2013

Dimethylated sulphur compounds in Acropora corals: antioxidant response and implications for climate regulation

Elisabeth Suzanne Marie Deschaseaux

Southern Cross University
Dimethylated sulphur compounds in *Acropora* corals: antioxidant response and implications for climate regulation

Elisabeth Deschaseaux
Bachelor of Applied Science (Hons)

Submitted in fulfilment of the degree of
Doctor of Philosophy

20th of December 2013
“Life clearly does more than adapt to the Earth. It changes the Earth to its own purposes. Evolution is a tightly coupled dance, with life and the material environment as partners. From the dance emerges the entity Gaia.”

James Lovelock
DECLARATION

I, Elisabeth Deschaseaux certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other University.

I acknowledge that I have read and understood the University’s rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University (as they may be from time to time).

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Acropora corals have been recognized as a significant source of dimethylsulphide (DMS), a climatically active sulphur gas that contributes to the formation of clouds when oxidised to sulphate aerosols in the atmosphere. In zooxanthellate corals, DMS can be sourced from biogenic dimethylsulphonylpropionate (DMSP) and/or dimethylsulphoxide (DMSO) from Symbiodinium, with DMSP also being produced by the animal host. DMS(P)(O) are suspected to act as antioxidants through the scavenging of reactive oxygen species (ROS) that accumulate during oxidative stress, with DMS and DMSP possibly oxidising to DMSO in the presence of ROS. This thesis investigated the production of these dimethylated sulphur compounds (DSC) in the holobiont and axenic cultures of Symbiodinium isolated from Acropora corals, under basal conditions and environmental stress (e.g. temperature, light, salinity, air exposure) with the aim to assess their role as climate regulators and antioxidants. A chamber experiment revealed that concentrations of both DMS and DMSP decreased under thermal stress in the thermo-sensitive Symbiodinium clade C1, suggesting that these sulphur compounds were consumed intracellularly, possibly as a result of DMS(P) oxidation by ROS. To test this hypothesis, coral tissue removal methods for the optimal extraction of antioxidants from coral tissue were compared, with air blasting determined to be the most efficient. Similarly, two chemical reduction methods were compared for optimised quantifications of DMSO in coral tissue. Methods were greatly correlated and no categorical conclusion was drawn on the preferential suitability of one method over the other. Further experiments established a correlation between enhanced DMSO production and an up-regulation of the antioxidant capacity in Acropora aspera under environmental factors that led to oxidative stress, indicating that the DMSP-based antioxidant system was involved in the antioxidant regulation of the coral holobiont. A pilot bubbling chamber experiment conducted on Acropora pulchra showed a link between the oxidation of coral-derived gaseous DMS and the occurrence of freshly nucleated aerosol nanoparticles, reinforcing the role of DMS derived from Acropora
corals as a potential source of secondary aerosols over coral reefs. Together, these results indicated that the production of coral-derived DMS could participate in aerosol formation over *Acropora* dominated coral reefs if not consumed as a response to oxidative stress. This study provided new insights into the production of DSC by reef-building corals and their symbiotic microalgae and showed that the coral biogenic sulphur cycle was likely to be affected under future climate change scenarios, with possible consequences on climate regulation over coral reefs.
ACKNOWLEDGEMENTS

First and foremost, I want to thank my wonderful partner Renaud for keeping me sane, for his love, endless support, and encouragements throughout this major milestone of my life. Renaud, this would never have been possible without you. “Merci d’avoir toujours cru en moi.”

I would also like to sincerely thank my main supervisor, Associate Professor Graham Jones, for his guidance, availability, financial support, endless editing and genuine kindness throughout my studies. I am also extremely thankful to my secondary supervisor, Dr Myrna Deseo, for the help and support that she has been willing to give me before she even officially became involved in my supervision. I am also very grateful to my third supervisor, Professor Bradley Eyre, for his moral and financial support, and to my last but not least supervisor, Professor Peter Harrison, for his guidance, faith and immense kindness.

A big thank you to Hilton Swan, my colleague and loyal field-work companion, for his advice, shared knowledge and friendship. You have been like a fifth supervisor to me Hilton and I have learnt so much from you.

I would also like to thank Professor Ron Kiene for welcoming me into his laboratory, for his genuine kindness and for his help editing the manuscripts on which we share authorship. Many thanks also to Victor Beltran for guiding me through the genotyping process and for his help finalising our joined manuscript.

Thanks to Anna Scott, Ross Hill and Stefanie Pontasch for our collaboration at the NMSC. It was such a pleasure to work with you and I hope that opportunities will come up again in the future.
I would also like to thank the very special friends who I have met during the three and half years of my studies at Southern Cross University (SCU): Mick, Jody, Andrew, ‘Licia, Paul, Lea, Melissa, Paddy, Tyler, Sergio, Perrine, Denis, Ashley, Judith, Cecile, Kwanho, Meggie, Shimano, Mathieu, John and Kai. You have been like family to me and you have made every single shared time a fun and distracting moment that has helped me keeping sane. A special thank you to my forever friends: Loute, Amelia, Momo, N’Helene, Caro, Max, Shinta, Thomas, Sarah and Liz for their love and constant support, even from a distance. Love you guys.

Thanks to the entire Centre for Coastal Biogeochemistry crew for welcoming me among them. Thanks also to the Heron Island Research Staff for their assistance in the field. Many thanks to the administrative and technical Staff of the School of Environment, Science and Engineering (Sonia Weiss, Rosi Brown and beloved Delva Smith who passed away this year).

Thanks to the SCU Division of Research for the Australian Postgraduate Award that has helped supporting my research, and to the travel allowance that sponsored my collaboration with Professor Ron Kiene.

I would also like to thank my entire family for helping me shape into the person that I am today. « A mon père, pour avoir financé mon Honours et grâce à qui je fais cette thèse aujourd’hui, pour son amour, sa joie de vivre, et son soutien permanent. A ma mère, pour son amour inconditionnel, son éternelle confiance en moi, et pour m’avoir donné le goût à la culture. A mon frère pour la merveilleuse personne qu’il est et a toujours été, et sans qui je ne serais pas la moitié de la personne que je suis aujourd’hui. A ma soeur de « Noël Canélonie », celle avec qui j’ai grandit et tout vécue, celle qui me connait par cœur et que j’aime par-dessus tout. A mon Nico et mon Olive, mes frères de coeurs, que j’adore et qui me manquent tellement. A Dodo, ma deuxième maman qui s’est toujours occupée de moi comme si j’étais sa propre fille. A ma Babylou. A mon Daminous, Pipolette et Thomas-chou. Mon grand filleul, tu as été mon rayon de soleil et ma plus grande source de motivation pendant ces derniers mois de thèse. A ma soeur Sissi, que j’ai enfin rencontré cette année, et que j’aime déjà si fort. A Sully, Siouxi et
And finally, thanks to Byron Bay for its wonderful atmosphere, to music and my guitar for being a great source of inspiration, to Kiva Spa for providing a haven of peace among the stress and wildness of research, and to life in general for mysteriously always taking me where I belong.

*Elisabeth Deschaseaux*
PREFACE

This thesis was prepared in fulfilment of the requirements for the degree of Doctor of Philosophy. It reports on the research that was undertaken from the 20th of April 2010 to the 20th of December 2013 within the School of Environment, Science, and Engineering at Southern Cross University, Lismore, New South Wales, Australia. Principal supervisor was Associate Professor Graham Jones, with co-supervision from Dr Myrna Deseo, Professor Bradley Eyre and Professor Peter Harrison. During this study, extensive field collections and laboratory investigations have been conducted involving collaboration with researchers from the Australian Institute for Marine Science (AIMS), Townsville, the Dauphin Island Sea Laboratory (DISL), Alabama and the Queensland University of Technology (QUT), Brisbane. Results from this thesis have been presented at the 12th International Coral Reef Symposium (ICRS), 2012, Cairns, Australia.

This thesis comprises seven chapters, five of which are currently published.

Chapter 1 Introduction introduces the study in the context of the relevant literature and highlights the aims and objectives of the research. It also presents the sites and specimens that were studied and collected throughout this research.

Chapter 2 Comparative response of DMS and DMSP concentrations in Symbiodinium clades C1 and D1 under thermal stress has been published in the Journal for Experimental Marine Biology and Ecology in June 2014. This chapter investigates the relative capacity of two Acropora-associated Symbiodinium clades that exhibit different thermal tolerances to produce dimethylsulphide (DMS) and dimethylsulphoniopropionate (DMSP) under thermal stress. This manuscript has been prepared in co-authorship with Dr Victor Beltran, Assoc. Prof. Graham Jones, Dr
Myrna Deseo, Hilton Swan, Prof. Peter Harrison and Prof. Bradley Eyre and it is also presented in its published form in Appendix 3.

Chapter 3 *Air blasting as the optimal approach for the extraction of antioxidants in coral tissue*, has been published in the Journal of Experimental Marine Biology and Ecology in July 2013. This chapter compares two common methods for the removal of tissue in hard corals in their capacity to efficiently extract antioxidants from coral tissue. The methods are described and the outcomes of the comparison are discussed. This manuscript has been published in co-authorship with Dr Myrna Deseo, Kellie Shepherd, Assoc. Prof. Graham Jones, and Prof. Peter Harrison and it is also presented in its published form in Appendix 4.

Chapter 4 *Dimethylsulphoxide (DMSO) in biological samples: a comparison of the TiCl₃ and NaBH₄ extraction methods* has been published in Marine Chemistry in June 2014. This chapter provides a comparison of the two most commonly used methods for dimethylsulphoxide (DMSO) analysis in coral tissue and other biological samples and discusses the benefits and drawbacks of each method. This chapter also reports the relative DMSO concentrations in coral tissue samples, macroalgae and phytoplankton. This manuscript has been prepared in co-authorship with Prof. Ronald Kiene, Assoc. Prof. Graham Jones, Dr Myrna Deseo, Hilton Swan, Lisa Oswald and Prof. Bradley Eyre and it is also presented in its published form in Appendix 5.

Chapter 5 *Effects of environmental factors on the production of dimethylated sulphur compounds and their potential role in the antioxidant system of the coral holobiont* has been published in Limnology and Oceanography in January 2014. Based on the assumption that DMS, DMSP and DMSO act as antioxidants in corals through the active scavenging of reactive oxygen species, this chapter investigates the production of these dimethylated sulphur compounds in the coral holobiont under various environmental stressors (e.g. temperature, light, salinity and air exposure), and discusses of their potential role in the antioxidant capacity of the coral holobiont. This manuscript has been published in co-authorship with Assoc. Prof. Graham Jones, Dr Myrna Deseo,
Chapter 6 Can corals form aerosol particles through volatile sulphur compound emissions? is a modified version of a manuscript that has been published as part of the Proceeding of the 12th ICRS in July 2012. Based on the hypothesis that DMS produced by reef-building corals could play a role in climate regulation, this chapter investigates the production of DMS and DMSP by the reef-building coral Acropora pulchra and the presence of these two sulphur compounds in coral reef waters. This chapter also evaluates the potential of coral-derived atmospheric DMS to oxidise to non-sea-salt aerosol particles that could contribute to climate regulation. However, this study only constitutes a pilot experiment for future research as results presented are preliminary and discussion invites for further investigations. This manuscript has been written in co-authorship with Assoc. Prof Graham Jones, Dr Branka Miljevic, Prof. Zoran Ristovski, Hilton Swan and Dr Petri Vaattovaara and it is also presented in its published form in Appendix 7.

Chapter 7 Synthesis and future work provides a summary and synthesis of Chapters 2 – 6 and discusses of the implications of the main outcomes of this research for climate regulation in a context of climate change. This chapter also provides suggestions for future research.

In addition, another two peer-reviewed co-authored manuscripts have been published during the course of this PhD candidature: one as part of the 12th ICRS Proceeding, with Hilton Swan as first author; and the other one in the Wold Academy of Science, Engineering and Technology Proceeding, with Petri Vaattovaara as first author. These publications are presented in Appendix 8 and 9 respectively.

Appendix 1 and 2 provides the calibration data for Chapters 2 – 6 and signed statements of the contribution of others, respectively.
**AUTHOR CONTRIBUTION STATEMENT**

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<td>Thesis writing</td>
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<tr>
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<td>ED (75%), GJ (7%), MD (2.5%), PH (2.5%), BE (2.5%), RK (2.5%), HS (2.5%), KS (1%), VB (1%), BM (1%), ZR (1%), PV (1%) and LO (0.5%)</td>
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<tr>
<td>Obtaining funding</td>
<td>GJ (80%), BE (15%), PH (2.5%), MD (2.5%)</td>
</tr>
<tr>
<td>Overall responsibility</td>
<td>ED (100%)</td>
</tr>
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**Author declaration**

I, **Elisabeth Deschaseaux**, the author of this thesis, certify that the contributors and conflicts of interest statements included in this thesis are correct and have been approved by all contributors.

Signature:  
Date: 01/12/13
I agree with the contents of the thesis; to being listed as a contributor; and to the conflicts of interest statement as summarised. I have had access to all the data in the study and accept responsibility for its validity.

Assoc Prof Graham Jones  Signature:   Date: 6/12/2013

Dr Myrna A. Deseo  Signature:   Date: 09/12/2013

Prof Bradley Eyre  Signature:   Date: 09/12/2013

Prof Peter Harrison  Signature:   Date: 18/12/2013

Signed statements from each of the following contributors are included in Appendix 2:

Prof Ronald Kiene
Hilton Swan
Kellie Shepherd
Dr Victor Beltran
Dr Branka Miljevic
Prof Zoran Ristovski
Dr Petri Vaattovaara
Lisa Oswald
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LIST OF ABBREVIATIONS

AOC   AntiOxidant Capacity
CCN   Cloud Condensation Nuclei
Chl $a$  Chlorophyll $a$
CLAW  Charlson-Lovelock-Andreae-Warren
DMS   DiMethylSulphide
DMS$_a$  Atmospheric DMS
DMS$_g$  Gaseous DMS
DMS$_w$  Dissolved DMS
DMSO  DiMethylSulphOxide
DMSP  DiMethylSulphonioPropionate
DMSP$_d$  Dissolved DMSP
DMSP$_p$  Particulate DMSP
DMSP$_t$  Total DMSP
DMSP$_{t/d}$  Total and dissolved DMSP
DMS(P)(O)  DMS, DMSP and/or DMSO
DNA   DeoxyriboNucleic Acid
DSC   Dimethylated Sulphur Compounds
ESH   Ethanethiol or ethyl mercaptan
FSW   Filtered SeaWater
GC    Gas Chromatograph
GP-FPD  GC with Flame Photometric Detector
GC-MSD  GC with Mass Selective Detector
GC-PFPD  GC with Pulsed FPD
GBR   Great Barrier Reef
GBRMPA  Great Barrier Reef Marine Park Authority
He   High purity Helium
L:D   Light:Dark
MES   Methyl Ethyl Sulphide
MeSH   Methanethiol or methyl mercaptan
nss   non-sea-salt
ORAC   Oxygen Radical Absorbance Capacity
RSD   Relative Standard Deviation
ROS   Reactive Oxygen Species
SCU   Southern Cross University
SD   Standard deviation
SE   Standard error
SSCP   Singled Stranded Conformation Polymorphism
SST   Sea Surface Temperature
CHAPTER 1

Introduction
The ecology of reef-building corals, importance and threats

Coral reef ecosystems in the current context of climate change

The Earth has always been the stage for climate fluctuations from basic seasonal variations to glacial-interglacial cycles (Harley et al., 2006). However, a rise in greenhouse gas concentrations associated with increased human activities since the industrial revolution has amplified and accelerated global warming, a phenomenon known as anthropogenic climate change (Hofmann et al., 2009). Increasing evidence of climate change has been found and its occurrence is now considered inevitable, with possible consequences for the whole earth system (IPCC, 2007; Serreze, 2010; IPCC, 2013). More specifically, marine ecosystems are expected to suffer from variations in sea surface temperature (SST), ultra-violet (UV) radiation, pH, salinity and nutrient limitation effects due to increased stratification, which are expected to occur at an unprecedented rate (Meier, 2002; Harley et al., 2006; Brierley and Kingsford, 2009).

Coral reefs are fascinating and complex ecosystems of which 20% have already been seriously damaged and an additional 50% are under constant human pressure and imminent threat of collapse (Wilkinson, 2004). Although coral reefs represent only 0.2% of the world ocean area (Smith, 1978), great environmental and financial importance has been attributed to these ecosystems (Hoegh-Guldberg, 1999; Harrison and Booth, 2007). The three-dimensional structure of coral reefs and their high productivity provide a habitat and an active source of food and nutrients for many thousands of species of invertebrates, fish, reptiles, birds and marine mammals, representing 25% of the marine biodiversity (Harrison and Booth, 2007). From an economical and sociological point of view, more than 500 million people draw their food and resources from coral reef ecosystems (Hoegh-Guldberg, 2011). The Great Barrier Reef in Australia, which represents 17% of the global coral reef area, is one of the world’s premier tourist locations, and its global economic value has been estimated at more than AUD$5 billion per year (Harrison and Booth, 2007), which is worth 2.5 times more than the commercial fishing and aquaculture industry for all Australia (Australian Government Fisheries, 2012). In light of these statistics, we can estimate
the impact that damage to coral reefs will have all around the world from an ecological, social and economical point of view.

Increasing SST and ocean acidification are currently considered the main threats for coral reef ecosystems as (i) the fragile algal symbiosis on which corals rely for their nutritional requirements is highly temperature dependent, and (ii) the chemistry and precipitation of calcium carbonate that constitutes the skeleton of reef-building corals intimately depends on pH and CO₂ concentrations (Hoegh-Guldberg et al., 2007). During the 20th century, global SST has gradually increased by 0.74°C and pH has decreased by 0.1 pH unit, conditions that could compromise the future of coral reef ecosystems around the globe, which are predicted to disappear by 2100 (Pockley, 1999; Hoegh-Guldberg et al., 2007; IPCC, 2007).

The coral-zooxanthellae symbiosis
Scleractinian corals are also known as “hard corals”, and within this group are colonial forms that have interconnected polyps produced by growth and division from an original single polyp, and solitary corals that exist as single polyps of various sizes (Harrison and Booth, 2007; Wallace, 2008). Coral polyps are composed of two cellular tissue layers separated from each other by a mostly acellular layer of mesoglea: the epidermal tissues consist of the outer epidermis facing the surrounding sea water and the aboral epidermis that lies adjacent to and secretes the calcium carbonate aragonite exoskeleton (also called calicoblastic layer), while the interior of the polyp is formed by the gastrodermis (Figure 1) (Harrison and Booth, 2007). Ecologically, scleractinian corals can be divided into reef-building (hermatypic) corals that create coral reefs, and ahermatypic corals that do not contribute significantly to reef formation (Harrison, 2011). Hermatypic corals usually live in symbiosis with zooxanthellae dinoflagellate microalgae and are termed “zooxanthellate” corals, whereas other corals do not contain these symbionts and are termed “azooxanthellate” corals (Harrison and Booth, 2007). The photosynthetic zooxanthellae, which are endosymbionts of the genus Symbiodinium (Trench, 1979; Douglas, 2003), are contained in vacuoles within the cytoplasm of the host gastrodermic cells with usually one but up to five zooxanthellae per host cell (Gates et al., 1992). The total density averages several millions of
endosymbiotic cells per cm$^2$ of coral tissue (Fitt et al., 2000). The coral-zooxanthellae symbiosis is described as a mutualistic association where both the host coral and symbiont benefit from the association. Zooxanthellae provide corals with nutrients, signalling molecules and photosynthetic carbon, which can correspond to more than 90% of the coral’s nutritional requirements in some species (Muscatine and Porter, 1977; Trench, 1993). Zooxanthellar pigments that give the corals their brown colour are also known to protect both the host and the symbiont against excessive sunlight and UV radiation exposure (Harrison and Booth, 2007). In return, coral polyps provide a suitable shelter for the algae against predation and a significant source of ammonium and carbon dioxide needed during photosynthesis (Trench, 1993; Yellowlees et al., 2008). However, the mutual benefits of the symbiosis have recently been called into question with a new theory stipulating that far from benefiting from the symbiosis, zooxanthellae might be kept under captivity and exploited by the parasitic corals (Wooldridge, 2010). Another study has discussed the possibility that the coral-Symbiodinium relationship could be both mutualistic and parasitic depending on the symbiont taxon (Lesser et al., 2013). Still, the limited understanding of the biochemistry behind the coral-zooxanthellae association does not allow to draw conclusions on this fringing view.
Figure 1: Diagram illustrating the architecture of a coral polyp.

Rowan and Powers (1991a) made crucial genetic discoveries concerning the taxonomy of zooxanthellae involved in symbiosis with various marine invertebrates by identifying several taxa from different genetic lineages. Nine phylogenetic clades of zooxanthellae, indexed from A to I, have now been identified, with each containing many sub-clades (Pochon and Gates, 2010). More than one type of zooxanthellae can be in symbiosis with a single host and one single host species can associate with different clades of zooxanthellae (Rowan and Powers, 1991b). Coral species are generally associated with two clades of zooxanthellae, one of which is usually dominant and the other in lower densities which is termed the “background” clade. These clades may be involved in a mechanism of “symbiont shuffling” during environmental stress, where the more resistant background clade takes over the dominant sensitive symbiont (Berkelmans and Van Oppen, 2006). Another mechanism of symbiont-host combination is referred to as “symbiont switching”, which opens the possibility of the recombination of the host with new symbionts acquired from the environment as a consequence of environmental pressure (Baker, 2003). Although these two recombination mechanisms may be restricted to certain coral species only (Goulet, 2006; Stat et al., 2009), they
introduce the concept of “ecospecies” according to which coral colonies can supposedly respond differently to environmental pressures depending on the type of zooxanthellae they associate with (Buddemeier et al., 2004). Therefore, the breakdown of the coral *Symbiodinium* symbiosis, which has been linked to the limited ability of the zooxanthellae to cope with environmental stress exposure (Harrison and Booth, 2007), might also be an adaptive mechanism of expulsion of the algae by the coral preceding re-colonization of the animal by a more resistant clade of zooxanthellae (Buddemeier and Fautin, 1993; Ware et al., 1996; Baker, 2001). In the Great Barrier Reef (GBR), Australia, reef-building corals predominantly associate with clade C and D (Ulstrup and Van Oppen, 2003; Mieog et al., 2007), with clade D being more resistant to thermal stress than clade C (Glynn et al., 2001; Rowan, 2004; Tchernov et al., 2004). As such, coral colonies that have experienced repeated coral bleaching events often associate with clade D symbionts (Baker et al., 2004). This observation also suggests that coral resistance to thermal stress may be slightly increased with time through the adaptive association of the host with more tolerant symbionts (Berkelmans and Van Oppen, 2006). A recent study revealed that the different levels of thermo-resistance across sub-clades of *Symbiodinium* were linked to the presence of critical compounds such as heat-stress proteins, superoxide dismutase or photosystem II D1 protein (Fitt et al., 2009), although more research is still needed to understand the full extent of this process. However, it is important to note that some physiological trade-offs such as a reduction in coral growth rates (Abrego et al., 2009) or potential disease susceptibility (Littman et al., 2010) can be associated with thermo-tolerant *Symbiodinium* clades.

_Coral bleaching, a breakdown of the symbiosis that might lead to death_

Coral bleaching is defined as the loss of symbiotic zooxanthellae and/or photosynthetic pigments by the coral (Iglesias-Prieto et al., 1992; Brown, 1997; Hoegh-Guldberg, 1999) and can result in starvation of the animal host from a lack of photosynthetic compounds (Perez et al., 2001). Coral bleaching is known to be associated with changes in environmental conditions that lead to stress, including changes in SST (Hoegh-Guldberg and Smith, 1989), solar radiation (Hoegh-Guldberg and Smith, 1989), reduced salinity (Goreau, 1964), bacterial and other infections (Goreau, 1964; Kushmaro et al., 1996) with increased temperature and light considered to be the main
drivers of bleaching (Hoegh-Guldberg, 1999; Douglas, 2003). Coral bleaching does not always lead to coral death; however, it may become fatal following elevated and extended periods of stress exposure (Hoegh-Guldberg, 2011).

In the case of increased sunlight intensity, bleaching is often a consequence of the loss of pigments by the zooxanthellae with pale corals resulting in pale symbionts, whereas during high temperature stress, coral bleaching is often a direct consequence of algal density reduction, with pale corals resulting in a direct loss of pigmented zooxanthellae (Hoegh-Guldberg and Smith, 1989). From a cellular perspective, three main mechanisms are considered to result in coral bleaching: degradation of zooxanthellae (e.g. loss of photosynthetic pigments), release or digestion of endosymbiont cells from the host cells (Downs et al. 2009), or release or degradation of gastrodermal cells containing the endosymbionts (Brown et al., 1995; Titlyanov et al., 1996). For example, temperature-associated bleaching has been identified to induce the release of host cells containing endosymbiotic zooxanthellae, with free host cells disintegrating rapidly in the seawater, leaving behind free zooxanthellae (Gates et al., 1992).

The first coral bleaching event observed was reported at Birdley Reef, Florida in 1911 (Meyer, 1914), while the first “mass coral bleaching” event, where bleaching was widespread on many reefs, was observed in the 1980’s in both the GBR and along the coast of Panama (Glynn, 1983, 1984; Harriott, 1985; Oliver, 1985). The years of 1998 and 2002 have been reported as the warmest years in climate records and were both associated with strong El Nino Southern Oscillation events and mass coral bleaching (Berkelmans and Oliver, 1999; Wilkinson et al., 1999; Berkelmans et al., 2004). During the Southern Hemisphere summer of 2008-2009, the GBR again experienced anomalously high SSTs combined with heavy rainfall and run-off that have resulted in mass coral bleaching and increased diseases along the central and northern areas of the GBR Marine Park (GBRMPA, 2010). In the current era of global warming, coral bleaching is therefore considered as becoming more severe, more frequent and more widespread with time (Hoegh-Guldberg, 1999; Wilkinson et al., 1999).
Coral bleaching and oxidative stress

During normal aerobic metabolism, organisms produce reactive oxygen species (ROS) such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), hydroxyl radicals (HO\textsuperscript{•}) and superoxide radicals (O\textsubscript{2}•) (Griffin and Bhagooli, 2004), which are normally managed by a series of specialised enzymes called antioxidants that scavenge and convert ROS to non-reactive species (Winston, 1991; Martindale and Holbrook, 2002). In the coral-zooxanthellae symbiosis, ROS are produced by both cnidarian hosts and endosymbiotic algae during normal aerobic metabolism and photosynthesis (Dykens and Shick, 1982; Dykens et al., 1992; Lesser, 1996; Mydlarz and Jacobs, 2004, 2006). However, as a consequence of environmental variations such as thermal stress, excessive exposure to solar radiation or pollution, the production of ROS will increase and become harmful for the organism, leading to oxidative stress (Lesser, 2006; Richier et al., 2006).

The dissociation of the coral symbiosis is believed to be linked to oxidative stress in both the coral tissue and endosymbiotic zooxanthellae (Lesser, 1997; Downs et al., 2002; Flores-Ramirez and Linan-Cabello, 2007; Csaszar et al., 2009). Parallel studies have also shown a clear relationship between thermal stress induced bleaching and an impairment of the photosynthetic system of zooxanthellae and more precisely the CO\textsubscript{2} fixation mechanism, Calvin cycle and photosystem II (Iglesias-prieto et al., 1992; Fitt and Warner, 1995; Warner et al., 1996; Jones et al., 1998b), which are known to be the site of intense ROS production. Consequently, it was suggested that the clade-specific sensitivity to environmental stressors could be linked to the clades’ capacity to respond to oxidative stress, which most likely relies on the stability of the chloroplast membranes in the presence of ROS (Tchernov et al., 2004). Realistically, environmental stress acts on several levels within both the animal host and endosymbiotic macroalgae, leading to a dysfunction of the symbiosis, which eventually results in bleaching.

The coral holobiont

In addition to the mutualistic relationship that takes place between the coral host and the endosymbiotic microalgae, a wide range of bacteria, viruses, fungi, endolithic algae and archae are abundant in the tissue and mucous layer of the animal host, and together
these constitute the coral holobiont (Wegley et al., 2004; Rosenberg et al., 2007; Marhaver et al., 2008). This microbial community is a source of nutrients, photosynthesis products, nitrogen fixation, and in some cases provides resistance against infection that confer benefits to the coral host (Rosenberg et al., 2007). Similarly to the mechanisms of symbiont “shuffling” or “switching” that can take place during and after bleaching events, emerging hypotheses known as the “Coral Probiotic Hypothesis” (Reshef et al., 2006) and the “Hologenome Theory of Evolution” (Rosenberg et al., 2007) stipulate that adaptive responses also exist within the coral holobiont where a recombination of the symbiotic microorganism populations can occur under environmental pressure, potentially leading to a more advantageous partnership for the host. Evidence is growing that such mechanisms can occur under environmental stress leading to bleaching (Bourne et al., 2008). This switch in the microbial population allows for a much faster adaptation than mutation and selection that take place over many years and generations (Reshef et al., 2006). However, mutualism is not the only outcome of the coral holobiont combination as a shift within the microbial community can sometimes lead to disease and hence compromise the health of the whole animal (Bourne et al., 2009).

The production of dimethylated sulphur compounds in the marine environment

*Dimethylsulphide (DMS), the main source of marine sulphate aerosols*

Although DMS is a trace sulphur compound, it is the most abundant volatile sulphur species in seawater and is the largest input of biogenically derived sulphate aerosols into the marine boundary layer (Andreae and Crutzen, 1997; Liss et al., 1997). DMS is mainly produced by either cleavage of dimethylsulphonio propionate (DMSP) or reduction of dimethylsulphoxide (DMSO), which are both biogenically produced through the marine sulphur cycle (Turner et al., 1988; Hatton et al., 1996). When sea-air exchange occurs, due to chemical and physical factors such as wind and wave action (Liss and Merlivat, 1986; Wu, 1996; McGillis et al., 2000), part of the dissolved pool of oceanic DMS, approximately 10% (Malin et al., 1992), diffuses to the atmosphere where it is oxidised to sulphur dioxide (SO₂) that can further oxidise to sulphuric acid (H₂SO₄), which can then condense into sulphate (SO₄²⁻) aerosol particles (Andreae et
al., 1985; Andreae et al., 2003). These non sea-salt (nss) aerosol particles can, when combined with ultra-fine sea-salt and marine primary organic aerosols (Hawkins and Russell, 2010), seed cloud condensation nuclei (CCN), which can contribute to the formation of clouds (Shaw, 1983) (Figure 2). Although nss sulphate aerosols can also have an anthropogenic origin over the continent, they are mainly sourced from biogenically produced DMS over the remote and unpolluted oceans (Andreae et al., 1983; Falkowski et al., 1992). Natural fluxes represent about 50% of the total gaseous sulphur fluxes to the atmosphere, with the natural sulphur cycle still dominating in the Southern Hemisphere (Charlson et al., 1987).

Figure 2: Schematic illustration the chemistry of sea-to-air DMS fluxes.

*Dimethylsulphoniopropionate, the main precursor of DMS*
DMSP constitutes the predominant source of DMS (Liss et al., 1997) and it is mostly found in photosynthetic organisms such as higher plants and various species of marine algae (Keller et al., 1989; Malin and Kirst, 1997; Jones et al., 1998a; Stefels, 2000), although not in every taxa (Archer et al., 2002). However, a recent study has
demonstrated that DMSP was produced by both the endosymbiotic microalgae and host tissue in zooxanthellate corals, which represents the first evidence of DMSP production within the animal kingdom (Raina et al., 2013). The cleavage of DMSP that generates DMS happens either through enzymes known as DMSP-lyases that are encoded by different genes so far identified as dddL and dddP (Todd et al., 2009; Kirkwood et al., 2010) or through other distinct enzymatic pathways such as the one involving the dddD gene (Todd et al., 2007). These “DMSP dependant genes are most likely widespread among marine organisms as DMSP-lyase activity and other DMSP metabolic pathways have been found in phytoplankton (Stefels and Dijkhuizen, 1996; Niki et al., 2000), macroalgae (Steinke and Kirst, 1996; Malin and Kirst, 1997), bacteria (Desouza and Yoch, 1995a; b) and fungi (Bacic et al., 1998; Bacic and Yoch, 1998). DMSP-lyases are believed to be membrane-bound and located extracellularly (Stefels and Dijkhuizen, 1996; Steinke and Kirst, 1996), although little is known about the exact operational mode of these proteins, especially in light of the recent genetic discoveries that have highlighted the existence of several classes of DMSP-lyases. The vast diversity in metabolic pathways in DMSP metabolism also shows that DMSP constitute a rich source of volatile sulphur and labile carbon for the marine microbial food web.

