in all other experimental treatments, these numbers were much lower (3.1 to 7.6%). Some groups were present in all the treatments, such as the Cyanobacteria, however this group increased in numbers as the experiment progresses from an average of 6.2% in wild and T0 samples, to 7.2% after 48 h and up to an average of 19.8% after 7 d, suggesting, like with the Planctomycetes, an experimental effect rather than temperature effect to bacterial communities.

In contrast, the Bacteroidetes are present in all samples with fairly consistent numbers throughout, from 2.1% in samples from 30°C treatment after 48 h to 9.7% in wild and T0 samples. Interestingly, while the Firmicutes represented an average of 4.0% in T0 and wild samples, 8.8% in other 23°C and 10.5% in 26°C samples, they represented an average of 30.4% of the bacterial community in 30°C samples. This abundance is concentrated in samples at 30°C after 48 h, in which Firmicutes represent 44.3% of the total bacterial community.
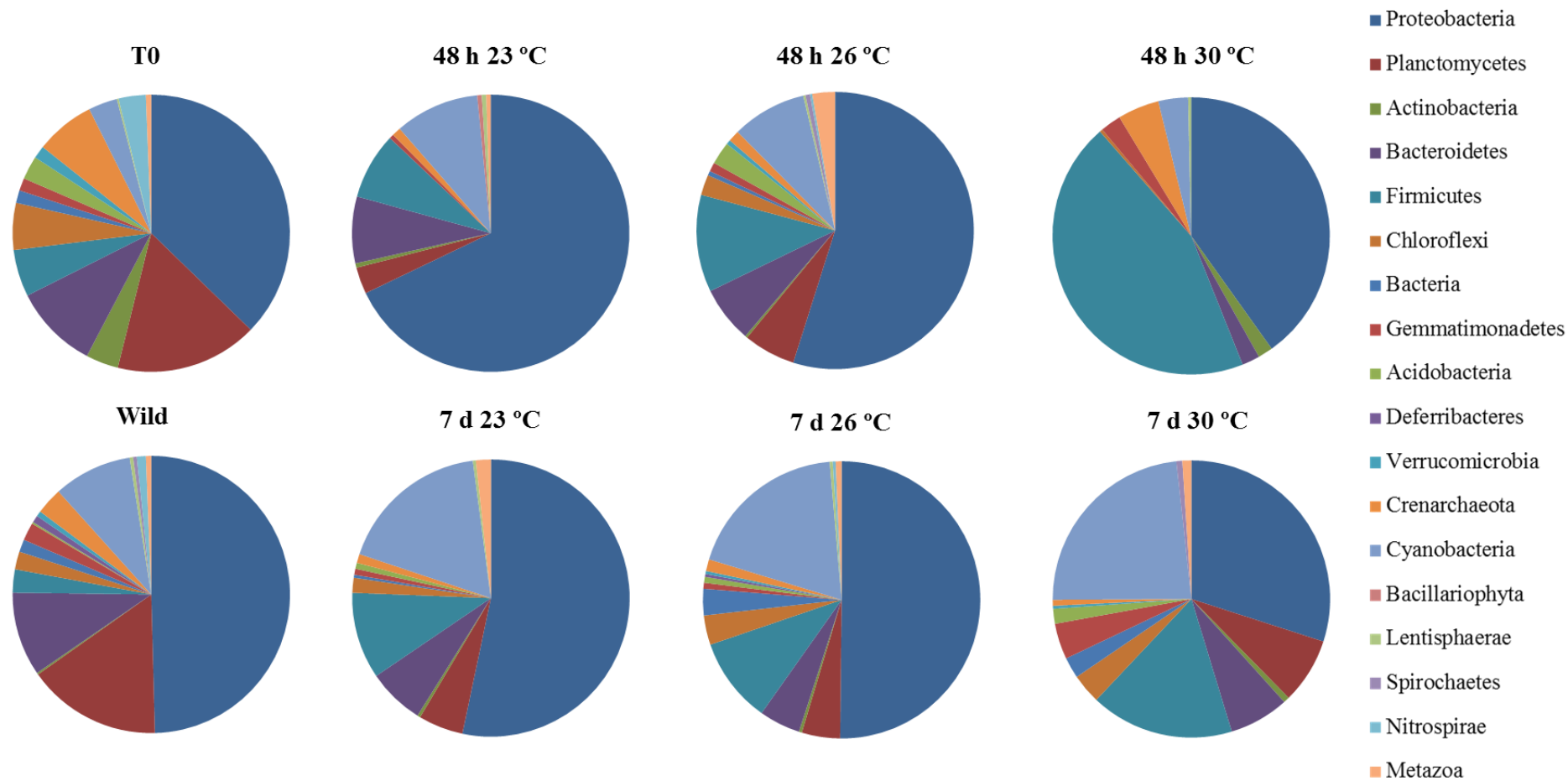
Figure 4.9 Phylum level analysis of resampled 454-pyrosequencing data for 97 sequences per sample. Bacteria group relate to sequences with most common affiliations with uncultured bacterium.

While for some differences seen at higher phylogenetic levels (Phylum) between samples there is no one OTU responsible, such as in the Planctomycetes, where a number of OTUs contribute towards a higher abundance in Wild and T0 samples, other differences in Phyla abundance in temperature and time treatments are due to one OTU. OTU 7748 was found to be most closely related to an *Endozoicomonas sp.* (FJ357696) in a NCBI BLAST search, a bacterium isolated from the sea cucumber *Apostichopus japonicus*. In *S. pistillata* 454-libraries, this OTU makes up between 2.4 and 51% of sequences per sample. Hits to *Endozoicomans sp.* (FJ357696; 97%) make up an average of 7 and 11% of hits in T0 and Wild samples, 51 and 26% in 23°C (48 h, 7 d), 34 and 25% in 26°C (48 h, 7 d) and 2 and 6% in 30°C (48 h, 7 d). This suggests that this group is present in natural bacterial communities associated with *S. pistillata* and increase in experimental conditions, except for at 30°C.

The OTU responsible for the main change seen in the abundance of Cyanobacteria in *S. pistillata* (OTU 5887) was found to be most similar to an uncultured Cyanobacteria (GQ348629; 99%) in the NCBI database. Sequences most closely relating to this OTU made up an average of 2 and 6% of sequences in T0 and Wild samples, 5 and 8% in 23°C (48 h, 7 d), 5 and 13% in 26°C (48 h, 7 d) and 2 and 14% in 30°C (48 h, 7 d), suggesting that this group become more prevalent over the experimental time period, in all temperature treatments. Another OTU (5126) found in abundance in all samples was responsible for differences in the abundance of Firmicutes found in *S. pistillata*. This OTU most closely related to a *Geobacillus* sp. sequence (JN867331, 99%) in the NCBI database. These sequences made up an average of 5 and 2% of hits in T0 and Wild samples, 5 and 6% in 23°C (48 h, 7 d), 7 and 7% in 26°C (48 h, 7 d) and 31 and 12% in 30°C (48 h, 7 d), suggesting that both the 30°C treatment has a marked effect on this OTU and that otherwise, this group is relatively stable throughout.

4.3.8 Coral stress as determined by zooxanthellae counts and chlorophyll production

Assessment of zooxanthellae numbers and chlorophyll per zooxanthellae were carried out as a measure of temperature stress to the coral holobiont. Due to time restrictions this investigation was only carried out for T0 samples and samples taken after 7 d exposure to temperature treatments. Figure 4.9 shows the results of this analysis with no significant differences between chlorophyll (μg) per zooxanthellae in any of the treatments (ANOVA). The same is true of zooxanthellae numbers, standardised to surface area of coral fragments. Although a drop in zooxanthellae numbers is apparent in coral fragments from 26°C and 30°C treatments, the results are not significant (ANOVA). The wide variation seen in the results suggests that the methodology used to assess surface area may not be accurate enough to use to standardise these variables.
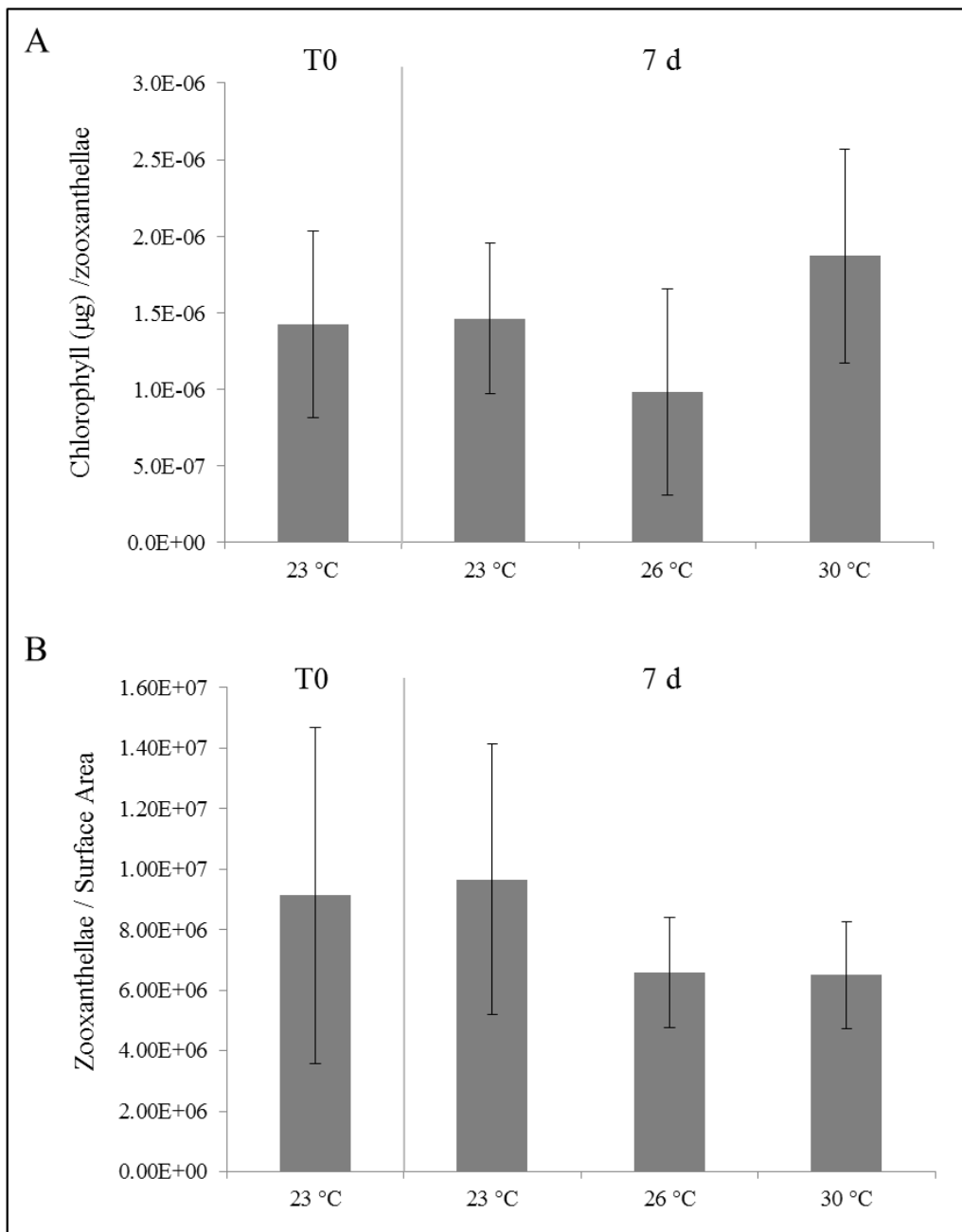
Figure 4.10 Average chlorophyll (µg) per zooxanthellae in *S. pistillata* fragments analysed at T0 and after 7 days incubation at different temperature treatments (A) and average zooxanthellae counts standardised to surface area of *S. pistillata* fragments at T0 and after 7 days at different temperature treatments (B). Replicates vary per treatment (T0: n = 5; 7 d, 23 °C: n = 2; 7 d, 26 °C: n = 4; 7 d, 30 °C: n = 3) Error bars represent standard deviation.

4.4 Discussion

The production of AHLs in coral-associated bacteria (Golberg et al., 2011), free-living marine bacteria (Romero et al., 2011) and bacteria from a range of other marine sources (Gram et al., 2002; Taylor et al., 2004; Mohammed et al., 2008; Huang et al., 2007) as well as evidence that AHLs can be detected in a range of cnidarians (Chapter 3) suggest that quorum sensing may play an important role in marine bacterial communities. This study provides the first evidence that the ability of marine bacteria to communicate by employing quorum sensing in their natural environment may be affected by increasing sea surface temperatures.

4.4.1 Presence and potential role of AHLs in *S. pistillata*

The presence and stability of a number of putative AHLs in *S. pistillata* tissue in the range of 10 μM AHL and with bacterial numbers in the range of 6×10^8 (corresponding to 5 mg of host protein) suggests that both bacteria and signal are present at high enough concentrations to assume that AHL signalling is being employed to allow phenotypic change and the regulation of behaviours in *S. pistillata* bacteria, as discussed in 3.4.1 (Kaplan & Greenberg, 1985; Wang & Leadbetter, 2005). As a result these signals may play an important role in the maintenance of these communities and the health of the coral holobiont.

The interaction between a signal and its receptor is very specific, which allows intraspecies communication in mixed communities (Rice et al., 1999). However, recognition of universal communication signals synthesised by different bacterial species (interspecies communication) have also been found to play a role in mixed species communities (Riedel et al., 2001; Steidle et al., 2001; Valle et al., 2004). Although AHLs may be primarily intended and used by bacteria for communication with their own species, and not between two different species (Jayaraman & Wood, 2008), there are many examples of transcriptional activator proteins that accept AHLs of different chain lengths to their cognate molecule. There are also examples of bacteria that can

detect AHL signals without themselves being producers, which is why they are useful as bioreporters (Brelles-Mariño & Bedmar, 2001). Two core proteins, the LuxI-type protein (the AHL synthase) and the LuxR-type protein (the response regulator) are at the heart of AHL-QS systems (except in *Vibrio harveyi* (LuxM AHL synthase) and in *Pseudomonas fluorescens* (putative HdtS AHL synthase)) but in 45 out of 68 genomes investigated by Case et al. (2008), more LuxR than LuxI homologs were found. This suggests that many bacteria may have evolved to detect the signalling molecules derived from other bacteria within the same community. This may also be the case in *S. pistillata*, with a stable signal consistently produced, possibly from a core group of bacteria, which may select for behaviours that help maintain the stable bacterial communities within *S. pistillata*.

4.4.2 Decrease is putative AHLs with temperature stress

Although this study showed a conserved AHL profile in all fragments, the marked reduction in signals at 30°C after 7d exposure suggests that temperature stress had a severe impact on the presence of AHL molecules from *in situ S. pistillata* associated bacteria. Yates et al. (2002) has provided observations of increased pH-dependent hydrolysis of AHL signals when temperatures are increased from 22 to 37°C in cell cultures of *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis*. pH-dependent hydrolysis, causing the lactone ring to open and the AHL to be inactivated would decrease the detection of these molecules on TLC plates. This suggests that increased temperatures may be directly affecting the stability of signalling molecules produced by *S. pistillata* associated bacteria. This is supported by the continued, although reduced, detection of conserved signalling profiles in coral fragments kept at 30°C, suggesting that bacteria are still producing signals but that their stability in the extracellular environment has been reduced.

High temperatures however, may also work directly to inhibit AHL signals from being produced by certain bacteria. AHL production has been shown to vary with temperature in

bacteria such as *Erwinia carotovora* (Hasegawa et al., 2005) and *Y. pseudotuberculosis* (Atkinson et al., 2006). In *S. pistillata* fragments, as well as a decrease in the two main putative AHLs, a third (spot 1), was only found in 23°C treatments (and one 26°C fragment) suggesting that this signal is either more stable at lower temperatures or that it is not produced at higher temperatures. A similar effect was seen in *Vibrio* strain R-21415 which produces two additional small chain AHLs at 18°C and not at 30°C (Tait et al., 2010). The study by Tait et al. (2010) also found the detection of AHLs from a number of marine *Vibrio* spp. known to associate and cause disease in a number of corals to be temperature dependent. It established that while some *Vibrio* spp. produce less AHLs in high temperatures (30°C) e.g. *V. harveyi* (R-21446) and *V. shiloi* (LMG 19703), AHL production remains constant with increasing temperature in other strains e.g. *V. campbellii* (R-21441) and *V. coralliilyticus* (LMG 20984T). There is also evidence that temperature can affect the level of AHL production positively (Hasegawa et al., 2005), all of which suggests that the lack of AHLs detected at high temperature treatments in *S. pistillata* fragments may not be due to temperature degradation alone.

Indeed, temperature has been found to play a more direct role in vibrio pathogenicity (Oh et al., 2009) with recent evidence that increased temperatures cause significant increases in the number of transcriptional regulators detected in *V. coralliilyticus* (Vc450), including quorum sensing proteins. This study also showed that the Vc450 proteome exhibits up-regulation of numerous QS proteins at 27°C, while analysis of AI-1 and AI-2 signalling also reveal significant temperature effects, indicating that temperature has a direct effect of Vc450 QS (Kimes et al., 2012). With the diverse communities of bacteria associated with coral tissue and the recent evidence that 30% of these produce AHL signals (Golberg et al., 2011), one would expect a range of changes to AHL signals detected from *S. pistillata*. Instead, this study shows an overall decrease in signal production. However, it is possible that the detection of smaller quantities of AHLs or potentially more localised signalling is beyond the bounds of the *A. tumefaciens* biosensor used.

4.4.3 Decrease in bacterial numbers with temperature stress

The decrease in bacterial numbers associated with *S. pistillata* tissue in 26°C and 30°C treatments after 7 d suggest that *S. pistillata* bacterial communities may be vulnerable to heat stress. Previous studies have found *S. pistillata* to be extremely susceptible to heat stress (Loya et al., 2001). Investigations have concluded this vulnerability is the result of a number of factors including the clade of *Symbiodinium* present, unrepaired DNA damage (lack of essential host proteins), limited tissue biomass and the depletion of mucus reserves found at 32°C (Fitt et al., 2009). The absence of mucocytes in the epidermis after 5 days at 32°C (Fitt et al., 2009) and thus the depletion of mucus reserves seen in this species may have a knock on effect to the number of bacteria within *S. pistillata*, as bacteria are known to associate with the mucus of corals in large numbers (Koren & Rosenberg, 2006). As coral mucus is thought to be a possible first line of defence for coral species (Brown & Bythell, 2005; Ritchie, 2006), its depletion may also be the reason for the shift in community seen in the 30°C treatments in this study. Further, the depletion of bacterial numbers in the 30°C treatment after 7 d may contribute to the striking reduction in AHLs detected, which may have a potential effect on bacterial phenotypes such as biofilm formation and antibiotic production, as the control of phenotypic change by QS is a density dependent phenomenon (Fuqua et al., 1994).

4.4.4 Bacterial communities associated with *S. pistillata*

The decrease in bacterial numbers and detection of AHL signalling seen in this study was accompanied by a marked change in the bacterial community associated with *S. pistillata* tissue; however these changes were not restricted to the high temperature treatment. There are a limited number of studies looking at the bacterial diversity associated with *S. pistillata*, but one study has shown a large variation in the composition of *S. pistillata* bacterial communities from Kenting, in the far south of Taiwan, and suggests that seasonal and geographical variations in individual coral colonies are vital drivers of bacterial community structure. However, the effect of species specificity, although weak, is still detectable (Hong et al., 2009).

In this study, the dominance of Gammaproteobacteria is not a surprise, as this class of Bacteria is found in abundance in a number of coral species (e.g. Bourne & Munn, 2005), as is the genus *Endozoicomonas* within this class (discussed in chapter 2 and 3). Hong et al. (2009) also found this group in abundance. The Gammaproteobacteria are known producers of AHLs; in one study 90% of all bacterial isolates from coral tissue testing positive for quorum sensing belonged to the Proteobacteria, with 90% of these belonging to the Gammaproteobacteria (Golberg et al., 2011). As such, it follows that there are abundant and conserved AHL signals detected with the presence of this class of Bacteria. To date, there is no evidence that members of the genus *Endozoicomonas* have the ability to produce AHLs. Other phyla of bacteria were also found throughout all the samples, such as the Cyanobacteria and the Bacteroidetes. Members of these phyla were also found in chapter 3, in association with *Anemonia viridis* tissue; as discussed previously, members from both these groups have recently been found to produce one or more AHL signals (Huang et al., 2008; Sharif et al., 2008).

Comparing these bacterial communities to those found in *S. pistillata*, similar patterns emerge. In the winter the two major groups that Hong et al. (2009) detected were the Gammaproteobacteria and the Bacilli, which is consistent with the present study. In the summer *S. pistillata* was found to harbour more variation in their bacterial communities, however Gammaproteobacteria, Cyanobacteria and Bacilli remained three of the four most represented groups (Hong et al., 2009).

4.4.5 Changing bacterial communities: experimental and temperature effect

This study shows differences in the bacterial community associated with *S. pistillata* and finds these to be related to both temperature and experimental conditions. Experimental conditions resulted in an increase in Gammaproteobacteria in 23 and 26°C treatments, a decrease in the Planctomycetes, an increase in the Cyanobacteria and an increase in multivariate dispersion over the experimental period. This suggests that the experimental conditions employed in this

experiment were different to the natural conditions in the coral nursery and that great care needs to be taken to assure that experimental conditions imitate the natural environment as far as possible. Due to time constraints in Israel this experiment had to proceed as it was, however in future work greater acclimatization time needs to be enforced to ensure the minimal impact on the coral and its bacterial communities.

The most notable shift seen due to temperature treatments was the increase in Firmicutes after 48h in the high temperature treatment. Firmicutes have also become a dominant group in the sponge *Rhopaloeides odorabile* when subjected to temperature stress of 33°C for three days, when not found in control treatments (Webster et al., 2008). In this previous study, the authors suggest that this sponge has a temperature threshold of between 31-33°C at which temperature becomes critical to the partnership between the sponge and a known Alphaproteobacterial symbiont (Webster et al., 2008). They hypothesise that this group is either highly sensitive to temperature stress or that aggressive alien microbial populations are able to outcompete native bacterial species over this short time period. The same may be true for the *Endozoicomonas* genus found in a number of coral species and the vulnerability may be due in part to a break down in quorum sensing, as established in this study.

Alien microbial populations, restricted under normal conditions, may be allowed to dominate with temperature stress for a range of reasons; however the increase in Firmicutes in this study and others suggests that they may be more resistant to temperature stress. Some Firmicutes are known to produce endospores which allow resistance to high temperatures (Munn, 2011) which may allow them to take hold in niches where others may suffer. They are also known to produce extracellular enzymes and antimicrobials (Priest, 1977), which may allow them to gain a foot-hold under adverse conditions. An increase in this phylum has also been found associating with corals suffering from Black Band disease (Cooney et al., 2002; Frias-Lopez et al., 2004).

Members of the Bacilli, a class within the Firmicutes, are known to degrade AHLs in many environments, including natural marine biofilm communities (Tait et al., 2009). Quorum quenching (QQ) is a term that describes mechanisms that inactivate QS communication systems (Dong et al., 2001, 2007). AHLs generally produced by Gram negative bacteria can be degraded by Gram positive bacteria using two main groups of enzymes, AHL lactonases and AHL acylases (Decho, 2010). In this study members of the genus *Geobacillus*, found to be the top contributor to increases in this phylum, have been found to degrade a wide variety of AHLs at a wide range of temperatures (Seo et al., 2011). The decrease in AHLs present in high temperatures observed in this study may therefore in part be due to an increase in QQ by *Geobacillus*.

In terrestrial examples (Dong et al., 2002; Uroz et al., 2003), all the characterised isolates from soil (n=800) with strong QQ activity belonged to the *Bacillus* genus (Dong et al., 2002). AHL degraders belonging to both the Bacilli and Alphaproteobacteria were also isolated from the tobacco rhizosphere, but again only Bacilli could be isolated from the same soil (D'Angelo-Picard et al., 2005). From the marine realm, Romero et al. (2010) have recently shown 14.4% of isolates from marine sources to eliminate or significantly reduce N-hexanoyl-L-homoserine lactone activity. Among these, 15 strains (out of 24) were able to inhibit N-decanoyl-L-homoserine lactone activity as well, belonging to nine different genera including members of the Firmicutes. Lactonase activity seems to be widespread in the genus *Bacillus* (Dong et al., 2002) however the acidification of media after AHL degradation in the isolate *Bacillus circulans* showed no recovery of C12-HSL activity and minimal amounts of C4-HSL (Romero et al., 2011). This indicates a type of enzymatic activity different to that described so far for this genus (Romero et al., 2011). The increase in Firmicutes and particularly in the genus *Geobacillus* seen in this study suggest that when assessing temperature stress and the breakdown of natural coral-associated bacterial communities in the future, investigations need to focus not only on the natural bacterial communities and the effect of temperature on quorum

sensing, but also on the bacteria invading the coral niche and the role of quorum quenching in their success.

4.4.6 Importance of employing a number of molecular community analyses

This study has highlighted the importance of using multiple methods for assessing microbial community change. In this study DGGE analysis showed no difference between treatments, accept for T0 samples which clustered together away from the other treatments, while 454-pyrosequencing showed distinct differences in community structure. Similar effects have been seen recently when employing DGGE to look at changes in bacterial communities associated with crustose coralline algae (Webster et al., 2010); in that study universal 16S rRNA gene primers and DGGE resulted in banding patterns too dense for adequate resolution, while DGGE with *rpoB* gene primers showed no difference in temperature treatments. However, a more detailed analysis using 16S rRNA clone libraries revealed distinct shifts between temperature treatments.

DGGE is a good tool for investigating community composition to give a high number of replicates for statistical analysis. However, the high diversity seen in some marine bacterial communities can result in multiple, weak bands that often don't separate well on the gel, making it impossible for gel analysis software to pick up changes in bacterial communities. In this study use of 454-pyrosequencing, based on 97 sequences per sample, provided a greater insight as to how bacterial profiles associated with *S. pistillata* change with temperature and which members of the bacterial community are affected.

4.4.7 Overall stress to the coral holobiont: zooxanthellae and the production of chlorophyll

Bleaching or the loss of pigmentation due to a decrease in the number of symbiotic algae (zooxanthellae) has been largely correlated with elevated sea surface temperatures (Glynn, 1993; Brown, 1997) in reef-building corals and has been observed where average daily sea water temperatures exceed the mean summer temperature by as little as 1–2°C (Hoegh-Guldberg & Salvat, 1995; Brown et al., 1996; Jones et al., 1997). In this study, no significant difference in zooxanthellae numbers was seen between the treatments. Similar results have previously been found in *S. pistillata* from the Lizard Island, Great Barrier Reef, Australia, when fragments were subjected to 30°C (Hoegh-Guldeberg & Smith, 1989), suggesting that 30°C is below the threshold of severe expulsion of zooxanthellae in this species. Hoegh-Guldberg and Smith (1989) found significant differences at 32°C, with increased mortality after 4 days. There was also no significant drop in chlorophyll per zooxanthellae in this study; again echoing the study by Hoegh-Guldberg and Smith (1989). Authors have suggested that the loss of algal chlorophyll may be a secondary effect resulting from long-term exposure to elevated sea water temperature and high light (Hoegh-Guldberg & Smith, 1989; Jones et al., 1997). Interestingly, the change seen in AHL-signalling and in the bacterial community associated with *S. pistillata* at 30°C occurs prior to the threshold for mass expulsion of zooxantheallae documented with temperature stress in *S. pistillata*.

4.4.8 Temperature variability compared with real world scenarios

The temperatures used in this experiment, 23°C, 26°C and 30°C correspond to average winter temperatures, average summer temperatures and peak summer temperatures, respectively (Israel National Monitoring Program, Gulf of Eilat Meteorological Station; unpublished data). In 2009 and 2010 sea temperature was recorded at 1.79 m depth, within 200 m of where corals were collected and ranged from 22.3–29.8°C and 21.8–29.8°C, respectively. Temperatures in this experiment varied by approximately 2°C during each 24 hour cycle, which corresponds to variation seen in their natural environment. However, the high temperature treatment in this

experiment increased above values seen in the natural environment to a maximum of 31.27°C on the last day of this experiment, due to the difficulty of controlling water temperature in such small bodies of water (10 L) and with varying air temperatures. This may have had some effects on the *S. pistillata* fragments, the bacterial communities associated with them and hence the results presented in this chapter. However, with predicted scenarios for sea surface temperature increasing from 1.8 to 4.0°C in the 21th century (IPCC, 2007), experiments such as this are imperative to understand future pressures to coral populations.

The short-term rate of change in seawater temperatures is also likely to influence the stress response in the coral and their bacterial communities. Temperatures were increased from 23°C to experimental temperatu res over a week, corresponding to approximately 0.5°C/day for the 26°C treatment and 1°C/day for the 30°C treatment. *S. pistillata* fragments the Gulf of Eilat are subjected to change in temperature of approximately 2°C/day, however temperature changes sustained by *S. pistillata* fragments can increase by 2.8°C within 24 hours (2010 data; Israel National Monitoring Program, Gulf of Eilat Meteorological Station; unpublished data).

4.4.9 Looking to the future

It is likely that increases in sea surface temperature directly affect both the host and microbial components, along with symbiotic partnerships critical to health of the holobiont (Webster et al., 2008). This study has shown a complicated change in bacterial community dynamics due to temperature stress, with decreases in bacterial numbers and detection of AHL signalling and a change to the bacterial community. In another study, investigations of temperature stress of 32°C for five days on *S. pistillata* fragments showed that even with a further five days recovery time at control temperatures (28°C), more than half had died, showing recovery to be limited in this species (Fitt et al., 2009). A similar study looking at temperature stress in crustose coralline algae showed no recovery of photosynthetic capacity after 7 days at 32°C but did after the same time at 31°C (Webster et al., 2010), suggesting strict temperature thresholds for the overall

health of the holobiont. It is likely that conserved bacterial communities associated with marine invertebrates have strict temperature thresholds too, after which their normal function is jeopardized. This study provides evidence that one of the reasons normal function may cease is due to the breakdown of QS at high temperatures.

With predictions of future increases in sea temperature, it is imperative to understand further the dynamics of bacterial populations with temperature stress and the mechanisms behind the breakdown of AHL signalling. This is especially relevant if this breakdown occurs prior to overall coral stress, as seen with the lack of mass expulsion of zooxanthellae from *S. pistillata* fragments in this experiment. Indeed, there is some evidence that the loss of bacterial symbionts due to elevated SST can have significant implications on the health of the host (Webster et al., 2008). Furthermore, short term studies (24h) have found that microbial community changes in sponges exposed to 33°C did not recover after returning to ambient temperatures (27°C) for 27 days, which indicates a potentially permanent breakdown of associations (Webster et al., 2008). The importance of bacterial communities to a number of marine invertebrates is only now being considered and their possible fragility due to their specific associations and temperature thresholds are only beginning to be unveiled (Webster et al., 2010). Future investigations need to focus on the breakdown of QS mechanisms at high temperatures to better predict and possibly mitigate against changes in bacterial communities and their invertebrate hosts.

