ABSTRACT

Title of Dissertation: DIMETHYSULFONIOPROPIONATE (DMSP) AND DMSP-LYASE IN CNIDARIAN ALGAL SYMBIOSES.

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Dimethylsulfoniopropionate (DMSP) is a multifaceted sulfur compound produced by several groups of marine phytoplankton, macroalgae and higher plants. Additionally, DMSP cleaving enzymes (most of which are thought to be DMSP-lyases) are known to exist in many species of marine phytoplankton, macroalgae and bacteria. Endosymbiotic dinoflagellate microalgae (genus *Symbiodinium*) of reef-building corals produce high intracellular levels of DMSP. The existence of DMSP-lyase(s) in *Symbiodinium* was, until recently, unknown. The function(s) of the DMSP/DMSP-lyase system in cnidarian-algal symbioses is poorly understood. Chapter one introduces coral symbioses, DMSP and the potential roles of the DMSP/DMSP-lyase system in cnidarian-algal symbioses. Chapter two describes the first evidence for *in vivo* DMSP-lyase activity in several isolated *Symbiodinium*

strains, revealing varying levels of DMSP and DMSP-lyase activity. These results prompted further characterization of *Symbiodinium* DMSP-lyases. Enzyme assay optimization and substrate kinetics experiments found the measured activity of DMSP-lyase enzymes to be affected by permeabilization buffers, pH, temperature and potential oxidative stress effects (chapter three). Prior to investigations of field-collected intact corals, methods for DMSP analyses were optimized in the laboratory to address the inherent complexities of the coral holobiont. This work compared several preparation techniques for the analysis of particulate (algae only, $DMSP_p$) and total (coral tissue and algae, $DMSP_t$) DMSP in several species of stony corals (chapter four). Field-collected corals in chapter five showed $DMSP_p$ and $DMSP_t$ responses when exposed to the oxidative stressor, copper. The second field study (chapter six) describes how $DMSP_p$ and $DMSP_t$ concentrations within five prominent Bermudian corals changed with water depth. Finally, chapter seven presents a synthesis examination of the potential functional attributes and significance of the DMSP/DMSP-lyase system in cnidarian-algal symbioses. The factors influencing variable DMSP production and accumulation as well as differences in DMSP-lyase activity are discussed in light of methodological limitations, the biology and physiology of symbiont and coral, *Symbiodinium* phylotype and environmental variables. The results of this research highlight the existence of DMSP-lyases in *Symbiodinium* and provide insight into the partitioning of DMSP in cnidarian-algal symbioses, furthering our understanding of the production and potential turnover of DMSP while recognizing the limitations inherent in such investigations.

DIMETHYLSULFONIOPROPIONATE (DMSP) AND DMSP-LYASE IN CNIDARIAN-ALGAL SYMBIOSES

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

The essence of this work is dedicated to all who risk truly exploring, knowing and embracing what it means to LIVE THE LIFE YOU LOVE. Dare to dream in brilliant colors you have never seen.

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Chapters two and five, entitled "Dimethylsulfoniopropionate (DMSP) lyase activity in different strains of the symbiotic alga *Symbiodinium microadriaticum"* and *"*Alterations in dimethylsulfoniopropionate (DMSP) levels in the coral *Montastraea franksi* in response to copper exposure", respectively, were previously published in the journals Marine Ecology Progress Series and Aquatic Toxicology (respectively). Kind permission has been granted from these original publishers to reproduce the research herein. Written permissions may be found in the appendix.

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Namasté.

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Chapter 1: Introduction

The coral reefs of the world are fascinating ecosystems that provide a unique, accessible connection with nature for those who explore the shallows of many tropical oceans. For scientists, reefs provide insight into nearly every discipline, from physiology and chemistry to conservation and biodiversity. Reefs are indicators of ocean and ecosystem health and therefore, it is not surprising that studies continue to focus on multiple aspects of both coral polyps and their symbiotic microalgae to understand how these intricate symbioses might respond to climate change and other stressors. Coral reefs are extremely important as they provide many significant ecosystem services worldwide; crucial functions threatened by global warming, ocean acidification and exacerbated by local stressors. Reefs provide critical socioeconomic resources through, for example, fisheries, coastline protection, tourism, biochemical research and biological diversity, thus impacting oceanic and terrestrial landscapes as well as human populations around the world.