_Dimethylsulphoxide, both a precursor and end-product of DMS_

DMSO is another precursor of DMS when reduced in marine algae and sulphate-reducing bacteria through a poorly-understood reduction pathway that might depend on DMSO reductases (Spiese et al., 2009). DMSO was for the first time reported in the marine environment in 1980 (Andreae, 1980; Andreae et al., 1983) and for a long time, the only source of DMSO in seawater was considered to be from the photo-oxidation of DMS in the euphotic zone of the water column according to the following equation:

\[
2 \text{DMS} + 1 \text{O}_2 \rightarrow 2 \text{DMSO} \quad \text{(Brimblecombe and Shooter, 1986)}
\]

However, further studies have shown that bacteria could also play an important role in the oxidation of DMS to DMSO in seawater (Zeyer et al., 1987; Taylor and Kiene, 1989; Visscher and Vangemerden, 1991; Zhang et al., 1991). More recent studies have demonstrated the presence of particulate DMSO in phytoplankton (Simó et al., 1998;
Lee and de Mora, 1999a; Hatton and Wilson, 2007), indicating that DMSO is also biogenically produced, possibly through DMS oxidation in the presence of ROS (Sunda et al., 2002). Decreasing dissolved DMSO concentrations in coastal waters during winter could therefore be explained by a decrease in photo-emission as well as from reduced phytoplankton and microbial activity (Lee and de Mora, 1999b). Being both a precursor of DMS and an end product of DMS oxidation, DMSO constitutes a key compound in the marine sulphur cycle that should deserve more attention in future research.

The environmental roles of dimethylated sulphur compounds

_DMS, a potential climate regulator_

Shaw (1983) formulated the hypothesis that the formation of biogenic sulphur aerosols that are sourced from the oxidation of sulphur gases in the atmosphere could potentially affect the climate by altering the Earth’s albedo and thus cause cooling. In 1987, four authors named Robert Charlson, James Lovelock, Meinrat Andreae and Stephen Warren formulated an hypothesis, now better known as the CLAW hypothesis (which is an acronym of the authors’ surnames), whereby biogenic marine DMS, as the major source of CCN over the oceans, could be involved in a negative feedback effect on elevated temperature and sunlight (Charlson et al., 1987). Their theory was that global warming would increase the production of DMS derived from phytoplankton, which would increase atmospheric DMS emissions and CCN formation in the marine boundary layer, increasing the cloud albedo over the ocean and reducing the effect of solar radiation and temperature on phytoplankton, with this cooling in turn leading to reduced DMS production through a climate feedback loop.

Under the current climate change scenarios, DMS could potentially help counteract the adverse effects of CO₂ and other greenhouses gases that are accumulating in the atmosphere at an unprecedented rate (Charlson et al., 1987). Interestingly, the melting of Antarctic sea ice has been shown to release substantial concentrations of DMS and DMSP in the water column, that could constitute a dissolved pool for potential DMS emissions to the atmosphere (Trevena and Jones, 2006), a phenomenon that once again
could support a feedback loop on global warming. Similarly, high DMSO cellular permeability has been associated with high temperature (Liu et al., 1997) indicating that the pool of dissolved DMSO is increasing with increasing temperature, constituting another source for potential atmospheric DMS emissions, with a possibly enhanced negative feedback effect on temperature. However, the CLAW hypothesis is still being debated (Quinn and Bates, 2011) as ongoing laboratory-based research and modelling studies over the past twenty years (Ayers et al., 1991; Prospero et al., 1991; Hertel et al., 1994) have failed to validate a direct biological control of DMS over cloud formation in the open ocean. Even though an absence of evidence does not constitute a refutation of the hypothesis, it nevertheless highlights the complexity of the biochemical loop and atmospheric processes on which it relies.

The multi-functionality of dimethylated sulphur compounds

Trends in the geographical and seasonal distribution of dimethylated sulphur compounds (DSC) suggest that ecological factors such as temperature, pH, salinity and/or light availability could influence DSC production in the marine environment (Turner et al., 1988; Van Alstyne and Puglisi, 2007; Vila-Costa et al., 2008). Experimental studies have reported that DMSP and DMS concentrations could directly be affected by salinity (Reed, 1983; Vairavamurthy et al., 1985; Dickson and Kirst, 1986), temperature (Kirst et al., 1991; Karsten et al., 1992), pH (Archer et al., 2013; Arnold et al., 2013), light and nitrogen availability (Keller and Korjef-Bellows, 1996; Slezak and Herndl, 2003; Harada et al., 2009). In light of these observations, DMSP has been hypothesised to serve multiple physiological and biological functions including osmo- and thermo-regulation (Stefels, 2000). Other studies have also shown that DMSP and DMS could act as a chemical defence for marine plankton and macroalgae against herbivores (Wolfe et al., 1997; Van Alstyne et al., 2001; Van Alstyne and Houser, 2003), but also as a chemo-attractant in a whole range of marine species (DeBose et al., 2008; Seymour et al., 2010; Knight, 2012). Based on its physicochemical properties and on several experimental studies, DMSO is also believed to act as a cryoprotectant and osmo-regulator (Simó et al., 1998; Lee and de Mora, 1999a). Finally, an antioxidant function has been proposed for all three dimethylated sulphur compounds (Lee and de Mora, 1999a), which are considered to actively scavenge ROS (Sunda et
In the presence of free oxygen radicals, DMS and DMSP are expected to oxidise to DMSO, which can further oxidise to methane sulphinic acid (MSNA). Increased DMSP production has been associated with increased ROS levels in *Symbiodinium* (McLenon and DiTullio, 2012) and increased ascorbate peroxidase activity in marine algae, the main antioxidant in chloroplasts (Sunda et al., 2002). DMSP and DMSO concentrations were also positively correlated with the antioxidant beta-carotene in upwelled seawater (Riseman and DiTullio, 2004), supporting the antioxidant hypothesis for DMS and its precursors. This DMSP-based antioxidant system is now thought to play an important role in the complex antioxidant system of marine algae, with each compound possibly acting at various rates and reacting preferentially with different ROS (Sunda et al., 2002).

**The turnover of dimethylated sulphur compounds in the marine sulphur cycle**

As cell membranes are impermeable to charged ions and since DMSP is a zwitterion, carrying both a positive and a negative charge, it is very unlikely that DMSP leaks through cell membranes during normal metabolism (Dacey and Blough, 1987). However, significant pools of free DMSP have been found in seawater (Wakeham et al., 1987; Turner et al., 1988; Trevena et al., 2000; Trevena et al., 2003; Trevena and Jones, 2006). Although particulate DMSP concentrations might have been overestimated due to non-optimised filtration techniques used at the time (see Kiene and Slezak, 2006), these results suggest that part of the particulate (intracellular) DMSP pool is released into the water column. This most likely occurs when there is a loss of cell integrity during either cell lysis or phytoplankton grazing (Dacey and Wakeham, 1986; Bratbak et al., 1995). For that reason, DMSP has been described as a good biomarker of cell breakage during grazing (Kiene and Slezak, 2006). It has been suggested that viruses may also be involved in the lysis of algae and phytoplankton cells, which indicates that intense microbial activity can exacerbate the release of intracellular DMSP into the water column (Malin et al., 1992; Bratbak et al., 1995). Once in seawater, DMSP constitutes an important carbon source for bacteria that can degrade this sulphur compound into DMS, however, only a small proportion of DMSP is cleaved into DMS through the microbial cycle (Visscher et al., 1992; Ledyard and
DMSP has also been shown to form DMS in the presence of hydroxide ions (Dacey and Blough, 1987). However, at the pH of seawater, which is currently ranging from 7.9 – 8.3 in the open ocean (IPCC, 2007), the half life of DMSP was estimated at 8 years, indicating that hydroxylation is unlikely to be the major pathway for DMS production in seawater, which most likely relies again on DMSP-lyase activity.

There is a contradiction in the literature in regards to the relative concentrations of DMSO and other dissolved dimethylated sulphur species, as several studies show that DMSO is present in the water column in greater concentration than DMS or DMSP (Hatton et al., 1996; Simó et al., 1997; Broadbent and Jones, 2006), whereas other results have shown that levels of both DMS and DMSP were greater than that of DMSO (Gibson et al., 1990; Lee and de Mora, 1999a). Such differences in the relative abundance of DMSO have been explained by diurnal and seasonal variations of DMSO (Lee and deMora, 1996). Due to its biochemical property as a dipolar aprotic hydroscopic substance, DMSO is known to permeate easily through membranes of healthy cells and into the surrounding seawater (Hatton and Wilson, 2007), which is possibly another reason why relative DMSO and DMSP concentrations are more difficult to estimate. This might also be the reason why particulate DMSO had not been detected earlier in marine organisms. Once in seawater, DMSO can be reduced to DMS during microbial activity that previously played a role in the DMSO production (Zinder and Brock, 1978; Jonkers et al., 1996; Van Bergeijk and Stal, 1996). DMS is an uncharged, volatile and hydrophobic compound that is therefore believed to diffuse through cell membranes much more easily than DMSP and DMSO (Sunda et al., 2002).

In light of the literature review, the complex redox loop that is believed to occur between dimethylated sulphur compounds as part of the marine sulphur cycle is summarised in Figure 3.
Reef building corals: a unique platform for DMSP production and atmospheric DMS emissions

Evidence through time
Inspired by the discovery of high concentrations of aerosol particles over the GBR (Bigg and Turvey, 1978) that could play a role in the CLAW hypothesis (Charlson et al., 1987), an expedition was carried out in the South Pacific Ocean to measure the concentrations of dissolved DMS and DMSP in reef waters, together with atmospheric DMS concentrations (Jones et al., 1994). Levels of both DMS and DMSP were found to increase with proximity to reefs, indicating that coral reef ecosystems might be a potential source for DMSP production and atmospheric DMS emissions. In parallel, this research reported for the first time that coral tissue of Acropora spp. collected from the GBR contained significant amounts of DMSP (Broadbent, 1993; Jones et al., 1994). However, the cycling of dimethylated sulphur compounds in lagoon waters was first interpreted as a consequence of coral production, grazing, phytoplankton growth and
benthic microalgae activity, and the dominance of one source over the other was not
established (Broadbent and Jones, 2006). Subsequently, other studies showed that
corals were a greater source of DMSP than benthic macroalgae (Broadbent et al., 2002)
and that coral mucous contained the highest concentrations of DMS and DMSP ever
found in the marine environment (Broadbent and Jones, 2004), suggesting that corals
were the predominant source of dimethylated sulphur compounds in coral reef waters.
Later research has shown that the high levels of atmospheric DMS measured over the
GBR, Coral Sea, Gulf of Papua, Salomon Sea, and Bismarck Sea were correlated with
areas of high coral reef biomass (Jones and Trevena, 2005), suggesting that coral reefs
around the world were a potential source for atmospheric DMS emissions.
Subsequently, a recent chamber experiment on isolated Acropora spp. was the first
study to establish a direct link between coral DMS production and its potential as a
source of atmospheric DMS emissions (Fischer and Jones, 2012). Finally, in situ
measurements of atmospheric DMS levels over the Heron Island reef flat, southern
GBR, has established in comparison with past research that coral reefs constitute a
greater source of atmospheric DMS emissions than temperate marine ecosystems
(Swan et al., 2012). Together, these observations led to the hypothesis that coral-
derived DMS could influence the climate on a local scale when emitted to the
atmosphere and oxidised to sulphate aerosols that could contribute to CCN formation,
eventually increasing the cloud-cover over coral reef waters (Jones and Ristovski,
2010) (Figure 4).
Figure 4: The negative feedback effect of coral-derived DMS on SST within coral reef ecosystems (modified from Jones and Gabric, 2006).

In 2009, the first reported aerosol particle formation events over the GBR were observed during hours of high solar intensity (Modini et al., 2009), which the authors hypothesised were sourced from high DMS emissions over the reef. However, no direct link between coral-derived DMS emissions and aerosol formation has been established to date. Pristine coral reefs within or near the Western Pacific Warm Pool have had fewer reported bleaching events relative to reefs in other regions, a phenomenon that could be due to an “ocean thermostat” mechanism that acts to depress warming beyond certain temperature thresholds (Kleypas et al., 2008). Over the past 50 to 60 years, SSTs in the warmest parts of the world oceans have increased to a lower extent than in other surrounding marine areas, suggesting that there is a threshold to SSTs. It was suggested that there could be a link between this phenomenon and abundant atmospheric DMS emissions sourced from reefs, with atmospheric DMS emissions contributing to enhanced cloud cover and cooling on a local scale (Fischer and Jones, 2012). A recent study has provided more evidence of a direct relationship
between cloud cover and SSTs over the GBR, with cloud cover being responsible for more than 30% of the variation in SSTs and SSTs being inversely responsible for more than 20% of the variation in cloud cover (Leahy et al., 2013). These correlations were found to be enhanced during strong El Niño and La Niña events, highlighting the presence of a cloud cooling effect on coral reefs under thermal and UV stress.

*Factors influencing DMSP and DMS production in coral reef waters*

DMS is oxidised by hydroxide radicals during daylight hours and by nitrate radicals at night (Staubes and Georgii, 1993). As nitrate radicals are less effective oxidants than hydroxide radicals, it is usually expected that a build-up of atmospheric DMS occurs at night as reported for the Atlantic, Arctic and Antarctic regions (Staubes and Georgii, 1993). However, an increase in atmospheric DMS and dissolved dimethylated sulphur compounds was reported during mid-day in reef waters of the GBR during the summer months (Broadbent and Jones, 2006), suggesting that DMS production in coral reef waters relies on benthic primary productivity through photosynthesis, with this process being highly influenced by light and sea surface temperatures. This observation was later confirmed by similar research that showed that dissolved DMS and DMSP concentrations in the GBR were positively correlated with SSTs (Jones et al., 2007).

Tide is another factor that has been shown to influence concentrations of dissolved DMS and DMSP in coral reef waters (Broadbent and Jones, 2006), with low tides that exposed the reef to the atmosphere resulting in enhanced atmospheric DMS emissions (Jones and Trevena, 2005; Jones et al., 2007; Swan et al., 2012).

Based on the assumption that the expulsion and lysis of zooxanthellae would result in an increase in dissolved DMSP and DMS in reef waters (Jones et al., 1994; Hill et al., 1995), it was hypothesised that heat-stress associated coral bleaching might result in enhanced atmospheric DMS emissions, potentially leading to a negative feedback effect on elevated temperature on a local scale. However, field-based and chamber experiments have shown that dissolved DMS production in reef waters decreased as a consequence of thermal stress beyond bleaching thresholds (Jones et al., 2007), which could possibly result in a shut down in atmospheric DMS emissions (Fischer and Jones, 2012). This would potentially result in decreased aerosol formation and enhanced
therm and UV stress, eventually exacerbating coral bleaching. In agreement with Sunda et al. (2002) who first showed that DMS and DMSP were active ROS scavengers in marine phytoplankton, Jones et al. (2007) hypothesised that the decrease in DMS production by corals under bleaching conditions might be linked to the use of DMS and DMSP as antioxidants in coral tissue during stress exposure. **However, no conclusive study on the exact role of dimethylated sulphur compounds in corals exposed to environmental stress has been conducted to date.**

DMSP production in corals: a complex and multi-partner mechanism

In plants, algae, bacteria, and fungi, DMSP is known to be synthesized from methionine through at least three different biochemical pathways (Stefels, 2000; Wirtz and Droux, 2005). Cysteine is the first sulphur amino acid sourced from assimilatory sulphate reduction (Charlson et al., 1987) and can be converted into homocysteine, which can subsequently be methylated to methionine, eventually leading to DMSP formation (Stefels, 2000). Whereas photosynthetic organisms, bacteria and fungi can synthesize cysteine and methionine, animals usually acquire these amino acids through their diet or via symbiosis (Wirtz and Droux, 2005). DMSP was thus presumed to be exclusively synthesised by photosynthetic organisms at the time this sulphur compound was first reported in coral tissue (Jones et al., 1994; Hill et al., 1995). Consequently, DMSP concentrations in corals were often expressed as per zooxanthellae or cell biovolume, assuming that the endosymbiotic algae were the sole source of DMSP in corals. Evidence through time tends to support this assumption as amounts of DMSP per zooxanthellae biovolume were found to be similar to those of free-living dinoflagellates in three different reef coral species (*Montipora verrucosa, Pocillopora damicornis*, and *Porites compressa*) (Hill et al., 1995), and intra-specific differences in DMSP concentrations in corals were found to be linked to the zooxanthellae morphology (Broadbent et al., 2002). Another study conducted on DMSP among various species of cnidarians showed that DMSP concentrations always correlated with the symbiont density, indicating that DMSP was sourced from the symbiosis (Van Alstyne et al., 2006). In 2009, a comparative study conducted on zooxanthellate and aposymbiotic anemone species (that do not harbour zooxanthellae) showed that DMSP was exclusively present in zooxanthellate specimens, providing evidence that the symbiont
was the sole source of DMSP in *Aiptasia pallida* (Van Alstyne et al., 2009), which suggested that this phenomenon would most likely be applicable to other symbiotic cnidarians. However, contradictions are also found in the literature since DMSP concentrations found in lab cultures of coral-isolated zooxanthellae were lower compared to concentrations found in hospite, suggesting that polyps also contain DMSP that they either produce or accumulate from prey (Jones et al., 1994; Hill et al., 1995). Relative measurements of DMSP concentrations in endosymbiotic zooxanthellae and animal tissue revealed that important portions of DMSP were contained in the animal fraction, which some authors interpreted as a translocation from the symbiont to the host (Yost et al., 2012). The recent discovery by Raina et al. (2013) has challenged outcomes of past research by showing that not only endosymbiotic zooxanthellae but the animal host could produce DMSP in zooxanthellate reef-building corals. These authors provided genomic-based evidence that the coral host possesses key genes involved in the enzymatic synthesis of DMSP, with these genes possibly being derived from horizontal gene transfer or ancient coding DNA. Aposymbiotic corals had long ago been found to have the ability to synthesize methionine (Fitzgerald and Szmant, 1997), although it is not yet known if this is by the same pathway as used by photosynthetic organisms (Van Alstyne et al., 2009) nor if methionine is the best candidate precursor for DMSP production by the coral host.

More recent studies have shown that different clades and sub-clades of endosymbiotic zooxanthellae could produce different concentrations of DMS and DMSP (Yost and Mitchelmore, 2009; Steinke et al., 2011), which indicates that the coral capacity to produce dimethylated sulphur compounds relies on the type of symbiont they associate with. *However, it is not known how DMS and DMSP production among different clades of Symbiodinium would vary under environmental stress.* DMSP has also been shown as an important source of carbon for coral-associated bacteria and could even influence their preferential association with the coral host (Raina et al., 2009; Raina et al., 2010). Since bacteria are present at high concentrations in coral tissue and mucous, the production and turnover of DMS and DMSP in corals are most probably influenced by the presence of symbiotic microorganisms, and supposedly vary depending on the type of microbial population. Another study also revealed that certain types of coral-
associated fungi contained a gene encoding for a type of DMSP-lyase and therefore had the capacity to convert DMSP into DMS (Kirkwood et al., 2010). Although authors suggested that the opportunistic pathogen had acquired this gene from proteo-bacteria by horizontal gene transfer, their results suggest that DMSP consumption and DMS production can be affected by the presence of fungi in corals. Together these observations make the coral holobiont a complex and unique platform for the production and turnover of dimethylated sulphur compounds.

**Aims and objectives**

Based on the knowledge gaps that were highlighted in the literature (bold and italic) in regards to the marine biogeochemical sulphur cycle within coral reef ecosystems, this research has aimed to address the following objectives:

1) Provide a better understanding of the production of DMSO by corals, a key dimethylated sulphur compound in the marine sulphur cycle that remains poorly understood to date.

DMSO in the coral holobiont, like in other marine algae, is believed to be both a precursor of DMS, when reduced through enzymatic processes (Spiese et al., 2009), and an end-product of DMS oxidation during ROS scavenging linked to oxidative stress (Sunda et al., 2002). Therefore, this dimethylated sulphur compound is a significant source and sink for DMS and it constitutes a key element in the complex redox loop that composes the biogenic sulphur cycle of corals and marine algae. However, little is known about the production, role and turnover of this key sulphur compound.

2) Evaluate the potential of coral-derived DMS emissions to lead to CCN formation in coral reef ecosystems

It has been established that corals had the potential to produce DMSP and DMS, with dissolved DMS production resulting in high atmospheric DMS emissions over coral
reefs, especially during the day and during low tides that expose the reef to the atmosphere (Jones et al., 1994; Jones and Trevena, 2005; Jones et al., 2007). Marine biogenic DMS is known to contribute to the formation of sulphate aerosols that are CCN precursors and therefore could possibly lead to a cooling effect on a local scale (Charlson et al., 1987). Although high aerosol particle formations events have been observed in proximity to coral reefs (Bigg and Turvey, 1978; Modini et al., 2009), no direct link has been established between coral-DMS production and aerosol particle formation.

3) Investigate the role of dimethylated sulphur compounds as antioxidants in the coral holobiont.

Dimethylated sulphur compounds are believed to act as antioxidants in corals and marine algae by actively scavenging ROS that accumulate under oxidative stress, with DMSP and DMS possibly oxidising to DMSO, which can get further oxidised in the presence of ROS (Sunda et al., 2002). Given that the fate of DMS and other dimethylated sulphur species might be to preferentially oxidise in response to oxidative stress, it could explain the decrease in DMS production and the possible shut-down in atmospheric DMS emissions in corals when they are exposed to temperatures beyond bleaching thresholds (Jones et al., 2007; Fischer and Jones, 2012). However, the biochemical role of dimethylated sulphur compounds in corals is still to be assessed.

4) Examine the production of dimethylated sulphur compounds by the coral holobiont in response to environmental stress in order predict variations in the biogeochemical marine sulphur cycle under future climate change scenarios.

Coral reefs are facing increased stress associated with anthropogenic climate change, ocean acidification pollution and overfishing, leading to alarming trends in coral decline worldwide (Hoegh-Guldberg et al., 2007). These ecosystems are not only important for their biodiversity, biomass abundance and economical values (Harrison and Booth, 2007), but they might also be a significant source of atmospheric DMS emissions that could be part of a negative feedback on global warming (Jones and
Ristovski, 2010). Consequently, the loss of coral reef ecosystems under increasing human pressures could not only be dramatic in ecological and financial terms but could also exacerbate climate change. Understanding how the coral production of DMS and other dimethylated sulphur compounds will vary in response to environmental stressors is therefore essential to assess and predict the future of coral reefs under imminent climate change.

5) Compare the relative production of dimethylated sulphur compounds among different clades of *Symbiodinium* that exhibit different thermal tolerance in response to thermal stress.

Different clades of dinoflagellate algae of the genus *Symbiodinium* that associate with corals are known to react differently to environmental variables such as temperature, providing the corals with different resistance to environmental stress (Buddemeier et al., 2004). It has been established that different DMSP concentrations and different DMSP-lyase activity, could be found across different clades of *Symbiodinium* (Yost and Mitchelmore, 2009; Steinke et al., 2011; Yost and Mitchelmore, 2012). Although no link could be established between DMS(P) concentrations and thermo-tolerance in *Symbiodinium* under basal conditions (Steinke et al., 2011), it is not known how DMS(P) production can vary in *Symbiodinium* clades that exhibit different thermal tolerance as a response to thermal stress.

**Study sites**

This study was conducted at two locations: the Heron Island reef, southern GBR region, Australia, which is where most coral collections were undertaken (Chapters 3-6), and Nelly Bay reef, Magnetic Island, central GBR region, Australia, where specimens used in Chapter 2 were collected.

Heron Island is a coral cay of 800 m x 300 m that is located in the southern GBR, approximately 80 km off the Queensland coast (Figure 5). Heron Island is surrounded by a 26 km² reef lagoon of which about a quarter is dominated by *Acropora* corals.
(Scopelitis, 2011). The Heron Island reef is managed by the GBR Marine Park Authority (GBRMPA), which regulates collection of specimens from the Scientific Research Zone (Figure 5 – shaded area) following the granting of a scientific permit for coral collection.

Nelly Bay reef is a fringing reef located on the southeast shoreline of Magnetic Island, which is a 52 km² continental island from the central GBR, located 8 km off the Queensland coast (Figure 5). Coral collections at Nelly Bay are also managed by GBRMPA and require a permit.
Figure 5: Map of Heron Island and Nelly Bay reefs located in southern and central GBR respectively. The shaded area around Heron Island represents the Scientific Research Zone where coral collection can be undertaken appending permit acquisition.
Coral specimens

*Acropora* coral species collected for this research were *A. tenuis* (Figure 6), *A. millepora* (Figure 7), *A. aspera* (Figure 8) and *A. pulchra* (Figure 9). Collection methods are described throughout Chapters 2-6.

**Figure 6:** Photos of the five colonies of *Acropora tenuis* used for isolating clade C1 *Symbiodinium* as described in Chapter 2.

**Figure 7:** Photo of the three colonies of *Acropora millepora* used for isolating clade D1 *Symbiodinium* as described in Chapter 2.
Figure 8: Photo of nubbins of *Acropora aspera* branches as described in Chapter 4 and 5.

Figure 9: Photos of *Acropora pulchra* in bubbling chamber experiment before (A) and after bubbling was applied (B) as described in Chapter 6.
CHAPTER 2

Comparative response of DMS and DMSP concentrations in *Symbiodinium* clades C1 and D1 under thermal stress

Unformatted published paper is shown in Appendix 3

Abstract

Coral-associated *Symbiodinium* are known to produce DMS and DMSP, two biogenic sulphur compounds that potentially play a role in the algal antioxidant system and climate regulation. Coral resistance to bleaching and oxidative stress partly depends upon the capacity of their symbionts to respond to environmental stressors, with DMS and DMSP possibly contributing to this response. Under increasing sea surface temperatures, zooxanthellate corals might dominantly associate with more thermally tolerant clades of *Symbiodinium*, of which the nutritional and biochemical contribution to the holobiont remains unknown. Here, the production of DMS and DMSP under increasing temperature (from 26°C to 31°C) was investigated over 6 days in axenic cultures of two *Symbiodinium* clades (C1 and D1) that are known to exhibit different thermal tolerances. Regardless of the temperature treatment, clade C1 was a greater producer of DMS and DMSP. An increase in temperature up to 5°C did not substantially affect DMS and DMSP production in the thermo-tolerant clade D1. However, thermal stress induced enhanced consumption of both DMS and DMSP in the more thermally-sensitive clade C1, potentially indicating the use of sulphur compounds as antioxidants. Together, these results suggest that thermal stress differentially affects the biogenic sulphur cycle of *Symbiodinium* clades that exhibit different thermal tolerances, with possible consequences for reef-building corals under future climate change scenarios.
Introduction

DMS is the largest input of biogenically derived sulphur into the marine boundary layer (Liss et al., 1997). Although still controversial (Quinn and Bates, 2011), DMS is suspected to take part in a climate feedback that lowers global temperatures through its oxidation into sulphate aerosols that acts as precursors for CCN, which in turn increase cloud albedo (Charlson et al., 1987). DMS is mostly synthesised through the enzymatic cleavage of its main precursor, dimethylsulphoniopropionate (DMSP), in various species of marine algae including coral-associated Symbiodinium (Yost and Mitchelmore, 2009). Zooxanthellate reef-building corals have been found to produce high amounts of DMS (Broadbent and Jones, 2004; Broadbent and Jones, 2006; Fischer and Jones, 2012), with high levels of sulphate aerosols over reefs (Modini et al., 2009), indicating that coral reefs could be significant sources of CCN precursors over the GBR (Deschaseaux et al., 2012; Swan et al., 2012), potentially affecting local climate.

The conversion of DMSP into DMS can be catalysed by several types of enzymes so far identified as DMSP-lyases (Kirkwood et al., 2010) or acyl CoA transferase (Todd et al., 2007), that have been found in phytoplankton (Stefels and Dijkhuizen, 1996), macroalgae (Steinke and Kirst, 1996), Symbiodinium (Yost and Mitchelmore, 2009), bacteria (Desouza and Yoch, 1995a), and/or fungi (Bacic and Yoch, 1998), making DMSP a considerable source of sulphur and carbon, not only for photosynthetic species that synthesise it but also for opportunistic organisms. The various pathways through which DMSP can be metabolised highlight the complexity of the dimethylated sulphur cycle. Specific coral associated bacteria have been found to play an essential role in the conversion of DMSP into DMS (Raina et al., 2009), with both sulphur compounds being involved in structuring bacterial communities in corals (Raina et al., 2010). Thus, the production and turnover of DMS and DMSP in the coral holobiont rely on complex interactions between the host, symbionts and microbial populations.

Nine phylogenetic clades of Symbiodinium, indexed from A to I, have now been identified, with each containing many subclades (Pochon and Gates, 2010). Depending on the type of Symbiodinium they associate with, zooxanthellate corals can potentially
respond differently to environmental stress (Buddemeier et al., 2004). Thus, coral resistance to bleaching, which corresponds to a breakdown of the symbiosis (Iglesias-prieto et al., 1992), depends upon the type of coral-symbiont combination. Under predicted climate change scenarios (IPCC, 2007), coral reefs might become dominated by more thermally tolerant symbionts such as clade D *Symbiodinium* as a means of adaptation (Jones et al., 2008). However, little is known about the benefits and drawbacks of such symbiosis against the association with the presently dominant and more thermally sensitive clade C (Stat and Gates, 2011). Recent studies have highlighted that the association with more thermally tolerant clade D could potentially increase coral susceptibility to diseases (Littman et al., 2010) and slow down growth rate (Abrego et al., 2009).

Temperature is one of the main stressors associated with coral bleaching (Douglas, 2003), which is known to be intimately linked with oxidative stress and the build up of ROS in the coral holobiont (Lesser, 1996; Downs et al., 2002). As a protective mechanism against oxidative damage, a wide range of antioxidants (e.g. catalase, superoxide dismutase, glutathione-S-transferase, ascorbate peroxidise, etc) is produced in corals by either the host or the symbionts (Ramos and Garcia, 2007). DMS and DMSP have been shown to actively scavenge ROS in marine algae (Sunda et al., 2002), which implies that they could be playing an important role in the coral holobiont under bleaching conditions. Although concentrations of DMS and DMSP have been shown to be strain-specific in cultures of *Symbiodinium*, no clear trend with thermo-tolerance has been established (Yost and Mitchelmore, 2009; Steinke et al., 2011). Moreover, the capacity of *Symbiodinium* clades with different thermal tolerances to produce DMS and DMSP under thermal stress has not yet been investigated. In this study, we hypothesised that the symbiont resistance to increasing temperature could be linked to its capacity to produce DMS and DMSP. We therefore measured the production of DMS and DMSP under thermal stress in two clades of *Symbiodinium* (C1 and D1) that exhibited different thermal tolerances with the aim to test this hypothesis. Baseline temperature was similar to *in situ* temperature at the time of collection (26°C) and the temperature range was selected (26°C to 31°C) as it fitted within the worldwide bleaching thresholds (Jokiel and Brown, 2004) and severe climate change scenarios (IPCC, 2007). In order to focus
exclusively on the synthesis of DMS and DMSP by coral-associated _Symbiodinium_, it was decided that this study should be conducted in axenic conditions using antibiotics.