CHAPTER 5

THE EFFECT OF INCREASED TEMPERATURE ON BACTERIAL COMMUNITY DYNAMICS, QUORUM SENSING AND ANTAGONISTIC INTERACTIONS IN THREE SPECIES OF CORAL (*Discosoma* sp., *Sinularia* sp., and a gorgonian)

5. 1 Introduction

Despite knowledge of the diverse and species-specific nature of bacterial communities associated with corals, the direct function of many bacterial associates remains largely unknown, as do the dynamics of coral-associated bacterial communities during temperature stress and disease. Shifts in bacterial community structure have been observed following coral bleaching and disease (as previously discussed) but while a number of studies have implicated bacteria as aetiological agents in disease (e.g. Ben-Haim & Rosenberg, 2002), other investigations have found no specific agent. This suggests that while some diseases may have specific bacteria associated with their progression, others may be the result of the breakdown of the natural bacterial community. Many of these diseases have been correlated with temperature stress, however to date it is unclear whether this stress directly affects the bacterial communities, which then contributes to changing the health state of the coral or whether changes in bacterial communities are merely symptomatic of unhealthy corals (Kvennefors et al., 2012).

Increasing evidence suggests that coral-associated bacteria may employ specific phenotypes, such as the production of antibiotics, that help maintain coral health (Ritchie, 2006; Nissimov et al., 2009) and that this may be depressed in times of bleaching/ disease (Ritchie, 2006). As shown in Chapter 4, it was also observed that the detectable concentration of bacterial quorum sensing (QS) molecules, acyl-homoserine lactones (AHLs) declined in extracts from temperature-stressed *Stylophora pistillata*. As previously discussed, QS has been shown to regulate phenotypes which may be important in maintaining coral bacterial associates and possibly coral health and as a result the loss of QS signals could potentially cause severe changes to coral-associated bacterial communities. This decrease in the detection of QS in coral fragments may be a direct result of increasing temperature or it may be the result of a decrease in bacterial numbers or changing bacterial consortium (as seen in Chapter 4). A changing bacterial consortium may impact the detection of AHLs due to a decrease in AHL producers or

an increase in bacteria known to break down AHLs (quorum quenchers). Further, temperature could affect this changing bacterial consortium directly or temperature could affect another part of the holobiont e.g. the production of host compounds or host metabolism, leading to an altered bacterial community.

Evidence suggests that both bacterial associates and the coral itself produce compounds that have the ability to affect bacteria (Kvennefors et al., 2008; 2006; Shnit-Orland et al., 2009; Vizcaino et al., 2009). Some or all of the factors mentioned above could lead to an environment that allows the proliferation of potentially harmful bacteria due to a decrease in competition or a decrease in antimicrobial activity. In addition, growth of specific bacteria may be promoted or less favoured through the provision of secondary metabolites by the coral holobiont (Ritchie, 2006; Kvennefors et al., 2012). This study further investigates the detection of AHL signal molecules in corals subjected to temperature stress in order to confirm previous patterns and to gain further clarity of the effects of increasing temperatures on the dynamics of coral-associated bacterial communities.

The aims of this study were to investigate the effect of experimental increases in temperature on the detection of AHLs and *in situ* degradation by AHL-lactonolysis in three coral species; *Sinularia* sp. (Octocorallia, Alcyonacea), *Discosoma* sp. (Hexacorallia, Corallimorpharia) and a gorgonian (Octocorallia, Gorgonacea) (Figure 5.1C) and link these to bacterial dynamics. The ability of crude coral extracts to degrade synthetic AHLs (quorum quenching; QQ) was also investigated and linked to the ability of coral extracts (including the bacterial fraction) to inhibit pathogens.

5.2 Methods

5.2.1 Coral collection, fragmentation and maintenance

Ten large rocks containing >10 *Discosoma* colonies and four colonies of both the *Sinularia* and gorgonian were purchased from the Tropical Marine Centre and delivered to the National Marine Aquarium (NMA), Plymouth, UK in February, 2011. Due to the lack of temperature control rooms, this experiment was carried out in winter to avoid unwanted temperature shifts during the experiment.

On arrival at the NMA, all corals were placed in holding tanks at 25°C with running, filtered seawater and left for one week to recover from transportation. After a week, gorgonian and *Sinularia* sp. colonies were sectioned into smaller fragments to provide replicates for the experiment. Gorgonian fragments were cut with pliers into ~ 7cm fragments and a small section of tissue (~ 1.5 cm) was removed from the internal skeleton at the exposed end of the fragment. The internal skeleton was then pushed into resin and placed on a plastic mount to set, taking care to avoid the exposed tissue touching the mount. *Sinularia* colonies were also cut into ~ 7 cm fragments with pliers and placed, exposed side down, onto a bed of epibiont free rocks. All fragments were left to heal and attach to their new substrate in holding tanks for two weeks prior to the experiment. All fragments had only one exposed area of tissue at their base, which were fully healed by the start of the experiment.

Following two weeks in the holding tanks, coral fragments were transferred to the experimental set up seen in Figure 5.1A; consisting of three large water baths (Figure 5.1A 1-3) and three smaller experimental tanks per water bath (35 L; Figure 5.1B). Coral fragments were distributed in the experimental tanks using a random number table. Water circulation was maintained with water pumps fitted inside each experimental tank. Due to facility limitations at the NMA, this

set up was a closed-system; with two thirds water change occurring daily from header tanks, using a basic siphon system.
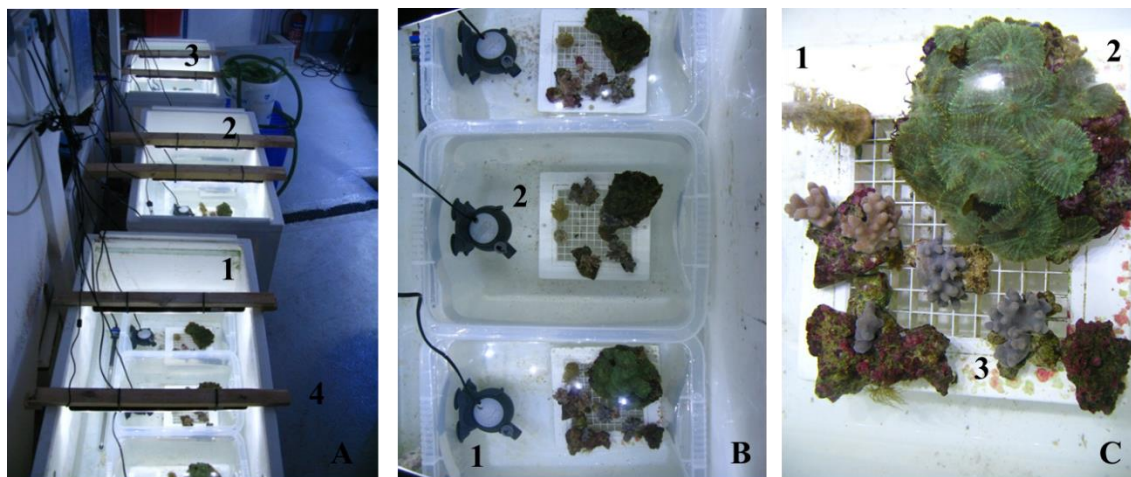
Figure 5.1 Experimental setup at the National Marine Aquarium consisting of three water baths (A 1-3) with light provided by two strip lights per bath (A4). Coral fragments on stands (B2) were situated in three experimental tanks per temperature (B) with water pumps to provide water circulation (B1). gorgonian (C1), *Discosoma* sp. (C2) and *Sinularia* sp. (C3) can be seen in C.

After a further week of acclimatisation in experimental tanks, water bath temperatures were raised gradually by 0.5°C every day until experimental temperatures of 27°C (5 days) and 30°C (10 days) were reached, using heating rods situated in the water baths, but not in the experimental tanks themselves. This system allowed experimental tank temperature to be raised uniformly over a few hours each day, rather than adjusting the temperature of the experimental tanks with almost immediate effect. Three header tanks were acclimatised at the same rate to ensure there was no difference between experimental tank water and header tank water to avoid temperature shock to corals. After experimental treatments were reached, coral samples were taken from the ambient temperature treatment (T0). Further coral samples were taken after 48 hours (48 h; T1), 7 days (7 d; T2) and 14 days (14 d; T3).

Due to the closed-system nature of this experiment, temperature (°C), salinity (ppt) and pH were recorded every day prior to the 2/3 water change using a hand-held YSI 63 meter. Two water samples (200 ml) were also taken for nutrient analysis every week prior to coral sampling and water change. These samples were kept on ice and returned to the laboratory where one of them was filtered (0.2 µm; Whatman); both samples frozen at -20°C until further analysis.

5.2.2 Coral tissue removal and processing

At T0, fragments of *Sinularia* (n=3) and gorgonian (n=3) and individual *Discosoma* colonies (n=3) were removed from the ambient temperature treatment. A further three fragments/ colonies of *Sinularia* and *Discosoma* were removed from each temperature treatment at time points T1 to T3. The gorgonian was only re-sampled at T3 due to the numbers of healthy fragments available after initial transportation. All samples were selected using a random number table.

Coral samples were removed from experimental tanks and immediately washed three times in 0.2 µm filtered, sterile phosphate buffered saline (PBS; pH6.5) and the tip of a branch of each fragment (~ 1.0 cm^3) was removed aseptically, weighed and placed in liquid nitrogen immediately for analysis of bacterial DNA. DNA was extracted following the method described in 4.2.2. The remainder of the tissue was then weighed, placed in a Falcon tube and homogenised with 5 ml of sterile PBS until no tissue was visible. Total volume of homogenate was recorded, kept on ice and returned to Plymouth Marine Laboratory for further analysis (~ 30 min).

Tissue samples were kept on ice at all times. On arrival at the laboratory, DNA samples were stored at -80°C. Homogenised samples were vortexed for 30s; 100 µl was removed for total protein measurements and stored in the dark at 4°C. A 2 ml sample was removed and kept on

ice for plate assays (see below). The remaining sample was then centrifuged at 3, x000g for 5 min at 4°C and the supernatant was removed to a clean 50 ml Falcon tube; 100 µl was removed for host protein measurements and kept in the dark at 4°C until analysis. To assess the ability of the coral homogenate to degrade AHLs, a further 0.5 ml was removed. Immediately 50 µl of this sub-sample was placed in four wells of a 96-well plate and a further four wells were prepared with a 1:10 dilution of the homogenate (5 µl supernatant and 45 µl of sterile PBS). A 2 µl sample of synthetic AHL (OC10; 2 µM) was added to three of the four wells for each sample (and the 1/10 dilution) and incubated for 3h at 25°C, 27°C and 30°C (depending on which temperature treatment the sample came from) to keep homogenate (including bacterial fraction) under the same experimental temperatures. The 96-well plates were then frozen at −80°C until further analysis (see below).

The remaining pellet was re-suspended in 2ml sterile PBS, homogenised and centrifuged at 3,000 xg for 5 min at 4°C. The supernatant was removed and pooled with the previous supernatant. The remaining pellet was then re-suspended in 1ml sterile PBS, homogenised, re-centrifuged and then re-suspended in 1 ml PBS. A 100 µl sample was removed for zooxanthellae counts, as in section 4.2.2. Remaining sample was centrifuged as above and the supernatant was removed; treated and analysed for chlorophyll as described in 4.4.2. The remaining supernatant was split into two equal halves for the extraction of AHLs.

5.2.3 Dichloromethane extraction of AHLs

HCl (1 M) was added to half of the supernatant until pH 2 was reached, checked by pH meter, and incubated overnight at 30°C with shaking (200 rpm) to reform the lactone ring of AHLs that had been degraded by lactonolysis. To the second half of the supernatant, 10 ml dichloromethane (DCM) was added and AHL extraction was carried out as described in section 4.2.3. This process was repeated the following day for the acidified samples. To assess the

presence of AHL-lactonolysis degradation in coral homogenate the results from the acidified and non-acidified samples were compared.

5.2.4 Analysis of types of bacteria present and Burkholder diffusion assays for bacterial interactions

A 2 ml sample of coral homogenate (kept on ice) was vortexed and 60 µl was spread on two replicate plates of blood agar (5% sterile horse blood added to molten sterile marine salts agar (MSA; 1g yeast extract (Sigma); 5 g bactopeptone (BD); 15 g bactoagar (BD); 1 L aged, filtered seawater; cooled to 45°C) and skimmed milk agar (5 g of skimmed milk powder in 50 ml dH$_2$O sterilised at 121°C for 15 min added to 1 L molten sterile MSA; cooled to 45°C) . A dilution series was created with a further 100 µl of the homogenate using sterile PBS and 60 µl of 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread onto sterile MSA without additions.

For Burkholder diffusion assays to assess bacterial interactions, 400 µl of homogenate was incorporated into 5 ml soft-top MSA (as above, using 6.5 g agar per litre) and poured onto a base layer of 15 ml MSA. Controls consisted of soft-top agar without coral extract. Once set, 10 µl of overnight cultures (grown in MSB at 20°C) of the potential marine pathogens *Vibrio coralliilyticus, V. shiloi, V. splendidus, V. tubiashii* and *V. communicaris* (adjusted to an OD of 0.2 at 590 nm) were spotted onto the plates. Another set of plates was prepared in the same way using cultures of the terrestrial pathogens *Pseudomonas aeruginosa 8626, E. coli 10777, E. coli 10418, Staphlococcus aureus 1803* and *S. aureus BRL1555* (grown in nutrient broth (Oxoid, NB) at 37°C) using a base layer of nutrient agar (NA, Oxoid) and a soft-top layer (NA with 6.5g agar per litre). Plates were kept at room temperature and after 3 days the size of colonies, and presence and size of swarming and extracellular products was recorded and compared to controls. Numbers of bacteria present on skimmed milk and horse blood agar were also recorded.

5.2.5 Normalisation of fragments for quantification of other parameters: total protein and host protein

Within 48 h, total and host protein was measured using Bradford quick start dye reagent in flat bottom multi well plates using the manufacturer's protocol as in. Protein values were taken in triplicate, with a blank of sterile PBS as in 3.2.5 with an R^2 of 0.97.

5.2.6 Zooxanthellae counts and chlorophyll quantification

After 24 h at 4°C chlorophyll content of samples was analysed as described in section 4.2.7. Samples were re-diluted if chlorophyll values exceeded 1.0 optical density (OD). Samples for zooxanthellae counts were vortexed, diluted 1:10 with sterile PBS and placed in a Neubauer haemocytometer. Zooxanthellae were counted in four corner squares of the haemocytometer for each sample. This process was repeated twice for each sample, giving 8 counts.

5.2.7 Visualisation of AHLs from coral tissue

Dried extracts were re-suspended in 50µl acetonitrile, vortexed and applied to RP18 F_{254} thin-layer chromatography (TLC) plates (20x20 cm; VWR International). The amount of sample applied was normalised to 1.5 mg (*Discosoma* sp. and gorgonion) and 10 mg (*Sinularia* sp.) total protein. Differences in the quantity of protein standardised against was a result of amount of tissue/ protein present in whole extracts and the assay was carried out and analysed as described in 3.2.7 using the biosensor *Agrobacterium tumefaciens* NTL4 (Fuqua & Winans, 1996) and synthetic AHL standards as markers; 5ul of stock containing 5µM *N*-oxohexanoyl-L-homoserine lactone (OC6), 0.2µM *N*-oxohexanoyl-L-homoserine lactone (OC8) and 0.5mM of *N*-oxodecanoyl-L-homoserine lactone (OC10).

5.2.8 AHL degradation assays

A 20 ml culture of *E. coli* pSB1075 was grown overnight in LB broth containing 10 μg/ml tetracycline antibiotic at 30°C. The OD (600 nm) was measured and bacteria pelleted by centrifugation (4,000 xg, 5 min). The bacteria were washed in sterile PBS and the OD was adjusted to 1.0. The 96-well plates containing sample extracts were removed from the freezer and 200 μl of washed biosensor cells were added to all the wells. Plates were incubated for 4 h at 30°C. Relative light units (RLU) measurements were recorded with a decrease in light output being indicative of AHL degradation. Optical density (600 nm) was also measured to ensure extracts had not restricted biosensor growth.

5.2.9 Denaturing gradient gel electrophoresis (DGGE) analysis

A nested PCR approach was used to amplify 16S rRNA for DGGE using the method described in 2.2.3. Due to the presence of multiple bands in *Discosma* sp. amplifications, PCR bands were purified by gel extraction using the Wizard® SV Gel and PCR Clean-Up System (Promega). PCR products were all checked for similar intensity of bands on agarose gels and DGGE was performed using the INGENYphorU System (Ingeny, Netherlands) as described in 3.2.4. Bands were cut out for sequencing as described in 3.2.4.

5.2.10 Nutrient analysis of water samples

Nutrients were analysed using a Bran and Luebbe AAIII segmented flow colorimetric autoanalyser by Malcolm Woodward at Plymouth Marine Laboratory. Using previously described methods, measurements of phosphate (Zhang & Chi, 2002), nitrate (Brewer & Riley, 1965), nitrite (Grasshoff, 1976) and ammonia (Mantoura & Woodward, 1983) were recorded.

5.2.11 Statistics

Minitab (v6) was used to analyse differences between treatments. Where possible, data were normalised to allow analysis with ANOVA. However, where data could not be normalised using standard transformations the non-parametric Kruskal-Wallis test was used to rank data and assess differences between treatments. PRIMER (v6) was used for multivariate analysis of DGGE data, using non-multidimensional scaling and hierarchical clustering to look for differences in bacterial communities between samples. Covariates role in *Sinularia* sp. AHL profiles were assessed using a Type I sums of squares covariate PERMANOVA.

5.3 Results

5.3.1 Experimental conditions

Average temperatures for the three treatments over the experimental period were 25.4°C (25°C), 27.44°C (27°C) and 29.7°C (30°C) as shown in Figure 5.2. Positioning the heating rods in the water baths kept experimental tank temperature stable over the 14 d experiment and enabled temperature to be raised gradually in experimental tanks. Kruskal-Wallis tests show no differences between temperatures in replicate treatments.
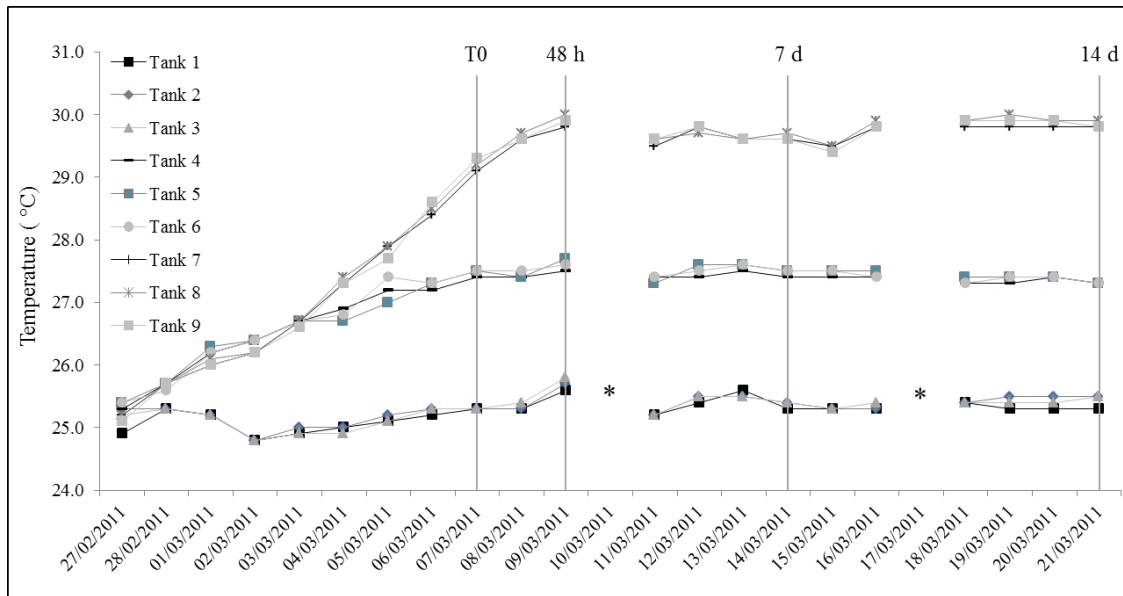
Figure 5.2 Temperature data for experimental period as recorded by hand-held YSI 86 meter daily before water change. Sampling points are highlighted and * denotes days temperature was not recorded. 5/3/2011 and 6/3/2011 data points were measured using a thermometer due to equipment failure.

Salinity (ppt) and pH were also recorded throughout the experimental period (figure 5.3). A Kruskal-Wallis test assessed the equality of sample medians for the three treatments at 30.8 ppt (25°C), 30.9 ppt (27°C) and 31.2 ppt (30°C) and indicates a difference between treatments (H=17.13, p<0.001). No significant differences were found between time points. Figure 5.3A shows the variability in salinity, which suggests that more evaporation is occurring in higher temperature tanks, increasing salinity. Kruskal-Wallis analysis of pH data showed no differences between temperature treatments, but between time points (H=20.28, p<0.001). Figure 5.3B, indicates that there were some differences in pH over time. pH measured before YSI equipment failed shows a much lower pH than that measured after March 7. This is likely to be due to calibration error of the equipment after failure. pH was relatively stable throughout the experimental period, however on March 9, the pH was seen to be variable over experimental tanks ranging from pH 8.04–8.28. As this was not seen elsewhere in the data set it may be that this difference relates to the water provided for the experiment on the previous day.

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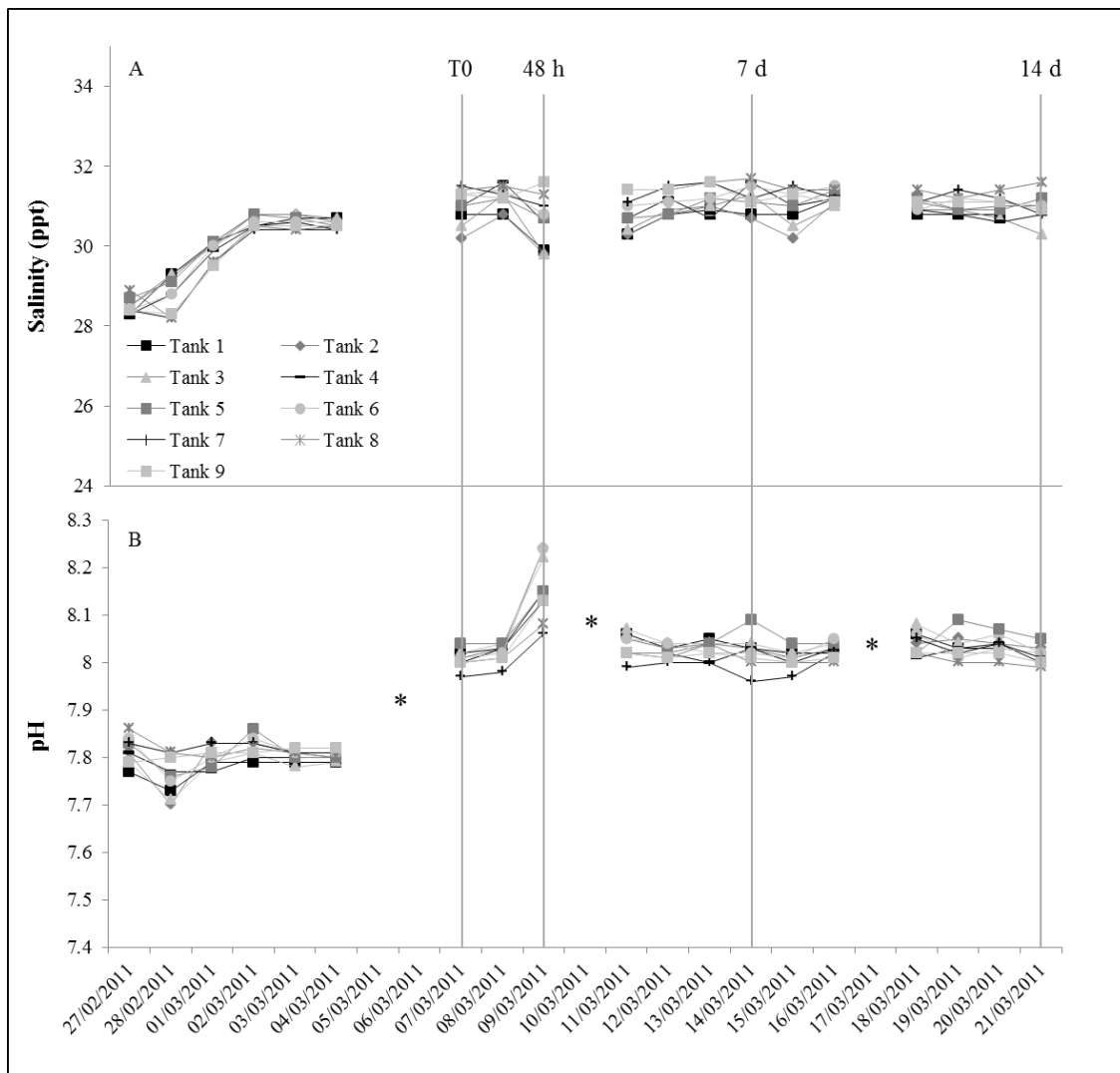
Figure 5.3 Salinity (A) and pH (B) data for experimental period as recorded by hand-held YSI 86 meter daily before water change. Sampling points are highlighted and * denotes days data was not recorded.

Light data was not measured throughout the experiment, but was tested prior to commencing the experiment and averaged 415 PAR ($\mu E.m^{-2}.s^{-1}$). This is within the ideal range for aquarium kept corals (Borneman, 2008) and no significant differences were found between treatments (ANOVA).

Due to the closed-system nature of this experiment water samples were taken for nutrient analysis before each sampling point and prior to water changes. Filtered and unfiltered samples

were tested; however, unfiltered samples are compared here for a higher degree of accuracy. As seen in Figure 5.4, some differences between time points and temperature treatments occurred, as well as high levels of nitrate ($\sim$ 60 μM) which is typical of Cattewater (Plymouth harbour water; Woodward pers. comm.). Significant differences were also seen between nitrate concentrations across time points (ANOVA, loge(loge) transformed, F=0.024, p<0.005), with Tukey tests confirming a number of differences, including 48 h samples, with significantly higher readings (average 83.7 μM) and all the rest (63.3, 64.2 and 58.3 μM, respectively), as well as between 7 d and 14 d samples (Figure 5.4D; * indicate main differences).

Differences in ammonium concentration were found between time points (ANOVA, $\sqrt{\sqrt{}}$ transformed, F=9.43, p< 0.005), with Tukey tests confirming samples taken at 7 d to have higher concentrations than all other sampling points. Average values of 1.52 μM were found at 7 d compared to other time points (average T0, 0.5 μM; 48 h and 14 d, 0.61 μM) with much higher variability between replicate tanks, especially at 30°C (Figure 5.4A).

No significant differences were found in phosphate concentration between time points or temperature treatments, with an average of 1.27 μM phosphate (Figure 5.4B). However, significant differences were seen in nitrite concentrations (Figure 5.4C). Significant differences in nitrite concentration were observed between temperature treatments (ANOVA, $\sqrt{}$ transformed, F=13.11. p<0.005) with Tukey tests confirming the 30°C treatment to have significantly higher nitrite at an average of 0.42 μM compared to 0.19 μM and 0.29 μM in 25°C and 27°C treatments, respectively. No significant differences were found between time points.
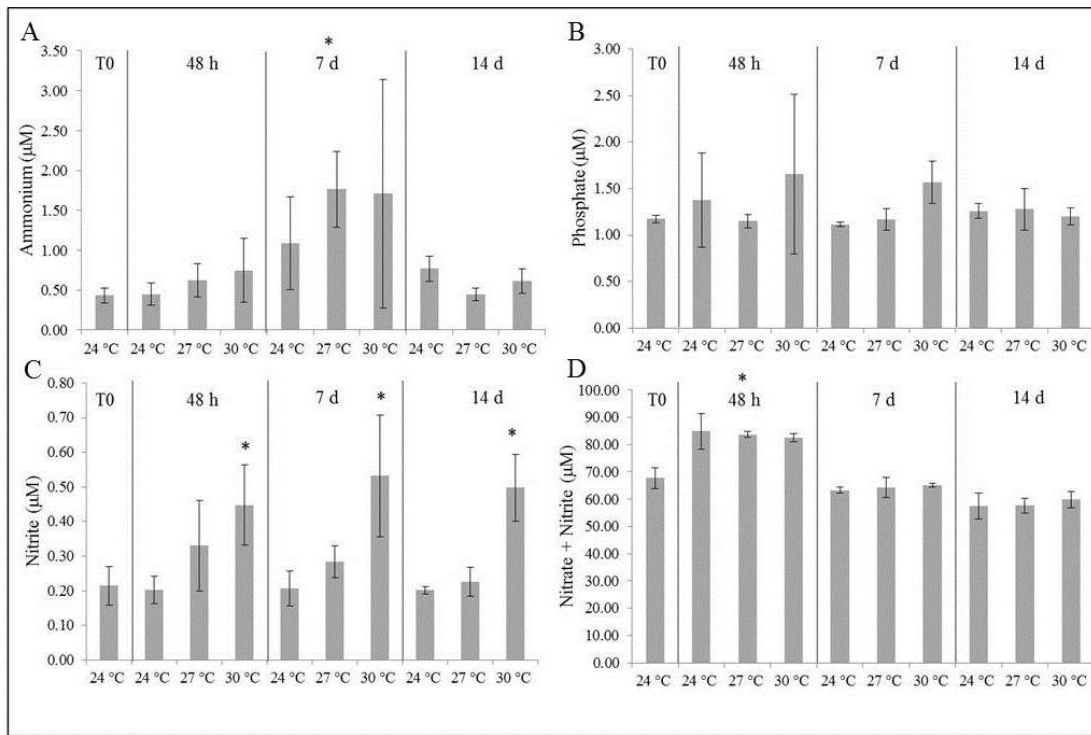
Figure 5.4 Summary of nutrient analysis data over experimental period. Data from three replicates within in each treatment have been averaged with error bars representing standard deviation. Stars represent samples with statistically different nutrient levels compared to the rest of the data points.

5.3.2 Normalising data to wet weight and protein content

Due to the physiology of the corals in this experiment, measuring surface area was not a realistic option as a tool for standardising all other parameters. Therefore, wet weight, total and host protein were measured for all the coral samples. Because of the need for care handling samples to avoid disturbing surface bacterial communities, weighed samples still had considerable moisture on their surface. Therefore, correlations between wet weight and total protein content were relatively low with R^2 values of 0.247 for *Discosoma* individuals, while *Sinularia* and gorgonian fragment correlations were much higher at 0.596 and 0.725, respectively. Correlations between total and host protein were relatively high in all coral species with R^2 values of 0.556, 0.668 and 0.725 for *Discosoma* individuals, *Sinularia* and gorgonian

fragments, respectively. As a result of little difference between host and total protein and questions surrounding accuracy of wet weight, total protein was used to standardise all other parameters in the following analysis. However, due to the amount of tissues available and as mentioned in section 5.2.7, many of the following analyses were standardised to different quantities of total protein depending on coral species (*Discosoma* sp. and gorgonian 1.5 mg; *Sinularia* sp., 10 mg) to allow for maximum detection of AHLs (i.e. more sample added to TLC plates) and comparison between AHL concentration and other parameters.

