Production and fate of dimethylsulfoniopropionate (DMSP) in reef-building corals and its integral role in coral health

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Abstract

Bacteria play crucial roles in most biogeochemical cycles in the oceans because of their high abundance and metabolic capabilities. Each square centimetre of coral surface harbours between 10^6 and 10^8 bacterial cells, and significantly, bacterial assemblages tend to be highly specific to their coral host. Although the phylogenetic diversity and dynamics of coral-associated bacterial communities have been studied for more than a decade, their ecological and functional roles in the coral holobiont are still poorly understood. The taxonomic composition of these bacterial communities is likely to be greatly influenced by chemicals produced by coral hosts, as well as by their endosymbiotic algae *Symbiodinium*. Dimethylsulfoniopropionate (DMSP) is a ubiquitous compound found within reef-building corals and is a central molecule in the marine sulfur cycle, particularly as a precursor to the climate-regulating gas dimethylsulfide (DMS). Marine bacteria are the primary organisms that degrade DMSP into DMS, and consequently play a critical role in linking the marine environment and the atmosphere in the global sulfur cycle. To date, the role of these organic sulfur compounds in the metabolism of coral-associated bacteria has not been investigated. Consequently, this thesis aims to provide new insights into the roles of DMSP in corals, and more specifically in coral-bacterial associations, with a particular focus on the production and metabolism of this sulfur molecule.

To investigate the roles of DMSP in corals, I developed a new direct approach to accurately and rapidly quantify DMSP and one of its breakdown products, acrylate, based on quantitative nuclear magnetic resonance (qNMR) spectroscopy (Chapter 2). This method overcomes inaccuracies associated with indirect methods that convert DMSP to DMS and measure this volatile molecule. The method was tested on a range of coral genera, and enabled simultaneous and direct quantification of multiple molecules from the same extract, as well as rapid processing with high reproducibility. Thus large numbers of samples can be processed in short time periods. The method was successfully applied to environmental samples and provides the first baseline information on diel variation of DMSP and acrylate concentrations in the coral *Acropora millepora.* The lack of diel variation found raises questions about the role of endosymbiotic dinoflagellates in DMSP biosynthesis in corals.

Reef-building corals are among the most prolific DMSP producers in the ocean, but their DMSP production has been attributed entirely to the activities of their algal symbiont, *Symbiodinium*. Combining chemical, genomic and molecular approaches, I show that coral juveniles from the genus *Acropora* produce DMSP in the absence of associated microalgae (Chapter 3). DMSP levels increased through time (by up to 54% over 6 days) in coral juveniles raised without access to photosynthetic symbionts. Increased DMSP levels in juvenile and adult corals exposed to experimentally elevated temperature treatments suggest a role for DMSP in thermal stress responses. Discovery of coral orthologs of two algal genes recently identified in DMSP biosynthesis suggests that corals possess the enzymatic machinery necessary for DMSP production. My findings overturn the current paradigm that photosynthetic organisms are the sole biological source of DMSP, and highlight a direct role for corals in climate regulation.

In order to investigate the influence of DMSP and DMS on coral-associated bacteria, the bacterial communities of two coral species, *Acropora millepora* and *Montipora aequituberculata*, were characterized by both culture-dependent and molecular techniques (Chapter 4). Three genera, *Roseobacter*, *Spongiobacter*, and *Alteromonas*, which were isolated on media with either DMSP or DMS as the sole carbon source, comprised the majority of bacterial communities in these two corals based on both clone library and pyrosequencing approaches. Bacteria capable of degrading DMSP represented 37% of the communities in *Montipora* and between 67 and 92% in *Acropora*. These results demonstrate that DMSP and potentially DMS act as nutrient sources for coral-associated bacteria, and that these sulfur compounds are likely to play a role in structuring bacterial communities in corals. Exploration of the publically available metagenome databases revealed that genes implicated in DMSP metabolism are abundant in the viral component of coral-reef-derived metagenomes, indicating that viruses can act as a reservoir for such genes (Chapter 4).

The metabolic potential of bacteria in pure culture does not necessarily reflect their metabolic activities within the coral holobiont, therefore I used state-of-the-art imaging techniques (NanoSIMS), coupled with analytical chemistry approaches, to determine linkages between DMSP-synthesising

Symbiodinium and DMSP-degrading bacteria (Chapter 5). DMSP-degrading bacteria were co-incubated with *Symbiodinium* cells previously grown in a medium with isotopically labelled sulfate as sole sulfur source. This experiment confirmed that the sulfur used for DMSP biosynthesis comes from sulfate assimilation in *Symbiodinium* and enabled visualization of sulfur isotope hotspots adjacent to *Symbiodinium* cells that correlated with the location of bacteria observed with electron microscopy. These results confirm the role of coral-associated bacteria in the sulfur cycle and constitute the first empirical evidence of the bacterial assimilation of *Symbiodinium* secondary metabolites *in vivo*.

Bacterial communities associated with healthy corals have been suspected to produce antimicrobial compounds that inhibit the colonization and growth of invasive microbes and potential pathogens; however, antimicrobial molecules derived from coral-associated bacteria have not been identified. In chapter 6, I describe the isolation of an antimicrobial compound produced by *Pseudovibrio* sp., a bacterium commonly associated with reef-building corals and able to degrade dimethylsulfoniopropionate (DMSP). Bioassay-guided fractionation and spectroscopic techniques, including NMR and mass spectrometry (MS), identified the antimicrobial as tropodithietic acid (TDA), a sulfur-containing compound likely derived from DMSP metabolism. TDA was produced in large quantities by *Pseudovibrio* spp. and prevented the growth of two known coral pathogens, *V. coralliilyticus* and *V. owensii*, at very low concentrations (0.5 µg/mL) in agar diffusion assays. Its production was significantly reduced at temperatures causing thermal stress in corals, indicating a role for DMSP-metabolizing bacterial communities in coral disease prevention under ambient temperatures and the potential disruption of this protection during thermal stress events.

In summary, this thesis presents novel information on the production and fate of DMSP in reef-building corals. It identifies the coral animal as a DMSP producer, provides corroborative evidence of the important role of DMSP for numerous coral-associated bacteria using both *in vitro* and *in vivo* approaches, and isolates an antimicrobial compound likely derived from DMSP metabolism. Together, these results constitute the first comprehensive study of DMSP in reef-building corals and underscore the remarkable contribution of this molecule to coral health.

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Chapter 1: General introduction

The importance of sulfur and its biogeochemical cycle in coral reefs

A portion of this chapter is published as:

Raina, J.B., Dinsdale, E.A., Willis, B.L., and D.G. Bourne (2010) Do the organic sulfur compounds DMSP and DMS drive coral microbial associations? *Trends in Microbiology* **18**:101-108.

1.1. The marine sulfur cycle

Biogeochemical cycles involve the transport and transformations of chemical elements between living (biotic) and nonliving (abiotic) compartments, and are intrinsically linked to the abundance and distribution of organisms. Six elements undergoing cycling are essential for living organisms, constituting more than 95% of their biomass: carbon, nitrogen, hydrogen, oxygen, phosphorus, and sulfur (Johnson and Risser 1975, Fagerbakke et al. 1996). In addition, biogeochemical cycles are central to global climate regulation (Post et al. 1990, Galloway 1998). Although the carbon cycle has been studied extensively for its influence on climate, an ocean-atmosphere linkage occurs in the sulfur cycle, and as far-reaching consequences for local climate regulation (Charlson et al. 1987, Andreae 1990, Ayers and Gras 1991). Although geochemical aspects of the sulfur cycle are well known, biogenic processes involved in sulfur cycling are comparatively less well studied. Understanding the sources and sinks of major chemical elements, like sulfur, as well as the biological, chemical and physical processes regulating them are fundamental to understanding interactions within and between ecosystems.

The ocean represents one of the largest reservoirs of sulfur on Earth and the marine sulfur cycle has a significant influence on atmospheric chemistry and climatic processes (Andreae 1990, Sievert et al. 2007). In the photic zone, the largest quantities of sulfur are present as dissolved sulfate, which constitutes the main source of exogenous sulfur for phytoplankton and marine algae (Stefels 2000). Most of this sulfur is assimilated by these organisms into the sulfur-based amino acids cysteine and methionine, and ultimately recycled as dimethylsulfoniopropionate (DMSP) (Stefels 2000). DMSP represents up to 10% of the carbon fixed by marine primary producers in the photic zone (Archer et al. 2001, Simo et al. 2002) and is also the precursor of the volatile dimethylsulfide (DMS). DMS is a gas responsible for the largest natural flux of sulfur into the atmosphere, thus exerting considerable influence on atmospheric chemistry (Sievert et al. 2007). In the atmosphere, DMS is oxidized into aerosol particles that induce the formation of clouds and increase their reflectivity, thereby playing an important role in reducing light levels and water temperatures over marine

ecosystems (Ayers and Gras 1991, Andreae and Crutzen 1997). DMSP production by marine photosynthetic organisms is well documented, and phytoplankton species belonging to prymnesiophyte, chrysophyte and dinoflagellate taxa (Stefels et al. 2007) are believed to produce more than half of the biogenic sulfur emitted to the atmosphere each year (Andreae 1990). Recently, significant concentrations of DMSP and DMS were recorded in invertebrates harbouring symbiotic dinoflagellates, such as scleractinian corals and giant clams (Broadbent et al. 2002, Van Alstyne et al. 2006), suggesting that coral reefs might play a substantial role in sulfur cycling.

1.2. A sulfurous smell over coral reefs

The importance of coral reefs in the carbon and nutrient biogeochemical cycles of shallow, tropical waters is well established, but their contribution to biogenic sulfur cycling has not been investigated (Broadbent et al. 2002). Coral reefs are typically confined to oligotrophic waters with extremely low phytoplankton densities; consequently, their productivity is largely dependent on the cycling of nutrients and trace elements by reef-associated bacterial communities. In this type of ecosystem, benthic organisms, particularly those containing symbiotic dinoflagellates (*Symbiodinium* spp.), dominate primary production (Broadbent et al. 2002). The densities of symbiotic dinoflagellates present in coral tissues are equivalent to those recorded in phytoplankton blooms (Van Alstyne et al. 2006). As dinoflagellates are among the biggest producers of DMSP, and high intracellular concentrations of this compound have been found in cultured *Symbiodinium* (Keller et al. 1989), it is reasonable to predict that symbiotic dinoflagellates might play an integral role in sulfur cycling in reef waters, but this has not been empirically demonstrated to date.

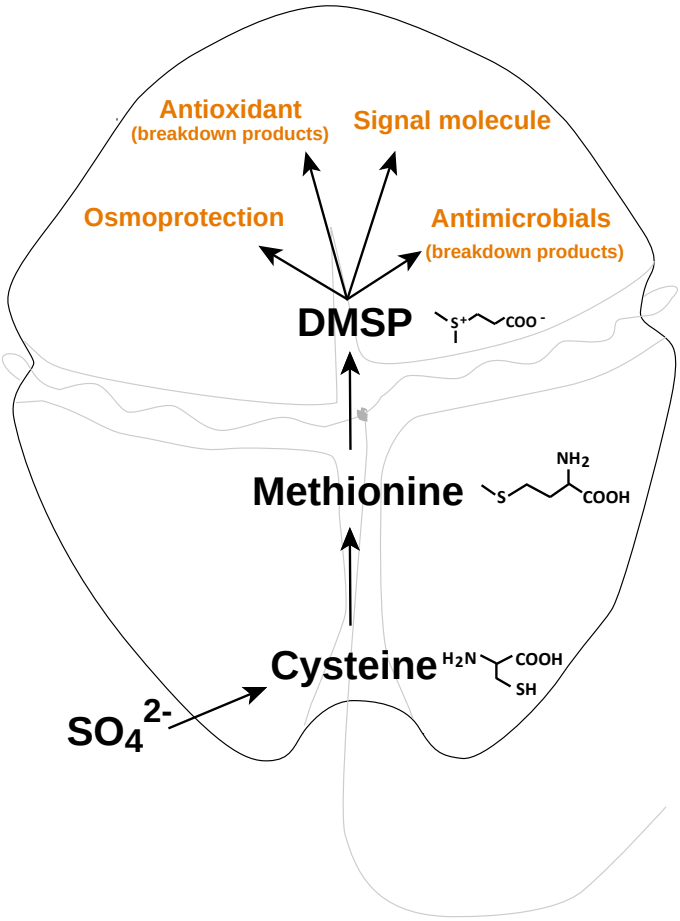

Figure 1.1: Production of DMSP in marine dinoflagellates and its possible functions (in orange) for these organisms.

In the oceanic system, DMSP produced by planktonic dinoflagellates (Figure 1.1) is released into the surrounding seawater through exudation, grazing or viral infection (Christaki et al. 1996, Laroche et al. 1999, Evans et al. 2007). An important fraction of the released DMSP is converted to DMS through a process mainly mediated by bacteria (Figure 1.2) (Moran et al. 2004). However, most of the DMS produced is consumed by bacteria before reaching the atmosphere (Kiene and Bates 1990). Bacteria are highly abundant in corals (Rohwer et al. 2001), although their role in the physiology and health of the coral holobiont (a complex symbiosis between the coral animal, endosymbiotic algae and an array of microorganisms) is still poorly understood. The species-specific nature of coral-microbial associations suggests that microbial communities provide benefits to their hosts, such as fixation and passage of nitrogen and carbon to other members of the coral holobiont (Williams et al. 1987, Shashar et al. 1994, Rohwer et al. 2002, Lesser et al. 2004, Lema et al. 2012),

along with production of secondary metabolites (e.g. antibiotics) that inhibit growth of potentially pathogenic microbes (Castillo et al. 2001, Ritchie 2006). As corals produce high levels of DMSP and DMS, and metabolism of these compounds typically involves bacteria, it is reasonable to predict that coral-associated bacteria might be involved in the cycling of these compounds. However, this possibility was unexplored before I commenced this PhD study.

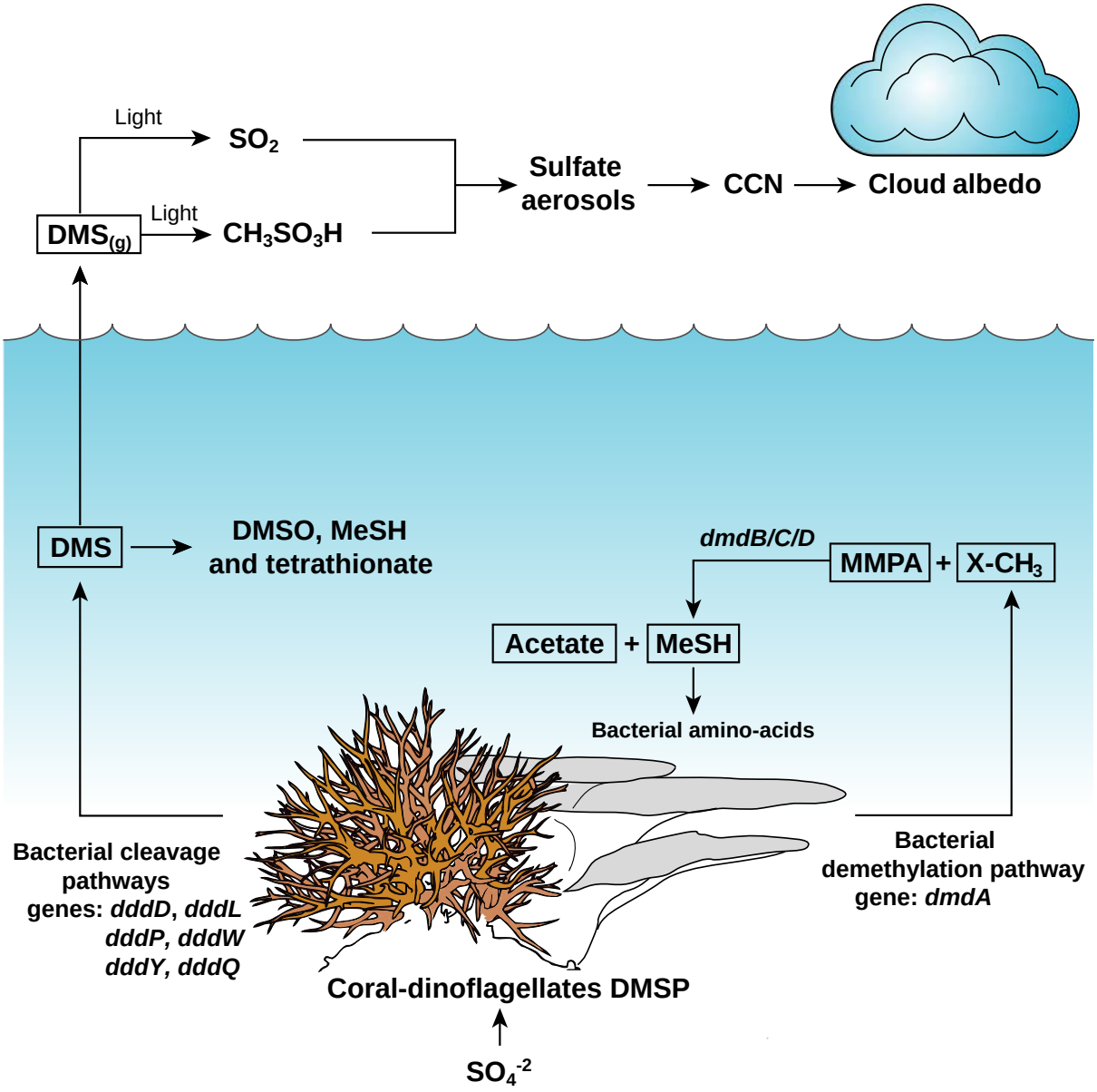

Figure 1.2: Following its production by *Symbiodinium*, dimethylsulphoniopropionate (DMSP) is subjected to bacterial degradation following two general routes: *i*) the demethylation pathway, leads to the production of methylmercaptopropionate (MMPA) and ultimately methanethiol (MeSH); *ii*) whereas the cleavage pathway produces dimethyl sulphide (DMS) and either acrylate or 3-hydroxypropionate. DMS can then be further transformed by DMS-degrading bacteria or released into the atmosphere, where it can be converted to sulphate aerosols, enhancing the formation of cloud condensation nuclei (CCN). MeSH, methanethiol; DMSO, dimethyl sulfoxide; X, tetrahydrofolate.

1.3. Production of methyl-sulfur compounds on coral reefs

Despite variability between species, DMSP concentrations measured in corals are typically one to three orders of magnitude greater than values obtained for benthic algae (Broadbent et al. 2002, Van Alstyne and Puglisi 2007). Furthermore, DMSP concentrations measured in coral 'mucus ropes' (i.e. patches of mucus present at the sea surface during low tides) (Broadbent and Jones 2004) are one order of magnitude greater than the highest levels measured from highly productive polar waters of Antarctica (Fogelqvist 1991, Trevena et al. 2000, 2003). Similarly, concentrations of the breakdown product DMS in mucus ropes are the highest on record (Broadbent and Jones 2004). These extremely high concentrations of DMSP and DMS measured in coral mucus suggest that coral reefs play a disproportionately larger role in global sulfur cycling than would be predicted from their geographically relatively modest distribution.

Studies conducted in the Northern Great Barrier Reef (GBR) revealed that atmospheric DMS concentrations increase during daytime and are positively correlated with tidal height (Jones and Trevena 2005, Broadbent and Jones 2006). A seasonal and diurnal study carried out on One Tree Island reef (Southern GBR), showed an increase in both atmospheric and dissolved DMS concentrations during summer (Broadbent and Jones 2006). This increase in DMS was directly linked to higher levels of dissolved DMSP in reef waters, probably as a result of a seasonal increase in the photosynthetic activity of corals and benthic algae (Broadbent and Jones 2006). Moreover, dissolved and atmospheric DMS concentrations were three times lower after a coral bleaching event (i.e. loss of the symbiotic dinoflagellate partner, *Symbiodinium*, from coral tissues) (Jones et al. 2007). The same pattern of diminished DMS concentrations following coral bleaching was observed in closed chamber temperature experiments involving the coral *Acropora formosa and Acropora intermedia* (Jones et al. 2007, Fischer and Jones 2012), providing evidence that corals are likely driving seasonal variations in DMS production observed in reef waters (Broadbent and Jones 2006).

1.4. The role of microorganisms in the methyl-sulfur cycle

1.4.1.Microbes involved in DMSP degradation

The chemical stability of DMSP in seawater (pH 8.3) is eight years (Dacey and Blough 1987), however the majority of DMSP released into seawater appears to be degraded and taken up by bacteria (Howard et al. 2006, Howard et al. 2008, Tripp et al. 2008). This molecule acts as a sulfur and carbon source for a wide range of microorganisms, contributing 50-100% of the total sulfur requirements for heterotrophic bacteria in the surface oceans (Kiene et al. 2000). DMSP-consuming bacteria use at least seven degradation routes (Howard et al. 2006, Todd et al. 2007, Curson et al. 2008, Todd et al. 2009, Todd et al. 2010, Curson et al. 2011, Todd et al. 2011): the demethylation pathway, which converts approximately 70% of dissolved DMSP into methyl-mercaptopropionate and ultimately to methanethiol and acetaldehyde (Howard et al. 2006, Reisch et al. 2011), and six different cleavage pathways, each mediated by different enzymes, but all resulting in the formation of DMS (Figure 1.2).

The first gene involved in DMSP degradation to be characterized, *dmdA*, was identified in 2006 and encodes the enzyme for the first step in the demethylation pathway (Howard et al. 2006), whereas the other genes *dddD*, *dddL*, *dddP*, *dddW*, *dddQ* and *dddY* are involved in the degradation of DMSP into DMS (Todd et al. 2007, Curson et al. 2008, Todd et al. 2009, Todd et al. 2010, Curson et al. 2011, Todd et al. 2011). Orthologs of these seven genes have been found in all subdivisions of the Proteobacteria and in the Cytophaga–Flavobacterium cluster, confirming that the capacity to degrade DMSP is common to all major phylogenetic groups of marine bacteria (Howard et al. 2008, Todd et al. 2009). The capacity of marine bacteria to degrade DMSP is further supported by the isolation of a range of phylogenetically diverse bacteria from DMSP-enrichment cultures (Appendix A; Tables S1.1 and S1.2). The widespread capacity of marine microbes to metabolize DMSP and their preference for DMSP over other abundant sources of sulfur is remarkable, considering, for example, that seawater contains 10 million times more sulfate than DMSP (Kiene et al. 1999, Kiene et al. 2000). Some DMSP-degrading bacteria are also extremely abundant. For example, members of SAR11, the most

numerous and ubiquitous clade of marine bacteria, have an incomplete set of genes for assimilatory sulfate reduction and require reduced sulfur (such as DMSP) for growth (Tripp et al. 2008). Similarly, *Roseobacter* spp. (*Alphaproteobacteria*, *Rhodobacterales*) a taxon accounting for up to 30% of the bacterioplankton and dominating communities during phytoplankton blooms (Gonzalez and Moran 1997, Moran et al. 2007) display high chemotactic attraction towards DMSP (Miller et al. 2004). This could explain why *Roseobacter* spp. are early colonisers of coral larvae from *Pocillopora* species, a group of corals that vertically transmit DMSP-producing *Symbiodinium* to their larvae during reproduction (Apprill et al. 2009). Therefore, DMSP could act as a primary chemical cue enabling the establishment of bacterial communities associated with corals.

1.4.2.Bacteria involved in the degradation of DMS

The importance of DMS was revealed in 1972, when it was identified as the missing gaseous compound that enables the steady-state flow of sulfur between marine and terrestrial environments, making DMS emission a key step in the global sulfur cycle (Lovelock et al. 1972). Concentrations of this volatile compound are typically two orders of magnitude lower in the atmosphere than in ocean surface waters, resulting in a net flux of sulfur (estimated to be between 15 and 40 million tons per year) from the oceans to the atmosphere (Andreae and Crutzen 1997, Kettle and Andreae 2000, Bentley and Chasteen 2004). DMS flux to the atmosphere was thought to be the major removal pathway of this compound from the ocean; however, more recent studies have revealed that between 50% and 80% of the DMS produced is directly consumed by bacteria (Kiene and Bates 1990).

Degradation of DMS has been described for a range of aerobic and anaerobic prokaryotes (Visscher and Taylor 1993), and several very abundant DMSP-degrading bacteria can also degrade DMS (Appendix A; Table S1.2). However, to date, all known DMS-degrading organisms belong to the Proteobacteria class. Two degradation pathways have been identified in these organisms: (*i*) the aerobic pathway, in which DMS is degraded by an NADH-dependant monooxygenase to form methanethiol and formaldehyde (De Bont et al. 1981, Suylen et al. 1986); and (*ii*) the

methyltransferase pathway, which does not use oxygen as a substrate and therefore allows growth on DMS with nitrate or nitrite as the electron acceptors (Visscher and Taylor 1993). However, the genes encoding DMS monooxygenase, DMS methyltransferases and other key enzymes involved in DMS degradation (Schafer 2007) have not been identified. Owing to the lack of genetic markers targeting the genes encoding these enzymes, culture-based techniques have been used to identify the DMS degraders. As only a small percentage of marine bacteria can grow on artificial culture media, it is probable that only a similarly small percentage of the DMS-degrading bacteria have been isolated.

1.4.3.Coral-associated bacteria

In nutrient-poor coral reef environments, bacteria are extremely dependent on organic compounds produced by photoautotrophic organisms, such as the coral endosymbiont *Symbiodinium* (Ritchie and Smith 2004). The photosynthetic products that are released into coral tissues lead to mucus production and potentially govern the microbial communities present in corals (Ritchie and Smith 2004). A detailed understanding of the bacterial communities closely associated with corals is only beginning to emerge (Rohwer et al. 2002, Wegley et al. 2007, Bourne et al. 2009), and the nature of their interactions with the coral host remains an important research question.

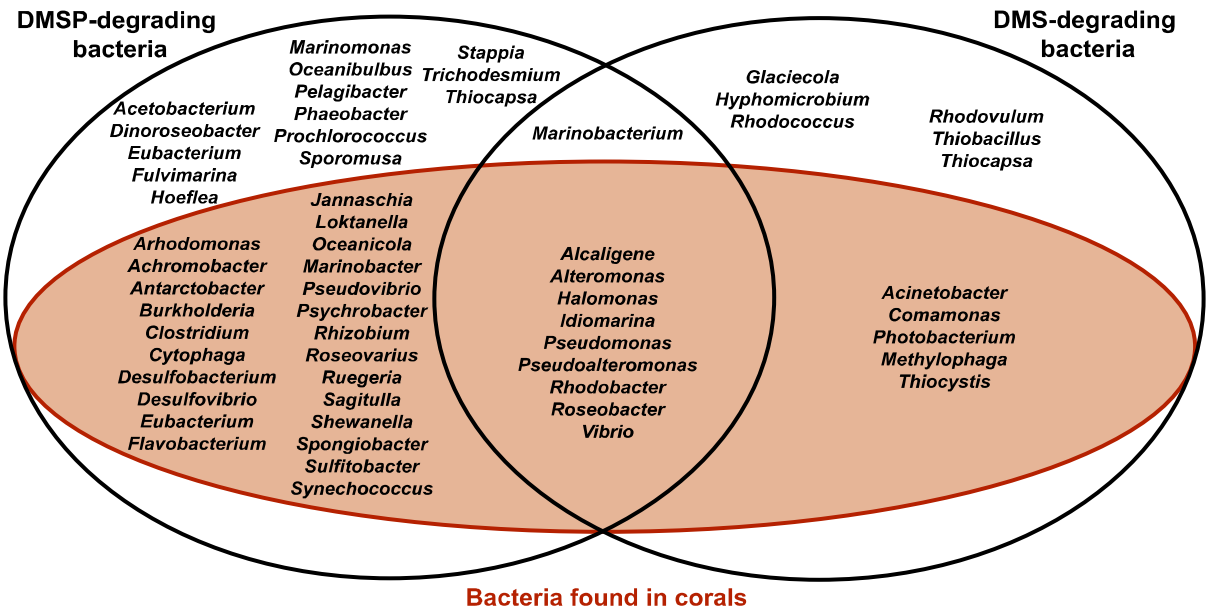

Figure 1.3: Comparison of marine bacterial taxa involved in degradation of DMSP/DMS with those associated with corals. The bacterial genera implicated in the degradation of DMSP (left oval) and DMS (right oval) in the water column overlap extensively with the bacterial taxa found in corals (orange oval). Further details, including references, can be found in Appendix A; Tables S1.1 and S1.2.

To explore the links between sulfur metabolism and coral-associated microbial communities, I completed an extensive survey comparing bacterial species associated with corals to those implicated in the degradation of DMSP and DMS. I found that more than 65% of the bacterial genera known to be involved in DMSP/DMS metabolism have also been reported to be associated with corals (Figure 1.3, Appendix A; Table S1 and S2 (Raina et al. 2010)). This survey indicates that corals harbour a large number of bacterial strains potentially involved in the metabolism of methylated sulfur compounds, suggesting that these compounds might be important in structuring coral-associated microbial communities.

1.5. Putative roles of DMSP and DMS in the coral host

1.5.1. Reported roles of DMSP and DMS

DMSP is involved in several protective physiological functions within phytoplankton and macroalgal cells, including osmotic regulation and cryoprotection, which may explain its high concentrations in bacterial communities in polar waters (Trevena et al. 2000). It is also an important

signal molecule in the marine environment, acting as a foraging cue for herbivorous fishes (DeBose et al. 2008) and attracting a diverse array of bacteria (Seymour et al. 2010). DMSP reacts rapidly with reactive oxygen species such as hydroxyl radicals (·OH), and therefore can be considered an effective cellular scavenger of damaging free radicals (Sunda et al. 2002). However, DMS and acrylate, the breakdown products of DMSP, are even more effective ·OH scavengers, being 20–60 times more reactive than DMSP in laboratory-based conditions (Sunda et al. 2002). Therefore, these three compounds, (DMSP, DMS and acrylate) acting together, might constitute an extremely effective antioxidant system (Sunda et al. 2002). Enzymatic cleavage of DMSP could substantially increase antioxidant protection of cells, highlighting an important metabolic function of bacterial degradation (Sunda et al. 2002). Extremes of ultraviolet radiation, CO_2 and iron limitation, plus high H_2O_2 and ·OH levels, have been correlated with a significant increase in concentrations of cellular DMSP and its breakdown products in marine algae and anemones (Lesser and Shick 1989, Butow et al. 1998, Okamoto et al. 2000, Sunda et al. 2002). Furthermore, antimicrobial activities are frequently associated with the presence of DMS and acrylate in both bacteria and phytoplankton (Sieburth 1960, 1961). Laboratory experiments revealed that millimolar concentrations of both DMS and acrylate reduced bacterial growth and they were most effective when applied in combination (Slezak et al. 1994, Evans et al. 2006).

1.5.2. Relevance of these roles for corals

1.5.1.1. <u>Antioxidant function</u>

It remains unknown whether an antioxidant mechanism involving DMSP and its breakdown products is present in corals; however, the high light intensity of reef environments and the presence of coral-associated bacteria able to cleave DMSP suggest that such a mechanism is possible and potentially important for corals. Loss of *Symbiodinium* during coral bleaching events could result in a decrease in associated DMSP production, possibly amplifying physiological stress to the coral host and potentially increasing rates of coral mortality.

1.5.1.2. Antimicrobial properties

To maximize the uptake of prey and nutrients, most coral species have a high surface area to volume ratio. However, this condition might also maximize exposure to potentially pathogenic bacteria. Although lacking a sophisticated immune system, corals have previously been reported to inhibit the growth of some invasive bacterial species (Geffen and Rosenberg 2005, Ritchie 2006, Geffen et al. 2009). The lower pH (around 7.7) of coral mucus compared with the surrounding seawater (~8.2) (Wild et al. 2005) indicates the presence of acidic compounds in mucus, and acrylate seems a likely candidate to explain this phenomenon. Coral mucus and tissues contain high concentrations of DMS and acrylate and could therefore play a significant role in the prevention of pathogenic bacterial colonization.

1.6. Study aims and objectives

My over-arching goal in this study was to investigate the roles of the methyl-sulfur compounds DMSP and DMS in the coral holobiont, and more specifically in coral-bacterial associations, with a particular focus on the production and metabolism of these two sulfur molecules. To accomplish this goal, I addressed the following five specific objectives:

1. **To accurately quantify DMSP production in corals by developing a direct technique that enables rapid and precise measurement of DMSP.** Current methods measure DMSP indirectly *via* its transformation into DMS. Indirect measurement techniques are not suitable for coral samples since they naturally contain high concentrations of DMS. Development of a direct technique using nuclear magnetic resonance (NMR) spectroscopy would significantly advance the field and was critical to enabling DMSP quantification in corals in my subsequent experiments.

2. **To determine the impact of thermal stress on the production of DMSP in coral juveniles and adults.** Understanding the impact of thermal stress on DMSP production by corals

provides the first insights into whether DMSP is likely to play a significant role in coral stress responses. Comparison of DMSP production under thermal stress between adults with established photosymbionts and newly settled juveniles lacking photosymbionts untangles the respective contribution of the coral host and its photosynthetic symbionts to the DMSP pool produced by coral reefs.

3. **To examine the potential roles that the sulfur compounds DMSP and DMS play in the maintenance of coral-associated bacterial communities and the health of the coral holobiont.** By using culture-dependant techniques as well as molecular tools ranging from clone libraries to metagenomic analyses, this study will determine if these two compounds provide important nutrient sources for coral-associated bacteria, significantly advancing current understanding of how coral-associated bacterial communities are structured and the role they play in coral health.

4. **To visualise the translocation and metabolism of DMSP between the coral endosymbiont *Symbiodinium* and coral-associated bacteria.** Using nano-scale secondary Ion Mass Spectrometer (NanoSIMS) to detect, visualise and localise compounds at the single cell level, I will follow labelled DMSP molecules synthesised *in vivo* by *Symbiodinium* and their uptake by coral-associated bacteria. This study will provide corroborative evidence of the central role of DMSP for coral-associated bacteria and the first visualisation of interactions between *Symbiodinium* and bacteria.

5. **To explore the function of DMSP in coral defence against pathogens.** Isolation of the first antimicrobial molecule produced by coral-associated bacteria, growing on DMSP as sole the carbon source, highlights the significant functional role that DMSP-degrading bacteria play in the health of the coral host.

1.7. Thesis structure

The five objectives listed above are addressed in Chapters 2 to 6. In Chapter 2, I introduce a new technique allowing quick and direct quantification of DMSP in coral samples. I test the applicability of the method to a range of coral species and investigate potential variations in DMSP concentration throughout a 24 hour period. In Chapter 3, I characterise the production of DMSP by the common reef-building coral *Acropora millepora* at different life-stages in controlled aquarium experiments. This chapter clarifies the respective roles of the coral animal and its photosynthetic symbionts in DMSP production and investigates the effect of thermal stress on the production of this molecule. In Chapters 4, 5 and 6, I investigate the fate of DMSP in the coral holobiont and its usage by coral-associated bacteria. More specifically, in Chapter 4, I examine the role of this molecule as a nutrient source, isolating coral bacteria capable of using DMSP to sustain their growth and estimating the abundance of DMSP-degrading consortia using molecular profiling. Chapter 5 underpins findings in Chapter 4, using state-of-the-art imaging techniques to visualise *in vivo* the bacterial uptake of DMSP produced by *Symbiodinium* associated with corals. This chapter provides additional support for the critical role that this molecule plays in structuring bacterial assemblages associated with corals. In Chapter 6, I investigate the functional role of DMSP-degrading bacteria and more specifically their antimicrobial properties. The isolation of a sulfur-based antimicrobial molecule provides new insights into the fate of DMSP in corals and the role that this molecule plays in coral health. Lastly, in Chapter 7, I discuss results obtained in the above chapters in an ecological context and synthesise discoveries from this complete body of work into a conceptual framework following the fate of sulfur in the coral holobiont.

Chapter 2: Direct measurement of dimethylsulfoniopropionate (DMSP) in reef-building corals

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2.1. Introduction

The compound dimethylsulfoniopropionate (DMSP) and its breakdown products dimethylsulfide (DMS) and acrylate have numerous functions in both marine and terrestrial ecosystems. In the marine environment, DMSP is an important signaling molecule, attracting a diverse array of bacteria (Seymour et al. 2010) and acting as a foraging cue for herbivorous fishes (DeBose et al. 2008) and marine birds (Cunningham et al. 2008, Nevitt 2008). In addition, DMSP has been involved in osmoregulation in marine algae (Kirst 1996) and cryoprotection in ice algae (Kirst et al. 1991). Acrylate has significant antimicrobial properties (Sieburth 1961) and, in combination with DMS and DMSP, this group of compounds constitutes an effective antioxidant system in marine algae (Sunda et al. 2002). Moreover, DMS has been the focus of considerable attention because of its possible role in climate regulation. DMS diffuses from seawater into the atmosphere where it is subsequently oxidized to form non-sea-salt sulfate aerosol particles, such as sulfur dioxide (SO_2) (Charlson et al. 1987, Andreae and Crutzen 1997). These aerosols enable the condensation of water molecules and can significantly affect solar radiation and sea surface temperature over coral reefs (Deschaseaux et al. 2012, Fischer and Jones 2012).

Although DMSP concentrations in sea water are usually in the nM range (Kettle et al. 1999), concentrations reported from reef-building corals are several orders of magnitude higher, with values in coral mucus as high as 54 µM (Broadbent and Jones 2004). These high concentrations of DMSP in corals are assumed to be due to their endosymbiotic microalgae (*Symbiodinium* spp.) (Broadbent et al. 2002, Broadbent and Jones 2004, Van Alstyne et al. 2006). Overall, DMSP concentrations appear to be highly variable between coral species (Van Alstyne and Puglisi 2007), potentially reflecting differences between their associated *Symbiodinium* clades, which are also known to vary in DMSP concentration (Steinke et al. 2011). The reasons for the production and accumulation of DMSP in coral-*Symbiodinium* symbioses have not been elucidated, nor has the effect of anthropogenic stressors on the concentration of this compound *in situ* been fully addressed. The capacity to readily quantify DMSP is a critical prerequisite for addressing these fundamental physiological questions.

Traditionally, DMSP quantification in corals has been achieved with gas chromatography (GC). This method has the advantage of being highly sensitive, with a detection limit of 1 nmol (Yost and Mitchelmore 2010); however, DMSP has to be measured indirectly, *via* alkaline hydrolysis to DMS. DMS is volatile and unstable when stored improperly (Sulyok et al. 2001), and is itself naturally present in high concentrations in coral samples (Broadbent et al. 2002). Thus, the *in vitro* conversion of DMSP into DMS for quantification purposes potentially introduces a bias, mixing natural DMS content with DMS produced in the alkaline hydrolysis step. These issues, inherent to this indirect method of measurement can be solved using a direct measurement technique, such as that developed by Spielmeyer *et al.* (Spielmeyer and Pohnert 2010). In this direct measurement technique, derivatization of DMSP with 1-pyrenyldiazomethane followed by reverse phase ultra high performance liquid chromatography coupled with mass spectrometry (UPLC-MS) enabled direct determination of DMSP levels in marine algae (Spielmeyer and Pohnert 2010). Another unexplored technique for directly detecting DMSP is nuclear magnetic resonance (NMR) spectroscopy (Chudek et al. 1987).