**Material and methods**

*Field collection, tissue extraction and cultures*

Colonies of _Acropora millepora_ (three) and _Acropora tenuis_ (five) were collected in shallow waters of Nelly Bay reef, Magnetic Island, Townsville, QLD (19°09.777’S/146°51.227’E) in October 2011. Coral colonies were brought back to shore in seawater and kept in a flow-through seawater holding tank for four days until processed. Coral colonies were carefully washed under deionised water for less than 1 min to remove macroalgae and other organisms that could have sheltered within the coral branches. Tissue from each colony was extracted by air blasting in 150 mL of sterile filtered (0.2 µm) seawater (FSW). Tissue slurries were sub-sampled for genotyping and sub-samples were kept at –20°C until analysis. Remaining slurries were filtered through a 60 µm mesh to remove coarse coral tissue, mucous and big pieces of skeleton. Lysates were homogenized using a tissue homogenizer (model Pro250, Perth Scientific Equipment) at 17,000 rpm for 2 min and filtered a second time using the same 60 µm mesh filter. Homogenates were centrifuged twice at 1,600 g for 7 min to remove excess coral tissue. Resuspended zooxanthellae from _A. millepora_ as well as from _A. tenuis_ were each combined and inoculated into 4.8 L of sterile IMK media (Wako Chemicals USA, Richmond, VA, USA) to represent the two initial stock cultures. The cell density of initial stock cultures was determined by performing 16 random counts on a haemocytometer and the culture with the greatest cell density was diluted for standardisation. The cell density of final stock cultures was determined at the start of the experiment using the same technique following dilution and was 1.47 x 10^5 cells mL^-1 for _A. millepora_ and 1.21 x 10^5 cells mL^-1 for _A. tenuis_, which was within the normal range for optimal cell growth (Yost and Mitchelmore, 2009). Antibiotics (neomycin, penicillin, streptomycin, nystatin, 100 µg mL^-1) and germanium dioxide (GeO₂) (50 µmol L⁻¹) were added to the stock cultures (Santos et al., 2011) in order to obtain axenic and unialgal cultures, respectively (Divan and Schnoes, 1982; Fernandes et al.,
2011). Each symbiont culture was also sub-sampled for genetic clade identification and sub-samples were kept at – 20°C until analysis.

**Genotyping**

Cultured sub-samples were thawed and centrifuged at 10,000 g for 10 min, and the supernatant discarded. A scoop of acid-washed glass beads and 200 µL of Milli-Q water were added to each pellet and samples were disrupted for 1 min using a mini beadbeater (biospec products, Australia). DNA was isolated using a commercial kit (Plant DNA Mini Kit, BIOLINE) following manufacturer’s instructions. DNA from each culture was used as a template to amplify the ITS1 region using zooxanthellae specific primers (van Oppen et al., 2001). Amplicons were separated and visualized, along with known standards, using single-stranded conformation polymorphism (SSCP) assay. *A. millepora* and *A. tenuis* isolated clades were found to be of genotype D1 and C1, respectively, as expected from previous research at this location (Van Oppen et al., 2001; Beltran et al., 2012).

**Study design**

Each stock culture was divided into six volumes of 800 mL and placed into individual 1 L hermetic chambers leaving a 200 mL headspace. Chambers were designed so lids had one air inlet and two outlets: one being connected to gold-wool tube to trap gaseous DMS that was emitted to the chamber headspace (Kittler et al., 1992) and one connected to an on/off valve for liquid sampling of DMS and DMSP in the cultures (Figure 10). Only the headspace was mixed using an air pump to avoid mechanical disturbance of the *Symbiodinium* cultures which could have occur by aerating the water column (Rogers and Davis, 2006). However, the air stream that agitated the surface of the cultures was simulating wind action as naturally occurring in the environment. Brand new tubing was used for aeration in an effort to maintain sterility. Two temperature treatments were applied throughout the experiment: a control treatment (where the temperature was maintained at 26°C) and an experimental treatment (where the temperature was ramped from 26°C to 31°C with a 1°C increase per day). Chambers were distributed so clades D1 and C1 were in triplicate in each temperature treatment. A chamber containing 800 mL of IMK media was included with the increasing
temperature treatment to serve as an analytical blank for both DMS and DMSP. A 12:12 h Light:Dark (L:D) cycle was applied throughout the entire experiment with an average light intensity of 40 ± 5 μmol photons.m⁻².s⁻¹ measured between the surface and bottom of chambers during light exposure. Such levels of light intensity were selected because (i) they have been proven to efficiently maintain C1 and D1 in culture for extended periods of time, and (ii) they are similar to what a coral from Magnetic Island will experience due to the proximity to the shore that makes most part of the year a turbid environment.

**Figure 10:** Design of hermetic chambers used for continuous gas measurements and aqueous sampling in *Symbiodinium* cultures.

*Sampling and preservation*

At the end of each 12 h L:D cycle, the air pump was turned off and the gold tubes were collected, capped with aluminium foil, sealed with Para Film and stored in dark ambient conditions in air-tight containers until analysis for gaseous DMS (DMS₇) that was conducted within few weeks following the sampling. Clean regenerated gold tubes were connected to each chamber and the air pump was turned back on to collect further samples. The total air volume sampled from each chamber was estimated by
individually measuring the flow rate from each chamber using a high-precision digital air flow meter (Alltech, Deerfield, IL 60015, USA; P/N 4700) at the beginning and end of each sampling period. Cultures were manually stirred before each round of sampling by carefully shaking chambers so cells would be evenly distributed in solution. Chambers were sub-sampled through the water outlet for total DMSP (DMSP\textsubscript{t} = dissolved + particulate DMSP), dissolved DMSP (DMSP\textsubscript{d}) and dissolved DMS (DMS\textsubscript{w}), cell density and cell size measurements. DMSP\textsubscript{d} samples were carefully filtered by applying a “drop by drop” procedure through 0.45 µm syringe filters (Sartorius stedim biotech, Ministart, non-sterile). On a random round of sampling, DMSP\textsubscript{d} samples were also sampled a second time through Whatman GF/F filters using an all-plastic filtration tower until the first 3 mL were filtered by small-volume gravity drip filtration (Kiene and Slezak, 2006) in order to calculate the error factor of hand filtering versus gravity drip filtration across 12 samples. DMSP samples were acidified to pH < 2 with 10\% HCl and stored in amber glass bottles kept at ambient temperature, whereas DMS\textsubscript{w} samples were acidified and immediately sealed in crimp cap vials. Particulate or intracellular DMSP (DMSP\textsubscript{p}) concentrations were calculated by subtracting DMSP\textsubscript{d} from DMSP\textsubscript{t}. Cell density was determined from quadruplicate subsamples of 100 µL by measuring absorbance at 750 nm (Power Wave plate reader; Bio-Tek, Australia) as a proxy for the number of cells (Pomati et al., 2003). This method was calibrated against direct cell counts as previously described. The cell diameter of 20 random cells was determined under the microscope at a magnification of 200x using a stage micrometer.

**Analysis of sulphur compound**

Gaseous DMS trapped on the gold-wool tubes was quantified using a purge and trap assembly attached to a Varian gas chromatograph (GC) (CP-3800) fitted with a pulsed flame photometric detector (PFPD) and a dual eight-port/six-port two-position manual valve switching system (Swan, 2000). A permeation tube of methyl ethyl sulphide (MES, 115 ng mL\textsuperscript{-1} at 35°C, VICI Metronics, USA) was permanently connected to the eight-port valve and flushed with a constant flow of high purity helium (He) at 10 mL min\textsuperscript{-1}. A known concentration of MES was added as an internal standard onto each gold-wool tube by switching the eight-port valve onto either the small loop (0.4 mL) or
large loop (0.8 mL) position. Each gold-wool tube, connected via the six-port valve, was heated in a thermal desorber at 350°C for 25 min and the sample was collected in a cryogenic trap cooled in liquid nitrogen with a constant flow of He at a rate of 25 mL min⁻¹. After 25 min, the six-port valve was switched to the “inject” position and the cryogenic trap was transferred into a water bath at room temperature. The DMS and MES internal standard then passed via the GC column (BP1, 30 m x 0.32 mm x 4 µm film thickness) to the PFPD detector at a flow rate of 1.8 mL min⁻¹ of He. Chromatographic peaks were integrated using Star chromatography workstation software (version 6.41) and DMS concentrations were expressed relative to the volume of air sampled. Seven-point MES calibration curves were used for DMSᵣᵣ calibration at the beginning and end of each sample analysis (Appendix 1). DMSPᵥₒ samples were converted into DMS in crimped cap vials using 1 mL of 5 mol L⁻¹ sodium hydroxide (NaOH) per 3 mL of sample (Broadbent, 1993; Zhou et al., 2009; Yost and Mitchelmore, 2010). Vials were immediately crimped and samples were let to sit for a minimum of 20 min for the reaction to be complete. DMSPᵥₒ samples, along with DMSᵣᵣ samples, were analysed with a purge and trap technique on a Shimadzu GC (model 2014) fitted with a flame photometric detector (FPD) operated at 175°C, and a chromosil 330-packed GC column with 8 x 1/8 inch OD Teflon (FEP) tubing (Kiene and Gerard, 1994). Crimp-cap vials were sparged with He at a constant flow rate of 100 mL min⁻¹. The GC oven was used isothermally at 60°C with He as carrier gas at a flow rate of 25 mL min⁻¹. DMSP stock standard solution was made by diluting a known quantity of DMSP powder (DMSP·HCl analytical reagent of assumed 100% purity, Research Plus, Inc.) into acidified Milli Q water (final pH < 2). DMSP standards and blanks were prepared in the exact same way as DMSᵣᵣ and DMSPᵥₒ samples. Seven-point DMSP calibration curves and three blanks were run daily at the beginning and end of DMSᵣᵣ and DMSPᵥₒ sample analysis for calibration and contamination test, respectively (Appendix 1).

Statistical analysis

Prior to analysis, assumption of normality was tested for each variable and transformations were applied when necessary. A mixed effects model ANOVA with repeated measures was fitted individually to each variable, which included temperature,
clade and time as fixed factors, using SPSS Statistics 17.0. Bonferroni adjustments were applied for multiple comparisons. Filtration methods for DMSP\textsubscript{d} measurements were statistically compared using a two-tail paired \textit{t}-test. All data are expressed as mean ± SE.

\textbf{Results}

\textit{Method development for measurements of the dissolved DMSP (DMSP\textsubscript{d}) fraction}

DMSP\textsubscript{d} concentrations were on average 54.5\% lower (p < 0.001) when samples were carefully hand-filtered through 0.45 µm syringe filters using a drop by drop technique compared to samples that were filtered by gravity filtration through GF/F as described in Kiene and Slezak (2006) (Figure 11). The averaged coefficient of variation (CV) across clades and treatments was 17.9\% for hand filtration versus 15.3\% for gravity filtration. These results suggested that either (i) small cell fractions containing some DMSP\textsubscript{p} could pass through GF/F filters (0.7 µm mesh) but were removed using smaller poresize (0.45 µm) syringe filters, (ii) more DMSP\textsubscript{p} was drained out of algal cells by gravity filtration compared to hand-filtering, or (iii) the difference in filter material (glass fiber versus polycarbonate) was involved as polycarbonate filters have very regular pores while glass fiber filters are a mesh of irregular sized spaces. Ultimately, since lower DMSP\textsubscript{d} concentrations were obtained by hand-filtering with 0.45 µm syringe filters, it was concluded that this technique allowed for a finer separation of the DMSP\textsubscript{d} fraction from DMSP\textsubscript{p}. This method was therefore consistently used throughout this experiment. Although the pool of DMSP\textsubscript{d} did not vary significantly throughout the experiment (data not presented), the DMSP\textsubscript{d} fractions were used to calculate DMSP\textsubscript{p} concentrations.
Figure 11: Comparison of filtration methods at the 12 h time point. Error bars = SE, n = 3.

Cell size and density

Clade D1 had significantly larger cells than clade C1, with a mean at time zero of 9.09 ± 0.23 μm for clade D1 and 8.18 ± 0.08 μm for clade C1 (p ≤ 0.001) (Figure 12). Cells shrank to their minimal size 72 h after *Symbiodinium* cultures were transferred into hermetic chambers, after which cell diameter progressively expanded until the end of the experiment. Although this pattern was observed in both clades and under both temperature treatments, it was slightly less significant for clade D1 under control temperature (p ≤ 0.01, Figure 12A) compared to clade C1 and clade D1 under increasing temperature (p ≤ 0.001, Figure 12). However, temperature had no significant effect on cell diameter across the entire data set (p > 0.05). Cell density did not vary throughout the experiment (Table 1).
Figure 12: Cell diameter for clade D1 (A) and C1 (B) over six days of experiment and across two temperature treatments: 26°C (control) and ramping from 26 °C to 31 °C (experimental). White and shaded areas represent periods of light and dark respectively. Error bars = SE, n = 3.
Table 1: Cell density for clade C1 and D1 over six days of experiment and across two temperature treatments: 26°C (control) and ramping from 26 °C to 31 °C (experimental) expressed in number of cells x 10^6 mL⁻¹ ± SE, n = 3.

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 control</td>
<td>1.52 ± 0.11</td>
<td>1.76 ± 0.05</td>
<td>1.54 ± 0.11</td>
<td>1.69 ± 0.06</td>
<td>1.82 ± 0.14</td>
<td>1.66 ± 0.10</td>
</tr>
<tr>
<td>C1 control</td>
<td>1.51 ± 0.11</td>
<td>1.64 ± 0.06</td>
<td>1.35 ± 0.11</td>
<td>1.41 ± 0.02</td>
<td>1.66 ± 0.13</td>
<td>1.69 ± 0.04</td>
</tr>
<tr>
<td>D1 experimental</td>
<td>1.75 ± 0.17</td>
<td>1.80 ± 0.43</td>
<td>1.46 ± 0.07</td>
<td>1.69 ± 0.07</td>
<td>2.18 ± 0.11</td>
<td>1.75 ± 0.04</td>
</tr>
<tr>
<td>C1 experimental</td>
<td>1.57 ± 0.12</td>
<td>1.54 ± 0.06</td>
<td>1.21 ± 0.14</td>
<td>1.34 ± 0.19</td>
<td>1.83 ± 0.2</td>
<td>1.56 ± 0.06</td>
</tr>
<tr>
<td>Time (hour)</td>
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<td>96</td>
<td>108</td>
<td>120</td>
<td>132</td>
<td>144</td>
</tr>
<tr>
<td>D1 control</td>
<td>1.22 ± 0.10</td>
<td>1.86 ± 0.02</td>
<td>1.51 ± 0.07</td>
<td>1.48 ± 0.01</td>
<td>1.58 ± 0.17</td>
<td>1.89 ± 0.1</td>
</tr>
<tr>
<td>C1 control</td>
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<td>1.60 ± 0.11</td>
<td>1.23 ± 0.12</td>
<td>1.27 ± 0.05</td>
<td>1.48 ± 0.12</td>
<td>1.55 ± 0.03</td>
</tr>
<tr>
<td>D1 experimental</td>
<td>1.33 ± 0.28</td>
<td>1.85 ± 0.10</td>
<td>1.33 ± 0.12</td>
<td>1.44 ± 0.03</td>
<td>1.58 ± 0.11</td>
<td>1.94 ± 0.11</td>
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<tr>
<td>C1 experimental</td>
<td>1.31 ± 0.09</td>
<td>1.52 ± 0.07</td>
<td>1.14 ± 0.13</td>
<td>1.09 ± 0.12</td>
<td>1.28 ± 0.15</td>
<td>1.59 ± 0.26</td>
</tr>
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Intracellular or particulate DMSP (DMSP$_p$)

Initial DMSP$_p$ concentrations were 50.7 ± 4.09 fmol cell$^{-1}$ or 140 ± 16.6 mmol L$^{-1}$ cell volume (CV) for clade D1 and 63.0 ± 4.67 fmol cell$^{-1}$ or 220 ± 20.1 mmol L$^{-1}$ CV for clade C1 (Figure 13A-B), with significantly greater DMSP$_p$ concentrations in clade C1 than in clade D1 throughout the experiment (p ≤ 0.001). Because DMSP$_p$ concentrations expressed per cell and biovolume followed the same trend throughout this experiment, only concentrations per biovolume are reported. Fluctuations in DMSP$_p$ concentrations were similar between the two clades of Symbiodinium, with concentrations gradually decreasing over time regardless of the temperature treatment. In clade D1, no significant difference in DMSP$_p$ concentration was observed between temperature treatments (p > 0.05, Figure 13A) whereas in clade C1 significantly more DMSP$_p$ was found under control conditions than under increasing temperature 60 h, 108 h and 144 h after the start of the experiment (p ≤ 0.01, Figure 13B).
Figure 13: Particulate DMSP concentrations for D1 (A) and C1 (B) over six days of experiment and across two temperature treatments: 26°C (control) and ramping from 26 °C to 31 °C (experimental). White and shaded areas represent periods of light and dark respectively. Significant differences in concentrations between control and experimental temperature are shown by an asterisk. Error bars = SE, n = 3.

DMS concentrations: dissolved (DMS_w) and gaseous (DMS_g)
Significantly more DMS_w (p < 0.001) and DMS_g (p < 0.05) was measured in cultures of clade C1 than of clade D1 (Figures 14-15), with concentrations fluctuating similarly between clades throughout the experiment. In clade D1, DMS_w concentrations did not vary between the control and increasing temperature treatment (p > 0.05, Figure 14A); however, significantly more DMS_w was found in clade C1 under control conditions
than under the increasing temperature treatment 84 h, 132 h, and 144 h after the start of the experiment (p ≤ 0.001, Figure 14B). In clade D1, significantly more DMS$_g$ was measured in the increasing temperature treatment than in the control 60 h, 72 h and 108 h after the start of the experiment (p < 0.05), with an emission peak of DMS$_g$ being recorded 96 h after the start of the experiment in the increasing temperature treatment (Figure 15A). An emission peak of DMS$_g$ was also observed in clade C1 96 h after the start of the experiment but under both the control (p ≤ 0.01) and increasing temperature treatments (p ≤ 0.001), with DMS$_g$ concentration being significantly greater under control conditions than under increasing temperature at this point in time (p ≤ 0.001) (Figure 15B). DMS$_w$ and DMS$_g$ concentrations did not follow equilibrium, which is possibly due to (i) the applied air stream enhancing gas partitioning from the aqueous phase into the air phase or (ii) the viscosity of the Media used for the cell cultures.
Figure 14: Dissolved DMS (DMSw) concentrations for D1 (A) and C1 (B) over six days of experiment and across two temperature treatments: 26°C (control) and ramping from 26 °C to 31 °C (experimental). White and shaded areas represent periods of light and dark respectively. Significant differences in concentrations between control and experimental temperature are shown by an asterisk. Error bars = SE, n = 3.
Figure 15: Gaseous DMS (DMSg) concentrations for D1 (A) and C1 (B) over six days of experiment and across two temperature treatments: 26°C (control) and ramping from 26 °C to 31 °C (experimental). White and shaded areas represent periods of light and dark respectively. Significant differences in concentrations between control and experimental temperature are shown by an asterisk. Error bars = SE, n = 3.
Discussion

Clade genotyping
Although *A. millepora* and *A. tenuis* specimens from the outer reefs along the GBR can harbour a variety of symbionts, this study confirmed that the main composition of zooxanthellae in corals from Magnetic Island was very stable (Beltran et al., 2012). The genotyping of both fresh isolates and cultures showed that extracted symbionts sources from *A. millepora* and *A. tenuis* exclusively retained a D1 and C1 pattern respectively, with no noticeable multiple bands that could have indicated the presence of more than one clade per coral host.

Signs of physiological impairment
The diameter of cultured *Symbiodinium* increased over time for both clade C1 and clade D1 and for both temperature treatments (Figure 12), although changes were less significant in clade D1 under control conditions (Figure 12A). An absence of culture growth in both the control and increasing temperature treatment was also observed for both *Symbiodinium* cultures (Table 1). While a gain in cell volume combined with culture growth might suggest enhanced cell division (Fitt and Trench, 1983), a continued increase in cell size in cultures that are in stationary phase generally indicates DNA damage and oxidative stress (McLenon and DiTullio, 2012). In addition, cell shrinking as observed 72 h after the start of the experiment might suggest a loss of cell membrane integrity that was most likely due to stress exposure (Dunn et al., 2002). Future research might seek to verify this statement using techniques such as electron microscopy (Dunn et al., 2012) or lysosomal stability assays (Deschaseaux et al., 2011). These results therefore suggest that both clade cultures were decaying throughout the experiment regardless of the temperature treatment, although clade D1 under temperature control showed less signs of impairment, most likely suggesting an overall greater resistance of clade D1 to stress exposure.

Particulate DMSP (Figure 13) and dissolved DMS (Figure 14) gradually decreased over time following the 72 h time point for both clades and under both temperature treatments, suggesting additional stress exposure to the *Symbiodinium* cultures. Since
the decrease in DMSP_p was not accounted for by an increase in DMS_w, it is possible that part of the DMSP_p fed the DMSO pool (Sunda et al., 2002). Culture health could have been affected by a multitude of factors with the following three being the most important: (i) the antibiotic treatment which can sometimes negatively affect cell growth in some sensitive strains of Symbiodinium in culture (Santos et al., 2001; Yost and Mitchelmore, 2009), although similar cocktails of antibiotics have previously been used in long-term C1 and D1 cultures with no noticeable effect on growth or survival (Beltran et al., 2012); (ii) treatment with GeO₂, and/or (iii) repeated agitations of the chambers prior to each sampling as mechanical disturbances can have adverse effects on the normal development of cultured Symbiodinium (Rogers and Davis, 2006). The results of this experiment might therefore have been affected by additional factors; however, only effects that are relevant to differences between temperature treatments and clades are discussed below.

**DMSP and DMS production in Symbiodinium under thermal stress**

Throughout the experiment, significantly greater DMSP and DMS_w concentrations were consistently found in clade C1 than in clade D1 (Figure 13-14), suggesting that the thermal-sensitive clade C1 could be an overall greater producer of DMS(P) and/or possibly had enhanced DMSP-lyase activity than the thermal-tolerant clade D1. This observation could have interesting implications for the role of DMS and DMSP in the thermostolerance of different Symbiodinium clades. However, it is important to keep in mind that DMS(P) concentrations and DMSP-lyase activity can vary greatly from one species to the other (Niki et al., 2000), and that no clear link has been established between thermo-tolerance and DMS(P) concentrations when investigating various types of Symbiodinium (Yost and Mitchelmore, 2009; Steinke et al., 2011).

Significantly greater intracellular DMSP concentration was found in clade C1 under the control temperature compared to the increasing temperature treatment 60 h, 108 h and 144 h after the start of the experiment (Figure 13B). Although this was only from a few points in time, this result suggests enhanced DMSP consumption in clade C1 under thermal stress, possibly as a means of thermostolerance as shown in marine algae under low temperatures (Kirst et al., 1991; Karsten et al., 1992). Thermally-induced DMSP
consumption most likely happens through either (i) DMSP-lyase activity (Mohapatra et al., 2013), which should consequently result in DMS formation or (ii) enhanced ROS scavenging by DMSP under oxidative stress (Lesser, 1996; Sunda et al., 2002). Lower dissolved DMS concentrations were also recorded in clade C1 under increasing temperature compared to the control 84 h, 132 h and 144 h after the start of the experiment, when temperature had increased by 3°C and 5°C respectively (Figure 14B). This was coupled with a significantly lower emission of gaseous DMS 96 h after the start of the experiment, when temperature had then increased by 4°C (Figure 15B). These results therefore suggest enhanced DMS and DMSP consumption in clade C1 under thermal stress, which could support the hypothesis of DMS(P) oxidation as a consequence of oxidative stress (Sunda et al., 2002). However, because the DMSO pool was not measured in this experiment, the possibility of the involvement of DMSO in the DMS(P) turnover still remains speculative at this stage.

In clade D1, significantly less DMS\textsubscript{g} was emitted under increasing temperature than in the control 60 h, 72 h and 108 h after the start of the experiment (Figure 15A); however, no significant difference in DMSP\textsubscript{p} or DMS\textsubscript{w} concentration was observed between the two treatments for this clade (Figure 14A). Although significant, this difference in DMS\textsubscript{g} concentrations in clade D1 was thus not supported by the DMSP\textsubscript{p} and DMS\textsubscript{w} data, which makes this data even harder to interpret than for clade C1, which showed few but relatively consistent significant differences in DMS(P) concentrations between the two treatments. Because the pattern observed in clade D1 was only significant for gaseous DMS emissions, it is possible that other mechanical factors might have altered the aqueous to air phase transfer in the hermetic chambers baring clade D1 under control temperature (e.g. higher flow rate).

Initial DMSP\textsubscript{p} concentrations recorded for both clades D1 (50.7 ± 4.09 fmol.cell\textsuperscript{-1} or 140 ± 16.6 mmol.L\textsuperscript{-1}.CV) and C1 (63.0 ± 4.67 fmol.cell\textsuperscript{-1} or 220 ± 20.1 mmol.L\textsuperscript{-1}.CV) were in agreement with the concentration range previously reported among different clades and strains of cultured Symbiodinium (Table 2), although clade D1 had one of the lowest pre-experimental DMSP\textsubscript{p} concentration recorded to date. Previous research conducted on a wider diversity of Symbiodinium clades showed that no clear link
existed between thermal tolerance and intracellular DMSP concentrations (Steinke et al., 2011). However, it is interesting to note that clade D1, a subclade of the most thermally tolerant clade known to date (clade D) (Rowan, 2004; Berkelmans and Van Oppen, 2006) also registered one of the lowest DMSPₚ concentration ever reported in *Symbiodinium*.

Although unlikely, it is still conceivable that bacterial and/or viral contamination (either from the symbiont isolation procedure or external sources such as the chambers, connectors or tubing) could have contributed to the DMS(P) consumption in this chamber experiment. Axenicity was not monitored throughout the experiment because empirical tests have shown that the cocktail of antibiotics used in the cultures opted for a wide spectrum of action under standard concentrations (Santos et al., 2011; Beltran et al., 2012). The stability of these antibiotics has been verified for a minimum of 3 to 5 days at 37°C (Sigma, product information sheet), with considering the short-term experiment (6 days) and the low temperature range (26°C-31°C), the antibiotics were most likely effective and degradation unlikely. However, for future experiments, it is recommended that axenicity be tested by 16S PCR or plating of an aliquot of the culture over an IMK-agar media at the beginning and end of the experiment to demonstrate that bacteria are no longer developing and have therefore been inactivated by the antibiotic treatment.
Table 2: Summary of intracellular DMS and DMSP concentrations reported across various clades of cultured *Symbiodinium* to date. Concentrations are expressed as per mean ± SE except for values reported by Yost and Mitchelmore which are expressed as mean ± SD.

* DMSP concentrations and corresponding standard errors sourced from McLenon and DiTullio (2012) are estimates based on graphed values at time zero averaging both temperature treatments.

** DMSP concentrations sourced from Yost and Mitchelmore (2009) were expressed in mmol per litre of cell volume (CV) based on averaged cell diameters that were reported in their study for each clade. Respective standard deviation was estimated by calculating the square root of the squared relative standard deviation of both the cell diameters and DMSP concentrations expressed in fmol per cell.

- : not provided

<table>
<thead>
<tr>
<th><em>Symbiodinium</em> type</th>
<th>[DMSP] (fmol cell⁻¹)</th>
<th>[DMSP] (mmol.L⁻¹.CV)</th>
<th>[DMS] (mmol.L⁻¹.CV)</th>
<th>Thermal tolerance</th>
<th>Source</th>
</tr>
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<tr>
<td>Clade D1</td>
<td>50.7 ± 4.09</td>
<td>140 ± 16.6</td>
<td>-</td>
<td>Very high</td>
<td>Current study</td>
</tr>
<tr>
<td>Clade C1</td>
<td>63.0 ± 4.67</td>
<td>220 ± 20.1</td>
<td>-</td>
<td>Low</td>
<td>McLenon and DiTullio (2012)</td>
</tr>
<tr>
<td>Clade B – strain 1633</td>
<td>~ 68 ± 5.8</td>
<td>~450 ± 115</td>
<td>-</td>
<td>Low</td>
<td>McLenon and DiTullio (2012)</td>
</tr>
<tr>
<td>Clade A1 - strain CCMP 2464</td>
<td>-</td>
<td>225 ± 3.5</td>
<td>4.1 ± 1.22</td>
<td>High</td>
<td>Steinke et al. (2011)</td>
</tr>
<tr>
<td>Clade A13 - CCMP 2469</td>
<td>-</td>
<td>194 ± 19</td>
<td>2.1 ± 0.37</td>
<td>Very low</td>
<td>Steinke et al. (2011)</td>
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<tr>
<td>Clade A2 - Mf</td>
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<td>158 ± 3.8</td>
<td>0.3 ± 0.06</td>
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<td>Clade B1 - CCMP2463</td>
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<td>0.5 ± 0.22</td>
<td>Low</td>
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<tr>
<td>Unspecified - strain 373</td>
<td>-</td>
<td>7.6 ± 2.3</td>
<td>-</td>
<td>-</td>
<td>Yost and Mitchelmore (2009)</td>
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<tr>
<td>Unspecified - strain 374</td>
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<td>4.7 ± 2.1</td>
<td>-</td>
<td>-</td>
<td>Yost and Mitchelmore (2009)</td>
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<td>Clade E- strain 421</td>
<td>201 ± 138.9</td>
<td>353 ± 70 **</td>
<td>-</td>
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<tr>
<td>Clade A - strain 828</td>
<td>122.6 ± 60.7</td>
<td>192 ± 51 **</td>
<td>-</td>
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<td>Yost and Mitchelmore (2009)</td>
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<td>81.4 ± 42.9</td>
<td>128 ± 54 **</td>
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<td>-</td>
<td>Low</td>
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<tr>
<td>Clade B - strain1633</td>
<td>329.9 ± 193.2</td>
<td>686 ± 59 **</td>
<td>-</td>
<td>Low</td>
<td>Yost and Mitchelmore (2009)</td>
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**Implications for the GBR**

DMS is a volatile sulphur substance that could take part in a climate feedback that decreases solar radiation and sea surface temperatures (SSTs) (Charlson et al., 1987) by being involved in the formation of sulphate aerosols and formation of CCN, leading to increased low-cloud cover over the GBR (Jones and Trevena, 2005; Modini et al., 2009; Leahy et al., 2013). Although fundamental gaps remain in our understanding of this complex mechanism, especially with regard to the potential negative feedback effect of DMS on global warming (Quinn and Bates, 2011), as DMS oxidation in the atmosphere occurs fairly rapidly (1 day on average) (Kloster et al., 2006) the likelihood of local CCN formation over hotspots of DMS production such as coral reefs remains strong. A decrease in gaseous DMS emission under thermal stress in the thermal sensitive clade C1 of *Symbiodinium* could lead to reduced DMS emissions under predicted rises in SSTs associated with global warming (IPCC, 2007) in the Great Barrier Reef (GBR) that is currently clade C dominant (Stat and Gates, 2011). This could eventually result in reduced cloud cover and a positive feedback on regional climate in the GBR as previously pointed out by Fischer and Jones (2012). As a consequence of symbiont shuffling under increasing SSTs (Berkelmans and Van Oppen, 2006), reef-building corals might start associating with more thermally tolerant symbionts such as clade D, of which the beneficial mutualism with their coral host is still being discussed (Stat and Gates, 2011). Since clade D1 has been identified as one of the lowest producers of DMSP among *Symbiodinium* spp (Table 2), reduced DMS emissions might also be expected if the GBR shifts toward a clade D dominance. However, more work needs to be conducted on the potential of both clade C and D to produce DMS and DMSP under the current climate change scenarios, both in culture and in hospite, as biochemical efficiency of symbionts can be different when associated with the host (Stat et al., 2008), especially in light of the recent discovery that coral polyps also have the capacity to produce DMSP (Raina et al., 2013). However, based on the recent finding by Raina et al. (2013), the coral host will most likely increase its DMSP production under thermal stress, which could counterbalance the previously discussed prediction of a decrease in DMS(P) production/emission in coral reef ecosystems, unless coral cover is being lost globally (Hoegh-Guldberg, 1999).
**Conclusion**

This experiment demonstrated the effect of increasing temperature on the production of DMS and DMSP in axenic cultures of two *Symbiodinium* clades with different thermal tolerances. While in the thermally-tolerant clade D1 DMSP was not greatly affected by an increase in temperature, in clade C1, which is known to be more sensitive to variations in temperature, both DMS and DMSP were consumed at a higher rate under thermal stress. Given that DMS and DMSP are ROS scavengers under oxidative stress, these results suggested that the DMSP-based antioxidant system was possibly involved in the response to thermal stress in clade C1. Variations in DMS and DMSP concentrations between these two clades suggested differences in the production of DMSP and DMSP-lyase potential activity in *Symbiodinium*. Even though this is speculative at this stage, the comparative capacity of clade C1 and D1 to produce DMS and DMSP as a response to thermal stress could have implications for the GBR in the current context of climate change.
Air blasting as the optimal approach for the extraction of antioxidants in coral tissue

Unformatted published paper is shown in Appendix 4

Abstract

To accurately measure the antioxidant capacity in corals, it is essential to optimally extract antioxidants from coral tissue. Here, we compared the two most commonly used methods for coral tissue extraction, air blasting (similar to Water-Pik method) and grinding, with or without sonication, to optimally extract antioxidants in corals. By air blasting, the coral tissue was stripped off the skeleton with a stream of compressed air into a polyethylene bag containing a known volume of buffer, whereas by grinding, both the coral tissue and skeleton were crushed using a mortar and pestle into a powder and homogenised in buffer. The antioxidant capacity (AOC) in coral homogenates was measured using the oxygen radical absorbance capacity (ORAC) assay. We found that air blasting was the most appropriate sample preparation method to use as it allowed for a more efficient and complete extraction of antioxidants from coral tissue, without the need for a second extraction step.
Introduction

Measuring the AOC of biological samples rather than targeting individual antioxidant compounds has been recognised as more cost-effective and convenient (Huang et al., 2005). Additionally, when testing the antioxidant function of specific compounds and their role in the overall antioxidant system of biological samples, measuring the AOC becomes essential (Minh et al., 2011). However, to accurately measure the AOC of biological samples, it is necessary to efficiently and optimally extract the totality of antioxidants present in the tissue of living organisms.