5.3.3 Detection, temperature and time effects on putative AHLs in *Sinularia* sp.

TLC of coral fragments detected a range of putative AHLs in *Sinularia* fragments in both acidified and non-acidified conditions (Figure 5.5). Five different spots, running various distances relative to the standard were observed in *Sinularia* extracts. All spots ran below or alongside the OC8 standard (Figure 5.5A) with an exception of those seen in figure 5.5F, suggesting that bacteria associated with this species of coral produce mainly long-chain AHLs. Interestingly however, not all control samples (T0) showed a similar profile of putative AHLs (Figure 5.5A-G).
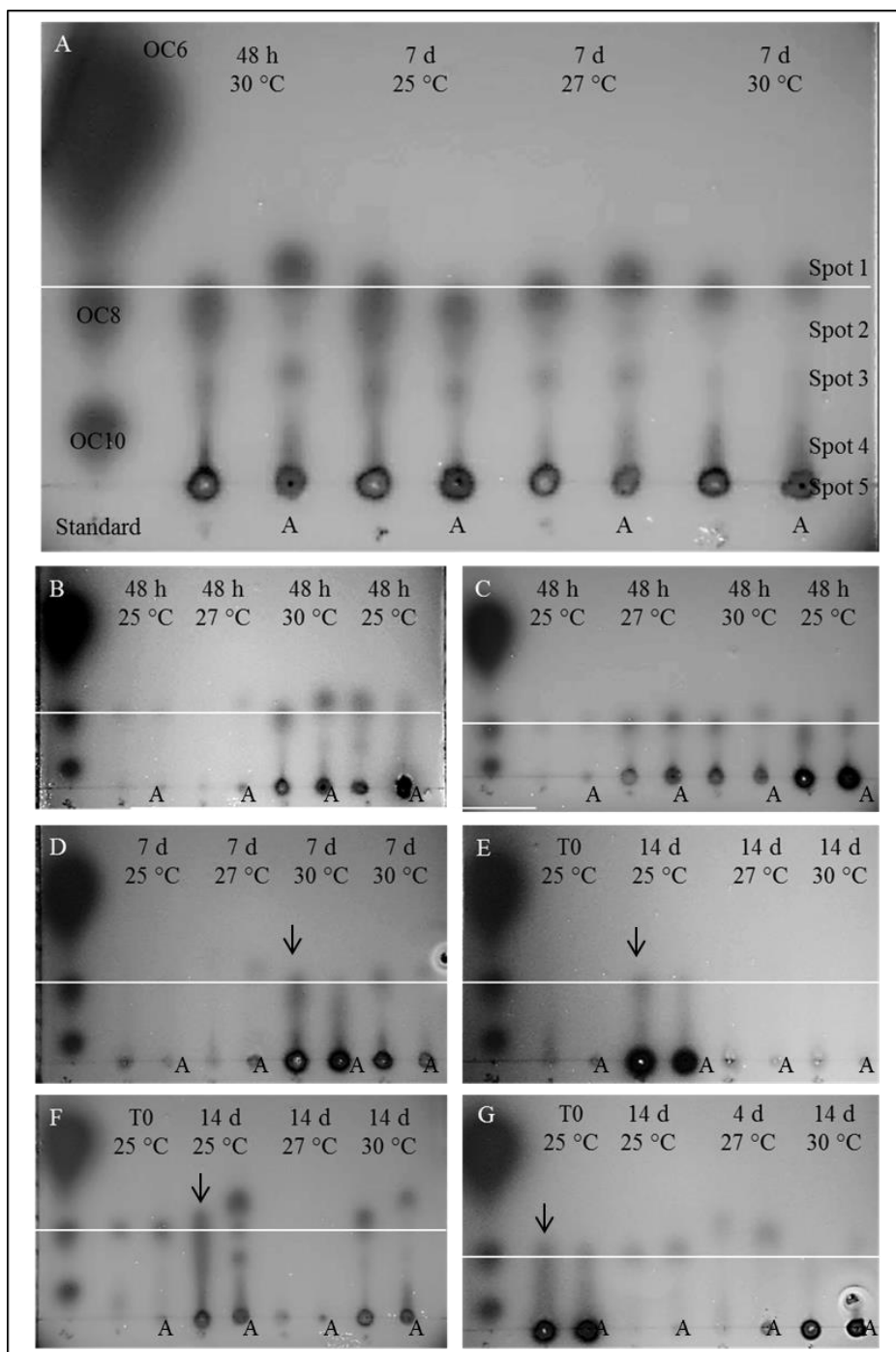
Figure 5.5 Reverse thin-layer chromatography profiling of AHLs extracted from *Sinularia* fragments. In image A, from left to right, first column (standard) shows 5 µl of AHL synthetic standard made up of 5 µM *N*-oxohexanoyl-L-homoserine lactone (OC6), 0.2 µM *N*-oxohexanoyl-L-homoserine lactone (OC8) and 0.5 mM of *N*-oxodecanoyl-L-homoserine lactone (OC10) and dichloromethane extract corresponding to 10.0 mg total protein from *Sinularia sp.* from different treatments (labelled). Each sample has two columns, the first un-acidified samples (to the left) and the second acidified samples (A). Spots are labelled for further analysis and white line denotes distance travelled by standard OC8. Images B – G show all other *Sinularia* extracts, with the same set up as TLC plate shown in image A.

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Putative AHLs were not detected from all *Sinularia* extracts and in those extracts that had detectable quantities of AHLs, variability in number and density of spots was apparent. Figure 5.6 shows the average relative area of spots detected from extracts maintained at different temperatures and removed for analysis at different time points. A few spots were hard to measure in area-density analysis as signals had tails and ran in to each other (labelled with an arrow on figure 5.5). The resting place of putative AHLs from *Sinularia* extracts were also not uniform, showing slight variations in the migration of different extracts (as seen in chapter 3). This includes acidified samples, which are seen to run further than non-acidified samples (labelled A on figure 5.5). Slight changes in migration distances could be due to composition of extracts or could be due to the presence of a modified form of AHL. The former was assumed to allow for further analysis.

Standard deviation error bars in Figure 5.6 show the variability seen within *Sinularia* replicates. Using a non-parametric Kruskal-Wallis test no significant differences were found for overall AHL production between temperature treatments or time points in acidified or non-acidified samples. When looking at individual putative AHLs, there were no significant differences between time points or temperature treatments for acidified and non-acidified samples, except between time points for spot 2 (Kruskal-Wallis, H=8.10, p<0.05), but showing no significant difference between temperature treatment. Interestingly, no difference between acidified and non-acidified samples suggests there is little pH-lactonolysis occurring *in situ*. The Kruskal-Wallis test for spot 2 calculated sample medians for the four time points at 20.0 (T0), 11.0 (48h), 18.0 (7d) and 12.7 (14d) and suggests that T0 and 7 d samples have higher production of spot 2 than other time points.

To assess the difference between acidified and non-acidified samples as a proxy for the hydrolysis of the lactone ring (one type of degradation of AHLs) the relative area of biosensor

induction in acidified samples was subtracted from non-acidified samples. Using a non-parametric Kruskal-Wallis test, no significant differences in percentage of ring-open forms between samples from different time points and temperature treatments were found. Looking at spots individually; all Kruskal-Wallis tests were non-significant, suggesting no temperature or time effect on percentage of ring open forms.
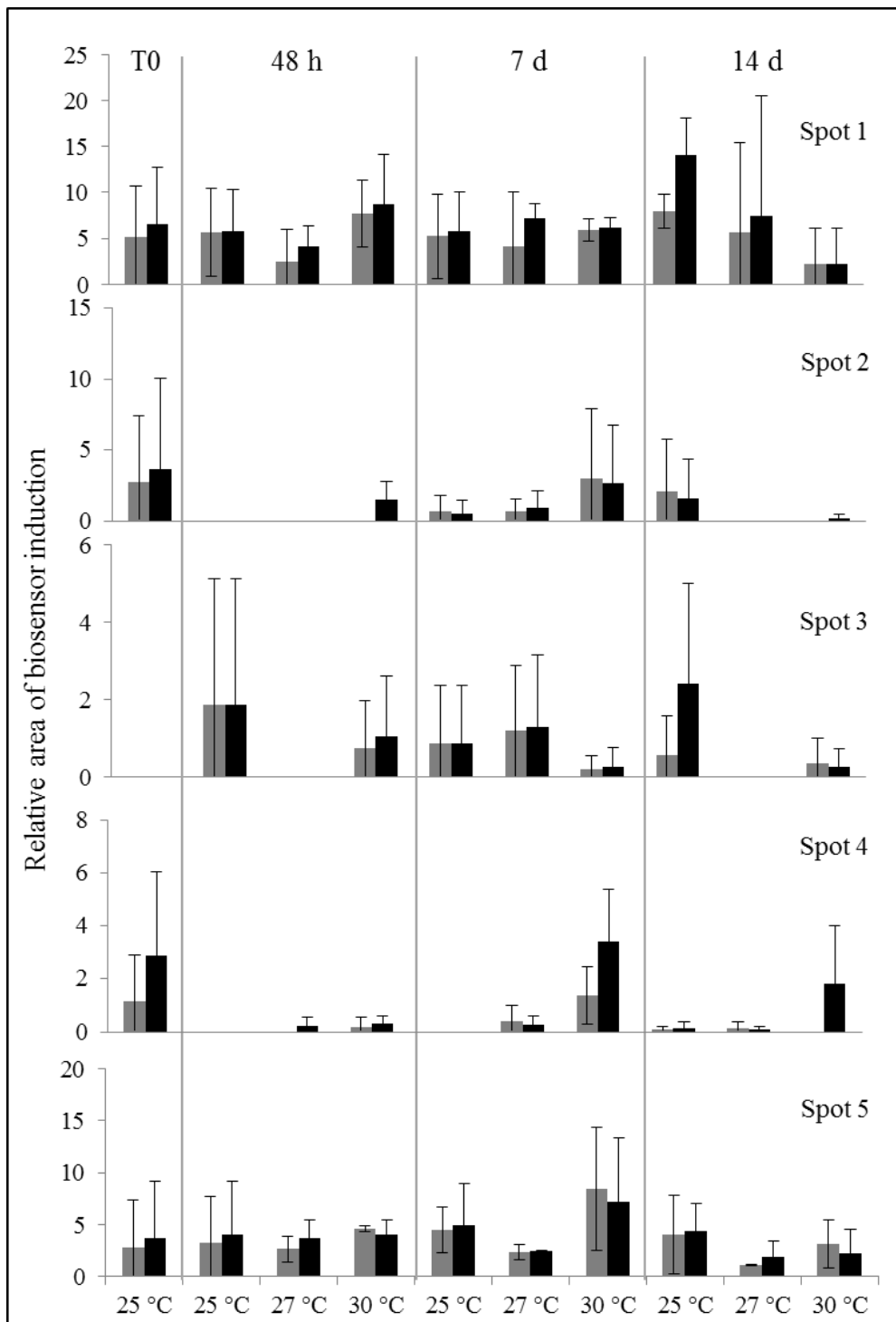
Figure 5.6 Analysis of reverse thin-layer chromatography using area analysis of spots from *Sinularia* extracts (Image Pro Plus). Amounts correspond to AHL detected from 10.0mg total protein compared to area of blue colouration induced by OC8 synthetic standard at concentration of 2.5 ng. Error bars represent standard deviation, grey bars represent non-acidified samples and black bars represented acidified counterparts.

5.3.4   Detection, temperature and time effects on putative AHLs in *Discosoma* sp.

In contrast to *Sinularia* extracts, the *A. tumefaciens* bioreporter detected three different spots in *Discosoma* extracts, all of which also ran below or alongside the OC8 standard and most of which were consistent throughout T0 samples (Figure 5.7). Interestingly, *Discosoma* extracts ran uniformly on TLC plates, with all of the corresponding spots settling along the same horizontal line, unlike *Sinularia* extracts.
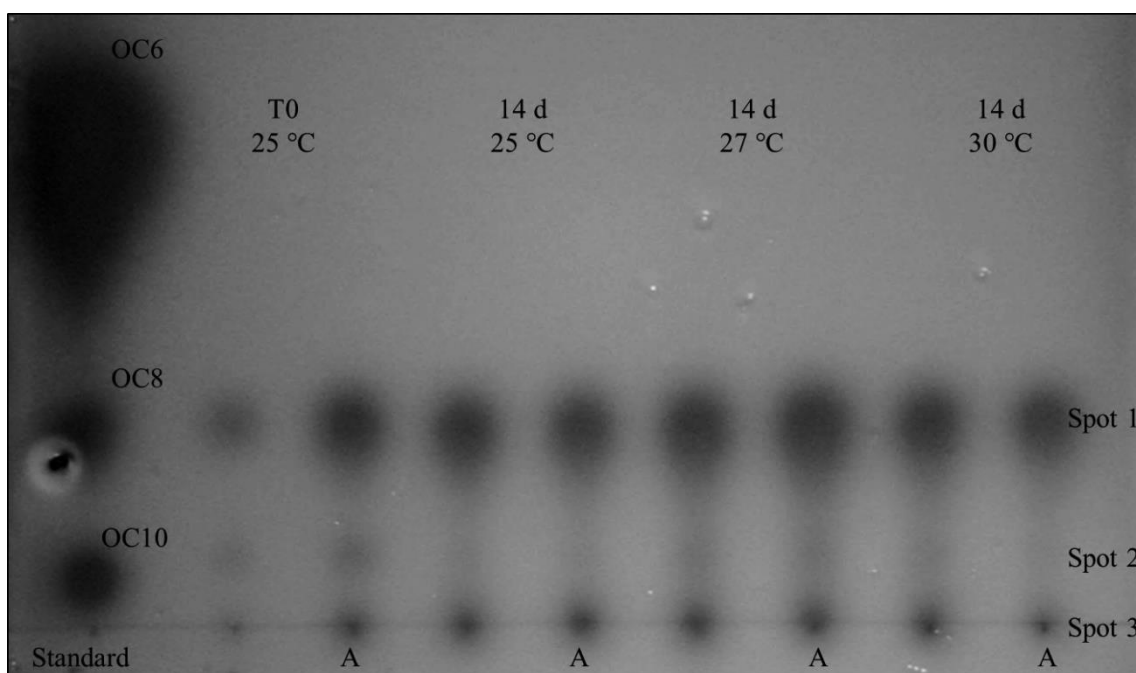
Figure 5.7 Reverse thin-layer chromatography profiling of AHLs extracted from *Discosoma* fragments. From left to right, first column (standard) shows 5 µl of AHL synthetic standard made up of 5 µM *N*-oxohexanoyl-L-homoserine lactone (OC6), 0.2 µM *N*-oxohexanoyl-L-homoserine lactone (OC8) and 0.5 mM of *N*-oxodecanoyl-L-homoserine lactone (OC10) and dichloromethane extract corresponding to 1.5 mg total protein from *Discosoma* fragments from different treatments (labelled). Each sample has two columns, the first un-acidified samples (to the left) and the second acidified samples (A). Spots are labelled for further analysis.

Putative AHLs were detected from all *Discosoma* extracts and remained relatively stable throughout all temperatures and time points. Figure 5.8 shows the average relative area of spots detected from extracts maintained at different temperatures and removed for analysis at different time points. Unfortunately, some of the TLC images acquired for this data set were lost due to computer error and thus have not been included in Figure 5.7 or in further analysis. However, by eye, similar profiles were seen in all replicates unaccounted for in further analysis; while without these replicates, the slight variability in the abundance of putative AHLs detected was unaccounted for. No significant differences were found between temperature, time points or the presence of AHLs in acidified or non-acidified samples, except for spot 3 (acidified) which showed significant differences with time (ANOVA, $F=3.34$, $p<0.05$), however Tukey tests found no significant differences between treatments. When assessing the amount of hydrolysis of the lactone ring found in *Discosoma* individuals, no significant differences in amount of ring-open forms for spots 2 and 3 at different time points and temperature treatments were seen (ANOVA). However significant differences were found between time points for spot 1 (ANOVA, $F=5.61$, $p<0.01$). Tukey tests showed samples to have less ring open forms at 48 h and 7 d.
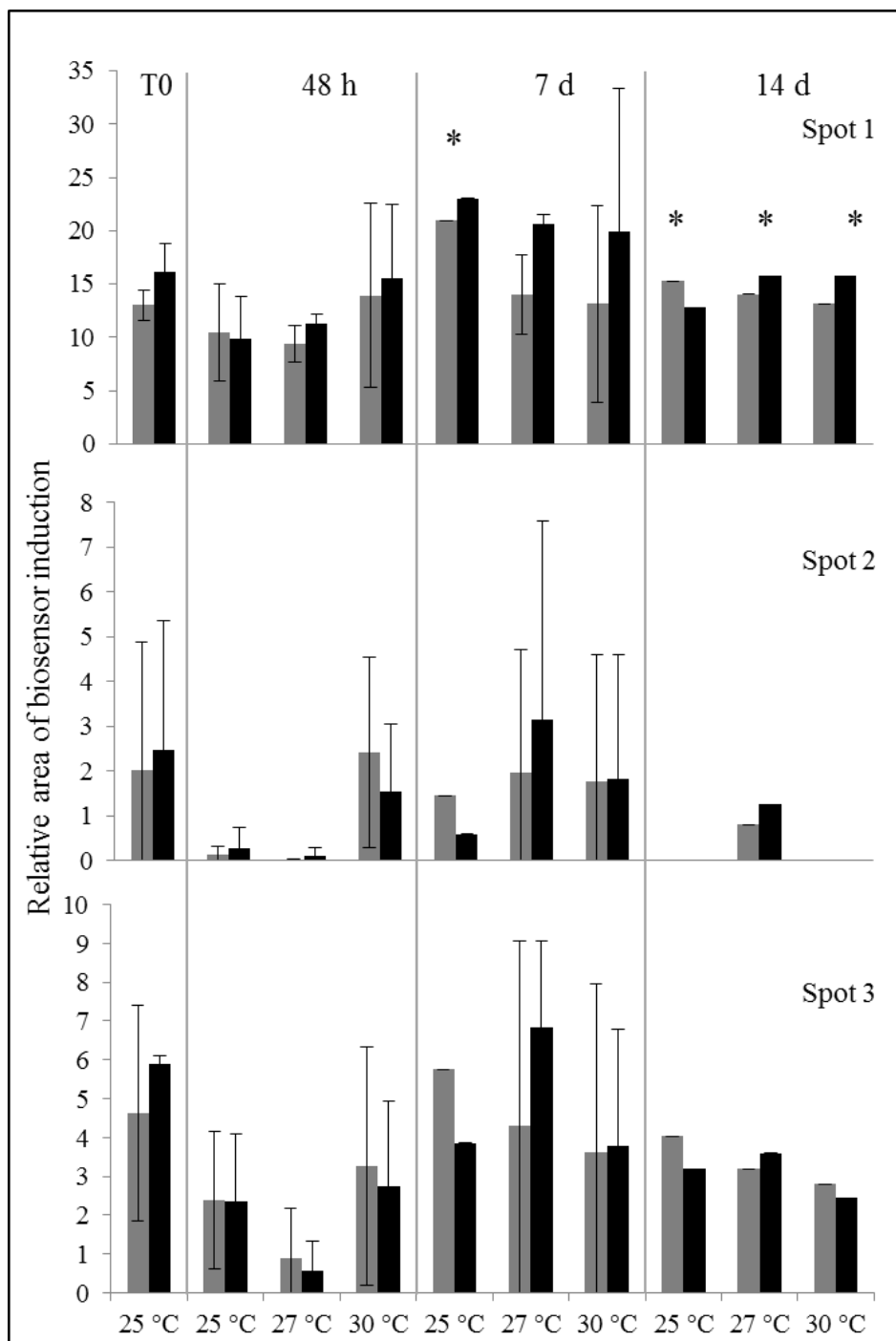
Figure 5.8 Analysis of reverse thin-layer chromatography using area analysis of spots from *Discosoma* extracts (Image Pro Plus). Amounts correspond to AHL detected from 1.5 mg total protein compared to area of blue colouration induced by OC8 synthetic standard at concentration of 2.5 ng. Error bars represent standard deviation, grey bars represent non-acidified samples and black bars represented acidified counterparts. * represent treatments where replicates are missing.

None of the extracts from the gorgonian fragments contained detectable quantities of putative AHLs on TLC plates.

5.3.5 Detection of quorum quenching: temperature and time effects

Quorum quenching was assessed by investigating the ability of coral extracts (including their bacterial communities) to degrade synthetic AHLs, standardised by total protein, over 3h, at which time the presence of AHLs was tested with the *E.coli* pSB1075 biosensor. Average results for full strength samples can be seen in Figure 5.9. No significant differences were found between time points or temperature treatments for *Sinularia* fragments (Kruskal-Wallis). However a significant difference was found between time points for *Discosoma* colonies (ANOVA, $F=10.83$, $p<0.01$), with differences between T0/ 48 h with 7 d/ 14 d samples. The decrease of AHL degradation below zero in these samples suggests an increased production of AHLs, above the synthetic AHLs found in controls. Significant differences can also be seen between temperature treatments for *Discosoma* colonies, with colonies kept at 27°C and 30°C, degrading less AHL than at 25°C (ANOVA, $F=6.06$, $p<0.01$). Significant differences were also found between T0 samples and 25°C treatments at 7 d and 14 d (ANOVA, $F=5.90$, $p<0.05$). Like the *Sinularia* sp. results, no significant differences were found between time points or temperature treatments for the gorgonian investigated.
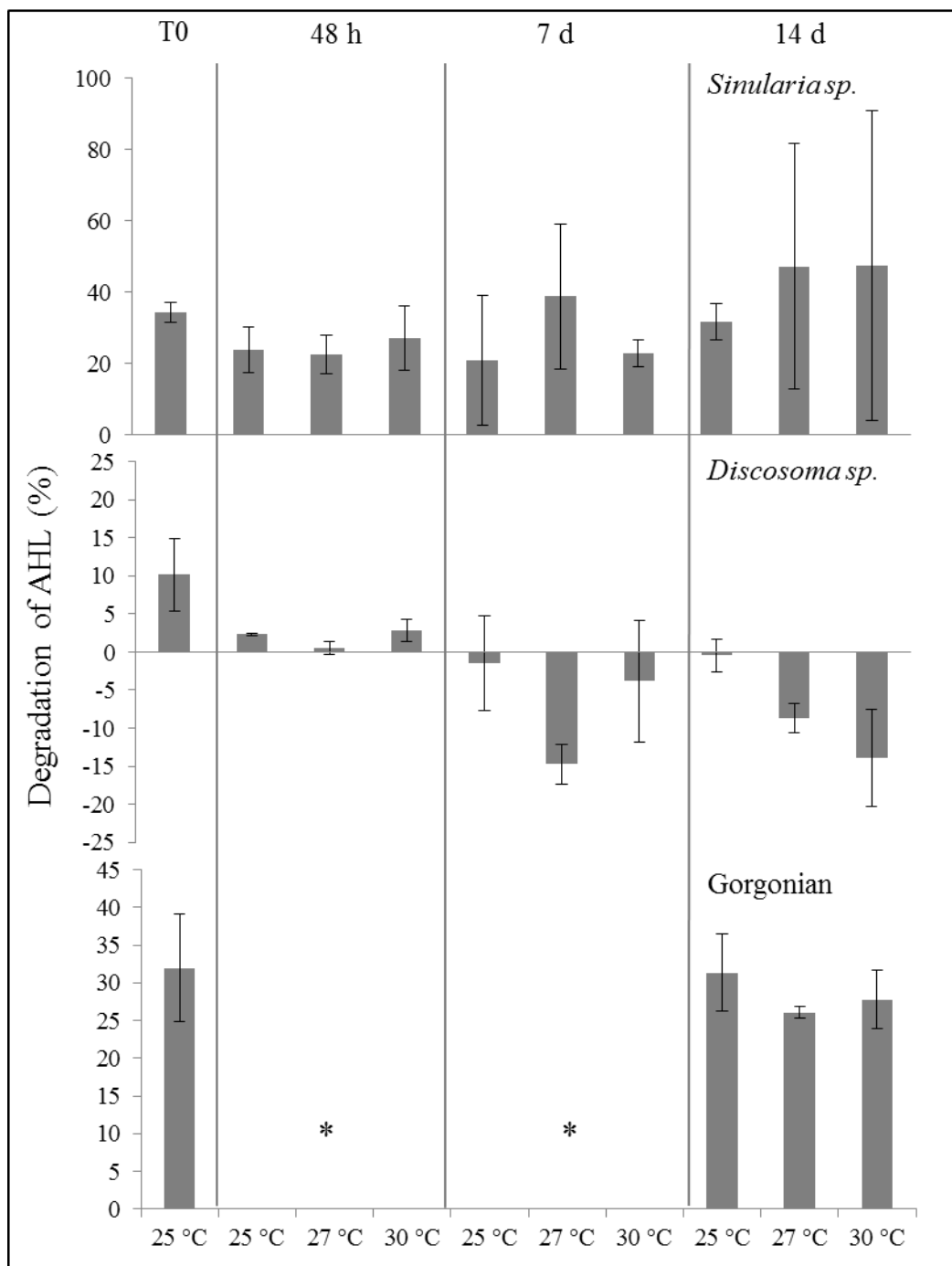
Figure 5.9 Average degradation of synthetic AHLs by coral tissue (homogenised) kept at 25°C, 27°C and 30°C over two weeks exposure. Values represent degradation per 1.5 mg protein for *Discosoma* sp. and the gorgonian, while *Sinularia* sp. values represent 10 mg protein.

5.3.6 Antagonism of known pathogens by coral extracts

To link changes in the detection of putative AHLs and the ability of coral extracts to degrade synthetic AHLs to the ability of coral extracts (including bacteria present) to defend against potential pathogens Burkholder diffusion assays seeded with coral extract were employed. Growth characteristics of a number of coral and human pathogens, including size of colony and presence and increase of extracellular products and swarming were recorded and compared to control plates with no coral extract present. Examples of these assays can be seen in Figure 5.10.
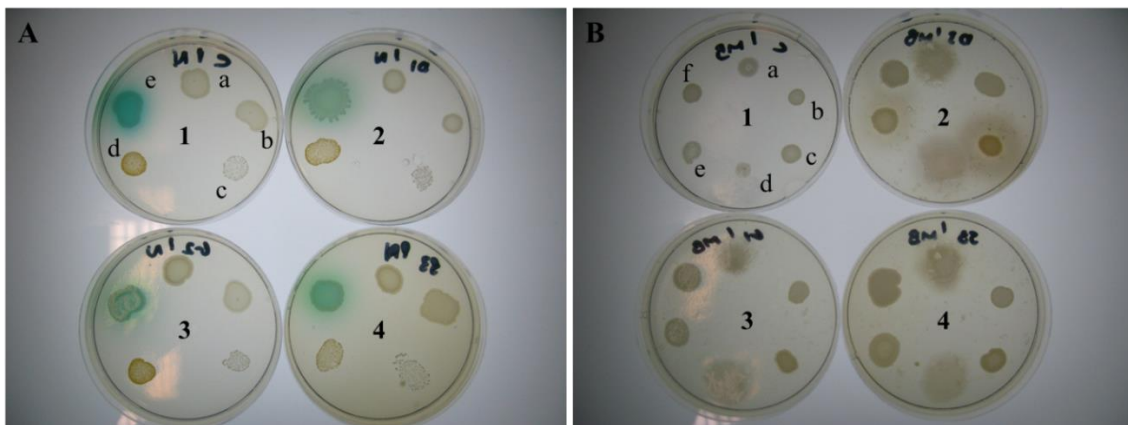
Figure 5.10 Burkholder diffusion assays testing for antagonism and control of known pathogens or potentially harmful bacteria by live coral extracts with temperature stress. Image A shows samples from T0 including a control (1), *Discosoma* extract (2), gorgonian extract (3) and *Sinularia* extract (4) with bacterial spots *E.coli* 10777 (a), *E.coli* 10418 (b), *S. aureus* 1803 (c), *S. aureus BRL* (d) and *P. aeruginosa* (e) on LB agar. Image B also shows samples from T0 in the same order (1-4) with bacterial spots *V. coralliilyticus* (a), *V. shiloi* (b), *V. tubiashii* (c), *V. harveyi* (d), *V. splendidus* (e) and *V. communicaris* (f). Examples of increased swarming from control (A1e) can be seen in A2e, increased growth from control (B1a) can be seen in B2a and increased production of extracellular products from control (B1c) can be seen in B2c.

A number of interesting and dynamic changes in the growth of bacterial strains were seen when grown in the presence of coral extracts maintained at 25°C, 27°C and 30°C over the experimental period (Table 5.1). These changes were diverse, with no overall correlations in the data, which indicates the complicated nature of the coral holobiont and the interactions it has with potential pathogens. However, one major trend shows that while size of colonies of human pathogens decreased in the presence of any coral extract from control treatments (T0; 25°C) (when compared to control plates; except *Pseudomonas aeruginosa* in the presence of *Discosoma* sp. extract), all marine *Vibrio* sp. colonies had increased growth (with an exception of *V. tubiashii* in the presence of gorgonian extract). This suggests that while coral extracts may contain compounds which deter the growth of human pathogens, marine vibrios may use compounds present to support growth or that human pathogens are affected by salinity of coral homogenate.

Table 5.1 Summary of antagonism assays using *Sinularia sp*. (A), *Discosoma sp*. (B) and gorgonian (C) supernatant in agar. Circle size represents average difference in colony size of selected potential pathogens from control plates. Small circles represent an average of 0–0.5 cm change between replicated samples (n=6 (3x2), medium circles 0.5–1.0 cm change, large circles 1.0–1.5 cm change and extra-large circles over 1.5 cm change. Black circles represent an average increase in colony size when compared to the control, while clear circles represent a decrease. Light grey circles represent a small increase to swarming behaviour and dark grey circles represent a large increase in swarming behaviour of colonies (from control).

		V. coralliilyticus	V. shiloi	V. tubiashii	V. harveyi	V. splendidus	V. commucaris	E.coli 10777	E. coli 010418	S. aureus 1803	S. aureus BRL	P. aeruginosa
T0	24 °C						-					
	24 °C											
7 d	27 °C											
	30 °C											
	24 °C			-	-	-						
14 d	27 °C			-	-	-						
	30 °C			-	-	-						
	24 °C						-					
21 d	27 °C						-					
	30 °C						-					

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		V. coralliilyticus	V. shiloi	V. tubiashii	V. harveyi	V. splendidus	V. commucaris	E.coli 10777	E. coli 010418	S. aureus 1803	S. aureus BRL	P. aeruginosa
T0	24 °C			EP 0.80		EP 0.18	-					
7 d	24 °C			EP 0.45		EP 0.53	EP 0.73					
	27 °C	EP 0.267		EP 0.48		EP 0.48	EP 0.52					S
	30 °C			EP 0.29		EP 0.29	EP 0.22					
14 d	24 °C	EP 0.25		-	-	-	EP 0.27					
	27 °C			-	-	-	EP 0.13					
	30 °C	EP 0.18		-	-	-						HS
21 d	24 °C						-					
	27 °C						-					
	30 °C				EP 0.5	EP 1.0	-					

		V. coralliilyticus	V. shiloi	V. tubiashii	V. harveyi	V. splendidus	E.coli 10777	E. coli 010418	S. aureus 1803	S. aureus BRL	P. aeruginosa
T0	24 °C	⬤ (large grey)	●	○	⬤ (large grey)	●	○	○	○	○	
	24 °C	○	◉ (grey)	○		●	●	●	○	●	○
21 d	27 °C	◉ (grey)	○	○	●	●	●	●	○	●	○
	30 °C	○ (light)	○	○	○	●	●	●	○	●	○

Sinularia extracts have a range of effects on the growth of potential pathogens, with *V. coralliilyticus* and *V. shiloi* showing the greatest changes out of the marine pathogens. *V. shiloi* colonies increased in size significantly (ANOVA, F=6.28, p<0.01, Tukey tests) from T0/ 48h samples (average 0.4 and 0.45 cm bigger than control colonies) to 7/14 d samples (average 1.93 and 1.26 cm larger than control colonies). Colonies also increased swarming behaviour with most significant increases observed at 30°C after 7 d (average 2.75 cm) and 27°C after 14 d (average 2.38 cm). However, no significant increase in size was observed for temperature treatments. *V. coralliilyticus* colonies increased above control plate growth at T0 by an average of 1.18 cm with continued average increase after 48 h (see Table 5.1). However, after 7 d and 14 d growth of *V. coralliilyticus* declined at 25°C compared to continued increase at 27°C and 30°C, with significant differences found between temperature treatments (ANOVA F=3.41, p<0.05; Tukey tests indicating differences between 25°C and 27°C). Like *V. shiloi*, *V. coralliilyticus* also increased average size of colony to 2.52 cm (bigger than control) after 14d of exposure to 27°C.