NMR spectroscopy has been used extensively for both the identification and quantification of chemicals from complex mixtures (Taggi et al. 2004, Pierens et al. 2005, Motti et al. 2009). Since the area under a ^{1}H NMR signal (the integral) is directly proportional to the number of protons giving rise to that signal, it allows very precise quantification of the compound of interest (Pauli 2001). The error of quantitative NMR (qNMR) has been reported to be less than 2% when acquisition parameters are optimized (Malz and Jancke 2005). NMR has a number of unique advantages over UPLC-MS and GC, as it is a non-destructive technique, allowing samples to be recovered for further analysis or purification if needed. It also allows simultaneous quantification of multiple compounds from the same extract without the addition of internal standards. Furthermore, it is highly reproducible and the analysis time required per sample is relatively short (between 1 and 15 minutes) (Silvestre et al. 2001), enabling extensive replicate numbers and complex experimental designs.

Here, I apply qNMR to samples of reef-building corals from the Great Barrier Reef (GBR) to: (*i*) determine the optimal parameters needed to ensure precise and accurate quantification of DMSP and acrylate, (*ii*) assess the applicability of the proposed method in a variety of reef-building coral genera, and (*iii*) determine the influence of diel circadian rhythms on DMSP and acrylate concentrations in the common reef-building coral *Acropora millepora*, demonstrating the suitability of the method for analyzing large numbers of samples.

2.2. Methods

2.2.1. Sample collection and analysis for method development

Colonies of *A. millepora* (*n*=3) were collected from Davies Reef, Great Barrier Reef, Australia (18°05' S/147°39' E) and single fragments (nubbins) were extracted into 2 mL of deuterated methanol (CD_3OD) for 2 minutes. CD_3OD was chosen for its ability to solubilize DMSP (Chudek et al. 1987) which allowed immediate analysis of the samples. The extracts were spun down, transferred directly into 5 mm Norell 509-UP NMR tubes and analyzed immediately by ^{1}H NMR. ^{1}H NMR spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer (Bruker, Germany) with a TXI cryoprobe, referenced using CD_3OD (δ_H 3.31). Spectra were acquired spinning at 298 K, using the standard Bruker zg pulse program.

2.2.2.Stability and recovery of the compound

To monitor the stability of the measured compounds, three CD_3OD extracts in NMR tubes were stored at -20°C for 24 hours, and then left at room temperature (25°C) on a laboratory bench for an additional 24 hours and ^{1}H NMR spectra were sequentially re-acquired at four different time points. The technique's ability to recover a known amount of DMSP was also assessed. Three extracts were spiked with 14 µL of 50 mM DMSP in 700 µL extracts (leading to a 1 mM increase in DMSP concentrations). ^{1}H NMR spectra were acquired before and after spiking to estimate the percent of recovery.

2.2.3. Sample collection for comparative survey of coral genera

Samples of 17 different genera of corals, including 18 different species (*n*=1 sample per species) were collected from Davies Reef (central GBR) using a small bone-cutters. Collected samples were handed over to a boat attendant and immediately preserved in liquid nitrogen.

2.2.4. Sample collection and experimental design for diel circadian rhythm study

Colonies of *A. millepora* (n=9) were collected from Trunk Reef, Great Barrier Reef, Australia (18°17' S/146°53' E), transferred to the outdoor aquarium facility at the Australian Institute of Marine Science (Townsville, Queensland, Australia) and acclimated for 2 weeks prior to starting the experiment. The colonies were arranged in three different aquaria (n=3 colonies per aquarium) and were exposed to natural sunlight. The amount of solar irradiance reaching the colonies was adjusted using 50% light reduction shade cloth to mimic the high light (midday irradiance 1200 μmol photons m^{-2} s^{-1}) and clear water environment of the collection site (3m depth, mid-shelf reef flat).

Samples were collected every two hours over a 24 hour period starting at midnight. The time points preceding sunrise (06.30 am) and following sunset (05.30 pm) were monitored every hour. At every sampling time, one coral fragment (nubbin) was collected per coral colony (n=9 colonies) and immediately snap-frozen and stored in liquid nitrogen until analysis.

2.2.5. qNMR analysis

Based on the results from the method development, samples from the comparative genera and the circadian rhythm studies were extracted using the following protocol: coral fragments were extracted in 5 mL of HPLC-grade methanol (CH_3OH) for 3 hours with sonication at room temperature, followed by a second extraction with an additional 1 mL of CH_3OH (for 10 min). The two extracts were pooled and dried using a vacuum centrifuge (Savant). The dried extracts were resuspended in a mixture of deuterated methanol (CD_3OD, 750 μL) and deuterium oxide (D_2O, 250 μL), vortexed to solubilize the compounds and then centrifuged to pelletize the debris. A 700 μL

aliquot of the particulate-free extract was transferred into a 5 mm Norell 509-UP NMR tube and analyzed immediately by ^{1}H NMR.

^{1}H NMR spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer (Bruker, Germany) with a TXI cryoprobe, referenced using CD_3OD (δ_H 3.31). Spectra were acquired spinning at 298 K, using the standard Bruker zg pulse program, with a sweep width of 12ppm (7184 Hz), a 90° pulse to maximize sensitivity, a relaxation delay of 35 sec, receiver gain of 16, 2 dummy scans, 16 acquisition scans and 64 k data points corresponding to an acquisition time of 4.6 s. Quantification was performed using the ERETIC method (Electronic REference To access *In vivo* Concentrations) (Akoka and Trierweiler 2002). This technique electronically generates an external reference signal during the data acquisition, which is calibrated using stock solutions of 4 mM acrylate and DMSP. The data were Fourier transformed with exponential filtering (em), line broadening of 0.7 Hz and no zero filling (SI = 32 k). The spectra were phased manually and the baseline automatically corrected with a fifth-order polynomial. After calibration, the concentrations of acrylate and DMSP in the NMR sample were determined by comparing the signal intensities of well resolved non-exchangeable protons ($\underline{CH_2}$=$CHCO_2^-$ centered at 6.15 ppm for acrylate and $(\underline{CH_3})_2SCH_2CH_2CO_2^-$ centered at 2.95 ppm for DMSP) in a 0.20 ppm window against the intensity of the reference signal (through signal integration) (Akoka and Trierweiler 2002).

2.2.6. Surface area calculation

Coral skeletons remaining after extraction were dried overnight under vacuum (Dynavac FD12) and their surface area calculated using a wax dipping technique (Veal et al. 2010). The surface area of each individual fragment was used to normalize the qNMR data.

2.3. Results and Discussion

2.3.1. Compound identification using NMR spectroscopy

^{1}H NMR spectra of the direct CD_3OD extract of *A. millepora* contained several well-resolved signals (Figure 2.1). Three multiplet signals (δ_H 5.71, 6.13 and 6.20 ppm, C$\underline{H}_2$=C$\underline{H}$-) diagnostic of acrylate protons were observed as described previously (Tapiolas et al. 2010). Two triplet signals at δ_H 3.45 and 2.72 ppm (2×C$\underline{H}_2$), and a singlet signal at δ_H 2.95 ppm (2×C$\underline{H}_3$) were indicative of DMSP. Furthermore, a singlet signal at δ_H 2.09 ppm (2×C$\underline{H}_3$) corresponded to DMS; while a singlet at δ_H 2.69 ppm established the presence of DMSO (2×C$\underline{H}_3$), the chemical oxidation product of DMS. The presence of DMSP, DMS, acrylate and DMSO was confirmed by comparison with 1D and 2D spectra of the commercially available compounds and by spiking experiments. Spiking was also used to estimate the extent of matrix effects on the position of the diagnostic signals and to determine the method's ability to recover a known amount of DMSP added to coral extracts (Appendix B; Table S2.1). The DMSP recovery was 97.1% (±1.3), which is in line with previous error estimations using qNMR, reported to be approximately 2% when acquisition parameters are optimized (Malz and Jancke 2005).

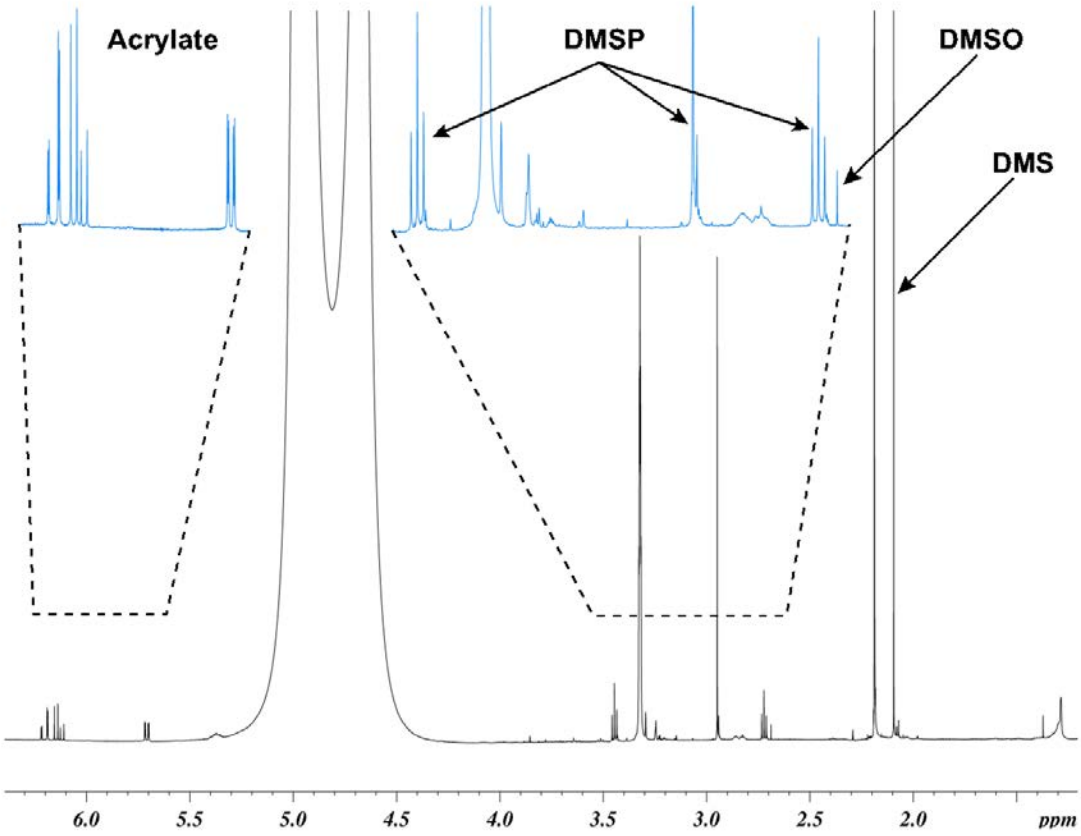

Figure 2.1: ^{1}H NMR spectrum of *Acropora millepora* in CD_3OD showing the position of the DMSP, DMS, DMSO and acrylate signals.

Although the NMR spectra allowed clear identification of these four compounds, only DMSP and acrylate were stable after extraction (Appendix B; Table S2.2). DMSP is relatively stable in seawater, with a half-life of approximately eight years (Dacey and Blough 1987). However, the volatility of DMS and its rapid oxidation into DMSO upon exposure to oxygen and light (Figure 2.2) did not permit a precise quantification of these two molecules and consequently only DMSP and acrylate were subsequently quantified.

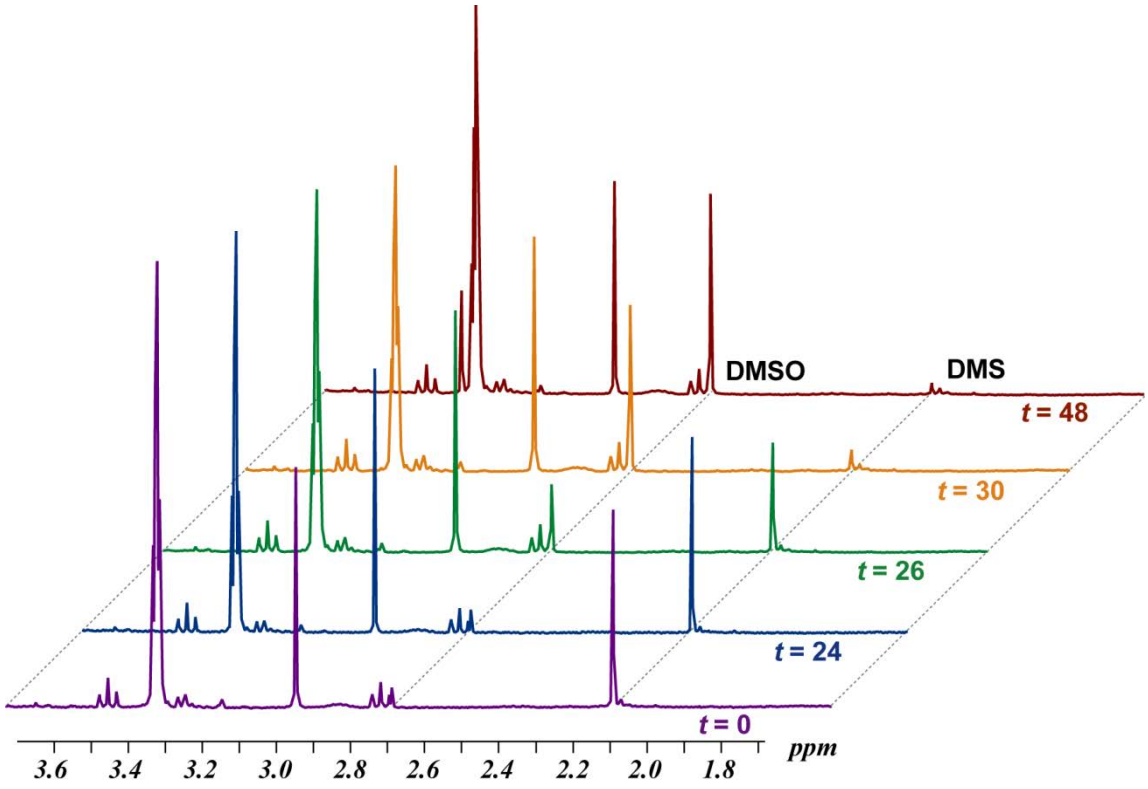

Figure 2.2: ^{1}H NMR spectra of the same *Acropora millepora* extract in CD_3OD through time. The sample was kept at -20°C for 24 hours and subsequently at room temperature (25°C). Note conversion of DMS into DMSO starting after the sample was left at 25°C (from 24 hours onward).

2.3.2. Quantification method development

Based on the above optimization, a mixture of CD_3OD and D_2O was used in the acquisition of ^{1}H NMR spectra to disperse and enhance the resolution of the diagnostic signals. The 90° pulse length and the *T*1 relaxation times for both acrylate and DMSP were determined prior to the quantification to give the best signal-to-noise ratio. Regions containing the two downfield signals from acrylate (6.00 - 6.20 ppm), and the singlet signal arising from DMSP (2.94 - 2.97 ppm) were selected for integration (Figure 2.1).

2.3.3. DMSP and acrylate detection and quantification across coral genera

DMSP was unambiguously detected in 15 coral species, with the highest concentration measured in *Acropora millepora* and the lowest in *Merulina ampliata*. In these corals, at least two of the three DMSP proton signals were clearly visible and well resolved (Table 2.1). However, in four

species (*Goniastrea aspera*, *Porites cylindrica*, *Diploastrea heliopora*, *Hydnophora exesa*), the resolution of the signals was poor, due to the presence of overlapping signals from other compounds (Figure 2.3) which did not enable DMSP quantification. The DMSP concentrations measured were comparable to those reported using classic GC methods (Broadbent et al. 2002, Wilson et al. 2002, Van Alstyne et al. 2006), ranging from 0.03 to 2.47 nmol/mm^2. In comparison, acrylate was observed in 16 of the 18 GBR coral species. The lack of other signals from co-extracted compounds in the region between 6.20 ppm and 5.50 ppm of the ^{1}H NMR spectra facilitated the identification and quantification of this molecule. Interestingly, acrylate concentrations in *Acropora* and few other branching corals, such as *Echinopora* spp. and *Porites cylindrica* were consistently one order of magnitude greater than those of DMSP. This result correlates well with previous measurements on *A. millepora* (Tapiolas et al. 2010) and further suggests that either the turn-over of acrylate is slower than DMSP or that it is stored in these corals for an unknown purpose. These results show that quantitative ^{1}H NMR spectroscopy is a suitable technique to investigate both DMSP and acrylate concentrations in a large array of reef-building corals, including the commonly studied genera *Acropora*, *Pocillopora*, *Seriatopora*, *Stylophora* and *Montipora*.

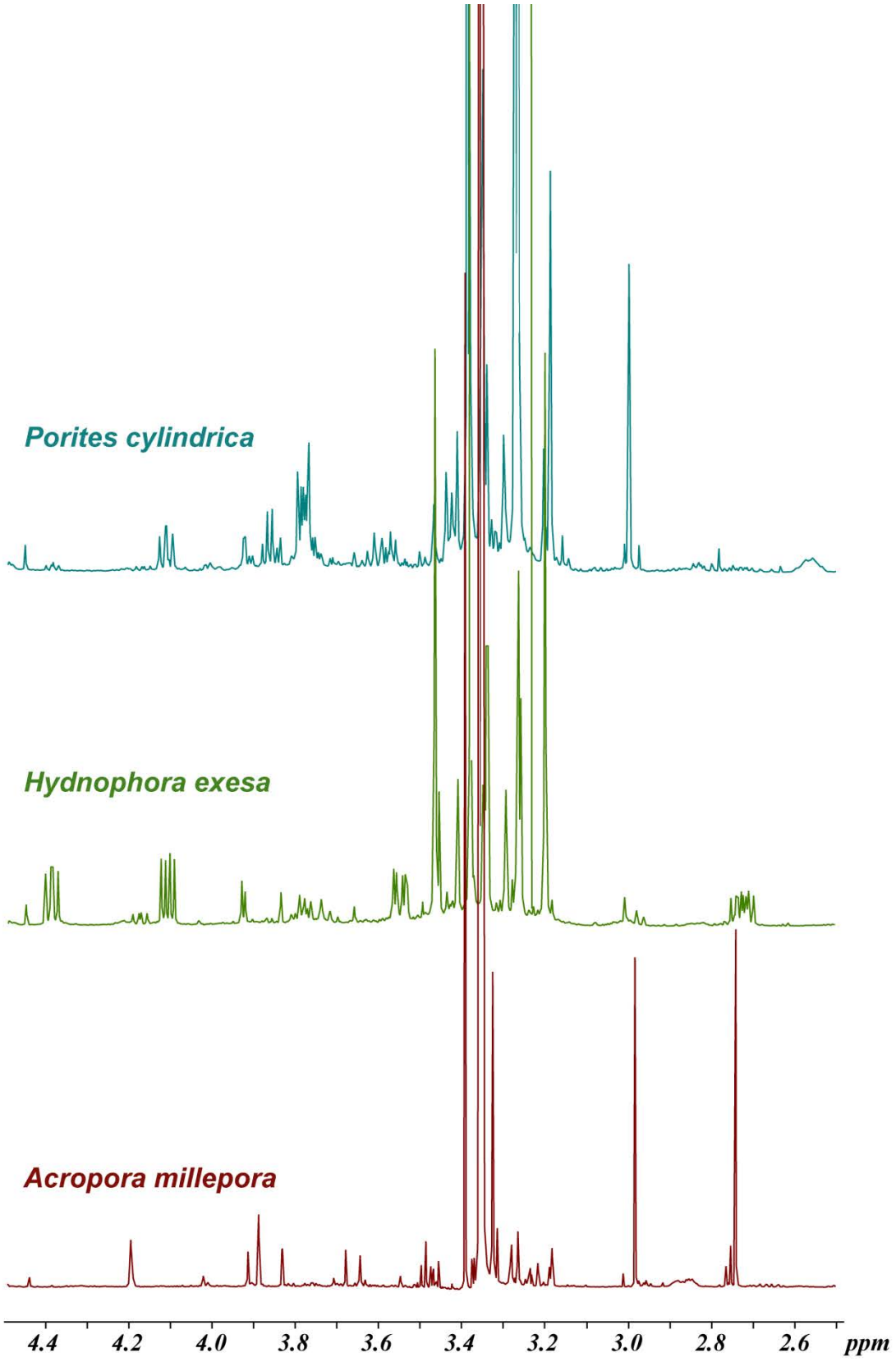

Figure 2.3: ^{1}H NMR spectra of three different reef-building coral species, *Acropora millepora*, *Hydnophora exesa* and *Porites cylindrica*. Note the large number of overlapping signals in *Porites cylindrica* and *Hydnophora exesa*, making DMSP quantification difficult.

Table 2.1: Measurements of DMSP and acrylate in 18 species of hard corals from the Great Barrier Reef. ND: not detectable.

Family	Species	DMSP (nmol/mm^2)	Acrylate (nmol/mm^2)
Faviidae	*Diploastrea heliopora*	ND	ND
Faviidae	*Platygyra sinensis*	0.355	0.936
Faviidae	*Goniastrea aspera*	ND	0.253
Faviidae	*Echinopora spp.*	0.467	8.235
Acroporidae	*Acropora millepora*	2.473	15.223
Acroporidae	*Montipora spp.*	0.092	0.387
Pocilloporidae	*Seriatopora hystrix*	0.362	0.083
Pocilloporidae	*Pocillopora damicornis*	0.333	0.035
Pocilloporidae	*Stylophora pistillata*	0.774	0.130
Poritidae	*Porites spp.*	0.271	1.083
Poritidae	*Porites cylindrica*	ND	2.945
Merulinidae	*Merulina ampliata*	0.042	2.588
Merulinidae	*Hydnophora exesa*	ND	0.759
Agariciidae	*Pachyseris spp.*	0.080	0.803
Euphyllidae	*Physogyra lichtensteini*	1.048	0.330
Fungiidae	*Fungia spp.*	0.353	ND
Mussidae	*Symphyllia recta*	1.517	0.171
Oculinidae	*Galaxea fascicularis*	0.156	1.457

2.3.4. DMSP concentrations in A. millepora throughout the day measured by qNMR

To explore potential daily fluctuations in DMSP levels in corals and establish a clear baseline for variations in relation to time of sampling, DMSP concentrations in *A. millepora* were measured over a 24 hour period. This experiment also allowed the suitability of the qNMR technique for the analysis of large numbers of samples to be tested. Despite the large number of samples collected (n=135), less than 48 hours of work were required to process and analyze all samples. Contrary to my expectations, DMSP concentrations did not change in response to high light conditions that are known to increase photosynthetic activity and oxidative stress in corals (Figure 2.4) (Sunda et al. 2002). the data showed that DMSP concentrations were not influenced by light or potential diel metabolic patterns, but remained constant throughout the 24 hour period, ranging between 2.3 and 3.6 nmol/mm^2 (Figure 2.4). Similarly, acrylate did not display a diel light-related pattern, even though greater variability in its concentration was observed. As with the comparison between different coral

genera, acrylate concentrations were one order of magnitude greater than DMSP over the course of the experiment, ranging from 27.2 to 42.6 nmol/mm^2. The absence of light influence on both DMSP and acrylate concentrations could reflect adaptation of colonies to their high light environment, as revealed by oxidative and photochemical stress measurements conducted on the same colonies (A. Lutz, unpublished results). In summary, under "normal" conditions, light levels do not influence DMSP concentration and temporal variability is minimal in *A. millepora*. This implies that samples could be collected at any time of the day in future studies investigating DMSP concentrations in *Acropora*.

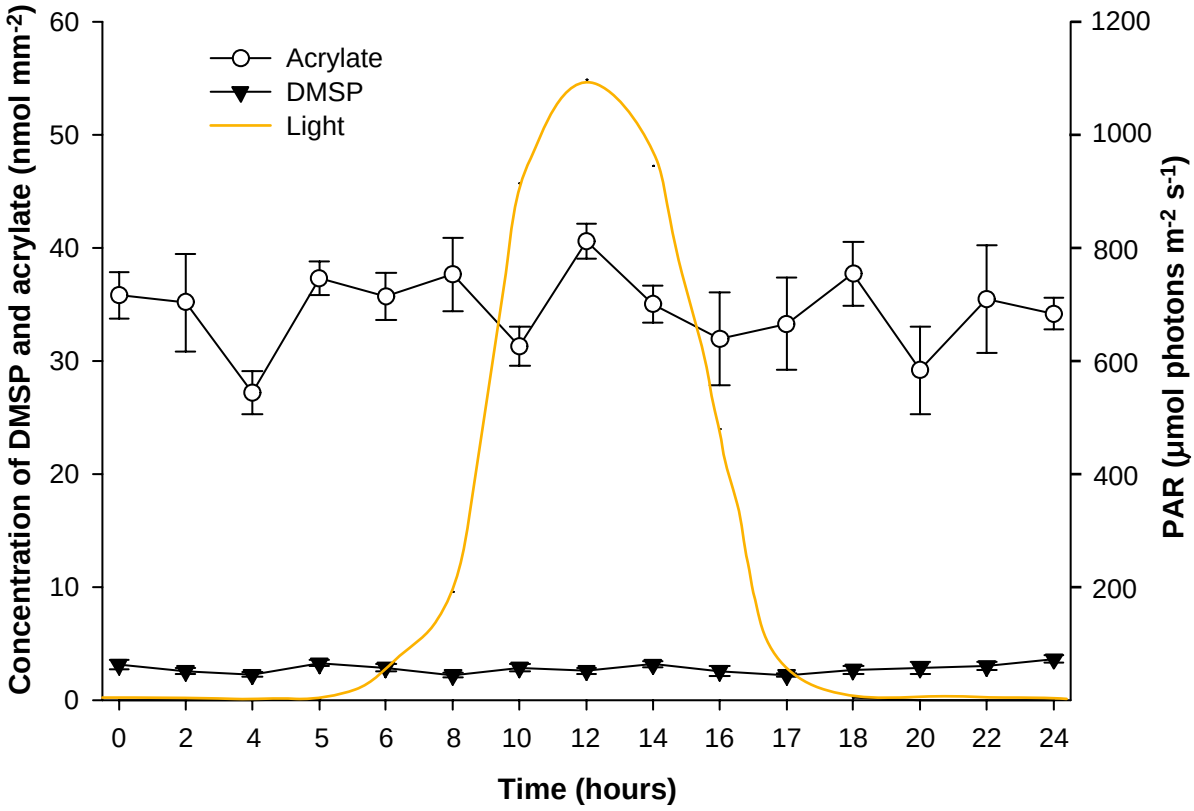

Figure 2.4: Concentration of DMSP and acrylate in *A. millepora* throughout a day. Photosynthetically active radiation (PAR; 400-700 nm) are indicated on the right-hand axis. DMSP and acrylate concentrations remained consistent over the diurnal cycle with 2.3-3.6 nmol mm^{-2} and 27.2 – 42.6 nmol mm^{-2} respectively.

2.3.5. Conclusion

Quantitative NMR allows for the direct and simultaneous quantification of DMSP and acrylate in a wide range of reef-building corals. It has several advantages over measurement methods using GC: it allows direct measurements of DMSP without chemical conversion, reducing the potential risks of sample degradation or concentration artifact through DMS conversion. Furthermore, it allows for the quantification of several compounds simultaneously in a relatively short amount of time, without complex sample preparation. Therefore, qNMR is an efficient and rapid method for quantifying DMSP and acrylate in corals and the method presented here could easily be optimized to quantify these compounds in other DMSP producing organisms.

Chapter 3: DMSP biosynthesis by an animal: implication for reef-building corals in a warming world

3.1. Introduction

Only a few classes of marine algae and some species of higher plants have been reported to produce DMSP, the main producers belonging to phytoplanktonic prymnesiophyte and dinoflagellate taxa (Keller et al. 1989, Scarratt et al. 2002). Marine invertebrates that harbour photosynthetic symbiotic dinoflagellates (*Symbiodinium* spp.), such as reef-building corals, sea anemones and giant clams, also produce DMSP (Broadbent et al. 2002, Van Alstyne et al. 2006). To date, the highest concentrations of DMSP and its breakdown products DMS and acrylate have been recorded in reef-building corals, making coral reefs one of the most important ecosystems in the world in terms of DMSP production (Broadbent and Jones 2004, Tapiolas et al. 2010). However, since it is widely accepted that the production of DMSP is restricted to photosynthetic organisms (Karsten et al. 1990, Kiene et al. 1996, Malin and Kirst 1997, Stefels 2000, Otte et al. 2004), it has been assumed that DMSP produced by corals and other marine invertebrates is derived from the high density of *Symbiodinium* present in their tissues. However, evidence that the total amounts of DMSP recorded in corals are consistently higher than levels present in *Symbiodinium* cells alone (Hill et al. 1995, Yost and Mitchelmore 2010, Yost et al. 2012) raise the possibility of a cryptic source of DMSP in reef-building corals.

Here, I investigate DMSP production in two life history stages of corals from the genus *Acropora*: *i*) coral juveniles prior to the acquisition of photosynthetic symbionts, and *ii*) adult colonies with established endosymbiotic populations of *Symbiodinium*, under both ambient and elevated temperatures constituting thermal stress. Corals in the genus *Acropora* are the most abundant reef-building organisms in the Indo-Pacific region (Veron 2000) and as broadcast-spawning species, they acquire *Symbiodinium* from their surrounding environment following larval development (Harrison et al. 1984). The *Symbiodinium*-free coral larvae of these species provide a unique opportunity to investigate *Symbiodinium*-independent production of DMSP by the coral animal, enabling us to elucidate the roles of both corals and their photosymbionts in DMSP production in marine ecosystems.

3.2. Methods

3.2.1.Adult corals

3.2.1.1. Thermal stress experiment

Acropora millepora colonies (*n*=10) were collected from Pelorus Island, Great Barrier Reef, Australia (18°33' S/146°29' E) and transferred to the Australian Institute of Marine Science. Coral colonies were fragmented to give a total of 24 fragments, each comprising approximately 25 branches (nubbins). Fragments were arranged in eight indoor tanks in a randomized bloc design, resulting in the allocation of 12 coral fragments to each of the control and thermal stress temperature treatments (27°C and 32°C respectively). All tanks were continuously supplied with fresh 1 µm filtered seawater (FSW), which was maintained at 27°C (±0.1°C) *via* computer control using a flow-through system at a rate of 1.5 liters min^{-1}. UV-filtered lights were mounted above each tank and provided an average underwater light intensity of 350 µE over a 12:12 h light/dark cycle (400 W metal halide lamps, BLV, Germany), typical of light intensities recorded at the collection site. The fragments were acclimatized for two weeks prior to starting the experiment. Seawater temperatures in four tanks were slowly and continuously ramped to 32°C (±0.05°C) over a 7 day period, *via* computer control, whilst the remaining four control tanks were maintained at 27°C for the entire duration of the experiment.

Coral nubbins were sampled four times during the experiment: before any temperature changes when both treatments were at 27°C (t = -7); once the 32°C target temperature had been reached in the thermal stress treatment (t = 0); after 5 days at 32°C, when the first physiological effects of temperature stress were visible (t = 5); and after 10 days at 32°C when all colonies in the 32°C treatment were completely bleached (t = 10). At each time point, one coral nubbin (approximately 50 mm in length) was collected from each coral fragment (*n*=24) and immediately transferred to a tube containing 2 mL of HPLC-grade methanol for qNMR analysis. Another coral nubbin was collected from each coral fragment (*n*=24) to evaluate *Symbiodinium* densities. In addition, one coral nubbin was collected at each time point from 4 different coral fragments, transferred directly into fixative (1.25% glutaraldehyde + 0.5% paraformaldehyde in 0.2 µm-FSW)

and stored at 4°C until processed for structural investigations by transmission electron microscopy (TEM).

3.2.1.2. Quantitative NMR analysis

The coral nubbins were extracted in methanol for 2 h with sonication followed by a second extraction with an additional 1 mL of HPLC-grade methanol for 10 min. The two extracts were pooled and dried using a vacuum-centrifuge then resuspended in a mixture of deuterium oxide (D_2O, D 99.8 %, 250 µL) and deuterated methanol (CD_3OD, D 99.8 %, 750 µL) (Cambridge Isotope Laboratories, Andover, MA, USA). A 700 µL aliquot of the particulate-free extract was transferred into a 5 mm Norell 509-UP-7 NMR tube (Norell Inc., Landisville, NJ, USA) and analyzed immediately by ^{1}H NMR.

^{1}H NMR spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer with TXI 5 mm probe and quantification performed using the ERETIC method (Akoka and Trierweiler 2002). This technique generates an internal electronic reference signal, calibrated using commercial stock solutions of 4 mM acrylate and DMSP. The concentrations of DMSP and acrylate were determined by integration of their respective signals in a 0.10 ppm window.

3.2.1.3. Symbiodinium densities

Freshly collected coral nubbins were airbrushed (80 lb.in^2) in individual plastic bags in 4 mL of 0.2 µm-FSW. The slurry was homogenized to breakdown aggregates and centrifuged at 3000 rcf. The supernatant was removed and the pellet resuspended in 1 mL of 10% formalin. Homogeneous extracts were placed on a hemocytometer (depth 0.1 mm) and *Symbiodinium* cells were counted under a light microscope (8 technical replicates were averaged per sample).

3.2.1.4. Symbiodinium genotype

In hospite Symbiodinium populations of each experimental coral fragment were characterized based on sequence differences in the nuclear ribosomal DNA internal transcribed spacer 1 region using single-strand conformation polymorphism (SSCP) analysis (van Oppen et al. 2001). Total DNA was extracted using a modified protocol (Wilson et al. 2002). *Symbiodinium* ITS1 region was amplified with fluorescently labelled Sym ITS1 PCR primers. Genotype was determined using SSCP with known reference samples running alongside experimental samples and scored manually using gel images.

3.2.1.5. Surface area calculation

Coral skeletons remaining after samples were extracted for qNMR analyses and *Symbiodinium* densities were lyophilized overnight and their surface area determined using a wax dipping technique (Veal et al. 2010). The surface area of each individual nubbin was used to normalize the qNMR and *Symbiodinium* data.

3.2.1.6. Transmission electron microscopy (TEM)

Fixed coral nubbins were decalcified in a formic acid:fixative mixture (1:3), with the solution changed every 12 h until complete dissolution of the skeleton. Three individual polyps per sample were post-fixed in osmium and subsequently dehydrated with increasing concentrations of ethanol followed by dry acetone. Dehydrated samples were infiltrated in increasing concentrations of Araldite resin before being cured for 24 h at 60°C. Longitudinal sections 90 nm thick were collected on copper grids and imaged at 120 kV in a JEOL 2100 TEM (Tokyo, Japan).

3.2.1.7. PAM fluorometry measurements

Photosystem II (PSII) photochemical efficiency was measured with a Diving-PAM (Walz Gmbh, Germany) on three random nubbins per coral fragment. Minimum and maximum fluorescence

(F_O and F_M) were recorded daily, two hours before the start of the light cycle. PS II photochemical efficiency was expressed as maximum quantum yields ($(F_M-F_O)/F_M$) = (F_V/F_M).

3.2.1.8. Data analyses

All data were square root transformed and no significant tank effect was detected for the DMSP, acrylate and PAM fluorometry data (Nested ANOVA, $p>0.05$) (Statistica 7, Statsoft, Tulsa, USA). Repeated measures ANOVA were carried out on the time series data, (data met all assumptions of the test). Simple main effect tests (Quinn and Keough 2002) were used to compare the results between the two temperature treatments, and between temperatures at each time point (Table S1). This statistical technique was used to minimize the number of multiple comparisons (focusing only on comparisons of interest), decreasing the likelihood of type I error.

3.2.2. Coral juveniles

3.2.2.1. Sample collections

Colonies of *Acropora millepora* (*n*=10) and *A. tenuis* (*n*=3) were collected from Orpheus Island, Great Barrier Reef, Australia (18°34' S/146°30' E) and transferred to the Australian Institute of Marine Science outdoor aquarium facility 4 days prior to the predicted spawning event in November 2011. One hour before spawning, the colonies were isolated in 70 L tanks with 1 µm-FSW. Gametes were collected from the surface of these tanks and fertilized in separate 70 L tanks with FSW. After fertilization, embryos were gently rinsed three times by transferring to new containers and were subsequently transferred to 500 L tanks (containing 0.5 µm-FSW) where they were kept through larval development. After 12 days, *Symbiodinium*-free coral larvae were collected using a 1 µm mesh net and washed three times in 0.2 µm-FSW. Larvae were subsequently settled in sterile 6-well plates (8 plates per species, 40 larvae per well; each well filled with 10 mL of 0.2 µm-FSW). Eight hours after settlement, the plates were separated between two temperature regimes: 4 plates per species were incubated at 27ºC (control temperature), and the other 4 plates were ramped to 32ºC over 5 hours (thermal stress treatment); all plates were maintained in the dark (in order to prevent the growth of

potential photosynthetic organisms). Settled juveniles were incubated at their respective temperature treatments and six random wells were sampled every 2 days. The size of the sampled juveniles was measured using a motorized stereomicroscope with a high resolution camera (Leica MZ16A and Leica DFC500, Leica Microsystems, Wetzlar, Germany); sizes did not vary significantly between day 2 and day 6 with an average size of 0.79 mm^2 (±0.05) for *A. millepora* juveniles and of 0.82 mm^2 (±0.07) for *A. tenuis* juveniles.

3.2.2.2. qNMR analysis

After the required incubation time, seawater was removed from each well with a pipette, and a sterile cotton bud was used to soak up any residual seawater, taking care not to disturb the settled juvenile corals. Juveniles in six wells were extracted by adding 300 µL of deuterated methanol (CD_3OD) to each well, followed by 30 s of gentle shaking; 200 µL of this extract was transferred into a 3 mm Bruker MATCH NMR tube and analyzed immediately. In addition, negative control wells without settled juveniles were extracted following the same procedure. The concentrations of DMSP and acrylate were normalized initially to the number of settled coral juveniles in the respective well. They were then normalized to the averaged surface area of the juveniles (note that juveniles were approximated to perfect circles).

3.2.2.3. DNA extractions and PCR amplification

At each time point, the contents of two wells were scraped into a 2 mL tube using a scalpel blade, snap frozen with liquid nitrogen, and used for total DNA extraction, according to methods in Bourne and Munn (Bourne and Munn 2005). Multiple sets of primers were subsequently used to target different taxonomic groups: *Symbiodinium* (van Oppen et al. 2001), coral (Suzuki et al. 2008), and general primers for the algal 23S rDNA plastid (Sherwood et al. 2008) and chloroplast DNA (Taberlet et al. 1991) (Table S3). The PCR consisted of 1 µL of DNA template (dilution series from 1 to 10^{-5} of the original concentrations), 10 µL of buffer containing dNTP and $MgCl_2$ (Bioline, London, UK), 1.5 µL of each primer (10 µM), and 0.5 µL of *Taq* polymerase (Bioline, London, UK), adjusted

to a final volume of 50µL with sterile MilliQ water. Amplified PCR products were visualized by electrophoresis on 1% agarose gel stained with ethidium bromide.