Two sample preparation methods have commonly been used for tissue extraction when measuring antioxidants in corals: grinding (Griffin and Bhagooli, 2004; Richier et al., 2008) and air blasting (Levy et al., 2006; Flores-Ramirez and Linan-Cabello, 2007; Ramos and Garcia, 2007; Palmer et al., 2009; Linan-Cabello et al., 2010) which is a modification of the Water-Pik method by Johannes and Wiebe (1970). However, neither of these methods has been tested for optimised extraction of antioxidants to date.

In this study, measurements of AOC from the supernatant of two successive extractions of coral tissue homogenates prepared by air blasting or grinding were compared as a function of sonication time, with the aim to evaluate whether the sample preparation and sonication time had an influence on the extraction of antioxidants from coral tissue. This was performed using the ORAC assay. Although the ORAC method measures the antioxidant capacity against peroxyl radicals and not all reactive oxygen species (e.g. superoxides and singlet oxygen), it still remains a good indicator of the total antioxidant capacity of biological samples.

Materials and Methods

Coral collection

Three colonies of *Acropora pulchra* were collected within the scientific research zone of Heron Island (23°26'35.80"S/151°54'44.23"E), Southern Great Barrier Reef, Australia. The colonies were brought back to the Heron Island Research Station and
kept in a flow-through seawater holding tank for five days to recover. Nubbins of approximately 4 cm each were collected from each of the 3 coral colonies, frozen at – 80°C and brought back within two days to Southern Cross University (SCU) in a dry shipper loaded with liquid nitrogen. Frozen nubbins were stored at – 80°C immediately after arrival. Ten coral nubbins were randomly selected as a representative subsample of the three coral colonies and five nubbins were randomly assigned to each sample preparation method tested: air blasting or grinding, followed by sonication.

**Tissue extraction**

The air blasting method consisted of placing each coral nubbin into a thick polyethylene (PE) bag (10 cm x 15 cm) and adding 10 mL of 75 mmol L\(^{-1}\) sodium phosphate buffer, pH 7.4, at 4°C. Coral tissue was stripped off the coral skeleton using an air gun connected to a source of compressed air (flow rate 11 L min\(^{-1}\)) inside the PE bag to prevent tissue loss by splattering. When blasting is considered imprecise, a 10 - 200 µL pipette tip can be placed on the tip of the air gun to narrow the air stream (flow rate 13 L min\(^{-1}\)) and penetrate the small pores and crevices within the nubbin skeleton. Once the tissue was fully removed, the skeleton was removed and the PE bag was sealed and shaken to collect splattered tissue into the homogenate, which was collected into a 15 mL centrifuge tube by cutting the PE bag open. The grinding method consisted of crushing unthawed coral nubbins (tissue and skeleton) using a mortar and pestle. The pulverized material was transferred into a 15 mL centrifuge tube using a spatula and homogenized in 10 mL of 75 mmol L\(^{-1}\) sodium phosphate buffer, pH 7.4, at 4°C.

Each tissue homogenate from both of the sample preparation methods (air blasting or grinding) was divided into four aliquots that were processed as follows: unsonicated, sonicated for 5 min, 10 min and 20 min in a chilled sonicating bath (Soniclean 160T, Soniclean Pty Ltd, Australia). All aliquots were centrifuged at 10,000 g for 15 min at 4°C (Sigma 4K15, Quantum Scientific Pty Ltd, Australia). Supernatants (S1) were kept aside for AOC measurement.

The residual tissue pellets were resuspended in 100% dimethylsulfoxide DMSO (1 mL), which is a non-volatile solvent useful in extracting antioxidants (Gorinstein et al., 2006)
and processed similarly to the first extraction step described above. DMSO was not used in the first extraction step as it can alter protein quantification (Tjernberg et al., 2006). After centrifugation, the supernatants (S2) were also collected for AOC measurements. Both S1 and S2 were frozen at – 80°C until the analysis for AOC and protein content.

**Antioxidant capacity and protein content measurements**

Tissue extracts (S1 and S2) were analysed for AOC using the ORAC assay (Ou et al., 2001), which is based on hydrogen-atom transfer reactions (Huang et al., 2005). The antioxidant scavenging activity of coral tissue was measured against peroxyl radical induced by AAPH (2,2'-azobis(2-methylpropanimidamide) dihydrochloride, Aldrich 440914, 25 mmol L⁻¹) using fluorescein (disodium-2(3-oxo-6-oxidoxanthan-9-yl)benzoate, Sigma Aldrich 166308, 0.25 µmol L⁻¹) as a probe. The fluorescence decay at λ<sub>ex</sub> 485 nm and λ<sub>em</sub> 535 nm was measured using a Wallac Victor2 1420 Multilabel counter (PerkinElmer, Turku, Finland). Epicatechin ((-)-epicatechin, Sigma E-1753, 0.17 mmol L⁻¹) was used as a positive control and Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Fluka Chemika 56510, 100 µmol.L⁻¹) as the reference standard (Appendix 1). Protein concentrations were determined using the colorimetric Biuret assay (Dustin, 1950) using the Biuret reagent (0.8% w/v NaOH, 0.9% w/v sodium potassium tartrate, 0.19% copper sulphate and 0.5% potassium iodine) and bovine serum albumin (BSA) as the calibration standard (Appendix 1). Samples were incubated at room temperature for 20 min after adding the reagent and absorbance was determined at 550 nm on the Wallac Victor2 1420 Multilabel counter. The ORAC values were expressed as micromoles of Trolox equivalent and standardised to protein content (µmol TE mg⁻¹ protein).

**Statistical analysis**

Distribution of the dataset was right skewed when tested for normality and therefore square-root transformations were applied prior to the analysis. A linear mixed effects model was fitted to the transformed dataset with Bonferroni adjustments for multiple comparisons, using AOC as a variable and using methods (air blasting versus grinding), supernatant fractions (pellet or supernatant) and sonication times (0, 5, 10 and 20 min).
as fixed factors. The least significant 3-way and 2-way interactions were eliminated and the principles of marginality were respected.

**Results**

A significant 2-way interaction between the supernatants (S1 and S2) and methods (air blasting and grinding) was found (df=49, p<0.001) (Figure 16), which indicated that the measurements of AOC from coral tissue homogenates significantly depend upon the sample preparation method used. Regardless of the extraction method, significantly lower levels of AOC (df=49, p<0.05) were measured in S2 than in S1. However, significantly greater AOC (df=49, p<0.05) was found in the supernatant from the second extraction (S2) of the ground material compared to S2 from air blasted tissues where no AOC was detected. Sonication time was found to play a non-significant role (df=49, p>0.05) in the extraction of antioxidants in coral tissue.

![Graph showing AOC measurements](image)

**Figure 16:** Comparison of AOC measurements in supernatant 1 (S1) and supernatant 2 (S2) of coral tissue homogenates extracted by either grinding or air blasting as a function of sonication time. Error bars = SE, n = 5.
Discussion

The two most common methods used for coral tissue removal are: (i) grinding, and (ii) air blasting, which is an adaptation of the Water-Pik method (Johannes and Wiebe, 1970). In the Water-Pik method coral tissues are blasted with water, often resulting to an uncontrolled dilution of the tissue homogenates. In this study, air blasting was used to detach coral tissues from the skeleton directly into a pre-specified volume of buffer, resulting in more concentrated tissue homogenates.

This study provides the first comparison of these two sample preparation methods in their capacity to efficiently extract antioxidants from coral tissue homogenates and to allow optimal measurements of the AOC in corals. The two methods were found to significantly differ in terms of optimal antioxidant extraction from coral tissue homogenates. Results showed that regardless of the sonication time, the air blasting method followed by 15 min of centrifugation at 10,000 g allowed complete extraction of antioxidants into the supernatant of coral tissue homogenates, whereas some antioxidants remained in the tissue pellets of samples that had been processed by grinding. These results indicated that the antioxidants were exhaustively extracted in the first extraction step of tissue collected by air blasting whereas a significant and unknown portion of antioxidants probably remained trapped in the skeletal debris of the ground coral nubbins. Air blasting was therefore found to be the most suitable method to use when measuring the AOC of coral tissue extracts. Even though sonication time was not found to influence the extraction of antioxidants from coral tissue, a sonication step of a minimum of 5 min is still recommended as it could help with the extraction of proteins from coral and symbiont cells (Woods et al., 2011), as protein content was used to normalise the AOC in tissue extracts (Palmer et al., 2009).
CHAPTER 4

**Dimethylsulphoxide (DMSO) in biological samples: a comparison of the TiCl$_3$ and NaBH$_4$ reduction methods using headspace analysis**

Unformatted published paper is shown in Appendix 5

Abstract

DMSO is a sulphur compound that can result from the oxidation of biogenic DMS in marine algae and bacteria; with DMSP being the main precursor of DMS. The two most commonly used methods for the analysis of DMSO in seawater and biological samples consist of its chemical reduction to DMS by either titanium trichloride (TiCl₃) or sodium borohydride (NaBH₄), with subsequent measurement of derived DMS by gas chromatography. Here, these two methods have been compared for the quantitative analysis of DMSO in the zooxanthellate coral Acropora aspera and in two species of marine algae (Ulva intestinalis and Ulva lactuca) using headspace analysis on DMSO-derived DMS. Reduction by NaBH₄ or TiCl₃ in biological samples yielded highly linear calibrations ($R^2 \geq 0.99$) and excellent repeatability ($RSD = 6.17\%$ and $4.32\%$ for TiCl₃ and NaBH₄ respectively, $n = 10$). In coral samples, although a strong linear correlation was generally obtained between the two reduction methods ($R^2 = 0.8451$, $p < 0.001$, $n = 72$), the regression slope of 0.6 indicated that DMSO concentrations were either underestimated with NaBH₄ reduction or overestimated with TiCl₃. Reduction with TiCl₃ yielded lower values than NaBH₄ at DMSO concentrations $< 0.6 \mu mol \text{ L}^{-1}$, whereas TiCl₃ gave higher values than NaBH₄ when DMSO was $> 2 \mu mol \text{ L}^{-1}$. The reasons for these significant differences remain unclear at this stage and we therefore cannot draw conclusions on the preferential suitability of one reducing agent over the other for coral DMSO analysis. In macroalgae samples, significantly lower DMSO concentrations were obtained with NaBH₄ than with TiCl₃ for DMSO concentrations averaging 0.6 $\mu mol \text{ L}^{-1}$ and 0.8 $\mu mol \text{ L}^{-1}$ for U. intestinalis and U. lactuca respectively. The difference between reduction methods in the analysis of DMSO across macroalgae and coral samples was interpreted as a difference in taxa or in sample preparation, although this needs to be further investigated. Corals were found to contain more DMSO than macroalgae with similar DMSP concentrations.
**Introduction**

DMSO was reported for the first time in the marine environment in 1980 (Andreae, 1980) and was later identified as the most dominant dissolved dimethylated sulphur species in the Mediterranean Sea (Simó et al., 1997). While several studies have reported higher concentrations of DMSO than those of both DMS or DMSP (Hatton et al., 1996; Simó et al., 1997), other studies have shown that DMSO concentrations were lower than those of DMS and DMSP in seawater (Gibson et al., 1990). However, such differences in the proportions of these sulphur compounds could be explained by diurnal and seasonal variations of DMSO (Lee and de Mora, 1999).

Marine DMSO was initially thought to be derived mainly from the photo-oxidation of DMS in the euphotic zone of the water column (Brimblecombe and Shooter, 1986); however marine bacteria were later found to play an important role in this reaction (Hatton et al., 2012). Other studies have demonstrated the presence of particulate (cellular) DMSO in phytoplankton (Hatton and Wilson, 2007; Simó et al., 1998), indicating that this oxidised sulphur species is also biologically produced. Given that DMSO can easily permeate through cell membranes (Hatton and Wilson, 2007), the particulate pool should be a source for the dissolved pool, but relatively little is known about the cycling of DMSO in marine systems.

DMS oxidation to DMSO through the scavenging of reactive oxygen species (ROS) appears to be the main source of DMSO synthesis in marine algae (Sunda et al., 2002). This mechanism is expected to be enhanced under oxidative stress (Husband et al., 2012), when ROS accumulate within the organism as a consequence of stress exposure (Lesser, 2006). DMSO can also be biologically reduced to DMS through enzymatic reactions that most likely involve reductases (Spiese et al., 2009). Thus, DMSO is a key compound in the complex redox loop that is involved in the marine sulphur cycle as it can be both an end-product of DMS oxidation and a precursor of DMS, which potentially plays an important role in climate regulation (Charlson et al., 1987).
Dissolved DMSO has been found in trace concentrations (ranging from 1 nM to 70 nM) in rainwater and freshwater environments (Andreae, 1980), and at a higher concentration range (1-200 nM) in seawater (Gibson et al., 1990; Lee and Wakeham, 1988). Although particulate DMSO concentrations have been quantified on multiple occasions in seawater (Hatton, 2002; Riseman and DiTullio, 2004; Simó et al., 1998), only a few studies have measured intracellular DMSO concentrations within specific organisms (Table 3). Moreover, relative particulate concentrations across species are often difficult to compare as they are frequently normalised to different parameters among studies (e.g., fresh weight, cell density, cell volume, surface area, etc).
Table 3: Particulate DMSO concentrations and DMSO:DMSP ratios in biological samples across various studies and normalisations. DMSO reduction method is indicated. FW = fresh weight. CV = cell volume.

<table>
<thead>
<tr>
<th>Source (year)</th>
<th>biological sample</th>
<th>normalisation</th>
<th>[DMSO]</th>
<th>DMSO:DMSP (%)</th>
<th>DMSO reduction method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simó et al. (1998)</td>
<td>Phytoplankton</td>
<td>fmol cell⁻¹</td>
<td>3.84 ± 2.56</td>
<td>4</td>
<td>enzymatic method Hatton et al. (1994)</td>
</tr>
<tr>
<td>Husband and Kiene (2007)</td>
<td>Macrophyte</td>
<td>µmol g⁻¹ FW</td>
<td>0.25 - 0.6</td>
<td>1.6 – 4</td>
<td>TiCl₃</td>
</tr>
<tr>
<td>Spiese et al. (2009)</td>
<td>Phytoplankton</td>
<td>mmol L⁻¹ CV</td>
<td>0.034 - 8.4</td>
<td>0.053 - 30.3</td>
<td>TiCl₃</td>
</tr>
</tbody>
</table>
Because of its importance in the algal antioxidant system and marine sulphur cycle, more research is required on DMSO and therefore the choice of analytical techniques for DMSO measurements in biological samples needs to be further examined. To date, DMSO has mainly been quantified by gas chromatography following its chemical reduction to DMS using either TiCl$_3$ (Del Valle et al., 2007; Harada et al., 2009; Husband and Kiene, 2007; Husband et al., 2012; Kiene and Gerard, 1994; Rellinger et al., 2009; Spiese et al., 2009; Vila-Costa et al., 2006) or NaBH$_4$ (Riseman and DiTullio, 2004; Sciare and Mihalopoulos, 2000; Sciare et al., 2002; Simó et al., 1996; Simó et al., 1997; Simó and Vila-Costa, 2006; Ui et al., 2004; Vila-Costa et al., 2008; Wang et al., 2013), with other studies using the enzyme-based reduction method developed by Hatton et al (1994) (Hatton and Wilson, 2007; Simó et al., 1998) or the proton nuclear magnetic resonance technique (Zeyer et al., 1987). Although TiCl$_3$ and NaBH$_4$ are both in common use for the analysis of DMSO, their relative efficiencies have not been extensively assessed. Moreover, comparisons among studies that have used these two techniques independently suggested that reduction of DMSO with NaBH$_4$ could result in significantly higher DMS concentrations than with TiCl$_3$ (Spiese et al., 2009). Here we tested both reducing agents with the aim to assist future research in the selection of the most appropriate method for DMSO measurements in biological samples and to facilitate comparisons of DMSO analysis between studies that have used these reduction methods independently.

Material and methods

Collection, preservation and DMSP analysis of coral-zooxanthellae samples

Colonies of the zooxanthellate coral _Acropora aspera_ were collected at low tide from the Heron Island reef flat (23°26′46.19″S/151°54′46.35″E), Australia. In order to obtain different levels of DMSO production, five sets of twelve random nubbins representing an even mix of colonies were assigned to experimental treatments simulating environmental stress over set periods before being snap frozen in liquid nitrogen (see details of experimental protocol in Deschaseaux et al., 2014). Another set of 12 nubbins was immediately snap frozen as controls under basal conditions (unexposed to stress) following collection. Samples were kept at - 80 °C until the coral tissue (containing
both coral and algal cells, as well as most likely bacteria and fungi that are often present in corals) was extracted by air blasting in 10 mL of 75 mmol L\(^{-1}\) sodium phosphate buffer (Deschaseaux et al., 2013). This generated what is termed the coral blastate. Although essential to provide preliminary information on how coral samples had been handled prior to analysis, the outcome of experimental treatments will not be commented on in this manuscript as it is already been discussed in another article (Deschaseaux et al., 2014). Instead, we focus on the quantification of DMSO by two different approaches using TiCl\(_3\) and NaBH\(_4\).

Prior to DMSO analysis all samples were analysed for DMSP. A 1 mL subsample of the coral blastate was diluted to 10 mL with MilliQ water and purged with high purity nitrogen (N\(_2\)) for 10 min at a flow rate of 100 mL min\(^{-1}\) to remove free DMS. Following addition of 1 mL of 10 mol L\(^{-1}\) NaOH to each aliquot, vials were immediately sealed with gas-tight septa (Agilent Technology, PTFE/Silicone septa, P/N 5183-4477) and crimp capped for subsequent DMSP analysis that was conducted within two weeks of sample preservation.

Coral DMSP samples were processed by headspace analysis using a GC (Agilent Technologies 6890N) with a Mass Selective Detector (MSD) (Agilent Technologies 5973N) operated in scan mode coupled with a Gerstel multipurpose sampler (MPS 2XL). Headspace injection (1 mL) was performed using a 2.5 mL syringe set at 95°C at an injection speed of 500 \(\mu\)L s\(^{-1}\). The GC injector temperature was set at 280°C and the injection was made with a split ratio of 25:1. Volatiles were separated using a capillary column (BPX, 50 m, 0.22 mm x 1 µm film thickness, SGE) with He as carrier gas at a constant flow rate of 1.1 mL min\(^{-1}\). The oven temperature was programmed from 35°C (held for 8.0 min) to 180°C at a temperature ramp of 80°C per min and held at 180°C for 2 min. Compounds were identified by reference to MS library database and data were processed using MSD ChemStation Software (Version D.02.00.275, Agilent Technologies). DMSP stock standard solution was made by diluting a known quantity of DMSP powder (DMSP.HCl reference material WR002 of certified purity 90.3 \(\pm\) 1.8% w/w, National Measurement Institute, Sydney, Australia) into acidified MilliQ water (final pH < 2) and was kept at \(-20°C\). DMSP standards and blanks were prepared...
daily in the exact same proportions of phosphate buffer, MilliQ water and NaOH as the coral samples. Seven-point calibration curves and three blanks were run daily at the start and end of the sample analysis for calibration and contamination tests respectively under the exact same analytical conditions as for samples (Appendix 1). The value of blanks was subtracted from all measurements.

Because the DMSO analysis in coral samples was conducted on a Shimadzu GC coupled with a Flame Photometric Detector (FPD) (see analytical details below), which is a more sensitive detector than the MSD used for the DMSP analysis, samples that had been treated with NaOH for DMSP analysis were diluted 10- or 100-fold into 75 mmol L\(^{-1}\) sodium phosphate buffer, pH 7.4, following DMSP sample processing. Diluted samples were purged with \(N_2\) for 10 min to remove the DMS that was generated during the alkaline treatment of DMSP. The absence of DMS following purging was verified on twenty random samples by purge-and-trap analysis as follows: vials were individually connected to the GC-FPD by piercing septa with (1) the outlet needle (Terumo corporation, sterile needle, 1.10 x 38 mm) that was in line with a cryogenic Teflon loop dipped into liquid nitrogen and (2) the inlet needle (Cadence Science, Septum penetration needle, 0.2 x 152.4 mm) which was delivering a high purity He flow of 100 mL min\(^{-1}\). If DMS was present, it was purged out of the vials and cryo-trapped for 3 min at this flow rate and subsequently measured by transferring the cryo-loop into a hot water bath (~60\(^{\circ}\)C). A Nafion dryer (Perma Pure, MD-050-48 P2) was placed upstream of the cryotrap using a drying flow of 100 mL.min\(^{-1}\) to avoid ice blockage during the purge due to moisture that could build up in the cryogenic loop (Simó et al., 1996). Once this verification step had attested of the absence of DMS following the 10 min purge, one mL aliquots were transferred into two sets of crimp-cap glass vials of either 14 mL (used for TiCl\(_3\)) or 70 mL (used for NaBH\(_4\)). The larger volume vial was used for the NaBH\(_4\) treatment because it better accommodated the pressure generated due to vigorous hydrogen production during DMSO reduction by NaBH\(_4\) (Ui et al., 2004) (see equation [2] below).
Collection, preservation and DMSP analysis of macroalgae samples

Algal tissue was collected from two different species of green macroalgae: *Ulva intestinalis* and *Ulva lactuca*, from rocky platforms at Dauphin Island (30°15'4.55"N/88° 4'46.24"W), USA. The tissue was quickly dried on absorbent paper, divided into fragments of 1 – 4 mg of which 10 replicates were placed in two sets of crimp-cap glass vials of either 14 mL or 70 mL volume capacity for future reduction by TiCl$_3$ or NaBH$_4$ respectively as per reasons mentioned above. One mL of 0.2 µm filtered seawater and 1 mL of 5 mol L$^{-1}$ NaOH was added on top of each algal fragment and vials were immediately sealed with gas-tight septa (Fischer & VWR, P/N 16171-651) and crimp capped for DMSP analysis that was conducted on the same day.

Macroalgae DMSP samples were analysed on a Shimadzu GC-FPD (Model 2014) by direct headspace injections (100 µL from 14 mL headspace vials and 500 µL from 70 mL headspace vials) using gas tight syringes (SGE Analytical Science, Australia) of the appropriate volume onto a chromosil 330-packed column of 8 x 1/8” OD Teflon. Detector and oven temperatures were set at 175°C and 60°C respectively, with a carrier flow of high purity He at 25 mL min$^{-1}$. DMSP stock standard solution (DMSP.HCl analytical reagent of assumed 100% purity, Research Plus, Inc) was made similarly as previously described for the DMSP analysis on the GC-MSD. Standards and blanks were prepared in 0.2 µm filtered seawater and processed in the exact same way as for samples. Seven-point calibration curves and three blanks were run daily at the start and end of the sample analysis for calibration and contamination tests respectively under the exact same analytical conditions as for samples (Appendix 1). The value of blanks was subtracted from all measurements. Once algal samples had been processed for DMSP analysis, they were purged with high purity N$_2$ for 10 min and neutralised with 800 µL of 10% HCl (pH ~ 7.4) prior to DMSO reduction and subsequent DMS measurements.
DMSO in biological samples: a comparison of two methods

Reduction

DMSO reduction by TiCl₃ is believed to perform with subsequent production of DMS, titanium, chlorine, and water, according to equation [1]:

\[
C₂H₆SO + 2 TiCl₃ + 2 HCl \rightarrow C₂H₆S + 2 Ti \,(s) + 4 Cl₂ \,(g) + H₂O \quad [1]
\]

The reaction was induced by adding 200 µL of 20% w/w TiCl₃ acidic solution (Fischer Scientific, AC42915-1000) to each 14 mL headspace vial that was immediately sealed (Fischer & VWR septa, P/N 16171-651) and crimp capped. Samples were heated at 50°C for one hour in a water bath and allowed to cool to room temperature until analysis (Kiene and Gerard, 1994).

DMSO reduction by NaBH₄ is believed to occur with subsequent production of DMS, hydrogen, sodium, boron, and water according to equation [2]:

\[
C₂H₆SO + NaBH₄ \rightarrow C₂H₆S + H₂ \,(g) + Na \,(s) + B \,(s) + H₂O \quad [2]
\]

The reaction was performed by adding 5 mg to 15 mg of ground up Co-NaBH₄ pellets (90% cobalt doped NaBH₄, Sigma Aldrich, P/N 247677) to each 70 mL headspace vial. As stated by Simó et al. (1996), a transient DMS-boranes adduct might be produced as an intermediate of the DMSO reduction by NaBH₄, with the cleavage of the adduct being facilitated by addition of acid. The presence of metal ions such as Co(II) has been shown to facilitate the reduction of DMSO by NaBH₄ (Simó and Vila-Costa, 2006; Ui et al., 2004), possibly because it prevents the formation of the adduct and the reaction resumes to boron formation. However, the exact chemical process on which this reaction relies has not been examined at this stage.

Vials were immediately sealed, crimp capped and kept under ambient conditions until analysis. In DMSO standards that had been reduced to DMS by NaBH₄, a simple test using a 50 mL gas-tight syringe to pierce the septum of 70 mL headspace vials in which
the reaction occurred showed that excess pressure did not vary substantially inside the vials (16 mL ± 0.70 mL, n = 5).

Analysis
Once DMSO reduction to DMS by either TiCl₃ or NaBH₄ was complete, coral and macroalgae tissue samples were analysed by direct headspace injections (100 μL from 14 mL headspace vials and 500 μL from 70 mL headspace vials) on the Shimadzu GC-FPD, using the same mode and conditions as previously described for DMSP analysis in macroalgae samples. DMSO standards were made by direct dilutions of a stock DMSO solution (Fischer Scientific BP321). Standards and blanks were prepared in the exact same proportions of phosphate buffer or 0.2 μm filtered seawater as used for coral and macroalgae samples. Seven-point calibration curves and three blanks were run daily at the start and end of the sample analysis under the exact same analytical conditions as for samples for calibration and contamination tests respectively (Appendix 1).

Reaction time, repeatability, and influence of pH
Following reduction by either TiCl₃ or NaBH₄, the reaction time of both reduction methods was evaluated by repeated analyses of concentrated DMSO standards (7 μmol L⁻¹) on the GC-FPD at different time intervals until maximal response was obtained by gas chromatography. In order to estimate the repeatability of each DMSO reduction method by headspace analysis on biological samples, five repeats of two random coral samples were also performed on the GC-FPD following either TiCl₃ or NaBH₄ reduction. Slight variations in pH resulted from the 10-fold or 100-fold dilutions of concentrated coral samples (initial concentration 1 mol L⁻¹ NaOH) that were performed prior to DMSO analysis. In order to evaluate the effect that these variations in pH could have on the analysis of DMSO samples following TiCl₃ or NaBH₄ reduction, calibration curves of DMSO standards prepared in phosphate buffer (with no base added, pH 7.4) were plotted and compared to calibration curves plotted for two sets of DMSO standards prepared in the same proportions of NaOH as in diluted coral samples (0.1 mol L⁻¹ NaOH, pH 12, and 0.01 mol L⁻¹ NaOH, pH 7.5).
Normalisation of DMSP and DMSO concentrations

In *A. aspera*, symbiont density and volume of algal cells were determined by performing 8 replicate counts of each coral sample on a haemocytometer using a compound microscope (Olympus CX21) (magnification of 100X) and measuring the cell diameter of 20 random algal cells using a stage micrometer (magnification of 200X). Chlorophyll a (Chl a) content was quantified by extracting filtered coral subsamples in 90% acetone at –20 °C overnight in dark conditions. Thawed acetone extracts were sonicated and centrifuged for 15 min and the absorbance was measured by spectrophotometry (GBC UV/VIS 916 spectrophotometer) at 664 nm, 665 nm and 750 nm wavelengths before and after adding 0.1 mol L⁻¹ HCl using a standard method (APHA, 1998). As described above, fresh weights of *U. intestinalis* and *U. lactuca* were estimated at the start of the sample preparation. The concentration estimates of Chl a in *Ulva sp.* per gram of fresh weight of macroalgal tissue that was determined in Beer and Israel (1986) were used to convert the *U. intestinalis* and *U. lactuca* fresh weight estimates to Chl a values for normalisation of DMSP and DMSO content.

Statistical analysis

Concentrations of reduction products using either TiCl₃ or NaBH₄ were compared using a paired two-tailed t-test in *A. aspera* samples and an unpaired two-tailed t-test in *U. intestinalis* and *U. lactuca* samples. Correlation coefficients and corresponding p-values were established in the two-tailed analysis of TiCl₃ versus NaBH₄.

Results & Discussion

Main analytical findings –TiCl₃ versus NaBH₄ reduction in coral samples

DMSO reduction and direct headspace analysis of the DMS reaction product showed strong linearity in calibration (R² ≥ 0.99) and robust repeatability (4.32% for TiCl₃ and 6.17% for NaBH₄) for repeated measures of the same two samples (n = 10) using both reducing agents. The reduction to DMS of the most concentrated DMSO standard used for this study was complete in 30 min in 4% TiCl₃ v/v (200 µL of 20% TiCl₃ in a 1 mL sample) and in 3 min for a NaBH₄ concentration range of 0.45% and 1.35% w/v (5 - 15 mg of 90% NaBH₄ in a 1 mL sample) (Fig 18). This indicated that both reactions...
happened relatively fast, with reduction by NaBH$_4$ allowing for more rapid subsequent DMS analysis by gas chromatography.

![Graph showing DMSO (nmol) vs Time (min) for NaBH$_4$ and TiCl$_3$.](image)

**Figure 17:** Chromatographic response obtained as a function of time for DMSO reduction to DMS using either TiCl$_3$ or NaBH$_4$. The response is expressed as per nmoles of DMS. The vertical dashed lines on the graph indicate the time after which the reduction reaction was complete. Error bars = SE, $n = 5$.