V. harveyi showed significant differences in growth with time and temperature (Kruskal-Wallis, H=7.74, p<0.05; H=6.92, p<0.05 respectively) suggesting significantly less growth at 30°C and significantly more growth in T0 samples. All other marine vibrios in all treatments were maintained at less than 0.5 cm larger than control, with little increase to swarming. In contrast, a decline in growth was seen in all human pathogens compared to controls, with increases seen only after 48h at 30°C for *E. coli* 10777, *E. coli* 010418 and *P. aeruginosa* and at 14 d at all temperatures for *E. coli* 10777, *E. coli* 010418 and *S. aureus* 1803. Significant differences were found between samples from 14 d and the rest for these three strains (ANOVA F=23.81, p<0.001; F=4,31, p< 0.05; F=12.68, p<0.001 respectively).

In contrast, pathogens grown on Burkholder diffusion assays seeded with *Discosoma* extract showed different patterns. Primarily, swarming present in *V. coralliilyticus* at T0 and after 48 h

was not present after 7 d or 14 d. Growth also decreased significantly from all other time points in all temperature treatments to below control levels after 7 d (ANOVA, F=14.22, p<0.01) and although increased above the control after 14 d, was still reduced. *V. shiloi* had complicated reactions to *Discosoma* extracts but with no significant differences found between time and temperature treatments (ANOVA). However, increases in colony size after 48 h at 30°C were apparent as were increases in colony size after 7 d at 25°C and after 14 d at 25 and 27°C. Significant time effects on colony growth were seen for *V. harveyi*, with less growth after 14 d (ANOVA, F=15.55, p<0.001). While there was little change in colony size for any of the other *Vibrio* spp., *V. tubiashii* and *V. splendidus* produced extracellular products at T0 and after 48 d, which were only present at 30°C after 14 d for *V. splendidus*.

With respect to the human pathogens, patterns mimicked those of the *Sinularia* sp. tests. Growth was inhibited below control levels and only increased significantly after 14 d for *E. coli* 10777 (ANOVA, 2+log~e~transformed, F=8.73, p<0.001) and *S. aureus BRL* (Kruskal-Wallis, H=19.22, p<0.001), increasing more at 30°C. Further, while no swarming was recorded in any treatment for *P. aeruginosa* with *Sinularia* sp. extract, swarming was recorded in all *Discosoma* sp. treatments (except 25°C, 48 h) with more swarming occurring after 7 d and 14 d and a decrease in growth occurring at 25°C after 48 h and 7 d (ANOVA, F=6.22, p<0.005). Gorgonian extracts showed similar patterns for human pathogens, increasing in size at all temperatures after 14d, while marine pathogens showed some increases in growth.

5.3.7 Presence of proteolytic bacteria, haemolytic bacteria and *Vibrio* spp.

To further confirm patterns in Burkholder diffusion assays the presence of *Vibrio* spp. in coral extracts at different temperatures as well as the presence of haemolytic and proteolytic bacteria were assessed Unfortunately, total cultural bacterial counts were made impossible due to the colonisation of fungal spores on all the marine agar plates and thus counts of proteolytic and

haemolytic bacteria as well as *Vibrio* spp. were standardised to total protein content. Representative plates can be seen in Figure 5.11.

No significant differences were found in Kruskal-Wallis tests between numbers of proteolytic, haemolytic or vibrio colonies associated with *Sinularia* extracts from any time points or temperature treatment. However Figure, 5.12 shows increases in proteolytic bacterial colonies at 25°C after 48 h and 14 d, although the huge variation seen in these treatments render the results insignificant (Kruskal-Wallis, H=4.77, p=0.092). Haemolytic bacteria were found in all samples (except for after 14 d at 27°C) and showed some increases in certain treatments (25°C 28h and 14 d; 27°C 48 h and 30°C 7 d), however this was not correlated with temperature or time. Very few vibrio colonies grew throughout the experiment on *Sinularia* extracts, only present at 25°C after 48 h and at 30°C after 7 d.
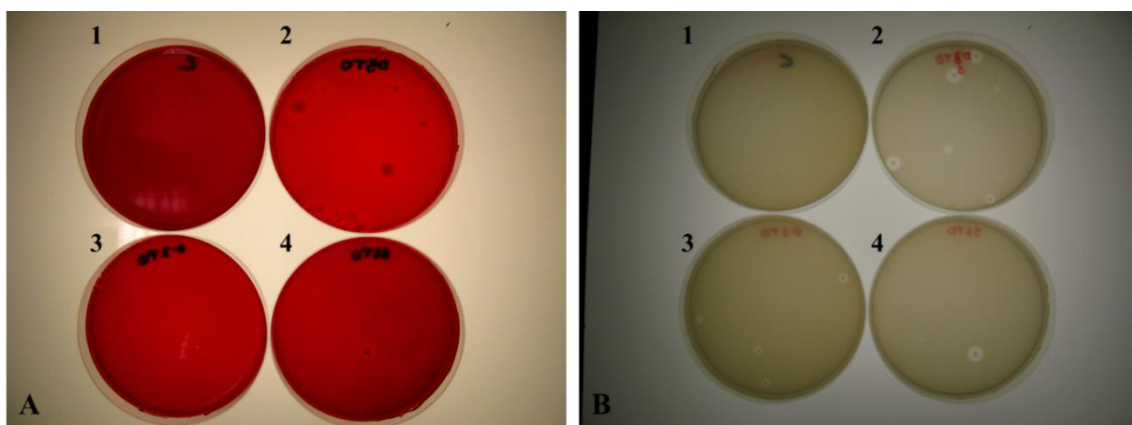
Figure 5.11 Bacterial assays to determine number of haemolytic (A) and proteolytic (B) bacteria present in coral extracts with temperature stress. Images show a control (1) and T0 samples from the *Discosoma* sp. (2), the Gorgonian (3) and the *Sinularia* sp. (4).

In contrast, large increases were observed in the presence of haemolytic and proteolytic bacteria grown on *Discosoma* extracts after 14 d at 30°C. However, again this was not related to temperature or time point alone and was insignificant due to the huge variability between samples. The same was found in haemolytic and proteolytic bacteria grown on gorgonian extract kept at 30°C for 14 d, however with significant results (Kruskal-Wallis, H=6.45, p<0.05; H=6.07, p<0.05, respectively).
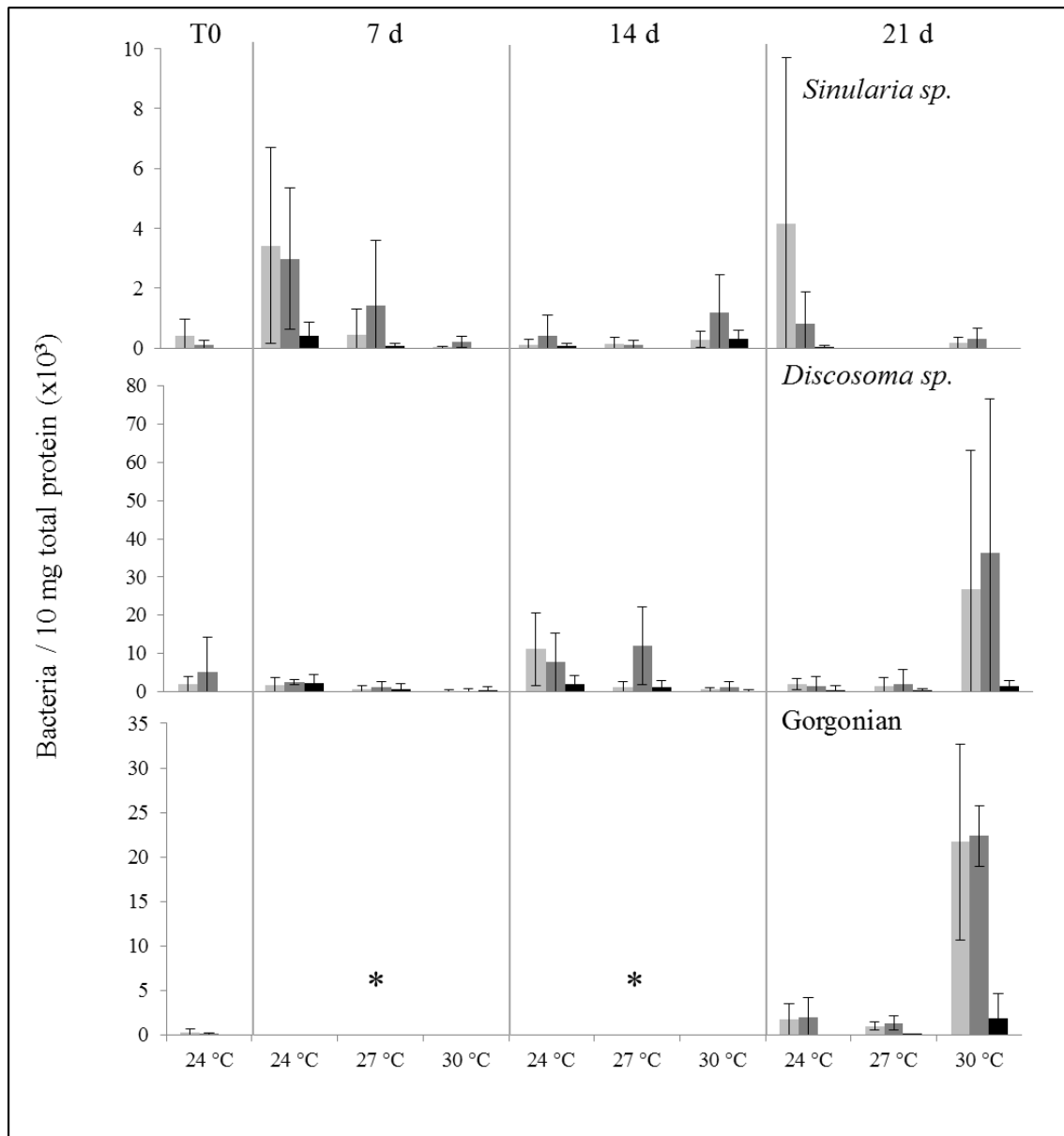
Figure 5.12 Number of culturable *Vibrio* spp. (black bars), haemolytic (dark grey bars) and proteolytic (light grey bars) colonies from coral extracts standardised to total protein content. Error bars represent standard deviation of three replicates. * denotes where no coral extracts were tested.

5.3.8 Bacterial community profiles using PCR-DGGE of 16S rRNA gene

Analysis of bacterial community structure in coral fragments was carried out for *Sinularia* sp. and *Discosoma* sp. only. *Sinularia* sp. PCR-DGGE can be seen in Figure 5.13; the bands successfully sequenced and their BLAST information are shown in Table 5.2. A number of bands were found in over 10 of the samples including successfully sequenced bands (Table 5.2)

and a number of un-sequenced bands including 148.49 (11), 200.87 (13), 207.04 (11), 212.18 (11), 216.09 (12), 221.09 (11), 246.49 (14) and 263.43 (11) (labelled in Figure 5.13). Multi-dimensional scaling and hierarchical clustering analysis was carried out to assess similarities in bacterial community structure between temperature treatments and time points using a Bray-Curtis resemblance matrix (Figure 5.14). Cluster analysis using a SIMPROF test shows no significant differences in community structure between any of the samples, as can be seen in the MDS plot with all the samples clustering together and no distinction between times and temperatures (Figure 5.14). However, in a SIMPER analysis looking at species contribution towards similarities within groups, band 221.09 contributes 60.61% similarity between T0 samples and contributes 17.55% to similarity between 23°C samples. In contrast, the top contributor at 27°C is the uncultured Bacteroidetes (GQ274072) and at 30°C, an uncultured bacterium (JF229945). However the uncultured Cyanobacterium (JF733403) is within the top three contributors for all temperature treatments, as well as at 7 d and 14 d time points but does not contribute highly in T0 and 48h samples. This suggests that although there is no significant change to community structure, some smaller shifts in bacterial community are occurring.
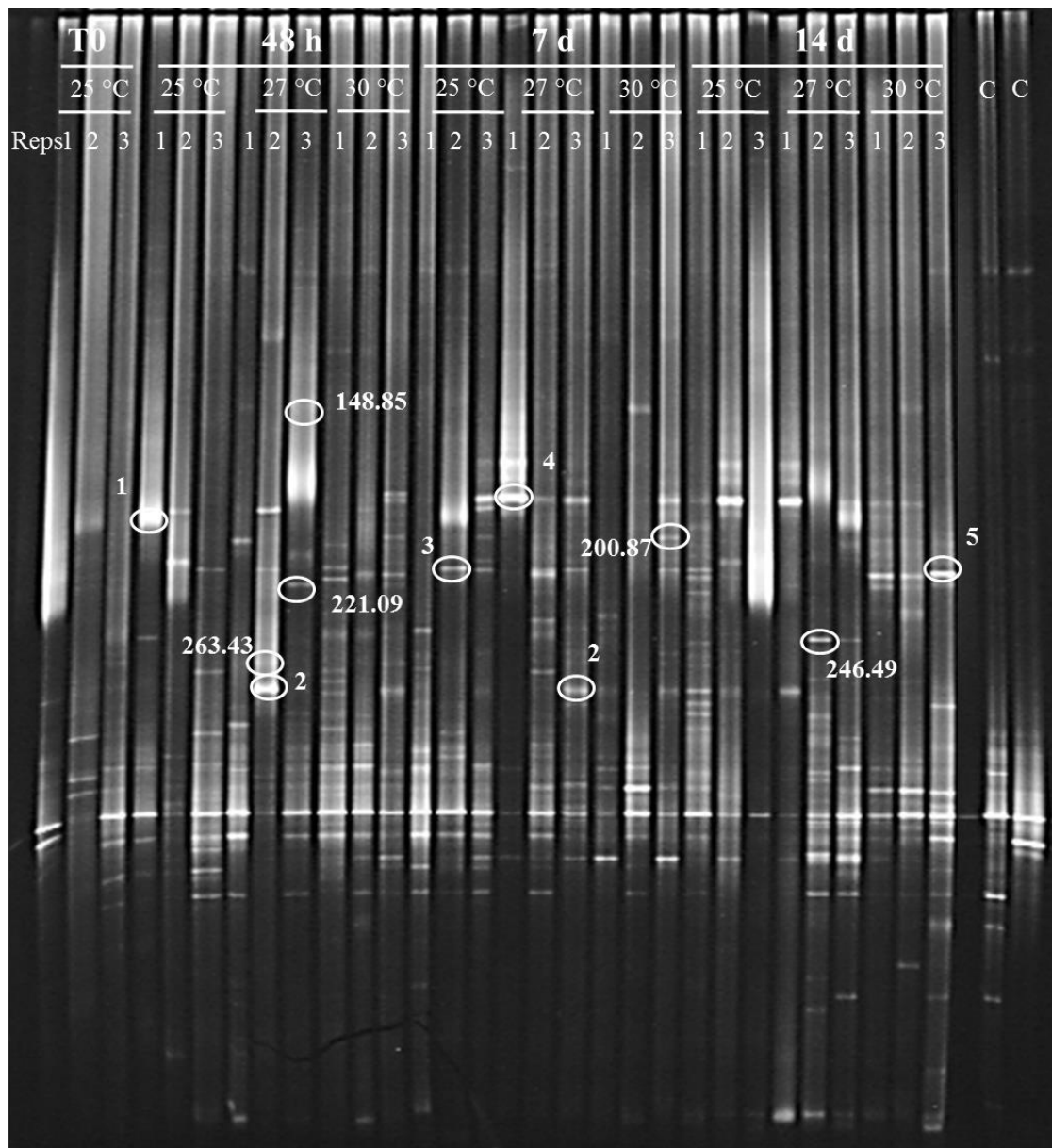
Figure 5.13 Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments extracted from *Sinularia* sp. DNA samples. Labels at the top of the image relate to sampling time and temperature treatment with lanes corresponding to replicates under each time. Circles and numbers (1-5) correspond to bands successfully excised, re-amplified, sequenced and run through the NCBI database to identify prominent bands (see table 5.2 for BLAST information). Other prominent bands appearing in >10 samples are also circled with their relative position on the gel labelled.
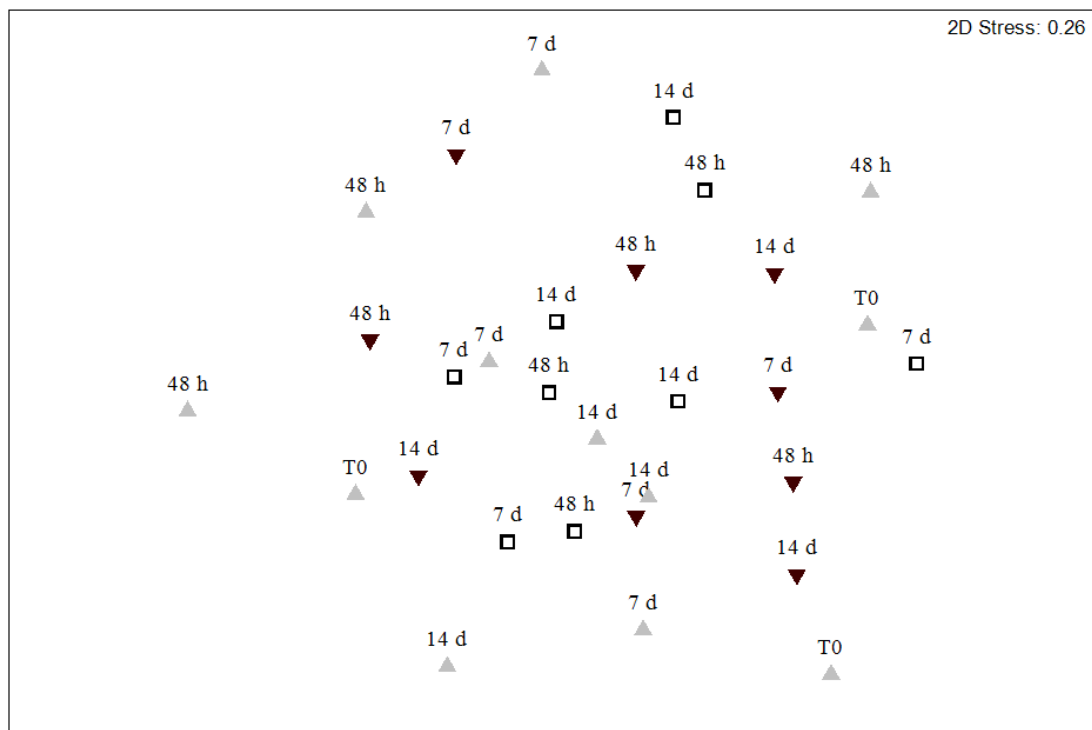
Figure 5.14 MDS comparing banding profiles of 16S rRNA gene fragments amplified *Sinularia* sp. fragments based on a Bray-Curtis resemblance matrix of presence and absence data. Labels represent time point and symbols represent temperature treatment 25°C (grey triangles), 27°C (black triangles) and 30°C (squares).

In contrast, more specific changes were seen between temperature treatments in *Discosoma* sp. bacterial community profiles. The *Discosoma* sp. PCR-DGGE can be seen in Figure 5.15 and BLAST information for successfully sequenced bands in Table 5.2. A number of sequenced bands were found in over 10 of the samples in addition to a number of un-sequenced bands including 145.77 (13) and 179.84 (28) (labelled in Figure 5.15).

Multi-dimensional scaling and hierarchical clustering analysis was carried out as for *Discosoma* samples (Figure 5.16). Cluster analysis using a SIMPROF test shows significant differences in community structure between group to the left and the right of the MDS plot (Figure 5.16). This plot shows many of the 27°C samples to the right of the plot (black triangles) with 23°C samples clustered to the left (grey triangles) and 30°C samples spread across both groups.

However at 60% similarity 30°C samples cluster together away from 25°C samples. In a bubble plot analysis to establish the presence of certain bands in different samples, it was observed that the uncultured Mollicute (EF137401), the uncultured Chloroflexi (EF07623) and the uncultured bacterium (JQ415965) were found in the left hand group (on the MDS plot) and in the two 30°C samples on the right, whereas the uncultured marine bacterium (GU317735), the uncultured Bacteroidetes (EU544843), and the uncultured coral-associated bacterium (FJ930173) were found across both groups. See Figure 5.17 for examples. This suggests that although the community changed, key groups were still present throughout. A number of un-sequenced bands were responsible for the separation of the right-hand group in the MDS plot (n=5).
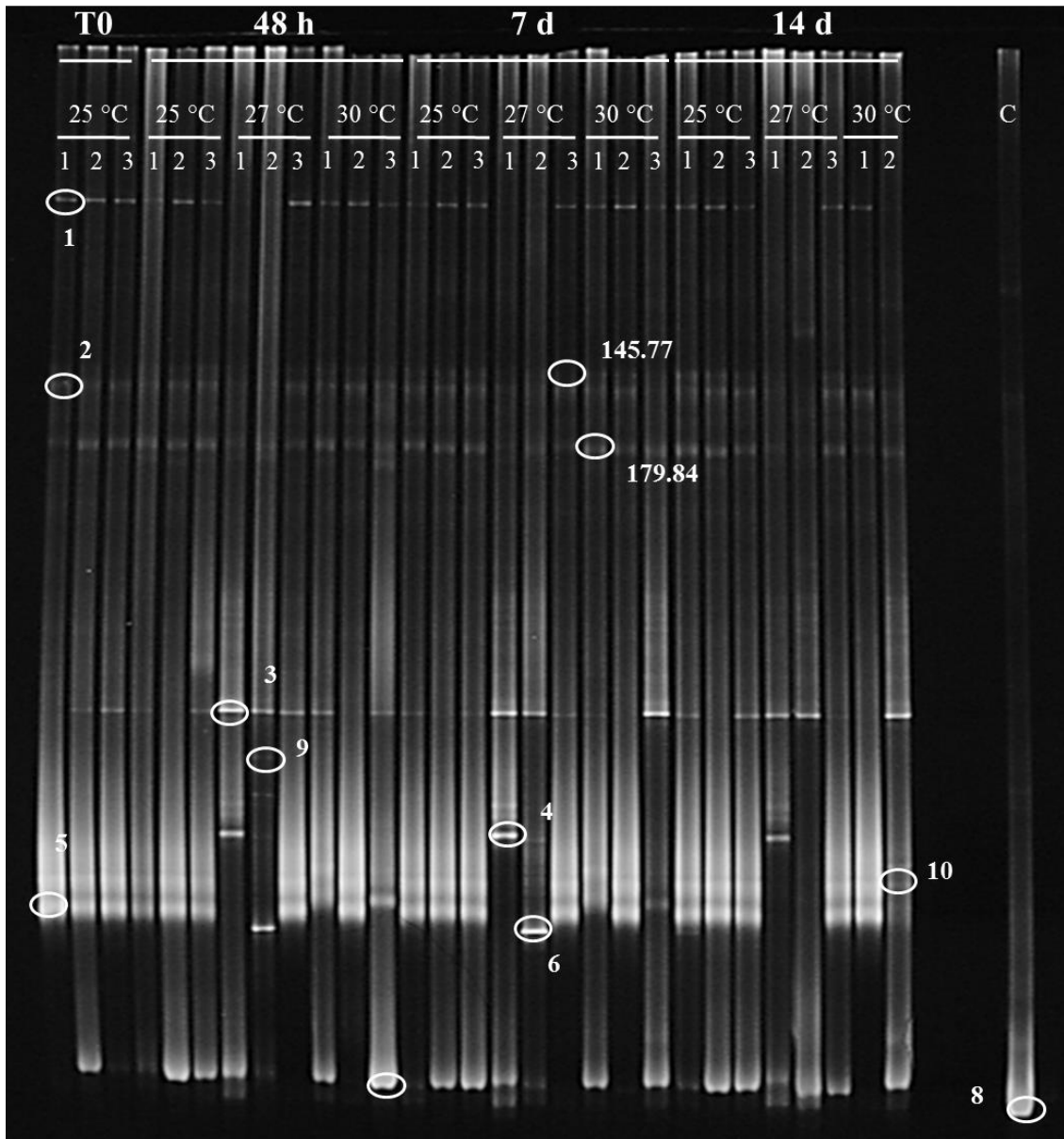
Figure 5.15 Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments extracted from *Discosoma* individuals DNA samples. Labels at the top of the image relate to sampling time and temperature treatment with lanes corresponding to replicates under each time. Circles and numbers (1-10) correspond to bands successfully excised, re-amplified, sequenced and run through the NCBI database to identify prominent bands (see table 5.2). Other prominent bands appearing in >10 samples are also circled with their relative position on the gel labelled.
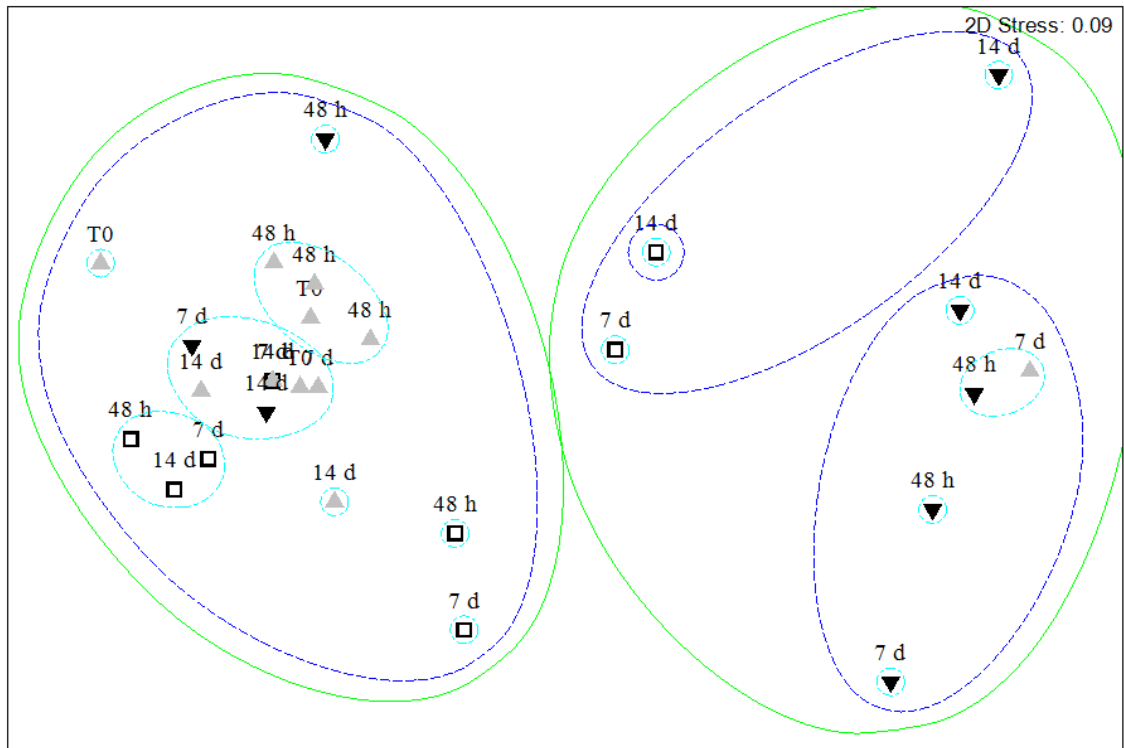
Figure 5.16 MDS comparing banding profiles of 16S rRNA gene fragments amplified *Discosoma* individuals based on a Bray-Curtis resemblance matrix of presence and absence data. Labels represent time point and symbols represent temperature treatment 25°C (grey triangles), 27°C (black triangles) and 30°C (squares). Clusters denote similarity between samples at 20, 40 and 60% based on a hierarchical clustering analysis.
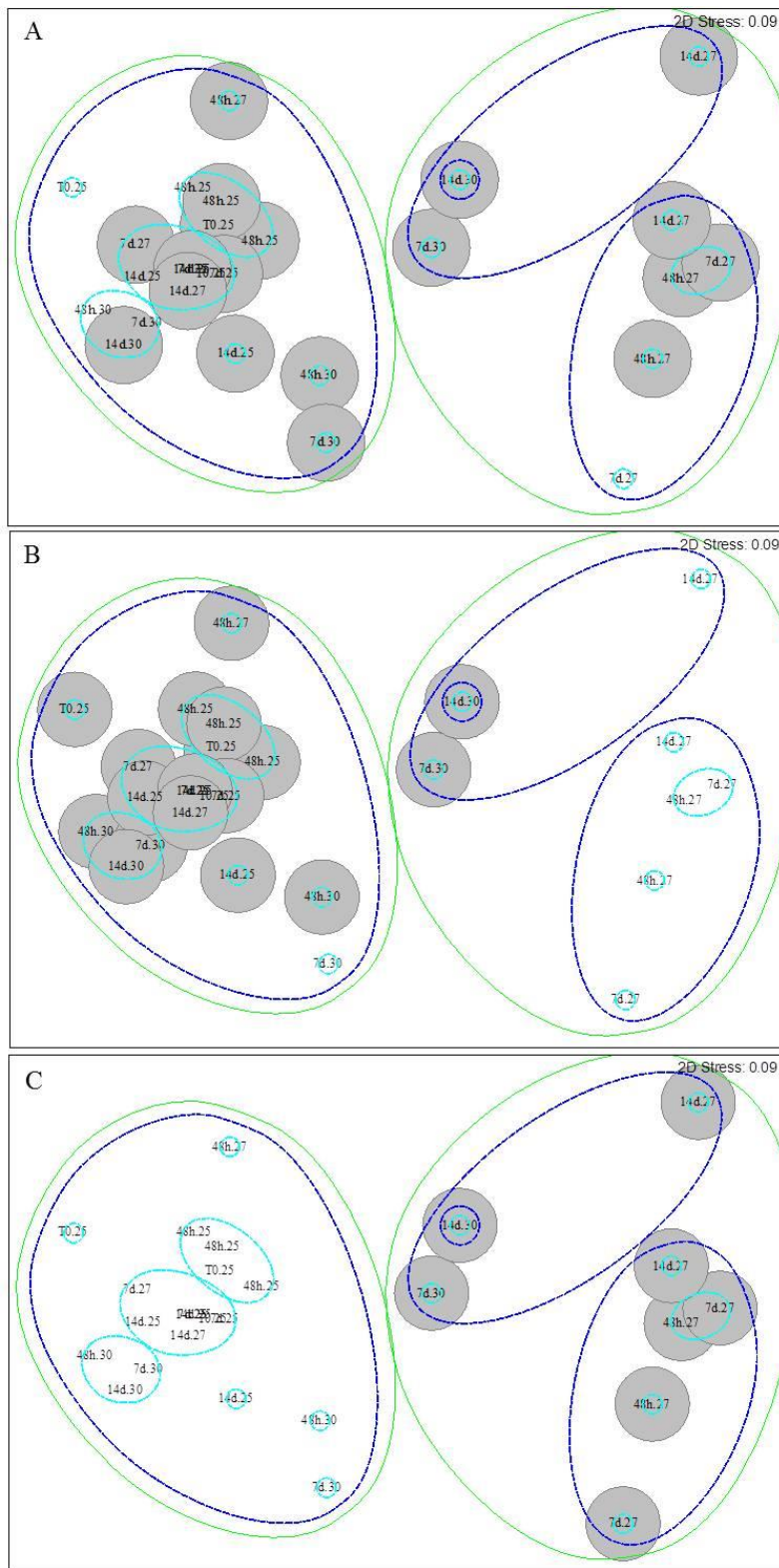
Figure 5.17 MDS as above (Figure 7.16) showing examples of bacterial species spread across MDS groupings, representing the uncultured bacterium (JQ415965; A), the uncultured Bacteroidetes (EU544843; B) and an unknown band (299.01; C).