3.2.2.4. Identification of candidate genes

Orthology between coral and diatom genes was inferred based on best reciprocal BLAST hits (Moreno-Hagelsieb and Latimer 2008). Orthologs in other species were identified using release 5 (March 2011) of the OrthoMCL database (Feng et al. 2007).

3.2.2.5. Data analyses

Repeated measure ANOVA were performed on the normalized DMSP and acrylate concentrations (data met all assumptions of the test, except the acrylate data from *Acropora tenuis* that violated sphericity assumption; in that unique case, Greenhouse-Geisser correction was applied to the degrees of freedom). Simple main effect tests (Quinn and Keough 2002) were then used to compare the results from the temperature treatment within species (Table S2).

3.3. Results

3.3.1. DMSP production in adult corals

Adult colonies of *A. millepora* (*n*=12 for each treatment) were subjected to ambient (27°C) or thermal stress (32°C) conditions during a 17 day experiment, during which their DMSP and acrylate contents were measured using quantitative nuclear magnetic resonance (qNMR) (23). All colonies contained only *Symbiodinium* type C2 (GenBank Accession AF380552 (25)) (Single strand conformation polymorphism (SSCP) profiles from all samples were single bands identical to the reference). After 5 days of thermal stress, cell counts revealed a 25% decrease in *Symbiodinium* cell numbers within coral tissues (Figure 3.1A). Transmission electron microscopy (TEM) showed that all *Symbiodinium* cells observed within coral tissues (*n*=1304) were structurally compromised (Figure 3.2). Unexpectedly, DMSP concentrations in thermally stressed coral tissues increased significantly in

comparison to controls (ANOVA, n=12, p<0.005; Figure 3.3 and Appendix C; Table S3.1), despite the substantial degradation and loss of *Symbiodinium* cells.

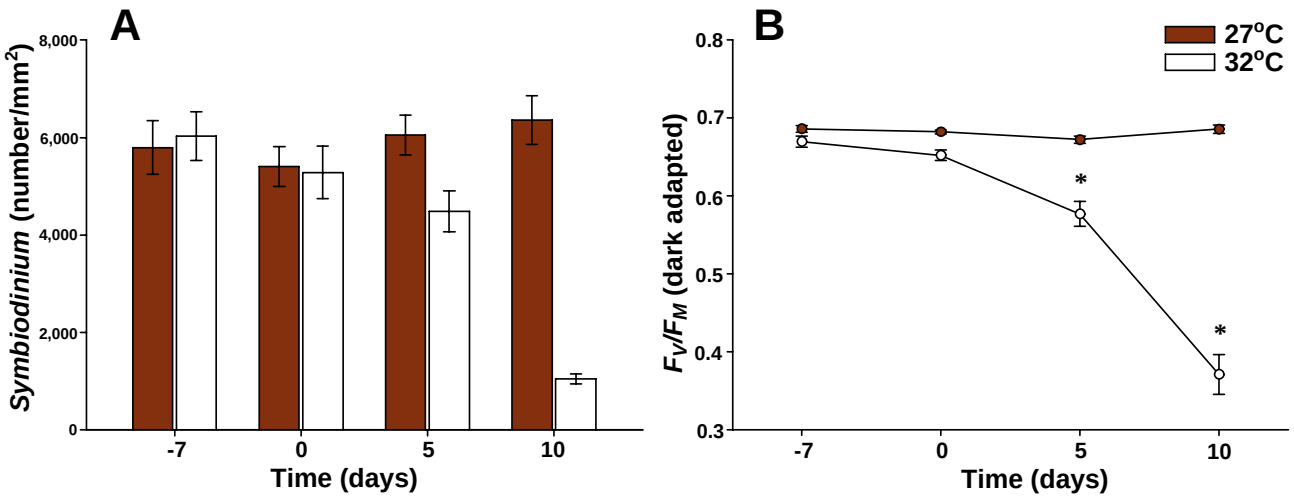

Figure 3.1: Density and photosynthetic efficiency (mean±SE) of *Symbiodinium* cells within adult colonies of the coral *Acropora millepora* maintained under control (27°C) or thermal stress (32°C) conditions for 10 days. (*A*) Density of *Symbiodinium* cells in the same coral fragments through time. (*B*) Comparison of photosystem II photochemical efficiency (maximum quantum yields: F_V/F_M) through time (repeated measure ANOVA, *p < 0.001; post-hoc simple main effect test, *p < 0.01). See also Appendix C; Table S3.1.

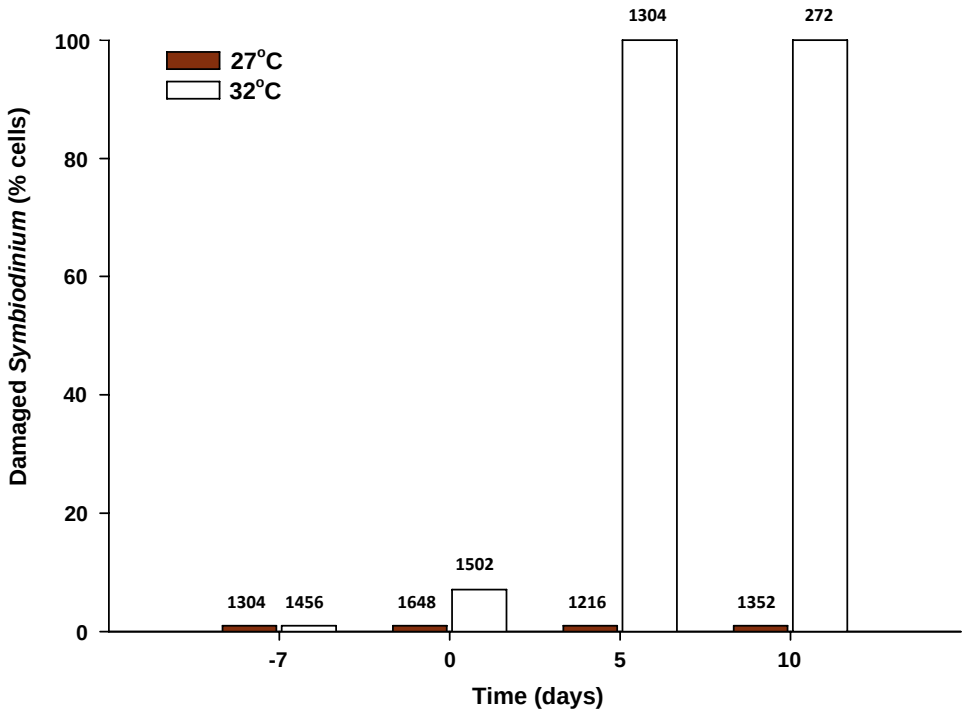

Figure 3.2: Structurally damaged *Symbiodinium* cells (%) within adult colonies of the coral *Acropora millepora* maintained under control (27°C) or thermally stressed (32°C) conditions. The numbers above the bars refers to the total number of *Symbiodinium* cells observed.

After 10 days of thermal stress, the number of *Symbiodinium* cells remaining in coral tissues was reduced to 16% of their original density (Figure 3.1A and Figure 3.3A-C) and structural damage increased. In particular, thylakoid membranes within chloroplasts were completely disrupted and general cell structure was lost in all cells examined (*n*=272) (Figure 3.2 and 3.4). Consistent with these observations, Pulse Amplitude Modulation (PAM) fluorometry measurements revealed significant reductions in the photochemical efficiency (F_V/F_M) of photosystem II in thermally stressed corals (ANOVA, *n*=12, $p<0.005$; Figure 3.1B). Despite the severe degradation and depletion of algal symbionts from coral tissues, thermally stressed corals contained 68% more DMSP and concomitantly 36% less acrylate than control colonies (Figure 3.3), suggesting the existence of an alternative source of DMSP production in coral tissues, which dramatically escalates DMSP production in response to thermal stress.

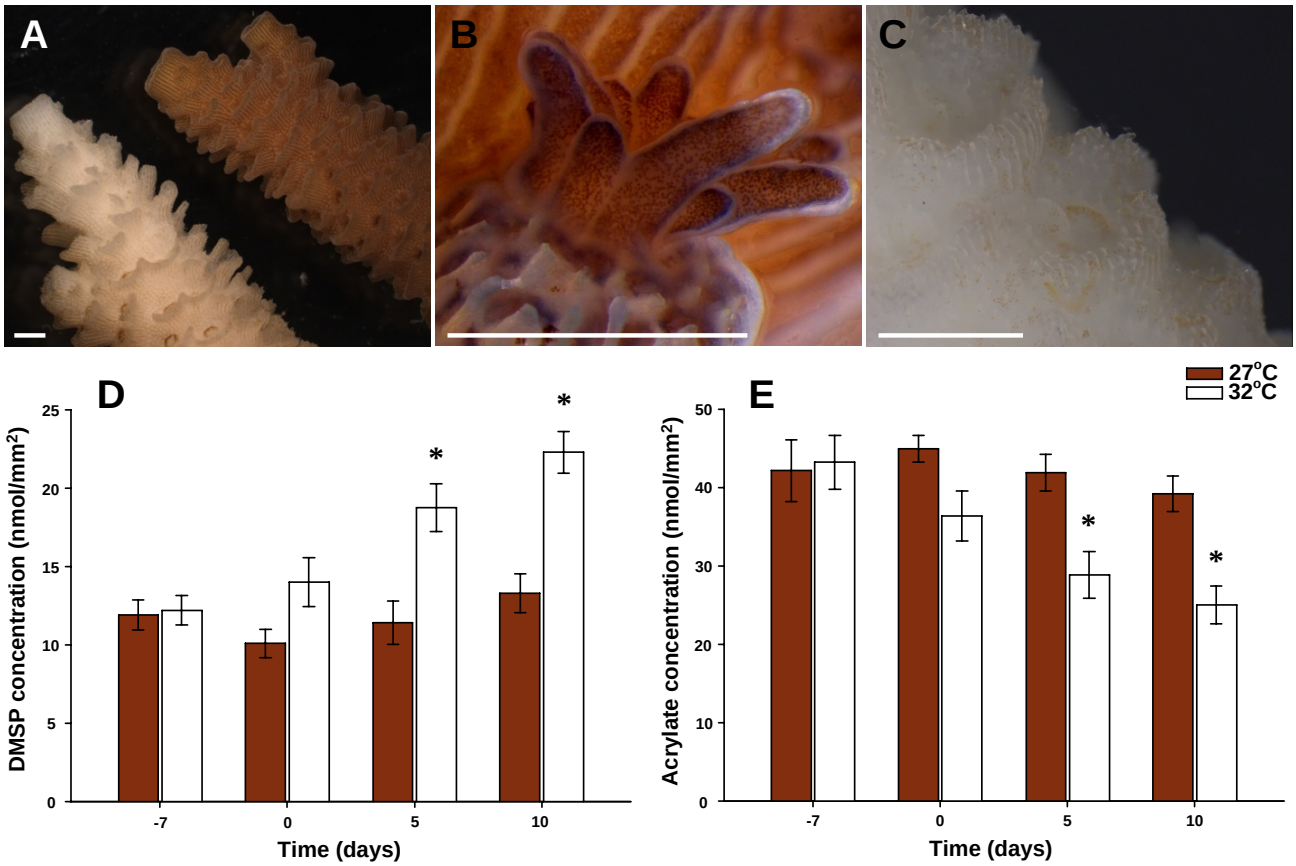

Figure 3.3: Effects of thermal stress on adult colonies of the coral *Acropora millepora*. Temperature was raised gradually over 7 days to 32°C, mimicking a realistic thermal stress scenario, therefore thermal stress commenced on day 0. The total duration of the experiment was 17 days. (*A*) Micrograph of representative nubbins showing visual differences in the density of *Symbiodinium* cells present in the tissues of *A. millepora* maintained under control (27°C, brown, right) or thermal stress (32°C, white, left) conditions for 10 days. (*B-C*) Higher magnification micrographs of coral polyps showing visual differences in tissue color as intracellular *Symbiodinium* densities decline between (*B*) 27°C and (*C*) 32°C (scale bars: 1 mm). (*D*) Changes in DMSP concentration (mean±SE) in adult corals (n=12) exposed to control (27°C) and thermal stress (32°C) treatments (ANOVA simple main effect test, $F_{1,22}$=10.79, *p<0.005). (*E*) Corresponding changes in acrylate concentration (mean±SE) in adult corals (n=12) exposed to control (27°C) and thermal stress (32°C) treatments (ANOVA simple main effect test, $F_{1,22}$=8.4, *p<0.01).

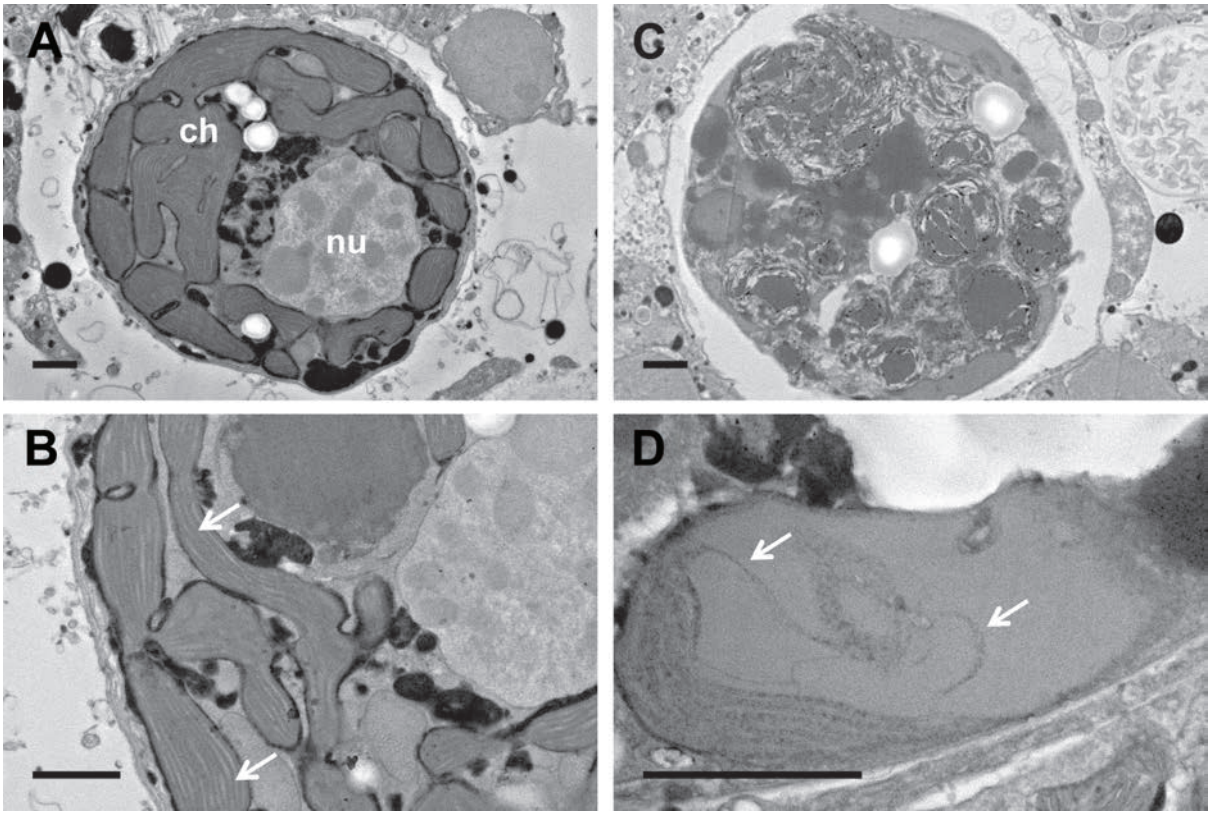

Figure 3.4: Representative transmission electron micrographs showing the effects of thermal stress on the internal structure of endosymbiotic *Symbiodinium* cells associated with the coral *Acropora millepora.* (*A-B*) *Symbiodinium* cells after 10 days at 27°C, showing intact cell structures and intact thylakoid membranes of chloroplasts (arrows), the photosynthetic centre of cells; (*C-D*) *Symbiodinium* cells after 10 days at 32°C, showing structurally degraded cells with highly disrupted thylakoid membranes (arrows). Scale bars: 1 µm. nu: nucleus, ch: chloroplast.

3.3.2.DMSP production in juvenile corals without photosynthetic symbionts

To further investigate the role of *Symbiodinium*-independent DMSP production in *Acropora*, DMSP and acrylate concentrations were also measured in juveniles from two coral species, *A. millepora* and *A. tenuis*, lacking photosynthetic symbionts. The absence of any photosynthetic organisms in these coral juveniles was confirmed using five different DNA markers, ranging from *Symbiodinium*-specific to universal 23S rRNA plastids primers, targeting all known lineages of microalgae and cyanobacteria. A lack of any detectable amplification was observed for all markers (Appendix C, Table S3.3). Despite the complete absence of photosynthetic microalgae, high concentrations of DMSP were recorded in all coral juvenile samples (Figure 3.5).

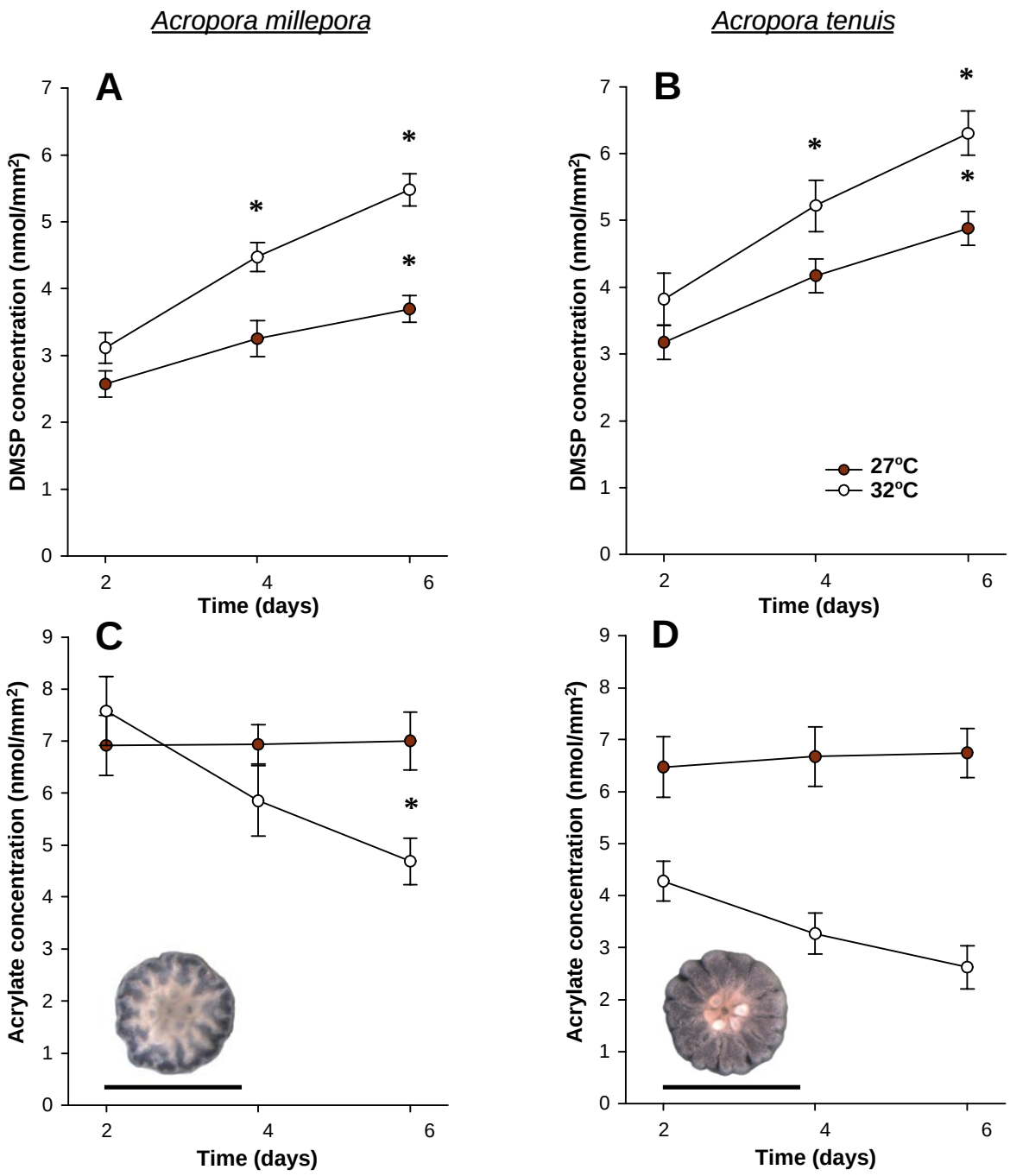

Figure 3.5: Changes in DMSP and acrylate concentrations (mean±SE) in coral juveniles lacking photosynthetic symbionts (n=6) through a six-day period after coral settlement. Patterns are compared for two thermal regimes: ambient (27°C, blue) and thermal stress (32°C, red), and for the coral species (*A*) *Acropora millepora* and (*B*) *Acropora tenuis*. DMSP concentrations increase significantly through time in the juveniles exposed to ambient temperature (ANOVA Simple main effect test, $F_{2,20}$=6.30, *p<0.01 for *Acropora millepora* and $F_{2,20}$=7.51, *p<0.005 for *A. tenuis)*. Furthermore, DMSP concentrations in coral juveniles exposed to elevated temperature (32°C) for 6 days were significantly higher than controls kept at ambient temperature (27°C) (Simple main effect test, $F_{1,10}$=27.68, *p<0.0005 for *A. millepora* and $F_{1,10}$=10.44, *p<0.01 for *A. tenuis*). Conversely, acrylate concentrations decreased significantly in juveniles of the corals (*C*) *Acropora millepora* and (*D*) *Acropora tenuis* when exposed to elevated temperature (Simple main effect test, $F_{1,10}$=5.58, *p<0.05 for *A. millepora* and $F_{1,10}$=61.68, *p<0.0005 for *A. tenuis*). Scale bar: 1mm.

Repeated sampling over a six day period after larval settlement revealed that initially high concentrations of DMSP in coral juveniles continued to increase significantly over time (ANOVA, n=6, p<0.005; Figure 3.5 and Appendix C; Table S3.2). At ambient water temperatures (27°C), mean DMSP concentration in *A. millepora* juveniles increased by 44% (i.e. by 1.1 nmol/mm^2) in four days. A similar trend was observed in *A. tenuis*, with mean DMSP concentration increasing by 54% (i.e. by 1.7 nmol/mm^2). These DMSP concentrations measured in non-photosynthetic coral juveniles are approximately half of those present in adult colonies.

As found in temperature experiments with adult corals, thermal stress resulted in significant increases in DMSP levels in juvenile corals (ANOVA, n=6, p<0.005; Figure 3.3 and Appendix C; Table S3.2), with *A. tenuis* and *A. millepora* showing 30% and 48% increases in DMSP concentrations, respectively, after six days of exposure to elevated temperatures (see Figure 3.5A-B and Appendix C; Table S3.2). Conversely, concentrations of the DMSP breakdown product acrylate decreased in both species, with thermally stressed juveniles containing 33% (*A. millepora*) and 61% (*A. tenuis*) less acrylate than juveniles at ambient temperature after six days (Figure 3.5C-D).

3.3.3.Identification of genes involved in DMSP synthesis in corals

A recent study of the diatom *Fragilariopsis cylindrus* identified candidate genes for each of the four steps of the DMSP biosynthesis pathway (Lyon et al. 2011). In an attempt to identify the possible molecular mechanisms underlying DMSP production in corals, we searched for potential orthologs of these genes in the comprehensive molecular resources available for two corals, the *A. millepora* transcriptome (Moya et al. 2012) and the *A. digitifera* genome (Shinzato et al. 2011). Two genes previously identified in diatoms had orthologs in both of these coral species (Appendix C; Tables S3.4). These genes encode a NADPH-reductase and an AdoMet-dependant methyltransferase, which mediate the second and third steps of the biosynthesis process respectively (Figure 3.6). The orthologous relationship between the coral and diatoms genes is supported by best reciprocal blast

hits, and their mapping to the same OrthoMCL clusters (Feng et al. 2007): OG5_131390 and OG5_156314 for the reductase and methyltransferase, respectively.

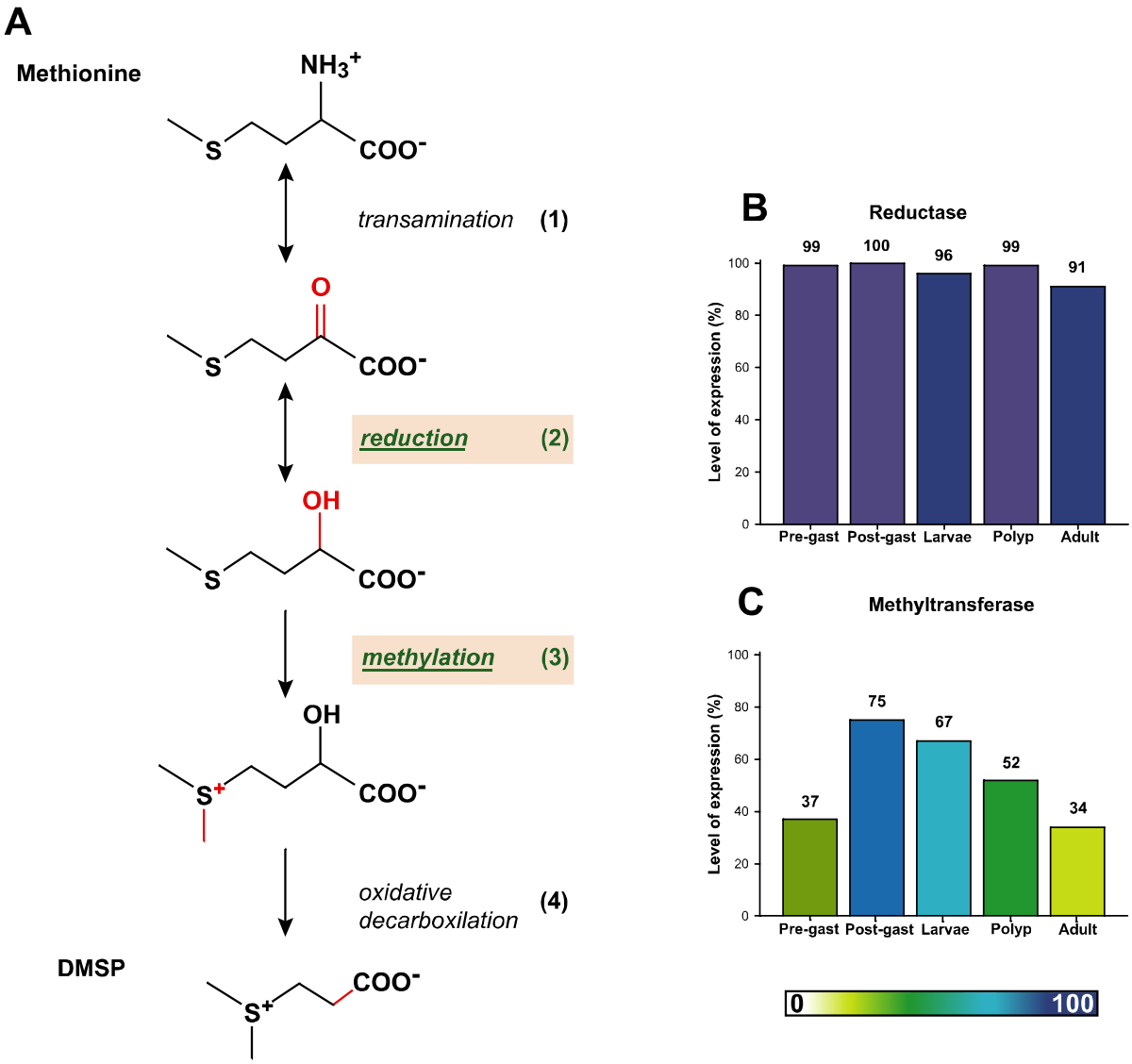

Figure 3.6: *(A)* Pathway of DMSP biosynthesis present in marine algae (Gage et al. 1997). The first two steps are reversible. The proposed diatom genes encoding the second and third steps (in green) have orthologs in *Acropora millepora* and *Acropora digitifera* genomes (see Appendix C; Table S3.4). *(B-C)* Color coded gene expression percentiles of the two putative genes involved in DMSP biosynthesis in *Acropora millepora* though five coral life-stages, from embryos (Pregast: pregastrula; Postgast: postgastrula) to adults, based on transcriptomic data from Moya et al. (Moya et al. 2012).

The reductase enzyme mediates a reversible step in the DMSP biosynthesis process (Gage et al. 1997, Stefels 2000) (Figure 4). The gene encoding this enzyme is highly expressed throughout all life history stages of *A. millepora* (Moya et al. 2012) (Figure 3.6A) and the phylogenetic distribution

of its OrthoMCL cluster revealed 45 orthologs spread throughout all Kingdoms (Appendix C; Figure S3.1), in line with the widespread presence of this enzyme among plants that do not produce DMSP (Stefels 2000). In contrast, the methyltransferase has been shown to mediate a non reversible step regulating intracellular DMSP levels and is believed to be specific to this pathway (Stefels 2000). The methyltransferase gene has high expression levels in early coral life stages but its expression decreases after settlement and remains relatively low in adult corals (Moya et al. 2012) (Figure 3.6C). This expression pattern could reflect the establishment of symbiosis with DMSP-producing *Symbiodinium* around the time of settlement. The OrthoMCL cluster corresponding to this methyltransferase has an unusually sparse phyletic pattern with only nine orthologs, including seven in photosynthetic organisms and two in other eukaryotes (Appendix C; Figure S3.1).

We also searched for potential homologues of the diatom genes putatively involved in DMSP synthesis in a comprehensive transcriptome assembly available for *Symbiodinium* (Bayer et al. 2012). Interestingly, we identified two *Symbiodinium* sequences belonging to the same OrthoMCL clusters as the methyltransferase and reductase identified in diatoms and corals (Appendix C; Table S3.4 and Figure S3.1). This suggests that the function of these enzymes in DMSP synthesis may be conserved between diatoms, alveolates, green plants and corals.

3.4. Discussion

Contrary to the current paradigm, which assumes that DMSP biosynthesis is limited to photosynthetic organisms, this study reveals that corals (Kingdom: Animalia) also produce this compound and were up-until-now a cryptic source of DMSP in coral reefs. Coral juveniles that did not harbour symbiotic photosynthetic organisms contained high DMSP concentrations, approximately half the concentrations measured in symbiont-bearing adult corals. Moreover, DMSP concentrations in juveniles increased significantly through time, demonstrating unambiguously that the DMSP levels measured were not simply inherited from parent colonies but produced by coral juveniles growing in the absence of photosynthetic symbionts.

This result sheds new light on the relevance and importance of corals as DMSP producers in coral reef ecosystems, highlighting the need to consider the influence of these animals in the global sulfur cycle. These data also explain previous discrepancies reported in the literature between the total amount of DMSP measured in corals and levels measured in *Symbiodinium* alone, which are typically two to three times lower (Hill et al. 1995, Yost and Mitchelmore 2010, Yost et al. 2012). Interestingly, DMSP concentrations are well-correlated with symbiont densities in giant clams (Hill et al. 2000), and undetectable in sea anemones lacking photosynthetic symbionts (Van Alstyne et al. 2009). Therefore, corals are likely to be the exception, rather than the rule, in terms of DMSP production by marine invertebrates harboring photosynthetic symbionts.

Thermal stress of juvenile corals triggered a significant increase in DMSP production and a simultaneous decrease in acrylate concentrations. Similar patterns have been reported in marine algae subjected to a variety of stressors, with significant increases in intracellular DMSP concentrations correlated with exposure to ultraviolet radiation, CO_2 and iron limitation, and high levels of reactive oxygen species (Sunda et al. 2002). This response relates to the antioxidant properties of DMS and acrylate, the breakdown products of DMSP. During thermal stress, the production of reactive oxygen species by coral mitochondria (and *Symbiodinium*, when present) increases, damaging coral cells (Weis 2008). DMS and acrylate are very efficient scavengers of hydroxyl radicals and other reactive oxygen species (Sunda et al. 2002); thus the observed decrease in acrylate concentrations in coral juveniles during thermal stress is a likely consequence of its reactivity with reactive oxygen species. This suggests that DMSP and its breakdown products fulfill an important role in coral stress responses, presumably reducing cellular damage from reactive oxygen species.

Similar experimental results with adult corals, the building blocks of coral reefs and therefore the most ecologically relevant life history stage in terms of DMSP production, further corroborate the conclusion that enhanced DMSP production in response to elevated temperatures may be an important mechanism for ameliorating thermal stress. Adult colonies of *A. millepora* exposed for ten days to a

temperature 2°C above their upper thermal threshold, showed a two-thirds increase in DMSP concentration and a simultaneous halving in acrylate concentration within their tissues. These trends are in close accordance to those observed in coral juveniles lacking photosymbionts. Remarkably, despite an 84% reduction in their *Symbiodinium* cell density, DMSP concentrations increased. The *Symbiodinium* cells remaining were metabolically dysfunctional, as they had suffered extensive structural damage (including disruption of the thylakoid membranes, where the first stage of photosynthesis takes place) and their photosynthetic efficiency was significantly reduced. Previous reports investigating the effect of thermal stress on *Symbiodinium* in corals concluded that these symptoms are characteristic of an advanced stage of necrosis (Strychar et al. 2004). In further support of our conclusion that *Symbiodinium* were not contributing significantly to DMSP production, previous experiments on *Symbiodinium* cultures have shown that DMSP content per cell volume decreases under thermal stress (McLenon & DiTullio 2012).

Taken together, experimental results for juvenile corals lacking photosymbionts and thermally stressed adult corals with dysfunctional photosymbionts provide conclusive evidence that the observed increases in DMSP concentrations in thermally stressed corals cannot be attributed to the activity of *Symbiodinium* cells. These results identify the coral animal as responsible for increased DMSP production and imply that this production is not restricted to juvenile life-stages, but also occurs at high levels in adult reef-building colonies subjected to thermal stress.

Studies have shown that marine algae produce DMSP from methionine *via* a pathway that involves the successive action of four different enzymes (Gage et al. 1997), but until recently, very little was known about genes involved in the biosynthesis of DMSP. The recent identification of candidate genes in a diatom is a significant step toward the functional characterization of this pathway in marine algae (Lyon et al. 2011). Remarkably, two of the four genes identified in diatoms have clear orthologs in *Symbiodinium* and corals. The homologous genes are potentially involved in the second and third steps of DMSP biosynthesis and encode, respectively, a reductase and a sulfur-

methyltransferase enzyme. Significantly, the sulfur-methyltransferase mediates the committing step of DMSP synthesis and has been shown to regulate intracellular levels of DMSP in algae (Ito et al. 2011). The presence of these genes in coral genomes further supports the conclusion that DMSP is synthesized directly by coral species in the genus *Acropora*.

A survey of the distribution of aerosol particles over Australia identified the Great Barrier Reef (GBR) as a considerable emission hotspot (Bigg and Turvey 1978), and sulfur aerosols were identified as a major constituent of these particles (Modini et al. 2009). The GBR is the largest biological structure on the planet and the release of these particles along its 2600 km length would constitute a major source of cloud condensation nuclei. During summer, the northern regions of the GBR (from 10 to 18°S) experience considerably lower solar radiation than the south based on significant increases in cloud cover (Masiri et al. 2008). This result can be ascribed to the south-easterly winds prevailing along the GBR that carry sulfur aerosols emitted by the reefs northwards (Jones and Trevena 2005). Coral reef-derived sulfur aerosol emission might therefore play a central role in cloud formation in areas of the world with large coral densities, such as the GBR and the Coral Triangle.

In summary, the novel finding of this study - that two common coral species produce large amounts of DMSP in the absence of photosynthetic partners - has major implications across physiological, ecological and biogeochemical scales. Notably, DMSP production originating from corals is important at the scale of: *i*) coral physiology, because concentrations of DMSP and its antioxidant breakdown product acrylate are strongly affected by thermal stress, indicating a role for these molecules in coral-stress responses; *ii*) coral reef ecology, because the large concentrations present in both juveniles lacking photosymbionts and adults with non-functional photosymbionts indicate that corals contribute extensively to the DMSP pool produced by this ecosystem; and *iii*) the global sulfur cycle, because coral-derived DMSP production is significant and likely to be integral to sulfur aerosol production from the marine environment in areas of the world where coral cover is still

high. Considering predicted increases in coral mortality worldwide caused by anthropogenic stressors, the associated decline in sulfur aerosol production from coral reefs may further destabilize local climate regulation and accelerate degradation of this globally important and diverse ecosystem.

Chapter 4: Coral-associated bacteria and their role in the biogeochemical cycling of sulfur

A portion of this chapter is published as:

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4.1. Introduction

DMSP plays a fundamental role as sulfur and carbon source for marine bacteria (Sievert et al. 2007, Reisch et al. 2011) and can be metabolized by these organisms *via* multiple pathways (Howard et al. 2006, Todd et al. 2007, Curson et al. 2008, Todd et al. 2009, Todd et al. 2010, Curson et al. 2011, Todd et al. 2011). The most widespread pathway among bacteria converts DMSP into methanethiol, and enables bacteria to assimilate the reduced sulfur (Howard et al. 2006, Reisch et al. 2011). The other pathways cleave DMSP into DMS (plus acrylate in some case) (Todd et al. 2007, Curson et al. 2008, Todd et al. 2009, Todd et al. 2010, Curson et al. 2011, Todd et al. 2011), the DMS produced by these enzymes is then released into the surrounding water (Kiene et al. 2000). Prior to the 1980s, diffusion of DMS from the oceans to the atmosphere was thought to be its major removal route from the marine system (Lovelock et al. 1972, Yoch 2002). More recently, however, it has been estimated that between 50 and 80% of the DMS produced by DMSP-degrading bacteria is degraded directly by other types of bacteria (Simo et al. 2002, Simo 2004), although the identity of the bacteria involved has not been extensively studied.

In oligotrophic environments, such as coral reefs, bacteria depend on organic compounds produced by primary producers and reef-building corals (Ritchie and Smith 2004) and these compounds may determine the composition of coral-associated bacterial communities (Ritchie and Smith 2004, Rosenberg et al. 2007b). The presence of high levels of DMSP and DMS in reef-building corals suggests that they are likely to harbor bacterial species capable of metabolising these compounds. To investigate the potential of these methyl-sulfur compounds to drive coral-associated microbial communities, we used them as sole carbon sources to isolate bacteria from two common coral species (*Montipora aequituberculata* and *Acropora millepora*) and directly compared these microbial isolates with coral-associated microbiota identified using culture-independent analyses. We also investigated the distribution and abundance of the genes involved in DMSP degradation within metagenomic datasets derived from a range of marine and terrestrial environments, to evaluate their relative roles in these ecosystems and their potential relevance to coral reefs.