This study is the first reported example of DMS analysis by headspace following DMSO reduction by NaBH$_4$ and linearity in calibrations demonstrated that crimped cap vials with large headspace volumes (70 mL) allowed for a build up of overpressure into the vial (see Material and Methods section) without perturbing the conversion of DMSO to DMS or causing losses of DMS (which would have presumably led to variability). Headspace analysis of DMS derived from DMSO reduction by TiCl$_3$ had previously been conducted on samples that contained DMSO concentrations at or above the micromolar level (Kiene and Gerard, 1994) because it was found to avoid reaction product interferences (most likely due to Cl$_2$ gas production – see equation [1]) when using purge-and-trap sample concentration. Our study confirms that DMSO reduction by TiCl$_3$ followed by headspace analysis of the DMS product is analytically robust.
The concentrations of DMSO determined by using either TiCl₃ or NaBH₄ reduction to DMS and subsequent headspace analysis showed a significant correlation between the two methods for the entire coral dataset ($R^2 = 0.8451$, $p < 0.001$, $n = 72$) (Figure 18). This indicated that both reducing agents were generally consistent in determining relative DMSO concentrations in coral samples when using direct headspace analysis. However, the slope of the model I linear regression for DMSO concentrations obtained with NaBH₄ versus TiCl₃ reduction was 0.6. This indicates that DMSO concentrations tend to be either underestimated with NaBH₄ or overestimated with TiCl₃ in coral blastate samples. The intercept of the best fitted trend line crossed the y axis at 0.41 μmol L⁻¹, which suggests that one of the two reduction methods was slightly less accurate than the other. Although such differences were observed, they do not allow to draw conclusions on the preferential suitability of one reducing agent over the other for the analysis of DMSO in coral samples.

![Graph showing correlation of DMSO concentrations](image)

**Figure 18:** Correlation of DMSO concentrations in coral blastate samples following reduction by either TiCl₃ or NaBH₄.

When statistical analysis was conducted on the entire coral sample dataset, results indicated that there was no significant difference between the two reduction methods ($p > 0.05$, $n = 72$) (Figure 19). However, when DMSO concentrations were below
0.6 μmol L\(^{-1}\) (0.6 nmol of DMS in 1 mL sample), analysis using TiCl\(_3\) gave significantly lower values than with NaBH\(_4\) \((p < 0.001, n = 30)\). On the other hand, when DMSO concentrations were greater than 2 μmol L\(^{-1}\) (2 nmol of DMS in 1 mL sample), significantly greater values were found for TiCl\(_3\) compared with NaBH\(_4\) \((p < 0.002, n = 5)\). It remains unknown at this stage why such differences were observed, especially since calibrations were linear and repeatability was high for both reduction methods. Once again, this denotes that one reduction reaction prevailed the other although no definite conclusion can be drawn at this stage.

![Graph showing DMSO concentrations in coral blastate samples following reduction by TiCl\(_3\) and NaBH\(_4\) \((n = 72)\). The sample numbers are only representative of the order in which samples were analysed.](image)

**Figure 19:** DMSO concentrations in coral blastate samples following reduction by TiCl\(_3\) and NaBH\(_4\) \((n = 72)\). The sample numbers are only representative of the order in which samples were analysed.

*The influence of pH and purging on the analysis of DMSO in biological samples*

Although neutralisation with acid was found to be an essential step in the reduction of DMSO by NaBH\(_4\) after DMSP analysis involving NaOH additions up to 1 mol L\(^{-1}\) (Rismanan and DiTullio, 2004), variations in pH from 7.5 to 12 due to the presence of 0.01 to 0.1 mol L\(^{-1}\) NaOH from DMSP analysis in coral samples, was found to have no significant effect on the subsequent analysis of DMSO regardless of the reduction method used \((p > 0.05, n = 6)\) (Figure 20). This indicates that the variation in pH did not interfere with the DMSO analysis and therefore cannot explain the differences between reduction methods in coral samples.

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Figure 20: The influence of pH on the analysis of DMSO standards prepared in phosphate buffer (pH = 7.5) compared to DMSO standards prepared with the same proportions of NaOH as used in diluted coral samples (pH = 7.4 or pH=12) using either NaBH₄ (A) or TiCl₃ (B) as a reducing agent.

Coral versus macroalgae samples, the TiCl₃ and NaBH₄ reduction methods
Consistent with what has been observed for the entire coral dataset, significantly lower DMSO concentrations were obtained with TiCl₃ than with NaBH₄ in coral samples under basal conditions ($p < 0.001$, $n = 12$), in which DMSO concentrations were below 0.6 $\mu$mol L⁻¹ (Figure 21A). However, significantly greater DMSO concentrations were also obtained when using TiCl₃ than when using NaBH₄ in freshly collected...
*U. intestinalis* (*p* < 0.002, *n* = 10) and *U. lactuca* (*p* < 0.001, *n* = 10) for average DMSO concentrations of 0.6 μmol L⁻¹ and 0.8 μmol L⁻¹ respectively (Figure 21B). It is unclear why those results were obtained with macroalgae whereas no significant difference between the two reduction methods was found in coral samples for this concentration range (*p* > 0.05, *n* = 37 = 72 – 30 – 5) (Figure 19). Although speculative, this could be due to the difference in taxa and/or the way biological samples were processed. While coral samples were extracted into homogenized blastates, algal samples were only divided into 1–4 mg fragments of fresh tissue. This could have interfered with the way the TiCl₃ and NaBH₄ reduction reactions occurred within the different biological samples.

![Graph A](image)

![Graph B](image)

**Figure 21:** DMSO concentrations obtained following TiCl₃ and NaBH₄ reduction in coral blastate samples under basal, non-stressed, conditions (*A. aspera, n* = 12) and in macroalgae (*U. intestinalis* and *U. lactuca, n* = 10). Concentrations are expressed in μmol L⁻¹ ± standard error.
Particulate DMSO concentrations across various species

This study also provided a comparison of particulate DMSO concentrations across various types of photosynthetic organisms and showed that between the two reduction methods, an average of 10.1 ± 1.21 nmol (mean ± SE) of DMSO per µg of Chl \( a \) was found in freshly collected *A. aspera* samples compared to 0.48 ± 0.08 nmol µg\(^{-1}\) Chl \( a \) in *U. intestinalis* and 0.54 ± 0.04 nmol µg\(^{-1}\) Chl \( a \) in *U. lactuca* (Table 2). Although Chl \( a \) content in algal tissue greatly varies across species and seasons (Fernandez-Baco et al., 1998; Guo et al., 2011), these results clearly indicate that reef-building corals contain about 20 times more DMSO than macroalgae with a similar range of DMSP concentrations (Table 4). Both the symbionts and coral-associated bacteria could be responsible for the oxidation of DMS to DMSO (Hatton and Wilson, 2007; Raina et al., 2009; Sunda et al., 2002; Vila-Costa et al., 2006). The unique interaction that exists within the coral holobiont, involving host tissue, symbiotic microalgae, bacteria and other associated microbial populations, is most likely accounting for higher levels of DMSO in coral tissue. Moreover, since coral reefs are often subject to environmental pressure leading to oxidative stress (Downs et al., 2002; Flores-Ramirez and Linan-Cabello, 2007; Lesser, 1996; Linan-Cabello et al., 2010) and since DMSO is an end-product of oxidation by ROS (Sunda et al., 2002), elevated DMSO concentrations were expected in corals.
Table 4: Particulate DMSO and DMSP concentrations measured in coral samples (*A. Aspera*, *n* = 12) under basal conditions and macroalgae samples (*U. intestinalis* and *U. lactuca*, *n* = 10). CV = cell volume, FW = fresh weight. Concentrations are expressed as ± standard error.

<table>
<thead>
<tr>
<th></th>
<th>[DMSO]</th>
<th>[DMSP]</th>
<th>DMSO:DMSP</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>fmol cell⁻¹</td>
<td>mmol L⁻¹ CV</td>
<td>nmol µg⁻¹ Chl a</td>
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<tr>
<td><strong>A. aspera</strong></td>
<td></td>
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<tr>
<td>NaBH₄</td>
<td>22.3 ± 1.69</td>
<td>33.5 ± 2.46</td>
<td>12.7 ± 1.58</td>
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<tr>
<td>TiCl₃</td>
<td>13.4 ± 1.09</td>
<td>20.0 ± 1.57</td>
<td>7.54 ± 0.84</td>
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<td><strong>U. intestinalis</strong></td>
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<tr>
<td>NaBH₄</td>
<td>0.16 ± 0.05</td>
<td>0.08 ± 0.01</td>
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<tr>
<td>TiCl₃</td>
<td>0.79 ± 0.12</td>
<td>0.37 ± 0.06</td>
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<tr>
<td><strong>U. lactuca</strong></td>
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<tr>
<td>NaBH₄</td>
<td>0.12 ± 0.01</td>
<td>0.05 ± 0.005</td>
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</tr>
<tr>
<td>TiCl₃</td>
<td>0.95 ± 0.06</td>
<td>0.45 ± 0.03</td>
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Conclusion

This work is the first report of a comparison conducted on the two most commonly used reduction methods for the analysis of DMSO in biological samples using headspace analysis. It showed that these two techniques were strongly correlated in coral samples even though some significant differences were observed under certain concentration ranges. However, these differences did not allow us to draw categorical conclusions on the preferential suitability of one reducing agent over the other, which suggests that future work should probably be comparing the TiCl₃ and NaBH₄ reduction methods to a third DMSO analytical technique. Differences across reduction methods as observed between coral and macroalgae samples could be taxa-specific or due to differences in sample preparation. This study was also the first reported example of headspace analysis applied to the NaBH₄ reduction method and showed strong linearity and repeatability similarly to the reduction method with TiCl₃. Zooxanthellate corals were found to be much greater producers of DMSO than marine macroalgae with equivalent DMSP concentrations.
Effects of environmental factors on dimethylated sulphur compounds and their potential role in the antioxidant system of the coral holobiont

Unformatted published paper is shown in Appendix 6

Abstract

Biogenic DMS and its main precursors, DMSP and DMSO, are potential scavengers of reactive oxygen species in marine algae and these dimethylated sulphur compounds (DSC) could take part in the algal antioxidant system. In this study, a link between the DSC production and the AOC of Acropora aspera reef coral was investigated under a range of environmental factors (temperature, light, salinity, and air exposure) that can lead to oxidative stress in the coral holobiont. Enhanced DMS(P)(O) production occurred under experimental conditions, indicating that DSC are potential biomarkers of stress level in coral tissue. Differences in concentrations and partitioning as a response to different treatments suggest that DSC production and turnover undergo different biochemical pathways depending on the type and severity of environmental stress. Osmotic pressure and light depletion led to an up-regulation of the coral AOC that was correlated with a significant increase in DMSO:DSC ratio. These results, combined with a positive correlation between the AOC and DMSO concentrations under these two treatments, suggest that the DMSP-based antioxidant system is involved in the overall antioxidant regulation of the coral holobiont. Enhanced DMS production coupled with an increased DMS:DSC ratio under increased temperature indicated that thermal stress triggers DMS formation in coral tissue. Considering the role that DMS can have in both climate regulation and the DMSP-based antioxidant system, our findings highlight the need to further examine the fate of DSC in coral reef environments under scenarios of increasing sea surface temperatures.
Introduction

DMS is the most abundant volatile sulphur compound in seawater and the largest input of biogenically derived sulphur into the marine boundary layer (Andreae and Crutzen, 1997). Its main precursor, DMSP, is mostly synthesised by photosynthetic marine algae; however, it has recently been demonstrated that both the endosymbiotic algae and animal host in corals produce this sulphur compound (Raina et al., 2013). DMSP is a zwitterion that does not easily permeate through cell membranes during normal metabolism, but it can diffuse into the surrounding media when there is a loss of membrane integrity through grazing or cell lysis (Dacey and Blough, 1987). DMSP can be enzymatically cleaved into DMS by DMSP-lyases that have been found in various organisms including various types of marine algae, fungi and bacteria (Stefels, 2000), making DMSP a considerable source of volatile sulphur and labile carbon for the microbioal food web.

DMSO is another biogenic precursor of DMS when it is reduced by marine algae and sulphate-reducing bacteria through a poorly-understood reduction pathway that might depend on reductases (Spiese et al., 2009). DMSO is also an end-product of DMS oxidation by ROS in marine algae (Sunda et al., 2002). Due to its biochemical property as a dipolar, aprotic, hydroscopic substance, DMSO is known to permeate easily through membranes of healthy cells, which is possibly the reason why DMSO is usually found in lower intracellular concentrations than DMSP (Hatton and Wilson, 2007). Similarly, being uncharged, volatile, and more hydrophobic, DMS is believed to diffuse through cell membranes more easily than both DMSP and DMSO (Sunda et al., 2002).

Particular interest has been given to DMS over the past two decades because this sulphur compound may exert a negative feedback effect on global warming (Charlson et al., 1987), although this is debated (Quinn and Bates, 2011). According to the CLAW hypothesis, DMS is oxidised to non-sea-salt sulphate aerosols that can contribute to the formation of cloud condensation nuclei, increasing the albedo of tropospheric clouds over the ocean and locally lowering solar radiation and sea surface temperatures (Charlson et al., 1987). In parallel, a wide-range of biological functions has been
proposed for DMSP, and/or DMSO, such as osmoregulation (Reed, 1983), thermoregulation (Kirst et al., 1991), chemo-attraction (DeBose et al., 2008), and chemoprotection against grazers (Wolfe et al., 1997). Both DMS and its precursors are suspected to act as antioxidants, since all three DSC can actively scavenge ROS (Sunda et al., 2002), and particularly hydroxyl radical (OH•), which is the most harmful ROS known (Gill and Tuteja, 2010). In all likelihood, DMSP serves multiple physiological functions (Stefels, 2000) that are still not completely understood.

During normal metabolism, phototrophs produce ROS, through both cellular respiration and photosynthesis, which makes these organisms much more susceptible to oxidative damage than aerobic heterotrophs (Gill and Tuteja, 2010). These ROS are normally in balance with a range of antioxidants that scavenge and convert them to non-reactive species (Csaszar et al., 2009). However, this balance can shift towards oxidative stress under environmental stress, either through an excessive accumulation of ROS or a depletion in antioxidants that can cause damage to proteins, lipids, carbohydrates, DNA, and the entire photosynthetic system, ultimately leading to cell death (Martindale and Holbrook, 2002; Gill and Tuteja, 2010). Increased DMSP production has been associated with increased ROS levels in *Symbiodinium* (McLenon and DiTullio, 2012) and increased ascorbate peroxidase activity in marine algae (Sunda et al., 2002), the main antioxidant in chloroplasts, which is a major site for ROS generation. DMSP and DMSO concentrations were also positively correlated with the antioxidant beta-carotene in upwelled seawater (Riseman and DiTullio, 2004), supporting the antioxidant hypothesis for DMS and its precursors. This DMSP-based antioxidant system is now thought to play an important role in the complex antioxidant system of marine algae, with each compound possibly acting at various rates and reacting preferentially with different ROS (Sunda et al., 2002).

The coral host, its associated microbial community, and its endosymbiotic algae, of which density averages several millions of cells per cm² of coral tissue (Fitt et al., 2000), constitute the coral holobiont, with each partner being a substantial source of DSC in corals (Raina et al., 2009; Fischer and Jones, 2012; Raina et al., 2013). This study concentrated on the production of DSC under potential environmental stressors.
(elevated temperature, direct sunlight, reduced salinity, air exposure and light depletion) in *Acropora aspera*, a common branching reef-building coral of the GBR, Australia. The AOC was examined as a whole rather than targeting specific antioxidants, with the aim to understand the role that DSC play in the complex antioxidant system of the coral holobiont. Since an up-regulation of the antioxidant system is expected in acclimating organisms under oxidative stress (Lesser and Shick, 1989), stress exposure should lead to an increase in the AOC in coral tissue. In parallel, if the DMSP-based antioxidant system contributes to the coral AOC, then DMS, and DMSP are expected to quickly oxidise to DMSO through the scavenging of ROS under oxidative stress (Sunda et al., 2002). However, oxidative stress and production of ROS can also vary greatly with respect to the type of treatment, its dosage and/or the duration of exposure (Martindale and Holbrook, 2002). Therefore, the extent of environmental stress and the time of exposure are important factors to consider.

**Methods**

*Coral collection and experimental design*

Three colonies of *Acropora aspera* were collected on the 28/02/2012 at 16:30 local time at low tide (low versus high depth range: 0.2 - 3.1 m) within the scientific research zone of the Heron Island reef flat (23° 26' 46.19" S / 151° 54' 46.35" E) using a hammer and chisel, and transported to the Heron Island Research Station (HIRS) in Nally bins that were filled with seawater collected *in situ*. Salinity, light and temperature at the collection site were recorded using a portable Vital Sine refractometer (SR-6) and an underwater light and temperature probe respectively from the depth of the coral colonies (approximately 50 cm). Physical parameters at the collection site are reported in Table 5. Colonies were immediately nubbinised to approximately 4 cm long fragments that were randomly cut using hand shears. A first mix of 12 nubbins (4 per coral colony) were immediately snap frozen in liquid nitrogen and kept at – 80°C as untreated controls. The remaining nubbins were left to acclimate at the HIRS in an outdoor flow-through seawater holding tank for 40 h before the start of the experiment. Shade cloths attenuating sunlight by approximately 50%, were placed over the holding tank during hours of direct sunlight in order to regulate UV exposure. A mix of 4 nubbins per
colony were randomly assigned to five experimental treatments that aimed to mimic acute environmental stress: T\textsubscript{1} – elevated temperature (increased from 28°C to 31°C over 4 days and maintained at 31°C for 3 days); T\textsubscript{2} – direct sunlight exposure (daily maximum of approximately 350 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) for 7 days); T\textsubscript{3} – reduced salinity (27‰ for 2 days); T\textsubscript{4} – short-term air exposure mimicking low tide effects (exposure to air for 5 h), and T\textsubscript{5} – light depletion (daily maximum of approximately 5 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) for 3 days) (Table 5). Two sets of 12 nubbins (4 per colony) were kept as experimental controls under monitored control conditions (28°C, 35‰, and approximate daily maximum of 150 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) in either flow-through seawater holding tanks (as representative controls for T\textsubscript{2}, T\textsubscript{4}, and T\textsubscript{5}) or aerated enclosed tanks kept in flow-through water baths (as representative controls for T\textsubscript{1} and T\textsubscript{3}) for the entire time of the experiment (7 days). Light and temperature were monitored every 10 min using HOBO data loggers (Onset, United States of America), and salinity was measured using a portable Vital Sine refractometer (SR-6). At the end of each experimental treatment, nubbins were immediately snap frozen in liquid nitrogen and kept at –80°C for future tissue extraction.
Table 5: Summary of measured light, temperature, and salinity levels over time for the different experimental treatments and unexposed controls. PAR = photosynthetic active radiation (µmol photons m\(^{-2}\) s\(^{-1}\)).

<table>
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<tr>
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Tissue extraction and sample preservation

Frozen tissue was extracted by air blasting in 10 mL of 75 mmol L⁻¹ sodium phosphate buffer, pH 7.4 (16.7% NaH₂PO₄ Sigma Aldrich S-8282 and 83.3% Na₂HPO₄ Sigma Aldrich S-7907) using an air gun connected to an air cylinder as this was the most appropriate method for antioxidant extraction from coral tissue (Deschaseaux et al., 2013). Since DMSP and DMSO are enzyme regulated and DMS is a highly volatile compound, it is possible that the balance of DSC in the samples could have been altered during the extraction procedure, which likely released enzymes. However, since all samples were extracted and preserved the same way throughout the experiment, concentrations among treatments should be comparable. Tissue lysates were transferred into centrifuge tubes, manually shaken for homogenization and kept on ice while sub-sampled. A one mL aliquot was sonicated for 5 min in a chilled water bath, centrifuged at 10,000 g for 15 min and supernatant was subdivided into two 500 µL subsamples for AOC and protein content measurements. Another 1 mL subsample was diluted to 5 mL for symbiont density and cell size measurements. Another 3 mL subsample was diluted to 30 mL with MilliQ water and divided into three 10 mL aliquots. One aliquot was hand-filtered through Whatman Grade GF/F glass fibre filters for Chl a content analysis and filters were frozen at – 80°C. The second 10 mL aliquot was purged with high purity N₂ for 10 min at 100 mL min⁻¹ to remove free DMS derived from coral tissue while the third 10 mL aliquot was left unpurged. One mL of 10 mol L⁻¹ NaOH was added to both purged and unpurged aliquots (final pH > 14) and vials were immediately sealed with gas-tight septa impermeable to DMS (Agilent Technologies, PTFE and Silicone septa, 5183-4477) and crimp capped. Purged aliquots contained only DMSP-derived DMS that was generated during the alkaline treatment whereas unpurged aliquots contained both free and DMSP-derived DMS. Tissue DMS concentrations were therefore obtained by calculating the difference between purged and unpurged aliquots. A diagram illustrating the different extraction and analytical steps is shown in Figure 22.
**Analysis of sulphur compounds**

Purged and unpurged samples were analysed for total DMSP and DMS content in coral tissue within two weeks of sample preservation. Analysis was performed on a GC-MSD (Agilent Technologies 6890N; 5973N) operated in scan mode coupled with a Gerstel multipurpose sampler (MPS 2XL) configured for headspace analysis with a 2.5 mL syringe. The syringe temperature was set at 95°C and the injection volume used was 1000 µL with an injection speed of 500 µL s⁻¹. The GC injector temperature was set at 280°C and the injection was made with a split ratio of 25:1. DMS was eluted using a capillary column (5% Phenyl Polysilphenylene-siloxane, BPX5, 50 m, 0.22 mm x 1 µm
film thickness, Scientific Glass Engineering) with He as the carrier gas at a constant flow rate of 1.1 mL min⁻¹. The GC oven temperature was programmed from 35°C (held for 8 min) to 180°C at a ramp rate of 80°C per min and held at 180°C for 2 min. DMS was identified by reference to the MS library database (National Institute of Standards and Technology (NIST98) mass spectra library), and data were processed using ChemStation software (version D.02.00.275, Agilent Technologies). DMSP. HCl standards (certified reference material WR002, purity 90.3 ± 1.8% mass fraction, National Measurement Institute, Sydney, Australia) and blanks were prepared with the same proportions of phosphate buffer, MilliQ water and NaOH as for DMS and DMSP samples. Seven-point calibration curves and three blanks were run daily at the start and end of the sample analysis for calibration and contamination tests respectively under the exact same analytical conditions as for samples (Appendix 1).

DMSO concentrations were measured from pre-purged aliquots that were diluted following 10- or 100-fold into 75 mmol L⁻¹ sodium phosphate buffer (pH 7.4) and purged with N₂ for 10 min to remove the DMS that was generated during the alkaline treatment of DMSP samples. Two hundred μL of 20% w:w TiCl₃ (Sigma Aldrich) was added to 1 mL of diluted subsample in glass vials that were immediately sealed with gas-tight septa (VWR, 16171-651) and crimp capped before being heated in a 50°C water bath for 1 h (Kiene and Gerard, 1994). After samples had cooled down to room temperature, headspace analysis was performed by direct injection on a Shimadzu GC-FPD (model 2014) set at 175°C, and a 20 cm x 0.32 cm OD Teflon (FEP) column packed with Chromosil 330. The GC oven was used isothermally at 60°C with a He carrier flow of 25 mL min⁻¹. DMSO data were processed using the GCsolution software (version 2.32.00, LabSolutions, Shimadzu). DMSO standards (Fisher Scientific BP321) and blanks were prepared in the same proportions of phosphate buffer, MilliQ water and NaOH as used for the samples. Seven-point calibration curves and three blanks were run daily at the start and end of the sample analysis for calibration and contamination tests respectively under the exact same analytical conditions as for samples (Appendix 1).
**AOC and Protein content**

Samples for AOC and protein concentrations were analysed using the ORAC assay (Ou et al., 2001) and Biuret assay (Dustin, 1950) respectively, as described in Deschaseaux et al. (2013) (Chapter 3). Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Fluka Chemika 56510, 100 μmol.L⁻¹) was used as a reference standard in the ORAC assay and AOC values were expressed per micromole of Trolox equivalent (TE) and standardised to protein content in each sample.

**Cellular parameters and surface area measurements**

Eight replicate cell counts of the diluted tissue homogenates were performed on a haemocytometer using a compound microscope (Olympus CX21) at a magnification of 100X for symbiont density measurements and the diameters of 20 random cells were measured at a magnification of 200X using a stage micrometer. Filters for Chl a analysis were homogenised and extracted overnight at –20°C in 90% acetone. Samples were thawed, sonicated and centrifuged for 15 min. Absorbance of the acetone extract was measured by spectrophotometry (GBC Scientific Equipment, UV visible 916 spectrophotometer) at 664 nm, 665 nm, and 750 nm wavelengths before and after adding 0.1 mol L⁻¹ HCl. Chl a concentration was determined using the equation from Standard Methods (APHA, 1998). Remaining nubbin skeletons used for coral tissue extracts were cleaned of skeleton debris and remaining tissue in concentrated sodium hypochlorite and left to dry for several hours. The surface area of each bleached nubbin was measured using the hot wax method (Chancerelle, 2000) (Appendix 1).

**Statistical treatment and analysis of variates**

Variates were tested for normality and data were logarithmically transformed when the assumption of normality was violated. A student t-test was performed to compare the values obtained for cellular parameters, DSC and AOC between untreated controls and experimental controls. No significant difference was found in the AOC levels, Chl a content, cell density, cell size, and DMS, DMSP, and DMSO concentrations (p > 0.05) between unexposed and experimental controls. As such, only values of unexposed controls are presented as they provide a more accurate view of natural levels.
A one-way ANOVA was fitted to the dataset using Bonferroni adjustments for pair-wise comparisons between treatments. Pearson’s correlation coefficients and corresponding $p$-values were established for each treatment between AOC and DMSO as well as between DMSO and reduced sulphur compounds using a two-tailed test.

Due to an accidental loss in information, the effective sample size of this study may be smaller than the 72 samples employed in the analysis because it is unknown which nubbins were collected from which colonies. Because the resulting $p$-values from the analyses are likely to be smaller by an unknown amount, our approach was to interpret the results of the analyses very conservatively using a more rigorous probability criterion for significance of $p \leq 0.01$ and to calculate standard error based on the number of colonies ($n = 3$). Averaged values are reported as mean ± SE throughout the manuscript.

**Results**

*Chlorophyll $a$, symbiont density, and cell size*

Symbiont density significantly decreased by approximately 50% under elevated temperature ($p < 0.001$) and direct sunlight ($p < 0.001$) (Figure 23A). Although the amount of Chl $a$ per cell did not vary among treatments ($p > 0.01$, data not shown), Chl $a$ concentration per square cm of coral surface area decreased significantly by around 50% under reduced salinity ($p \leq 0.01$) and light depletion ($p \leq 0.01$) (Figure 23A). Symbiont cells shrank significantly when corals were exposed to reduced salinity (~ 6% decrease, $p < 0.001$), air exposure (~ 3% decrease, $p \leq 0.01$), and light depletion (~ 3% decrease, $p \leq 0.01$) (Figure 23B).
Figure 23: Symbiont density and Chl a concentration (A) along with cell diameter (B) for each treatment. Error bars = SE, n = 3. An asterisk indicates significant differences relative to controls.
**Dimethylated sulphur compound**

Concentrations of DMS, DMSP, and DMSO were normalized to Chl \(a\), protein concentrations, coral surface area, zooxanthellae cell number, and cell volume (Table 6). However, the \(p\)-values reported in the text are those obtained for concentrations expressed per mg of protein because protein concentration is the most representative proxy for live tissue, including the host, symbiont and microbial fractions that constitute the coral holobiont. DMSP levels were significantly greater under elevated temperature \((p < 0.001)\), direct sunlight \((p < 0.001)\), and air exposure \((p \leq 0.01)\) relative to the control (Table 6). However, the DMSP:DSC ratio under direct sunlight \((78 \pm 1\%)\) and air exposure \((71 \pm 7\%)\) was similar to the controls \((72 \pm 3\%)\) \((p > 0.01)\), whereas it was significantly reduced under elevated temperature \((39 \pm 5\%)\), reduced salinity \((40 \pm 5\%)\), and light depletion \((51 \pm 3\%)\) \((p < 0.001)\) (Figure 24). DMS concentrations increased significantly under elevated temperature \((p < 0.001)\) and direct sunlight exposure \((p < 0.001)\) (Table 6) however, the DMS:DSC ratio exclusively increased under thermal stress \((36 \pm 4\%)\) compared to the controls \((6 \pm 2\%)\) (Figure 24). DMS was undetectable in coral tissue under reduced salinity, air exposure, and light depletion (Table 6, Figure 24), and therefore both DMS concentrations and the DMS:DSC ratio were found to decrease significantly under these three treatments compared to the controls \((p < 0.001)\). DMSO concentrations significantly increased as a response to stress exposure, regardless of the experimental treatment \((p < 0.001)\) (Table 6). However, the DMSO:DSC ratio only increased under reduced salinity \((60 \pm 5\%)\) and light depletion \((49 \pm 3\%)\), compared to a DMSO:DSC ratio of \(22 \pm 2\%\) in the controls (Figure 24).
Table 6: Total DMS, DMSP, and DMSO concentrations normalized to different measured parameters. Concentrations in the different units are given for each individual treatment and controls. Concentrations are reported as mean ± SE, with the exception of DMS concentrations that were below the detection limit (BDL). Numbers in bold are those that were significantly different ($p < 0.01$) relative to the controls.

<table>
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<th>Units DMS</th>
<th>Units DMSP</th>
<th>Units DMSO</th>
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<td>Controls</td>
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Figure 24: Partitioning of relative DMS, DMSP, and DMSO concentrations in the coral holobiont expressed in ratios of DMS(P)(O):DSC (%) for each treatment.

Correlations between DMSO, DMSP, and DMS

A positive correlation was found between DMS and DMSO concentrations under elevated temperature ($r = 0.762, p \leq 0.01$) (Figure 25A). DMSP and DMSO concentrations were positively correlated under direct sunlight ($r = 0.827, p < 0.001$), reduced salinity ($r = 0.936, p < 0.001$), air exposure ($r = 0.984, p < 0.001$), and light depletion ($r = 0.938, p < 0.001$) (Figure 25B-E). DMS concentrations fell below the detection limit under reduced salinity, air exposure, and light depletion (Figure 25, Table 6), so no relationship could be established between DMS and DMSO for those treatments.
Figure 25: Significant correlations between ln-transformed DMSO concentrations and ln-transformed DMS or DMSP concentrations normalised to protein concentrations under elevated temperature (A), direct sunlight (B), reduced salinity (C), air exposure (D), and light depletion (E).
Antioxidant capacity and DMSO

Significantly greater AOC was found in corals that were exposed to reduced salinity ($p < 0.001$) and light depletion ($p < 0.001$) (Figure 26). Coral AOC was correlated with DMSO concentrations under direct sunlight exposure ($r = 0.856, p < 0.001$), reduced salinity ($r = 0.777, p \leq 0.01$), and light depletion ($r = 0.838, p < 0.001$) (Figure 27A-C).

Figure 26: AOC of the coral holobiont for each treatment. Error bars = SE, $n = 3$. An asterisk indicates significant differences relative to controls.
Figure 27: Significant correlations between log-transformed AOC and In-transformed DMSO concentrations, both normalised to protein concentrations, under direct sunlight (A), reduced salinity (B) and light depletion (C).
Discussion

DSC as biomarkers of environmental stress in corals

DMS, DMSP, and DMSO were found at different concentrations in coral tissue depending upon the environmental treatment to which corals were exposed (Table 6). Specifically, DMSO concentrations significantly increased under experimental conditions regardless of the type of variables. This response suggests that ecological and physical factors such as temperature, light availability, salinity and exposure to air at low tides, influence the production and turnover of these sulphur compounds in the coral holobiont, making DSC substantial biomarkers of stress exposure in corals, with DMSO being the most indicative of all because it significantly increased under each treatment.