In a SIMPER analysis, showing similarity within and dissimilarity between groups, the uncultured marine bacterium (GU317735) came within the top three species for contributing to similarity within all temperature treatments (13.52–15.08%) and time points (11.43–17.7%), suggesting the importance of this species in *Discosoma* bacterial communities. Further, this band remained constant throughout all but three samples from 27°C and 30°C treatments after 7 d and 14 d, possibly suggesting the beginning to the decline of these bacteria. This bacterium contributed to 17.7% of similarity at T0 and only 11.43% of similarity after 14 d. Band 179.84 was also found within the top three contributors towards similarity within all temperature treatments (14.53-19.38%) and time points (14.57-17.7%). For temperature treatments the third group differed between the uncultured Chloroflexi (EF07623) at 23°C and 30°C (13.37 and 14.53% respectively) and the uncultured Bacteroidetes (EU544843) at 27°C (16.52%). For the time points, the uncultured Chloroflexi (EF07623) helps defines similarity in T0 and 48h samples (17.7 and 9.57% respectively), while the uncultured coral-associated bacterium (FJ930173) does the same in 7 d and 14 d samples (14.24 and 14.12 respectively). This suggests that there is a shift in important contributors over time and temperature but that the presence of top contributors remains relatively stable.

Table 5.2 Most common affiliations in the NCBI database for bands successfully excised, re-amplified and sequenced from 16S rRNA PCR-DGGE of DNA extracts from *Sinularia* sp. and *Discosoma* sp..

Origin	Band number	Frequency in samples	Top blast hit	Accession number	Sequence similarity (%)
Sinularia sp.	1	14/30	*Geobacillus sp.*	JN867331	98
Sinularia sp.	2	18/30	uncultured Bacteroidetes	GQ274072	93
Sinularia sp.	3	12/30	uncultured bacterium	JF229945	99
Sinularia sp.	4	15/30	uncultured Cyanobacterium	JF733403	98
Sinularia sp.	5	11/30	uncultured bacterium	HQ225311	99
Discosoma sp.	1	23/30	uncultured Chloroflexi	EF076231	92
Discosoma sp.	2	27/30	uncultured marine bacterium	GU317735	91
Discosoma sp.	3	22/30	uncultured Bacteroidetes	EU544843	85
Discosoma sp.	4	1/30	uncultured Bacillus	AB550471	97
Discosoma sp.	5	18/30	uncultured Mollicutes	EF137401	82
Discosoma sp.	6	3/30	uncultured Deltaproteobacterium	EF414153	94
Discosoma sp.	7	23/30	uncultured coral bacterium	FJ930173	98
Discosoma sp.	8	Control	*Deftia sp.*	JF274927	99
Discosoma sp.	9	7/30	uncultured Bacillus	AB550471	97
Discosoma sp.	10	21/30	uncultured bacterium	JQ415965	99

5.3.9 Assessment of overall coral stress with zooxanthellae counts and chlorophyll analysis

To gain a measure of the effects of temperature and experimental time on the coral holobiont, the numbers of zooxanthellae and the amount of chlorophyll produced in the coral tissue were investigated. *Sinularia* fragments (Figure 5.18A) showed significant differences between 25°C and 30°C temperature treatments (ANOVA; F=4.12, p<0.05; Tukey tests) with 25°C treatments having significantly more zooxanthellae. There was also a time effect with T0 having significantly more zooxanthellae associated with *Sinularia* tissue than after 48 h and 14 d (ANOVA; F=4.89, p<0.01). Although there was no effect of temperature on chlorophyll

production by zooxanthellae, there was a significant difference found between samples from 7 d and 14 d (ANOVA; F=4.85, p<0.01). In comparison, no differences were found with time or temperature in *Discosoma* samples (Figure 5.18B). However, the numbers of zooxanthellae associated with *Discosoma* individuals was extremely variable, possibly suggesting that using protein content to standardise for this parameter is not viable.
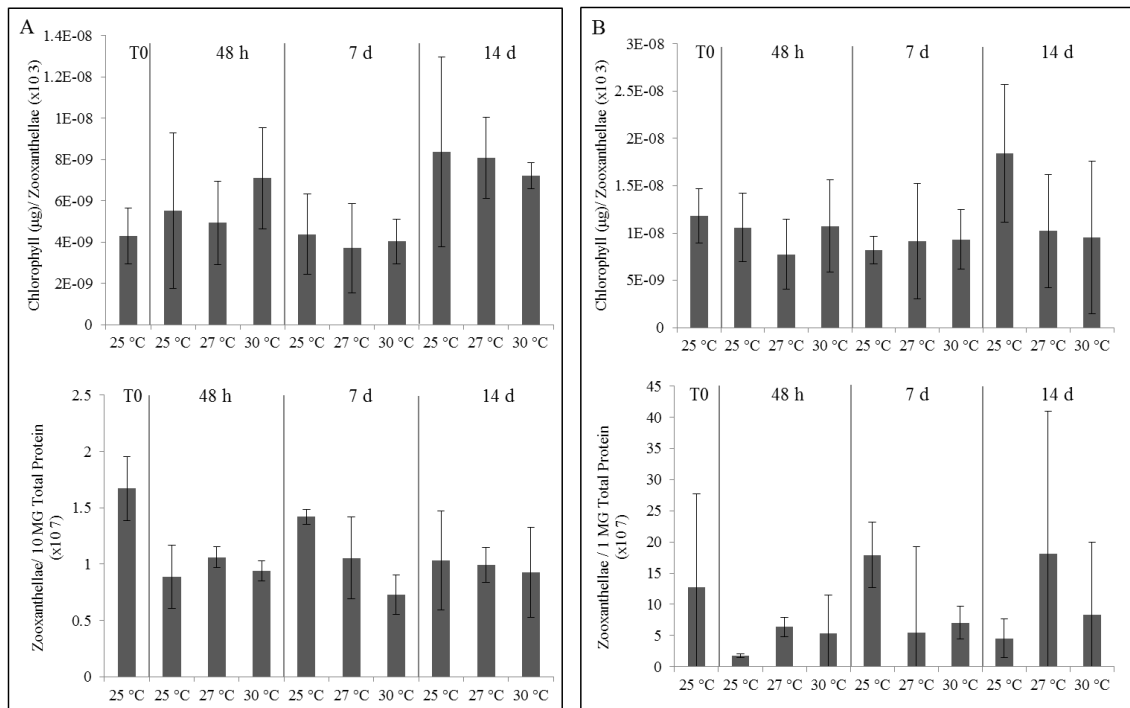
Figure 5.18 Average chlorophyll production per zooxanthellae and average zooxanthellae numbers per 1mg total protein for S*inularia* fragments (A) and *Discosoma* individuals (B) over the experimental period.

5.3.10 Effects of covariates on AHL production and degradation in *Sinularia* sp. fragments

Due to the lack of trends in AHL production with both temperature and time, further correlations between signalling in *Sinularia* sp. and the rest of the factors measured including parent colony, feeding behaviour of coral fragments, differences in water chemistry, tank effects, number of zooxanthellae, chlorophyll production of zooxanthellae, the ability of coral

extracts to degrade AHLs and protein content were investigated. Effects of time point and temperature (fixed factors) upon AHL signalling profiles was tested using Type I sums of squares covariate PERMANOVA with normalised environmental factors as covariates. To avoid issues of confounding type I error and multiplicity of P-values, covariates were added one at a time to the model. None of these covariates generated significant interactions with the main effects or were significant linear predictors of AHL signalling. RELATE also tested for a relationship between AHL-signal profiles and DGGE banding patterns, but with no significant results.

5.4 Discussion

Understanding the ecology and function of coral-associated bacterial communities has become increasingly important as a result of the global deterioration of coral species due to climate change (Bourne et al., 2009) and disease (Ward & Lafferty, 2004). With evidence that bacterial associates and the coral itself produce compounds that have the ability to affect bacteria (Kvennefors et al., 2008; Nissimov et al., 2009; Ritchie, 2006; Shnit-Orland et al., 2009; Vizcaino et al., 2010), a greater understanding of how bacteria interact with their host and how increasing temperature leads to an environment that allows proliferation of potentially harmful bacteria is needed. As a result, this study brought together a number of methodologies to attempt to further investigate the effects of temperature on AHL signalling, AHL degradation and bacterial dynamics in three species of coral. The three species of coral used in this study will be discussed separately.

5.4.1 Bacterial dynamics in an alcyonacean soft coral: *Sinularia* sp.

The presence of a number of putative AHLs in the *Sinularia* sp. tested supports the potential role for AHLs in signalling. However, the lack of stability of AHL profiles seen in this species was different to all the corals tested to date (chapters 3 & 4), with the NTL4 bioassay detecting

no putative AHLs in some fragments, unrelated to temperature or time treatments (except for a significant increase in spot 2 at T0 and 7 days). In addition, a number of tail shapes were seen in TLC profiles suggesting the presence of a 3-oxo- or 3-hydroxy substitution on the AHL compound (Gram et al., 2002; Golberg et al., 2011), which may represent a difference in the functional and taxonomic specificity of the AHL compared to one with the same migration difference without a tail (Hmelo et al., 2009).

Statistical analysis has shown no correlation with any of the covariates measured, which suggests that in this coral, bacterial signalling is not stable and is not affected by nutrients, feeding behaviour of corals, parent colony, tank effect, zooxanthellae numbers or bacterial community patterns or standardisation to protein content. This study also showed no significant change in AHL-degradation, bacterial community or presence of proteolytic or haemolytic bacteria or of *Vibrio* sp. over temperature treatments or time points, suggesting that temperatures in the ranges seen in this experiment have no effect. S*inularia flexibilis* has been found to be rarely overgrown with bacteria, a trait attributed to the presence of many diterpenes that this coral produces (Aceret et al., 1995; Aceret et al., 1998). Some of these diterpenes have been shown to have antibacterial properties (Aceret et al., 1998), suggesting that this genus of coral may not harbour as diverse a community as found in other corals and may not rely on the bacterial component for certain phenotypes to maintain the coral holobiont, as suggested in other species.

However, there is also some evidence that some bacteria isolated from *Sinularia* sp., such as *Arthrobacter nicotianae*, may have an ecological role in deterring marine biofilm-forming bacteria such as *Vibrio harveyi* (Radjasa & Sabdono, 2008) as well as isolates closely related to a *Pseudovibrio* sp., a member of the Alphaproteobacteria and a *Microbulbifer* sp. showing activity against strains of known multi-drug resistant bacteria; *S. aureus*, *E. coli* and *Enterobacter* sp. (Sulistiyani et al., 2010). This evidence suggests that there are bacteria found

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on the surface of some *Sinularia* sp. with antibacterial activity; therefore it is possible that its bacterial population is controlled differently to other corals, with mechanisms that are not affected by increasing temperatures. Indeed, differences in the prevalence of antibacterial activity by coral isolates have been found between corals (Kelman et al., 2006; Shnit-Orland et al., 2009), authors suggesting that different mechanisms of antimicrobial activity may be at play in different species. However, to date there have been no studies investigating the stability of such bacterial communities with *Sinularia* sp. in the natural environment and thus they may not be considered associates. It is also possible that 30°C is below the threshold of stress for this species. While some changes in zooxanthellae numbers were observed in this experiment, this was not replicated over all the time points. *Sinularia* sp. in the wild have been found to have intermediate levels of bleaching tolerance to elevated temperatures, surviving prolonged exposures at 32°C but dying within 24h at 34°C (Strychar et al., 2005). However, information regarding temperature-induced bleaching of alcyonacean soft corals is very limited (Strychar et al., 2005). As a species of coral whose bacterial community does not appear to be affected by temperature to the same degree as others (chapter 4 & 5), *Sinularia* and other alcyonacean soft corals merit further investigation in comparison to other more vulnerable coral species.

5.4.2 Bacterial dynamics in a gorgonian

The lack of activation of the biosensor by gorgonian extracts would suggest that this species of coral has very little bacterial activity associated with it, however the amount of soft tissue available for extraction was minimal in this species and although extracts were standardised to the same protein concentration as the *Discosoma* sp., the physiology of these two species is very different. Some gorgonian corals are known to have very thin coenchymes (Wahle et al., 1983; Coma et al., 1995) and less mucus secretory cells (Mullen et al., 2004) which is likely to mean less space and/ or nutrients available for bacterial colonisation. Although species of gorgonian have been found to have a conserved bacterial community associated with their tissue, which is altered during disease (chapter 2), it may be that these communities act on microscales within

the coral holobiont and thus concentrations of AHLs present in tissues are relatively low compared to that seen in other corals.

In contrast, crude gorgonian extracts were found to be extremely high degraders of AHLs, at approximately 30% throughout the experimental period, with no differences found at high temperatures and with a much more dilute sample than the *Sinularia* sp.. This suggests that this species may employ efficient QQ as a method for controlling QS. Future research should determine the source of such high levels of AHL degradation in this species (whether from the host or the natural bacterial community). This is particularly important in light of the conserved bacterial communities found in *Eunicella verrucosa* containing high percentages of particular genera of bacteria (*Spongiobacter* sp. and *Endozoicomonas* sp.), also found associating with a number of other marine invertebrates (Martinez-Garcia et al., 2006; Schuett et al., 2007) and corals (Bourne et al., 2007; Yang et al., 2010; Kurahashi & Yokoto, 2006; Littman et al., 2009).

Finally, the sustained ability of gorgonian extracts to degrade AHLs seen at 30°C is particularly interesting considering the increase in proteolytic, haemolytic and *Vibrio* spp. seen after 14 days at 30°C. Although the coral holobiont has a continued ability to degrade AHLs as crude slurry, potentially harmful bacteria are still capable of settling upon this species of coral, suggesting that AHL-QQ is not enough to deter settlement of invasive bacteria. However, it is also be possible that these bacteria have settled on the surface of the gorgonian fragment, not employing QS to do so and that they will not be able to express virulence genes as reaching their 'quorum' is kept in check by QQ activity of the holobiont.

5.4.3 Bacterial dynamics in a Corallimorph: *Discosoma* sp.

5.4.3.1 Presence and temperature effects on AHLs

The presence of a number of putative AHLs in *Discosoma* individuals confirm previous patterns of detection found in a range of other cnidarians (shown in Chapters 3 & 4). This stability of AHL signalling suggests the presence of a core bacterial community and potentially an important role for the long-chain AHLs in this *Discosoma* sp.. Interestingly these signals remained stable in all temperature treatments, suggesting that increased temperatures to an average of 27.4°C and 29.7°C, above the control of 25.4°C, does not directly affect the ability of these coral bacterial associates to produce AHL signals. However, differences seen in the quantity of spot 3 at different time points suggest that there may be some fluctuations in the AHL-producing community over time. In addition, there was no difference to the overall quantity of AHLs detected between acidified and non-acidified samples at various temperatures. However, less pH lactonolysis was seen at 48h and 7d for the short-chain AHL detected (spot 1). This may be due to the greater pH ranges seen between tanks at these time-points, causing slight deviations to the amount of AHL being affected by pH-lactonolysis. Bacterial AHL signals can be inactivated by pH-lactonolysis at pH-levels above 7 (Rasmussen & Givskov 2006), which leads to opening of the lactone ring and inactivation of signals, especially of short-chain AHLs. Yates and colleagues (2002) showed this pH-lactonolysis to be temperature dependent in *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis*, with increased degradation when temperatures were raised from 22 to 37°C. Further, Tait and colleagues (2005) observed AHLs to be degraded more rapidly in seawater from 5°C to 20°C, but at 37°C degradation reached similar levels to 20°C samples; although no data are available for temperatures in between. However, this study suggests that there is no effect of temperature, within these ranges, on pH-dependent lactonolysis and suggests that even small fluctuations in pH may affect at least short-chain AHLs, which are known to be more susceptible to pH-lactonolysis (Yates et al., 2002). Further the presence of oxo and hydroxyl groups, which may contribute to the quantity of short-chain AHLs seen in *Discosoma* extracts could be responsible

for the differences seen, as they are known to be more prone to pH-lactonolysis than their un-substituted forms (Hmelo et al., 2009).

5.4.3.2 Decreasing degradation of AHLs by *Discosoma* sp. extracts with time and temperature

Although this study showed little change in AHL signalling within *Discosoma* extracts, the ability of *Discosoma* extracts to degrade AHLs was significantly reduced in 27°C and 30°C treatments as well as with the experiment's progression. This study suggests that quorum quenching (QQ) or the degradation of AHLs by *Discosoma* extracts is affected by increasing temperatures and experimental conditions. As no significant change in AHLs was observed, the potential for these effects to be a result of increased quantities of AHLs in certain extracts is unlikely. However, it may be possible that increases in AHLs (above control values), may be the result of methodological differences. While AHL production profiles on TLC plates were concentrated in dichloromethane extractions, for degradation assays synthetic AHLs were added to coral homogenate (including bacteria). It is possible that the presence of AHLs simulated the production of AHLs in the bacteria present in the sample, resulting in values above the control, however the increase is surprising considering the small amount of homogenate used.

Such QQ could be a characteristic of *Discosoma*-associated bacteria or of the coral host. QQ has recently been found to occur in 23% of 284 extracts of marine organisms from the Great Barrier Reef, including in corals (Skindersoe et al., 2008), however the origin of this degradation was not investigated. According to published data, most of the known QS inhibitors have been isolated from bacteria, but this may be due to sampling effort (Dobretsov et al., 2009). Bacterial AHL signals can be supressed by the degradation of QS signals (Dong et al., 2002) but also via the inhibition of signal generation (Rasmussen & Givskov, 2006) or DNA transcription (Zhang & Dong, 2004). As already discussed, bacterial AHLs can be inactivated by pH-lactonolysis, but also by two main groups of enzymes identified in number of bacterial species; AHL-lactonase and AHL-acylase as well as by novel oxidoreductase activity as described by Uroz

and colleagues (2007). AHL-lactonases catalyse ring opening in AHLs by hydrolysing the lactone ring of AHLs rendering them inactive (Dong et al., 2002; Dong & Zhang, 2005), of which one, AiiA, has been found in members of the genus *Bacillus*, in *Arthrobacter* sp. and in *Agrobacterium tumefaciens* (Dong et al., 2002). AHL-acylases in comparison cleave the acyl chain from the lactone ring also rendering signals inactive (Zhang & Dong et al., 2004). Although the ecological function of these enzymes is not yet understood, they are thought to interrupt QS in other species to mediate the competition for space and food (Dobretsov et al., 2009), to help regulate their own QS (Sio et al., 2006) and to utilise signals from other bacteria as sources of carbon, nitrogen and energy (Flagan et al., 2003; Uroz et al., 2007). However, it has been suggested that the true role of lactonases is to disrupt γ-butyrolactone signalling by the actinomycetes in soils (Khan & Farrand, 2009). In addition, both lactonases and acylases have homology to the antibiotic resistance gene products for beta-lactams (Dong et al., 2007) and cephalosporin acylases (Dong et al., 2007; Park et al., 2005), respectively. In the marine environment, information on the prevalence of QQ in bacteria is scarce except for two recent studies, where Romero and colleagues (2011) showed that 14.4% of strains isolated from dense marine microbial communities were able to reduce AHL activity, many of which were confirmed as a result of enzyme activity, and Tait and colleagues (2009), who show AHL-degrading bacteria quenching AHL signals in neighbouring bacteria which in turn was found to modulate *Ulva* zoospore settlement. The high QQ found in all of the corals tested in this study corroborates these studies and suggests an ecological role for bacterial QQ in the marine environment.

However, eukaryotes also have the ability to affect QS. While it is more likely that AHL-degradation has been detected in this study, the possibility that a compound produced by the corals may have been interfering with the bioreportor used in this study should be noted. A number of eukaryotes are known to produce enzymes and AHL mimics that degrade or effect bacterial QS signals. Enzymes have been found to be produced by human airway epithelial cells (Chun et al., 2004) and include enzymes such as porcine kidney acylase I (Xu et al., 2003). In

the red alga *Delisea pulchra* the halogenated furanones were the first example of interference with bacterial signalling (Rasmussen et al., 2000). These molecules are now known to competitively bind to the nascent polypeptide chain of AHL receptors (Lux-R), preventing correct folding of receptor proteins and increasing turnover (Manefield et al., 2002), consequently they cannot bind AHLs and the positive feedback loop for producing more AHLs is not activated, disrupting AHL production and QS regulation (Koch et al., 2005). Manoalide monoacetate from the marine sponge *Luffariella variabilis* has also been found to disrupt bacterial QS but to date the mode of action has not been determined (Skindersoe et al., 2008). Other eukaryotes mimic the activity of AHLs, such as compounds produced by the unicellular chlorophyte, *Chlamydomonas reinhardtii* and can have a stimulatory, inhibitory or additive effect on QS (Teplitski et al., 2004). However, there is some doubt that these molecules function to disrupt QS as the nature of the molecules remains unknown. It is thought that all these compounds help to control the types of bacteria that colonise the surfaces of hosts such as *D. pulchra*, which can switch the bacterial community associated with its fronds from being dominated by Gram-negative bacteria to Gram-positive bacteria (Dworjanyn et al., 1999; 2006).

The effect of temperature and experimental conditions on the ability of *Discosoma* extracts to degrade AHLs could therefore be three-fold; the stability of QQ enzymes or compounds from bacteria or coral host could be affected, as could the ability of the coral host or bacteria to produce QQ enzymes or compounds, however never before has the effect of temperature on AHL degradation been investigated. The change in QQ could also be the result of a changing bacterial community. This could potentially lead to the proliferation of a range of bacteria and allow QS signals, including those controlling virulence factors, to reach their 'quorum threshold' in potential pathogens. However, information on QQ processes in the marine environment is scarce (Romero et al., 2011). Hmelo & Van Mooy (2009) suggest that increased degradation of AHL signals in seawater as opposed to artificial water may be due to the presence of QQ activity but further research needs to address these unknowns to further

understand the progression of bacterial community breakdown with temperature stress and the importance of this finding on coral health.

5.4.3.3 Bacterial communities associated with *Discosoma* sp. with time and temperature

To link the changes in AHL degradation and patterns in AHL signalling to the bacterial community, denaturing gradient gel electrophoresis was carried out with Sanger sequencing to identify the main bacterial groups present, alongside plate assays to determine numbers of culturable proteolytic and haemolytic bacteria and *Vibrio* sp. across the treatments. DGGE profiles of bacterial communities associated with *Discosoma* sp. suggest a shift in the bacterial community after only 48h under experimental conditions in some fragments. Although statistically different bacterial communities were observed in some individuals from 27°C and 30°C a number of OTUs were found across these communities such as a band identified as closely related to the uncultured bacterium isolated from the coral *Porites compressa* (FJ930173) with secondary similarity to a member of the Alphaproeobacteria isolated from an *Acropora* sp. (AB571945). A band closely related to an uncultured Bacteroidetes (EU544843) and a band closely related to another uncultured marine bacterium (GU317735) were also found across the groups. The first member of Alphaproteobacteria found to utilise quorum sensing was a *Roseobacter* sp. isolated from marine snow (Gram et al., 2002). QS is now known to be widespread in this class, with 36 out of 67 Alphaproteobacteria testing positive for QS in a more recent study (Wagner-Döbler et al., 2005). AHL-signalling has lately also been observed in the Bacteroidetes, as already discussed (Huang et al., 2008; Romero et al., 2010). The stability of core members of the bacterial community associated with *Discosoma* tissue and the presence of a relatively stable AHL-profile throughout this study suggests that temperature ranges from 25.4 to 29.7°C do not disrupt the coral holobiont enough to see a change in bacterial community or AHL-signalling profiles.

There were distinct changes in the ability of extracts to degrade synthetic AHLs after 48h, which relates to changes in communities seen in DGGE profiles. Enzymatic degradation of AHLs has recently been found in nine difference genera of marine bacteria including members of the Alpha- and Gamma-proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Romero et al., 2011), members of which were identified from sequenced DGGE bands in this study. The decrease to QQ seen prior to any change in QS suggests that an initial breakdown in *Discosoma*-associated bacterial communities may be the result of changes in QQ potential of the holobiont, which may initiate further changes to bacterial communities and QS. *Discosoma* individuals also see an increase in proteolytic and haemolytic bacteria after 14 d at 30°C suggesting that the coral holobiont may not be capable of keeping potential pathogens at bay. Bacteria producing proteolytic enzymes have been found to increase in diseased coral tissue (Hall-Spencer et al., 2007) and proteolytic activity has been diagnosed as a possible component of the aetiology of some coral diseases, such as White Syndrome (Sussman et al., 2008). In a number of bacteria, protease expression is positively regulated by quorum sensing, including *Erwinia carotovora* (Jones et al., 1993; Pirhonen et al., 1993) and *Pseudomonas aeruginosa* (Gambello et al., 1991; Jones et al. 1993), which suggests that changes in QQ may be one factor that affects the ability of *Discosoma* sp. to deter potentially harmful bacteria from settling, in an otherwise controlled environment.

5.4.3.4 Antagonism of *Discosoma* extracts on potential pathogens

Using Burkholder assays to determine antagonistic interactions between coral associated bacteria and potential pathogens has been used in other studies (e.g. Rypien et al., 2010). In this study, assays were altered to include crude coral extract in agar plates to determine growth of the potential pathogens in the presence of extracts. The result of this study adds weight to the complex nature of the coral holobiont and the range of responses that different bacteria will have to them. However, an increase in growth was seen in two human pathogens at 25°C and more so at 27°C and 30°C after 14 d, where at all other time points all human pathogens were

inhibited, which links in with the decrease to QQ at all temperatures after 14d and the increase in proteolytic bacteria seen at 30°C. Potential marine pathogens however grew above control levels suggesting that they utilise compounds within *Discosoma* extracts for growth. Mucus from the coral *Acropora millepora* has also been found to support the growth of many marine bacterial isolates, including potential pathogens (Kvennefors et al., 2012). Similarly, isolates from the marine environment not readily observed on coral grew well on sterilised coral mucus, particularly including *Vibrio* spp. (Sharon & Rosenberg, 2008) which implies that growth previously inhibited by the coral holobiont *in situ* is permitted once the mucus is removed. In their study, Sharon and Rosenberg (2008) sterilised the mucus, however disruption due to homogenisation may have had a similar effect. Depending on the pathogen, different patterns emerge which may be a result of differing virulence pathways and the way they are affected by the chemical makeup of the extracts. Further studies need to employ a range of techniques, including isolation of coral bacteria to test for the effects of sterile and non-sterile temperature treated coral extracts on naturally occurring bacterial isolates.

5.4.3.5 Zooxanthellae numbers and chlorophyll production as a proxy of host stress

As previously discussed, bleaching or the loss of pigmentation due to a decrease in the number of symbiotic algae (zooxanthellae) has been largely correlated with elevated sea surface temperatures (Glyyn, 1993; Brown, 1997) in reef-building corals and has been observed where average daily sea water temperatures exceed the mean summer temperature by as little as 1–2°C, as discussed previously. However, this study confirms previous results (chapter 4; Hoegh-Guldeberg & Smith, 1989) that no decrease in zooxanthellae numbers or chlorophyll production could be seen at 30°C, suggesting that 30°C may be below the threshold of severe expulsion of zooxanthellae in this *Discosoma* sp.. Further, using total protein content of *Discosoma* individuals to standardise zooxanthellae numbers may not be as accurate as assessing surface area, used by many other studies (chapter 4), hypothesised by the widely variable results seen in this study.

5.4.4 Limitations of experimental studies on assessing coral-associated bacterial populations

One of the overarching goals of marine ecology is to better understand the roles that environmental and anthropogenic factors have on the marine environment and the species that live within it. The use of experimental studies to investigate impacts of specific environmental factors on marine species are important, however have to be approached with care. Coral physiological status and environmental parameters such as irradiation and available nutrients are known to play a role in the stability and composition of the mucus layer (Brown & Bythell, 2005) and therefore it is likely that these also affect the bacteria associated with this layer. It is therefore not surprising that with the adaptation to aquarium conditions, Kooperman et al. (2007) observed a change in bacterial communities associated with *Fungia granulosa* kept in a closed-system aquarium environment when compared to natural colonies. They observed significantly lower diversity of bacteria in the aquarium setting and a loss of several groups, including the Actinobacteria and the Cyanobacteria as well as an increase in the Betaproteobacteria.

In this experiment increases in nutrients were seen above those that corals in their natural environment would be exposed to. Inorganic nutrients have been shown to exacerbate diseases of corals (Marubini & Davies, 1996; Fabricius, 2005; Voss & Richardson, 2006), such as yellow band disease of *Monastraea annularis* and *M. franksii* (Bruno et al., 2003) and black band disease (Voss & Richardson, 2006). Marine fungi and bacteria, including potential pathogens, are normally nitrogen limited and have been hypothesized to utilize dissolved inorganic nutrients that may result in increased growth rates. However, Bruno and colleagues (2003) have also shown that healthy sea fans in high nutrient treatments do not exhibit signs of disease, suggesting that enrichment alone is not enough to cause disease. In addition, an analysis of *Montastraea annularis* communities with increased nitrate, ammonia and phosphate showed no substantial change in coral mortality (Kline et al., 2006). Kuntz and colleagues (2005) have

suggested that the probability of mortality increases significantly over time, indicating that chronic stressors may be more harmful than acute stressors and showing that high nutrients did not directly kill corals (Kuntz et al., 2005). While there were some changes to the bacterial community associated with *Discosoma* individuals in this study there was no change in *Sinularia* fragments. In addition a number of bands were identified that remained relatively constant throughout the experiment, all of which suggests that the increased nutrients seen in this study did not have an overall effect on the bacterial communities present.