4.2. Methods

4.2.1.Sample collections

Three colonies of the corals *A. millepora* and *M. aequituberculata* were collected from Davies Reef, Great Barrier Reef, Australia (18°05'S/147°39'E) and maintained in aquaria at the Australian Institute of Marine Science (Townsville, Queensland, Australia). Five replicate samples of coral mucus were taken from each colony using sterile 50-ml syringes. Samples (1 ml) were used immediately for selective enrichment cultures, and the remaining volume was filtered through 0.22-µm-pore-size Sterivex filter columns (Durapore; Millipore), which were filled with 1.6 ml of lysis buffer (0.75 M of sucrose, 40 mM EDTA, 50 mM Tris-base; pH 8.3), and stored at -80°C for subsequent molecular analyses. Seawater samples (1 liter each) were also taken from the aquaria and processed in the same manner. Coral tissue slurry samples were obtained from five replicate coral fragments (25 mm in length, 60 to 70 polyps) from each colony and washed in autoclaved, 0.22-µm-pore-size-filtered artificial seawater (ASW) to remove loosely attached microbes. The samples were airbrushed (80 lb/in2) to form a slurry with 5 ml of ASW to remove coral tissues and associated microbes from the skeleton. This slurry was homogenized to break down aggregates and divided into 1-ml aliquots and either stored at -80°C for DNA extraction or used immediately for selective enrichment cultures. All corals appeared healthy when the samples were collected.

4.2.2.Isolation of bacterial strains using DMSP, DMS, and acrylate as carbon sources

After air brushing, the remaining coral skeleton was crushed by using a sterilized pestle and mortar. Dilution series in basal medium were performed for each of the five replicates of mucus, tissue slurry, and crushed skeleton samples for each species. Sterile 125-ml crimp-top vials were used for the dilution series, and the vials were inoculated with coral bacteria to a final volume of 10 ml. All vials were sealed by using sterile Teflon coated butyl rubber septa. Two different basal media (lacking a carbon source) were tested for their ability to support the growth of coral bacteria: a modified marine ammonium mineral salt (MAMS) medium and a modified ASW media (MASW) (Table 4.1). The carbon sources used for enrichments were DMSP (50 µM), DMS (50 µM), and acrylate (50 µM).

These carbon sources were added aseptically through the septa of crimp-top vials with a syringe and needle. Control bottles containing only the basal medium and the carbon source were set up, along with enrichment cultures, to account for the chemical breakdown of the carbon sources. Cultures were incubated at 28°C for 7 days, and the presence of DMS in the headspace gas was monitored by gas chromatography (GC) analysis. Enrichments were respiked daily with an additional dose (20 µM) of the carbon source to avoid the potential deleterious effect of high dosage concentrations. The degradation of acrylate was assessed by nuclear magnetic resonance (NMR) analysis.

Table 4.1: Basal media composition (in grams per liter) used for the isolation of DMSP-, DMS- and acrylate-degrading bacteria. The Marine Ammonium Mineral Salts medium was modified from Goodwin et al. (2001). All solutions were autoclaved separately before combining. SL10 trace metal solution from Widdel et al. (1983); Vitamin solution from Pfennig (1978).

Solution	Components (g/liter)	
	MAMS medium	MASW medium
1	NaCl (25), $(NH_4)_2SO_4$ (1), $CaCl_2 \cdot 2H_2O$ (0.2)	NaCL (25), MgSO4 · 7H2O (2.44), KCl (0.6), $NaNO_3$ (1), CaCl2 · 2H2O (0.3)
2	$MgSO_4 \cdot 7H_2O$ (1),$FeSO_4 \cdot 7H_2O$ (0.002), $Na_2MoO_4 \cdot 2H_2O$ (0.002)	KH_2PO_4 (0.5)
3	KH_2PO_4 (3.6)	K_2HPO_4 (0.9)
4	K_2HPO_4 (4.6)	NH_4Cl (0.267)
5	SL10 trace metal solution (1 ml/l)	SL10 trace metal solution (1 ml/l)
6	Pfennig's vitamin solution (1 ml/l)	Pfennig's vitamin solution (1 ml/l)

A 50-µl aliquot of each enrichment culture was spread onto the appropriate basal medium plate (MAMS or MASW) containing the appropriate carbon source (DMSP, DMS, or acrylate). DMS enrichment plates were kept in gas-tight jars, and 200 µM DMS was added to each jar. The jars were regularly vented and replenished with DMS (every 3 days). All enrichment plates were incubated at 28°C in the dark for 14 days. To isolate single strains capable of metabolizing the carbon source for growth, individual colonies were picked from isolation plates and resuspended in 10 ml of MAMS or MASW medium containing the appropriate carbon source (50 µM DMSP, 50 µM DMS, or 50 µM acrylate). Degradation of DMSP and DMS by the isolates was monitored by GC analysis, while the degradation of acrylate was assessed by NMR analysis. A 50-µl aliquot of each liquid culture was then inoculated back onto appropriate plates as described above to confirm the cultures formed

colonies with a consistent morphology. Again, a single colony was picked and grown in liquid culture as described above, and degradation of the carbon source was confirmed.

4.2.3.DNA extraction and purification

DNA was extracted from isolated single strain liquid cultures by using a DNA extraction kit (Promega, Madison, WI), according to the manufacturer's instructions and resuspended in 30 µl of sterile Milli-Q water. DNA from the seawater and coral mucus samples was extracted according to the procedure of Schauer et al. (2000), resuspended in 30 µl of Milli-Q water, and used directly for PCR amplification. DNA from the coral tissue samples was extracted according to a modified urea extraction protocol (Bourne and Munn 2005). Extracted DNA (30 µl) from coral tissues was purified to remove coextracted PCR-inhibitory humic and phenolic materials by passage through a 0.7% agarose gel, and DNA fragments larger than 2 kb were cut from the gel and purified by using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All DNA was resuspended in 50 µl of sterile Milli-Q water, quantified with a Nanodrop spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden), and stored at -20°C until required.

4.2.4.PCR amplification of bacterial 16S rRNA genes

Primers 63F and 1387R, specifically targeting a 1,300-bp section of the genomic bacterial 16S rRNA gene (Marchesi et al. 1998), were used for PCR amplifications. The PCRs included 2.5 µmol of each deoxyribonucleotide triphosphate and 5×PCR buffer containing $MgCl_2$, 10 pmol of each primer, and 0.25 µl of *Taq* DNA polymerase, adjusted to a final volume of 50 µl with sterile Milli-Q water. The reaction conditions were as follows: 94°C for 3 min; followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; and then a final extension of 72°C for 7 min. Amplified PCR products were visualized by electrophoresis on 1% agarose gel stained with ethidium bromide.

4.2.5.Clone library construction and sequencing

PCR products from seawater, coral mucus, and coral tissue samples were purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified DNA was ligated into a TOPO-TA cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and ligated vectors and inserts were sent to the Australian Genome Research Facility for clone library construction and sequencing. A total of 96 clones were sequenced from each library. Inserts of the wrong size and chimeric sequences were removed from subsequent analysis.

4.2.6.GC analysis

Determination of DMS in the vials was performed by injecting 50 µl of headspace gas into a GC-ECD gas chromatograph (HP GC-500; Hewlett-Packard, Palo Alto, CA) fitted with a 1-m-by-4 mm glass column containing Poropack-Q and using nitrogen as the carrier gas (flow rate, 30 ml/min) at 200°C. A flame ionization detector was used to detect the compound. A DMS standard (Fluka, Evry, France) was used to confirm retention times.

4.2.7.NMR analysis

High-pressure liquid chromatography-grade methanol (20 ml) was added to each vial containing the acrylate enrichments and subsequently transferred to a glass round-bottom flask. The mixtures were dried *in vacuo* using a Buchi rotary evaporator and qNMR were run as described in Chapter 2.

4.2.8.Data analysis

All sequence data were edited by using the Sequencher program (Gene Codes Corp.) for removal of the vector and primer sequences and assembled into a single file for BLAST search comparisons (http://www.ncbi.nlm.nih.gov/) to determine the closest sequence affiliation to reference organisms or previously sequenced 16S rRNA genes. Chimeric sequences (checked with the

CHECK_CHIMERA software of the Ribosomal Database Project (Maidak et al. 1996)) were removed from the analysis. Coverage values were calculated by the equation: $C = 1 - (n/N) \times 100$, where n is the number of unique clones, and N the total number of clones examined in the libraries. Simpson's (Magurran 1988) and Shannon-Weaver (Shannon and Weaver 1963) diversity indices were generated by using DOTUR software (Schloss and Handelsman 2005).

4.2.9.Nucleotide sequence accession numbers

The nucleotide sequences obtained in the present study have been deposited in GenBank database (http://www.ncbi.nlm.nih.gov/) under accession numbers FJ463226 to FJ463252 (isolates), and FJ809043 to FJ809713 (clone libraries).

4.2.10. Pyrosequencing

In order to test the robustness of the results obtained with clone libraries, 10 biological replicates of *A. millepora* were collected from Pelorus Island, Great Barrier Reef, Australia (18°33' S/146°29' E) and their DNA was extracted and purified following the protocol described above. Primers 63F and 533R, specifically targeting a 450-bp section of the genomic bacterial 16S rRNA gene (Engelbrektson et al. 2010, Bourne et al. 2013), were used for PCR amplifications as previously described. A total of 5 PCR reactions was performed per samples, replicate PCRs were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and pooled to generate more than 1 μg of amplicon sequences. PCR products were sequenced on a GS-FLX Titanium (454 Life Sciences, Branford, Connecticut, USA). Sequences were checked for chimerae using UCHIME version 3.0.617 (Edgar et al. 2011), denoised using Acacia (Bragg et al. 2012), normalized to 2,000 reads per samples and then parsed using the QIIME pipeline with default settings (Caporaso et al. 2010).

4.2.11. Occurrence of DMSP-degrading genes in environmental metagenomes

Genes implicated in DMSP degradation (*dmdA*, *dddD*, *dddL* and *dddP*) were compared with the content of 152 metagenomes derived from a variety of marine and terrestrial ecosystems (publicly

available from the San Diego State University: Center for Universal Microbial Sequencing, SCUMS database; http://scums.sdsu.edu/). These metagenomes were constructed by size fractionation of environmental samples, which provided microbial metagenomes (corresponding to bacteria and archaea) and viral metagenomes (derived mostly from bacteriophages). Only sequence hits with an E-value $<10^{-4}$ were considered. The gene sequences used for the comparison were described in (Howard et al. 2008, Todd et al. 2009) and consisted of 2097 *dmdA*, 59 *dddL*, 22 *dddD* and 120 *dddP*. Results were normalized to take into account the size difference between the metagenomes.

4.2.12. Phylogenetic analysis

A maximum-likelihood tree was constructed in ARB (Ludwig et al. 2004), with the James-Taylor-Thornton model for distance calculation, on the peptide sequences of *DmdA* used by Howard et al. (2008) and additional sequences coming from virus metagenomes of the SCUMS database. A column filter, focusing on 360 amino-acids shared between the different sequences was used to compare bacteria and viruses-derived sequences.

4.3. Results

4.3.1.Isolation of coral-associated bacteria degrading DMSP

Six different bacterial genera capable of metabolising DMSP were isolated from coral mucus, tissue, and skeleton of *A. millepora* and *M. aequituberculata*. Some bacteria were isolated more than once and were retrieved in enrichments inoculated from different coral derived samples (e.g., mucus, tissue, and/or skeleton). Of the two basal media formulations (MAMS and MASW), only enrichments grown on MAMS demonstrated the ability to degrade DMSP. This was confirmed by DMS formation detected by GC analysis in the culture headspace, typically 24 hours after culture inoculation. After 2 weeks, no DMS formation was observed in the headspace gas of the MASW enrichment cultures or in controls, hence these samples were not analysed further. The majority of isolates capable to degrade DMSP belonged to the *Gammaproteobacteria* class as determined by 16S rRNA gene sequence similarity, and included *Alteromonas, Arhodomonas*, *Idiomarina*, *Pseudomonas*, and *Spongiobacter*-

related organisms (Table 4.2). Organisms related to *Roseobacter* (*Alphaproteobacteria*) were also isolated.

Table 4.2: Bacteria isolated from DMSP, DMS and Acrylate enrichment cultures of mucus, tissue and skeletal samples of the corals, *Acropora millepora* and *Montipora aequituberculata* and PCR detection of genes homologous to *dddL* and *dddD* previously implicated in DMSP degradation. [1]Enrichment on MAMS media, [2] Enrichment on MASW media (Table 1), [3]Sequences were aligned to the closest relative using BLAST, the similarity was calculated without gaps taken into account.

Enrichment	Closest taxonomic related strain(s)[3]	Strains isolated from	Taxonomic description	Alignment (bp)	Sequence identity (%)
DMSP[1]	*Arhodomonas* sp. EL-201	*Acropora* mucus	*γ-Proteobacteria*	682/699	97
	Spongiobacter nickelotolerans	*Acropora* tissues		667/699	95
	Pseudomonas sp. CJ11075	*Montipora* tissues		666/670	99
	Idiomarina sp. PR53-12	*Acropora* tissues & skeleton		666/670	99
	Alteromonas sp. S1613	*Acropora* mucus, skeleton & *Montipora* tissues		700/700	99-100
	Roseobacter sp. SOEmb11	*Acropora* & *Montipora* tissues	*α-Proteobacteria*	685/700	98
DMS[2]	*Alteromonas* sp. S1613 and CF6-3	*Acropora* mucus, skeleton & *Montipora* mucus	*γ-Proteobacteria*	698/699	99-100
	Pseudoalteromonas ruthenica	*Acropora* tissues & skeleton		695/700	99
	Vibrio tubiashi	*Acropora* tissues		699/700	99
	Vibrio sp. 6G8 and 1G4	*Montipora* tissues, *Acropora* tissues & skeleton		700/700	100
	Photobacterium sp. 3F8	*Montipora* tissues		698/699	98
	Idiomarina sp. JL110-118	*Acropora* skeleton		700/700	100
Acrylate[2]	*Vibrio harveyi*	*Acropora* mucus *Acropora* tissues	*γ-Proteobacteria*	700/700	100
	Vibrio fischeri	*Acropora* tissues		700/700	100
	Vibrio fortis	*Acropora* mucus *Acropora* tissues		641/642	89-98
	Photobacterium sp. 3F8	*Acropora* tissues		694/695	99
	Halomonas sp. s2151	*Acropora* tissues		644/667	96
	Shewanella piezotolerans	*Montipora* tissues		676/698	96

4.3.2.Isolation of coral-associated bacteria involved in DMS metabolism

In contrast to enrichments grown on DMSP, only samples inoculated into MASW medium were able to completely deplete the headspace gas of DMS, generally within 3 to 4 days after inoculation. Samples inoculated into the other medium did not degrade DMS, even after 2 weeks, and were not analysed further. Six different bacterial genera demonstrated the ability to degrade DMS. All belonged to the *Gammaproteobacteria* class, and included *Alteromonas*, *Idiomarina*, *Photobacterium*, *Pseudoalteromonas*, and *Vibrio*-related organisms (Table 4.2). These isolates have not previously been implicated in the degradation of DMS; the present study therefore widens the identity of taxonomic microbial species able to metabolize this organic sulfur compound.

4.3.3.Bacterial 16S rRNA gene clone libraries

Comparison between bacterial 16S rRNA clone libraries from mucus and tissue samples of *A. millepora* and *M. aequituberculata* revealed species-specific differences. The bacterial assemblages from two independent *A. millepora* tissue samples were highly similar and were dominated by the *Gammaproteobacteria* class (86 and 88% of the total number of affiliated sequences) (Figure 4.1B). Similarly, libraries constructed from *M. aequituberculata* tissues were highly consistent, but were dominated by members of the *Alphaproteobacteria* (Figure 4.1A) (62 and 57% of affiliated sequences). Clones derived from mucus samples of both *A. millepora* and *M. aequituberculata* were markedly different from their tissue counterpart and exhibited a higher proportion of sequences that belonged to unidentified bacterial groups (Figures 4.1C and D).

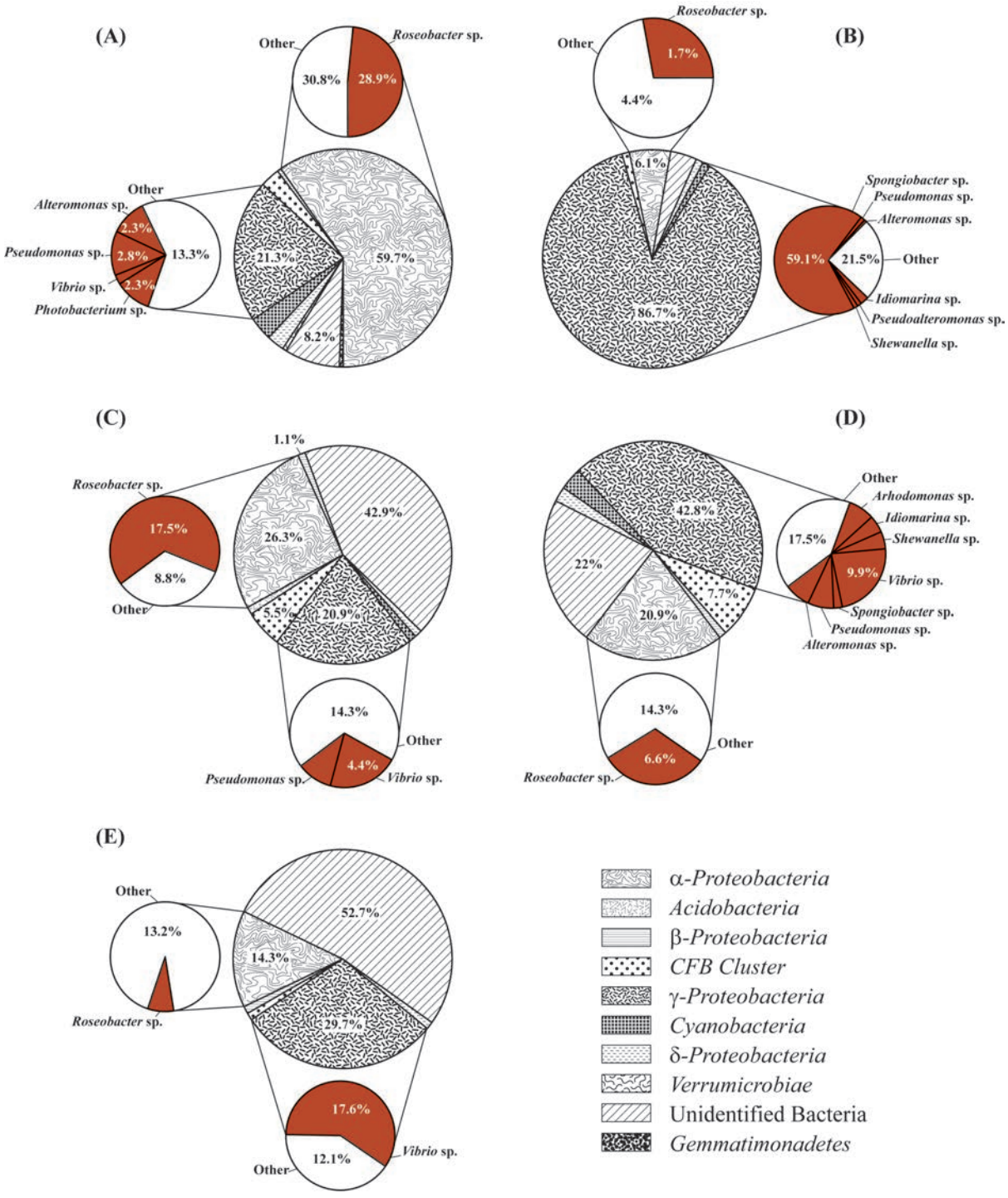

Figure 4.1: Composition of the clone libraries. (A) *M. aequituberculata* tissues (181 clones); (B) *A. millepora* tissues (172 clones); (C) *M. aequituberculata* mucus (91 clones); (D) *A. millepora* mucus (91 clones); (E) seawater libraries (91 clones). The large pie charts represent the contents of the libraries at the class level. The smaller pies represent the percentage proportions of the different isolates in red (at the genus level). Replicate tissue libraries were highly similar, and therefore clone sequence data were pooled for this figure.

4.3.4. Comparison between isolates and retrieved clone library sequences

All bacterial isolates that could metabolize DMSP and DMS were present in the clone libraries. *Spongiobacter* and *Roseobacter*-related organisms represented a large fraction of the clone sequences retrieved from the libraries. *Spongiobacter*-affiliated sequences accounted for 59% of the *A. millepora* tissues libraries and *Roseobacter* accounted for 28% of the *M. Aequituberculata* tissues, 15% of the *M. aequituberculata* mucus, and 10% of the *A. millepora* mucus libraries. The other bacterial isolates represented a smaller fraction of the clone library sequences (ranging between 1 and 10%) (Figure 4.1).

4.3.5. Pyrosequencing data

Since clone libraries are limited both in their coverage of the bacterial communities and the number of replicates they allow, high-throughput sequencing was undertaken on 10 biological replicates of *A. millepora* tissues. This approach enabled the retrieval of 2,000 reads per samples, vastly improving the coverage of the bacterial assemblages. The 10 replicates exhibited very consistent bacterial communities, dominated by the *Gammaproteobacteria* class (Figure 4.2), similarly to the tissues clone libraries. These bacterial assemblages contained high proportions of bacterial isolates metabolizing DMSP or DMS (between 77.9 and 92.1% of the reads per samples), the most abundant being *Spongiobacter* (*Endozoicomonas*) and *Roseobacter*-related organisms (70.7 to 74.8% and 2.3 to 7.6% respectively).

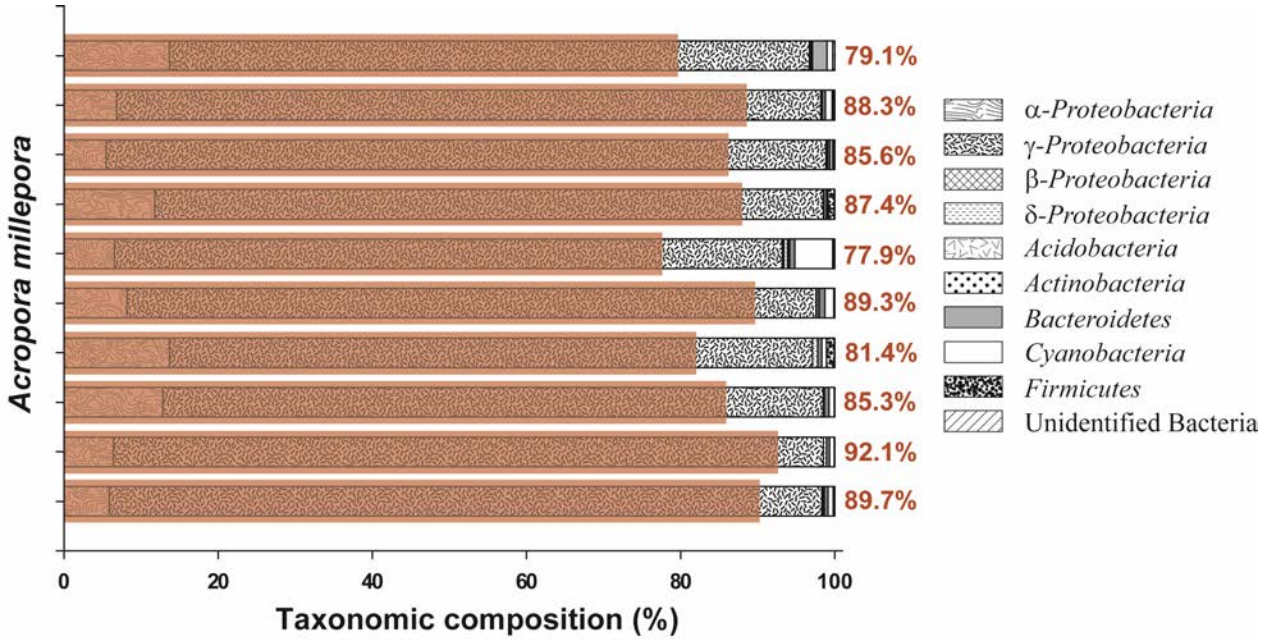

Figure 4.2: Composition of the 16S rRNA gene sequences retrieved from 10 different biological replicates of *Acropora millepora* tissues using pyrosequencing. The proportion of the different DMSP/DMS isolates is represented in red.

4.3.6. Occurence of DMSP-degrading genes in environmental metagenomes

Sequences putatively encoding DMSP-degrading enzymes were identified in 26 of the 152 metagenomes (Figure 4.3 and Appendix D; Table S4.1). The *dmdA* gene, the most widespread gene for DMSP degradation in the ocean (Howard et al. 2006, Howard et al. 2008), was also predominant in the SCUMS metagenomes; being present in 16 microbial metagenomes including that of the coral *Porites astreoides* (Wegley et al. 2007) and metagenomes from coral reef water (Dinsdale et al. 2008b). Not surprisingly, *dmdA* was also highly abundant in DMSP-enriched seawater samples (Mou et al. 2008). Two other genes, *dddP* and *dddD* were also observed in coral reef water samples and DMSP enriched seawater.

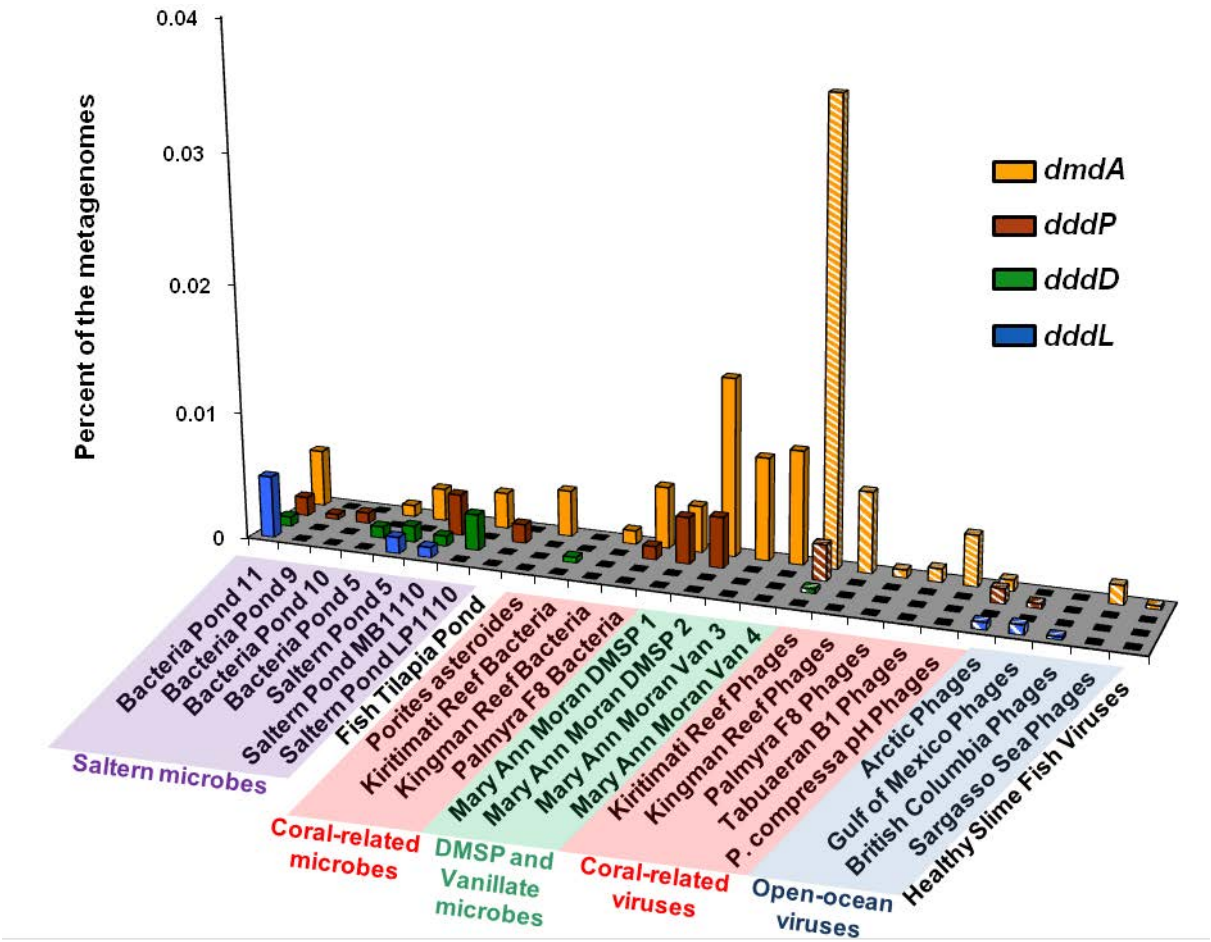

Figure 4.3: The presence of genes for DMSP degradation in marine metagenomes. The plain bars represent the microbial metagenomes, whereas the dashed bars represent the viral metagenomes (sequence notations from the SCUMS database can be found in Appendix D; Table S4.1); the abundance of genes is normalized to the size of the metagenomes.

Unexpectedly, these four DMSP-degrading genes were also well represented in viral metagenomes. The *dmdA* gene in particular, was present in viral particles collected from coral reef water of the Line Islands in the central Pacific (Kiritimati, Tabuaeran, Palmyra and Kingman) (Dinsdale et al. 2008b). Indeed, the viral fraction of the Kiritimati reef sample contained twice as many *dmdA* sequences as the microbial fraction in the DMSP-enriched water samples (Figure 4.3). To determine if all *dmdA* sequences from bacteriophages (virus infecting bacteria) had a common origin, a subset of these sequences was translated and incorporated into a phylogeny analysis of bacterial DmdA protein sequences (Howard et al. 2008), revealing that the viral sequences were phylogenetically diverse, with orthologs occurring in the SAR11- and *Roseobacter*-clusters as well as the *dmdA* clade C (Figure 4.4). Because most of the bacteriophages known are specific to the bacterial

taxa they infect (Kutter and Sulakvelidze 2004), it is probable that these viral *dmdA* genes were originally captured by the phages from their cognate host bacteria. The presence of phylogenetically diverse *dmdA* sequences in viral metagenomes indicates that multiple gene transfer events might have occurred between bacteria and their associated phages.

Figure 4.4: Maximum-Likelihood tree showing the diversity of DmdA orthologs and comparing the viral (red) and the bacterial derived protein sequences (black).

4.3.7.Isolation of coral-associated bacteria involved in acrylate metabolism

Enrichment cultures derived from *A. millepora* mucus and tissues metabolised acrylate twice as fast as cultures derived from *M. aequituberculata*. A total of six isolates were recovered from coral enrichment cultures, and belonging to the *Gammaproteobacteria* class. The isolates included organisms related to *Photobacterium*, *Halomonas*, and *Shewanella* species, as well as different vibrios, including *V. fortis*, *V. harveyi*, and *V. fischeri* (Table 4.2). All isolates showed rapid metabolism of acrylate, with complete depletion of the compound 7 days after inoculation (Figure 4.5). All isolates showed phylogenetic affiliation to sequences recovered from the coral clone libraries, with members of the *Vibrio* genus in particular being a component of the *A. millepora* (10%) and *M. aequituberculata* mucus (4%) libraries (Figure 4.1).

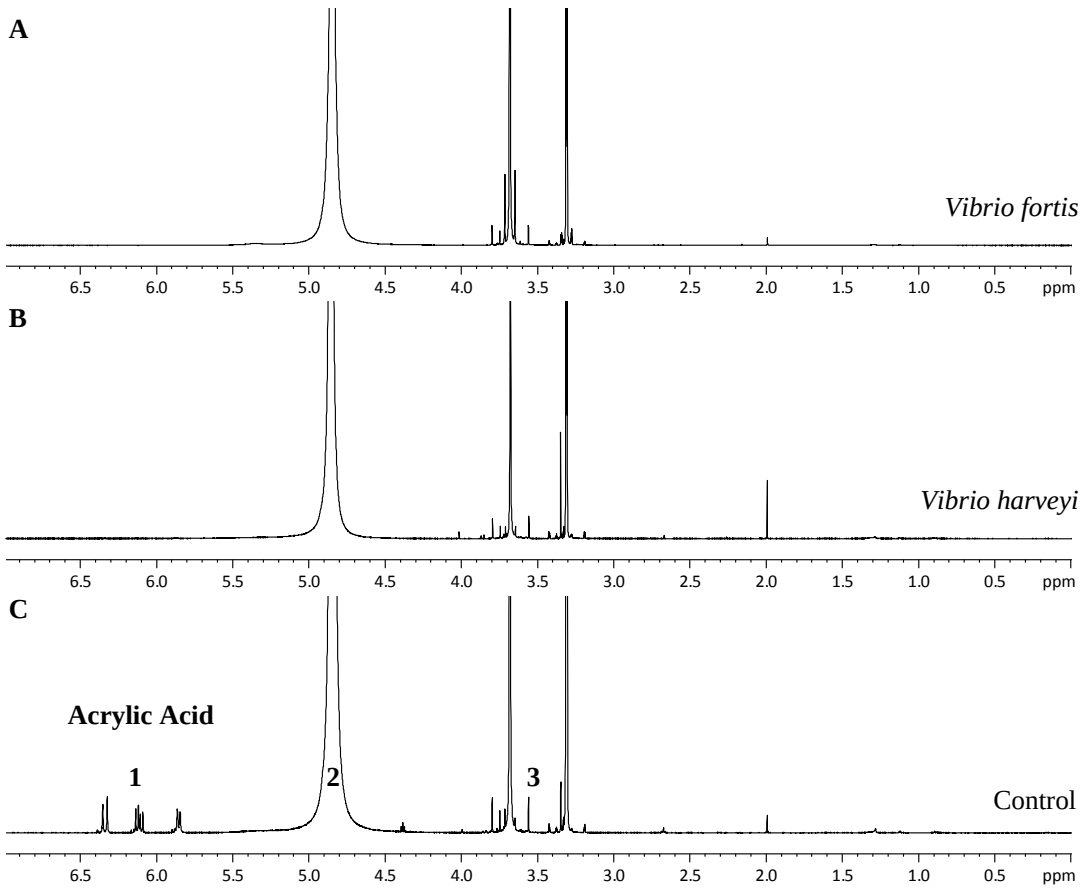

Figure 4.5: ^{1}H NMR spectra of acrylate enrichment (50 µM) in MASW medium with *Vibrio fortis* (A) or *Vibrio harveyi* (B), both isolated from *A. millepora* tissues, and control (C) 7 days after inoculation. The three peaks of the acrylate signal (position 1) are only visible in the control; the other peaks represent the water (position 2) and methanol (position 3).

4.4. Discussion

This study constitutes the first report of the isolation of DMSP/DMS-degarding bacteria from reef-building corals. The strains isolated represented a major component of the bacterial communities present in the two coral species investigated and it suggests that these two compounds might drive healthy coral-associated bacterial communities.

4.4.1.Bacterial strains metabolizing DMSP

The present study isolated six different bacterial genera capable of using DMSP as sole carbon source. Although *Alteromonas* (Ansede et al. 2001), *Pseudomonas* (Ledyard et al. 1993), and *Roseobacter* (Ledyard et al. 1993, Gonzalez et al. 1999) genera have previously been demonstrated to degrade DMSP, the *Spongiobacter*, *Arhodomonas*, and *Idiomarina* strains isolated from coral have never previously been directly implicated in the degradation of this compound. Since corals harbor both diverse and unique microbial diversity (Rohwer et al. 2001, Rohwer et al. 2002, Knowlton and Rohwer 2003, Rosenberg et al. 2007b), the discovery of new DMSP-degrading species is not surprising. Previously, the genus *Spongiobacter* has been reported in association with sponges (Mohamed et al. 2008), acidians (Martinez-Garcia et al. 2007), and corals (Bourne et al. 2008, Hansson et al. 2009). This genus represented more than half of retrieved sequences from *A. millepora* tissues and has previously been reported as a dominant species harboured by healthy reef-building corals (Bourne et al. 2008, Bourne et al. 2013).

Roseobacter-related organisms were isolated from both coral species sampled in the present study and represented a large component (28%) of sequences retrieved from the clone library derived from *M. aequituberculata* tissues. The ability of this bacterial genus to degrade DMSP has been extensively studied (Ledyard et al. 1993, Gonzalez and Moran 1997, Gonzalez et al. 1999, Wagner-Dobler and Biebl 2006), and it is known to dominate bacterioplankton communities in environments with high DMSP concentrations, such as phytoplankton blooms (González et al. 2000, Riemann et al. 2000) or polar waters (Wagner-Dobler and Biebl 2006). This genus has antibacterial activities against

a wide range of fish and invertebrate pathogens (Hjelm et al. 2004), is widely associated with corals (Rohwer et al. 2002, Bourne and Munn 2005, Bourne et al. 2008) and is likely involved in symbiotic relationships with cultured *Symbiodinium* (Ritchie 2011). Given its strong association with corals and the potential to produce antimicrobial compounds through the assimilation of DMSP, the *Roseobacter* genus is potentially central to coral health. Other isolated DMSP metabolizing bacteria, such as *Pseudomonas* and *Alteromonas* are also frequently reported from coral diversity studies and associate with a variety of different coral species (Bourne and Munn 2005, Ritchie 2006, Kooperman et al. 2007, Bourne et al. 2013).

4.4.2. Bacterial strains metabolizing DMS

Despite the importance of DMS degradation mediated by bacteria (Simo 2004), bacterial populations and the pathways involved in the metabolism of this compound are still poorly understood. The dominant population of DMS-degrading organisms in the enrichments affiliated with members of the *Alteromonas*, *Idiomarina*, *Photobacterium*, *Pseudoalteromonas,* and *Vibrio* genera. The ability of these bacteria to degrade DMS has not been reported previously, which emphasizes the need for a greater understanding of bacteria capable of degrading this compound and reducing the amount of DMS reaching the atmosphere.

4.4.3. Coral-associated microbial communities

Overlap between the bacterial diversity detected in coral clone libraries/pyrosequencing and bacteria implicated in methyl-sulfur metabolism highlights the potential importance of DMSP and DMS in structuring coral associated bacterial communities. Previous studies have shown that some bacterial genera are associated with corals from geographically separated locations (Rohwer et al. 2001, Littman et al. 2009), and some of these genera can metabolize DMSP and DMS. For example, *Pseudomonas*, *Roseobacter* and *Spongiobacter* (*Endozoicomonas*) genera are ubiquitous in corals (Rohwer et al. 2001, Rohwer et al. 2002, Bourne and Munn 2005, Koren and Rosenberg 2006, Kooperman et al. 2007, Bourne et al. 2008, Bourne et al. 2013), providing further support that some

bacterial populations implicated in DMSP/DMS degradation are conserved between coral species, and the availability of these methyl-sulfur compounds may drive the specificity of these coral-bacterium associations.

4.4.4. Genes for DMSP degradation in the environment

The recent availability of metagenomic datasets from a wide range of environments provides an opportunity to assess the presence of genes of interest in specific ecosystems and, therefore, the potential importance of metabolic processes linked to these genes in the associated bacterial communities. The presence of DMSP-degrading genes in all coral derived metagenomes present in the database suggests the utilisation of this molecule to support bacterial growth in this ecosystem and further support our hypothesis that methyl-sulfur compounds play a significant role in driving coral bacterial associations.