DMSP concentrations increased significantly in nubbins of *Acropora aspera* that were exposed to elevated temperature, direct sunlight and air exposure, with the greatest concentration being recorded under UV and tidal stress (Table 6). Maximum intracellular concentrations under direct sunlight as opposed to low intracellular DMSP concentrations under light depletion suggest that DMSP production of the coral holobiont is intimately linked to photosynthesis in the symbiotic microalgae. A loss of symbiotic zooxanthellae in response to increased temperature and direct sunlight (Figure 23A) indicated that bleaching occurred under these two treatments (Brown, 1997). Together, these results suggest that DMSP production is significantly enhanced during bleaching of reef-building corals. This observation is particularly interesting considering that endosymbiotic zooxanthellae, whose density is reduced during bleaching, are likely contributing to half of the DMSP pool in the coral holobiont. High DMSP concentrations associated with bleaching could reflect increased DMSP production by the host (Raina et al., 2013) or a change in the relative abundance of clades through either switching or shuffling (Baker, 2003), with the newly dominant clade being a greater producer of DMSP than the clade it replaced (Steinke et al., 2011). However, this study did not investigate clade genotyping.
DMSP concentration did not vary significantly under reduced salinity and light depletion compared to controls (Table 6). Considering the osmotic properties of DMSP and the photosynthetic requirement for its synthesis (Stefels, 2000), low concentrations were expected under these two treatments. Results suggest that DMSP might have been expelled extracellularly in order to re-establish cellular osmotic balance as a response to reduced salinity and that DMSP formation might have been restricted under light depletion. Cell shrinkage under reduced salinity (Figure 23B) suggests a loss of cell integrity, which could have facilitated the diffusion of osmolytes such as DMSP through cell membranes. Similarly, decreased Chl a concentration per coral surface area under light depletion (Figure 23A) suggested a lower yield for photosynthesis and therefore for DMSP synthesis for this treatment. Since an increase in Chl a is expected under light limitation in photoadapted organisms (Greider et al., 2006), these results therefore indicate that photoadaptation had not occurred under this treatment.

DMS concentrations increased in response to elevated temperature and direct sunlight, although DMS concentration under thermal stress was more than 10 times that recorded under direct UV exposure (Table 6). Similarly, DMS as a fraction of total DSC in the coral tissue mainly increased as a response to elevated temperature (Figure 24). Together, these results suggest that a rise in temperature and direct sunlight exposure trigger DMS production in the coral holobiont, with temperature being a strong driver of DMS formation. Under reduced salinity, air exposure, and light depletion, DMS levels fell below the detection limit (Figure 24, Table 6), indicating either (i) enhanced DMS consumption or loss from coral tissue or (ii) inhibited DMS production, with the former suggesting either DMS oxidation to DMSO (Sunda et al., 2002) or direct DMS discharge to the surrounding media. The increase in DMSO:DSC ratio under reduced salinity and light depletion (Figure 24) suggests that DMS was preferentially oxidised to DMSO under these treatments. On the other hand, the steady DMSO:DSC ratio under air exposure in proportion to the controls (Figure 24) could indicate inhibited DMS production or DMS tissue-to-air exchange. The latter interpretation coincides with observations of enhanced atmospheric DMS emissions in coral reefs following low tide (Jones and Trevena, 2005; Jones et al., 2007; Swan et al., 2012).
Disparities observed in DSC partitioning and concentrations between treatments are most probably due to complex biochemical interactions that take place in the coral holobiont under environmental stress (Lesser, 2006; Thurber et al., 2009). As previously mentioned, such biochemical processes could involve photosynthesis, bleaching, or symbiont recombination but could also implicate coral-associated bacteria that constitute a determinant factor in the coral sulphur cycle during both basal conditions and stress exposure (Bourne et al., 2008; Raina et al., 2009). It is also important to acknowledge that the treatments used in this study should be considered acute stressors that might occur more frequently and over longer periods of time under future climate change scenarios. The production and consumption of DSC are likely changing at variable rates and amplitude over time and in response to the severity of these diverse factors. However, the implication of changes we observed in the production of DSC under stress remains speculative at this stage.

The DMSP antioxidant system in the antioxidant response of the coral holobiont

The response to oxidative stress depends upon the type of treatment and the severity of oxidative damage that has been caused to the organism (Martindale and Holbrook, 2002). In organisms responding to stress exposure, an accumulation of ROS is expected to stimulate the antioxidant system and to therefore increase AOC values, which was observed under reduced salinity and light depletion in this experiment (Figure 26). However, AOC represents a net balance between ROS scavenging and antioxidant production. Therefore, if antioxidant production is low (e.g. unstressed organisms) or if ROS scavenging rate is greater than antioxidant production (e.g. organisms not coping with stress), then AOC levels would be low. On the other hand, if ROS scavenging rate is low with respect to the antioxidant production rate (e.g. organisms coping with stress exposure), then AOC values would be high. Although stressors and time of exposure in this experiment were carefully determined to be as realistic as possible with what corals could naturally experience in their natural environment, therefore leading to stress that organisms will cope with, AOC results should be carefully interpreted.

The AOC was similar to that of unexposed controls under elevated temperature, direct sunlight and air exposure, environmental factors to which reef-building corals are
naturally exposed (Jimenez et al., 2012), (Figure 26). In contrast, under reduced salinity and light depletion, the AOC increased significantly (Figure 26), indicating oxidative stress and ROS accumulation under these two treatments (Lesser and Shick, 1989). Cell shrinkage under reduced salinity (Figure 23B) and decreased Chl \(a\) concentration per coral surface area under light depletion (Figure 23A) confirmed physiological impairments under these treatments.

Increased AOC levels under reduced salinity and light depletion (Figure 26) correlated with a significant increase in the DMSO:DSC ratio in these two treatments (Figure 24), suggesting that DMSO production in coral tissue is intimately linked to the up-regulation of the coral AOC. This observation was further supported by positive correlations between the AOC and DMSO concentrations under reduced salinity and light depletion (Figure 26B,C). Since DMSO can result from DMS and DMSP oxidation through the scavenging of ROS (Sunda et al., 2002), our results collectively suggest that the DMSP-based antioxidant system plays a substantial role in the overall antioxidant response of the coral holobiont to oxidative stress.

Although DMSP and DMSO concentrations were significantly higher under direct sunlight than in the controls (Table 6), the relative partitioning of DSC was similar to that of controls (Figure 24). Similarly, the AOC obtained under direct sunlight did not vary from the controls (Figure 26). However, a positive correlation was established between the AOC and DMSO concentrations for this treatment (Figure 27A). These results suggest that DMSO production may be involved in the maintenance of the antioxidant system of the coral holobiont during photosynthesis, when high production of ROS, and particularly singlet oxygen and superoxide radicals, occurs (Gill and Tuteja, 2010). The oxidation of reduced sulphur compounds and especially DMS, which is highly reactive towards singlet oxygen (Sunda et al., 2002), can be expected during photosynthesis. Considering that DMSO might accumulate at higher cellular concentrations than DMS (Sunda et al., 2002), this result also suggests that DMSO formation during photosynthesis will constitute a second shield against oxidative stress as it can be further oxidised in the presence of ROS.
The relative speciation of DSC

The low permeability of cell membranes to DMSP makes this compound a relatively stable biomarker to measure in coral tissue under normal metabolism compared to both DMS and DMSO, which should freely pass through cell membranes (Sunda et al., 2002). Due to these differences in properties, DSC most likely operate at different levels within cell compartments and membranes, with their relative abundance reflecting the needs of the cells and the ability for these compounds to be generated in areas where ROS are synthesised or accumulated. However, during environmental stress leading to cell impairment, cell loss (bleaching) and/or symbiont recombination, the relative cellular permeability of DSC becomes compromised (Dacey and Blough, 1987). Moreover, because the coral holobiont comprises coral tissue, endosymbiotic cells, and a vast microbial community, with each partner potentially being involved in the production, consumption, and turnover of DSC (Raina et al., 2013), this adds to the difficulty in trying to understand the DMSP-based cycle in corals.

Under elevated temperature, the high DMS concentration was likely sourced from DMSP as indicated by a significant decrease in the DMSP:DSC ratio and a significant increase in DMS:DSC under this treatment (Figure 24). This result suggests that increasing temperature triggers enhanced DMSP-lyase activity in the coral holobiont. However, it is important to keep in mind that DMS concentration under this treatment could reflect a snapshot of accumulated DMS within the holobiont. The positive correlation that was observed between DMS and DMSO under elevated temperature (Figure 25A) suggests that some of this DMS was probably converted to DMSO by ROS or other oxidation pathways. However, it might also indicate that part of the DMS came from DMSO reduction (Spiese et al., 2009). However, without turnover data we cannot draw categorical conclusions about the source and fate of DMS under increased temperature.

DMSO and DMSP concentrations were correlated under most variables (Figure 25B-E), suggesting that DMSP is the main source of DMSO in coral tissue under environmental stress. However, direct oxidation of DMSP to DMSO is chemically unlikely to by-pass DMS formation (Van Alstyne et al., 2001; Wolfe et al., 2002). These correlations
therefore most likely indicate DMSP cleavage into DMS, which was then oxidised to DMSO. Since DMS is highly reactive towards OH• (Sunda et al., 2002), it is unlikely to accumulate in coral tissue under enhanced oxidative stress and excessive ROS production, but to be quickly oxidized to DMSO and other oxidised sulphur species. This possibility is supported by the absence of DMS under reduced salinity, air exposure, and light depletion (Figure 24, Table 6). On the other hand, the high DMS:DSC ratio under elevated temperature could be interpreted as DMS in coral tissue fulfilling other functions than as an antioxidant under this stressor and/or it could constitute a pool for DMS emissions to the surrounding media.

Levels of DMSP measured under thermal, UV, and air exposure in this study were among the highest concentrations yet reported in corals (Broadbent et al., 2002; Yost et al., 2012), especially when expressed per symbiont cell or cell volume (Table 6). However, under environmental stress, corals tend to produce copious amounts of mucous (visual observations), which has been shown to contain the highest concentrations of DMS and DMSP in the marine environment (Broadbent and Jones, 2004). Hence, data obtained in these treatments most likely represent accumulated DMSP in mucous, symbiont cells, and coral tissue and are therefore more accurate when normalised to protein or square cm of coral surface area than to cell, cell volume or Chl a content.

**Implications for the Great Barrier Reef**

About 10% of the DMS that is biosynthesised in the ocean is emitted to the atmosphere (Malin et al., 1992), possibly leading to an increase in cloud cover and a negative feedback effect on sea surface temperature and incident solar radiation (Charlson et al., 1987); although this mechanism is much more complex than first outlined in the CLAW hypothesis (Quinn and Bates, 2011). In the present study, increased temperature was found to be an important driver of DMS production in the coral holobiont, as indicated by a substantial increase in DMS concentrations under thermal stress (Table 6) reaching a DMS:DSC ratio of 36 ± 4% compared to 6 ± 2% in the controls (Figure 24). This finding could have direct relevance to the ongoing debate that coral reefs under thermal stress could contribute to a local feedback effect on climate (Deschaseaux et al., 2012),
as enhanced coral DMS production could be expected to lead to enhanced DMS discharge into the water column, increasing the dissolved pool for potential atmospheric emissions over coral reefs. While in this study no measurements were made of dissolved or gaseous DMS, chamber experiments conducted on Acropora formosa (Jones et al., 2007) and Acropora intermedia (Fischer and Jones, 2012) showed a decrease in both dissolved and atmospheric DMS as a result of temperature increase. Unless the contradiction between these studies and the current experiment were due to species-specific variability, taken together these results suggest that the DMS produced by the coral holobiont under thermal stress does not leach out of to the surrounding media but instead is stored in the coral tissue, most likely to fulfil other biological functions. Further experiments are therefore needed to understand the fate of enhanced DMS production in coral tissue under scenarios of increasing sea surface temperature, and their affect on atmospheric DMS production derived from the coral holobiont over the GBR.
CHAPTER 6

Can corals form aerosol particles through volatile sulphur compound emissions?

Unformatted published paper is shown in Appendix 7

Abstract

*Acropora* dominated coral reefs are a substantial source of atmospheric dimethylsulphide, one of the most abundant reduced sulphur gases present in the marine boundary layer. DMS is believed to act as a climate regulator of solar radiation and sea surface temperatures through the formation of non-sea-salt sulphate aerosols and CCN, although this regulation has not yet been demonstrated. A bubbling chamber experiment was conducted on coral reef seawater containing a branch of *Acropora pulchra*, to investigate whether the coral-generated DMS$_g$ could be oxidised to non-sea-salt sulphate aerosols under treatment with UV light and O$_3$. Results indicated that *A. pulchra* produced significant amounts of DMSP and dissolved DMS although DMS$_g$ emissions in the chamber headspace were reduced by the presence of the coral, probably from the build up of the coral mucous in the chamber following bubbling, which might have retained the bulk of DMS$_g$ produced by the coral. Significant amounts of suspected carbon disulphide (CS$_2$) and ethanethiol (ESH), other sulphur gases that could be involved in CCN formation, were also measured in the bubbling chamber, possibly from coral production. However, no conclusion was drawn on these sulphur gases as both their origin and formal identification are still to be determined. A decrease in DMS$_g$ in the presence of UV light and O$_3$ followed by an occurrence of freshly nucleated nanoparticles (<10nm) suggested that this sulphur compound was oxidised and could potentially participate in aerosol particle formation. The study provided insights into the production of sulphur compounds by *Acropora* dominated coral reefs and how reef aerosol emissions could potentially impact on local climate.
Introduction

According to the CLAW hypothesis, increasing atmospheric dimethylsulphide emissions sourced from phytoplankton under anthropogenic warming can oxidise to non-sea-salt (nss) sulphate aerosols which go on to form CCN, increasing the albedo of stratocumulus clouds and locally lowering solar radiation and SST in the ocean (Charlson et al., 1987). Although supported by several studies (Ayers et al., 1991; Modini et al., 2009), the CLAW hypothesis has never been verified. On the other hand, oxidised organic compounds can also participate in the formation of newly-formed secondary aerosol particles (Vaattovaara et al., 2006).

Research has shown that zooxanthellate corals, through both their symbiotic microalgae and animal tissue, produce DMSP (Raina et al., 2013), the main precursor of DMS, and can be a source of DMS\textsubscript{a} in coral reefs (Fischer and Jones, 2012). Continuous monitoring of SST in coral reefs worldwide has shown that pristine reefs within or near the Western Pacific Warm Pool have had fewer reported coral bleaching events relative to reefs in other regions of the world possibly because of an “ocean thermostat” mechanism that acts to depress warming beyond certain SST thresholds (Kleypas et al., 2008). Research on DMS and aerosol formation suggest that oxidation of atmospheric DMS (DMS\textsubscript{a}) from reefs could form nss-sulphate aerosols and thus could contribute to this phenomenon (Jones and Trevena, 2005; Jones et al., 2007; Modini et al., 2009; Jones and Ristovski, 2010).

Bubble bursting from breaking waves in the ocean is the primary source of sea-air exchange and sea spray aerosol production in the atmosphere, constituting a possible source for CCN formation (Modini et al., 2009). Hydroxyl radicals in the atmosphere, which are formed as a consequence of photo-dissociation of ozone (O\textsubscript{3}) by solar UV, are responsible for the oxidation of gaseous precursors such as DMS into atmospheric aerosols (Andreae and Crutzen, 1997). Thus, bubble bursting, O\textsubscript{3} and UV radiations are three major components in the emission and oxidation of DMS\textsubscript{a} to aerosol particles.
Between May and June 2011, the CORACE-1 (CORal Reef Aerosol Characterization Experiment-1) campaign was conducted on Heron Island coral cay as a collaborative research project between QUT (Queensland University of Technology, Brisbane), SCU (Southern Cross University, Lismore) and UEF (University of Eastern Finland, Kuopio) in order to ascertain whether DMS_a sourced from coral reefs could, during bubble bursting and under O_3 and UV radiations, contribute to the formation of aerosol particles and hence potentially influence local climate. For more background information refer to Swan et al. (2012) in Appendix 8.

The overall aim of this chapter was to evaluate the potential of a coral branch to contribute to the formation of aerosol particles which could grow to CCN. The specific objectives of this chapter were to (i) ascertain whether the coral branch was a source of dissolved DMS(P) and gaseous DMS, precursors of CCN (ii) assess whether the gaseous constituents emitted from the seawater containing the coral branch could form aerosol particles when treated with UV and O_3 that mimicked natural atmospheric processes.

**Material and Methods**

*Study design and terminology*

A bubbling chamber experiment was conducted on seawater collected from the Heron Island Reef flat (23°26'35.80"S/151°54'44.23"E) in which a 26 cm x 15 cm branch of *A. pulchra*, a common reef-building coral of the Indo-Pacific and GBR (Veron, 2000), was immersed in seawater for four days (coral seawater) 24 h following the coral collection (Figure 9A). Three control bubbling chamber experiments were also conducted over four days on coral reef seawater collected at high tide (high tide seawater), low tide (low tide seawater) and from the Heron Island’s tap seawater system that pumps water directly from the reef flat (reticulated seawater). A control air sample (CAS) was also taken from the “aquaria room” in which the experiment was held. In order to simplify the terminology used for this experiment, “high tide seawater”, “low tide seawater” and “reticulated seawater” are referred to as “control seawaters”.

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Bubble bursting (~6L min⁻¹), simulating sea-air exchange of volatile substances present in the seawater, was intermittently applied to the four types of seawater using dried and filtered compressed air. The air flushed out of the bubbling chamber intermittently went through a particle filter (pore size 100-160 µm) before reaching the reaction chamber in which UV light (UVA, 300-400 nm, 40 W) and O₃ (200-500 ppb) treatments were also applied intermittently. When filtration was applied, the bubble burst primary particles were removed leaving the gaseous compounds to enter the reaction chamber (~5L min⁻¹). When applied, UV and O₃ were simulating and accelerating oxidation processes that may occur to DMSₐ and to other organic compounds that are released from the reefs to the atmosphere during sea-air exchange.

**Sampling**

Two types of samples were collected during the experiment: gaseous samples from the headspace of the bubbling chamber simulating atmospheric sulphur emissions from the reef; and water samples from the bubbling chamber, simulating the dissolved sulphur compounds produced within the water column from the reef. Gaseous samples collected onto gold-wool tubes (Kittler et al., 1992) were taken from either the top of the bubbling chamber (before the air was flushed through the reaction chamber) or from the reaction chamber’s outlet. Fifty mL water samples were collected into a beaker from an on/off tap that was located at the bottom of the bubbling chamber containing 10 L of seawater. In the coral seawater experiment, the coral branch that was placed at the bottom of the hermetic chamber was completely submerged in seawater. Both air and water samples were collected in various conditions: (i) before and after bubbling, (ii) with or without air filtration, (iii) with or without UV and O₃ treatments and (iv) before and after the coral branch was placed in and taken out of the chamber (coral seawater only). Air samples were collected for DMSₕ as well as for suspected CS₂ and ESH (Swan, 2000). Water samples were collected for total organic and inorganic carbon (TOC and TIC) (Williams et al., 1993), Chl a (Clesceri et al., 1998), DMSₜ and DMSP₁ (Curran et al., 1998). Temperature, salinity, conductivity and pH were monitored throughout the experiment using Eutech EcoScan CON/pH 6 probes.
Number and size of primary and secondary particles were determined using a SMPS (scanning mobility particle sizer) (Surawski et al., 2010). Particle chemical properties (composition, volatility, hygroscopicity, oxidised organic fraction) were determined using an Aerodyne ToF-AMS (time-of-flight aerosol mass spectrometer), VH-TDMA (volatilisation and humidification tandem differential mobility analyser) (Fletcher et al., 2007) and UFO-TDMA (ultrafine organic tandem differential mobility analyser) (Vaattovaara et al., 2005) that were placed in-line with the reaction chamber and bubbling chamber. A summary of our experimental design is presented in Figure 28.

![Experimental design of the bubbling chamber experiment conducted at Heron Island, May-June 2011, on coral seawater, high tide seawater (HTSW), low tide seawater (LTSW) and reticulated seawater (RSW).](https://example.com/figure28)

**Figure 28:** Experimental design of the bubbling chamber experiment conducted at Heron Island, May-June 2011, on coral seawater, high tide seawater (HTSW), low tide seawater (LTSW) and reticulated seawater (RSW).

**Analysis**

Gaseous sulphur samples were analysed with a purge and trap technique coupled to a GC-PFPD (Varian 3800) using a dual eight-port/six-port two-position manual valve switching system (Swan and Ivey, 1994). Thermal desorption of the gold-wool tubes was performed at 350°C for 25 min and sulphur compounds passed onto a BP1 30 m x 0.32 mm x 4 µm film column. Five point calibration curves were performed daily at the start and end of each analysis by injecting increasing volumes of MES onto the purge
and trap system using a MES permeation tube (Appendix 1). A known volume of MES was loaded onto each gold-wool tube prior to the analysis as an internal standard. Peaks were integrated on a Star chromatography workstation software (version 6.41) and calibrations were reported as per ng of sulphur as a function of SQRT of peak surface area. Samples for DMS\textsubscript{w} and DMSP\textsubscript{t} were processed in a similar way although using a single six-port valve electronically activated by a pneumatic switch. Acidified DMSP\textsubscript{t} samples were analysed following alkaline treatment with 10 mol L\textsuperscript{-1} NaOH that converts DMSP to volatile DMS (Broadbent, 1993) into a purge chamber connected to the GC-PFPD (note: values for DMSP\textsubscript{t} were corrected for free DMS\textsubscript{w} content). Acidified DMS\textsubscript{w} samples kept in crimped cap vials were purged using an inlet and outlet needles that were inserted to the bottom and headspace of the vial respectively. Purged samples passed through a -50°C moisture trap made of a condensor plunged in a mixture of methanol and dry ice that was designed to freeze moisture while retrieving DMS. Five point calibration curves were performed daily at the beginning and end of each analysis using DMSP standards (Research Plus, DMSP.HCl analytical reagent of assumed 100% purity) (Appendix 1). Analysis of TOC and TIC samples was carried out in duplicates using a total organic carbon analyser. Absorbance of Chl \textit{a} samples was determined in duplicates by spectrophotometry (APHA, 1998). Series of two-tailed unpaired t-tests were conducted to compare TOC/TIC and Chl \textit{a} concentrations within and across the various seawater experiments.

**Results**

Three main peaks (31 µmol L\textsuperscript{-1}, 39.6 µmol L\textsuperscript{-1} and 10.8 µmol L\textsuperscript{-1}) of DMSP\textsubscript{t} and an increasing concentration of DMS\textsubscript{w} (up to 0.3 µmol L\textsuperscript{-1}) were found in coral seawater containing the branch of \textit{A. pulchra}, whereas DMSP\textsubscript{t} and DMS\textsubscript{w} were not found in control seawaters (Figure 29). DMSP\textsubscript{t} concentrations in coral seawater followed an up and down pattern.
Figure 29: DMS\textsubscript{w} and DMSP\textsubscript{t} concentrations in coral seawater and control seawaters (RSW, HTSW, LTSW) during the bubbling chamber experiment. Shading shows bubbling periods. CO and CI stand for “coral out” and “coral in” respectively.

DMS\textsubscript{g} as well as two background sulphur compounds, suspected to be CS\textsubscript{2} and ESH, were detected in the headspace of the bubbling chamber (Figure 30). The concentrations of DMS\textsubscript{g} and suspected CS\textsubscript{2} and ESH measured in the “aquaria room” air were seven times lower than the averaged concentrations of these sulphur compounds contained in the bubbling chamber. Although suspected CS\textsubscript{2} (5.2 nmol m\textsuperscript{-3}) and ESH (2.8 nmol m\textsuperscript{-3}) were detected from the headspace of the chamber prior to applying bubbling, seawater-air exchange of DMS\textsubscript{g} only took place when bubbling was applied. Then 5.5 nmol m\textsuperscript{-3} of DMS\textsubscript{g} was measured in the headspace of the bubbling chamber containing the branch of \textit{A. pulchra}. However, five times more DMS\textsubscript{g} and twice as much suspected CS\textsubscript{2} were released from the coral seawater as soon as the coral branch was taken out of the chamber while maintaining constant bubbling.
Figure 30: Concentrations of sulphur gases in bubbling chamber experiments conducted on coral seawater and control seawaters (RSW, HTSW, LTSW). Concentrations of sulphur gases in the control air sample (CAS) are also shown. Arrows indicate times when the UV and O₃ treatment was applied. Shading shows bubbling periods. CO and CI stand for “coral out” and “coral in”, respectively.

Generally, DMS₈ concentrations in the headspace of the chamber were similar when purging control seawaters and coral seawater, after the coral branch had been taken out of the chamber. Suspected ESH was present in all experiments but in lower concentrations than both DMS₈ and suspected CS₂. Suspected CS₂ was found in all types of seawater except for low tide seawater. Of particular interest was the finding that DMS₈ and suspected CS₂ concentrations decreased as soon as UV and O₃ were applied to the reaction chamber. No decrease in suspected ESH was recorded as a result of UV and O₃ treatment.

A significant increase in Chl a and TOC as well as a decrease in TIC were found in coral seawater towards the end of the bubbling chamber experiment whereas Chl a, TIC and TOC concentrations remained insubstantially low in control seawaters (Table 7).
Temperature, salinity, conductivity and pH did not vary throughout the entire experiment (Table 8).
Table 7: Chl a, TIC and TOC values through time in bubbling chamber experiments.

* : significant value
- : missing values.

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<td>21:30</td>
<td>11:00</td>
<td>22:00</td>
<td>18:00</td>
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<tr>
<td>Chl a (µg/L)</td>
<td>0.12 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.15±0.02</td>
<td>-</td>
<td>6.22 ± 0.02*</td>
<td>0.50 ± 0.18</td>
</tr>
<tr>
<td>TIC (mg C/L)</td>
<td>29.6 ± 0.00</td>
<td>34.1 ± 1.47</td>
<td>31.7 ± 1.61</td>
<td>26.0 ± 0.00</td>
<td>18.7 ± 0.15</td>
<td>15.6 ± 0.34</td>
<td>14.1 ± 2.02</td>
<td>11.6 ± 0.32</td>
<td>9.16 ± 0.66</td>
<td>3.58 ± 0.41</td>
</tr>
<tr>
<td>TOC (mg C/L)</td>
<td>3.16 ± 0.00</td>
<td>3.37 ± 0.61</td>
<td>3.18 ± 0.24</td>
<td>7.72 ± 0.00</td>
<td>7.80 ± 0.30</td>
<td>8.68 ± 0.04</td>
<td>8.28 ± 0.21</td>
<td>8.64 ± 0.23</td>
<td>8.21 ± 0.33</td>
<td>0.95 ± 0.17</td>
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<tr>
<td>Chl a (µg/L)</td>
<td>-</td>
<td>0.99 ± 0.01</td>
<td>0.40 ± 0.05</td>
<td>-</td>
<td>0.48 ± 0.01</td>
<td>0.22 ± 0.03</td>
<td>0.31 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>0.26 ± 0.02</td>
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<tr>
<td>TIC (mg C/L)</td>
<td>6.27 ± 0.75</td>
<td>5.37 ± 0.85</td>
<td>12.6 ± 7.93</td>
<td>4.12 ± 0.61</td>
<td>1.36 ± 0.18</td>
<td>1.09 ± 0.41</td>
<td>0.34 ± 0.02</td>
<td>0.00 ± 0.00</td>
<td>0.37 ± 0.37</td>
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<td>TOC (mg C/L)</td>
<td>0.60 ± 0.01</td>
<td>0.57 ± 0.02</td>
<td>0.58 ± 0.02</td>
<td>0.75 ± 0.06</td>
<td>0.05 ± 0.05</td>
<td>0.11 ± 0.11</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>3.00 ± 1.00</td>
<td>0.34 ± 0.34</td>
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Table 8: Temperature, salinity, LDO and pH through time in bubbling chamber experiments.

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<td>21.1</td>
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<tr>
<td>LDO (mg/L)</td>
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<td>8.96</td>
<td>9.21</td>
<td>9.35</td>
<td>9.09</td>
<td>8.43</td>
<td>8.72</td>
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<td>8.08</td>
<td>8</td>
<td>8.02</td>
<td>8.01</td>
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<tr>
<td>Temperature (°C)</td>
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<td>22.9</td>
<td>22.7</td>
<td>22.3</td>
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<tr>
<td>LDO (mg/L)</td>
<td>8.95</td>
<td>9.02</td>
<td>8.64</td>
<td>8.65</td>
<td>8.73</td>
<td>8.87</td>
<td>7.26</td>
<td>8.67</td>
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The SMPS data showed that new particle formation occurs when gaseous components released into the chamber headspace upon bubbling were exposed to UV and O$_3$ (data not shown). These newly-formed particles were too small (count median diameter < 10nm) to be measured by the AMS and therefore their chemical composition has not been determined. The hygroscopicity and volatility profiles of these particles were lower and greater than for sulphates, respectively.

The UFO-TDMA measurements when bubbling, particle filtration, UV and O$_3$ were applied, showed that the freshly formed secondary particles included at least 50% oxidised organic compounds. The formed ultrafine particles were quickly further oxidised when they grew bigger in size, due to the production of high level of oxidants into the air.

**Discussion**

Bubbling chamber experiments conducted on *A. pulchra* and *Acropora*-dominated coral reef seawaters provided important information on the production of DMS$_a$ in coral reef ecosystems and its potential role in aerosol formation and climate regulation. DMSP was clearly produced by the coral *A. pulchra* and was rapidly cleaved into DMS$_a$, probably as a result of DMSP-lyase activity, by either the endosymbiont (Yost and Mitchelmore, 2009) and/or marine bacteria (Todd et al., 2007) present in the seawater.

The placement of the coral branch into the chamber resulted in a release in zooxanthellae as shown by an increase in Chl $a$ and TOC (Table 7). The up-down pattern in DMSP in coral seawater (Figure 29) most likely mimic a loss of symbiotic zooxanthellae from the coral branch (Iglesias-prieto et al., 1992) as well as DMSP-lyase activity in the water column which converts DMSP to DMS. The decrease in TIC (Table 7), which is usually a proxy for CO$_2$, suggested an increase in photosynthesis and/or changes in respiration in the water column. Coral bleaching was further suggested by observation of excessive cloudiness of the coral seawater (Figure 9B), probably linked to a build up in coral mucous and zooxanthellae in the bubbling chamber. Although the amount of mucous released by the coral was most likely due to
the bubbling process, the present results supports previous findings that corals produce significant amounts of DMS and DMSP in coral reef seawater through the release of coral mucous containing expelled zooxanthellae (Broadbent and Jones, 2004).

*Acropora*-dominated coral reefs at Heron Island have already been shown to be a significant source of DMS and DMSP (Jones et al., 2007; Fischer and Jones, 2012), and concentrations found in this experiment were similar to what is recorded in the literature (Figure 30). However, the presence of coral-reef-produced sulphur compounds suspected to be CS$_2$ and ESH was unexpected. Both CS$_2$ and ESH can play a role in the atmospheric sulphur cycle, with CS$_2$ oxidising into COS and SO$_2$, sulphate compounds that can influence CCN formation as well as global climate and are involved in the formation of acid rain (Yu et al., 2004; Kachina et al., 2006). Both compounds can be produced naturally in the environment (Watts, 2000) but can also be produced as a result of pollution such as coal burning, paper making and petroleum refining (Yu et al., 2004; Kachina et al., 2006). In this study, bubbling chamber experiments were conducted in a closed chamber and the compressed air that was pumped through the chamber to create bubble bursting was pre-filtered. Although concentrations of sulphur gases measured from the “aquaria room” were very low relative to that measured in the chamber headspace, it is still possible that these compounds could have originated from the handling of the water samples or from materials of which the chamber was made. Therefore, in regards to these possibilities and to the lack of replication used in this experiment, we cannot ascertain that CS$_2$ and ESH were a result of reef production. Moreover, gas chromatography retention time alone cannot be considered as an absolute means to claim the identity of an analyte. Therefore, a proper confirmation of identity, using spectral instrumental techniques, needs to be carried out for both suspected CS$_2$ and ESH. For instance, methanethiol (MeSH), also known as methyl mercaptan, has a similar retention time to ESH. In a recent chamber experiment, the headspace vapours emitted from purged seawater containing a branch of *Acropora aspera* were trapped on gas canisters that were analysed on a GC coupled with a mass spectrometer (Swan et al., in preparation). This experiment confirmed the presence of CS$_2$ and MeSH, although these compounds were found at much lower concentrations than DMS, but refuted the presence of ESH.
DMS$_g$ emissions were similar in coral seawater and control seawaters, suggesting that coral reef seawater was already highly concentrated with these sulphur compounds. However, it appears that the presence of the coral in the chamber inhibited the release of DMS$_g$ into the bubbling chamber headspace as increased DMS$_g$ concentrations were observed following the removal of the coral branch from the chamber. This observation suggested that coral mucous and tissue may have retained the bulk of DMS$_g$, which was released when the coral was removed from the chamber.