In a study by Kooperman and colleagues (2007), the most abundant bacterial group, the Alphaproteobacteria, remained the same when subjected to aquaria conditions, suggesting that aquaria experiments are still worthwhile to help establish the effects of anthropogenic factors on some of the main groups of coral-associated bacteria. However it is important to acknowledge that with a shift in community could come a shift in bacterial function, such as a change to the protection against pathogens (e.g. Rohwer et al., 2002). This is potentially seen with a decrease in Actinobacteria (Kooperman et al., 2007) which are known to produce many bioactive compounds (Magarvey et al., 2004; Fiedler et al., 2005; Jensen et al., 2005). This is echoed in the work of Kline and colleagues (2006) who found major increases in the abundance of bacteria when corals were maintained in aquaria. Further, Ainsworth and Hoegh-Guldberg (2009) observed penetration and proliferation of bacterial populations through the epithelium in corals kept for only 3d in aquaria, where they only observed bacterial aggregates in the gastrodermis of samples taken from the natural environment. As such, extreme care must be taken when interpreting bacterial population changes in corals maintained under aquaria conditions (Ainsworth & Hoegh-Guldberg, 2009).

5.4.5 Future directions

While care must be taken extrapolating the results presented here to the natural environment, this study reveals several important aspects of coral microbiology and hints at a complex web of

interactions within the coral holobiont that seems to be somewhat species specific. This study suggests that temperature is not directly affecting the production or stability of AHL molecules within the holobiont. Although, slightly different temperature regimes were used compared to Chapter 4, suggesting that it may be beneficial to carry out a graduated temperature study to assess the production and accumulation of AHL molecules and determine if there are temperature thresholds associated with the production/ successful accumulation of AHLs in the coral holobiont. This study also provides the first evidence of the high QQ ability of crude coral slurry, suggesting that the coral host or the natural bacterial community may control bacterial interactions though the degradation of AHLs and provides evidence that in the *Discosoma* sp. investigated this QQ decreases rapidly after 48 h in experimental conditions, which matches with a change in the bacterial community associated with this coral. This study gives us a more detailed insight into the potential bacterial dynamics that occur with temperature stress and suggests that more detailed experiments need to investigate the source of QQ in the coral holobiont and the effects of changing QQ profiles on coral-associated bacterial isolates to gain a more detailed picture of how these changes may affect some of the more abundant groups within each coral species during temperature stress.

CHAPTER 6

AHL PRODUCTION AND DEGRADATION IN CORAL-ASSOCIATED BACTERIA AND THE EFFECT OF TEMPERATURE MANUPULATIONS ON QS, QQ AND BACTERIAL ANTAGONISM

6. 1 Introduction

Understanding the mechanisms of environmental signal exchange and other factors that mediate different bacterial processes in the coral ecosystem is necessary to provide insight into the driving forces of coral ecology (Golberg et al., 2011). The work described in this thesis reveals for the first time that AHL-signal molecules are abundant and stable in coral extracts (chapter 3) and are affected by high summer temperatures in the coral *Stylophora pistillata* (chapter 4). Work in this thesis is supported by Golberg and colleagues (2011), who have also recently shown that 30% of bacteria isolated from a range of corals produce AHLs. Although the presence of AHL-signals in coral extracts and coral-associated bacteria has been established, the link between these signals and bacterial antagonism/ protection of the holobiont has not yet been made.

The degradation of these QS signals, known as quorum quenching (QQ) has recently been found in 23% of extracts from marine organisms from the Great Barrier Reef, including corals (Skindersoe et al., 2008); however the origin of this degradation was not investigated. Bacteria are known to degrade AHLs via the production of three different enzymes (chapter 5). Although the purpose of these enzymes is not fully understood, they are thought to mediate the competition for space and food (Dobretsov et al., 2009), to help regulate their own QS (Sio et al., 2006) and to utilise signals from other bacteria as sources of carbon, nitrogen and energy (Flagan et al., 2003; Uroz et al., 2007). The degradation of QS signalling could play an important role in maintaining coral-associated bacterial communities alongside QS, as well as potentially protecting against pathogens via the inhibition of their QS circuits. However this has yet to be investigated in corals and there is little data available on the prevalence of bacteria capable of interfering with QS in the sea (Romero et al., 2011).

One perturbation that could affect the coral-bacterial community is temperature (chapter 4). Temperature could indirectly affect the community by altering bacteria-bacteria interactions such as the production of antibiotics, and this may be mediated by disruption of QS. However, temperature could also affect the community as a regulator of growth (Madigan et al., 2008). Understanding the role of temperature on coral-associated bacteria is particularly interesting since many coral disease outbreaks are associated with elevated temperatures (Cerrano et al., 2000; Harvell et al., 2001; Kuta & Richardson, 2002).

Understanding quorum-sensing based AHL language in coral-associated bacteria, its link to bacterial antagonism and the effects that temperature has on the dynamic nature of both is imperative if we are to further understand the role of coral-associated bacteria and the effects temperature has on the stability of bacterial communities and through them, the coral host. The experiments described in this chapter investigate the variability of bacterial antagonism, AHL production and AHL degradation in bacterial isolates from *Eunicella verrucosa*, a *Discosoma* sp. and an *Acropora* sp. The effect of temperature on antagonism, QS and QQ in a selection of *E. verrucosa* isolates is also investigated.

6.2 Methods

6.2.1   Isolation of coral-associated bacteria

Eunicella verrucosa samples were collected from populations in Plymouth Sound in May 2008 and samples of a *Discosoma* sp. were taken from the National Marine Aquarium in October 2008. Coral samples were brought to the lab on ice, washed three times in sterile phosphate buffered saline (PBS; pH 8.0), placed in 2 ml sterile PBS and homogenised. A dilution series was created with the tissue slurry (to 10^{-5}) and dilutions were plated onto replicate marine salts agar plates (MSA; described in 5.2.4), $1/10^{th}$ MSA plates, seawater with coral extract agar plates (frozen in liquid nitrogen then ground to a powder, added to dH_2O then filtered (0.2 μm)),

thiosulphate citrate bile salts sucrose agar (TCBS; Oxoid) and starch casein agar (10 g starch, 1 g casein, 0.5 g K$_2$HPO$_4$, 20 g NaCl, 1 L aged filtered seawater (Whatman, 0.2 μm)). All contained 10 μg/ml cycloheximide to prevent fungal growth. Plates were incubated at room temperature for 30 d. After incubation, bacterial colonies were removed and re-plated onto fresh agar. This process was repeated until pure cultures were obtained for each isolate, and then all colonies were transferred to MSA plates. Bacterial isolates from an *Acropora* sp. were provided as pure cultures from the University of Bar Ilan in Israel, in March 2010.

6.2.2 Determination of bacterial 16S RNA gene sequences

Using newly plated pure bacterial cultures, one colony was removed from agar plates aseptically and placed in an microcentrifuge tube containing 50 μl sterile distilled water (dH$_2$0), heated for 5 min at 95°C to break open bacterial cells, and the lysed cells diluted 1:10 with dH$_2$0. PCR was carried out using 1 μl of diluted lysed cells and the 16S rRNA primers 9bfm (5' GAGTTTGATYHTGGCTCAG-3') and 1512uR (5' ACGGHTACCTTGTTACGACTT-3') (Mühling et al., 2008), and amplified using the same conditions described in 2.2.3. Successful amplifications were directly sequenced using a BigDye Terminator v3.1 cycle sequencing kit (ABI) and the M13F primer for the sequencing reaction. Sequences were analysed as in 2.2.4.

6.2.3 Screening for AHL-degradation of isolates

Pure cultures of coral-associated bacteria were grown in 25 ml of sterile marine salts broth (MSB; 21°C; 150 rpm) until an optical density (OD; 600 nm) of 0.6–0.8 was reached. Bacterial cells were pelleted (4,000 xg 5 min), the supernatant removed and retained, and remaining cells washed in sterile PBS (pH 6.8). The OD of bacteria to be tested was adjusted to 1.0 using sterile PBS. Synthetic AHL (50 ng OC10-HSL) was added to triplicate wells of a white, clear-bottomed 96 well-plate and 50 μl of washed cells were added. Three wells contained PBS as a cell-free control and three contained an overnight culture of *V. anguillarum*, used as a non-

degrader control (Tait et al., 2005), treated in the same manner as above. Plates were incubated at 30°C for 3 h.

After 3 h, a 20 ml culture of the biosensor *E. coli* pSB1075 (grown overnight in LB broth containing 10 μg/ ml tetracycline antibiotic; 30°C; 150rpm) was diluted 1:10 with fresh sterile LB broth containing 10 μg/ ml tetracycline and 200 μl added to each well. Plates were incubated again at 30°C for 3 h. Relative light units (RLU) were recorded using the Mithras plate reader (Berthold, Germany), measuring light output for 1 s; with a decrease in light output being indicative of AHL degradation. Optical density was also measured to confirm that extracts had not inhibited growth of *E. coli* pSB1075. For a select group of strains isolated from *E. verrucosa*, this assay was used on cultures grown at 17°C and 21°C.

6.2.4 Screening for AHL-production of isolates

Supernatant from the bacterial cultures (section 6.2.3) was adjusted to pH 2 with 1 M HCl and incubated overnight at 37°C (150 rpm). AHLs were extracted with dichloromethane (DCM, 10 ml), added to the supernatant, shaken vigorously for 1 min and centrifuged (2,000 xg; 5 min), then treated as described in section 4.2.3. Dried extracts were re-suspended in 200 μl acetonitrile and vortexed. A 50 μl aliquot of the extract was placed into three wells of a 96-well plate (with spacing to avoid light contamination from surrounding wells in the plate reader), and left to dry in a fume hood for 1 h. Once dried, biosensor *E. coli* pSB1075, *E. coli* pSB401 and *E. coli* pSB536 (grown overnight at 30°C, in LB broth containing 10 μg/ ml tetracycline, 10 μg/ ml tetracycline and 50 μg/ ml ampicillin antibiotic respectively) were diluted 1:10 with fresh LB media containing the respective antibiotics and 200 μl of each biosensor was added to one of the three wells. Biosensors were also added to blank wells and wells containing synthetic AHLs (2ul of stock containing 5 μM *N*-oxohexanoyl-L-homoserine lactone (OC6) for *E. coli* pSB401, 0.5mM of *N*-oxodecanoyl-L-homoserine lactone (OC10) for *E. coli* pSB1075 and 50 mM *N*-butanoyl-L-homoserine lactone (C4) for *E. coli* pSB536). Plates were incubated at 30°C for 6 h

and RLU measured as above. Again, for a select group of *E. verrucosa* isolates, these assays were conducted at both 17°C and 21°C.

6.2.5 Assays for antagonistic activities of *E. verrucosa* isolates

For diffusion assays to test antagonistic activity (Burkholder et al., 1966), one colony of each isolate from a freshly streaked agar plate was grown for ~ 40 h in 20 ml marine broth at 17°C or 21°C, depending on the temperature treatment. The OD was measured and samples were diluted to an OD of 0.5 by centrifuging as above and diluting in sterile marine salts broth (MSB). A soft agar lawn of 60 μl diluted bacterial culture in 5ml molten soft top agar (as above but with 0.65 g agar) was poured on MSA plates and left to set. Once cool, 10 μl of each bacterial culture was spotted onto the agar in a 3x3 grid for 150x15mm Petri plates. Control producer spots consisted of marine broth, while control lawns consisted of soft top agar with no bacterial strain added. Plates were incubated at 17°C or 21°C depending on the temperature treatment. After 72 h colony diameter and halo diameter were measured as well as presence of swarming and extracellular products. Antagonism was considered to occur when the diameter of zone inhibition was at least 1mm greater than the diameter of the colony formed. This cut-off was chosen as it represents a spatial scale relevant to bacterial interactions (Long & Azam, 2001; Rypien et al. 2010).

6.3 Results

6.3.1 Comparing *E. verrucosa* bacterial diversity using culture- and culture-independent methods

Thirty four bacteria were successfully isolated and sequenced from *E. verrucosa* tissue slurry. To compare the diversity of cultured isolates to molecular analysis, clone library data from different healthy fragments described in Chapter 2 have been added together. Isolates (Figure 6.1A) used for this study under-represent the community found by culture-independent analysis,

as determined by clone libraries (Figure 6.1B). Only eight orders of Bacteria were represented by isolates, compared with 25 orders found in the natural environment (clone library analysis). In addition, three orders, the Vibrionales, Rhizobiales and the Actinomycetales found in the culture collection were not represented in clone libraries.

However, many of the abundant taxa of Bacteria in the clone libraries were represented in the culture collection, including the Oceanospiirillaceae, however the unclassified *Spongiobacter* sp., the largest group in clone libraries, was not represented. In addition, looking at classes of Bacteria, the Gammaproteobacteria are represented in both libraries (47% and 71% for isolate and clone libraries, respectively), as are the Alphaproteobacteria (21% and 11%), the Betaproteobacteria (3% and 2%) the Firmicutes (12% and 1%) and the Bacteroidetes (3% and 2%), giving a good representation of the main bacterial classes found. However, other groups are absent in the culture collection, such as the Deltaproteobacteria and the Planctomycetes, whereas the Actinobacteria represent a major group in the culture collection, not seen in the clone libraries.
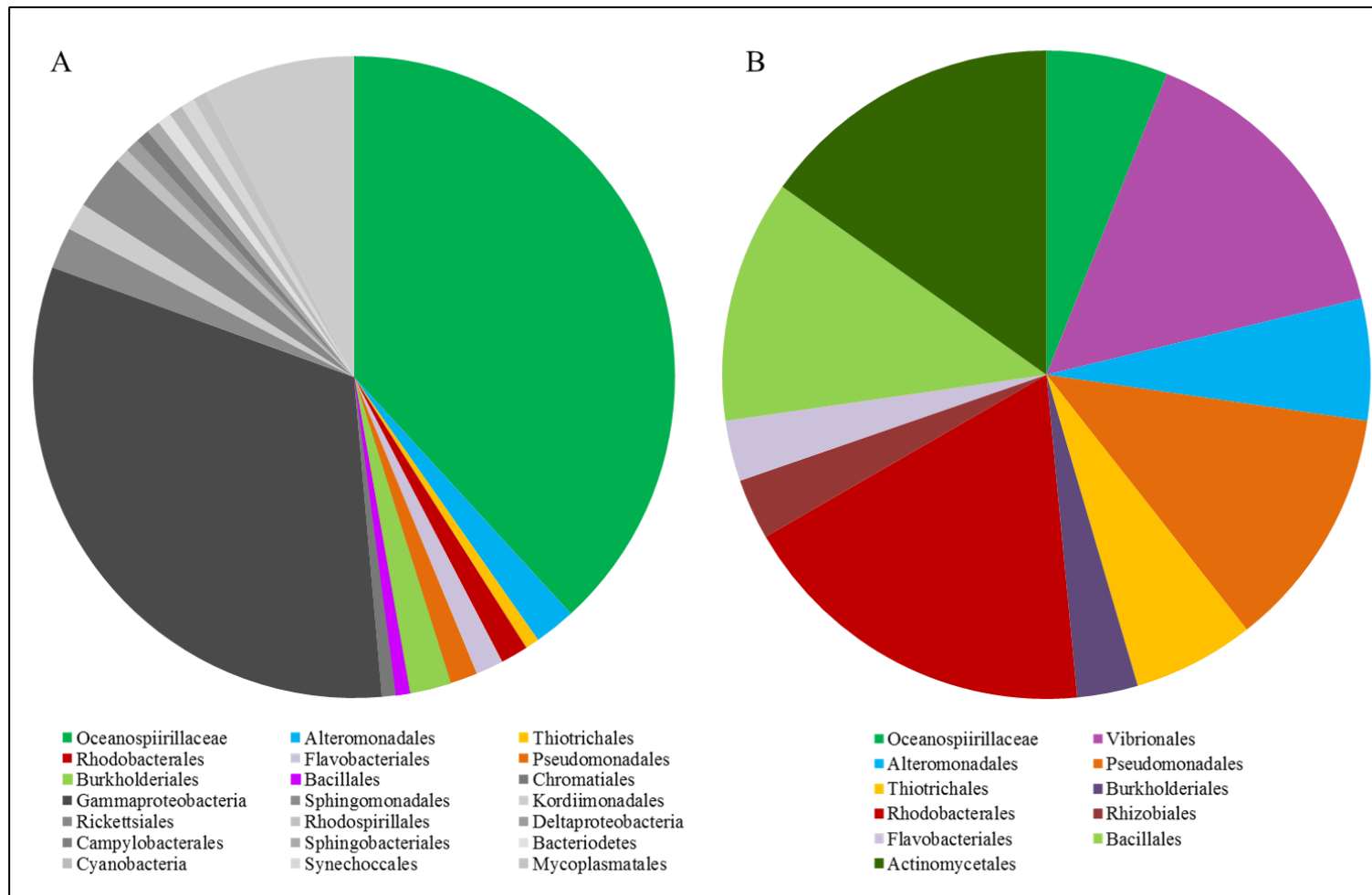
Figure 6.1 Bacterial taxa successfully isolated from *E. verrucosa* tissue (A) compared to average representation of bacterial classes in 16S clone libraries, summarised from chapter 2 data (B).

6.3.2 Variability in QS in *E. verrucosa*, *Discosoma* sp. *Acropora* sp. isolates

Forty-two isolates were successfully tested for AHL production; with the majority of these isolated from *E. verrucosa* (Table 6.1). Of all the isolates tested, 42.1% tested positive for the production of AHLs. When comparing percentages of isolates able to induce one of the three biosensors between coral species, 42.9% of *E. verrucosa* isolates tested positive, 30% of *Discosoma* sp. isolates, and 57.1% of *Acropora* sp. isolates induced one of three biosensors. Care should be taken interpreting these results however due to the differences in isolates sampled between the corals.

Investigating which of the main classes of bacteria represented in isolate libraries were producing these AHLs, the Alphaproteobacteria were found to produce the most AHLs, at 70% of those tested, whereas of the Gammaproteobacteria, only 35% were found to produce AHLs. Of the Betaproteobacteria one out of two and the lone Bacteroidetes isolate were also found to produce AHLs. Interestingly, this is the first time that AHL production has been found in a *Delftia* sp.. A further look at the Gammaproteobacteria reveals that 8 out of 20 of isolates belong to the *Vibrio* genus. Of these isolates, half were found to produce signals, leaving only 3 out of 12 (25%) of other Gammaproteobacteria as producers of AHLs.

Table 6.1 Summary of AHL biosensor assays for coral-associated bacteria, including places where biosensors indicated the presence of AHLs (+), where no AHLs were detected (-) and where consensus between replicates was not achieved (+/-). 'H' indicates genera that were found in visually healthy *E. verrucosa* tissue (from clone libraries; chapter 2) and 'D' indicates presence in diseased libraries.

Coral	Code	Class	Genus	pSB 536 C4	pSB 401 C6 - 8	pSB1142 C10 - 14
Eunicella	104	*Gammaproteobacteria*	*Halomonas*	-	-	-
Eunicella	105	*Gammaproteobacteria*	*Halomonas*	+	+	+
Eunicella	28	*Gammaproteobacteria*	*Neptunibacter*	-	-	-
Eunicella	401	*Gammaproteobacteria*	*Pseudoalteromonas*	-	-	-
Eunicella	500	*Gammaproteobacteria*	*Pseudomanas*	-	+	-
Eunicella	12	*Gammaproteobacteria*	*Pseudomonas* D	-	-	-
Eunicella	16.1	*Gammaproteobacteria*	*Psychrobacter* D	-	+	-
Eunicella	21	*Gammaproteobacteria*	*Vibrio* D	-	-	-
Eunicella	T1	*Gammaproteobacteria*	*Vibrio* D	+/-	-	-
Eunicella	27	*Gammaproteobacteria*	*Vibrio* D	-	+	-
Eunicella	83	*Gammaproteobacteria*	*Shewanella* D	-	-	-
Eunicella	308	*Alphaproteobacteria*	*Sulfitobacter* D	+	-	-
Eunicella	10.2	*Alphaproteobacteria*	*Roseobacter* D	-	+	+
Eunicella	18.2	*Alphaproteobacteria*	*Stappia* H	-	-	-
Eunicella	13	*Alphaproteobacteria*	*Kopriimonas*	-	-	-
Eunicella	45	*Alphaproteobacteria*	*Labrenzia*	+	-	+
Eunicella	54	*Actinobacteria*	*Branchybacter*	-	-	-
Eunicella	15	*Actinobacteria*	*Arthrobacter*	-	-	-
Eunicella	93	*Bacilli*	*Sporosarcinia*	-	-	-
Eunicella	82.2	*Betaproteobacteria*	*Delftia* H	-	+	-
Eunicella	18	*Flavobacteria*	*Zobellia*	-	-	+
Acropora	267.1	*Gammaproteobacteria*	*Vibrio* D	-	-	-
Acropora	45	*Gammaproteobacteria*	*Thalassomonas* D	-	-	-
Acropora	286	*Alphaproteobacteria*	*Ruegeria*	-	-	**+**
Acropora	46.2	*Alphaproteobacteria*	*Rhodobacter* H	+/-	-	**+**
Acropora	517.2	*Alphaproteobacteria*	*Thalassospiria*	-	**+**	-
Acropora	484	*Alphaproteobacteria*	*Paracoccus*	-	+	+
Acropora	269	*Bacilli*	*Bacillus*	-	-	-
Discosoma	151.2	*Gammaproteobacteria*	*Halomonas*	-	-	-
Discosoma	70.3	*Gammaproteobacteria*	*Marinobacter*	-	-	-
Discosoma	65.2	*Gammaproteobacteria*	*Rheinheimera*	-	-	-
Discosoma	144.1	*Gammaproteobacteria*	*Vibrio* D	+	-	-
Discosoma	140.2	*Gammaproteobacteria*	*Vibrio* D	-	-	+/-
Discosoma	32.2	*Gammaproteobacteria*	*Vibrio* D	+	-	-
Discosoma	151.1	*Gammaproteobacteria*	*Vibrio* D	+	-	-
Discosoma	2	*Alphaproteobacteria*	*Sphingomonadales* H	-	-	-
Discosoma	50.2	*Bacilli*	*Paenibacillus*	-	-	-
Discosoma	61.1	*Betaproteobacteria*	*Chromobacter*	-	-	-

These strains were all isolated from visually healthy coral tissue. However, when compared to the *E. verrucosa* clone libraries, very few were of genera found within the clone libraries from healthy tissue (indicated by 'H'; Table 6.1). Instead more correspond to genera found in diseased tissue libraries (indicated by 'D'; Table 6.1). Of these isolates, 50% tested positive for AHL production, the same percentage as AHL-producers found associating with healthy libraries.

Of the three biosensors used, each detected 7–9 isolates using the respective chain lengths of AHL (pSB536, C4; pSB401, C6-8; pSB1075, C10-14). While there was no pattern of signals used by the different classes of isolates, only one isolate tested positive in all biosensor assays; namely a *Halomonas* sp.. Only three others were detected by two biosensors and all others only tested positive in one biosensor. However, six out of 21 of *E. verrucosa* isolates utilised C6-8 AHLs and four of these were found in *E. verrucosa* clone libraries.

6.3.3   Variability in QQ in *E. verrucosa*, *Discosoma* sp. and *Acropora* sp. isolates

Fifty-five isolates were tested from the three species of coral for degradation of the synthetic AHL OC10-HSL, including 28 from *E. verrucosa*, 12 from an *Acropora* sp. and 15 from a *Discosoma* sp.. Results show 58.2% of these coral isolates to be capable of degrading more than 25% of OC10. However, only 23.6% are capable of degrading over 50% and only 9.1% of isolates degrade over 75% of OC10 (Table 6.2).

Table 6.2 Summary of degradation assays for coral isolates. Colour bars represent percentage degradation of synthetic AHL OC10 compared to control. Colours represent <25% ( ), 25–50% (░), 50–75% (▓) and >75% (■) degradation (Deg).

Coral	Code	Class	Genus	Deg
Eunicella	104	*Gammaproteobacteria*	*Halomonas*	▓
Eunicella	105	*Gammaproteobacteria*	*Halomonas*	
Eunicella	28	*Gammaproteobacteria*	*Neptunibacter*	
Eunicella	401	*Gammaproteobacteria*	*Pseudoalteromonas*	▓
Eunicella	500	*Gammaproteobacteria*	*Pseudomanas*	▓
Eunicella	12	*Gammaproteobacteria*	*Pseudomonas*	░
Eunicella	16.1	*Gammaproteobacteria*	*Psychrobacter*	░
Eunicella	301	*Gammaproteobacteria*	*Vibrio*	
Eunicella	21	*Gammaproteobacteria*	*Vibrio*	▓
Eunicella	T1	*Gammaproteobacteria*	*Vibrio*	░
Eunicella	27	*Gammaproteobacteria*	*Vibrio*	
Eunicella	83	*Gammaproteobacteria*	*Shewanella*	
Eunicella	24	*Alphaproteobacteria*	*Sulfitobacter*	
Eunicella	308	*Alphaproteobacteria*	*Sulfitobacter*	
Eunicella	10.2	*Alphaproteobacteria*	*Roseobacter*	
Eunicella	18.2	*Alphaproteobacteria*	*Agrobacterium*	
Eunicella	13	*Alphaproteobacteria*	*Kopriimonas*	
Eunicella	45	*Alphaproteobacteria*	*Stappia*	
Eunicella	45	*Alphaproteobacteria*	*Labrenzia*	
Eunicella	58	*Actinobacteria*	*Micrococcus*	░
Eunicella	54	*Actinobacteria*	*Branchybacter*	
Eunicella	15	*Actinobacteria*	*Arthrobacter*	■
Eunicella	59.2	*Actinobacteria*	*Rhodococcus*	■
Eunicella	81	*Actinobacteria*	*Rhodococcus*	■
Eunicella	32	*Bacilli*	*Bacillus*	■
Eunicella	89	*Bacilli*	*Bacillus*	░
Eunicella	82.2	*Betaproteobacteria*	*Delftia*	░
Eunicella	18	*Flavobacteria*	*Zobellia*	░

Coral	Code	Class	Genus	Deg
Acropora	265	*Gammaproteobacteria*	*Pseudomonas*	
Acropora	267.1	*Gammaproteobacteria*	*Vibrio*	▓
Acropora	288.2	*Gammaproteobacteria*	*Vibrio*	
Acropora	45	*Gammaproteobacteria*	*Thalassomonas*	░
Acropora	286	*Alphaproteobacteria*	*Ruegeria*	░
Acropora	479.2	*Alphaproteobacteria*	*Roseobacter*	░
Acropora	46.2	*Alphaproteobacteria*	*Rhodobacter*	
Acropora	8.2	*Alphaproteobacteria*	*Rhodobacter*	
Acropora	517.2	*Alphaproteobacteria*	*Thalassospiria*	░
Acropora	484	*Alphaproteobacteria*	*Paracoccus*	
Acropora	486.2	*Actinobacteria*	*Micrococcus*	
Acropora	269	*Bacilli*	*Bacillus*	░
Discosoma	151.2	*Gammaproteobacteria*	*Halomonas*	░
Discosoma	134.1	*Gammaproteobacteria*	*Marinobacter*	▓
Discosoma	65.2	*Gammaproteobacteria*	*Rheinheimera*	
Discosoma	210.2	*Gammaproteobacteria*	*Vibrio*	
Discosoma	140.2	*Gammaproteobacteria*	*Vibrio*	
Discosoma	32.2	*Gammaproteobacteria*	*Vibrio*	░
Discosoma	151.1	*Gammaproteobacteria*	*Vibrio*	░
Discosoma	147.2	*Gammaproteobacteria*	*Vibrio*	
Discosoma	129.2	*Alphaproteobacteria*	*Paracoccus*	▓
Discosoma	150.1	*Alphaproteobacteria*	*Ruegeria*	
Discosoma	2	*Alphaproteobacteria*	*Sphingomonadales*	■
Discosoma	64.2	*Alphaproteobacteria*	*Kopriimonas*	
Discosoma	154.2	*Actinobacteria*	*Micrococcus*	▓
Discosoma	50.2	*Bacilli*	*Paenibacillus*	
Discosoma	61.1	*Betaproteobacteria*	*Chromobacter*	

Comparing isolates from all three corals: the Gammaproteobacteria accounted for 43.6%, the Alphaproteobacteria 30.9% and the Actinobacteria for 12.3% of isolates tested, with the remaining isolates being members of the Bacilli, Betaproteobacteria and the Flavobacteria. When classes of bacteria were examined for the ability to degrade over 50% of the OC10, the Actinobacteria was highest with 57% of isolates from this class capable of degrading more than 50% of AHL. Twenty-five percent of the Gammaproteobacteria and only 12% of the Alphaproteobacteria could also degrade over 50% of AHL and of the Bacilli tested; 25%. However, both Betaproteobacteria and the lone Flavobacteria tested were found to degrade over 50% of the OC10.

Interestingly, when looking at the corals individually, the percentage of isolates able to degrade less than 25% of OC10 was similar in all corals, from 41.7–53.6% (Figure 6.2). However, differences can be seen in the number of isolates able to degrade more significant amounts of OC10. While the number of isolates able to degrade >75% of the AHL in *E. verrucosa* tissue was 14.3%, none of the isolates from the *Acropora* sp and only 6.7% from the *Discosoma* sp. indicated high degradation potential. In addition, while the number of isolates able to degrade over 50% of OC10 in *E. verrucosa* accounts for 28.6% and in *Discosoma* sp. accounted for 26.7%, only 8.3% of *Acropora* isolates tested could do so, with the majority of isolates only able to degrade 25-50% of OC10 present.
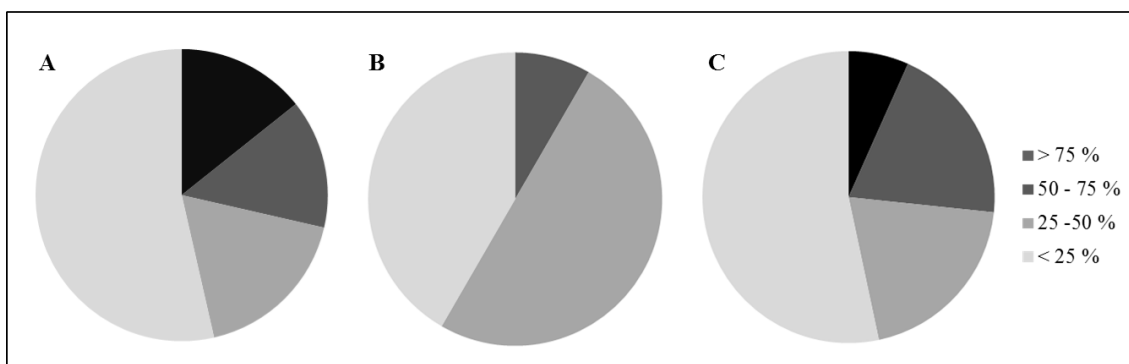
Figure 6.2 Percentage degradation of the synthetic AHL OC10 by bacteria isolated from *E. verrucosa* (A), *Acropora* sp. (B) and *Discosoma* sp. (C).