Surprisingly, DMSP-degrading genes were also identified in viral metagenomes, constituting the first report of the presence of these genes in viruses. The *dmdA* genes was highly abundant in viruses and in one instance more abundant that in the bacterial metagenomes. Bacteriophages have been shown to incorporate and carry extra pieces of bacterial DNA in their genomes, called 'morons', which might confer an evolutionary advantage (Hendrix et al. 1999, Mann et al. 2003). For example, cyanophages carry cyanobacterial genes that prevent photoinhibition of photosynthesis, thereby guaranteeing the photosynthetic activity of infected cells and ensuring the provision of energy required for their own replication (Mann et al. 2003). Mechanisms that exploit the bacterial metabolism have been reported for other bacteriophages (van Oppen et al. 2009), and it is likely that the additional function provided by 'morons' increases host cell fitness and helps support phage replications (Hendrix et al. 2000). Similarly, because DMSP acts as a carbon or sulfur source for a wide range of bacteria, the presence of DMSP degradation genes in phages potentially supports their replication. In particular, carrying genes capable of degrading these methyl-sulfur compounds into the

host might increase the fitness of the host through broader substrate utilisation potential, which, in turn, would increase the fitness of the phage.

Recently, Dinsdale et al. (Dinsdale et al. 2008a) demonstrated that a large number of bacterial metabolic capabilities were encoded within the associated viromes, suggesting that phage metagenomes provide a good representation of the functional diversity of associated bacterial metagenomes. The presence of genes for DMSP degradation in the genomes of viruses inhabiting high DMSP-producing environments (e.g. reef water, polar water and corals) supports this hypothesis. Phages might act as reservoirs of DMSP metabolism genes for bacterial communities, thereby influencing a wide range of processes, including short-term adaptation and long-term evolution of bacteria acting in the biogeochemical cycling of sulfur.

4.4.5.Acrylate, the forgotten story

High concentrations of acrylate, one of the DMSP breakdown products, have recently been reported in *A. millepora* (Tapiolas et al. 2010). The isolates derived from our coral samples were related to *Photobacterium*, *Halomonas*, *Shewanella* spp., and *Vibrio* spp. Acrylate degradation has been recorded previously for *Halomonas* (Johnston et al. 2008), and positive chemotactic responses toward this molecule were observed for *Vibrio* (Sjoblad and Mitchell 1979). *Vibrio*-related organisms have been implicated in several coral diseases (Kushmaro et al. 1996, Sussman et al. 2008), however they constituted a major component of the healthy coral libraries, representing up to 10% of the retrieved sequences in the *A. millepora* mucus. Similar results have been observed for the coral *Pocillopora damicornis*, with *Vibrio* species representing up to 38% of clone libraries (Bourne and Munn 2005), indicating that this group may constitute a natural part of healthy coral-associated microbial communities. Acrylate it is a powerful antimicrobial compound (Sieburth 1960, 1961) and might act as a selective filter for bacterial communities associated with other benthic organism (Noordkamp et al. 2000). However its role in coral health is unknown and requires further study.

4.4.6. Conclusion

Microbial communities associated with corals are highly diverse and have been reported to be species-specific, yet some bacterial genera are commonly associated with multiple coral species from geographically separated locations. The factors that drive these associations are poorly understood, although the passage of nutrients between the holobiont's symbiotic partners, including both *Symbiodinium* and associated microbial communities, will be important. The present study is the first to report the isolation of coral-associated bacteria that are capable of metabolizing DMSP and DMS. Three bacterial strains, *Spongiobacter*, *Pseudomonas*, and *Roseobacter* spp., represented between 36.8 and 92.1% of the bacterial communities associated with the species investigated, and all were able to metabolize these sulfur compounds. Additional analyses indicated a high abundance of some genes for DMSP degradation in coral-derived bacterial and viral metagenomes, highlighting the potential role of viruses in shuffling these genes to their bacterial host in coral reefs. These results demonstrate that DMSP, DMS, and acrylate can act as nutrient sources for coral-associated bacteria and that these compounds are likely to play a role in structuring the bacterial communities present in healthy corals.

Chapter 5: *In vivo* imaging of *Symbiodinium*-bacterial interactions using nanoscale secondary ion mass spectrometry

5.1. Introduction

The significant influence that the marine sulfur cycle exerts on atmospheric chemistry and climatic processes fully depends on the metabolic activities of microorganisms such as phytoplankton and marine bacteria (Andreae 1990, Sievert et al. 2007). Although interactions between DMSP-producing phytoplankton and DMSP-degrading bacteria are critical to sulfur cycling in the marine environment, there have been no studies that directly visualize sulfur exchanges between these two groups. Dinoflagellates are among the largest producers of DMSP on the planet (Keller et al. 1989, Scarratt et al. 2002), with this compound comprising more than 50% of their total cellular sulfur content (Matrai and Keller 1994, Keller et al. 1999). A large proportion of this DMSP production is exuded into the water column, where it can be used by marine bacteria to produce the climate-regulating gas dimethylsulfide (DMS) (Ayers and Gras 1991, Andreae and Crutzen 1997, Laroche et al. 1999, Todd et al. 2007) (Fig. 5.1). Reef-building corals that have both endosymbiotic dinoflagellates producing DMSP and bacterial communities capable of metabolising it provide a useful model system for exploring direct sulfur-cycling linkages between these two microbial groups.

In tropical oligotrophic waters, the provision of photosynthetically-derived secondary metabolites to the coral host by endosymbiotic dinoflagellates in the genus *Symbiodinium*, enables corals to flourish in these nutrient-poor environments (Muscatine 1990). The high densities of *Symbiodinium* present in coral tissues, coupled with the postulated role of the coral host in DMSP synthesis (see Chapter 3), jointly contribute to the extremely high DMSP concentrations recorded in reef-building corals. In addition to the role that DMSP plays in coral stress responses (see Chapter 3), DMSP is also highly likely to play an important role in coral-associated bacterial communities (see Chapter 4). It has been suggested that the specific bacterial assemblages associated with reef-building corals are involved in symbiotic relationships with the coral host (Rohwer et al. 2002, Bourne and Munn 2005, Rosenberg et al. 2007b), and also potentially with endosymbiotic *Symbiodinium* (Lesser et al. 2004, Olson et al. 2009, Ritchie 2011, Lema et al. 2012). However, direct visualisation of such interactions among corals and microbial members of the coral holobiont is lacking.

A large fraction of the bacterial consortia harboured by reef-building corals possesses the metabolic machinery to degrade DMSP (see Chapter 1 and 4). The presence of large concentrations of DMSP in corals, along with abundant bacterial communities capable of metabolizing it, strongly suggests that this molecule plays a central role in coral-bacteria interactions. Evidence that bacteria belonging to the *Alpha-* and *Gamma-Proteobacteria* classes, the dominant groups present in coral-associated bacterial communities, can metabolize DMSP in artificial culture media (see Chapter 4), suggests that DMSP is highly likely to influence the phylogenetic composition of the bacterial communities living in association with corals and might play a significant role in structuring coral-bacteria associations. However, this hypothesis is based on *in vitro* metabolic capabilities, which do not necessarily translate *in vivo*. It is therefore important to test this hypothesis *in vivo* in order to clearly elucidate the role played by DMSP in coral-bacteria associations.

Recent developments in high-resolution secondary ion mass spectrometry (NanoSIMS; Cameca, France), enable the incorporation of stable isotope substrate tracers in complex symbioses to be both quantified and visualised, providing an important tool for testing hypotheses about metabolic linkages between microbial partners in the coral holobiont. NanoSIMS has been applied to reef-building corals to investigate skeletal composition (Meibom et al. 2004, Meibom et al. 2008), as well as carbon (Clode et al. 2007) and nitrogen assimilation (Pernice et al. 2012) but has not yet been used to image and quantify the distribution of sulfur isotopes. In the environment, the most abundant sulfur isotope is ^{32}S (95%); however another stable isotope ^{34}S is far less abundant, representing only 4.2% of the total amount of sulfur. Enrichment of ^{34}S can therefore be used to trace sulfur exchange between *Symbiodinium* and coral-associated bacteria, providing a novel method for tracking the fate of sulfur in microbial interactions associated with corals (Figure 5.1).

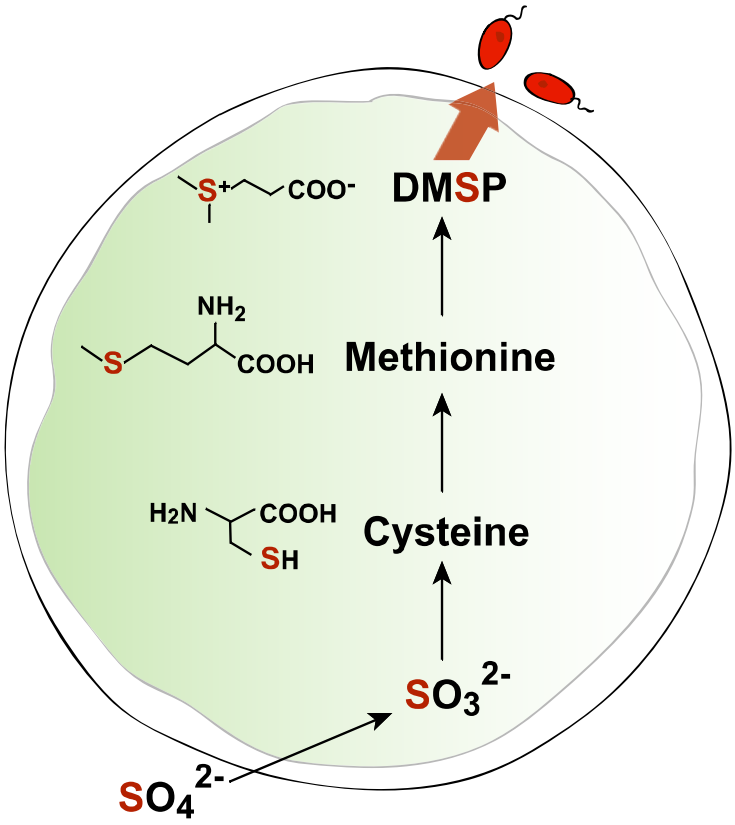

Figure 5.1: Hypothesized DMSP biosynthetic pathway in *Symbiodinium* spp. Sulfate (SO_4^{2-}) uptake from seawater is converted to sulfite (SO_3^{2-}), sulfur-based amino acids and finally DMSP. A portion of DMSP is then exuded from the cells and can be metabolized by some marine bacteria (sulfur atoms (S) and bacterial cells that have taken up sulfur are in red). For more details see Stefels (Stefels 2000).

The high solubility of DMSP in water (Stefels 2000) presents a unique set of challenges for studies of DMSP metabolism in corals. Usual NanoSIMS preservation techniques for biological samples involve chemical fixation of the tissue in aqueous glutaraldehyde, followed by multiple rinsing and dehydration steps (Wagner 2009). While glutaraldehyde stabilizes most proteins and amino acids, it does not prevent the extraction of highly soluble compounds like DMSP. Instead, the use of advanced preservation techniques, namely high-pressure freezing (Smart et al. 2010), followed by a water-free embedding procedure is required in order to effectively prevent the loss of DMSP from samples. Since coral skeletons must be decalcified in dilute acid, which would also extract DMSP into solution, NanoSIMS studies cannot be carried out on coral fragments. As a consequence, to successfully preserve DMSP in samples, the experimental system used in this study was simplified to focus solely on *Symbiodinium* and coral-associated bacteria.

Here, DMSP synthesis and translocation in *Symbiodinium* was investigated by: *i*) cultivating *Symbiodinium* cells in a medium containing $^{34}SO_4^{2-}$ as the sole sulfur source, *ii*) using mass-spectrometry (MS) and nuclear magnetic resonance (NMR) to assess the biosynthesis of ^{34}S labelled DMSP by *Symbiodinium* cells, and *iii*) visualizing the spatial distribution of ^{34}S within *Symbiodinium* cells and its potential uptake by coral-associated bacteria.

5.2. Methods

5.2.1.Isolation of Symbiodinium and bacteria

Cells of *Symbiodinium* type C1 used in this study were isolated from air-brushed tissues of the coral *Acropora tenuis*, which had been collected from Magnetic Island, Great Barrier Reef, Australia (latitude 19°10'S; longitude 146°50'E). Cells were sequentially washed three times (5 min at 1600 g) with 0.2 µm filtered seawater. Clean *Symbiodinium* cells were inoculated into sterile IMK medium (Wako Chemicals, Richmond, VA, USA) with the antibiotics penicillin (100 µg/mL), neomycin (100 µg/mL), streptomycin (100 µg/mL), nystatin (100 µg/mL), amphotericin (2.5 µg/mL), and GeO_2 (50 µM)) (Ishikura et al. 2004, De Santos et al. 2011). Cultures were genotyped by SSCP of the ITS1 region (van Oppen et al. 2001).

Two species of coral-associated bacteria were isolated from healthy coral colonies: *Pseudovibrio* sp. P12 from *Pocillopora damicornis* and *Pseudomonas* sp. A01 derived from *Montipora aequituberculata*. Both coral species were collected from Davies Reef, Great Barrier Reef, Australia (latitude 18°51'S; longitude 147°41'E) and maintained in aquaria at the Australian Institute of Marine Science (Townsville, Queensland, Australia) prior to strain isolation. Both strains are capable of metabolizing DMSP as sole carbon source and their isolation is described in detail in Chapter 4 for *Pseudomonas* sp. A01, and in Chapter 6 for *Pseudovibrio* sp. P12. *Escherichia coli* (*E. coli* W (ATCC 9637)), which is not associated with corals and not capable of degrading DMSP was also included in the study as a control strain.

5.2.2.Synthesis of labelled magnesium sulfate ($Mg^{34}SO_4$)

Magnesium sulfate ($Mg^{34}SO_4$) was synthesized from pure sulfur ^{34}S (purity > 98%, Cambridge Isotope, MA) following a two-step reaction:

(1) $6HNO_3 + {}^{34}S \rightarrow H_2{}^{34}SO_4 + 6NO_2 + 2H_2O$

(2) $H_2{}^{34}SO_4 + MgCO_3 \rightarrow Mg^{34}SO_4 + H_2O + CO_2$

Elemental sulfur ^{34}S (0.1069 g) was ground into a fine powder and transferred to a pear-shaped flask. Nitric acid (65%, 4 mL) was added to the flask and heated at 80°C for 5 hours. The temperature was subsequently raised to 130°C and maintained for an additional 24 hours in order to completely oxidise the nitric acid. The resulting sulfuric acid ($H_2{}^{34}SO_4$) was then converted to $Mg^{34}SO_4$ by adding magnesium carbonate ($MgCO_{3)}$ (0.2643 g), giving a yield of 0.3780 g. The solution was subsequently heated at 100°C until all water had completely evaporated. Elemental analysis of the dried crystals was carried out with an electron probe microanalyser (EPMA, Jeol JXA8200), equipped with an energy dispersive spectrometer (EDS), to confirm the production of $Mg^{34}SO_4$.

5.2.3.Symbiodinium growth and experimental conditions

Symbiodinium C1 cells were inoculated into sterile IMK medium (Wako Chemicals, Richmond, VA, USA) (starting density: 500 000 cells/mL) and incubated at 27°C for 18 days. LED lights were mounted above the culture, providing an average light intensity of 50 µE over a 14:10-hour light/dark cycle (AI Super Blue LED module 1003, IA, USA). Temperature and light intensities were monitored every 2 minutes for the entire duration of the experiment (using a HOBO UA-002-64, 64K temperature/light data logger).

After 18 days, *Symbiodinium* cells were inoculated into 5 different falcon tubes (5 mL per tube) in equal cell densities (1 500 000 cells/mL). The tubes contained sulfur-free artificial sea water (ASW) (24.72 g of NaCl, 0.67 g of KCl, 1.36 g of $CaCl_2 \cdot 2H_2O$, 4.66 g of $MgCl_2 \cdot 6H_2O$, 0.18 g of $NaHCO_3$, and 3.8 mL of modified ASP-8A solution (Table 5.1) in 1 L of MilliQ water). Magnesium sulfate ($MgSO_4 \cdot 7H_2O$, 6.29 g/L) was used as the sole sulfur source, with the sulfur atom being either

^{34}S (hereafter called ^{34}S-ASW) or ^{32}S (^{32}S-ASW) (Fig. 5.2). This sulfate concentration was adopted from recommended conditions for cultures of marine invertebrates (Karp and Solursh 1974). Tubes 1 to 4 were incubated in ^{34}S-ASW, whereas the control, tube 5, contained only ^{32}S-ASW. All treatments were incubated for a further 18 days (with growth media replaced every 5 days). *Symbiodinium* cell numbers were monitored every 3 days under a light microscope using a haemocytometer (depth 0.1 mm, 8 technical replicates were averaged per time point) and cell mortality assessed using a 0.05% (w/v) Evans Blue solution (Morera and Villanueva 2009).

Table 5.1: ASP-8A supplement composition used for *Symbiodinium* cultures modified from (Blank 1987).

Compound	Mass (g) to make stock	Stock volume (ml)	Stock (mM)	Volume of stock/L of media (ml)
$NaNO_3$	10.1988	40	3000	0.43333
Na_3NTA	10.284	40	1000	0.28
H_3BO_3	1.2366	40	500	0.36
KH_2PO_4	2.7218	40	500	0.296
Na_2EDTA	0.74448	40	50	1.8
NH_4NO_3	1.6008	40	500	0.03
Thymine	0.06305	40	12.5	0.256
$FeCl_3 \cdot 6\ H_2O$	0.5406	40	50	0.27
Pyridoxine (HCl)	0.08226	40	10	0.0097
Riboflavin	0.00301	40	0.2	0.05
Cyanocobalamin	0.00542	40	0.1	0.05

After 18 days, the medium in each tube was decanted and discarded. The remaining *Symbiodinium* cultures were rinsed three times with ^{32}S-ASW prior to the addition of bacteria. The ^{34}S-ASW was replaced by ^{32}S-ASW in tubes 1-4, in order to prevent direct bacterial uptake of $^{34}SO_4^{2-}$ from the medium. Tubes 1 and 2 were subsequently inoculated with the DMSP-degrading bacteria *Pseudovibrio* sp. P12 and *Pseudomonas* sp. A01, respectively; tube 3 with *E. coli*; tube 4 acted as a control without bacteria added; and tube 5 was never in contact with ^{34}S and acted as negative control

for sulfur isotope incorporation (Fig. 5.2). All bacteria strains were inoculated at a density of 10^6 cells/mL and samples collected six hours after bacterial inoculation.

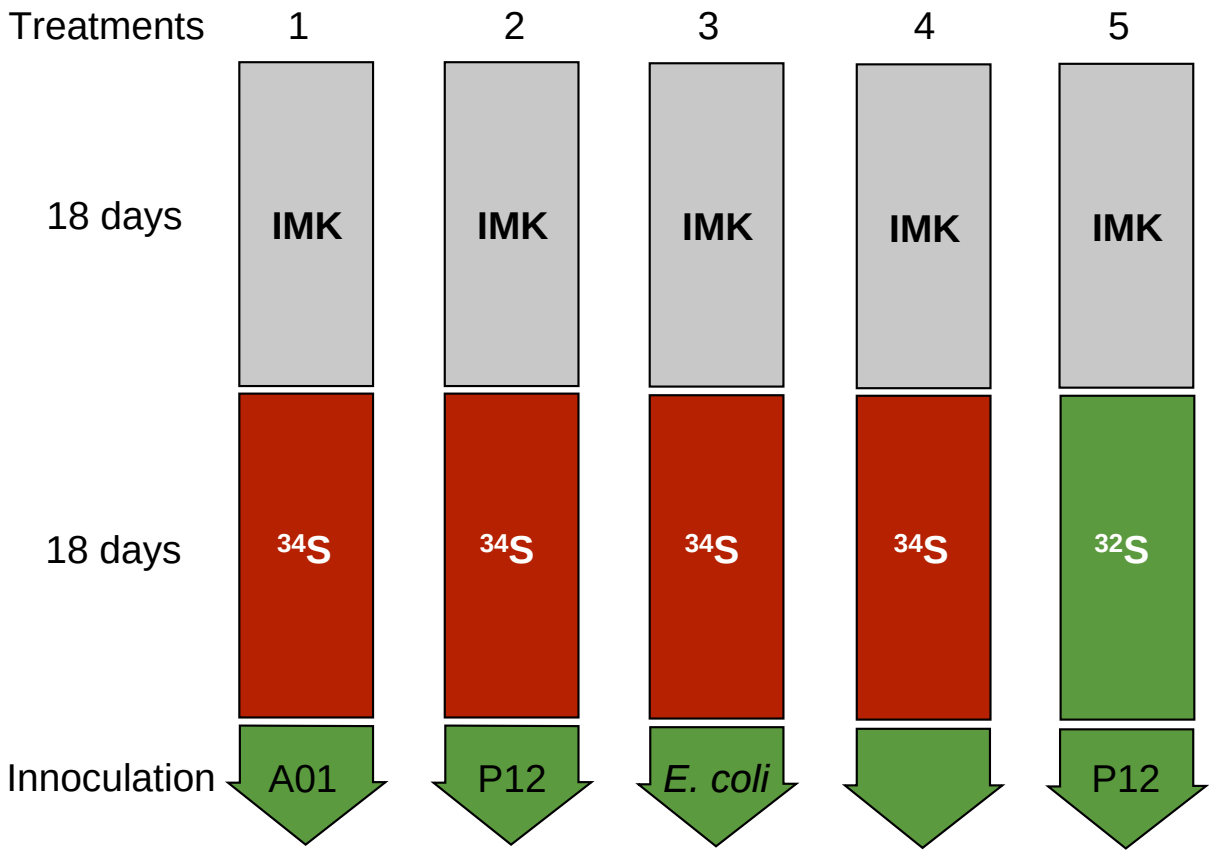

Figure 5.2: Sampling design showing culture conditions for the five treatments. After 18 days of incubation in IMK medium, *Symbiodinium* cells were transferred into 5 different tubes and further incubated into artificial sea water containing either $Mg^{34}SO_4$ (red, ^{34}S-ASW) or $Mg^{32}SO_4$ (green, ^{32}S-ASW). After a further 18 days, all treatments were rinsed with ^{32}S-ASW and inoculated with different bacterial strains for 6 hours.

5.2.4. Cryo-preservation

Because of the high solubility of DMSP in water, typical preservation techniques for NanoSIMS involving aqueous glutharaldehyde/paraformaldehyde fixative could not be used. An alternative cryopreparation method was therefore employed to preserve the DMSP in samples for NanoSIMS analysis: *Symbiodinium* cultures pre-incubated with bacteria (20 µL) were placed in drops on Thermanox strips (4 × 18 mm) in triplicate. After 10 minutes, the excess medium was carefully removed with filter paper and the strips were rapidly plunged into liquid nitrogen slush (liquid nitrogen under low vacuum). Samples required for structural imaging by electron microscopy (2 µL)

were also collected. These were deposited in a gold planchet and high-pressure frozen using a Leica EMPACT2 high-pressure freezer. Both sample types were stored in liquid nitrogen until required.

For analysis of DMSP by NanoSIMS, frozen samples were freeze-substituted over a molecular sieve in anhydrous 10% acrolein in diethyl ether, and warmed progressively to room temperature over 3 weeks in a Leica EM AFS2 automatic freeze-substitution unit. The temperature cycle was: 24 hours at -100°C, 168 hours at -90°C, 336 hours at -70°C, 24 hours at -20°C and a final step holding the cells at 0°C until required. This slow temperature ramp using freeze substitution was needed to avoid the formation of ice crystals within the cells as they thawed. These samples were subsequently infiltrated and embedded in anhydrous Araldite 502 resin, after which the thermanox strip was removed and the sample re-embedded and stored in a dessicator. For structural imaging, samples were freeze-substituted in 1% OsO_4 in acetone over 2 days and similarly infiltrated and embedded.

5.2.5. NanoSIMS sample preparation

Resin sections (750 nm thick) with embedded *Symbiodinium* cells were cut dry using a Diatome-Histo diamond knife on a Leica EM UC6 Ultramicrotome (Leica Microsystems, Wetzlar, Germany), mounted on a silicon wafer and coated with 10 nm of gold. Sections were imaged using a Cameca NanoSIMS 50 ion microprobe (Cameca, France) in order to quantify the distribution and abundance of the sulfur species ^{34}S and ^{32}S in the samples. The presence of sulfur within and outside *Symbiodinium* cells was assessed by simultaneously acquiring a mass image for ^{32}S and another one for ^{34}S. The incorporation and enrichment of the stable isotope ^{34}S was measured by determining the increase of $^{34}S/^{32}S$ ratio compared to the natural abundance ratio (0.0429). In addition, the sample structure was imaged in parallel to the sulfur measurements with the acquisition of the secondary ions $^{12}C_2$ and $^{12}C^{14}N$. The isotope ratio values are represented hereafter using a colour-coded transform (hue saturation intensity (HSI)) showing natural $^{34}S/^{32}S$ abundance levels in blue, and grading to high ^{34}S enrichment in pink.

Images were acquired at a 256 × 256 pixel resolution using a raster size of 20 µm. Detailed, high resolution images were also acquired using a raster size of 10 µm. A beam current of 100 pA, corresponding to a beam diameter of approximately 100 nm, was applied. Images were processed and analysed using the ImageJ software with the Open-MIMS plug-in. Quantitative data were extracted from the mass images through manually drawn regions of interest.

5.2.6.Transmission electron microscopy (TEM)

High pressure frozen samples for structural analysis were dehydrated with increasing concentrations of ethanol followed by dry acetone. Dehydrated samples were infiltrated with increasing concentrations of Araldite resin before being cured for 24 hours at 60°C. Longitudinal sections 90 nm thick were cut on a diamond knife, collected on copper grids and imaged unstained at 120 kV in a JEOL 2100 TEM (Tokyo, Japan).

5.2.7.High pressure liquid chromatography-mass spectrometry (HPLC-MS)

Six hours after bacterial inoculation, all *Symbiodinium* cultures were centrifuged (3000 g), medium was discarded and the pelletized cells were extracted with 5 mL of HPLC-grade methanol for 2 hours with sonication. Crude methanol extracts were then analyzed by reverse-phase (RP18) HPLC-MS in triplicate along with pure DMSP standards.

A 10 µl aliquot of sample was chromatographed by RP18-HPLC -MS using a Waters Alliance 2695 HPLC system comprising a quaternary pump, autosampler and photodiode array detector (200-400 nm) coupled to a Waters Micromass LCT Premier orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer. Separation was achieved on a Waters XBridge Shield RP18-HPLC column (3.5 µm; 2.1 × 100 mm) maintained at 20°C with a methanol (MeOH) and water linear gradient (20% MeOH:H_2O to 100% MeOH over 10 min at flow rate of 0.3 ml min^{-1}).

TOF-MS accurate mass measurements (scan-range *m/z* 100–1000 at 4GHz, resolution = 9500) were acquired using a electrospray ionization (ESI) source in W positive mode with the following operation parameters: capillary voltage: 3000 V; cone voltage: 80V; ion source temperature: 80 °C; desolvation temperature: 350 °C; cone gas flow: 10 L/hr; desolvation gas flow: 750 L/hr; ion energy: 33 V; acceleration voltage: 100 V. MassLynx software (version 4.1, Waters) was used for operating the HPLC-MS, as well as for data acquisition and processing.

5.2.8. Quantitative nuclear magnetic resonance (qNMR)

The methanol extract remaining after HPLC-MS analysis was dried using a vacuum-centrifuge and dissolved in a mixture of deuterium oxide (D_2O, D 99.8 %, 250 µL) and deuterated methanol (CD_3OD, D 99.8 %, 750 µL) (Cambridge Isotope Laboratories, Andover, MA, USA). A 700 µL aliquot of the particulate-free extract was transferred into a 5 mm Norell 509-UP-7 NMR tube (Norell Inc., Landisville, NJ, USA) and analyzed immediately by ^{1}H NMR.

^{1}H NMR spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer with TXI 5 mm probe and quantification performed using the ERETIC method (see Chapter 2). This technique generates an internal electronic reference signal, calibrated using commercial stock solutions of 4 mM acrylate and DMSP. The concentrations of DMSP and acrylate were determined by integration of their respective signals in a 0.10 ppm window.

5.3. Results

5.3.1. Labelled $MgSO_4$ synthesis

EDS spectra of the synthesized crystals revealed that Mg and S were the only elements present in the synthesized crystals (the instrument used cannot detect oxygen) and their mass ratio corresponded to the values expected for $MgSO_4$. Magnesium and sulfur represented 39.8% and 59.4%, respectively, of the mass in the compound (expected value: Mg=41.3% and ^{34}S=58.7%, when oxygen is not considered). Furthermore, the elongated crystal morphology observed by SEM (Fig.

5.3) was in accordance with previous $MgSO_4$ observations (Cullen and Baker 2002), confirming the identity of the synthesized compound.

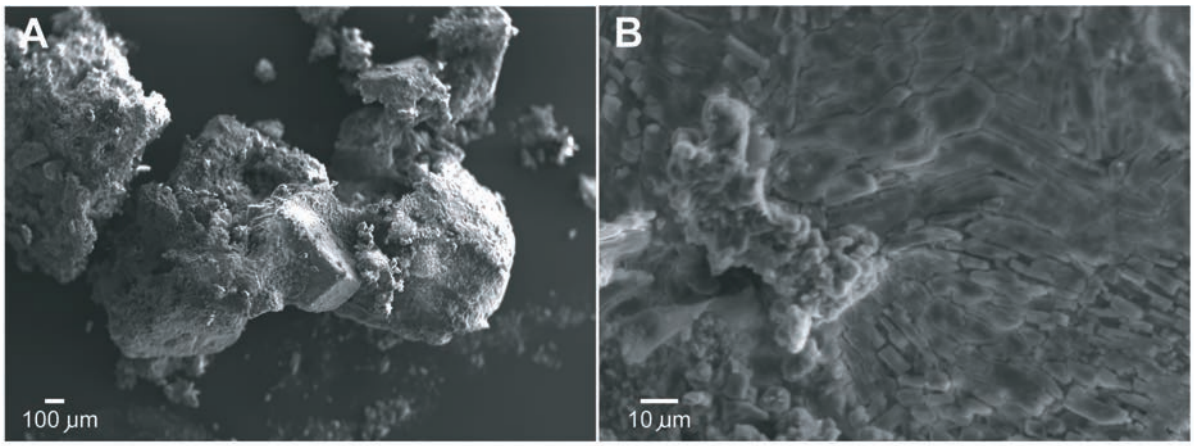

Figure 5.3: Scanning electron microscopy images showing crystal structure of the synthesized compound, $MgSO_4$.

5.3.2.Symbiodinium growth

Symbiodinium cells grew and divided actively in the ^{34}S-ASW. Their number nearly doubled during the 18 day incubation period, reaching 280,000 (±24,000) cells/mL by the end of the experiment (Fig. 5.4). The number of dead cells remained constant throughout the incubation, likely due to frequent medium changes (every 5 days) that removed floating cells from the culture.

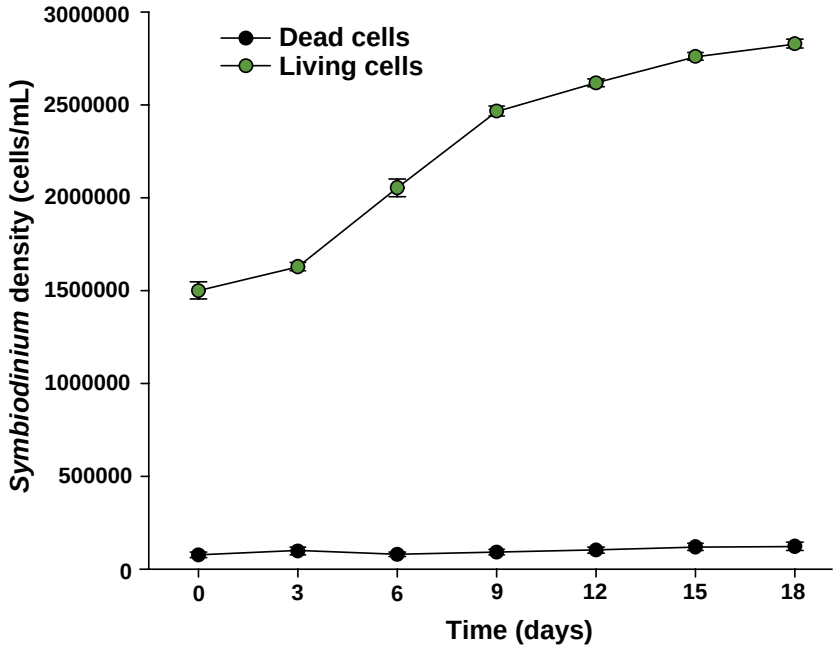

Figure 5.4: Growth kinetics of *Symbiodinium* cells (strain C1) incubated at 27°C in artificial sea water containing $^{34}SO_4^{2-}$ as sole sulfur source.

5.3.3.Incorporation

The production of ^{34}S DMSP by *Symbiodinium* cultures was confirmed using HPLC-MS. The natural abundance of ^{34}S in the environment is 4.29%, which correlates well with the amount of ^{34}S-DMSP detected in controls (5.01% of total DMSP) (Fig. 5.5A, Table 5.2). At the end of the experiment, six hours after all treatments were transferred into ^{32}S-ASW, all cultures that were initially incubated with ^{34}S-ASW were highly enriched in ^{34}S-DMSP (up to 62.64% of total DMSP) (Fig. 5.5B, Table 5.2). However, none of the DMSP precursors (the amino acids ^{34}S-methionine or ^{34}S-cysteine) could be detected in the samples. In addition, ^{32}S-DMSP was present in all ^{34}S-ASW incubated samples, at levels ranging from 37.36% to 55.19% of total DMSP (Table 5.2). Since the growth medium contained only traces of ^{32}S, these large amounts of ^{32}S-DMSP are likely to have been biosynthesized during the six hours preceding the sampling, when the *Symbiodinium* cells were transferred into ^{32}S-ASW prior to the addition of bacteria. Quantitative NMR revealed that the concentrations of total DMSP were significantly smaller in treatments with coral-associated bacteria present (T-Test, n=3, $F_{1,3}$=1.14, *p<0.005) compared to those with no bacteria or bacteria incapable of metabolising DMSP present (Table 5.2).

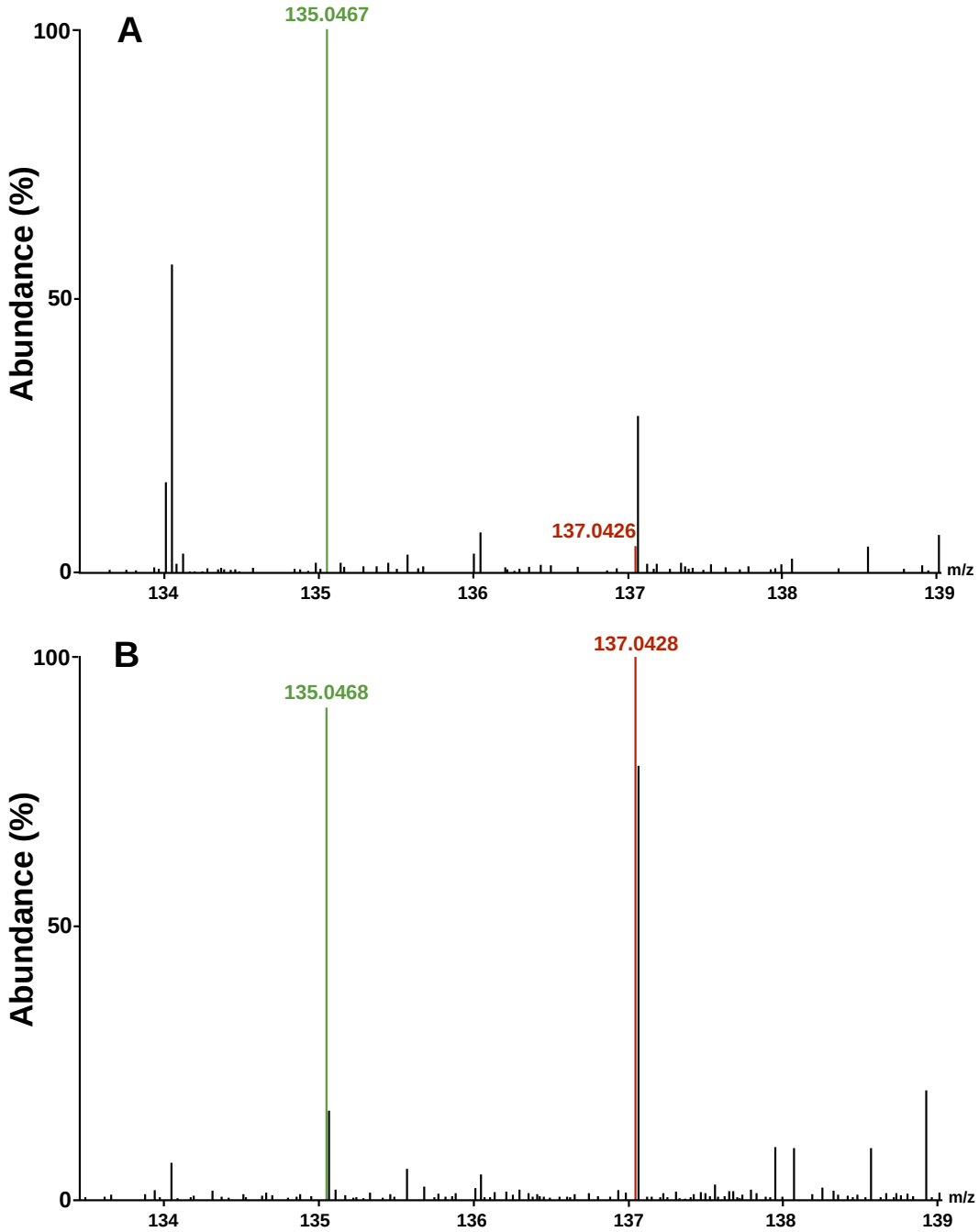

Figure 5.5: Representative HPLC-MS spectra showing the presence and relative abundance of ^{32}S DMSP (green) and ^{34}S DMSP (red) in methanol extracts derived from *Symbiodinium* cultures: (*A*) incubated with ^{32}S (treatment 5); (*B*) incubated with ^{34}S (treatment 3). Data were collected from *m/z* 133.5 to 139.0.

Table 5.2: DMSP concentrations in the five different *Symbiodinium* culture treatments, as measured by qNMR and HPLC-MS.