Bubbling was needed to transfer DMS$_w$ to the chamber headspace as no DMS$_g$ was measured from the headspace until bubbling was applied. However, the fact that suspected ESH and CS$_2$ were detected in the headspace of the chamber prior to applying bubbling suggests that either (i) these two sulphur compounds are more volatile than DMS, (ii) that they originated from a source of contamination within the chamber or (iii) that the observed compounds are not ESH and CS$_2$ but some other compounds with similar retention times. The Henry’s law constant of DMS, ESH and CS$_2$ averaged 0.52 mol L$^{-1}$ atm$^{-1}$, 0.29 mol L$^{-1}$ atm$^{-1}$ and 0.06 mol L$^{-1}$ atm$^{-1}$ respectively, which indicates that ESH and CS$_2$ will partition more readily from the aqueous phase into the gas phase than DMS (Sander, 1999). However, this does not exclude contamination as a source for these two compounds considering that these substances could be generated from either the plastic and/or rubber of the bubbling chamber, at least for CS$_2$ (Willoughby, 2003).

Although preliminary, of particular interest was the decrease of DMS$_g$ under UV and O$_3$ treatment which suggested that this sulphur compound could potentially oxidise in the atmosphere, participating in secondary aerosol particles and possibly CCN formation (Chin and Davis, 1993; Liss et al., 1997; Moore et al., 2011). Although the same pattern was observed for suspected CS$_2$, no conclusion can be drawn until this gas is properly identified and sources of contamination eliminated. In contrast, the fact that ESH remained constant after UV and O$_3$ exposure suggests that ESH does not contribute to the production of atmospheric aerosols. Because this experiment was the first of its kind, no other discussion on ESH as a potential source of aerosol particles
could be found in the literature. Once again, considering the lack of replication in this preliminary study and the potential for sources of contaminations within the experimental chamber, findings remain inconclusive at this stage.

It is worth noting that the newly-formed nucleation mode particles included a remarkable and even dominating fraction of oxidised organic compounds. Additionally, hygroscopicity data indicate that newly formed particles might not necessarily originate only from DMS and that other volatile organic compounds could also be responsible for particle formation and growth. The volatility profile of these particles indicates that sulphates were not the dominant component in these particles.

Further bubbling chamber experiments, as described in this paper, need to be conducted on *Acropora* corals and artificial seawater to (i) confirm the identity of ESH and CS$_2$ measured during this first bubbling chamber experiment, (ii) see if ESH and CS$_2$ are a result of coral production and (iii) confirm that biogenic DMS$_2$ and CS$_2$ are oxidised to aerosol particles and participate in forming CCN. By characterising aerosol particles formed in bubbling chamber experiments such as this one, as well as air masses over coral reefs, we are hoping to eventually ascertain whether reef aerosol emissions can affect local climate.
CHAPTER 7

Synthesis and future work
General conclusions

This work provided information on the production of DMS, DMSP and DMSO by reef-building *Acropora* corals and by different clades of *Symbiodinium* isolated from *Acropora* spp. under basal conditions and environmental factors that could lead to oxidative stress in the coral holobiont (e.g. temperature, light, salinity, and air exposure). The two major findings of this study relate to the differential potential of clade C1 and D1 to produce and consume DMS(P) under thermal stress and to the role of the DMSP-based antioxidant system in the overall antioxidant regulation of the coral holobiont. These findings could have significant implications for the GBR and coral reefs worldwide under a climatically changing world (IPCC, 2007).

Inspired by previous discoveries that reef-building corals could shut down their DMS production when exposed to temperatures beyond bleaching thresholds (Jones et al., 2007; Fischer and Jones, 2012), the potential of two *Symbiodinium* clades that exhibited different thermal tolerances to produce DMS and DMSP was investigated under thermal stress in cultures of freshly isolated symbionts from *A. millepora* and *A. tenuis* (Chapter 2). Although temperature stress induced unsubstantial variations in DMS(P) production in the thermo-tolerant clade D1, a consumption of both DMS and DMSP was observed in the thermo-sensitive clade C1, with clade C1 being an overall greater producer of DMS(P) than clade D1. Clade C is currently dominant along the GBR and other Indo-Pacific coral reefs (Stat and Gates, 2011). Since growing evidence of wide-spread changes in *Symbiodinium*-coral association has recently been documented (Chen et al., 2003; 2005a; 2005b; Lien et al., 2007; Hsu et al., 2012; Lien et al., 2013; Keshavmurthy et al., 2014), a shift in symbiont dominance from C to D can be expected for the GBR under extended thermal stress (Baker, 2003; Berkelmans and Van Oppen, 2006). As such, future climate change scenarios might lead to a decrease in DMS and DMSP in reef-building *Acropora* corals and possibly coral reef waters, either as a response of clade C1 to temperature increase, or through a shift in symbiont association toward a clade D dominance.
The decrease in DMS(P) in coral reef waters (Jones et al., 2007) and clade C1 Symbiodinium (Chapter 2) under severe and/or prolonged thermal stress was hypothesised to be linked to the use of DMS(P) as antioxidants in coral tissue and associated symbiotic microalgae under oxidative stress and enhanced ROS scavenging (Sunda et al., 2002). Before testing this hypothesis, efforts were made to investigate and select appropriate extraction and reduction methods for the optimal measurements of AOC and DMSO in coral tissue respectively. Air blasting was established as the most appropriate method, rather than tissue grinding, for the optimal extraction of antioxidants from coral tissue (Chapter 3). A comparison of DMSO quantification following its reduction by either TiCl3 or NaBH4 showed that reduction methods correlated greatly in coral tissue samples and no conclusion could be drawn on the preferential sustainability of one method over the other (Chapter 4).

Following method development, the role of DSC as antioxidants in reef-building corals was investigated in A. aspera under elevated temperature, direct sunlight, reduced salinity, light depletion and air exposure (Chapter 5). An up-regulation of the AOC was found to correlate with DMSO production under osmotic stress and light depletion, two acute stressors that led to oxidative stress. This indicated that the DMSP-based antioxidant system was probably involved in the overall antioxidant response of the coral holobiont, with oxidative stress leading to enhanced DMSO formation in coral tissue, most likely through DMS and DMSP oxidation in the presence of ROS (Sunda et al., 2002). Unexpectedly, elevated temperature induced enhanced production of DMS, DMSP and DMSO in the coral holobiont, with the DMS:DSC ratio significantly increasing as a response to thermal stress. The increase in DSC under increasing temperature in this study differed from observations reported with Symbiodinium clades C1 and D1 as previously described (Chapter 2). This contradiction could either reflect (i) the presence of different types of symbionts than clade C1 or D1, (ii) coral production, which can also be affected by thermal stress (Raina et al., 2013), or (iii) a different thermo-tolerance of the symbiont due to their different origins (southern versus central GBR). If it was in fact a reflection of coral production, working on the coral holobiont as a whole, rather than on isolated Symbiodinium should allow researchers to
predict more realistically how coral reefs will respond to climate change in terms of DMS(P) production.

In pilot bubbling chamber experiments conducted on *A. pulchra* and *Acropora*-dominated reef seawaters, the occurrence of newly formed aerosol particles correlated with a decrease in gaseous DMS from the headspace of the chamber following treatment with UV and O$_3$ (Chapter 6). Also preliminary, these results suggested that coral-derived atmospheric DMS emissions may have the potential to generate aerosol particles when emitted to the atmosphere and could therefore potentially contribute to CCN formation and regulation of solar radiation and sea surface temperatures as described in the CLAW hypothesis (Charlson et al., 1987). Although *in situ* measurements of atmospheric DMS emissions sourced from coral reefs, aerosol formation and influence on cloud albedo is still to be investigated, this study provided preliminary insights for coral-derived DMS to actively contribute to nucleation events such as the ones reported over coral reefs from the GBR (Modini et al., 2009).

In light of the overall findings of this study, where coral-derived DMS could be used as either a climate regulator in coral reefs when oxidised to sulphate aerosols in the atmosphere or as an antioxidant when oxidised to DMSO in coral tissue under oxidative stress, the obvious question is: “what will be the preferential pathway of coral-derived DMS under environmental stress in a climatically changing world?” Would it be consumed within the coral holobiont where it can fulfil immediate biochemical functions? Or would it be released to the water column where it can accumulate and be part of potential atmospheric DMS emissions that can possibly contribute to long-term climate regulation? As DMS is highly volatile, it is expected to leach out from the coral tissue whenever it is not involved in specific biochemical functions within the coral holobiont and/or when the net balance between DMS production and consumption becomes substantially positive. However, further work is needed to understand the fate of DMS in coral tissue under future climate change scenarios.

The imminent risk of substantial loss of coral reef ecosystems is of concern, and thus not only for obvious ecological, cultural and economic reasons (Hoegh-Guldberg,
2011), but also for the potential capacity of reef-building corals to control the climate on a local scale through DMS production (Jones and Ristovski, 2010, Chapter 6). The main findings of this research indicate that increasing SST and other environmental stressors are likely to affect the production of DSC in reef-building corals. However, an important question remains: would future climate change scenarios preferentially lead to a negative or a positive feedback on global warming (Fischer and Jones, 2012)? Quinn and Bates (2011) argued that no significant feedback, either negative or positive, of DMS water-to-air fluxes on global warming has yet been observed or verified. However, as climate is changing at unprecedented rates, it is possible that global and bigger scale changes in DMS biological production could affect the flux of DMS emissions to the atmosphere, with possible repercussions on CCN, cloud formation and the world climate. The main findings and remaining questions of this thesis are summarised in Figure 31.
Figure 31: Schematic highlighting the main findings and remaining questions of the Thesis.

Future work

Numerous gaps remain in our understanding of the turnover and fate of DSC in reef-building corals and coral reef waters. Future research should aim to establish a direct link between in situ coral-reef derived DMS emissions, nucleation of aerosols particles and variations in cloud-cover over the reef. This would validate the exact relationship between DMS emissions sourced from reef-building corals and direct consequences on the local climate.

The maze of processes that occur during mass bleaching events are extremely hard to simulate under artificial conditions, including recombination mechanisms that may or may not occur during recovery periods (Baker, 2003; Stat et al., 2009). For these reasons, it would also be essential to conduct long-term continuous DMS measurements over coral reefs and assess in situ DMS emissions preceding, during and following mass
bleaching events. This will help to predict the fate of atmospheric DMS emissions under climate change scenarios on a bigger scale.

It has been highlighted in this study that DSC concentrations in coral tissue can vary greatly depending on the type and severity of stress to which reef-building corals were exposed (Chapter 5). However, without turnover data, it is impossible to predict the preferential biochemical pathways that DMS(P)(O) will undergo under normal metabolism and stress exposure. Experiments using labelled sulphur, similar to what was conducted by Asher et al., (2011), should be undertaken to better understand the production, turnover and fate of DSC in reef-building corals and to estimate which proportion of these DSC is transferred to the surrounding environment, where they can become part of the dissolved pool and be involved in sea-air exchanges.

In addition to global warming, the other major threat for coral reefs under anthropogenic climate change is ocean acidification (Hoegh-Guldberg et al., 2007). In order to better predict the fate of DSC production and turnover in reef-building corals under future climate change scenarios, the effect of decreased pH, alone and combined with increasing temperature, should be investigated.

Recent efforts have been made to restore impaired coral reefs following cyclones or human degradations (Epstein et al., 2001; Edwards and Gomez, 2007). Once the direct implication of coral reefs on local climate have been fully evaluated, the potential of different reef-building corals to produce DMS(P)(O) when associated with different clades of Symbiodinium and different microbial communities should be investigated and the management implications of these scenarios evaluated.


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Calibration data
Figure 1: DMSP calibration curves for the quantification of DMSP$_{v}$ and/or DMS$_{w}$ using the GC-FPD (A), GC-PFPD (B) and GC-MS (C) as described in Chapter 2, 4, 5 and 6.
Figure 2: DMSO calibration curves for the quantification of DMSO by headspace analysis on the GC-FPD using TiCl₃ (A) and NaBH₄ (B) as described in Chapter 4.
Figure 3: MES calibration curve for the quantification of gaseous DMS and suspected gaseous CS₂ and ESH using the GC-PFDP by purge and trap analysis on range 8 as described in Chapter 2 and 6.

Figure 4: Trolox calibration curve for AOC quantification as described in Chapter 3 and 5.
Figure 5: Bovine serum albumin (BSA) calibration curve for the quantification of protein content in biological samples as described in Chapter 3 and 5.

Figure 6: Solid surface area calibration curve for the measurement of coral skeleton surface area as described in Chapter 5.
APPENDIX 2

Contribution of Others
I agree with being listed as a contributor of this thesis and to the conflicts of interest statement as summarised. I am a co-author of the manuscript entitled “Dimethylsulphoxide (DMSO) in biological samples: a comparison of the TiCl₃ and NaBH₄ extraction methods”, which has been re-submitted to Marine Chemistry after responding to comments from reviewers. I am also a co-author of the manuscript entitled “Effects of environmental factors on the production of dimethylated sulphur compounds and their potential role in the antioxidant system of the coral holobiont”, which has been re-submitted to Limnology and Oceanography after responding to comments from reviewers. I have had access to all the data in these studies and accept responsibility for their validity.

Prof Ronald Kiene

Signature: [Signature]

Date: Dec 16, 2013
I agree with the contents of the thesis; to being listed as a contributor; and to the conflicts of interest statement as summarised. I have had access to all the data in the study and accept responsibility for its validity.

Hilton Swan

Signature: [Signature]
Date: 16/12/2013
I agree with being listed as a contributor of this thesis and to the conflicts of interest statement as summarised. I am a co-author of the manuscript entitled “Air blasting as the optimal approach for the extraction of antioxidants in coral tissue”, which has been published in the Journal of Experimental Marine Biology and Ecology in July 2013. I am also a co-author of the manuscript entitled “Effects of environmental factors on the production of dimethylated sulphur compounds and their potential role in the antioxidant system of the coral holobiont”, which has been re-submitted to Limnology and Oceanography after responding to comments from reviewers. I have had access to all the data in these studies and accept responsibility for their validity.

Kellie Shepherd                      Signature:                      Date:  

17/12/2013
I agree with being listed as a contributor of this thesis and to the conflicts of interest statement as summarized. I am a co-author of the manuscript entitled “Comparative response of DMS and DMSP concentrations in *Symbiodinium* clades C1 and D1 under thermal stress”, which has been submitted to the Journal of Experimental Marine Biology and Ecology. I have had access to all the data in this study and accept responsibility for their validity.

Dr Victor Beltran   Signature:  

Date: 16/12/13
I agree with being listed as a contributor of this thesis and to the conflicts of interest statement as summarised. I am a co-author of the manuscript entitled “Can corals form aerosol particles through volatile sulphur compound emissions?” which has been published as part of the 12th International Coral Reef Symposium that was held in Cairn, Australia, from the 9th-13th of July 2012. I have had access to all the data in this study and accept responsibility for their validity.

Dr Branka Miljevic  Signature  Date: 16/12/2013
I agree with being listed as a contributor of this thesis and to the conflicts of interest statement as summarised. I am a co-author of the manuscript entitled “Can corals form aerosol particles through volatile sulphur compound emissions?”, which has been published as part of the 12th International Coral Reef Symposium that was held in Cairn, Australia, from the 9th-13th of July 2012. I have had access to all the data in this study and accept responsibility for their validity.

Prof Zoran Ristovski

Signature

Date: 16/12/2013
I agree with being listed as a contributor of this thesis and to the conflicts of interest statement as summarised. I am a co-author of the manuscript entitled “Can corals form aerosol particles through volatile sulphur compound emissions?”, which has been published as part of the 12th International Coral Reef Symposium that was held in Cairn, Australia, from the 9th-13th of July 2012. I have had access to all the data in this study and accept responsibility for their validity.

Dr Petri Vaattovaara

Signature

Date: 15.12.2013

Petri Vaattovaara, Kuopio, Finland
I agree with being listed as a contributor of this thesis and to the conflicts of interest statement as summarised. I am a co-author of the manuscript entitled “Dimethylsulfoxide (DMSO) in biological samples: a comparison of the TiCl₃ and NaBH₄ extraction methods”, which has been re-submitted to Marine Chemistry after responding to comments from reviewers. I have had access to all the data in this study and accept responsibility for their validity.

Lisa Oswald

Signature: [Signature]

Date: 12/17/2013
“Comparative response of DMS and DMSP concentrations in Symbiodinium clades C1 and D1 under thermal stress”
“Air blasting as the optimal approach for the extraction of antioxidants in coral tissue”

APPENDIX 5

“Dimethylsulphoxide (DMSO) in biological samples: A comparison of the TiCl₃ and NaBH₄ reduction methods using headspace analysis”

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APPENDIX 6

“Effects of environmental factors on dimethylated sulphur compounds and their potential role in the antioxidant system of the coral holobiont”

Publication removed due to copyright restrictions. The publisher's version is available at http://dx.doi.org/10.4319/lo.2014.59.3.0758 or http://www.aslo.org/lo/toc/vol_59/issue_3/0758.pdf
Dimethylated sulfur compounds in corals
Dimethylated sulfur compounds in corals
“Can corals form aerosol particles through volatile sulphur compound emissions?”
Can corals form aerosol particles through volatile sulphur compound emissions?

Elisabeth Deschaseaux¹, Graham Jones¹,², Branka Miljevic³, Zoran Ristovski³, Hilton Swan¹, Petri Vaattovaara⁴

¹ School of Environment, Science and Engineering, Southern Cross University, Lismore NSW 2480 Australia
² Marine Ecology Research Centre, Southern Cross University, Lismore NSW 2480 Australia
³ Queensland University of Technology, Brisbane QLD 4001 Australia
⁴ University of Eastern Finland, Kuopio, 70210 Finland

Corresponding author: e.deschaseaux.10@scu.edu.au

Abstract. Acropora dominated coral reefs are a substantial source of atmospheric dimethylsulphide (DMSₐ), one of the most abundant reduced sulphur gases present in the marine boundary layer. DMS is believed to act as a climate regulator of solar radiation and sea surface temperatures through the formation of non-sea-salt sulphate aerosols and cloud condensation nuclei (CCN), although this regulation has not yet been demonstrated. A bubbling chamber experiment was conducted on coral reef seawater containing a branch of Acropora pulchra, to investigate whether the coral-generated DMSₐ could be oxidised to non-sea-salt sulphate aerosols under treatment with UV light and O₃. Results indicated that A. pulchra produced significant amounts of dimethylsulphoniopropionate (DMSP) and dissolved DMS although emissions of DMSₐ in the chamber headspace were reduced by the presence of the coral, probably as a result of antioxidant activity in the coral tissue. Significant amounts of carbon disulphide (CS₂) and ethanethiol (ESH), other sulphur gases that could be involved in CCN formation, were also indicated in the bubbling chamber, most likely from coral production. A decrease in DMSₐ and CS₂ in the presence of UV light and O₃ followed by an occurrence of freshly nucleated nanoparticles (<10nm) suggested that these two sulphur compounds were oxidised and potentially participated in aerosol particle formation and thus could be involved in CCN formation and possibly climate regulation. The study provided insights into the production of sulphur compounds by Acropora dominated coral reefs with potential impact on local climate.

Key words: Dimethylsulphide, Coral, Aerosols, Climate.

Introduction

According to the CLAW hypothesis, atmospheric dimethylsulphide (DMSₐ) generated by phytoplankton is oxidised to non-sea-salt (nss) sulphate aerosols which go on to form cloud condensation nuclei (CCN), increasing the albedo of stratocumulus clouds and locally lowering solar radiation and sea surface temperatures (SST) in the ocean (Charlson et al., 1987). Although supported by several studies (Ayers et al., 1991; Modini et al., 2009), the CLAW hypothesis has never been verified. On the other hand, oxidised organic compounds can also participate in newly-formed secondary aerosol particle formation (Vaattovaara et al., 2006).

Research has shown that hard corals, through their symbiotic microalgae, produce DMSP, the main precursor of DMS (Jones et al., 1994; Hill et al., 1995), and emit DMSₐ in chamber experiments on Acropora (Fischer and Jones, 2012). Continuous monitoring of sea surface temperatures (SST) in coral reefs worldwide has shown that pristine reefs within or near the Western Pacific Warm Pool have had fewer reported coral bleaching events relative to reefs in other regions of the world possibly because of an “ocean thermostat” mechanism that acts to depress warming beyond certain SST thresholds (Kleypas et al., 2008). Research on DMS and aerosol formation suggests that oxidation of DMSₐ from reefs could form nss-sulphate aerosols and thus could contribute to this phenomenon (Jones and Trevena, 2005; Jones et al., 2007; Modini et al., 2009; Jones and Ristovski, 2010).

Bubble bursting from breaking waves in the ocean is the primary source of sea-air exchange and sea spray aerosol production in the atmosphere, constituting a possible source for CCN formation (Modini et al., 2009). Hydroxyl radicals in the atmosphere, which are formed as a consequence of photodissociation of ozone (O₃) by solar UV, are responsible for the oxidation of gaseous precursors such as DMS into atmospheric aerosols (Andreae and Crutzen, 1997). Thus, bubble bursting, O₃ and UV radiation are three major components in the emission and oxidation of DMS to aerosols.
Between May and June 2011, the CORACE-1 (Coral Reef Aerosol Characterization Experiment-1) campaign was conducted on Heron Island coral cay as a collaborative research project between QUT (Queensland University of Technology, Brisbane), SCU (Southern Cross University, Lismore) and UEF (University of Eastern Finland, Kuopio) in order to ascertain whether atmospheric DMS produced by coral reefs could, during bubble bursting and under O$_3$ and UV radiation, contribute to aerosol particle formation and hence potentially influence local climate (for more background information refer to Swan et al. 2012).

Material and Methods

Study design and terminology

A bubbling chamber experiment was conducted on seawater collected from the Heron Island Reef flat (23°26'35.80"S/151°54'44.23"E) in which a branch of Acropora pulchra, a widely spread coral in the Indo-Pacific and Great Barrier Reef (GBR) (Veron, 2000), was immersed (coral seawater). Three control bubbling chamber experiments were also conducted on coral reef seawater collected at high tide (high tide seawater), low tide (low tide seawater) and from the Heron Island tap seawater system that pumps water directly from the reef flat (reticulated seawater) (Fig. 1). A control air sample (CAS) was also taken from the “aquaria room” in which the experiment was held. In order to simplify the terminology used for this experiment, “high tide seawater”, “low tide seawater” and “reticulated seawater” are referred to as “control seawaters”.

Bubble bursting, simulating sea-air exchange of volatile substances present in the seawater, was intermittently applied to the four types of seawater using dried and filtered compressed air. The air flushed out of the bubbling chamber intermittently went through a particle filter before reaching the reaction chamber in which UV light (40W) and O$_3$ treatments were also applied intermittently. When filtration was applied, the bubble burst primary particles were removed leaving the gaseous compounds to enter the reaction chamber. When applied, UV and O$_3$ were simulating and accelerating oxidation processes that may occur to DMS$_a$ and to other organic compounds that are released from the reefs to the atmosphere during air-sea exchange.

Sampling

Two types of samples were collected during the experiment: air samples from the headspace of the bubbling chamber simulating atmospheric sulphur emissions from the reef; and water samples from the bubbling chamber, simulating the dissolved sulphur compounds produced within the water column from the reef. Air samples collected onto gold-wool tubes (Kittler et al., 1992) were taken from either the top of the bubbling chamber (before the air was flushed through the reaction chamber) or from the reaction chamber’s outlet (Fig.1). Both air and water samples were collected in various conditions: 1) before and after bubbling, 2) with or without air filtration, 3) with or without UV and O$_3$ treatments and, 4) before and after the coral branch was placed in and taken out of the chamber (coral seawater only). Air samples were collected for atmospheric sulphur compounds. Water samples were collected for total organic and inorganic carbon (TOC and TIC), chlorophyll-a, pheophytin, dissolved DMS (DMS$_a$) and DMSP. Temperature, salinity, conductivity and pH were monitored throughout the experiment.

Number and size of primary and secondary particles were determined using a SMPS (scanning mobility particle sizer). Particle chemical properties (composition, volatility, hygroscopicity, oxidised organic fraction) were determined using an Aerodyne ToF-AMS (time-of-flight aerosol mass spectrometer), VH-TDMA (volatilisation and humidification tandem differential mobility analyser) (Fletcher et al., 2007) and UFO-TDMA (ultrafine organic tandem differential mobility analyser) (Vaattovaara et al., 2005) that were placed in-line with the reaction chamber and bubbling chamber.

Analysis

Sulphur samples were analysed with a purge and trap technique coupled to a gas chromatograph (GC) with a pulsed flame photometric detector (PFPD) using a dual eight-port/six-port two-position manual valve switching system (Swan and Ivey, 1994). Acidified DMS$_a$ samples were analysed by alkaline hydrolysis in a purging chamber whereas acidified DMS$_a$ samples were analysed from the headspace (note: values for

Figure 1: Experimental design of the bubbling chamber experiment conducted at Heron Island, May-June 2011, on coral seawater, high tide seawater (HTSW), low tide seawater (LTSW) and reticulated seawater (RSW).
DMSP were corrected for free DMS<sub>o</sub> content. Analysis of TOC and TIC samples was carried out using a total organic carbon analyser. Absorbance of chlorophyll-a and pheophytin samples was determined by spectrophotometry (APHA, 1998).

**Results**

Three main peaks (31µM, 39.6µM and 10.8µM) of DMSP and an increasing concentration of DMS<sub>o</sub> up to 0.3µM were found in coral seawater containing the branch of *A. pulchra*, whereas DMSP and DMS<sub>o</sub> were not found in control seawaters (Fig. 2).

Although CS<sub>2</sub> (5.2 nmol/m<sup>3</sup>) and ESH (2.8 nmol/m<sup>3</sup>) were detected from the headspace of the chamber prior to applying bubbling, seawater-air exchange of DMS<sub>o</sub> only took place when bubbling was applied. Then 5.5 nmol/m<sup>3</sup> of DMS<sub>o</sub> was measured in the headspace of the bubbling chamber containing the branch of *A. pulchra*. However, five times more DMS<sub>o</sub> and twice as much CS<sub>2</sub> were released from the coral seawater as soon as the coral branch was taken out of the chamber while maintaining constant bubbling.

Generally, the DMS<sub>o</sub> concentration in the headspace of the chamber was similar when purging control seawaters and coral seawater, after the coral branch had been taken out of the chamber. ESH was present in all experiments but in lower concentration than both DMS<sub>o</sub> and CS<sub>2</sub>. CS<sub>2</sub> was found in all types of seawater except for low tide seawater.

Of particular interest was the finding that DMS<sub>o</sub> and CS<sub>2</sub> tended to decrease as soon as UV and O<sub>3</sub> were applied to the reaction chamber. No decrease in ESH could be recorded as a result of UV and O<sub>3</sub> treatment.

A significant increase in chlorophyll-a and TOC, as well as a decrease in TIC, were found in coral seawater towards the end of the bubbling chamber experiment whereas no variation was observed in control seawaters, and concentrations remained very low (data not shown). Temperature, salinity, conductivity and pH did not vary throughout the entire experiment.

The SMPS data showed that new particle formation occurs when gaseous components released into the chamber headspace upon bubbling were exposed to UV and O<sub>3</sub>. These newly formed particles were too small (count median diameter < 10nm) to be measured by the AMS and therefore their chemical composition has not been determined. The hygroscopicity and volatility profiles of these particles were lower and greater than for sulphates, respectively.

The UFO-TDMA measurements when bubbling, particle filtration, UV and O<sub>3</sub> were applied, showed that the freshly formed secondary particles included at least 50% oxidised organic compounds. The formed ultrafine particles were quickly further oxidised (aged) when they grew bigger in size, due to the production of high level of oxidants into the air.

**Discussion**

Bubbling chamber experiments conducted on *Acropora pulchra* and *Acropora* dominated coral reef water provided important information on the production of DMS<sub>o</sub> in coral reef ecosystems and its potential role in aerosol formation and climate regulation.

DMSP was clearly produced by the coral *Acropora pulchra*. The coral-produced DMSP was then rapidly cleaved into DMS<sub>o</sub>, probably as a result of DMSP lyase activity, by either the endosymbiont (Yost and Mitchelmore, 2009) or marine bacteria (Todd *et al.*, 2007) present in the seawater.

The pulsed production of DMSP in coral seawater may mimic coral bleaching events and the loss of symbiotic zooxanthellae (Iglesias-Prieto *et al.*, 1992). Eventually, mass release of zooxanthellae in the chamber led to an increase in chlorophyll-a and TOC (data not shown). The decrease in TIC, usually used as a proxy for CO<sub>2</sub>, also suggests an increase in coral bleaching and mortality through a decrease in respiration. This
conclusion was supported by observation of excessive cloudiness of the coral seawater, probably linked to a build up in coral mucus and zooxanthellae in the bubbling chamber. Thus, the present results confirmed previous findings that corals produce significant amounts of DMS and DMSP in coral reef seawater through release of coral mucus containing expelled zooxanthellae (Broadbent and Jones, 2004).

Acropora dominated coral reefs at Heron Island have already been shown to be a significant source of DMS and DMSP (Jones et al., 2007; Fischer and Jones, 2012), and concentrations found in this experiment were similar to what is recorded in the literature. However, the presence of coral-reef-produced CS₂ and ESH was unexpected. Both CS₂ and ESH play an important role in the atmospheric sulphur cycle, with CS₂ oxidising into COS and SO₂, sulphate compounds that can influence CCN formation as well as global climate and are involved in the formation of acid rain (Yu et al., 2004; Kachina et al., 2006). Both compounds can be produced naturally in the environment (Watts, 2000) but can also be produced as a result of pollution (Yu et al., 2004; Kachina et al., 2006). In this study, bubbling chamber experiments were conducted in a closed chamber and the compressed air that was pumped through the chamber to create bubble bursting was pre-filtered. Also, concentrations of sulphur gases measured from the “aquaria room” were very low relative to that measured in the chamber headspace. Therefore, it is unlikely that CS₂ and ESH came from external pollution and thus, are indicated to be a result of reef production.

However, gas chromatography retention time alone cannot be considered as an absolute means to claim the identity of an analyte and a proper confirmation of identity, using spectral instrumental techniques, needs to be carried out for both CS₂ and ESH.

Meanwhile, emissions of DMS₂, CS₂ and ESH were similar in coral seawater and control seawaters, suggesting that coral reef seawater was already highly concentrated with these sulphur compounds. However, it appears that the presence of the coral inhibited the release of DMS₂ and CS₂ into the bubbling chamber headspace, supporting the theory that under artificial stressful conditions DMS could be used as an antioxidant within the coral tissue (Sunda et al., 2002; Jones et al., 2007). However, increased concentrations of DMS₂ and CS₂ following the removal of the coral branch could suggest that the bulk of these gases could be entrained within the coral mucus and tissue and were released when the coral was removed from the chamber, constituting an increase in DMS₂ emitted from the water surface.

Bubbling was needed to transfer DMS₂ to the chamber headspace as no DMS₂ was measured from the headspace until bubbling was applied. However, the fact that ESH and CS₂ were detected in the headspace of the chamber prior to apply bubbling suggests that these two sulphur compounds are more volatile than DMS and thus could be more concentrated in the atmosphere relative to their production rates.

Of particular interest was the decrease of DMS₂ and CS₂ under UV and O₃ treatment which indicated that both sulphur compounds could potentially become oxidised in the atmosphere, participating in secondary aerosol particles and possibly CCN formation (Chin and Davis, 1993; Liss et al., 1997; Moore et al., 2011). In contrast, the fact that ESH remained constant after UV and O₃ exposure suggests that ESH does not contribute to the production of atmospheric aerosols.