6.3.4 Antagonism linked to QS and QSI in *E. verrucosa* isolates

Twenty-one isolates from *E. verrucosa* tissue were chosen to investigate links between antagonism of coral-associated bacteria and their ability to produce and degrade AHLs. Figure 6.3 shows a summary of these data. This study found no overall trends linking antagonism or susceptibility to AHL production or degradation (Kruskall-Wallis), indicating the complex nature of bacterial interactions within the coral holobiont. However, some trends were apparent that may become clearer given a larger dataset. The *Gammaproteobacteria* were least affected by the presence of other bacteria, with only 50 % forming halos in the presence of other bacteria, whereas in other classes almost all isolates were inhibited by a number of other bacteria. This can be seen most prominently in the *Actinobacteria*, where an *Arthrobacter* sp. was inhibited by 14 other isolates and the other two species were inhibited by six other bacteria. However, there was no pattern between classes with respect to isolates' inhibition of other bacteria, with the majority of isolates showing antagonism towards at least 1-5 other isolates. Interestingly though, only a *Vibrio* sp. matched high antagonism with high degradation of AHLs.
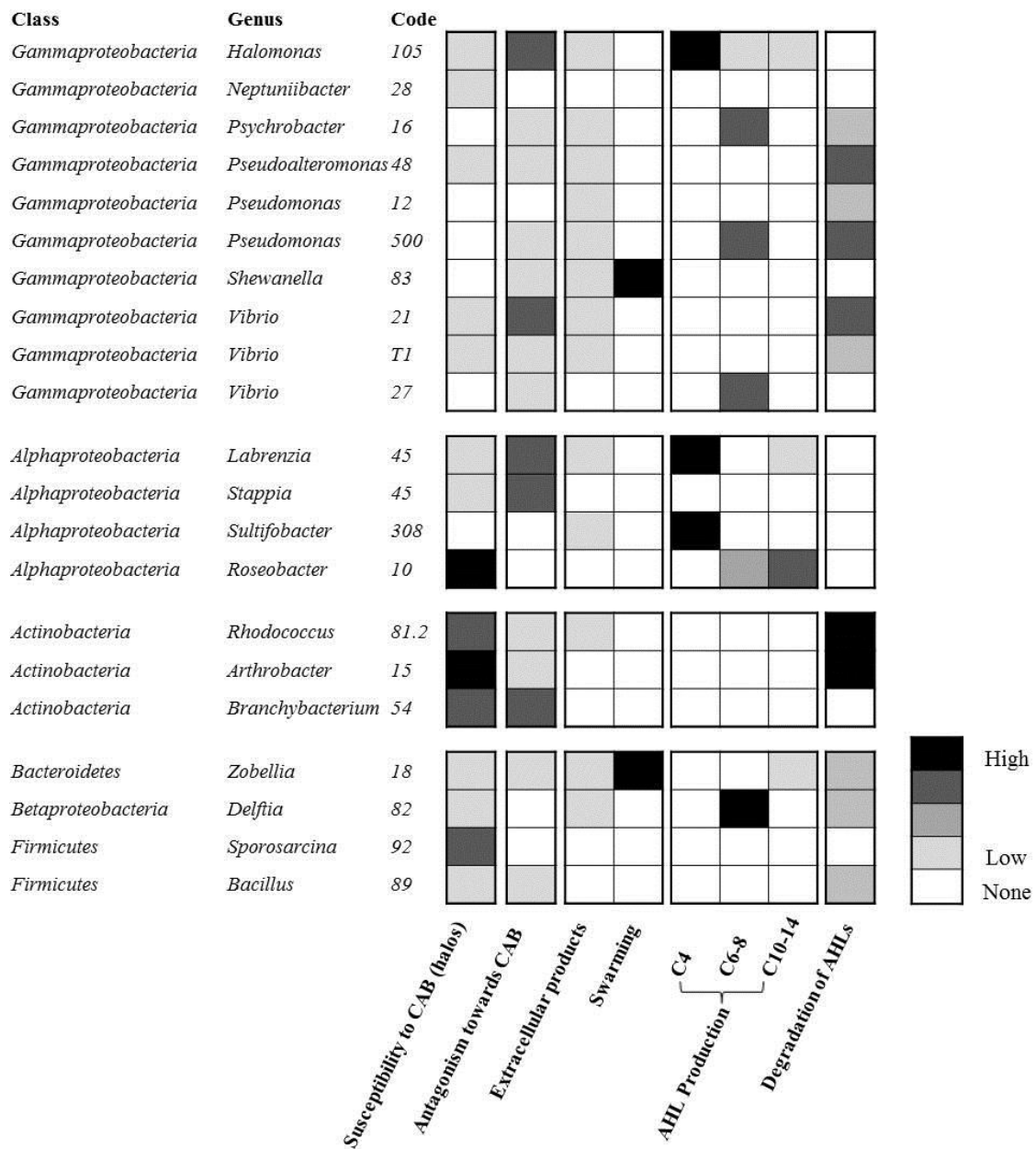
Figure 6.3 Summary of Burkholder assays at room temperature as a comparison with AHL production and degradation in *E. verrucosa* isolates. For susceptibility, antagonism and extracellular products of coral-associated bacteria high colouration (black) represents this interaction with >10 other bacterial strains, dark grey represents 5-10, light grey 1-5 and white denotes no interaction. For AHL production, colours represent high to low AHL production with white squares denoting no production. For degradation of AHLs black squares represent >75% degradation, dark grey 50-75% degradation and light grey 25-50% degradation.

Looking at the isolates individually, the *Halomonas* sp. was the only isolate to activate all three biosensors and it also inhibited the largest number of other *E. verrucosa* bacteria. No other bacterium was antagonistic towards as many isolates. Further, the only AHL producer (out of nine) to be susceptible to more than three other isolates was the *Roseobacter* sp., which was susceptible to 13 other bacteria, while five of the 12 none AHL-producers were susceptible to more than three strains.

6.3.5   Effects of temperature on antagonism between *E. verrucosa* bacterial isolates

The preliminary analysis of antagonism at 17°C and 21°C in this study suggests an effect of temperature on the interactions between coral-associated bacteria. Out of the 33 isolates tested 49% showed an increase in susceptibility to other isolates at 21°C, compared to 21% that showed an increase in susceptibility at 17°C (Figure 6.4). Eight isolates showed no inhibition by any other isolates, including five Gammaproteobacteria, two Alphaproteobacteria and a Firmicute. Such is the variety of bacterial reactions to temperature that a Kruskal-Wallis test showed no overall trend in temperature effect on susceptibility of isolates (H=3.61 p=0.057). The effects of temperature on antagonism of isolates was also variable, with 49% also showing an increase in antagonism towards other isolates with increased incubation temperatures, and four showing increased antagonism at 17°C, with no significant difference between temperature treatments found. However, significantly more bacteria were producing extracellular products (ECP) in the presence of lawn cultures at higher temperatures (H=12.70, p<0.001). While no significant differences were found between classes of bacteria in relation to antagonism towards other isolates or production of ECP, significant differences were found between the susceptibility of certain classes of Bacteria incubated at 21°C (Kruskal-Wallis; H=11.43, p<0.05), where no significant differences were seen at 17°C, showing the Gammaproteobacteria to be less inhibited by other bacterial strains at 21°C than any other classes.
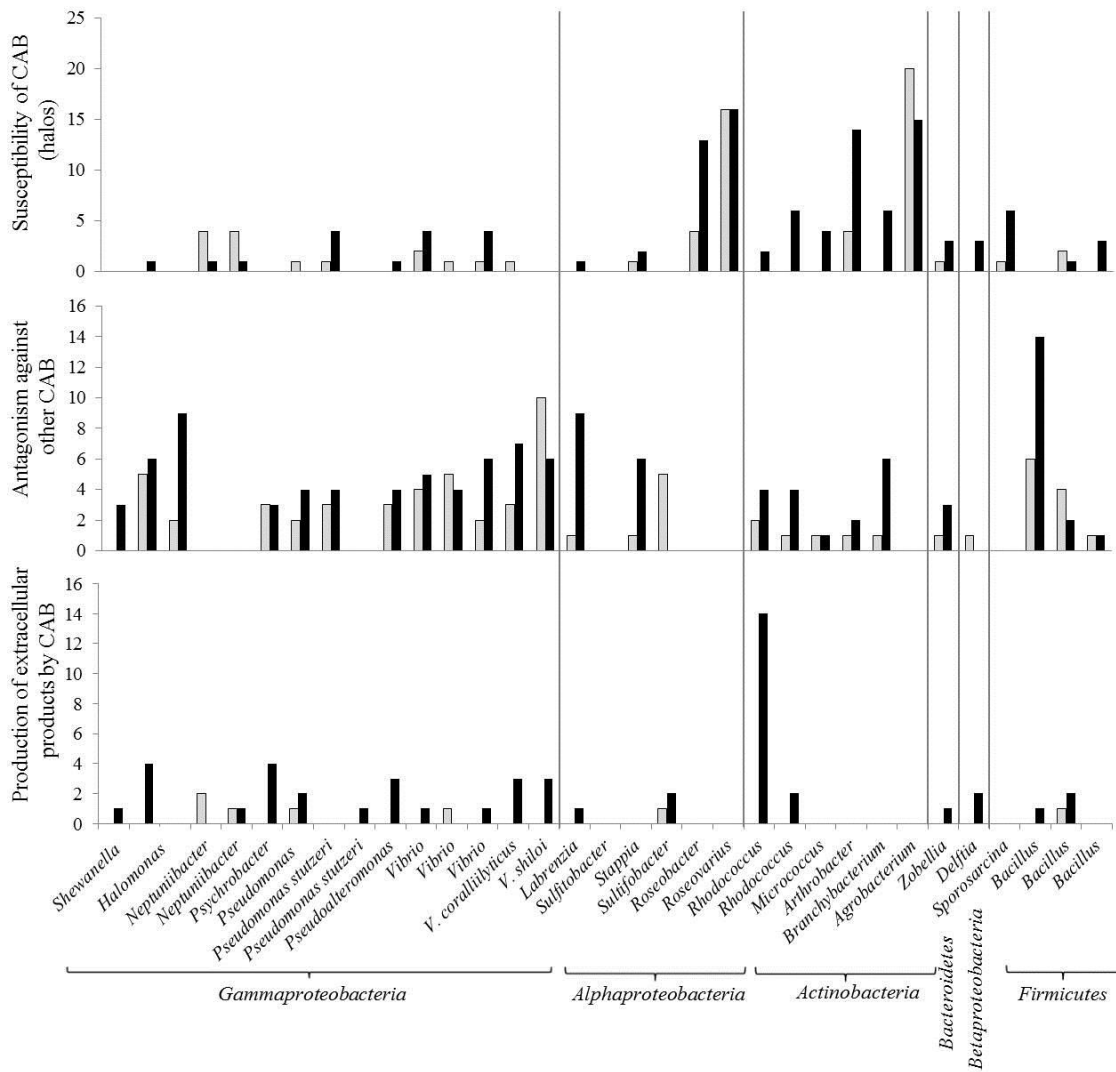
Figure 6.4 Summary of Burkholder diffusion assays in which cultures and plates were kept at 17°C (grey bars) and 21°C (black bars). Graphs represent the effect of temperature on isolates susceptibility to inhibition of growth by other *E. verrucosa* isolates (A), antagonism of isolates towards other *E. verrucosa* isolates (B) and the production of extracellular products by isolates at different temperatures (C).

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6.3.6 Effects of temperature on QS and QQ in *E. verrucosa tissue*; linked to antagonism

As a result of antagonism assays and results from previous experiments (Chapter 4), seven *E. verrucosa* isolates and two isolates closely related to known coral pathogens (*Vibrio coralliilyticus* and *V. shiloi*) were chosen to investigate the link between antagonism and AHL production and degradation at different temperatures. A summary of the results can be seen in Figure 6.5. This data shows that at 17°C and 21°C there were no consistent changes to AHL production for these nine bacterial isolates and instead strain by strain differences can be seen. Although quantity of signal being detected is variable, as seen by the error bars in figure 6.5, a decrease in AHLs detected by all three biosensors was found in *Pseudomonas* sp. and *V. coralliilyticus* sp. and a decrease in mid- and long-chain AHLs was seen in the *Roseobacter* sp.. Decreases in AHL production at 21°C can also be seen for *Labrenzia* sp. and *V. shiloi* (short-chain AHLs) and the *Sulfitobacter* sp. (long-chain AHLs). In contrast, increases in AHLs were detected by all three biosensors at 21°C for the *Halomonas* sp. and for *V. shiloi* for mid- and long- chain AHLs. The *Sulfitobacter* sp. also showed increases in short-chain AHLs. However, significant differences were only observed for the *Sulfitobacter* sp. (ANOVA; F=23.61; p<0.05).

There were also no overall significant changes in AHL degradation between temperature treatments. However, three out of nine of the isolates did increase their ability to degrade synthetic AHL OC-10 at 21°C (*Pseudomonas* sp., *V. shiloi* and *Zobellia* sp.), although no statistically significant increases were found (ANOVA). In contrast, the *Halomonas* sp. and the *Sulfitobacter* sp. tested showed decreases in AHL degradation at 21°C.
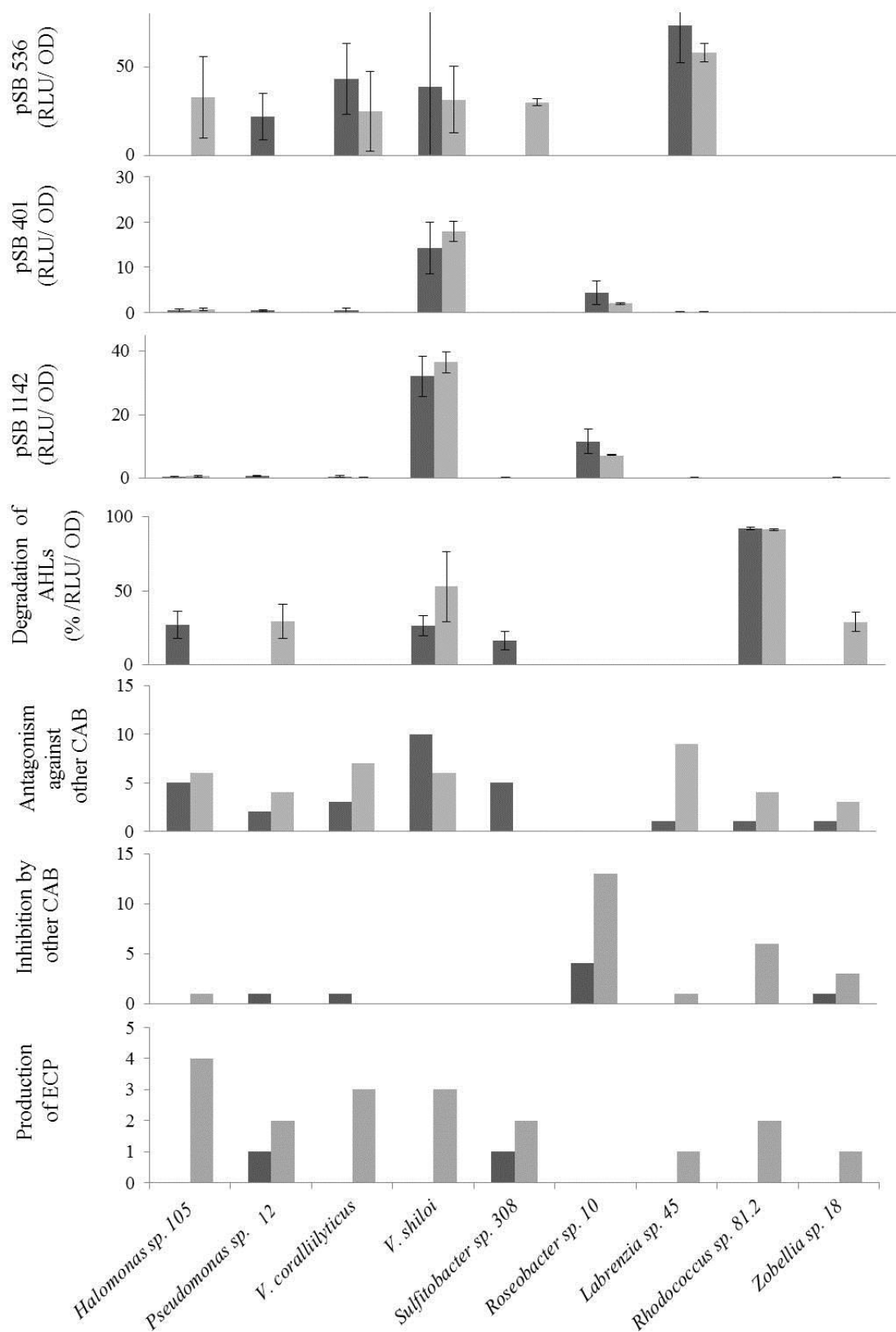
Figure 6.5 Summary of temperature experiments in which cultures and plates were kept at 17ºC (dark grey bars) and 21°C (light grey bars).

In two out of the three isolates showing an increase in degradation at 21°C, an increase in antagonism towards other isolates was also apparent, however small in some cases. In contrast, the opposite was seen for the *Sulfitobacter* sp. which showed a significant increase in antagonism and AHL degradation at 17°C. Linking AHL production to antagonism is less easy, especially as very few of these isolates were inhibited by many other isolates. However, all three of the species which were susceptible to inhibition by other isolates, namely the *Roseobacter* sp., *Zobellia* sp. and *Rhodococcus* sp. also showed no production of C4-AHLs, whereas the rest of the isolates did. Finally worth noting is the profile of AHL production and degradation exerted by *V. shiloi*, a known coral pathogen, which induced high levels of light output from all three biosensors, suggesting a wide range of signals are produced by this strain. It also showed high levels of AHL degradation and high levels of inhibition of other bacteria, while not being inhibited by any other isolates.

6.4 Discussion

6.4.1 QS and QQ in coral-associated bacteria

This study reveals the presence of AHL-mediated QS in a large proportion of coral-associated bacteria tested (40%) and suggests that AHL signalling between coral-associated bacteria may play an important role in structuring these communities and potentially deterring against pathogens. Evidence is beginning to accumulate on the importance of AHL-mediated QS processes in the marine environment, with approximately 10% marine isolates from the surface of the red algae *Delisea pulchra* producing AHLs (Maximilien et al., 1998) and 14.4% from a range of dense marine microbial communities (Romero et al., 2011). The presence of QS in coral bacteria has been corroborated by Golberg and colleagues (2011) in a recent study, who also found 30% of bacterial isolates from corals to produce AHLs. This higher proportion of AHL-QS in coral bacteria compared to other environments may simply be a result of the higher concentrations and variety of nutrients, allowing bacterial growth and reproduction which leads to positive feedback quorum initiation (Golberg et al., 2011); however, as suggested previously,

it has been suggested that the coral host may select its bacterial communities to benefit the coral holobiont (Ritchie, 2006) and may regulate its bacterial communities by interfering with QS regulatory pathways or producing AHL-mimics that can serve as a defence strategy against biofouling (e.g. Manefield et al., 1999), as discussed in chapter 5.

In this study, the percentage of AHL-QS in cultivated strains from each bacterial class was highest in the Alphaproteobacteria (70% of total), with fewer AHL-producers found in the Gammaproteobacteria (35% of total). Molecular studies have found both of these classes commonly in the coral holobiont (Long & Azam, 2001) and both have been found in abundance in *E. verrucosa* tissue (Chapter 2), suggesting a role for QS *in situ* in healthy coral-associated bacterial communities. In the only other study of QS in coral-associated bacteria, 90% of QS producers were associated with the Proteobacteria, but only 10% of these were Alphaproteobacteria and 90% were Gammaproteobacteria, however this study gives no information about the proportion of AHL-negative isolates in various bacterial classes (Golberg et al., 2011). In comparison, 60% of the Alphaproteobacteria isolated from different marine samples were able to activate AHL sensor strains (Wagner-Dobler et al., 2005) suggesting AHL-signalling is important in this class. A recent study has suggested that while the Gammaproteobacteria may be responsible for defence against pathogens and certain members of the Alphaproteobacteria may be involved more closely with nutrient turnover in the coral ecosystem (Kvennefors et al., 2012), suggesting that the high QS seen here may be related to controlling metabolism.

Of the members of the Gammaproteobacteria that tested positive for AHLs in this study, 57% were most closely related to the Vibrio genus, while only making up 40% of the Gammaproteobacteria in the culture collection, suggesting a higher proportion of QS in marine *Vibrio* spp. Many marine vibrios are known to employ diverse and complicated signalling systems for the control of phenotypes, with the production of a wide range of signals, including

AHLs, produced by the AHL synthases Lux I and also LuxM (so far, unique to vibrios), a small furanone-type molecule, autoinducer 2, and a *csqA*-dependent signal molecule, hydroxyketone(*S*)-3-hydroxytridecan-4-one. Different vibrios have been shown to have different combinations of these four QS systems, often controlled by complex regulatory hierarchies (Milton*,* 2006). The prevalence of AHL-QS in coral-associated vibrios is particularly interesting, as many members of the *Vibrio* spp. are known to be coral pathogens, whether of a primary or opportunistic nature, and QS is known to control virulence factors in this genus (Milton, 2006).

While many isolates tested positive for the production of AHL signal molecules, they only tested positive in one of three biosensor assays, suggesting that the majority of isolates employ one or a number of AHLs but of similar chain length. In addition, 7 out of 25 AHL-positive isolates induce pSB401, detecting mid-chain length AHLs, which have been found to be conserved and in abundance in many AHL profiles from crude coral extracts (Chapter 3, 4 and 5). This verifies previous findings in this thesis and suggests that a range of coral bacteria may utilize similar signals to allow detection and/ response to prevalent signals in the coral-holobiont. However one isolate, a *Halomonas* sp. from *E. verrucosa* tissue, tested positive in all three biosensor assays. The presence of a wide variety of AHL molecules might provide the ability to sense and regulate gene expression at different taxonomic levels, allowing for rapid responses to a range of environmental changes experienced by the coral ecosystem (Golberg et al., 2011). Interestingly, this species is a member of the class Gammaproteobacteria and the order Oceanospirillales. Members of this order are found in many coral libraries, such as *Endozoicomonas* spp., originally isolated from the encrusting pore coral *Montipora aequituberculata* (Yang et al., 2010) and found to dominate healthy *E. verrucosa* tissue. While there has been little work on this order of bacteria, as they are rarely isolated, this study suggests that members of this group may be important in maintaining the coral holobiont through multiple levels of quorum sensing control and deserve further investigation. However, it is not unusual for different signalling systems to be used between members of even the same genus,

exemplified by the vibrio genus (Milton, 2006), suggesting that investigations need to be carried out on those isolates found in abundance in the coral holobint.

Matching the prevalence of AHL production, this study shows for the first time the prevalence of QQ in coral-associated bacteria, with 23.6% of isolates capable of degrading over 50% of synthetic OC10 AHL. This and the high degradation potential (>75%) of 9.1% of isolates suggests that QQ could also play an important role in controlling coral-associated bacterial communities, especially with the prevalence of QS as discussed above. In this study bacteria from the classes Gammaproteobacteria, Alphproteobacteria, Bacilli, and Actinobacteria were found to degrade significant amounts of AHL, confirming the results of Romero et al. (2011), the only other study of AHL degradation in marine isolates, where degradation was found in 14.4% of isolates; including all bacterial classes studied here, and also in the Bacteroidetes.

Although QS and QQ processes have been known for some time in marine bacteria (Nealson et al. 1970; Givskov et al. 1996), little attention has been paid so far to their ecological significance in the marine environment, with no other direct evidence of QQ prevalence. However, indirect evidence concerning the high degradation of AHLs in seawater, related to the presence of QQ enzyme activity (Hmelo & Van Mooy, 2009) suggests that this process could be frequent in marine communities. In addition, Romero and colleagues (2011) have shown a strong effect of the origin of bacterial isolates on the percentage of isolates with QQ activity; finding a higher percentage of isolates from *Fucus vesiculosus* compared with isolates from biofilm and fish tank sediment. This suggests the high percentage of QQ activity may sustain the existence of strong microbial interactions in the eukaryote-prokaryote boundary that promote unique biological activities (Romero et al., 2011). Further, the diversity of bacteria found to exhibit QQ in this study and the study of Romero et al. (2011) is much higher than found in terrestrial environments, with only strains of *Bacillus* exerting QQ activity out of 800

strains tested from soil samples (Dong et al., 2002), suggesting the importance of QQ in dense marine bacterial communities.

6.4.2 Difference in prevalence of QS and QQ between coral species

This study suggests that QS and QQ activity may be more prevalent in certain species of coral and therefore possibly more important in the maintenance of certain bacterial communities. While a higher percentage of *Acropora* sp. isolates were able to activate AHL biosensors, compared to *E. verrucosa* and *Discosoma* sp. isolates, *E. verrucosa* and *Discocoma* sp. were found to have the highest percentage of isolates capable of degrading over 50% AHLs, while few *Acropora* sp. isolates were able degrade similar levels. Further, none of the *Acropora* sp. isolates were capable of degrading over 75% of AHLs (compared to 14.3% of *E. verrucosa* isolates). Interestingly, this suggests that different coral species may have different mechanisms for control of their bacterial associates.

Such differences are not surprising as the environments these isolates originate from are very different. Studies have shown that bacterial communities differ by coral species (Rohwer et al., 2002), as does their antimicrobial activity (Kelman et al., 2006; Shnit-Orland et al., 2009), which may be due to varying settlement surfaces or variations in the physical and chemical properties of the coral mucus (Sweet et al., 2011). Specific properties of mucus have been shown to differ by coral species (Brown & Bythell, 2005) and have been suggested to affect the formation of these microbial communities (Sweet et al., 2011). In addition to physiochemical properties, antimicrobial activity of the host/ resident microbial community has been implicated in determining coral-associated bacterial communities (Ritchie & Smith, 1995; Kooperman et al., 2007; Sharon & Rosenberg, 2008). All of these factors may also have an impact on the prevalence of QQ and QS bacteria associated with these corals. However, the differences in QS may also be a direct result of the prevalence of QQ in such bacterial communities, suggesting

that in certain corals the presence of QQ bacteria may determine the presence of species that rely on QS for settlement.

6.4.3 Bacterial antagonism linked to QS and QQ

With such prevalence of bacteria capable of QS and QQ it is likely that some of these signals and/ or the degradation of AHL-signals exert control on bacterial communities, possibly by exercising control over the expression of antimicrobial compounds as suggested by Ritchie (2006). This study reveals that interactions between coral bacterial associates are highly complex, with 71% of isolates showing inhibition of at least one other isolate and some affecting a broad range of isolates. However, with the small number (21) of isolates investigated here, these interactions cannot be linked to the presence of QQ or QS and reiterates the complex nature of bacterial interactions within the coral holobiont. While this study investigated AHL-signalling, as the most intensively studied and most prevalent form of bacterial QS, there are a range of other signalling systems, as discussed previously, that may play a role in bacterial antagonism in corals and this deserves further research. It is also possible that while the presence of QQ and QS bacteria in the coral holobiont may exert a range of control on bacterial communities, such as the production of antimicrobials, other control mechanisms cannot be assessed by simple plate assays investigating antagonism between isolates, such as the production and maintenance of biofilm communities. While it is likely that some bacteria are capable of affecting the abundance of others, which is supported by a metagenomic analysis on the coral *Porites asteroides* in which coral associate bacteria were shown to carry genes for 'waging chemical warfare' (Wegley et al., 2007), there is no evidence as of yet that these genes are under AHL-QS control or that such interactions are governed by the bacteria themselves instead of mediated by the coral host (Kvennefors et al. 2012). Further research needs to establish the role of the host in controlling bacterial phenotypes and the role of other bacterial signalling systems in coral-associated bacterial communities.

This study does however substantiate the high percentage of coral-associated bacteria that are capable of antagonism against other bacterial community members and for the first time shows the prevalence of this antagonism in bacteria associated with a cold-water Gorgonian coral. Previous reports from soft and scleractinian corals, have found associated bacteria to inhibit 25–70% of potential pathogens (Shnit-Orland et al., 2009), with 20 % capable of inhibition from *Acropora palmata* (Ritchie, 2006). However only one other study has shown antagonism against a range of other coral-associated bacterial isolates, finding 69.6% of bacteria from *Montastrea annularis* at 25°C to be antagonistic (Rypien et al., 2010). All these studies suggest that natural coral-associated bacterial communities are both competitive and dynamic community and are likely to employ QS and QQ in such interactions. Further, as in Chapter 2, these results suggest that bacteria associated with cold-water corals may also confer resistance for their host, like that now established in tropical scleractinian and soft corals. However, Kvennefors and colleagues (2012) have also found species associated with diseased tissue to have potent antimicrobial activity suggesting that they may hold a competitive advantage over the normal flora under the right conditions and establishing the importance of deciphering how these community interactions change with temperature.

Interestingly, this study also shows the Gammaproteobacteria to be the least inhibited by other bacteria, where other classes are strongly inhibited. The Gammaproteobacteria were also the only class that had more antagonism associated with it than it did inhibition by other isolates. However, care needs to be taken to distinguish between bacterial associates of corals and potential pathogens, both of which reside within the Gammaproteobacteria. Generally it was found that bacterial isolates with high antimicrobial activity were less likely to be inhibited, which indicates some level of microbial resistance and suggests there may be some level of competitive advantage associated with these bacteria. Similar results were found in bacterial isolates from *A. millepora* (Kvennefors et al., 2012), but in that study members of Pseudoalteromonas and ribotypes closely related to *V. coralliilyticus* displayed potent antimicrobial activity against other isolates and grew readily on detached coral mucus, but even

with this competitive advantage they were rare or absent on healthy colonies *in situ*. Kvennefors et al. (2012) suggest that bacterial communities are kept in check by a combination of host-derived and microbial interactions and postulate that members of the Gammaproteobacteria, the most abundant class of bacteria on many corals, may play a key role in maintaining stability of these bacterial communities.

6.4.4 Changes in antagonism of *E. verrucosa* isolates with temperature

Previous investigations into the effect of temperature on bacterial antagonism, QS and QQ are scarce. This study has revealed the complex nature of the bacterial communities that reside within *E. verrucosa* tissue, showing a complex set of responses to increasing temperatures. However, although there were no significant differences in antagonism towards other bacterial isolates between temperatures, 49% of isolates did show an increase in antagonism at 21°C. Significant increases can be seen in a number of isolates, including the *Halomonas* sp. and two *Vibrio* spp. from the Gammaproteobacteria, and two species of Alphaproteobacteria. Such increases suggest that these bacteria are either up-regulating genes to protect their communities or up-regulating virulence genes at higher temperatures. An increase in all these genera can be seen in libraries from diseased tissue (Chapter 2), suggesting the latter. However not found in clone libraries, the *Halomonas* sp. is a member of the Oceanospirillales which was decreased in diseased-tissue libraries. This suggests that while some bacteria may take advantage of the higher temperatures, others may up-regulate antagonism to try to sustain bacterial community structure.

However, increases seen in antagonism may also be the result of an increase in susceptibility of particular isolates. The effect of temperature in previous studies of tropical corals on antibacterial activity of coral bacteria has shown antagonism to be optimal at temperatures of 26°C with slight decreases at 30°C (Shnit-Orland et al., 2009). Ritchie (2006) also suggests antimicrobial compounds may be temperature sensitive, with little antibiotic activity occurring

in mucus collected from *A. palmata* during a period of increased sea water temperature (28–30°C), as opposed to colonies at normal temperatures (22-25°C). Although the temperatures tested in this study are far below previous studies, these are the temperatures these bacterial isolates were subjected to in their natural environment and thus are more relevant for this investigation. Interestingly, this study showed increases in antagonism at 21°C which suggests that there may be a temperature threshold upon which bacteria will up-regulate genes for antagonism to protect their communities, as well as a threshold past which they are no longer capable of doing so.