Treatment	DMSP (µmol)	^{32}S-DMSP (% of total)	^{34}S-DMSP (% of total)	^{32}S-DMSP (µmol)	^{34}S-DMSP (µmol)
^{34}S + *Pseudomonas* sp.	2.29	45.61	54.39	1.04	1.24
^{34}S + *Pseudovibrio* sp.	2.45	55.19	44.81	1.35	1.10
^{34}S + *Escherichia coli*	3.90	43.12	56.88	1.68	2.22
^{34}S + No bacteria	3.64	37.36	62.64	1.36	2.28
^{32}S + *Pseudovibrio* sp.	2.06	94.99	5.01	1.96	0.10

5.3.4. TEM observation

Transmission electron microscopy (TEM) showed that the cryopreservation technique used did not compromise the structural integrity of the *Symbiodinium* cells. All cells observed were well preserved, with chloroplasts, pyrenoids, mitochondria and other organelles appearing intact. In addition, bacteria were clearly visible around the cells (Fig. 5.6A-B) in all treatments except number 4 (Fig. 5.6C), where no bacteria were added.

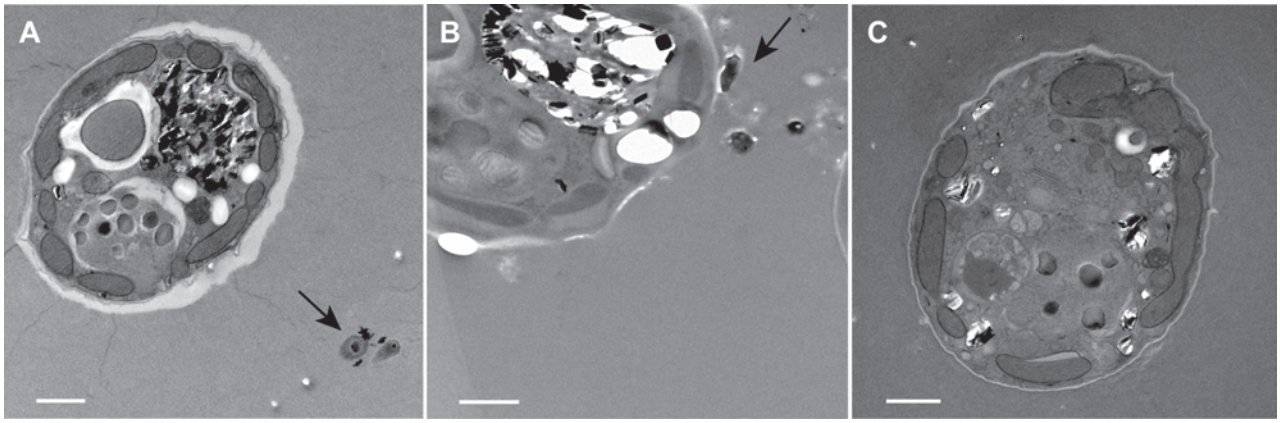

Figure 5.6: Representative transmission electron micrographs showing *Symbiodinium* cells from different treatments: (*A*) *Symbiodinium* and *Pseudomonas* sp. A01; (*B*) *Symbiodinium* and *Pseudovibrio* sp. P12; (*C*) control without bacteria. Arrows point to bacterial cells. Scale bars: 1 µm.

5.3.5. NanoSIMS imaging of Symbiodinium cells

Analysis of *Symbiodinium* cells in the control cultures with no ^{34}S enrichment (treatment 5) confirmed that their ^{34}S/^{32}S ratio (±SE), 0.0428 (±0.0007; *n*=10 regions), was highly similar to the natural abundance ratio (Fig. 5.7C and F). In contrast, *Symbiodinium* cells incubated for 18 days in

^{34}S-ASW were 27 times more enriched, giving an average value of 1.1549 (±0.1015; *n*=12 regions). In the two treatments where coral-associated bacteria were added (treatments 1 and 2), ^{34}S hotspots were recorded close to the *Symbiodinium* cell membranes (Fig. 5.7D and E). These hotspots measured 0.53 (±0.07) µm on average, which is consistent with the size of the bacterial cells observed with TEM (Fig. 5.6A and B) and their ^{34}S/^{32}S ratio was 3.8482 (±0.2851; *n*=10), which is 3 times more enriched than their neighbouring *Symbiodinium* cells and 90 times more than the cells in the control. The enrichment of these hotspots was also well illustrated by ^{34}S/^{32}S transects (Fig. 5.7G and H), which revealed the relative homogeneity of ^{34}S enrichment within *Symbiodinium* cells compared to the high values recorded on their periphery.

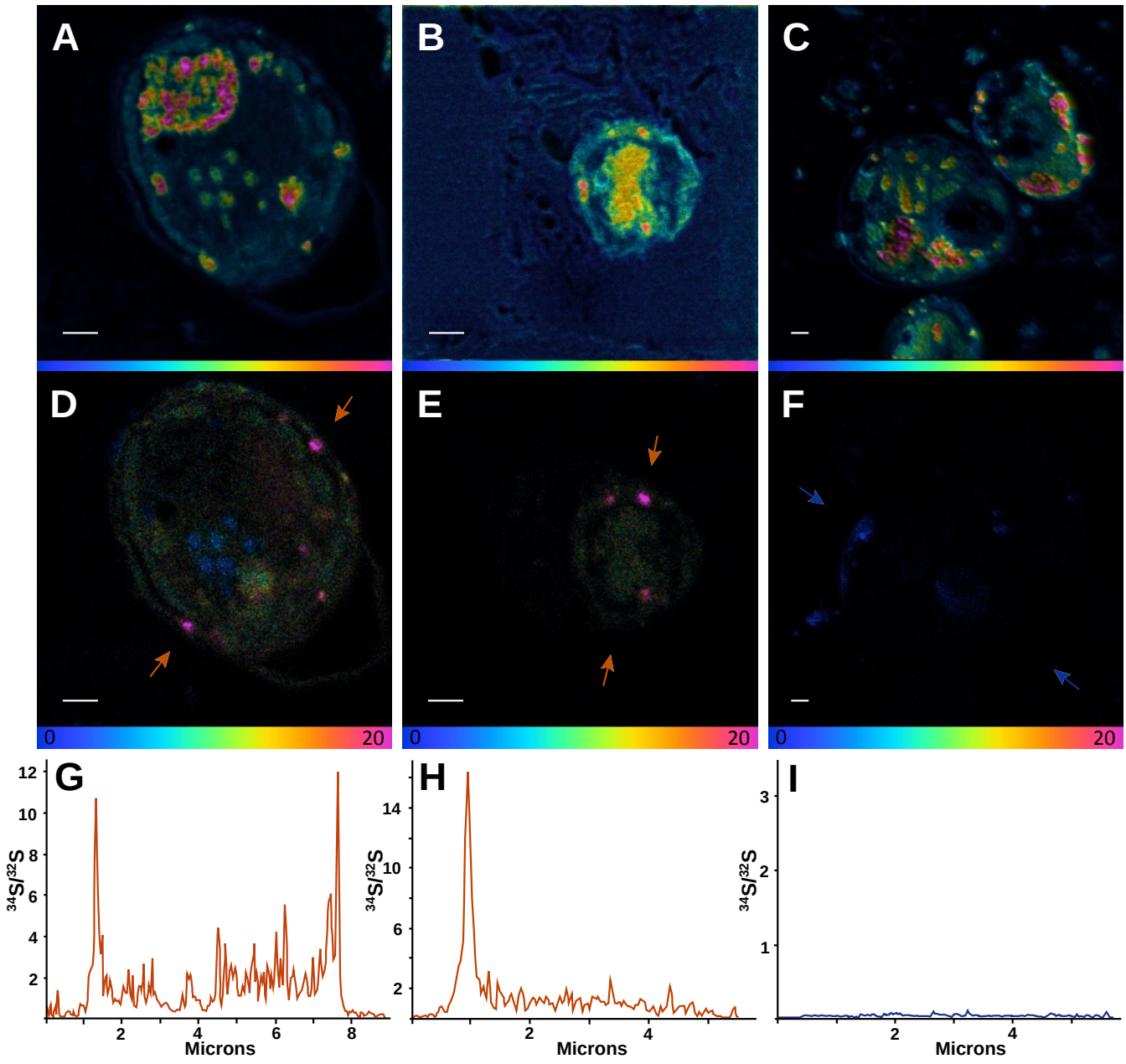

Figure 5.7: Representative NanoSIMS ion images of *Symbiodinium* cells exposed to ^{34}S- or ^{32}S-ASW for 18 days. (*A-C*): $^{12}C^{14}N/^{12}C_2$ mass images showing cellular structures for comparison with the same cells in images directly below. (*D-F*): $^{34}S/^{32}S$ ratio image of the same regions. These mass images are shown as HSI images where the colour scale indicates the value of the $^{34}S/^{32}S$ ratio, with natural abundance in blue, changing to pink with increasing ^{34}S levels. (*A* and *D*) contained the bacteria *Pseudomonas* sp. A01 (treatment 1), (*B* and *E*) contained the bacteria *Pseudovibrio* sp. P12 (treatment 2), (*C* and *F*) acted as negative control without prior enrichment with ^{34}S (treatment 5). Bars = 1 µm. (*G-I*): $^{34}S/^{32}S$ ratio transects from the regions between the orange arrows for (*D* and *E*), and between the blue arrows for (*F*).

5.4. Discussion

This study demonstrates that metabolic linkages occur between microbial members of the coral holobiont, specifically between the endosymbiont *Symbiodinium* and coral-associated, DMSP-degrading bacteria. A combination of HPLC-MS and qNMR confirmed the biosynthesis of high concentrations of ^{34}S-labelled DMSP by *Symbiodinium* in cultures. High resolution mapping of the ^{34}S tracer by a NanoSIMS ion microprobe documented high abundance of the tracer in micrometer-size particles closely associated with dinoflagellates that were consistent in size and position with bacteria. This represents the first demonstration of tight cycling of sulfur through DMSP biosynthesis and metabolism by microbial members of the coral holobiont.

Symbiodinium cells grew and divided normally in the medium containing $^{34}SO_4^{2-}$ as the sole source of sulfur, suggesting that *Symbiodinium* must have incorporated the sulfur isotope. Incorporation was verified at the end of the experiment, when high concentrations of ^{34}S-DMSP were found in *Symbiodinium* extracts using HPLC-MS and qNMR. These measurements confirm that the sulfur atom used for DMSP biosynthesis in *Symbiodinium* originates from inorganic sulfate incorporation (Stefels 2000). *Symbiodinium* cells in culture are known to exude proteins and sugars into the surrounding medium (Markell et al. 1992, Markell and Trench 1993), however the methionine and cysteine content of these exudates is extremely low (Markell and Trench 1993). DMSP is therefore the largest component of the ^{34}S exuded from *Symbiodinium* cells, and enriched $^{34}S/^{32}S$ ratios in NanoSIMS images can be confidently attributed to exuded DMSP.

NanoSIMS analysis confirmed that all *Symbiodinium* cells incubated with ^{34}S-sulfate were highly enriched in ^{34}S. Within these enriched *Symbiodinium*, the spatial distribution of the sulfur tracer was relatively heterogeneous, with some organelles such as the nucleus showing lower levels (but always above natural abundance); however, hotspots of much greater enrichment were detected outside *Symbiodinium* cells in the treatments inoculated with DMSP-degrading bacteria. These hotspots were at least 3 times more enriched in ^{34}S than levels within *Symbiodinium* cells and were

similar in size to bacterial cells observed with TEM. It is therefore highly likely that these hotspots represent coral-associated bacteria actively taking up DMSP excreted from *Symbiodinium* cells. This hypothesis is further supported by markedly lower concentrations of DMSP in those treatments inoculated with DMSP-degrading bacteria compared to treatments with either no bacteria or bacteria unable to degrade DMSP, indicating that DMSP was being actively metabolized.

The techniques used here represent a novel approach to follow metabolic exchange in symbiotic systems. By following the fate of ^{34}S, from its uptake by *Symbiodinium* as inorganic sulfate ($^{34}SO_4^{2-}$) to its biosynthesis and exudation as ^{34}S-labelled DMSP, this study goes a long way towards unravelling linkages in sulfur synthesis and metabolism between microbial members of the coral holobiont. However, the complete picture of *Symbiodinium*-bacteria interactions in terms of sulfur metabolism will only be obtained when the location of bacterial cells is unambiguously determined. In theory, this could be achieved by using fluorescent or other *in situ* hybridization techniques (*X*-ISH) to target bacteria of interest before using the same section for NanoSIMS, enabling bacterial phylogenetic identity to be correlated with their metabolic capabilities (Orphan et al. 2001, Kuypers and Jorgensen 2007, Behrens et al. 2008, Musat et al. 2008, Thompson et al. 2012). However, this approach was not feasible in this study because these procedures include numerous steps in aqueous buffers, which would extract soluble compounds such as DMSP. Similarly, correlation of structural data from TEM micrographs directly with NanoSIMS analysis, as done for microorganisms associated with plant roots (Clode et al. 2009), requires thin sections to be cut onto water, and could not be used here. One option for future studies is to grow the bacteria of interest overnight in a medium containing a ^{13}C or ^{15}N labelled sources prior to their inoculation with the *Symbiodinium,* allowing their co-localization with NanoSIMS. Alternatively, swimming coral larvae with and without *Symbiodinium* could be incubated in ^{34}S-ASW prior to inoculation of ^{13}C-labelled bacteria, providing a more realistic model in an intact symbiosis.

The present study opens up new avenues for exploring the functional role of coral-associated bacteria *in vivo*. The combination of NanoSIMS, HPLC-MS, qNMR, TEM and high-pressure freezing techniques represents a powerful approach, which in this study enabled: *i*) confirmation of the biosynthesis of high concentrations of ^{34}S-DMSP resulting from *Symbiodinium* assimilation of ^{34}S-labelled sulfate, *ii*) effective preservation of soluble DMSP in samples, *iii*) visualization and measurement of ^{34}S enrichment in *Symbiodinium* cells, and *iv*) determination of ^{34}S hotspots adjacent to *Symbiodinium* cells that correlate well with the size and position of bacteria observed with TEM. Taken together, these results constitute the first empirical evidence of the usage of *Symbiodinium* secondary metabolites by coral-associated bacteria *in vivo*. Results also emphasize the importance of DMSP for coral-associated bacteria and further support its role in structuring bacterial communities associated with corals.

Chapter 6: Identification of a sulfur-based antimicrobial produced by coral-associated bacteria

6.1. Introduction

Coral reefs are one of the most biologically diverse ecosystems on the planet (Pauley 1997, Bellwood and Hughes 2001, Knowlton 2001); however, despite the myriad of iconic reef fishes and coral groups, most of the biodiversity on coral reefs is invisible to the naked eye and of unknown functional significance. Each square centimetre of a coral's surface harbours several thousand bacterial species (Sunagawa et al. 2010) and up to 10^7 bacterial cells (Koren and Rosenberg 2006, Garren and Azam 2010), an abundance ten times greater, on average, than the surrounding sea water. These bacterial assemblages are often highly specific to their coral host and include large numbers of rare and sometimes even unique taxa (Sunagawa et al. 2010). Although the phylogenetic diversity and dynamics of coral-associated bacterial communities have been studied for more than a decade (Rohwer et al. 2001, Bourne and Munn 2005, Rosenberg et al. 2007a, Littman et al. 2009, Sunagawa et al. 2010), their ecological and functional roles in the biology and health of corals are still poorly understood.

Recent studies have started to unravel the roles that coral-associated bacteria and their interactions with their coral hosts are likely to play within the coral holobiont. For example, some members of the *Cyanobacteria*, *Rhizobiales* and *Vibrionaceae* taxa are likely to fix dissolved nitrogen, a particularly important process in oligotrophic environments such as coral reefs (Lesser et al. 2004, Olson et al. 2009, Lema et al. 2012). Others like *Roseobacter*, *Pseudomonas* and *Spongiobacter* can metabolize dimethylsulfoniopropionate (DMSP), an organic sulfur compound produced in large amounts by corals and suspected to play a role in structuring coral-associated bacterial communities (Raina et al. 2009, Raina et al. 2010). It has also been hypothesized that bacteria act as a line of defence against invasive pathogens, either by competing for space and occupying coral niches (Ritchie and Smith 2004), or by directly producing antimicrobial compounds that inhibit the growth of invasive microbes in coral mucus (Ritchie 2006).

In artificial culture conditions, approximately 25% of the cultivable coral-bacteria consortia produce antimicrobial compounds that prevent the growth of pathogenic micro-organisms (Ritchie 2006, Shnit-Orland and Kushmaro 2008). Several of these antimicrobial-producing taxa, such as *Pseudoalteromonas*, *Pseudomonas*, *Spongiobacter* and the *Roseobacter* clade are found in association with numerous coral species (Radjasa et al. 2008, Nissimov et al. 2009, Shnit-Orland and Kushmaro 2009, Rypien et al. 2010). However, production of secondary metabolites, such as antimicrobial compounds, in artificial culture medium does not necessary imply that they are also produced in corals.

Although the presence of antimicrobial defences in reef-building corals has been reported in numerous studies (Koh 1997, Geffen and Rosenberg 2005, Gochfeld and Aeby 2008, Geffen et al. 2009), attempts to isolate active compounds have been unsuccessful (Koh 1997, Kelman 2004). The most important issue that has hindered the identification of biologically active compounds in reef-building corals is their very low concentrations (Munro et al. 1999, Radjasa et al. 2008). The small amounts of these compounds per coral, coupled with possible geographic and seasonal variations in their production further complicate the isolation of these molecules. Therefore, it is currently impossible to determine the source of these antimicrobial compounds and to ascertain the role of associated bacteria in the defence of the coral host against pathogens.

My goal in this study was to apply new approaches for determining if bacteria produce antimicrobial compounds in the coral host, to enhance understanding of the functional roles of coral-associated bacteria in their natural environment. My specific objectives were to: *i*) identify coral-associated bacteria with antimicrobial activity; *ii*) isolate an antimicrobial compound produced by a pure culture of a common coral-associated bacterium; *iii*) evaluate the susceptibility of the coral pathogens *Vibrio coralliilyticus* and *Vibrio owensii* to the pure isolated compound; *iv*) compare the susceptibility of these pathogens when the antimicrobial-producing bacterium was grown under

ambient versus elevated temperatures; and *v*) investigate the natural abundance of this compound in coral extracts.

6.2. Materials and Methods

6.2.1. Bacterial isolation

Healthy colonies of the corals *Pocillopora damicornis*, *Acropora millepora* and *Montipora aequituberculata* (one colony per species) were collected from Davies Reef, Great Barrier Reef, Australia (latitude, 18°51'S; longitude, 147°41'E) and maintained in aquaria at the Australian Institute of Marine Science (Townsville, Queensland, Australia). Five replicate coral fragments (approximately 25 mm in length, containing 60 to 70 polyps) were collected from each colony and washed in sterile artificial seawater (ASW) to remove loosely attached microbes. Tissue slurries were produced by airbrushing (80 lb/in^2) each coral fragment into 5 mL of ASW to remove coral tissues and associated microbes. These tissue slurries were homogenized to break down tissue clumps, and a dilution series was plated immediately on minimal marine agar (1% bacteriological agar; 0.3% casamino acids; 0.4% glucose; in 1 L ASW [Instant Ocean]) (Hjelm et al. 2004). After 2 days of incubation at 28°C, single colonies were transferred into Marine Broth (Difco) and grown overnight. Liquid cultures were re-plated on minimal marine agar and the procedure was repeated until pure cultures were obtained.

6.2.2. Well diffusion assay with bacterial isolates

Fifty bacteria isolated from the coral tissue slurries of the 3 species combined were tested for growth-inhibitory activity against the known coral pathogens *Vibrio coralliilyticus* P1 (LMG23696) and *V. owensii* DY05 (LMG25443) in a well diffusion agar assay. In brief, the *Vibrio* strains were seeded into two different batches of minimal marine agar (after the agar temperature cooled to 40°C). Following solidification, wells (diameter 5 mm) were cut into the agar and loaded with 20 µL of dense overnight cultures of the test isolates grown in Marine Broth (MB; Difco) (28°C, 170 rpm). Plates were incubated at 28°C and observed every 24 h for a period of 72 h for inhibition zones.

Phaeobacter strain 27-4 was used as a positive antagonistic control on each plate because of its broad spectrum inhibitory activity against *Vibrio* (Hjelm et al. 2004, Bruhn et al. 2007).

6.2.3.DNA extraction, sequencing and analysis

One isolate named P12, from *Pocillopora damicornis*, produced the strongest growth-inhibitory activity against the two target *Vibrio* strains. Total DNA was extracted from a pure culture of P12 grown in Marine Broth (Difco, BD, Franklin Lakes, NJ) media using a Wizard genomic DNA purification kit (Promega, Madison, WI), as per manufacturer's instructions. The near complete 16S rRNA gene of the strain was PCR amplified with bacterial specific primers 63F and 1387R, as outlined in Marchesi et al. (1998). Amplified PCR products were visualized by electrophoresis on 1% agarose gel stained with ethidium bromide. The amplified DNA was dried in a vacuum centrifuge (Savant DNA 120) and sequenced (Macrogen, Inc., Seoul, Korea). The 16S rRNA gene sequence of isolate P12 was used for phylogenetic comparisons and Maximum Likelihood trees were constructed using the ARB software.

6.2.4.DMSP metabolic capabilities of the isolate P12

Two different minimal media were used to examine the DMSP metabolic capabilities of P12: a modified marine ammonium salt medium (MAMS) (Raina et al. 2009) lacking a carbon source, and a modified basal salt medium lacking a sulfur source (Fuse et al. 2000) (f25 g of NaCl, 0.7 g of KCl, 0.05 g of KH_2PO_4, 1 g of NH_4NO_3, 0.2 g of $MgCl_2 \cdot H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 0.005 g of FeEDTA, 1 g of Tris, 5 g of sodium succinate, 1.35 g of glucose in 1 L of distilled water). DMSP was added to both media (1 mM), acting either as the sole carbon or sulfur source. Five millilitres of each culture media were inoculated in triplicate with single P12 colonies and incubated at 28°C for 6 days. Negative controls containing only the basal media and DMSP were set up, along with the ones inoculated with P12, to account for possible chemical breakdown of DMSP. Bacterial growth was assessed *via* optical density measurement (NanoDrop, Thermo Fisher, Waltham, MA). DMSP metabolism was assessed by ^{1}H NMR, which involved adding MeOH (40 mL) to each culture tube

and subsequently dried the mixture *in vacuo* using a rotary evaporator (Buchi, Flawil, Switzerland). The dried extracts were resuspended in deuterated methanol (CD_3OD, 1 mL) and their ^1H NMR spectra recorded using quantitative NMR (see Chapter 2).

6.2.5. Preparation of crude extract for antagonist assays

An overnight culture of P12 (8 mL) was used to inoculate 4×250 mL of MB (total culture volume = 1L). Cultures were incubated for two days at 28°C (120 rpm) to reach stationary phase; the culture broth was then acidified to pH 2 with sulphuric acid before being extracted three times with ethyl acetate. The extract was washed three times with MilliQ water and the organic soluble layer dried *in vacuo* using a rotary evaporator (Buchi). It was then resuspended in MeOH (which was chosen for its ability to solubilise a wide range of compounds and its volatility, plus it is innocuous towards both *V. coralliilyticus* and *V. owensii*) and tested in well-diffusion assays to confirm the extraction of the antimicrobial compound.

6.2.6. Purification and characterization of active compound

Purification of the crude extract was carried out via solid phase extraction on a silica gel C18 flash column. Eleven fractions were eluted sequentially with: 20% Aq. methanol (MeOH), 40% Aq. MeOH, 60% Aq. MeOH, 80% Aq. MeOH, 90% Aq. MeOH, 100% Aq. MeOH, 20% dichloromethane (DCM)/MeOH, 50% DCM /MeOH, 100% DCM, 40% hexane/DCM, and 100% hexane. The fractions were dried and resuspended in MeOH (1 mg/mL of dry weight). Well diffusion assays were prepared as described above. On each plate, test wells were inoculated with 20 µL of each chromatographic fraction, whilst a control well was filled with 20 µL of MeOH and *Vibrio* growth monitored.
The active 80% MeOH fraction presented an intense yellow coloration. The fraction was concentrated and orange-red crystals precipitated. These crystals were labelled compound **1** (2.1 mg, 1.7 % organic extract).

6.2.7.NMR and FTMS analysis

Structure elucidation of compound **1** was achieved using Nuclear Magnetic Resonance (NMR) spectroscopy and Fourier Transform Mass Spectrometry (FTMS). ^{1}H and ^{13}C NMR spectra of compound **1** were acquired in a 5 mm 509-UP Norell NMR tube on a Bruker Avance 600 MHz NMR spectrometer (Bruker, Germany) with a TXI cryoprobe using standard Bruker pulse sequences. NMR spectra were referenced to residual ^{1}H and ^{13}C resonances in deuterated chloroform ($CDCl_3$).

High resolution mass spectra of compound **1** were measured with a Bruker BioApex 47e Fourier Transform Mass Spectrometer (FTMS) fitted with an Analytica of Branford ESI source; ions were detected in negative mode within a mass range *m/z* 200-1,000. Direct infusion was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 120 μl h^{-1}. The instrument was calibrated with methanolic trifluoroacetic acid (0.1 mg mL^{-1}). Proton and carbon NMR shifts measured, and the accurate mass of compound **1** were in accordance with data previously obtained for tropodithietic acid (TDA) (Kintaka et al. 1984, Brinkhoff et al. 2004).

Compound **1** was an optically inactive, orange-red microcrystalline solid. IR (film) ν_{max} 3420, 1660, 1280 cm^{-1}; UV (PDA, MeOH) λ_{max} 512 nm; ^{1}H NMR spectrum showed four signals (600 MHz, CD_3Cl): δ 7.12, 7.44, 7.45 and 16.70 ppm; ^{13}C NMR spectrum showed eight signals (150 MHz, CD_3Cl): δ 120.26, 131.99, 135.97, 138.67, 149.46, 168.71, 171.68, and 183.50 ppm; HRESIMS *m/z* found 210.9534 for $[M-H]^-$ (calculated for $C_8H_3O_3S_2^-$ 210.9529, Δ 2 ppm).

6.2.8.Temperature dependant activity

The activity of P12 grown at 32°C (upper limit of coral thermo-tolerance) was compared to the control incubated at 28°C. The two cultures were grown overnight in Marine Broth (Difco, BD, Franklin Lakes, NJ) at the two different temperatures, and their densities were measured based on their optical densities (NanoDrop, Thermo Fisher, Waltham, MA). Cell numbers were normalized prior to inoculation into agar wells, and their activities against the two pathogens were compared

using well-diffusion assays as described above. The same procedure was repeated using the pure active compound **1**: two vials containing equal concentrations (2 µM in MeOH) were incubated overnight at 32°C or 28°C and their antimicrobial activities were compared using well diffusion assay.

6.2.9. Genomic analyses

The presence of genes involved in DMSP breakdown (*dmdA*, *dddD*, *dddL*, *dddP*, *dddY*, *dddQ* and *dddW*) and TDA production (*tdaA-tdaF*) was investigated using the fully sequenced genome of *Pseudovibrio* sp. FO-BEG1 (KEGG genome T01669; isolated from a Caribbean coral), a strain sharing 100% sequence similarity with P12 based on its 16S rRNA gene sequence. The BLASTP function of the Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to determine the presence of the genes of interest.

6.2.10. Preparation of coral extracts

The coral species *Montipora aequituberculata, M. turtlensis, Pocillopora damicornis, Acropora millepora, A. muricata* and *Porites cylindrica* (one colony per species) were collected from Orpheus Island, Great Barrier Reef, Australia (latitude, 18°35'S; longitude, 146°20'E). The corals were airbrushed (80 lb/in^2) into 1µm filtered seawater (FSW) (total volume = 500 mL), acidified to pH 2 with sulphuric acid and the solution extracted three times with equal volumes of ethyl acetate. The crude extracts were washed with MilliQ water, dried and tested in well-diffusion assays as previously described for the bacterial isolate extracts. The active species were then fractionated as described above for the crude extract from P12. Each fraction was tested in well-diffusion assays and the active ones were screened for TDA presence using both ^{1}H NMR and FTMS.

6.3. Results

6.3.1.Isolate P12: antimicrobial production, taxonomy and metabolic capabilities

A total of 50 coral-associated bacterial isolates were obtained from tissue slurry homogenates of the three coral species. Twelve of the 50 strains that were tested against the two pathogenic *Vibrio* strains (*V. coralliilyticus* and *V. owensii*) inhibited growth of the pathogens in well diffusion assays. The bioactive isolate that exhibited the strongest *in vitro* activity against both pathogens, called P12, originated from *Pocillopora damicornis* and produced growth inhibition zones of 5 mm (±0.07 mm, n=20) against *V. owensii* and 2 mm (±0.09 mm, n=20) against *V. coralliilyticus*. The activity of P12 was temperature-dependent and was significantly reduced when grown at 32°C compared to 28°C (T-Test, n=20, $F_{2,38}$=1.25, *p<0.001 for *V. owensii* and $F_{2,38}$=3.45, *p<0.001 for *V. coralliilyticus*) (Figure 6.1). Based on its bioactivity, the isolate P12 was selected for bioassay-guided fractionation.

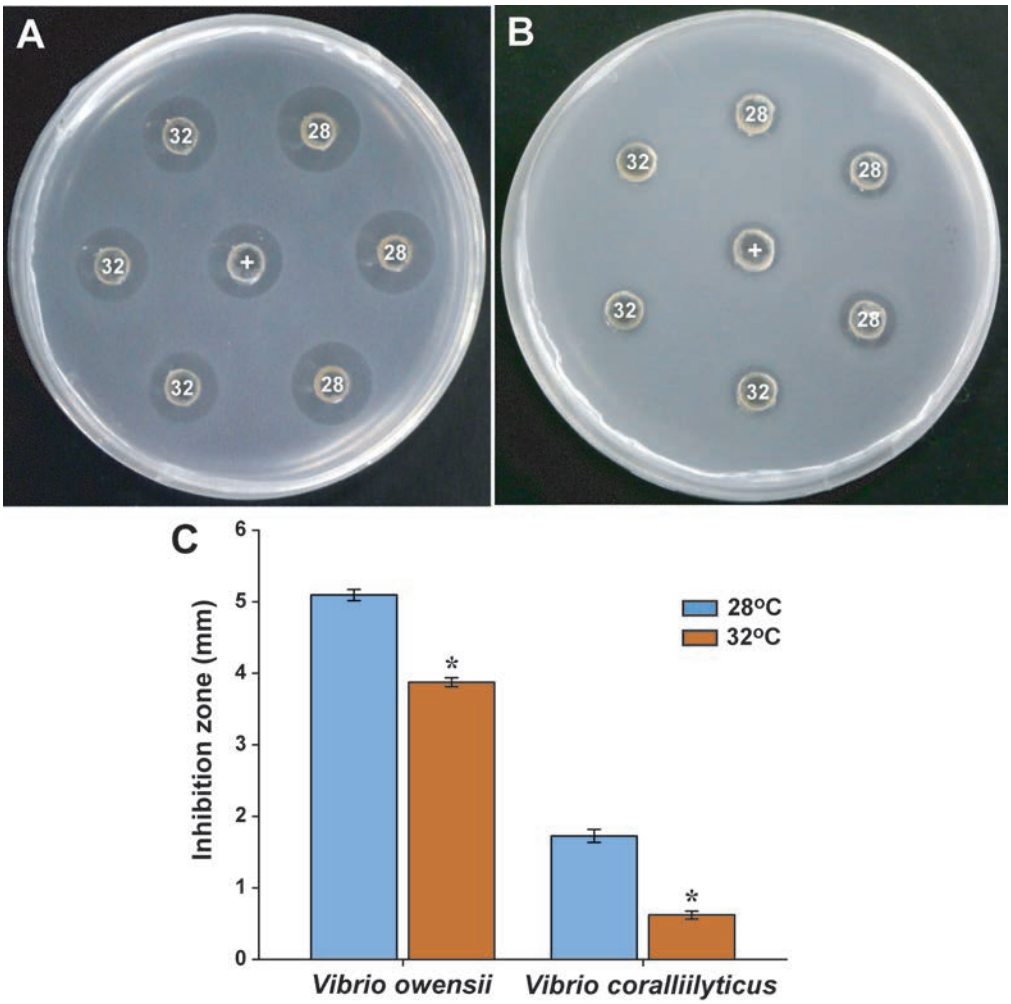

Figure 6.1: Representative well diffusion assays of P12 grown at two different temperatures (28°C and 32°C) and then inoculated onto agar plates with embedded: (*A*) *Vibrio owensii* or (*B*) *Vibrio coralliilyticus* [(+) Positive control: *Phaeobacter* sp. 27-4]. (*C*) Comparison of the radius of inhibition zones between the two temperature treatments (T-Test, n=20, $F_{2,38}$=1.25, *p<0.001 for *V. owensii* and $F_{2,38}$=3.45, *p<0.001 for *V. coralliilyticus*).

According to its 16S rRNA gene sequence, isolate P12 is an *Alphaproteobacterium* belonging to the *Rhodobacteraceae* family and the *Pseudovibrio* genus. Its closest fully described relative is *Pseudovibrio denitrificans* (100% identity to the type strain) (Figure 6.2). Like other *P. denitrificans* strains (Enticknap et al. 2006), P12 colonies formed brown mucoid colonies when grown on Marine Agar. The brown coloration was absent when the strain was grown on minimal marine agar, with colonies appearing white. This strain was able to grow in minimal media using DMSP as either a sole carbon or sole sulfur source. The complete utilisation of DMSP from the liquid media after 2 to 3 days of incubation, as well as the presence of its metabolic byproduct dimethylsulfide (DMS), were

confirmed by ^{1}H NMR, however acrylate, the other byproduct of DMSP metabolism, was not observed.

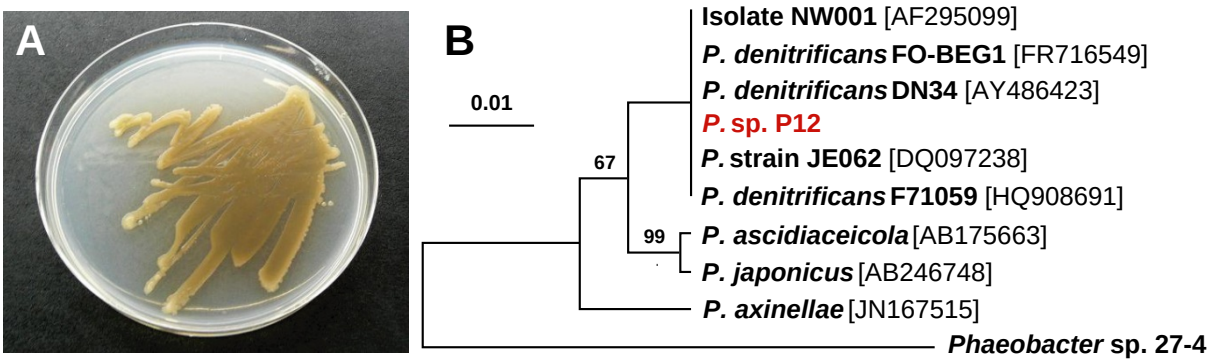

Figure 6.2: (*A*) *Pseudovibrio* sp. P12 colonies on Marine Agar (Difco, BD). (*B*) Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences showing the isolate used in this study (P12 in red) and closely associated *Pseudovibrio* spp. Note: the strain FO-BEG1 has been fully sequenced. *Phaeobacter* sp. 27-4 [AJ536669] was used as outgroup. Maximum parsimony bootstrap values (10,000 replicates) are given when different from 100. The scale bar indicates the number of substitution per nucleotide position.

Among the seven different DMSP degradation pathways currently identified, two gene orthologs, *dddD* and *dmdA*, involved in DMSP metabolism were identified in the genome of *Pseudovibrio denitrificans* strain FO-BEG1, which is closely affiliated to strain P12 (Table 1 and Figure 6.2). The *dddD* gene is involved in a pathway that converts DMSP into DMS without formation of acrylate (Todd et al. 2007), whereas *dmdA* encodes the sulfur assimilation route (Howard et al. 2006). The presence of these two genes supports the ^{1}H NMR measurements with observed production of DMS without acrylate formation following DMSP metabolism (*dddD* pathway); and the ability to use DMSP as sole sulfur source (*dmdA* pathway) (Table 6.1).

Table 6.1: *Pseudovibrio* sp. FO-BEG1 gene orthologs compared to known gene sequences involved in DMSP degradation and TDA biosynthesis.

Gene	Function	Best hit ortholog	E value	Accession number
dmdA	DMSP demethylation	Gammaproteobacterium HTCC2080 [MGP20802]	$2e^{-25}$	PSE_2975
dddD	DMSP cleavage	*Marinomonas* sp. MWYL1 [CP000749]	0	PSE_2912
tdaA	Transcriptional regulator	*Ruegeria* strain TM1040 [EF139200]	$9e^{-51}$	PSE_2264
tdaB	TDA biosynthesis	*Ruegeria* strain TM1040 [EF139201]	$4e^{-58}$	PSE_2263
tdaC	TDA biosynthesis	*Ruegeria* strain TM1040 [EF139202]	$1e^{-58}$	PSE_2261
tdaD	TDA biosynthesis	*Ruegeria* strain TM1040 [EF139203]	$2e^{-61}$	PSE_2260
tdaE	TDA biosynthesis	*Ruegeria* strain TM1040 [EF139204]	$1e^{-152}$	PSE_2259
tdaF	TDA biosynthesis	*Ruegeria* strain TM1040 [EF139205]	$6e^{-72}$	PSE_2247
paaI	Phenylacetate oxygenase	*Ruegeria* strain TM1040 [CP000376]	$1e^{-54}$	PSE_1788
paaJ	Phenylacetate oxygenase	*Ruegeria* strain TM1040 [CP000376]	$1e^{-42}$	PSE_1789
paaK	Phenylacetate oxydoreductase	*Ruegeria* strain TM1040 [CP000376]	$5e^{-66}$	PSE_1790
cysI	Sulfite reductase	*Ruegeria* strain TM1040 [CP000377]	$1e^{-169}$	PSE_1234
malY	Cystathionase	*Ruegeria* strain TM1040 [CP000377]	$1e^{-103}$	PSE_1673

6.3.2.Identification of antimicrobial compounds produced by P12

Well diffusion assays revealed that the crude extract from P12 retained the antimicrobial properties of the strain against both *Vibrio* species. Purification and subsequent chemical analysis of the active fraction using a combination of chromatographic and spectroscopic techniques revealed that the compound responsible for the antimicrobial activity was tropodithietic acid (TDA) (Brinkhoff et al. 2004) (Figure 6.3A). Approximately 2.1 mg (yield=1.7%) was recovered from 1 L of P12 culture and the identity of the molecule was confirmed using 2D NMR and FTMS (*m/z* 210.9534 for $[M-H]^-$). Eleven of the twelve genes involved in TDA production had clear orthologs in the *Pseudovibrio* sp. FO-BEG1 genome, confirming the presence of a genetic basis for the observed production (Table 6.1) (Geng et al. 2008).