It is worth noting that the newly-formed nucleation mode particles included a remarkable and even dominating fraction of oxidised organic compounds. Additionally, hygroscopicity data indicate that newly formed particles might not necessarily originate only from DMS and that other sulphur-containing gaseous precursors or other volatile organic compounds could also be responsible for particle formation and growth. The volatility profile of these particles indicates that sulphates were not the dominant component in these particles.

Further bubbling chamber experiments, as described in this paper, need to be conducted on Acropora corals and artificial seawater to 1) confirm the identity of ESH and CS₂ measured during this first bubbling chamber experiment, 2) see if ESH and CS₂ are a result of coral production and 3) confirm that biogenic DMS₂ and CS₂ are oxidised to aerosol particles and participated in forming CCN. By characterising aerosol particles formed in bubbling chamber experiments such as this one, as well as air masses over coral reefs, we are hoping to eventually ascertain whether reef aerosol emissions can affect local climate.

Acknowledgement
We would like to thank the Academy of Finland (Kaapio, Finland) and Southern Cross University (Lismore, Australia) including the Marine Ecology Research Centre, the centre for Biogeochemistry and the Division of Research for financial support. Thanks also to the Heron Island Research Station team for their assistance during the CORACE-1 campaign. We are also grateful to the GBR Marine Park Authority for being given permission to collect coral specimens.

Reference


Fletcher CA, Johnson GR, Ristovski ZD, Harvey M (2007) Hygroscopic and volatile properties of marine aerosol observed at Cape Grim during the P2P campaign. Environ Chem 4:162-171


APPENDIX 8

“Dimethylsulfide, climate and coral reef ecosystems”
Dimethylsulfide, Climate and Coral Reef Ecosystems

Hilton B Swan1, Graham B. Jones1,2 and Elisabeth Deschaseaux1

1School of Environment, Science and Engineering, Southern Cross University, Lismore NSW 2480, Australia
2Marine Ecology Research Centre, Southern Cross University, Lismore NSW 2480, Australia
Corresponding author: h.swan.11@scu.edu.au

Abstract. Dimethylsulfide (DMS) is the major biogenic source of atmospheric sulfur and is mainly derived from dimethylsulfiniopropionate (DMSP) produced by oceanic phytoplankton, marine algae and endosymbiotic zooxanthellae in reef-building corals. Although coral reefs occupy <1% of the global oceans, the potential source strength of DMS from these areas was found to be significant in comparison to other oceanic areas. In this study, healthy nubbins of Acropora valida and Acropora pulchra collected at Heron Island were examined to assess the source strength of DMS from these common coral species. Total DMS (free DMS and DMSP-derived DMS) measured in these corals was on average 3.6 µmol cm⁻² surface area. Sediment from the coral reef flat was found to release ~1000 times less DMS than the Acropora corals when compared by weight. Megatonnes of DMS are released from the oceans to the atmosphere annually, where it is oxidised to contribute to new nanoparticles that can lead to cloud condensation nuclei (CCN). These affect cloud microphysical properties and consequently the Earth’s radiation budget and climate. The results suggest emissions of DMS from coral reefs are significant and may affect regional climate. Notably strong DMS plumes of up to 13 nmol m⁻³ of air were detected above the coral reef flat during low tide when it was exposed at the end of the day under calm conditions. A seasonal comparison of atmospheric DMS concentrations determined at Heron Island with a temperate marine location showed the reef to be a greater source of DMS.

Key words: DMS, DMSP, Coral, Climate, Aerosol.

Introduction

DMS is the most abundant biogenic sulfur compound released from the oceans to the atmosphere (Andreae and Raemdonck, 1983) with recent global estimates at 28 Tg annually (Lana et al., 2011). The atmospheric lifetime of DMS is short (~1 day) because it is rapidly removed, mainly by hydroxyl radical, to form a variety of oxidized aerosol particles which affect solar radiation in the marine boundary layer (MBL) (Berresheim et al., 1990). Of particular importance is the oxidation product H₂SO₄ which can lead to the formation of new particles that may collectively affect the Earth’s radiation budget (Vallina and Simó, 2007). It is thought that a major source of CCN over the oceans is derived from DMS, and a link between phytoplankton, DMS and cloud albedo (reflectiveness) has been proposed (Charlson et al., 1987) which regulates temperature and climate.

Total aerosol particle concentrations over the Great Barrier Reef (GBR) were reported 34 years ago (Bigg and Turvey, 1978) to be on average 7 times higher than in typical maritime air. However, it was not until recently that Modini et al., (2009) reported what is believed to be the first direct observation of particle formation over the GBR, and concluded this to be potentially climatically significant. Reports of high levels of DMS in and around coral reefs of the GBR (Broadbent and Jones, 2004; Jones et al., 2007) have led to the belief that emissions of DMS from coral reefs are locally significant and could affect cloud-cover and the amount of incident solar radiation that a reef receives (Fischer and Jones 2012). This is supported by recent research that suggests an ocean thermostat involving sea surface temperature (SST) and cloud cover operates in the Western Pacific Warm Pool to the north of Australia, where coral reefs in this region may influence cloud cover keeping sea surface temperatures below 30°C, limiting coral bleaching events (Kleypas et al., 2008).

It has been shown that coral reefs are a source of DMS that is subsequently transferred to the atmosphere, and then oxidised to aerosol precursors of CCN such as non-sea-salt-sulfate, potentially forming cloud over reefs (Modini et al., 2009, Fischer and Jones, 2012). However, it has also been reported that corals may shut down DMS production when stressed by elevated SST and light levels, thus possibly reducing DMS derived aerosols and cloud cover in the marine boundary layer, increasing solar radiation levels over reefs and exacerabating coral bleaching (Fischer and Jones, 2012). The cause of this shut down in DMS emission could be linked to the use of these sulfur substances as antioxidants within stressed corals (Jones et al., 2007).
In marine ecosystems DMS and DMSP may also provide a mechanism to assist the flow of nutrients and energy via an infochemical role. It has been demonstrated that organisms as small as copepods will react to plumes of DMS injected into their surroundings (Steinke et al., 2006) and that DMS and DMSP increase the foraging success of zooplankton predators to find and capture prey (Seymour et al., 2010). Planktivorous reef fish will aggregate to DMSP experimentally deployed at biogenic concentrations (10^{-7} M) along a fringing coral reef (De Bose et al., 2008), while herbivorous reef fish can be agents of DMSP decomposition and DMS distribution (Dacey et al., 1994). Apex predators such as procellariiform seabirds can detect localised elevation in atmospheric DMS as an ocean surface foraging cue (Nevitt, 2011). DMS and DMSP thus appear to assist the process of predation, which is a dominant driving force on coral reefs required for the rapid and efficient recycling of nutrients and energy through these unique ecosystems (Hoegh-Guldberg and Dove, 2008). The abilities of reef fish to detect infochemical cues could however be affected by increased ocean acidification, which will apparently impair their olfactory discrimination and disrupt the predatory process (Dixon et al., 2010). This could greatly impact the biodiversity of coral reefs, and possibly lead to adverse climatic implications by affecting emissions of DMS to the atmosphere. The atmospheric and trophic processes outlined indicate that biogeochemical cycling, ecosystem health and climate are not mutually exclusive (Nevitt, 2011).

Material and Methods

This study was conducted at the Heron Island Research Station (23°26’34”S, 151°54’48”E) in the Capricornia Cays on the southern end of the GBR during May/June 2011. It formed part of the first Coral Reef Aerosol Characterisation Experiment (CORACE-1) with the aim to better characterize reef aerosol emissions and gauge their importance to regional climate (Deschaseaux et al., 2012, this issue).

Sampling and Analysis of Atmospheric DMS

Atmospheric DMS (DMS_a) was sampled through a 12.5 mm diameter tube using a low flow vacuum pump (~330 mL min^{-1}). The sample intake was above roof height at ~15 m ASL in a direct line of sight to the ocean. Each air sample was drawn through a high-capacity oxidant scrubbing filter and then through a Pyrex tube containing gold-coated glass wool, according to a previously reported procedure (Kittler et al., 1992). The air volumes sampled were measured using a high-precision gas meter (Toyo ML2500, Japan). These gold-wool chemisorption traps were sealed and stored for later analysis at SCU using the valving and cryostap configuration shown in Fig. 1. DMS was released from the gold-wool by thermal desorption at 350°C for 25 mins and sent to a cryotrap prior to analysis with a Varian CP3800 gas chromatograph equipped with a pulsed flame photometric detector (GC/PFPD). An internal standard of methylethylsulfide (MES) was added to each sample, via a certified permeation tube, and calibration was achieved using a certified DMS permeation tube (Dynacal, Vici Metronics, USA). Further details of this analysis procedure are described elsewhere (Swan and Ivey, 1994).

It has been shown that S-gases can be quantitatively adsorbed onto and thermally desorbed from metal surfaces such as Pd, Pt and Au (Kagel and Farwell, 1986), and this chemisorption technique using Au is a convenient means for the sampling of atmospheric DMS (Kittler et al., 1992). The recovery of DMS from the gold-wool traps has previously been found to be 97.0 ± 2.8 % (n = 49) provided their breakthrough volume of ~120 L of air is not exceeded (Swan and Ivey, 1994).

Figure 1: Configuration of the valving and cryogenic trap system used for the analysis of atmospheric DMS sampled at Heron Island.

Sampling and Analysis of DMS from Coral Nubbins and Sediment

Small nubbin tips (~0.5-1 g) were removed from healthy colonies of Acropora valida and Acropora pulchra (clade C2). Without delay, each nubbin was placed on paper to drain, weighed, and then transferred into a 20 mL capacity headspace (HS) vial containing 5 mL of 20% sodium sulfate acidified with 0.1% HCl, then crimp capped. Other samples were prepared in HS vials containing 5 mL of 20% sodium sulfate with the addition of one pellet of NaOH (~0.2 g = ~1M) to each vial. The samples treated with HCl were used to measure free DMS, while those treated with NaOH gave a measure of
total DMS (i.e. free DMS and DMSP-derived DMS via alkaline hydrolysis). Total DMSP (DMSP) was obtained from the difference of the NaOH and HCl treated samples. The surface areas of the nubbins were determined by hot wax displacement (Chancerelle, 2000) after analysis for DMS. Sediment, largely comprised of granular coral fragments, was also collected from below the live coral colonies. Approximately 5 g of this wet sediment was weighed into HS vials and prepared as described for nubbins.

These samples were analysed at the National Measurement Institute (NMI), Sydney, using static headspace gas chromatography with a mass spectral detector (HS/GC/MS). This equipment (Agilent G1888HS, 6890GC, 5973MSD) was operated in scan mode (range m/z 25-150) using multiple headspace extraction (Kolb and Ette, 2006). DMS was confirmed by its characteristic 70 eV mass spectrum at the expected GC retention time, and it was quantified by the sum of the responses from extracted ions m/z 62, 63, 64. A DMSP.HCl reference material (170.7 g mol$^{-1}$) of 90.3 ± 1.8% purity was prepared at the NMI from the reaction of excess DMS with acrylic acid in dry toluene followed by the addition of ethereal HCl to precipitate the product (Howard and Russell, 1995). Calibration was achieved by alkaline hydrolysis of the DMSP.HCl in 5 mL of 20% sodium sulfate to produce a number of suitable DMS standards in HS vials (Dacey and Blough, 1987). The chemical reaction that generates DMS is:

$$\text{DMSP.HCl + NaOH} \rightarrow \text{DMS + A + H}_2\text{O + NaCl},$$

where A is acrylate.

**Results**

Free DMS and total DMS released from coral nubbins was 9.6 ± 2.5 and 17.4 ± 3.8 μmol g$^{-1}$, respectively ($n = 7$). When related to coral surface area these concentrations are 1.5 ± 0.5 and 3.6 ± 1.7 μmol cm$^{-2}$, respectively. The increased relative standard deviation for these figures reflects the difficulty of determining the surface areas of small nubbins. A. valida and A. pulchra contained similar concentrations of DMS. Free DMS and total DMS measured in the sediment was 9.0 and 19.5 nmol g$^{-1}$, respectively.

The concentrations of DMS$_i$ from gold-wool samples taken between 30$^{\text{th}}$ May – 8$^{\text{th}}$ June 2011 were found to range from none detected to 13.1 nmol m$^{-3}$ (mean 2.3 nmol m$^{-3}$, n = 43, Fig. 2). The maximum DMS$_i$ was detected on the 6$^{\text{th}}$ of June around sunset under still conditions. This sample of 67.9 L of air was collected on a rising tide just after a low tide of 0.6 m when the coral reef platform was exposed. Another high DMS$_i$ of 12.8 nmol m$^{-3}$ was detected on the 3$^{\text{rd}}$ of June from a 47.0 L air sample, under similar calm conditions around sunset after a low tide of 0.4 m. The air temperature was 23°C when both these samples were collected.

**Discussion**

**DMS and DMSP production from Coral Reefs**

The analysis of small coral nubbins by static HS/GC/MS is a non-disruptive means to measure in-situ DMS in coral biomass. The procedures allowed determination of free DMS and DMSP, from the zooxanthellae and host cells. This study was used to assess methodology and gauge the level of DMS present in coral for further analysis by isotope dilution with deuterated internal standards (Smith et al., 1999). The results indicated that free DMS is a significant fraction of the potential DMS present in coral biomass in the form of DMSP, and may be generated enzymatically from the DMSP precursor as part of the symbiotic process. Coralline DMS appears to originate from the zooxanthellae symbionts rather than the invertebrate host (van Alstyne et al., 2008) where it may accumulate before release to the water column. The concentrations of free DMS and total DMS released from coral nubbins were approximately 10$^3$ times higher than in the sediment, which indicates that actively growing coral biomass is a far richer source of DMS than the benthos in the sediment.

On a cellular basis, *Symbiodinium* sp are a very rich source of DMS to coral reef ecosystems. By applying a winter mean *Acropora* sp zooxanthellae density of 3 x 10$^6$ cells cm$^{-2}$ (Moothien-Pillay et al., 2005) and a *Symbiodinium* (Clade C) cell diameter of 8.5 μm (spherical cell volume of 322 fl), the mean free DMS and DMSP, measured in this study is 0.5 and 0.7 pmol cell$^{-1}$, respectively. This equates to 1675 and 2299 nmol L$^{-1}$ (cell volume), respectively. These concentrations are within the broad range of 36 to 7590 nmol L$^{-1}$ (cell volume) reported by Broadbent et al (2002). It has been found that the concentrations of DMS and DMSP, and the production of DMS are strain-specific in cultures of *Symbiodinium* sp (Steinke et al., 2011), which provides one reason for the broad range of observed cellular concentrations.

![Figure 2: Atmospheric DMS concentrations derived from samples taken at Heron Island 30\text{th} May to 8\text{th} June 2011, with tide heights shown.](image-url)
Mucus exuded from *Acropora formosa* has been reported to contain 25.4 µM DMS, while mucus ropes contained 18.7 µM DMS and 54.4 µM DMSP (Broadbent and Jones, 2004). This data, along with measurements of coral nubbins from this study, are some of the highest concentrations of DMS and DMSP measured in any natural marine system. Coral reefs are therefore marine ‘power-houses’ for DMS and DMSP production, which are important components of the biogeochemical cycling of sulfur and carbon within these ecosystems.

**Atmospheric DMS: Comparisons and Patterns**

Significant emission of DMS to the atmosphere from coral reefs is expected to occur based on the concentrations of DMS released from the coral nubbins examined in this study. The two highest **DMS** at Heron Island of 12.8 and 13.1 nmol m⁻³ measured at the start of winter are similar to maximum summer **DMS** at the temperate location of Cape Grim in NW Tasmania (40°41'S, 144°41'E) during 1991 and 1992 using this technique (Gillett et al., 1993; Swan and Ivey, 1994). At Cape Grim, maximum **DMS** are associated with seasonal summer phytoplankton blooms, while **DMS** typically does not exceed 1 nmol m⁻³ during the dormant austral winter. At One Tree Reef (23°30'S, 152°06'E) **DMS** was found to range from 0.12 to a summer maximum of 23 nmol m⁻³ (mean 6.5 nmol m⁻³) in a seasonal study reported by Broadbent and Jones (2006). These seasonal and latitudinal comparisons indicate that more **DMS** originates from coral reefs than other temperate marine ecosystems.

It has been noted that **DMS** over coral reefs in the northern GBR, and the wider Coral, Solomon and Bismarck Seas to the northeast of Australia often increased after low tide which exposed the reefs to the atmosphere (Jones and Trevena, 2005). The tendency for elevated **DMS** at low tide or on the rising tide after low water was also observed from the coral reef flat at Heron Island (Fig. 2), which indicates that it is a point source for the emission of DMS. When the reef flat is exposed direct release of DMS to the atmosphere can occur and there is no need for wind to act as a sea-to-air transfer mechanism (Liss and Merlivat, 1986). A rising tide over the surface of the reef could also provide sufficient agitation to release DMS to the air without the need for wind to break the surface water film barrier. Consequently maximum **DMS** is expected to occur around low tide during still conditions when mixing and dilution of the coral reef point source emission with maritime air is least.

The observation of enhanced biogenic emissions associated with low tides is however not peculiar to coral reefs. It has been reported that an algal field on the Atlantic coast of Western Brittany (France) when uncovered at low tide was a notably more active source of **DMS** than when covered at high tide (Luce et al., 1993). At the Mace Head Atmospheric Research Station in western Ireland, elevated emissions of organo-iodine compounds from brown kelp have been found to coincide with low tides when the kelp is exposed (Carpenter et al., 2001). Like **DMS**, these halogenated organic emissions from kelp can lead to the formation of climatically relevant aerosol via photochemically produced oxidation products such as iodine monoxide (O’Dowd and Hoffman, 2005).

Factors such as surface seawater **DMS** concentrations, wind speed and direction, solar radiation intensity, atmospheric oxidant levels, conditions in the MBL, tides and SST all influence the observed **DMS** concentrations and thus its variability. Increased wind speed enhances emission of dissolved **DMS** to the atmosphere via wave and bubble-mediated action (Wanninkhof, 1992); air that has travelled over the oceans and particularly coral reefs will contain more **DMS** than air that has a back trajectory from the continent; high actinic flux leads to increased production of hydroxyl radical from **O₃** photolysis and thus daytime destruction of **DMS** (Ayers et al., 1995); the **DMS** sea-to-air transfer velocity is dependent on SST (Yang et al., 2011). Fig. 2 shows the high variability of **DMS** which may occur, where tide height is just one of many physical processes that can influence observed concentrations.

The processes controlling short-term fluctuations in **DMS** cannot be completely assessed using gold-wool chemisorption because it is a low frequency sampling technique. Therefore, as part of future CORACE studies, high time-resolved on-line **DMS** sampling coupled with continuous light, wind, **DMS** in seawater and tidal monitoring is planned to further understand the processes that control short-term variability in **DMS**. These measurements will also allow the sea-to-air flux of **DMS** from the Heron Island coral reef to be determined.

**Acknowledgement**

We would like to thank the CORACE team from QUT, staff at the HIRS for assistance with our experimental needs and the NMI for preparation of the DMSP.HCl reference material. This research was funded by the MERC at SCU and financial support was received from the SCU Division of Research. We are also grateful to the GBR Marine Park Authority for permits to collect corals and the two reviewers of this manuscript.

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“The contribution of sulphate and oxidized organics in climatically important ultrafine particles at a coral reef environment”
The Contribution of Sulfate and Oxidized Organics in Climatically Important Ultrafine Particles at a Coral Reef Environment

Petri Vaattovaara, Hilton B. Swan, Graham B. Jones, Elisabeth Deschaseaux, Branka Miljevic, Ari Laaksonen, Zoran D. Ristovski

Abstract—In order to investigate the properties of coral reef origin secondary organic aerosol, ethanol affinity to atmospheric nucleation mode particles (diameter < 15 nm) was measured at the Heron reef marine environment in the South Pacific Ocean during the first coral reef aerosol characterization experiment in May-June 2011 using an ultrafine organic tandem differential mobility analyser.

Our campaign study at Heron reef showed that the nucleation mode size particles (diameter = 10 nm) composition contain internally mixed sulfate and oxidized organic components in approximately equal proportion in sunny and still conditions around low tide time, indicating local biogenic sources. The produced secondary compounds and aerosols have potential to contribute to cloud condensation nuclei formation and properties that may affect local low-level cloud formation over the GBR. Additionally, primary marine sea-salt and organic material during windy conditions and anthropogenic/biogenic sources during continental air masses can affect the properties of these particles.

Keywords—Coral reef, DMS, particle composition, secondary organics.

I. INTRODUCTION

In regards to climate change, one of the largest uncertainties is related to aerosols and their direct and indirect (via clouds) connections to the Earth’s radiative balance and climate [1]. An important phenomenon associated with the climatically important direct and indirect aerosol effects is the formation of new nanometer-size particles. This phenomena consists of a set of processes that include the production of nanometer-size clusters from gaseous vapours, the growth of these clusters to detectable sizes (see e.g., [2]), and their growth to potential cloud condensation nuclei (CCN) and larger radiatively active sizes.

The aerosols typically consist of both organic and inorganic compounds whose relative ratios strongly depend on the environmental conditions where the interaction occurs, and the ratio of anthropogenic and biogenic gases and particles define the overall composition and properties. In order to more fully understand the climatic effects of atmospheric aerosols, the composition of freshly nucleated nucleation mode particles and Aitken mode size particles (i.e. ultrafine particles < 100nm) needs to be properly understood in each environment.

One of the most important environments is marine areas which cover around 70% of the Earth’s surface area. [3] suggested, using surface chlorophyll a concentrations (SeaWiFS satellite) as an indicator for the size of the plankton biomass and thus for overall marine productivity, that biologically productive marine regions are able to produce climatically important secondary particles worldwide (i.e. dimethylsulfide (DMS) and other organic vapours and their oxidation products), while so called marine deserts (typically 10-20 degree from equator) are not as productive. Depending on the availability of nutrients due to the strength of seasonal thermoclines and sunlight, the productivity in different parts of the ocean varies and thus the importance of these particles to regional climate also varies greatly.

Coral reefs form some of the most diverse ecosystems on the Earth, being one of those marine biologically active regions. They occupy less than 1% of the world's ocean surface but they provide a home for 25% of all marine species[4], including fish, mollusks, worms, crustaceans, echinoderms, sponges, tunicates and other cnidarians. They are most commonly found at shallow depths in tropical waters, but deep water and cold water corals also exist on smaller scales in other areas.

Stretching over 2600 km, a long way along the coast of Queensland in Australia, the Great Barrier Reef (GBR) is one of the largest and most important ecosystems in Oceania. The regional climatic importance of the GBR emissions was identified when Bigg and Turvey[5] reported 35 years ago that
mean concentrations of aerosol particles in maritime air masses between the Australian mainland and the GBR to be 1590 particles/cm³. Measurements made on the seaward side of the GBR were much lower (mean = 640 particles/cm³) and typical of remote marine aerosols. Since the CLAW hypothesis [6] suggested a feedback link in oceanic DMS emissions to the number of CCN and cloud albedo, the hypothesis of [6] has given a good reason to study DMS concentrations at biologically productive marine regions such as the GBR as a precursor for those particles. [7] noted on the northern GBR that atmospheric DMS (DMS₂) concentrations often increased after low tide when the reef was exposed to the atmosphere. On the southern GBR, at the Capricorn Bunk Group of reefs, [8] found a DMS₃ mean value of 6.5 ppt (ranging from 0.12 ppt to 23ppt). Additionally, [3] suggested the presence of significant secondary organic material in newly-formed secondary particles formed in biologically active marine areas. Importantly nearby the southern GBR at Agnes Water, [9] observed a strong new particle formation event when the air mass arrived from the GBR direction.

Understanding the role of clouds in regulating the temperature of the oceans and how that role changes is one of the biggest uncertainties for climate change researchers. A key feature in this regard is the formation and properties of CCN.

This in-situ coral reef 2011 study, the first coral reef aerosol characterisation experiment (CORACE-1), aimed to determine the contribution of sulfate and organic components to ultrafine (d<15nm) particles formation and growth in the southern GBR environment at Heron reef.

II. METHODS

A. The Measurement Site

This CORACE-1 study was conducted at the Heron Island Research Station (HIRS, 23°26′34″S, 151°54′48″E) on the southern end of the GBR on South Pacific Ocean during 26 May-14 June 2011.

HIRS is located on the Heron Island coastal area near the Tropic of Capricorn at the northern end of the Capricorn and Bunker group of reefs, 72 km north-east of Gladstone city (Queensland, Australia) and 539 km north of the state capital Brisbane. The island is situated on the western side of Heron Reef, a fringing platform reef of significant biodiversity, supporting around 900 of the 1,500 fish species and 72% of the coral species found on the Great Barrier Reef [10].

Heron Island is about 800 meters long and 300 meters at its widest, giving an area of approximately 16 hectares. The highest point, near the western tip, is 3.6 meters above sea level (ASL). A dune ridge along the southern shore rises some 3 meters ASL, lower dunes on the northeastern side are only about 1 meter above the sea [11]. The sampling inlet used was built 15 m ASL at the HIRS.

Unfortunately, another previously planned measurement site building at Mission Beach on the northern Queensland coast close to the GBR was totally destroyed by the strong austral summer cyclone Yasi (3.Feb.2011).
The organic volume fraction (OVF) was calculated based on the principle introduced in [3]. The OVF is obtained from the following comparison: the volume corresponding to the measured size OGF is compared with the volume corresponding to the OGF of a very high ethanol affinity compound. Typically, freshly formed secondary organics are moderately oxidized and they have a very high ethanol affinity. However, the presence of sulfate and oxidized organics also lead to low OGF organosulfates [18], therefore, the calculated OVF values are minimum estimates.

### III. RESULTS AND DISCUSSION

In order to investigate the properties of coral reef origin secondary organic aerosol (SOA), ethanol affinity to atmospheric nucleation mode particles (diameter $< 15$ nm) was measured in-situ at the Heron Island marine environment during CORACE-1 using the UFO-TDMA described. OGFs determined on 30 May and 3 June 2011 (ozone ca 22 ppb, RH~58%) under calm and sunny daytime conditions at low tide were 1.08 and 1.09, giving a minimum estimate of 46–52% (6% uncertainty) for the OVF of 10 nm particles. This finding was supported by an on-site coral seawater chamber experiment, where bubble-burst particles were filtered and then treated with UV and O$_3$. The chamber experiment showed that freshly formed secondary organics consisted of at least 50% moderately oxidized organic compounds [19]. Similar OVF values were measured for 10 nm particles under variable wind conditions at low tide on 26, 27 and 28 May and 13 June 2011 at Heron reef. However, air-masses during the days with higher wind speeds may also contain continuously derived compounds. A continuously polluted air-mass was measured on 31 May 2011 when air from 100 m height was received from the direction of Gladstone on the Queensland coast, which gave an OVF of 11% for 10 nm particles. Overall (Table I), the UFO-TDMA measurements typically showed that both sulfate and oxidized organics related volume fractions were close to 50% in new 10 nm particles during CORACE-1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Conditions</th>
<th>SOA (%)</th>
<th>Sulfates(%)</th>
<th>DMS$_x$ (ppt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26-28.5 and 13.6</td>
<td>variable winds</td>
<td>50</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>30.5</td>
<td>calm and sunny</td>
<td>46</td>
<td>54</td>
<td>-</td>
</tr>
<tr>
<td>31.5</td>
<td>continually</td>
<td>11</td>
<td>-</td>
<td>-</td>
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<td>3.6</td>
<td>influenced</td>
<td>52</td>
<td>48</td>
<td>12.8</td>
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<td>6.6</td>
<td>oxidation and UV</td>
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Additionally, during CORACE-1, the biological activity and the ability of coral reef waters to form atmospheric particles was additionally supported by gold-wool chemi-adsorption of DMSas. Under calm conditions just after low tide on 3 June 2011 a peak DMSa of 12.8 pptv was detected, and again under similar conditions on 6 June 2011 a peak DMSa concentration of 13.1 ppt was detected [20].

Previously, [9] found that the majority of the volume of Aitken mode size particles detected at Agnes Water, on the Queensland coast 50 km south of the GBR, could also be attributed to internally mixed sulfate and organic components. The majority of nucleation events observed at Agnes Waters occurred between 10:00-13:00 LT under high solar intensity (~1000 W m$^{-2}$) and low relative humidity (~50-60%) [9]. Generally, they assumed in the marine airmass new particle formation events at Agnes Water (a part of Pacific Ocean) that the particles consisted of around 60% sulfates and 40% organics by volume in 17 nm size during weak and 76% sulfates and 24% organics by volume in 22.5 nm size during strong nucleation events.

Along the Atlantic coast, a significant iodine oxide contribution to new particle formation may be derived from emission of iodine from brown seaweed ([20], [21]). However, local Australian studies suggest that the role of iodine in particle formation is likely to be minor over the GBR ([23], [24]). Those observations are supported by the fact that the brown seaweed contribution is relatively small compared to the amount of total GBR or Heron reef biota.

Instead, [25] highlighted high DMS and DMSO levels in coral mucus. Therefore, microalgae and the corals, and especially the mucus of corals could be the main sources for DMS and non-DMS volatile organic emissions which are necessary for SOA formation at coral reefs. Secondary compounds can also contribute to the composition and thus the properties of primary particles produced by bubble bursting which can potentially form CCN. A schematic of the potential for biogenic nucleation, Aitken and accumulation mode particle formation, growth, and composition during daylight hours at the Heron Island marine environment is shown in Fig. 2.

![Fig. 2 A schematic of potential biogenic nucleation, Aitken and accumulation mode particle formation, growth, and composition during daylight hours at the Heron reef marine environment](image-url)

Sunlight is required for nanoparticle formation from volatile organic compounds (VOC) such as DMS or isoprene.
Photochemically mediated oxidation of DMS results mainly in the formation of SO₂, although lesser amounts of methanesulfonic acid (MSA) are also produced [26]. Oxidation of SO₂ leads to sulfuric acid which may undergo nucleation with other gases such as neutralizing ammonia and oxidized organics to produce thermodynamically stable clusters of nanoparticles. These may grow further to radiatively and climatically important sizes with VOC oxidation products and iodine oxides to form atmospherically important SOA. Additionally, those secondary compounds can change the properties of primary organic material and sea salt, producing climatically relevant CCN [27], [28], [3], Fig. 2.

Importantly on the GBR scale, the importance of clouds in regulating (cooling) sea surface temperature (SST) over the GBR has recently been quantified for the first time [29].

IV. CONCLUSION

In order to investigate the properties of coral reef origin SOA, ethanol affinity to atmospheric nucleation mode particles (diameter < 15 nm) was measured in situ at the Heron Island marine environment in the South Pacific Ocean during CORACE-1 (May-June 2011) using an ultrafine organic tandem differential mobility analyser. Our 2011 campaign study at Heron reef showed that the composition of nucleation mode size particles (diameter < 15 nm) consisted of internally mixed sulfate and oxidized organic components in approximately equal proportion under sunny still conditions at low tide, thus indicating local biogenic sources. The sea-to-air release of secondary compounds and resulting aerosols can potentially contribute to cloud condensation nuclei formation that may contribute to local low-level cloud formation over the GBR while also affecting cloud properties. Additionally, primary marine sea-salt and organic material generated during windy conditions, together with anthropogenic/biogenic sources during continental air masses can affect particle and cloud properties. Generally, the aerosol processes that regulate the formation and properties of cloud condensation nuclei over large parts of the coral reef areas, and their effect on regional climate of these areas are purely understood. At biologically active marine regional scales, the particle formation can determine the population of aerosol particles that seed droplet formation in clouds[30]. These produced biogenic aerosol particles can therefore have a major influence on cloud properties and hence the climate and the hydrological cycle in biologically active marine areas such as the GBR region.

ACKNOWLEDGMENT

The CORACE-1 campaign was partly funded by the Marine Ecology Research Centre (SCU, Lismore, NSW) and the Australian Institute of Marine Science (Townsville, QLD). We wish to thank the HIRS staff for logistical assistance while we were at Heron Island. PV thanks the Finnish Academy and Emil Aaltonen foundation for a visiting grant, the Finnish Centre of Excellence Program and the Queensland University of Technology (Brisbane, QLD) for supporting research at Heron Island.

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