As discussed above, the Gammaproteobacteria showed significantly less susceptibility to other isolates than other classes. Eight isolates showed no inhibition by any isolates at either temperature, five of which were members of the Gammaproteobacteria. Interestingly, this is not the case at 17°C, with significant differences between classes only seen at 21°C which suggests that at 21°C the Gammaproteobacteria may resist inhibition, some of which also up-regulate genes for antagonism against other bacteria. As the Gammaproteobacteria are a class of Bacteria ubiquitously found to associate with healthy corals, further research into these findings are imperative to understanding more about coral health and the progression of disease.

6.4.5 Changes in QS and QQ with temperature linked to antagonism

Establishing differences between the presence of QS and QQ in coral-associated bacteria at different temperatures was deemed important after finding the deterioration in the detection of QS profiles in *Stylophora pistillata* fragments kept at 30°C (Chapter 4). In addition, a recent study showed the production of AHL molecules by a small number of *Vibrio* strains isolated from healthy and diseased coral tissue to be dependent on temperature, with AHL production decreasing in some cases and increasing in others (Tait et al., 2010). This study establishes the complex nature of the effects of temperature on bacterial species, however shows some significant difference between bacterial QS at 17°C and 21°C, but only for short-chain AHLs in

three of the species tested, which suggests high susceptibility of short-chain AHLs to temperature, as previously described by Tait et al. (2005). However, many isolates saw no significant change to AHL production. The same is true for QQ, with half of the isolates increasing their ability to degrade AHLs at 21°C but also showing two isolates decreasing in their ability. Interestingly, while the *Halomonas* sp. (related to known coral symbionts) shows decreases in its ability to degrade AHLs, the known coral pathogen *V. shiloi* shows an increase. Linking these results to those in Chapter 4, the temperature ranges chosen for each study were extremely different due to the different species investigated. However, it may be that bacteria have a range of temperature thresholds, as discussed earlier, that allow for an up-regulation of antagonism, as seen in this study; whereas at higher temperatures, this up-regulation is no longer possible, with a decrease in QS (Chapter 4) and a decrease in antagonism (Ritchie, 2006, Rypien et al., 2010). Linking these results to the susceptibility and antagonism of bacterial stains, very little susceptibility is seen, accept for those lacking in the production of short-chain AHLs. This suggests that the production of short chain AHLs may either help inhibit pathogens or may aid in the resistance to attack and warrants further investigation.

6.4.6 Culture based studies to assess QS and QSI in coral-associated bacteria

This study has shown that culture based studies of natural coral-associated bacteria only represent a fraction of the naturally occurring bacterial population. Assessing the differences between successfully cultured *E. verrucosa* isolates and sequences based on clone libraries from the natural environment shows that although many of the main orders represented in clone libraries are present in culture collections, others are not. In addition, other groups are over-represented in culture collections. It is well known that approximately 1% of bacteria from natural environments can be successfully cultured in the laboratory (Staley & Konopka, 1985), which suggests that the use of culture based studies to assess the prevalence of QS and QQ in coral-associated bacteria naturally occurring in the coral holobiont is somewhat limited. However, studies only using culture techniques are common in this field, even though many

studies have been unable to ascertain species dominant in natural coral microbial communities (Kvennefors et al., 2012). Further, taking bacteria out of their natural environment changes the chemical gradients they are subjected to, the nutrients that they can utilise and does not provide the competition with other bacteria they will be subjected to in the natural environment. Thus, studies such as this need to be approached with caution. However, due to the diverse nature of AHL synthases, designing primers to assess the prevalence of QS genes in mixed, dense bacteria communities is not presently an option. Further, the study of individual bacterial isolates allows investigations which can then be related to field observations and scaled up to bigger culture experiments to assess the role of bacterial communities in the coral environment, making this an important step on the road to understanding the role of these bacterial communities and how their role changes with environmental perturbations.

6.4.7 Conclusions

This study confirms the presence of AHLs in a number of coral-associated bacteria and for the first time establishes the prevalence of degradation of AHLs by coral-associated bacteria. This suggests that bacteria in the coral holobiont may play a major role in the maintenance of bacterial communities via the degradation of AHLs, possibly inhibiting the majority of bacteria from reaching their 'quorum' threshold and switching on genes not beneficial to the coral-holobiont. This study also suggests there may be a link between the production of short-chain AHLs and lack of susceptibility to inhibition by other isolates and that temperature has an effect on the ability of bacteria to both degrade AHLs and produce AHLs. Although suitable for this thesis, this work deserves further attention to delve further into the mechanisms behind these changes, what phenotypes this change in temperature may impact and which comes first – a breakdown of QS or QQ in coral-associated bacteria or a change in community as a direct or indirect result of environmental change.

CHAPTER 7

GENERAL DISCUSSION

7.1 Implications for the natural environment

7.1.1 Importance of QS and QQ in coral-associated bacteria

This thesis adds to the growing body of evidence that suggests an important role for QS and QQ in dense marine bacterial communities. It also suggests that both phenomena may be disrupted by increasing sea surface temperatures, in a strain dependent manner, which is likely to affect the roles of these bacteria in the coral holobiont and potentially the health of the holobiont itself. QS is known to control a number of phenotypes in genera known to infect corals, such as members of the *Serratia* genus, where QS controls surface spreading, production of antibiotics and exoenzymes, attachment to surfaces and timing of virulence gene expression (Van Houdt et al., 2007). The onset of bleaching in *Oculina patagonica* has been associated with the expression, at elevated temperatures, of a number of virulence factors, including adhesion that facilitated attachment of *V. shiloi* to *O. patagonica* (Toren et al., 1998), a peptide toxin (Banin et al., 2001) and a superoxide dismutase (Banin et al., 2003), although these factors have not yet been associated with QS. AHL-signalling has also been reported in a number of coral-associated vibrios, although the phenotypes these signals control have yet to be determined (Tait et al., 2010). However, Tait et al. (2010) showed that the majority of coral-associated vibrios produced constant or decreased quantities of AHLs with increasing temperature, matching the results from the small number of *E. verrucosa* isolates tested in this thesis, suggesting that temperature has an effect on the production of AHLs in a strain dependent manner. Recently, using whole genome sequencing of *Vibrio coralliilyticus* ATCC BAA-450, a bacterium associated with multiple coral diseases, Kimes et al. (2012) have demonstrated direct temperature regulation of virulence factors using proteomic analysis and bioassays. A number of virulence factors are up-regulated at 27 °C concurrent with phenotypic changes and independent of bacterial abundance. These factors include transcriptional regulators, one of which is quorum sensing and they hypothesise that the global transcriptional regulators, such as QS are driving the temperature modulation observed, with the Vc450 proteome exhibiting up-regulation of numerous QS proteins at 27 °C (Kimes et al., 2012). With evidence that

temperature can affect the level of AHL production positively (Hasegawa et al., 2005; Chapter 6) and negatively (Tait et al., 2010; Chapter 6), this suggests that with increasing sea surface temperatures a competitive advantage may be present for some bacterial species over others and that this advantage may be directly related to their regulation of quorum sensing genes.

With a potential imbalance in the up-regulation of QS with increased temperatures between natural bacterial communities and putative pathogens, disease outbreaks could become more prevalent in the future. In addition to this, with the decreases in AHL degradation seen in crude extracts of the *Discosoma* sp. tested at 30 °C (chapter 5) the likelihood of species that up-regulate QS reaching their 'quorum' threshold and switching on genes for virulence may increase. Also, the ability of one coral-associated vibrio (a *V. harveyi* strain) to decrease detection of AHLs by a bioreporter was found to be up-regulated at 30 °C and was also found to not be the result of the AHL degradation (Tait et al., 2010). This suggests that *V. harveyi* from diseased *A. millepora* may have a competitive advantage that decreases QS potential in other species. If, at the same time, the AHL degradation potential of native bacteria is temperature dependent, this may allow certain vibrios to be over represented in periods of high temperature.

7.1.2 Temperature thresholds for the normal functioning of bacteria-bacteria interactions within corals and other marine invertebrates

It is likely that many microbial symbionts have strict temperature thresholds, due to the specific functions they carry out in such a complex system. While decreases in AHL-degradation were seen for *Discosoma* sp. slurry over the experimental period and more so at 30 °C (Chapter 5), a number of *E. verrucosa* isolates were seen to increase their ability to degrade AHLs from 17 °C to 21 °C Chapter 6. Antagonism towards other bacteria also increased at 21 °C, although with the small sample size used, this could not be linked to AHL degradation. However, these results suggest that coral-associated bacteria may have temperature thresholds in which they can carry out normal function, in which they increase antagonism to defend their niche and after which

they are no longer capable of one or other of these roles, at which point opportunist pathogens can gain a foot hold. The slightly different temperatures used in Chapters 4 and 5, where a dramatic decrease to AHL-QS in *S. pistillata* was seen at high temperatures of 31.47 °C while no effect could be observed in the *Discosoma* sp. AHL profiles when corals were kept at high temperatures of 30 °C may be illustrating such a temperature threshold. Indeed Webster et al. (2008) found temperature thresholds for bacterial symbiosis with the sponge *Rhopaloeides odorabile* between 31 and 33 °C, which was critical to the partnership between the sponge and a known alphaproteobacterial symbiont, which has been previously linked to sponge health (Webster et al., 2002).

The evidence in this thesis that relatively low temperature shifts (17 – 21 °C) alter the ability of bacteria to produce AHLs and AHL degrading enzymes in bacteria from *E. verrucosa* suggests that QS and QQ may be quite vulnerable to temperature alterations in some species and thus may happen before community change occurs in corals; a finding that could not be determined in Chapter 5, where changes in the bacterial community were seen at the same time as a decrease in coral slurry AHL- degradation. Further research needs to establish which comes first, if this change becomes more extreme at higher temperatures, or if even small changes could result in a shift in community structure and a further decline in AHL signalling, as seen in Chapter 4. Many bacteria have now been identified with LuxR homologs where no corresponding AHL synthase was found, suggesting that certain bacteria "eavesdrop" on signalling within their niche. For example, *Escherichia coli* and *Salmonella typhimurium* contain a luxR homolog called sdiA that has been found to respond to exogenous AHLs and regulate virulence in *S. typhimurium* and biofilm formation in *E. coli* (Wang et al., 1991; Smith & Ahmer, 2003; Lee et al., 2007). This could allow certain coral-associated bacteria to mediate the formation of specific communities and regulate phenotypes of a range of bacteria through AHL-QS; thus, with slight changes to these signals, a whole range of phenotypes in the community could be affected, causing rapid and extensive changes in bacterial communities.

7.2 Future directions

While the results of this thesis have answered some questions about the prevalence of QS and QQ in coral-associated bacteria, the effect of temperature on the detection of these molecules/ enzymes, and how this relates to bacterial antagonism, much more work is needed to understand the breadth of roles of coral-associated bacteria or whether we can use such phenomena (QS and QQ) as mechanisms to mitigate coral disease in the future. The rest of this chapter proposes a number of further avenues to be explored and gives details of experiments that were out of reach in this thesis due to time constraints.

7.2.1 Future work should focus on susceptibility and alteration of natural bacterial communities, as well as putative pathogens.

While some coral diseases have been linked to the presence of specific pathogens (reviewed in Rosenberg et al., 2007) the causes of many coral diseases remain unidentified and controversial (Ritchie et al., 2001; Lesser et al., 2007). Even where potential pathogens have been identified, controversy still remains. Black band disease (BBD) provides an interesting example of the difficulties in understanding the progression of coral disease and any primary causative agent. A range of molecular studies have identified microorganisms within the disease band of BBD which suggests a complex structured microbial community and hints at disease being polymicrobial, first thought to be caused by a single pathogen. Despite almost 40 years of work, conditions that lead to the onset of lesions and causes of disease are still poorly understood. Environmental factors have been correlated with the progression and prevalence of disease although their exact mechanisms are difficult to clarify (Boyett et al., 2007; Voss & Richardson, 2006). In addition to this, marine *Vibrio* spp. have been associated with coral diseases world-wide (reviewed in Rosenberg et al. 2007), however they are also found in apparently healthy coral-associated communities (Bourne & Munn, 2005) and appear to have commensal functions (Chimetto et al., 2008; Shnit-Orland & Kushmaro, 2009). Tissue necrosis has also been found to occur with the simple addition of nutrients, altering the natural bacterial communities or

increasing the growth rates of opportunistic pathogens already present in the natural community (Kuntz et al., 2005).

This thesis reveals the conserved nature of bacterial signalling profiles and a potential role of QQ in maintaining bacterial populations. Knowledge that these communities and this signalling can rapidly change, prior to signs of disease, suggests that future research needs to focus on establishing the role of these associates and the factors which influence changes in their behaviour, instead of focusing on determining potential pathogens of coral disease. However, this area of research needs to be a two step process. The environmental conditions that mediate the infection of corals by pathogenic bacteria or switch native bacteria from a regime of symbiosis to pathogenesis needs to be understood to allow targeted efforts to mitigate coral disease at the right time, potentially reducing its impact. In addition, there is still a question mark as to whether QQ has a role in controlling the virulence of opportunistic pathogens. This could be investigated by testing the effect of the addition of QS blockers (Dobretsov et al., 2007) or purified AiiA protein (the recombinant *Bacillus* lactonase; Dong et al., 2000) on the production of virulence factors of coral pathogens.

7.2.2 What are the roles of each partner in the coral holobiont and what interactions mediate coral health?

One of the biggest questions associated with this field is whether coral-associated bacteria actually confer disease resistance to the host and, if so, which bacteria are responsible for this role. A number of experiments could establish such a role as shown recently by Alagely et al. (2011). Isolates already found to be antagonistic against other *Aiptasia pallida* associates could be inoculated back into the coral at the same time as a potential pathogen, as seen with this anemone (with *Serratia marcescens* as the pathogen; Alagely et al., 2011) and assessed for a reduction of disease symptoms. A number of these isolates reduced disease symptoms, suggesting that the native microbiota associated with invertebrates may be capable of producing

activities *in situ* that reduce susceptibility of marine invertebrates to opportunistic pathogens, one of which may be the prevalence of AHL degrading bacteria. To assess the role of QS a number of simple assays to screen for the expression of phenotypes commonly but not exclusively regulated by AHL-mediated gene transcription could be carried out alongside assays for the production of AHLs. Chong et al. (2012) have shown this with bacteria from activated sludge, in which exoenzyme production was frequently observed. The addition of AHLs was found to upregulate chitinase activity in an AHL-producing isolate related to *Aeromonas hydrophila*. AHLs produced by this strain were identified and genes *ahyI/R* and *chiA*, encoding AHL production and response and chitinase activity respectively, were sequenced, providing insight into the relationship between AHL-mediated gene expression and exoenzyme activity in activated sludge; ultimately creating opportunities to improve sludge performance. In coral-associated bacteria it would be worth matching the production of AHLs with bacterial antagonism, the presence of swarming, the production of proteases as well as their potential to degrade compounds such as DMSP, which was initiated in Chapter 6. To establish if these roles were controlled by quorum sensing, assays could be carried out by creating strains deficient in AHL production by inserting the lactonase gene *aiiA* (Dong et al., 2000) either on a plasmid or onto the chromosome of the strain of interest, blocking AHL-QS and investigating the sustained presence of particular phenotypes. Tait et al. (2005) used this method to establish the attraction of *Ulva* zoospores to *Vibrio anguillarum* bacterial biofilms producing and not producing AHLs. However, to determine processes under QS control, the most powerful method would be to create a signal-deficient mutant by deletion or interruption of the AHL synthase gene. To do this, genomic libraries of AHL producing strains would be introduced into an AHL lux-based biosensor strain that is activated by the AHLs produced by the bacterium (e.g. *E. coli* pSB1075 or pSB401). Recombinant clones producing AHLs would have the ability to produce bioluminescence. This technique has been used routinely for the identification of *luxI*-type genes in other bacteria (Swift et al., 1997). A signal-deficient mutant can then be engineered by interruption of the AHL synthase gene using an antibiotic resistance gene marker.

Alternatively, if the strain of interest can be transformed with a GFP-based AHL reporter plasmid, transposon mutagenesis (*e.g.* using Tn5) could be used to select for colonies unable to produce GFP. Mutation to the AHL synthase gene can be confirmed by rescue cloning of the affected genomic DNA.

Given that a large proportion of coral-associated bacteria are capable of producing AHL molecules it appears likely that they may serve as signals for communication between different species. To allow for *in situ* visualisation of AHL-mediated communication between individual cells, GFP-based monitoring strains could be developed using GFP-based AHL sensor plasmids that respond to a different spectrum of AHL inserted into AHL-negative derivatives of strains found stably in coral tissue. Such a process has been carried out with bacterial colonising the tomato rhizosphere (Steidle et al., 2001) and with GFP-tagged *V. anguillarum* to assess the settlement of *Ulva* zoospores on AHL producing cells (Tait et al., 2005). Similarly, for AHL degrading bacteria, if the active genes can be identified, 'infiltrator reporters' of signal degrading strains can be constructed by labelling with fluorescent proteins and/or GFP-tagged signal degrading reporter plasmids. These reporter strains could be introduced to corals in aquaria and the colonisation process tracked using microscopy. Combined with techniques such as fluorescent in situ hybridisation (FISH), this could give detailed information on the extent of QS activity *in situ* and the location of key signalling and degrading groups within coral microbial communities.

Such a study would give a better idea of the importance of QS in the coral holobiont and corroborate hypotheses in this thesis that signals are detected in high enough quantities in dense enough bacterial communities that they are likely to be used to control community phenotypes *in situ* coral communities (Chapter 3). Such investigations would also add to our knowledge about the location of particular concentrations of AHL production, confirming work by

Ainsworth et al. (2006; 2009) which shows bacteria to be concentrated in particular aggregates and would help to elucidate potential roles of these bacteria and their AHLs *in situ*.

It is more difficult to assess the role that the coral host and symbiotic zooxanthellae play in the control of native bacterial communities. Coral extracts could be assessed for the presence of compounds known to affect bacterial QS, such as the halogenated furanones found in the red alga *Delisea pulchra* (Rasmussen et al., 2000) or manoalide monoacetate found in the marine sponge *Luffariella variabilis* (Skindersoe et al., 2008). Extracts can also be frozen to assess the ability of the crude extract to inhibit the growth of putative pathogens without the bacterial portion of the holobiont, however this does not account for any inductive effect that pathogens/ commensal bacteria may have on the coral host.

Weis et al. (2008) and Bourne et al. (2009) have suggested that the coral holobiont represents an emerging model for studying invertebrate-microbial interactions and how climate drivers can disrupt the homeostasis of the holoiont, resulting in disease. As such, there is a call from many researchers to create a model in which to carry out such studies to allow for a better comparison between studies. This thesis demonstrates this with different reactions to temperature seen in AHL production, degradation and proportion of AHL producing and degrading bacteria found in each coral tested. *Aiptasia pallida* is a species that has been used to understand such interactions for over 30 years (Weis et al., 2008) and may be a good species to use for such a role. However, observations of soft corals from various reefs world-wide (*Sarcophyton* spp., *Sinularia* spp., *Dendronephthyas* pp. and *Lobophyton* spp.) have shown symptoms of bleaching recover quickly, rapidly colonizing local reefs (Obura et al., 2000; Rajasuriya et al., 2000; Smith et al., 2000; Wilkinson, 2000), as opposed to scleractinian corals, which appear to recover slowly (e.g. *Acropora* spp., *Favites* spp. and *Porites* spp. on the Australian Great Barrier Reef; Marshall & Baird, 2000). Therefore, the other side to this coin is that differences between coral phyla and world-wide distribution with respect to infection and recovery from disease is that

researchers need a number of models to test such interactions including a prevalent Scleractinian coral and potentially a soft-coral, such as the *Sinularia* sp. tested in this thesis, to allow comparisons between vulnerable and non-vulnerable species.

7.2.3 Do coral-associated bacteria have temperature thresholds past which normal functioning of these communities cease?

As a result of the changes in bacterial communities and the production and degradation of AHL signals, further research needs to investigate the effect of temperature on bacterial function in the holobiont. Experiments that were initiated in Chapter 6 of this thesis need to be carried out more comprehensively at various temperatures for all the *Discosoma* and *Acropora* sp. isolates. This will not only give information as to the variable effect of temperature stress between corals, but will also allow higher temperatures to be tested, which may reveal more dramatic changes to signalling or degradation. In addition, it will be important to carry out all the experiments mentioned in the previous section at different temperatures to give a more detailed picture of changing bacterial roles and communities with temperature stress, the role of QS and QQ in these communities and how temperature effects these molecules/ enzymes. In addition, it will be important to elucidate which occurs first, a change in signalling and degradation or a change in bacterial community to assess the potential role of QS and QQ in preventative measures of coral disease in the future. It will be important to carry out experiments looking at the effect of a range of temperatures on coral-associated bacterial function to expose the presence of temperature thresholds for the success of certain behaviours, such as antagonism and QS. Once this is established, the behaviour of isolates in mixed culture communities containing a putative coral pathogen could be investigated at a range of temperatures, looking at bacterial community structure and presence of QS and QQ with an increased number of timepoints than those seen in chapter 4 and 5 in this thesis. With such knowledge and if the role of QS and QQ in disease can be experimentally established, the inhibition of this QS by coral-associated bacteria and QS signal antagonists from corals could be investigated as a potential

biological control mechanism for coral disease. However, there is some debate as to the feasibility of such a broad scale mitigation technique and are discussed in the following section.

7.3 How feasible is the biological control of coral disease?

Many coral biologists have called for the development of strategies aimed at controlling coral disease as a result of the world-wide decline of coral reefs (Teplitski & Ritchie, 2009) and as a result various tools for coral disease management are being considered. BBD was the first disease that was proactively treated and involved the removal of the polymicrobial mats associated with its tissue by aspiration and sealing the diseased area with clay in the late 1980s (Teplitski & Ritchie, 2009). Interestingly, treated corals were only re-infected at a low rate (Hudson, 2000) however this process was time consuming and needed a significant investment in labour. Further, recent evidence suggests that six percent of staghorn coral genotypes are resistant to white band disease (WBD), representing the first evidence of host disease resistance in scleractinian corals and demonstrates that staghorn corals have an innate ability to resist WBD infection (Vollmer & Kline, 2008). This suggests that genotypes resistant to such a prolific disease may have a selective advantage over non-resistant genotypes, providing an effective means for the local recovery and persistence of staghorn coral populations. However, being poor sexual recruiters (Ritchie et al., 2001), having limited dispersal distances and slow growth rates, natural selection is not likely to keep up with the spread of coral disease in a rapidly changing environment. As such other tools need to be considered.

One such tool is the use of native mutualistic bacteria as a means of controlling coral diseases, however the methods by which this can be achieved are still in their infancy. Considering the diverse and species specific nature of coral-associated bacteria and their potential role in the coral holobiont, the use of such isolates as a potential method of biological control of coral disease is worth investigating. There is evidence from other environments that bacteria can

induce host defences and inhibit pathogens by producing biocides that inhibit growth or degrade their cell walls (Ravi et al., 2007; Balcázar et al., 2006); disrupt virulence, attachment or cell-cell communication in pathogens (Dong et al., 2000); and outcompete pathogens for available space and nutrients. It will be worthwhile to establish the presence of coral-associated bacteria that can do the same for putative pathogens of coral disease in light of the evidence for antagonism of coral-associated bacteria against other isolates and putative pathogens shown in this thesis and a range of other studies.

Indeed, studies of bacteria in other environments have found that the production of antibiotics by microbes under laboratory conditions correlates with biological control in the field. One example of the application of this method was shown by Ravi et al. (2007) in the control of vibriosis in trout hatcheries. *Paenibacillus* spp. and *Bacillus* sp. isolated from marine sediment were found to excrete an unidentified compound that inhibited the growth of pathogenic *Vibrio* sp. in culture; when added in mixed cultures to trout hatcheries, they inhibited 70 – 80 % of vibriosis disease (Ravi et al., 2007). The addition of *Bacillus subtilis*, *Roseobacter gallaeciensis* and *Pseudomonas aestumarina* (also known to secrete such a compound; Balcázar et al. 2007) to adult shrimp feed also inhibited 10 – 15 % of mortality in shrimp when challenged with a pathogenic *Vibrio* sp. (Balcázar et al., 2007). This suggests the potential use of coral bacteria known to inhibit a range of other isolates and pathogens as putative biological controllers of coral disease. Indeed, as noted above, Alagely and colleagues (2011) have recently shown that a range of bacteria isolated from the sea anemone *Aiptasia pallida* inoculated back in to *A. pallida* polyps at the same time as the coral pathogen *Serratia marcescens*, reduced the appearance of disease symptoms. This corroborates other studies and suggests that the native microbiota associated with marine invertebrates may be capable of producing activities *in situ* that reduce susceptibility of marine invertebrates to opportunistic pathogens, one of which may prove to be the QQ behaviour reported in this thesis.

However, ultimately the success of these agents will be dependent on their ability to be established within the host. In Chapter 2 of this thesis the breakdown of natural communities associated with *Eunicella verrucosa* tissue can be seen predisposing visual signs of disease, a finding backed up by a range of other studies (Bythell et al., 2002; Cooney et al., 2002; Frias-Lopez et al., 2002; Pantos et al., 2003; Gil-Agudelo et al., 2006; Bourne et al., 2007; Sussman et al., 2008). As such, it is likely that such bacterial isolates are vulnerable to environmental stress and thus would not gain a foot hold in an already disturbed coral holobiont. Although the use of these isolates for such a task should still be considered, investigations into other mechanisms for biological control may be more fruitful.

As discussed briefly in Chapter 3, the addition of synthetic AHLs and AHL-QS blockers have been found to change bacterial community composition and function in a number of environments (Valle et al., 2004; Dobretsov et al., 2007). An addition of 2 μM AHLs to activated sludge is capable of generating a change in both community composition with the transitional supplementation of a dominant functional member of the community with a different genus and function, increasing stability and extent of phenol degradation (Valle et al., 2004). In agriculture, QS pathogens have been disrupted when AHL signals were hydrolysed by lactonases, e.g. those produced by *Bacillus* sp. (Dong et al., 2000) or cleaved by acylases e.g. those produced by *Variovorax paradoxus* (Leadbetter & Greenberg, 2000), which have been identified on coral surfaces (Rohwer et al., 2002). However, the use of QS-blockers needs to be approached with care. Dobretsov et al. (2007) showed that QS-blockers affected a number of groups in mixed species biofilms with the most affected being the *Gammaproteobacteria*, the *Alphaproteobacteria* and the *Cytophagales*. As virulence is often controlled by QS, the disruption of QS has been suggested as a way to manipulate virulence-related behaviours (Skindersoe et al., 2008); however in diverse and mixed bacterial communities like the coral holobiont, the use of general QQ-blockers would risk disrupting other important behaviours in these communities.

Both prokaryotes and eukaryotes have been found to produce AHL-mimics which bind to receptors and block QS in some bacteria. Although this thesis has shown that crude coral extracts have the ability to degrade synthetic AHLs, it is so far unknown whether corals have the ability to block the process of QS through the production of mimics. An interesting avenue to explore would be to establish whether the coral host produces any compounds that deter bacteria or their QS signals, which are found to be degraded or up-regulated in times of stress, and whether they are species specific or broad-spectrum in nature, with the theory that an addition of these would allow native bacterial populations to exist while inhibiting putative pathogens. This in theory could be achieved as QS signal receptors are extremely specific and differ in their susceptibility to manipulation by signal antagonists (Rasmussen & Givskov, 2006; Skindersoe et al., 2008; Rajamani et al., 2008; Teplitski et al., 2004). One fascinating example of this is the free-living microalga *Chlamydomonas reinhardtii,* which produces AHL signal-mimics that inhibit some AHL signal receptors in bacterial pathogens and activate others (Rajamani et al., 2008; Teplitski et al., 2004). The coral host and potentially the symbiotic algae may similarly produce compounds which contribute towards structuring bacterial communities in this way.

In conclusion, understanding the cause of mortality and the progression of disease in coral species relies heavily on understanding the basic biology and function of all the members of the coral holobiont and how their functions and roles are disrupted in times of environmental stress. Priority should be given to building on the work presented in this thesis, which will reveal important knowledge that may lead to mitigation of some forms of coral disease in the future.

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Appendix 2: Primer sequences, MID tags and pool/sample identification for 454-pyrosequencing in Chapter 4

In Chapter 4, 454-pyrosequencing was carried out to assess the differences in bacterial communities associated with *Stylophora pistillata* fragments. PCR amplification of the 16S rDNA hypervariable region V4/V5 was performed with primer pools containing two forward and three reverse primers (Huse et al., 2010) with the following sequences:

V4-V5 518F: CCAGCAGCCGCGGTAAN

                    CCAGCAGCTGCGGTAAN

V5-V5 926R: CCGTCAATTCNTTTRAGT

                    CCGTCAATTCTTTTGAGT

                    CCGTCAATTTCTTTGAGT

Each sample was amplified using one of 12 primer pools and each pool was identifiable by a unique decamer multiplex identifier (MID) tag sequence, shown below:

1: ACGAGTGCGT

2: ACGCTCGACA

3: AGACGCACTC

4: AGCACTGTAG

5: ATCAGACACG

6: ATATCGCGAG

7: CGTGTCTCTA

8: CTCGCGTGTC

9: TAGTATCAGC

10: TCTCTATGCG

11: TGATACGTCT

12: TACTGAGCTA

MID sequences were taken from the Roche Technical Bulletin (TCB No. 005-2009) *"Using Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry – Extended MID Set"* (April 2009). These tags were used to enable multiplexing to enable sample multiplexing. The following table identifies which MID tags were assigned to which samples:

	1	**2**	**3**	**4**	**5**	**6**
Lane 1	T0 (1)	T0 (2)	T0 (3)	T0 (4)	T0 (5)	T0 (6)
Lane 2	48h 26 °C (2)	48h 26 °C (3)	48h 26 °C (4)	48h 26 °C (5)	48h 30 °C (1)	48h 30 °C (2)
Lane 3	48h 23 °C (5)	48h 30 °C (5)	7 d 23 °C (1)	7 d 23 °C (2)	7 d 23 °C (3)	7 d 23 °C (4)
Lane 4	7 d 26 °C (5)	7 d 30 °C (2)	7 d 30 °C (3)	7 d 30 °C (4)	7 d 30 °C (5)	

	7	**8**	**9**	**10**	**11**	**12**
Lane 1	Wild (1)	Wild (2)	Wild (3)	Wild (4)	Wild (5)	48h 26 °C (1)
Lane 2	48h 30 °C (3)	48h 30 °C (4)	48h 23 °C (1)	48h 23 °C (2)	48h 23 °C (3)	48h 23 °C (4)
Lane 3	7 d 23 °C (5)	7 d 26 °C (1)	7 d 26 °C (2)	7 d 26 °C (3)	7 d 26 °C (4)	7 d 30 °C (5)