The biosynthesis of TDA correlated with production of the brown pigmentation in the culture medium, as previously reported (Brinkhoff et al. 2004, Bruhn et al. 2005, Porsby 2010); however this pigment had no antimicrobial activity. Both pathogens were highly sensitive to TDA, with the pure compound inhibiting their growth at concentrations as low as 0.5 µg/mL (Figure 6.3B). In contrast to

the decrease in antimicrobial activity exhibited by *Pseudovibrio* sp. P12 after incubation at 32°C, TDA activity was not reduced after incubation at this temperature (Appendix E; Table S6.1).

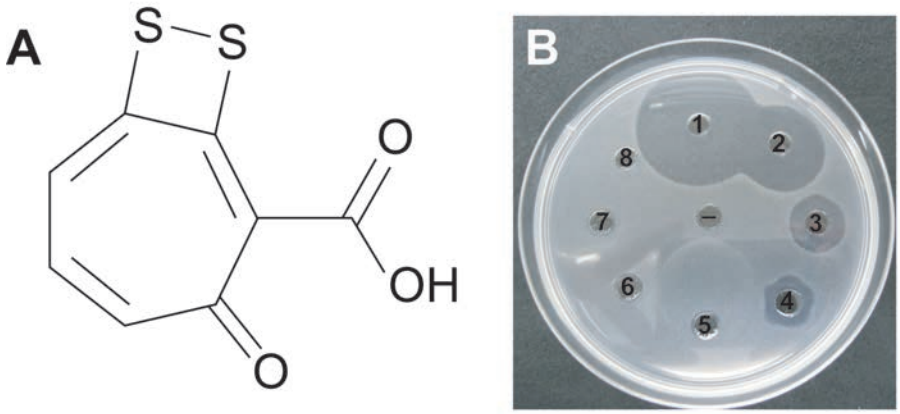

Figure 6.3: (*A*) Molecular structure of tropodithietic acid (TDA) as determined by ^{1}H and ^{13}C NMR spectroscopy. (*B*) Dilution series of pure TDA, showing zones of growth inhibition against *Vibrio coralliilyticus*: [(1): 500 µg/mL, (2): 50 µg/mL, (3): 5 µg/mL, (4): 500 ng/mL, (5): 50 ng/mL, (-) negative control with solvent only].

6.3.3.Investigating the presence of TDA in coral samples

Although extracts of all of the coral species investigated exhibited antimicrobial activity against the two pathogens, the inhibition zones were usually very small (1 mm on average). Only the non acroporid species, *P. cylindrica*, *M. aequituberculata*, *M. turtlensis* and *P. damicornis*, displayed high antimicrobial activity (ranging from 3 to 5 mm in radius). Extracts of these four species were fractionated and their chemical content was explored using both ^{1}H NMR and FTMS, however, I was not able to confirm the presence of TDA in any of the active fractions.

6.4. Discussion

This study represents the first successful isolation and identification of an antimicrobial compound produced by a coral-associated bacterium. Tropodithietic acid (TDA) was isolated from a pure culture of *Pseudovibrio* sp. P12 and inhibited the growth of two coral pathogens at ecologically relevant concentrations. These results indicate that this molecule could potentially be synthesized *in*

vivo by coral-associated bacteria to provide protective antimicrobial activity to the coral host and prevent colonisation by invasive bacterial species.

The strain P12 strongly inhibited the growth of two coral pathogens, *Vibrio coralliilyticus* and *V. owensii*, known to cause white syndromes (a collective term describing rapidly progressing tissue loss, exposing band-like areas of white skeleton) (Ben-Haim et al. 2003, Willis et al. 2004, Sussman et al. 2008, Ushijima et al. 2012). *Vibrio coralliilyticus* exhibits antimicrobial resistance to a wide range of commercial antibiotics and is also resistant to the activities of a large number of coral-associated bacteria (Shnit-Orland and Kushmaro 2009, Rypien et al. 2010, Vizcaino et al. 2010). Its antimicrobial resistance is considerably greater than other marine pathogens such as *V. parahaemolyticus* or *V. vulnificus*, and may contribute to its competitive advantage within the coral holobiont, as well as its ability to infect corals (Vizcaino et al. 2010). However, whilst *V. coralliilyticus* is resistant to many coral-associated bacteria, its growth was strongly repressed by the strain P12, underlining the important antimicrobial activity of this isolate.

The isolate P12 belongs to the bacterial genus *Pseudovibrio*, which has only recently been described (Shieh et al. 2004), although it is ubiquitously found in association with healthy sponges (Webster and Hill 2001, Hentschel et al. 2003, Thiel and Imhoff 2003, Enticknap et al. 2006) and corals (Ritchie 2006, Radjasa et al. 2008, Nissimov et al. 2009, Rypien et al. 2010, Sulistiyani et al. 2010, Vizcaino et al. 2010). *Pseudovibrio* is thought to be involved in symbiotic relationships with various organisms, as supported by evidence that it is vertically transmitted in large densities by adult sponges to their larvae (Enticknap et al. 2006) and its presence is required for the growth of the sulfur-oxidizing bacteria *Beggiatoa* in culture (Schwedt 2011). Furthermore, its genome is organized similarly to that of *Rhizobia*, a well-characterized symbiotic bacterium (Enticknap et al. 2006, Kennedy et al. 2009, Schwedt 2011). The full genome sequences of the *Pseudovibrio* strain closely related to P12 reveal the presence of genes involved in host-cell adhesion, interactions with eukaryotic

cell machinery, and production of secondary metabolites (Schwedt 2011), further suggesting that this bacterium is involved in symbiotic relationships with its hosts.

The *Pseudovibrio* genus is also known for its antimicrobial properties, especially against human pathogens such as *Mycobacterium tuberculosis, Bacillus cereus, Yersinia enterocolitica, Listeria monocytogenes* or methicillin-resistant *Staphylococcus aureus* (Sulistiyani et al. 2010, O'Halloran et al. 2011). To date, two active compounds have been isolated from different *Pseudovibrio* strains: heptylprodigiocin in *P. denitrificans* Z143-1 (Sertan-de Guzman et al. 2007) and tropodithietic acid (TDA) from *P. ascidiaceicola* D323 (Penesyan et al. 2011). In the present study, I isolated TDA from P12, a strain not closely related to *P. ascidiaceicola* D323, but sharing 100% sequence similarity based on the 16S rRNA gene with *P. denitrificans* strain FO-BEG1. The full genome sequence of this strain confirmed the presence of genes essential for TDA biosynthesis (Geng et al. 2008) and corroborated the observed TDA production with its genetic basis. TDA inhibits the growth of a wide range of marine pathogens (Bruhn et al. 2005, Bruhn et al. 2007) and is produced exclusively by bacteria from the *Roseobacter* clade, especially the genera *Phaeobacter, Silicibacter,* and *Ruegeria*, that are commonly associated with microalgae (Brinkhoff et al. 2004, Bruhn et al. 2005, Geng et al. 2008, Geng and Belas 2010, Porsby 2010).

Many members of the *Roseobacter* clade, including coral-associated isolates, have been implicated in sulfur cycling (Moran et al. 2003, Miller et al. 2004, Raina et al. 2010). Interestingly, TDA contains two sulfur atoms ($C_8H_4O_3\underline{S_2}$) and *Pseudovibrio* sp. P12 was able to use DMSP either as sole carbon or sole sulfur source, a common trait among *Alphaproteobacteria* and especially the *Roseobacter* clade (Bruhn et al. 2005, Wagner-Dobler and Biebl 2006). Bacteria from this clade preferentially metabolize DMSP rather than sulphate (SO_4^{2-}), despite the latter being between 10^6 to 10^7-fold more abundant in seawater (Kiene et al. 1999, Geng and Belas 2010). Genomic and spectrometric analyses revealed that DMSP metabolism in P12 (and related strains) occurs either *via* one of the cleavage pathways (encoded by the gene *dddD*) that release the climate-regulating

molecule DMS, or *via* the demethylation pathway (encoded by *dmdA*), by which the bacterium can retain the sulfur contained in DMSP molecules (Figure 6.4).

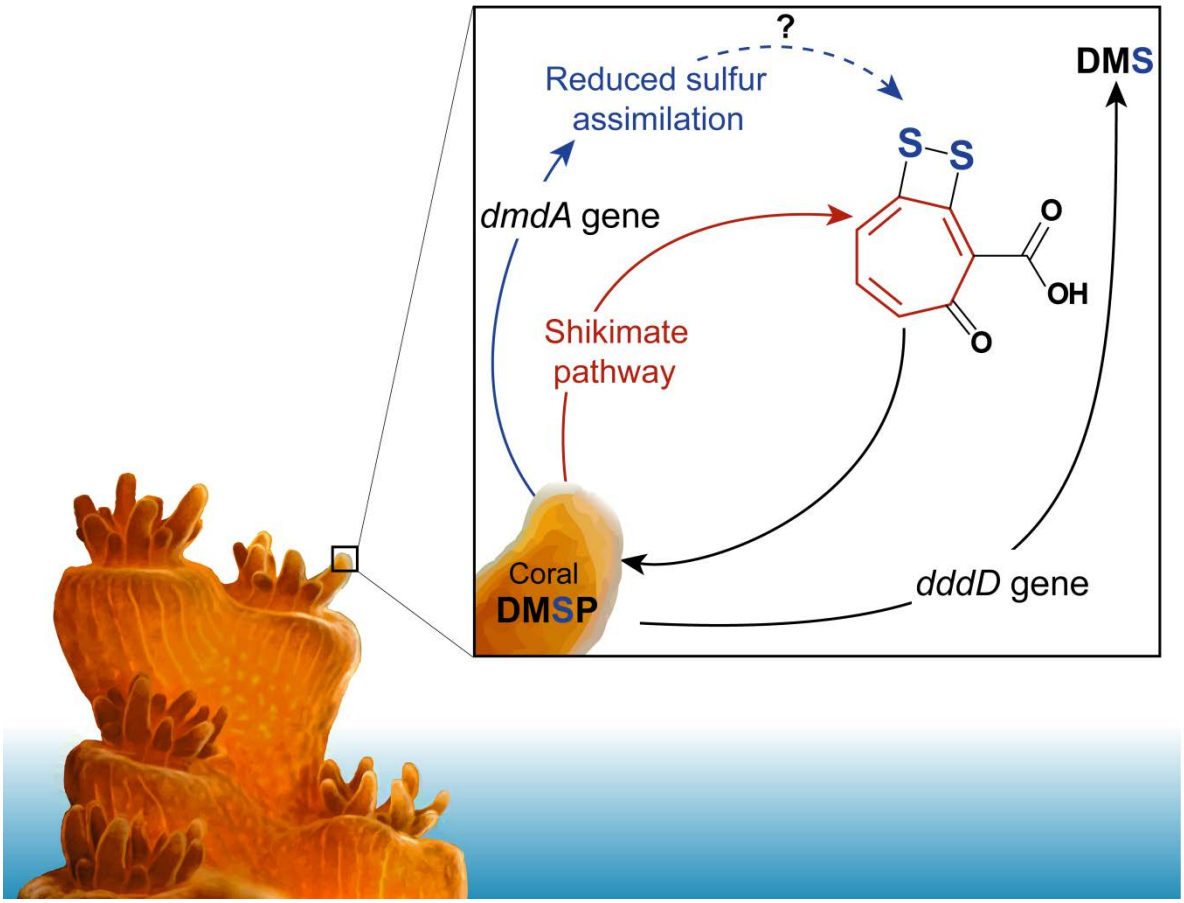

Figure 6.4: Potential pathways for DMSP degradation and TDA synthesis in *Pseudovibrio* sp. P12 aggregations present in corals (in close-up). The DMSP cleavage pathway encoded by *dddD* leads to DMS formation and subsequent loss of sulfur from the system, whereas the demethylation pathway encoded by *dmdA* leads to sulfur incorporation. The organic skeleton of TDA comes from the Shikimate pathway (red), whereas the sulfur atoms might come from DMSP metabolism *via* the demethylation pathway (blue).

The biosynthesis of TDA is not fully understood, but labelling studies have shown that its aromatic skeleton is derived from phenylacetyl-CoA produced by the shikimate pathway (Cane et al. 1992, Thiel et al. 2010). However, the sulfur donor allowing the incorporation of the two sulfur atoms into the TDA molecule is currently unidentified. It has been proposed that sulfur originating from DMSP metabolism might be used to synthesize TDA (Bruhn et al. 2005, Wagner-Dobler and Biebl 2006, Bruhn et al. 2007, Geng and Belas 2010, Porsby 2010). For example, DMSP increases TDA

synthesis two-fold in comparison to other sulfur sources (Geng and Belas 2010), suggesting that DMSP is a preferred source of sulfur for TDA biosynthesis. Even though it is likely that other sources of sulfur might be used to synthesize TDA (since artificial media like Marine Broth used in this study, do not contain DMSP, but rather the sulfur-based amino-acids cysteine and methionine) (Porsby 2010), it is highly likely that in DMSP-rich environments, such as reef-building corals, DMSP metabolism provides the sulfur needed to produce TDA *via* the demethylation pathway.

The activity of P12 against *V. coralliilyticus* sharply decreased at elevated temperatures (32°C), although the activity of TDA itself was not temperature-dependent. This reveals that the loss of antimicrobial activity observed for P12 at 32°C is not due to thermal sensitivity of TDA but to a decrease in its production. Decreased production of TDA during anomalously high sea temperatures could facilitate pathogen outbreaks in corals following thermal stress, especially since the virulence of some disease-causing bacteria (i.e. *V. coralliilyticus*) increase at 32°C (Sussman et al. 2008). Indeed, clear links have been identified between warm thermal anomalies and outbreaks of white syndromes (Bruno et al. 2007, Heron et al. 2010, Maynard et al. 2011), highlighting the likely contribution that TDA production makes to the disease resistance of corals.

I was not able to confirm the presence of TDA in coral sample homogenates derived from 6 coral species (*Montipora aequituberculata, M. turtlensis, Pocillopora damicornis, Porites cylindrica, Acropora millepora* and *A. muricata*) despite extensive efforts in terms of sample preparation, fractionation and testing. This result highlights a few issues that should be taken into consideration in future studies. First, TDA concentrations produced in the environment are predicted to be close to the detection limits of our instruments. In addition, the quantities of coral extracted in this study were minimal (due to the restricted availability of certain species) compared with classic examples from the literature, in which material extracted weighed as much as half a ton for a single species (Petit et al. 1983). Finally, the metabolites present in reef-building coral extracts are numerous and still poorly characterized. The resulting background noise adds significantly to the difficulty of detecting a

compound of interest present at trace level in the extracts. However, this negative result does not imply that TDA is not synthesized *in vivo*. *Roseobacters* are among the first bacteria to colonise the surface of marine microalgae and corals (Dang and Lovell 2000, Miller et al. 2004, Apprill et al. 2009), likely because of their chemotactic behaviour toward algal metabolites such as DMSP (Miller et al. 2004). Since *Roseobacter* species are highly abundant in numerous coral species, TDA biosynthesis in corals is therefore not unrealistic.

This study demonstrates that a common coral-associated bacterium, *Pseudovibrio* sp. P12, produces TDA, a potent antimicrobial compound that inhibits the growth of marine and coral pathogens, including *V. coralliilyticus*. The bacterium can use DMSP as a sole sulfur or carbon source and potentially uses this molecule for the biosynthesis of TDA. The production of TDA is greatly reduced at temperatures causing thermal stress in corals, providing a window of opportunity for the growth of pathogens that could lead to outbreaks of coral disease. These results provide additional evidence for the integral role of DMSP in structuring healthy, coral-associated bacterial communities and strongly suggest that these DMSP-metabolizing communities contribute importantly to the prevention of coral diseases.

Chapter 7: General discussion: Production and fate of DMSP in reef-building corals and its integral role in coral health

Research presented in this thesis significantly improves current understanding of sulfur cycling in coral reef ecosystems, in particular by determining the importance of sulfur compounds for coral-associated bacteria and conclusively establishing the functional roles of DMSP-metabolising bacteria, some of the most abundant bacterial taxa present in corals. In this concluding chapter, I synthesize results presented in this thesis to develop a broader view of coral-microbe interactions, and highlight the implications of these findings for both biologists and coral reef managers. I conclude by identifying future research directions that would further advance our understanding of coral microbiology and further emphasize the importance of bacterial symbioses in maintaining the health and fitness of reef-building corals.

7.1. From sulfate assimilation to DMSP production in reef-building corals

Despite the importance of DMSP in the marine sulfur cycle, its biosynthesis is still poorly understood (Stefels 2000). According to the current paradigm, DMSP is produced exclusively by photosynthetic organisms, especially marine phytoplanktonic taxa (Keller et al. 1999, Scarratt et al. 2002), and theoretical evidence suggests it arises from the assimilation of sulfate from seawater (Stefels 2000). However, much of our understanding of the processes involved in DMSP biosynthesis, from the uptake of dissolved sulfate to the incorporation of sulfur into DMSP, is derived from studies of higher plants (Stefels 2000). Results presented in Chapter 5, which documented uptake of isotopically labelled sulfate by coral-associated dinoflagellates in the genus *Symbiodinium* and subsequent integration of the sulfur tracer into DMSP molecules, confirm that the identity of the sulfur source used for DMSP biosynthesis in marine algae is indeed sulfate.

DMSP production in marine algae is believed to be enhanced by light and photosynthetic activity (Karsten et al. 1990, Simo et al. 2002), and in accordance with this belief, DMSP synthesis and primary production in free-living dinoflagellate assemblages are strongly correlated and both peak around noon (Simo et al. 2002). Although reef-building corals produce some of the highest DMSP concentrations recorded in the environment (Broadbent et al. 2002, Broadbent and Jones

2004), baseline data describing factors affecting DMSP synthesis have not been available. Results presented in Chapter 2 provide the first insights into the dynamics of DMSP synthesis in corals and show that during a "typical" day (average temperature and light levels), DMSP concentrations in corals are not influenced by light, remaining constantly high regardless the time of the day. This result was unexpected and raised questions about the role of endosymbiotic dinoflagellates in DMSP biosynthesis, which led me to investigate how DMSP concentrations are affected by temperature and light levels high enough to induce the disruption of the coral-dinoflagellate endosymbiosis.

A thermal stress experiment conducted on adult colonies of the coral *Acropora millepora* revealed an unexpected 68% increase in DMSP concentration in colonies subjected to increased seawater temperatures (32°C) (Chapter 3). Significantly, increases in DMSP occurred despite thermal stress causing major losses (i.e. bleaching) and degradation of DMSP-producing endosymbionts. To investigate further these confounding results, I performed experiments on early life-stages of corals devoid of photosynthetic endosymbionts. These additional studies unambiguously demonstrate that high DMSP concentrations are present in juveniles of two coral species (*A.millepora* and *A. tenuis*), and moreover, concentrations increased through time indicating active biosynthesis. This trend was even more pronounced when coral juveniles were subjected to elevated temperatures, suggesting that DMSP might contribute to mechanisms protecting coral tissues during thermal stress (Chapter 3). These multiple lines of evidence strongly indicate that the coral animal direct produces DMSP. This discovery is further supported by bioinformatic mining of two currently sequenced *Acropora* genomes, which confirmed the presence of two DMSP synthesis gene orthologs related to diatom genes recently implicated in DMSP biosynthesis (Lyon et al. 2011). These results overturn the current paradigm for DMSP production and confirm for the first time that the coral animal is a major source of DMSP in the marine environment. This poses further questions as to what other animals might also be sources of this important sulfur-based compound, a promising area for future research.

The direct production of DMSP by the coral host itself indicates that this compound plays an important ecological role in the coral holobiont. DMSP is a potent antioxidant (Sunda et al. 2002) and

could be an integral part of the coral tool-box to protect tissues from oxidative stress. This hypothesis would explain the strong increase in DMSP concentrations when both adults and juveniles were exposed to thermal stress (Chapter 3), as temperature stress is known to contribute to oxygen radical formation in both *Symbiodinium* cells and coral mitochondria. DMSP is also an important signal molecule in the marine environment (DeBose et al. 2008, Seymour et al. 2010) and many bacterial taxa are very strongly attracted to DMSP at concentrations similar to those present in *Acropora*. The production of DMSP by coral life stages still devoid of *Symbiodinium* might promote the early recruitment of important bacterial taxa, such as *Roseobacter* sp., suspected to play a central role in coral health (Miller et al. 2004, Apprill et al. 2009). Given the wide diversity of organisms using DMSP as a cue (ranging from bacteria, to fishes and birds (Nevitt and Haberman 2003, Cunningham et al. 2008, DeBose et al. 2008)), it also suggests that this molecule might be used to recruit other important members of the coral holobiont, further underlining the ecological advantage of DMSP biosynthesis by the coral animal. An important area for further research would be to investigate the role of DMSP in attracting and establishing microbial communities associated with corals.

Studies presented in this thesis provide important and novel information about the origin of the very high DMSP concentrations in corals, although many important questions about the biosynthesis of DMSP remain to be addressed. Future research relating to DMSP production in corals should determine if DMSP biosynthesis is common across reef-building corals or restricted to the genus *Acropora*. Comparisons of DMSP concentration across coral genera revealed striking heterogeneity (Chapter 2; (Van Alstyne et al. 2006, Van Alstyne and Puglisi 2007)), with *Acropora* consistently exhibiting higher levels than other taxa. Low levels of DMSP in some coral genera might indicate that they produce DMSP exclusively through their endosymbiotic algae *Symbiodinium*. A clear understanding of the full diversity of DMSP producers is needed to accurately model and estimate the contributions that corals make to the large DMSP-pool produced by coral reef ecosystems. Furthermore, the pathway(s) by which the coral hosts acquire the sulfur needed for DMSP biosynthesis remain to be elucidated; the assimilation of sulfate and its conversion to sulfide is

only carried out by microbes, fungi and photosynthetic organisms (Kredich 1996, Setya et al. 1996, Droux 2004, Wirtz and Droux 2005). Animals have to acquire sulfur-containing amino acids (cysteine and methionine) through their diet or potentially through endosymbioses with microbes or microalgae (Kredich 1996, Droux 2004). Previous studies have shown that corals lack an essential enzyme for cysteine biosynthesis (Shinzato et al. 2011). However they can produce methionine, the direct precursor of DMSP, in the absence of *Symbiodinium* (Fitzgerald and Szmant 1997), but neither the pathway involved nor the sulfur source have been elucidated. Since acroporid corals can produce both methionine and DMSP in the total absence of photosynthetic organisms (Chapter 3), it is likely that coral-associated microbes play a role in sulfate assimilation and its conversion into the amino acid cysteine. Consequently, the full sulfur cycle, from the uptake of sulfate to DMSP production, should receive more attention in corals, especially the role that coral-associated microbes might play in the synthesis of the essential amino acids cysteine and methionine.

7.2. The importance of DMSP for coral-associated bacteria

Bacteria play crucial roles in most of the biogeochemical cycles in the oceans because of their high abundance and metabolic capabilities (Azam and Malfatti 2007). Although they are 10 times more abundant in corals than in the surrounding sea water (Koren and Rosenberg 2006, Garren and Azam 2010), their functional roles in coral reefs remain largely unknown. Results from my thesis unambiguously implicate coral-associated bacteria in the sulfur cycle and demonstrate that the sulfur compounds DMSP and DMS support the growth of abundant bacterial taxa (Chapter 4 and 5). The isolation of coral-associated bacteria growing on DMSP and DMS as sole carbon source (Chapter 4), combined with confirmation of their capability to metabolise DMSP produced by *Symbiodinium* using state-of-the-art imaging techniques (Chapter 5), suggest that bacteria are integral components of DMSP metabolism in corals and that inter-kingdom linkages between microbial members of the coral holobiont, specifically between *Symbiodinium* endosymbionts and bacterial assemblages, are involved. This two-pronged approach, i.e. combining the isolation of methyl-sulfur degrading bacteria *in vitro* with the confirmation of their metabolic potential *in vivo*, confirms the functional role of these

critical members of the coral holobiont and emphasizes the importance of DMSP and DMS in structuring coral-associated bacterial communities.

Although DMSP/DMS degrading bacteria dominated microbial communities in *Acropora millepora* (comprising 67 to 85% of the bacteria present), they were less abundant in *Montipora aequituberculata* (37% of the bacterial assemblages) (Chapter 4). This observation might be due to the low bacterial diversity present in *Acropora*, with DMSP-degrading bacteria from the genus *Spongiobacter* (*Endozoicomonas*) typically dominate communities (Bourne et al. 2008, Littman et al. 2009). By contrast, the bacterial communities present in other coral species are often more diverse (Rohwer et al. 2002, Bourne and Munn 2005, Wegley et al. 2007). The difference between *Acropora* spp. and other coral genera at the bacterial level correlates well with marked divergences in the metabolites they produce (see Chapter 2, Figure 2.3). Indeed, *Acropora* spp. contain only a few compounds in large quantities (mainly DMSP, DMS, acrylate and lipids), whereas the metabolomes of other species are much more complex, with high concentrations of DMSP and DMS but also a plethora of undescribed molecules (Chapter 2). This could explain the large abundance of DMSP-degrading bacteria in *Acropora* but also clearly indicates that other compounds influence the taxonomic composition of the bacterial assemblages in non-acroporid corals. Future studies aimed at identifying the metabolites produced by other major reef-building coral genera will provide valuable information on the chemical landscapes that bacteria live within, enhancing our knowledge of the functional roles that bacteria associated with corals might perform.

Comprehensive understanding of the functional roles of bacteria within the coral holobiont depends on the capacity to isolate and cultivate coral-associated bacteria, but until recently, the vast majority of microbes from the environment were thought to be unculturable (Kirchman 2000, Rappe and Giovannoni 2003). In particular, artificial conditions intrinsic to culture media have been major impediments, for example the absence of specific compounds required for growth or extremely high substrate concentrations that enable fast-growing taxa to outcompete bacteria of interest (Zengler et

al. 2002). New high-throughput culturing techniques are now available, circumventing the flaws of traditional methods and enabling isolation of bacterial taxa previously defined as “unculturable” (Rappe et al. 2002, Zengler et al. 2002, Stingl et al. 2007). These new culturing techniques have not yet been applied to corals, but constitute a very promising tool to elucidate the full metabolic potential of the most abundant bacteria associated with corals, especially their potential role in vitamin and essential amino-acid syntheses, nitrogen fixation, and their involvement in the coral host digestive and immune systems.

7.3. The fate of DMSP in coral reef systems

Marine bacteria use at least two different routes to metabolise DMSP molecules. First, the sulfur and carbon content of DMSP can be used to sustain bacterial growth (Howard et al. 2006). Indeed, many marine bacteria appear to convert DMSP into methanethiol in order to assimilate the reduced sulfur (Reisch et al. 2011). In this thesis, a sulfur-based antimicrobial compound called tropodithietic acid (TDA) was isolated from a DMSP-degrading bacterium from the genus *Pseudovibrio* (Chapter 6), suggesting that sulfur from the DMSP molecule can be used to produce secondary metabolites capable of preventing the growth of coral pathogens. This result constitutes the first identification of an antimicrobial molecule produced by coral-associated bacteria and suggests that some DMSP-degrading bacteria play a critical role in the coral-host defence against pathogens. The production of TDA is mainly restricted to *Alphaproteobacteria* from the *Roseobacter* clade (Bruhn et al. 2005, Bruhn et al. 2007), which are well known for their DMSP-degrading capabilities and for their ubiquitous association with reef-building corals (Rohwer et al. 2001, Rohwer et al. 2002, Bourne and Munn 2005, Koren and Rosenberg 2006, Littman et al. 2009, Raina et al. 2009, Ceh et al. 2012, Bourne et al. 2013). Members of the *Roseobacter* clade represented nearly a third of the bacterial communities harboured by *Montipora aequituberculata* (Chapter 4) and similar abundances have been reported in other corals (Littman et al. 2009, Ceh et al. 2011). The widespread distribution of *Roseobacter* among coral species coupled with their high abundance indicate that TDA is likely produced in corals and might be used across multiple genera to prevent the growth of pathogens

(Chapter 5). Furthermore, the observed decrease of TDA production in *Pseudovibrio* at 32°C (Chapter 5) illustrates how pathogens can potentially overtake coral defences after thermal stress events (Miller et al. 2009).

The second way marine bacteria can metabolise DMSP is through a cleavage pathway ultimately releasing the gas DMS into the atmosphere (Todd et al. 2007, Curson et al. 2008, Todd et al. 2009, Todd et al. 2010, Curson et al. 2011, Todd et al. 2011). This metabolic route can also be found in coral-associated bacteria (Chapters 4 and 6) and explains the large quantities of DMS released from coral reefs (Broadbent and Jones 2004, Deschaseaux et al. 2012). Therefore coral associated-bacteria are involved in a crucial step linking the ocean and the atmosphere in the global sulfur cycle, and ultimately, in climate regulation processes through cloud formation. However, under predicted ocean warming scenarios (Harvell et al. 2002, Hansen et al. 2006), it is likely that the integral role that coral reefs play in ameliorating local climate throughout the Indo-Pacific region (see Chapter 3) will be dramatically decreased (Deschaseaux et al. 2012, Fischer and Jones 2012). DMS emissions from coral reefs decline sharply during thermal stress (Fischer and Jones 2012), possibly because of concomitant shifts in coral-associated bacteria and the loss of functionally important taxa (Bourne et al. 2008). These biogeochemical processes will be greatly affected by the projected global decline of corals over the coming century (Hoegh-Guldberg et al. 2007), highlighting another important reason why coral reef managers are critical to efforts aimed at mitigating future climate predictions.

7.4 A model for sulfur cyling in the coral holobiont

Results presented in Chapters 2-6 fill many gaps in current knowledge of sulfur cycling within the coral holobiont, as synthesised in the model presented in Figure 7.1. A multidisciplinary approach using a combination of metagenomic, metabolomic, and advanced imaging techniques, coupled with traditional microbiology and analytical chemistry techniques tracked the journey of a sulfur atom through the coral holobiont, from its uptake in the form of sulfate to the production of

DMSP and its subsequent release as antimicrobial or climate-regulating molecules (Figure 7.1). Together, these results constitute the first comprehensive study of DMSP in reef-building corals and underscore the remarkable contribution of this molecule in many aspects of coral health.

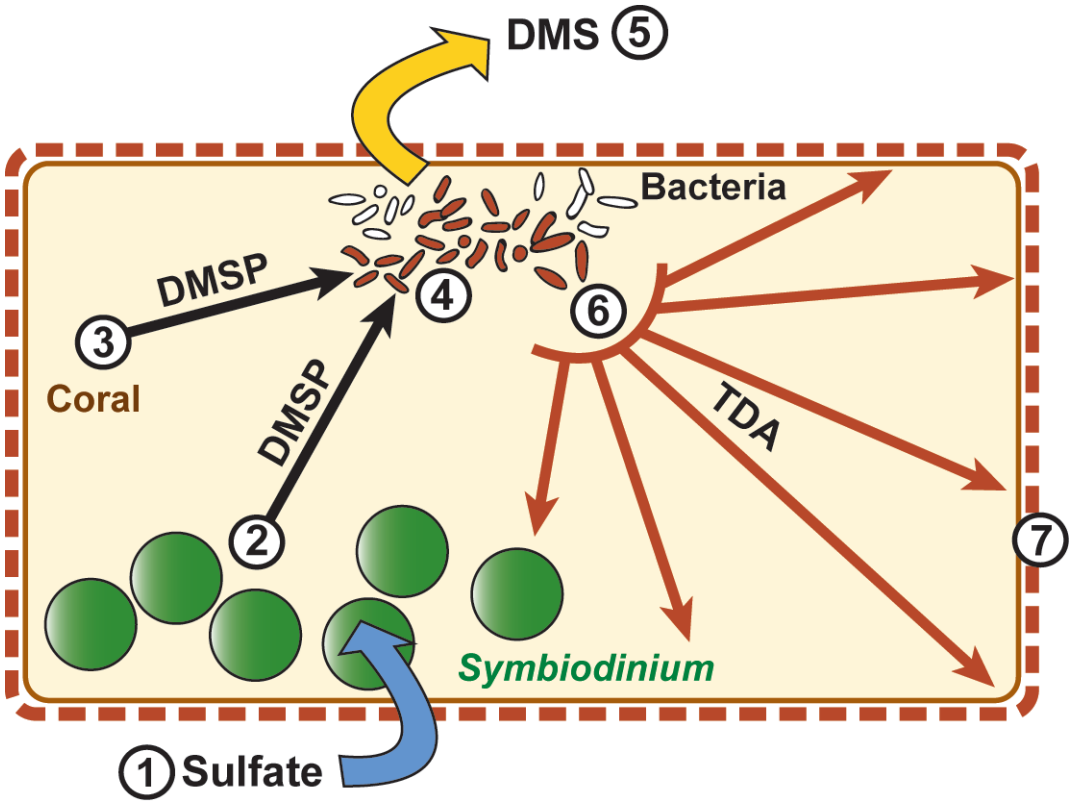

Figure 7.1: Schematic representation of interactions among the coral animal (tan rectangle), *Symbiodinium* cells (green circles) and associated bacteria (small multi-coloured rods) during sulfur cycling within the coral holobiont. The schematic synthesizes seven steps in sulfur cycling that summarise the main findings of my PhD research, i.e. 1) Sulfate is taken up by *Symbiodinium* cells, resulting ultimately in 2) the production of DMSP by these *Symbiodinium* cells. 3) Corals also produce DMSP, and the pool produced by the holobiont is then 4) metabolized by a large fraction of the associated bacterial communities. These bacteria can degrade DMSP to produce either 5) the climate regulating gas DMS, or 6) an antimicrobial compound called tropodithietic acid (TDA), which *Alphaproteobacteria* from the *Roseobacter* clade produce by incorporating sulfur from DMSP. Finally, 7) TDA produced by *Alphaproteobacteria* within the coral holobiont protects the coral host against potential pathogens.

7.5. Concluding remarks

Knowledge derived from my PhD study provides new insights into the sulfur cycle on coral reefs and identifies key coral-associated bacteria integral to this cycle. This research ultimately provides a stepping stone to gain a broader understanding of the role of microbes in coral reefs. Investigating the functional roles of bacteria in corals is still in its infancy but this field has the potential to progress rapidly and provide important scientific knowledge which will greatly enhance our understanding of symbiotic relationships involving multi-partite microbial associations. Understanding the complex microbial symbioses guaranteeing the health of reef-building corals and the external factors disrupting them is a prerequisite to elaborate new management strategies and enable the persistence of the most productive and biologically diverse marine ecosystem on the planet.

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Appendix A

Supporting tables for Chapter 1

Table S1.1: Bacterial taxa previously implicated in the degradation of the sulfur compound dimethylsulfoniopropionate (DMSP), and overlap with the bacteria found in corals (in bold).

Genus	Class	DMSP degradation	References	
			For DMSP degradation	For presence in corals
Acetobacterium	*α-Proteobacteria*	Degradation shown	(Jansen and Hansen 2001)	
Achromobacter	*β-Proteobacteria*	DMS production	(Miller et al. 1973)	(Bourne and Munn 2005, Bourne et al. 2008, Littman et al. 2009)
Alcaligene	*β-Proteobacteria*	Degradation shown	(DeSouza and Yoch 1995)	(Bourne and Munn 2005, Kooperman et al. 2007)
Alteromonas	*γ-Proteobacteria*	Degradation shown	(Raina et al. 2009)	(Frias-Lopez et al. 2002, Bourne and Munn 2005, Ritchie 2006, Kooperman et al. 2007, Raina et al. 2009)
Antarctobacter	*α-Proteobacteria*	Present in clone libraries from DMPS enrichments	(Zubkov et al. 2002)	(Wegley et al. 2007, Raina et al. 2009)
Arhodomonas	*γ-Proteobacteria*	Degradation shown	(Raina et al. 2009)	(Raina et al. 2009)
Burkholderia	*β-Proteobacteria*	*dddD*	(Todd et al. 2007)	(Wegley et al. 2007)
Clostridium	*Firmicutes*	Degradation shown	(Wagner and Stadtman 1962)	(Rohwer et al. 2001, Frias-Lopez et al. 2002, Rohwer et al. 2002)
Cytophaga	*Bacteroidetes*	FISH, present in clone libraries from DMPS enrichments	(Zubkov et al. 2002, Malmstrom et al. 2004)	(Frias-Lopez et al. 2002, Kooperman et al. 2007)
Desulfobacterium	*δ-Proteobacteria*	Degradation shown	(van der Maarel et al. 1993)	(Wegley et al. 2007)
Desulfovibrio	*δ-Proteobacteria*	Degradation shown	(van der Maarel et al. 1996)	(Klaus et al. 2007)
Dinoroseobacter	*α-Proteobacteria*	*dddL*, *dmdA*	(Curson et al. 2008, Howard et al. 2008)	
Eubacterium	*Firmicutes*	Degradation shown	(Jansen and Hansen 2001)	(Rohwer et al. 2001)
Flavobacterium	*Firmicutes*	FISH	(Zubkov et al. 2002)	(Kooperman et al. 2007, Littman et al. 2009)
Fulvimarina	*α-Proteobacteria*	*dddL*	(Curson et al. 2008)	

Hoeflea	*α-Proteobacteria*	*dddD*	(Howard et al. 2008)	
Halomonas	*γ-Proteobacteria*	Degradation shown	(Johnston et al. 2008)	(Beleneva et al. 2005, Cervino et al. 2008, Raina et al. 2009)
Idiomarina	*γ-Proteobacteria*	Degradation shown	(Raina et al. 2009)	(Littman et al. 2009, Raina et al. 2009)
Jannaschia	*α-Proteobacteria*	*dmdA*, *dddP*	(Howard et al. 2006)	(Kooperman et al. 2007)
Loktanella	*α-Proteobacteria*	*dddL*	(Curson et al. 2008)	(Littman et al. 2009)
Marinobacter	*γ-Proteobacteria*	*dddD*	(Howard et al. 2008)	(Bourne et al. 2008, Littman et al. 2009)
Marinomonas	*γ-Proteobacteria*	Degradation shown, *dddD*	(Ansede et al. 2001, Todd et al. 2007)	
Oceanibulbus	*α-Proteobacteria*	*dmdA*	(Howard et al. 2008)	
Oceanicola	*α-Proteobacteria*	*dddL*, *dddP*	(Curson et al. 2008)	(Littman et al. 2009)
Pelagibacter	*α-Proteobacteria*	*dmdA*	(Howard et al. 2006, Tripp et al. 2008)	
Phaeobacter	*α-Proteobacteria*	*dddP*	(Todd et al. 2009)	
Pseudomonas	*γ-Proteobacteria*	Degradation shown	(Ledyard et al. 1993)	(Rohwer et al. 2001, Frias-Lopez et al. 2002, Bourne and Munn 2005, Koren and Rosenberg 2006, Kooperman et al. 2007, Raina et al. 2009)
Pseudoalteromonas	*γ-Proteobacteria*	Present in clone libraries from DMPS enrichments	(González et al. 2000)	(Rohwer et al. 2002, Koren and Rosenberg 2006, Kooperman et al. 2007, Raina et al. 2009)
Psychrobacter	*γ-Proteobacteria*	Degradation shown	(Ansede et al. 2001)	(Cervino et al. 2008)
Rhizobium	*β-Proteobacteria*	*dddD*	(Todd et al. 2007)	(Kooperman et al. 2007)
Rhodobacter	*α-Proteobacteria*	*dddL*	(Curson et al. 2008)	(Rohwer et al. 2001, Kooperman et al. 2007)
Roseobacter	*α-Proteobacteria*	Degradation shown, FISH, *dddP*	(Ledyard et al. 1993)	(Bourne and Munn 2005, Koren and Rosenberg 2006, Littman et al. 2009, Raina et al. 2009)
Roseovarius	*α-Proteobacteria*	Degradation shown, *dmdA*, *dddP*	(Gonzalez et al. 2003, Howard et al. 2006, Todd et al. 2009)	(Littman et al. 2009, Raina et al. 2009)
Ruegeria	*α-Proteobacteria*	Degradation shown, *dmdA*	(Gonzalez et al. 2003, Howard et al. 2006)	(Lampert et al. 2006)
Sagittula	*α-Proteobacteria*	*dddD*	(Todd et al. 2007)	(Kooperman et al. 2007)

Shewanella	*γ-Proteobacteria*	Degradation shown	(Ledyard et al. 1993)	(Frias-Lopez et al. 2002, Bourne and Munn 2005, Kooperman et al. 2007)
Shigella	*γ-Proteobacteria*	Degradation shown	(Warren et al. 2007)	(Littman et al. 2009)
Sinorhizobium	*α-Proteobacteria*	*dddD*	(Todd et al. 2007)	
Spongiobacter	*γ-Proteobacteria*	Degradation shown	(Raina et al. 2009)	(Bourne et al. 2008, Littman et al. 2009, Raina et al. 2009)
Sporomusa	*Firmicutes*	Degradation shown	(Jansen and Hansen 2001)	
Stappia	*α-Proteobacteria*	*dddL*	(Curson et al. 2008)	
Sulfitobacter	*α-Proteobacteria*	Degradation shown, *dddL*	(Curson et al. 2008)	(Wegley et al. 2007)
Synechococcus	*Cyanobacteria*	Degradation shown	(Malmstrom et al. 2005)	(Rohwer et al. 2002, Bourne and Munn 2005)
Trichodesmium	*Cyanobacteria*	Degradation shown	(Taylor and Gilchrist 1991)	
Thiocapsa	*γ-Proteobacteria*	Degradation shown	(Jonkers et al. 1998)	
Vibrio	*γ-Proteobacteria*	Degradation shown	(Ansede et al. 2001)	(Bourne and Munn 2005, Bourne et al. 2008, Raina et al. 2009)

Table S1.2: Bacterial taxa previously implicated in the degradation of the sulfur compound dimethylsulfide (DMS), and overlap with the bacteria found in corals (in bold).

			References	
Genus	**Class**	**DMS degradation**	**For DMS degradation**	**For presence in corals**
Acinetobacter	*γ-Proteobacteria*	Degradation shown, DMS oxidation	(Horinouchi et al. 1997)	(Rohwer et al. 2001, Rohwer et al. 2002, Bourne and Munn 2005, Wegley et al. 2007, Littman et al. 2009)
Alcaligenes	*β-Proteobacteria*	Degradation shown	(Horinouchi et al. 1997)	(Rohwer et al. 2001, Bourne and Munn 2005, Kooperman et al. 2007)
Alteromonas	*γ-Proteobacteria*	Degradation shown	(Raina et al. 2009)	(Bourne and Munn 2005, Lampert et al. 2006, Raina et al. 2009)

Comamonas	*β-Proteobacteria*	DMS-oxidizing potential	(Horinouchi et al. 1999)	(Rohwer et al. 2001, Littman et al. 2009)
Glaciecola	*γ-Proteobacteria*	Degradation shown	(Schafer 2007)	
Halomonas	*γ-Proteobacteria*	Degradation shown	(Hirano et al. 2003)	(Beleneva et al. 2005, Ritchie 2006, Cervino et al. 2008)
Hyphomicrobium	*γ-Proteobacteria*	Degradation shown	(De Bont et al. 1981)	
Idiomarina	*γ-Proteobacteria*	Degradation shown	(Raina et al. 2009)	(Littman et al. 2009, Raina et al. 2009)
Marinobacterium	*γ-Proteobacteria*	Degradation shown	(Fuse et al. 2000)	
Methylophaga	*γ-Proteobacteria*	Degradation shown	(Zwart et al. 1996, Schafer 2007)	(Rohwer et al. 2002)
Photobacterium	*γ-Proteobacteria*	Degradation shown	(Raina et al. 2009)	(Koren and Rosenberg 2006, Raina et al. 2009)
Pseudoalteromonas	*γ-Proteobacteria*	Degradation shown	(Hirano et al. 2003)	(Raina et al. 2009)
Pseudomonas	*γ-Proteobacteria*	Degradation shown	(Zhang et al. 1991)	(Rohwer et al. 2001, Rohwer et al. 2002, Beleneva et al. 2005, Koren and Rosenberg 2006, Kooperman et al. 2007)
Rhodobacter	*α-Proteobacteria*	DMS as electron donor	(Hanlon et al. 1994)	(Rohwer et al. 2001, Kooperman et al. 2007)
Rhodococcus	*Actinobacteria*	Degradation shown	(Omori et al. 1995)	
Rhodovulum	*α-Proteobacteria*	Degradation shown	(Vogt and Fischer 1998)	
Roseobacter	*α-Proteobacteria*	Degradation shown	(Gonzalez et al. 1999)	(Rohwer et al. 2002, Bourne and Munn 2005, Koren and Rosenberg 2006, Raina et al. 2009)
Thiobacillus	*β-Proteobacteria*	Degradation shown	(Sivela 1980)	
Thiocapsa	*γ-Proteobacteria*	Degradation shown	(Visscher and Van Gemerden 1991)	
Thiocystis	*γ-Proteobacteria*	Degradation shown	(Zeyer et al. 1987)	(Wegley et al. 2007)
Vibrio	*γ-Proteobacteria*	Degradation shown	(Raina et al. 2009)	(Bourne and Munn 2005, Bourne et al. 2008, Raina et al. 2009)

Appendix B

Supporting tables for Chapter 2

Table S2.1: Percent of recovery of a spike of DMSP in coral extracts (14 µL of a 50 mM DMSP solution into a 700 µL extract, resulting in a theoretic addition of 1 mM of DMSP). Integration of diagnostic ^{1}H NMR signals for DMSP (2.98 – 2.96) in *Acropora millepora* CH_3OD/D_2O extracts. Spectra were all acquired using the following conditions described in the quantitative NMR method section.

Sample	Original concentrations (mM)	Spike (+1 mM)	Recovery (%)
1	3.065	4.014	94.9
2	3.055	4.049	99.4
3	3.073	4.044	97.1

Table S2.2: Stability study. Integration of diagnostic ^{1}H NMR signals for DMSP (3.50 – 3.40 and 2.95 – 2.92), DMS (2.10 – 2.07) and DMSO (2.683 – 2.674) in *Acropora millepora* CD_3OD extract from T_0 = 0.12 hrs to T_4 = 48 hrs. Spectra were all acquired using the following conditions: ns = 16, d1 = 1, rg = 9, p1 = 8.40. All spectra were referenced to residual CD_3OD (3.31 ppm), and baseline corrected using a polynomial degree = 5.

Time (hours)	Treatment (sequential)	Integration of diagnostic signals			
		DMSP CH_2 (2H)	DMSP CH_3 (6H)	DMS CH_3 (6H)	DMSO CH_3 (6H)
0	Initial extraction	2.51	7.41	4.73	0.38
24	Stored -20°C overnight	2.52	7.57	4.35	0.44
26	Exposed to light at 27°C	2.47	7.39	2.65	1.2
30	Exposed to light at 27°C	2.49	7.45	0.58	2.81
48	Exposed to light at 27°C	2.48	7.38	0.39	4.37

Appendix C

Supporting figures and tables for Chapter 3

Table S3.1: Sums of square (SS), mean squares (MS) and significance levels for ANOVAs of the thermal stress experiment on adult *Acropora millepora* corals: (*A*) DMSP concentrations, (*B*) acrylate concentrations, and (*C*) PAM data. Red lines show significant differences ($p<0.05$).

(A)

Effect	SS	df	MS	F	*p*
Intercept	1308.777	1	1308.777	2314.789	0
Temperature	11.056	1	11.056	19.555	0.000215
Time 0	0.0105	1	0.0105	0.018584	0.893047
Time 7	1.9423	1	1.9423	3.437699	0.077184
Time 12	6.1001	1	6.1001	10.79664	0.003374
Time 17	7.2047	1	7.2047	12.75168	0.001707
Error	12.439	22	0.565		
Time	8.787	3	2.929	11.272	0.000005
Time×Temperature	4.201	3	1.4	5.389	0.002226
27°C	1.5627	3	0.5209	2.003462	0.122017
32°C	11.4258	3	3.8086	14.64846	0
Error	17.151	66	0.26		

(B)

Effect	SS	df	MS	F	*p*
Intercept	3531.668	1	3531.668	3456.576	0
Temperature	14.419	1	14.419	14.112	0.00109
Time 0	0.065	1	0.065	0.063601	0.803235
Time 7	3.0065	1	3.0065	2.941781	0.100373
Time 12	8.5848	1	8.5848	8.4	0.008338
Time 17	10.1534	1	10.1534	9.934834	0.004626
Error	22.478	22	1.022		
Time	11.776	3	3.925	7.294	0.000269
Time×Temperature	7.391	3	2.464	4.578	0.005668
27°C	1.23	3	0.41	0.762082	0.519419
32°C	17.938	3	5.979	11.11338	0.000005
Error	35.521	66	0.538		

(C)

Effect	SS	df	MS	F	*p*
Intercept	59.38719	1	59.38719	38632.32	0
Temperature	0.14436	1	0.14436	93.91	0
Time 0	0.0006	1	0.0006	0.38961	0.538929
Time 7	0.00204	1	0.00204	1.324675	0.262124
Time 12	0.02226	1	0.02226	14.45455	0.000977
Time 17	0.29276	1	0.29276	190.1039	0
Error	0.03382	22	0.00154		
Time	0.16891	3	0.0563	103.23	0
Time×Temperature	0.17329	3	0.05776	105.91	0
27°C	0.00054	3	0.00018	0.327273	0.80568
32°C	0.34167	3	0.11389	207.0727	0
Error	0.036	66	0.00055		

Table S3.2: Sums of squares (SS), mean squares (MS) and significance levels for ANOVAs of the *Symbiodinium*-free juvenile experiment: (*A*) DMSP concentrations in *Acropora millepora*, (*B*) DMSP concentrations in *Acropora tenuis*, (*C*) acrylate concentrations in *Acropora millepora*, and (*D*) acrylate concentrations in *Acropora tenuis*. Red lines show significant differences ($p<0.05$).

(A)

Effect	SS	df	MS	F	*p*
Intercept	132.6612	1	132.6612	6959.5150	0.000000
Temperature	0.8075	1	0.8075	42.3610	0.000068
Time 2	0.0788	1	0.0788	4.1343	0.069420
Time 4	0.3015	1	0.3015	15.8185	0.002612
Time 6	0.5277	1	0.5277	27.6862	0.000367
Error	0.1906	10	0.0191		
Time	1.2373	2	0.6186	24.7230	0.000004
Time×Temperature	0.1007	2	0.0504	2.0120	0.159840
27°C	0.3160	2	0.1580	6.3099	0.007500
32°C	1.0220	2	0.5110	20.4073	0.000015
Error	0.5005	20	0.0250		

(B)

Effect	SS	df	MS	F	*p*
Intercept	162.4001	1	162.4001	6250.471	0
Temperature	0.5032	1	0.5032	19.368	0.001333
Time 2	0.08582	1	0.08582	3.30331	0.0991808
Time 4	0.17196	1	0.17196	6.618937	0.02776039
Time 6	0.27149	1	0.27149	10.44996	0.00898069
Error	0.2598	10	0.02598		
Time	1.5042	2	0.7521	19.869	0.000018
Time×Temperature	0.0261	2	0.013	0.344	0.712926
27°C	0.56773	2	0.283865	7.511245	0.00368833
32°C	0.96251	2	0.481255	12.7343088	0.00027113
Error	0.7571	20	0.037792		

(C)

Effect	SS	df	MS	F	*p*
Intercept	230.3004	1	230.3004	1830.6570	0.000000
Temperature	0.3525	1	0.3525	2.8020	0.125088
Time 2	0.0433	1	0.0433	0.3442	0.570427
Time 4	0.1583	1	0.1583	1.2583	0.288179
Time 6	0.7030	1	0.7030	5.5882	0.039685
Error	1.2580	10	0.1258		
Time	0.4889	2	0.2444	5.4270	0.013110
Time×Temperature	0.5521	2	0.2760	6.1290	0.008394
27°C	0.0010	2	0.0005	0.0111	0.988964
32°C	1.0400	2	0.5200	11.5479	0.000463
Error	0.9008	20	0.0450		

(D)

Effect	SS	df	MS	F	*p*
Intercept	903.6474	1	903.6474	589.2514	0
Temperature	94.6001	1	94.6001	61.6869	0.000014
Time 2	0.67816	1	0.67816	0.44221577	0.5210954
Time 4	1.82047	1	1.82047	1.1870953	0.30147489
Time 6	3.02883	1	3.02883	1.97504483	0.19021126
Error	15.3355	10	1.53355		
Time	2.8944	2	1.4472	1.118	0.34653
Time×Temperature	5.6668	2	2.8334	2.1888	0.13816
27°C	0.0105	2	0.00525	0.00405567	0.99595336
32°C	0.67052	2	0.33526	0.25899103	0.77437888
Error	25.8897	20	1.294485		

Table S3.3: Primer pairs used to target the genomic DNA of various microorganisms possibly responsible for DMSP production in order to assess their presence in coral juveniles. Mitochondrial DNA (mtDNA), chloroplast DNA (cpDNA), internal transcribed spacer (ITS), base pairs (bp).

Target organisms	Target region	Primer name	Amplicon size	Annealing temperature (°C)	Number of cycles	Reference	Amplification
Coral	mtDNA	RNS2/GR	700 bp	54	30	Suzuki et al. (2008)	Yes
Symbiodinium	ITS1	ITSFP/ITSRP	350 bp	59	30	Van Oppen et al. (2004)	No
Algae	23S rDNA plastid	p23SrVf1/Vr1	410 bp	Touchdown PCR (from 66 to 58)	35	Sherwood et al. (2008)	No
Photosynthetic organisms	cpDNA	a/b	Variable	55	35	Taberlet et al. (1991)	No
Photosynthetic organisms	cpDNA	c/d	Variable	55	35	Taberlet et al. (1991)	No
Photosynthetic organisms	cpDNA	e/f	Variable	55	35	Taberlet et al. (1991)	No

Table S3.4: Description of the reductase and methyltransferase sequences in diatoms and corals. Database for *A. digitifera* genome: http://marinegenomics.oist.jp/genomes/download?project_id=3 annotation version 1.0; for *A. millepora* transcriptome: NCBI Transcriptome Shotgun Assembly (TSA) (Moya et al. 2012); for Symbiodinium transcriptome (Bayer et al. 2012).

	Reductase	Methyltransferase
Fragilariopsis cylindrus	jgi|Fracy1|173405|estExt_Genewise1.C_220021	jgi|Fracy1|207357|estExt_Genewise1Plus.C_41074
Acropora digitifera	adi_v1.10730	adi_v1.21031
Acropora millepora	Cluster027405	Cluster022229
Symbiodinium (clade A, strain k8)	kb8_c41244	kb8_rep_c2522
Pfam domain	FMN_red (PF03358.8)	Methyltransf_7 (PF03492.8)
OrthoMCL cluster	OG5_131390	OG5_156314

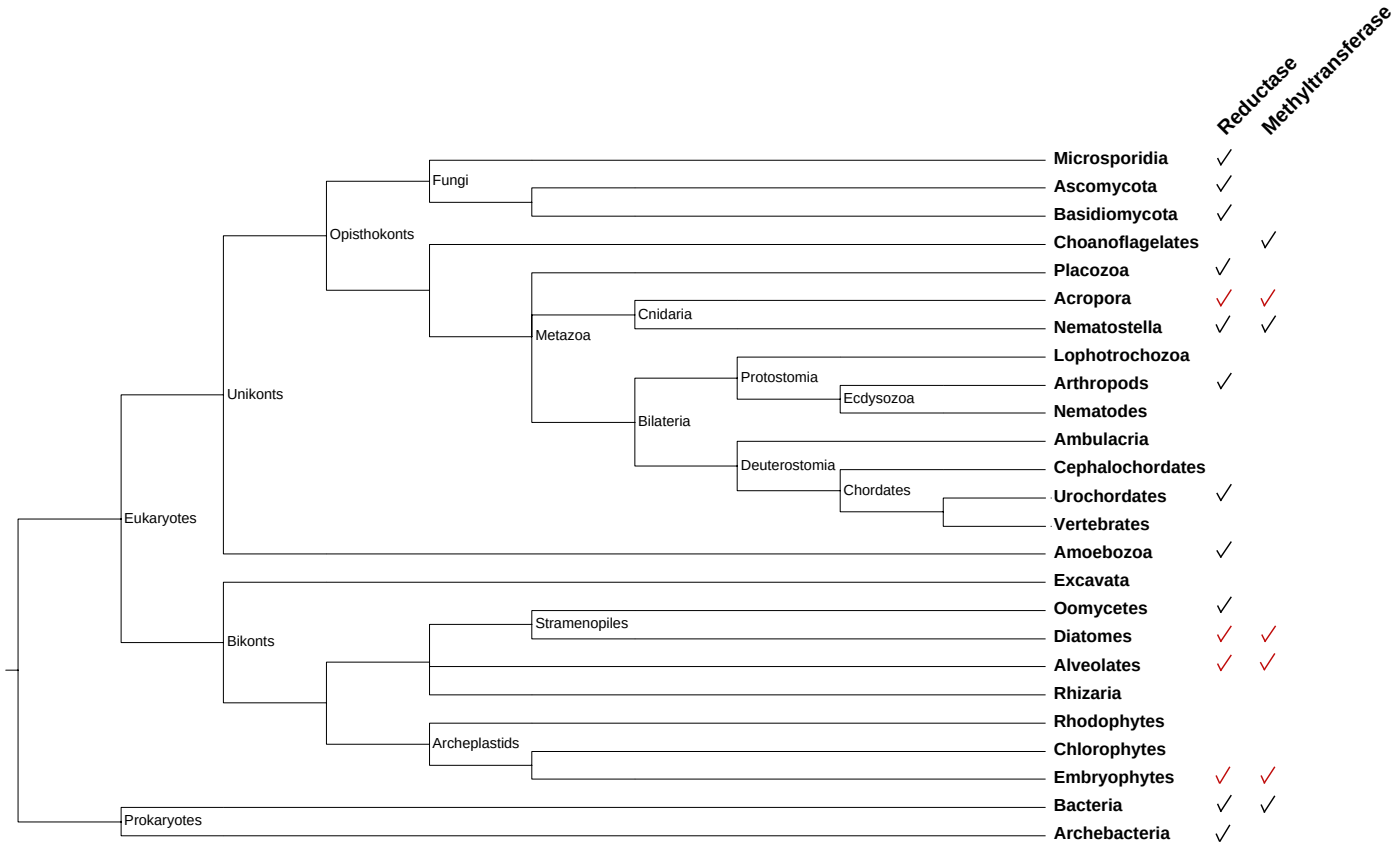

Figure S3.1: Phylogenetic distribution of the reductase and methyltransferase orthologs (OrthoMCL groups OG5_131390 and OG5_156314 respectively). Note the unusually sparse distribution of OG5_156314. In red: co-occurrence of these two enzymes happens predominantly in DMSP producing organisms. The only species of bacteria in the OrthoMCL database where these two enzymes occur simultaneously is the marine cyanobacterium *Synechococcus*. (Phylogeny based on (Parfrey et al. 2011, Derelle and Lang 2012)).

Appendix D

Supporting table for Chapter 4

Table S4.1: Metagenomic sequences from the SCUMS database producing significant alignment with sequences of genes implicated in DMSP-degradation (*dddD, dddL, dddP, dddR, dmdA*), see Figure 4.

Genes encoding enzymes for DMSP degradation		**Significant alignment in SCUMS database**	
Gene	**Source**	**Metagenome**	**Sequence name**
dddD	*Sagittula stellata*	Bacteria Pond 11	3681139
	Silicibacter Pomeroyi	Bacteria Pond 11	3703408
	Burkholderia phymatum	Bacteria Pond 5	3875960
	Rhizobium NGR234	Cow4 SID1015	12432207
	Dinoroseobacter shibae	Kirikimati Bacteria	5354770
	Burkholderia phymatum	Kirikimati phage	5471787
	Burkholderia phymatum	Saltern Pond 5	4734680
	Burkholderia phymatum	Saltern Pond LP1110	8344022
	Burkholderia phymatum	Saltern Pond MB1110	8505647
	Rhizobium NGR233	Sample 3. Soudan Red Stuff	717221
	Dinoroseobacter shibae	Sample 3. Soudan Red Stuff	905095
dddL	JCVI_READ_1093017122050	Arctic Phage	2314137
	JCVI_READ_1093017122050	Arctic Phage	2410768
	JCVI_READ_1093017122050	Arctic Phage	2500161
	JCVI_READ_1093017122050	Arctic Phage	2532205
	JCVI_READ_1095521066275	Bacteria Pond 11	3627313
	JCVI_READ_1093017122050	Bacteria Pond 11	3675932
	Oceanicola batsensis	Bacteria Pond 11	3676188
	JCVI_READ_1095521066275	Bacteria Pond 11	3736548
	JCVI_READ_1093017122050	Bacteria Pond 11	3744232
	JCVI_READ_1093017122050	Bacteria Pond 11	3745715
	JCVI_READ_1095521066275	Bacteria Pond 11	3750056
	JCVI_READ_1095521699328	Bacteria Pond 11	3784268
	JCVI_READ_1093017122050	Bacteria Pond 11	3788727
	JCVI_READ_1093017122050	Bacteria Pond 11	3807501
	JCVI_READ_1095521066275	Bacteria Pond 11	3860393
	JCVI_READ_1093017122050	Bacteria Pond 11	3865452
	Dinoroseobacter sp.	Bacteria pond 11	3661879
	JCVI_READ_1093017122050	BBC Phage	2021964
	JCVI_READ_1093017122050	Cow1 SID1012	11764215
	JCVI_READ_1093017122050	GOM Phage	1422779

	JCVI_READ_1093017122050	GOM Phage	1478032
	JCVI_READ_1095522123195	Saltern Pond 5	4655064
	JCVI_READ_1093017122050	Saltern Pond MB1110	8518791
dddP	*Roseovarius nubinhibens ISM*	Arctic Phage from world's oceans	2296455
	Roseovarius nubinhibens ISM	Arctic Phage from world's oceans	2362942
	Silicibacter sp. TM1040	Arctic Phage from world's oceans	2367586
	JCVI_PEP_1105119658689	Arctic Phage from world's oceans	2498429
	Roseovarius nubinhibens ISM	Arctic Phage from world's oceans	2513792
	JCVI_PEP_1105121008479	Arctic Phage from world's oceans	2781136
	Roseovarius nubinhibens ISM	Arctic Phage from world's oceans	2845736
	Silicibacter sp. TM1040	Arctic Phage from world's oceans	2938306
	JCVI_PEP_1105121008479	Bacteria pond 10	3671148
	JCVI_PEP_1105123494127	Bacteria pond 11	3816295
	Roseovarius nubinhibens ISM	Bacteria pond 11	3763744
	Roseovarius nubinhibens ISM	Bacteria pond 11	3847898
	Roseovarius nubinhibens ISM	Bacteria pond 11	3851959
	JCVI_PEP_1105123494127	Bacteria pond 9	3607275
	Roseovarius nubinhibens ISM	GOM Phage from world's oceans	1364115
	JCVI_PEP_1105152730411	Kiritimati Reef phage	5390766
	JCVI_PEP_1105152730411	Kiritimati Reef phage	5433454
	JCVI_PEP_1105152730411	Kiritimati Reef phage	5495532
	JCVI_PEP_1105152730411	Kiritimati Reef phage	5557429
	JCVI_PEP_1105152730411	Kiritimati Reef phage	5558814
	JCVI_PEP_1105129234453	Kiritimati Reef phage	5602053
	JCVI_PEP_1105152730411	Kiritimati Reef phage	5613573
	JCVI_PEP_1105133120683	Kiritimati Reef phage	5639520
	JCVI_PEP_1105161276095	Mary Ann Moran pt 1	6606238
	Silicibacter sp. TM1040	Mary Ann Moran pt 1	6616722
	Roseovarius nubinhibens ISM	Mary Ann Moran pt 2	6669848
	JCVI_PEP_1105133120683	Mary Ann Moran pt 2	6651987
	JCVI_PEP_1105152730411	Palmyra F8 Bacteria	6822704
	JCVI_PEP_1105086865837	Palmyra F8 Bacteria	6832624
	Rhodobacterales bacterium HTCC2150	Palmyra F8 Bacteria	6892790
	Roseovarius nubinhibens ISM	Saltern Pond MB1110	8531514
	Roseovarius nubinhibens ISM	Saltern Pond MB1110	8488116
	P. gallaeciensis 2.10	Saltern Pond MB1110	8497696
	Roseovarius nubinhibens ISM	Saltern Pond MB1110	8517833
	Roseovarius nubinhibens ISM	Tilapia pond microbes	9905177
dddR	*Silicibacter pomeroyi*	Arctic Phages	2309079
	Silicibacter pomeroyi	Arctic Phages	2368386

	Silicibacter pomeroyi	Arctic Phages	2373281
	Silicibacter pomeroyi	Arctic Phages	2682457
	Silicibacter pomeroyi	Arctic Phages	2801317
	Silicibacter pomeroyi	Arctic Phages	2824869
	Silicibacter pomeroyi	Arctic Phages	2869537
	Silicibacter pomeroyi	BBC phages	2134795
dmdA	JCVI_READ_1092256332829	Arctic Phage	2227575
	JCVI_READ_1092256332829	Arctic Phage	2436745
	Silicibacter Pomeroyi	Arctic Phages	2311726
	Silicibacter Pomeroyi	Arctic Phages	2496421
	Silicibacter Pomeroyi	Arctic Phages	2566452
	Silicibacter Pomeroyi	Arctic Phages	2763116
	JCVI_READ_1092257122408	Bacteria Pond 11	3679829
	Roseobacter sp. Awz	Bacteria Pond 11	3699676
	Roseovarius nubinhibin	Bacteria Pond 11	3714146
	JCVI_READ_1091138106770	Bacteria Pond 11	3716611
	Roseovarius nubinhibin	Bacteria Pond 11	3717065
	Roseovarius sp. 218	Bacteria Pond 11	3727882
	Roseovarius sp. 217	Bacteria Pond 11	3773057
	JCVI_READ_1091138106770	Bacteria Pond 11	3800591
	Roseovarius sp. 220	Bacteria Pond 11	3808671
	Roseovarius sp. 219	Bacteria Pond 11	3823849
	Roseobacter denitrificans	Bacteria pond 11	3710525
	Silicibacter Pomeroyi	Bacteria pond 11	3850696
	Roseovarius nubinhibin	Bacteria Pond 5	4014647
	JCVI_READ_1092994700309	Cow4 SID1015	12521204
	JCVI_READ_1092256235524	Healthy slime viruses	10333008
	JCVI_READ_1095458149684	Highborne Cay stromatolite bacteria	9124647
	JCVI_READ_1095458149684	Highborne Cay stromatolite bacteria	9170355
	JCVI_READ_1095975059571	Kingman Reef Bacteria	4878982
	JCVI_READ_1091140906836	Kingman Reef Bacteria	4960392
	Sargasso Sea	Kingman Reef Phage	5053150
	JCVI_READ_1092959713993	Kingman Reef Phage	5084374
	Sargasso Sea	Kingman Reef Phage	5110397
	JCVI_READ_1093006404075	Kingman Reef Phage	5113542
	JCVI_READ_1095403702145	Kingman Reef Phage	5067334
	JCVI_READ_1093012156421	Kingman Reef Phage	5073094
	JCVI_READ_1092343732701	Kiritimati Reef Phage	5364958
	Sargasso Sea	Kiritimati Reef Phage	5370124
	JCVI_READ_1092344085737	Kiritimati Reef Phage	5372837

	JCVI_READ_1092343724689	Kiritimati Reef Phage	5380036
	JCVI_READ_1092955209154	Kiritimati Reef Phage	5382467
	Sargasso Sea	Kiritimati Reef Phage	5386600
	JCVI_READ_1095975136586	Kiritimati Reef Phage	5390778
	JCVI_READ_1095460124329	Kiritimati Reef Phage	5394935
	JCVI_READ_1092960003247	Kiritimati Reef Phage	5408546
	JCVI_READ_1093011900577	Kiritimati Reef Phage	5410232
	JCVI_READ_1093017480083	Kiritimati Reef Phage	5415960
	JCVI_READ_1095975136586	Kiritimati Reef Phage	5419179
	JCVI_READ_1093017385112	Kiritimati Reef Phage	5419235
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5426680
	Sargasso Sea	Kiritimati Reef Phage	5427866
	Sargasso Sea	Kiritimati Reef Phage	5437405
	JCVI_READ_1095963235602	Kiritimati Reef Phage	5442722
	Sargasso Sea	Kiritimati Reef Phage	5444202
	SAR	Kiritimati Reef Phage	5444260
	JCVI_READ_1095978306459	Kiritimati Reef Phage	5448412
	JCVI_READ_1095978306459	Kiritimati Reef Phage	5448634
	JCVI_READ_1092343724689	Kiritimati Reef Phage	5450875
	JCVI_READ_1093011900577	Kiritimati Reef Phage	5454270
	JCVI_READ_1095978306459	Kiritimati Reef Phage	5458989
	SAR	Kiritimati Reef Phage	5460242
	JCVI_READ_1095462012136	Kiritimati Reef Phage	5464351
	JCVI_READ_1093017559476	Kiritimati Reef Phage	5467818
	JCVI_READ_1091143457749	Kiritimati Reef Phage	5467857
	JCVI_READ_1095949462115	Kiritimati Reef Phage	5471009
	JCVI_READ_1093015254418	Kiritimati Reef Phage	5479762
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5484366
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5486257
	SAR	Kiritimati Reef Phage	5487332
	JCVI_READ_1095460051668	Kiritimati Reef Phage	5487638
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5488176
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5489282
	JCVI_READ_1092955183754	Kiritimati Reef Phage	5489343
	JCVI_READ_1092955183754	Kiritimati Reef Phage	5489452
	SAR	Kiritimati Reef Phage	5497292
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5497433
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5498837
	SAR	Kiritimati Reef Phage	5503684
	JCVI_READ_1095978306459	Kiritimati Reef Phage	5503692

	JCVI_READ_1093011411295	Kiritimati Reef Phage	5506337
	JCVI_READ_1095462012136	Kiritimati Reef Phage	5507922
	JCVI_READ_1091139216945	Kiritimati Reef Phage	5507953
	JCVI_READ_1091143457749	Kiritimati Reef Phage	5508661
	SAR	Kiritimati Reef Phage	5524664
	JCVI_READ_1093015382958	Kiritimati Reef Phage	5524855
	JCVI_READ_1095901453524	Kiritimati Reef Phage	5530033
	JCVI_READ_1092961140792	Kiritimati Reef Phage	5533732
	JCVI_READ_1093017559476	Kiritimati Reef Phage	5535042
	SAR	Kiritimati Reef Phage	5537080
	JCVI_READ_1095403702145	Kiritimati Reef Phage	5539880
	SAR	Kiritimati Reef Phage	5547247
	SAR	Kiritimati Reef Phage	5547538
	JCVI_READ_1093015736344	Kiritimati Reef Phage	5548270
	JCVI_READ_1091141777680	Kiritimati Reef Phage	5548826
	JCVI_READ_1095403267546	Kiritimati Reef Phage	5550150
	JCVI_READ_1095349032352	Kiritimati Reef Phage	5554976
	JCVI_READ_1092256147551	Kiritimati Reef Phage	5555403
	SAR	Kiritimati Reef Phage	5555514
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5557969
	JCVI_READ_1095467559483	Kiritimati Reef Phage	5558665
	SAR	Kiritimati Reef Phage	5563865
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5568243
	JCVI_READ_1093017729705	Kiritimati Reef Phage	5568583
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5571604
	SAR	Kiritimati Reef Phage	5571909
	JCVI_READ_1092322703750	Kiritimati Reef Phage	5580420
	SAR	Kiritimati Reef Phage	5585149
	SAR	Kiritimati Reef Phage	5590075
	JCVI_READ_1095349032352	Kiritimati Reef Phage	5590889
	SAR	Kiritimati Reef Phage	5598291
	SAR	Kiritimati Reef Phage	5599434
	JCVI_READ_1092961140792	Kiritimati Reef Phage	5606886
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5607202
	JCVI_READ_1095462012136	Kiritimati Reef Phage	5607542
	JCVI_READ_1095462012136	Kiritimati Reef Phage	5607791
	JCVI_READ_1095467050886	Kiritimati Reef Phage	5610714
	SAR	Kiritimati Reef Phage	5615304
	JCVI_READ_1095978306459	Kiritimati Reef Phage	5615570
	JCVI_READ_1092963302515	Kiritimati Reef Phage	5616784

	JCVI_READ_1095403267546	Kiritimati Reef Phage	5624422
	JCVI_READ_1095978306459	Kiritimati Reef Phage	5628768
	JCVI_READ_1092344081569	Kiritimati Reef Phage	5629708
	JCVI_READ_1095349032352	Kiritimati Reef Phage	5630308
	JCVI_READ_1095978306459	Kiritimati Reef Phage	5632582
	JCVI_READ_1095898168770	Kiritimati Reef Phage	5634607
	JCVI_READ_1091140902113	Kiritimati Reef Phage	5637704
	JCVI_READ_1093011411295	Kiritimati Reef Phage	5643030
	SAR	Kiritimati Reef Phage	5646222
	JCVI_READ_1095462316992	Kiritimati Reef phage	5384108
	JCVI_READ_1095462316992	Kiritimati Reef phage	5414151
	JCVI_READ_1091140902113	Kiritimati Reef phage	5435754
	JCVI_READ_1092961104068	Kiritimati Reef Phage	5461958
	JCVI_READ_1092343724689	Kiritimati Reef Phage	5462587
	JCVI_READ_1092961104068	Kiritimati Reef phage	5537195
	JCVI_READ_1092961104068	Kiritimati Reef phage	5580608
	JCVI_READ_1092256577335	Kiritimati Reef phage	5583865
	JCVI_READ_1095390079788	Kiritimati Reef phage	5645255
	JCVI_READ_1092342060402	Lindas PA1	8015616
	JCVI_READ_1092342060402	Lindas PA1	8130545
	JCVI_READ_1092342060402	Lindas PA1	8178820
	JCVI_READ_1092342060402	Lindas PA1	8258760
	Roseobacter denitrificans	MAM1	6582535
	Silicibacter pomeroyi	MAM1	6613469
	Silicibacter pomeroyi	MAM2	6637164
	Silicibacter pomeroyi	MAM2	6642178
	Silicibacter pomeroyi	MAM2	6648037
	JCVI_READ_1093011411295	MAM2	6665189
	JCVI_READ_1093011411295	MAM2	6667004
	Roseobacter denitrificans	MAM2	6672527
	JCVI_READ_1092344085737	MAM2	6676947
	Silicibacter pomeroyi	MAM3	6686016
	Silicibacter pomeroyi	MAM4	6709772
	JCVI_READ_1093017480083	MAM4	6714003
	Silicibacter pomeroyi	MAM4	6714177
	JCVI_READ_1092970301894	Palmyra F8 Bacteria	6749262
	JCVI_READ_1095460124329	Palmyra F8 Bacteria	6772206
	JCVI_READ_1095901453524	Palmyra F8 Bacteria	6795397
	JCVI_READ_1092959727843	Palmyra F8 Bacteria	6824585
	JCVI_READ_1092961117404	Palmyra F8 Bacteria	6860162

	JCVI_READ_1091140902113	Palmyra F8 Bacteria	6885935
	JCVI_READ_1095456015748	Palmyra F8 Bacteria	6894768
	JCVI_READ_1093011411295	Palmyra F8 Bacteria	6901218
	JCVI_READ_1095403700801	Palmyra F8 Bacteria	6903119
	JCVI_READ_1092977802640	Palmyra F8 Bacteria	6953061
	JCVI_READ_1091140902113	Palmyra F8 Bacteria	6984981
	JCVI_READ_1095403267546	Palmyra F8 Bacteria	6986596
	JCVI_READ_1093017387794	Palmyra F8 Bacteria	6989949
	JCVI_READ_1093016261191	Palmyra F8 Bacteria	7004884
	JCVI_READ_1092351432286	Palmyra F8 Phage	7200751
	JCVI_READ_1095898168770	Palmyra F8 Phage	7312012
	JCVI_READ_1095390079788	Porites compressa pH treated viruses	11171008
	JCVI_READ_1095390079788	Porites compressa pH treated viruses	11179557
	JCVI_READ_1092344363543	Rios Mesquites Stromatolites bacteria	5676570
	JCVI_READ_1092256332829	Saltern Pond 5	4704428
	JCVI_READ_1092256332829	Saltern Pond 5	4750852
	Dinoroseobacter shibae	Saltern Pond LP1110	8399616
	JCVI_READ_1095515491117	Sample 3. Soudan Red Stuff	615019
	JCVI_READ_1095526022205	Sample 3. Soudan Red Stuff	673123
	JCVI_READ_1095526022205	Sample 3. Soudan Red Stuff	914800
	JCVI_READ_1095978306459	SAR Phage	4132730
	JCVI_READ_1092214963960	SAR Phage	4259065
	JCVI_READ_1095901449664	SAR Phage	4332849
	JCVI_READ_1091140844121	SAR Phage	4338095
	JCVI_READ_1095901434257	SAR Phage	4453793
	JCVI_READ_1093017387794	SAR Phage	4508167
	JCVI_READ_1093018642277	*Streptococcus iniae*	6247634
	JCVI_READ_1091145353780	Tabuaren B1 Phage	7640336
	JCVI_READ_1091140421823	Tabuaren B1 Phage	7648048
	JCVI_READ_1095403700801	Tabuaren B1 Phage	7881218
	JCVI_READ_1095403700801	Tabuaren B1 Phage	7931226

Appendix E

Supporting table for Chapter 6

Table S6.1: Comparison of the radius of inhibition zones exhibited by *Vibrio coralliilyticus* in contact to pure tropodithietic acid (TDA; 2µM in MeOH; pre-incubated overnight at two different temperatures) in well-diffusion assays.

Treatment	Clear zone (mm)
27°C	6
27°C	6.5
27°C	6
27°C	6
27°C	6.5
27°C	6
27°C	6.5
27°C	6.5
27°C	6
27°C	6
32°C	6.5
32°C	6
32°C	6.5
32°C	6.5
32°C	6
32°C	6
32°C	6.5
32°C	6
32°C	6
32°C	6.5
32°C	6.5