1.1 Cnidarian-algal symbioses

Historically, invertebrate-dinoflagellate symbioses are ancient, dating back to the mid-Triassic (Trench, 1997). The successful mutualistic symbiosis between the coral symbiotic dinoflagellates (commonly known as zooxanthellae; genus *Symbiodinium)*, and their coral hosts is achieved by internal nutrient cycling, enabling corals to grow and develop in environments that are typically nutrient-poor (Figure 1;

Muscatine and Porter, 1977). Photosynthetic products (mainly glycerol) are passed from symbiont to host and account for up to 95% of algal photosynthate (Muscatine, 1967; Trench, 1979; Muscatine et al., 1984). In return for the supply of glycerol and other metabolic products such as amino acids, algal symbionts receive inorganic nutrients (ammonium and phosphate) from host's metabolism, and a protected, well-lit environment in which they can thrive. The metabolism of coral colonies is based on two main components, photosynthesis and respiration. Respiration encompasses both that of algal symbionts and the coral animal, while photosynthesis by algae represents the other part of the overall energy budget (Stambler and Dubinsky, 2004).

Symbiodinium live within the cells of at least 5 different phyla: Cnidaria, Mollusca, Platyhelminthes, Porifera, and Protista (Stat et al., 2006). Coral *Symbiodinium* acquisition occurs by vertical (maternal) or horizontal (acquisition from the environment) transmission (Harrison et al., 1984; Harrison and Wallace, 1990). The genus *Symbiodinium* is composed of 8 divergent clades (or phylotypes) of symbiotic dinoflagellates, namely A-H (Figure 2a) (Rowan and Powers, 1991; Carlos et al., 1999; LaJeunesse and Trench, 2000; LaJeunesse, 2001; Pawlowski et al., 2001; Pochon et al., 2001, 2004). It is generally accepted that *Symbiodinium* clades are much more diverse than originally thought, comprised of several lineages representing species complexes (Santos, 2004; see Figure 2b for an example of the diversity within a single clade). Corals tend to have one dominant *Symbiodinium* species, but also have cryptic *Symbiodinium* species in low numbers (Trench, 1979, 1997; Goulet and Coffroth, 1997; LaJeunesse, 2001; Santos et al., 2001). Corals in the Caribbean predominantly harbor clades A-C (higher diversity of symbionts) while those in the Pacific harbor clade C, and have a higher diversity of coral hosts (Baker and Rowan, 1997; LaJeunesse, 2002; LaJeunesse et al., 2003, 2004). Evidence suggests that *Symbiodinium* may have evolved to occupy specific environmental niches determined by, for example, the depth at which their coral host grows. Evidence for this hypothesis includes a study of the vertical distribution of coral species and their association with symbionts adapted to maximize light capture at different depths and associated light intensities (Iglesias-Prieto et al., 2004).

1.2 Oxidative stress effects on coral reefs

Reef building corals live in environments within 1-2 °C of their critical upper temperature limits (Coles et al., 1976), beyond which susceptibility to bleaching and eventual death can occur. Coral bleaching is characterized by the loss of pigments from *Symbiodinium* that may or may not include the loss of the actual symbiotic algal cells (Figure 3). The bleached appearance of the remaining coral colony reveals a relatively transparent coral tissue covering a white calcium carbonate skeleton that may or may not survive the bleaching episode (Hoegh-Guldberg and Smith, 1989; Porter et al., 1989; Brown et al., 1995). Coral bleaching is widely considered to be a symptom of stress that can be elicited by numerous stress factors and is likely due to the synergistic effect of multiple factors. Stressors may include high and low water temperatures, high UV irradiation, bacterial/viral infection, lowered salinity, chemical contaminants and increased sedimentation (Stat et al., 2006 and references therein).

Furthermore, corals compromised by stress may be more vulnerable to numerous diseases that are known to affect nearly all coral species (Weil, 2004).

The onset of bleaching is thought to be associated with photoinhibition (Jones et al., 1998; Hoegh-Guldberg and Jones, 1999), hastened by high irradiance and/or UV light, with any stress agent (such as increased temperature, some pollutants) acting as a sensitizer (Stambler and Dubinsky, 2004). Temperature increase and duration have been used to accurately predict mass coral bleaching (Strong et al., 1996, 2000; Hoegh-Guldberg, 1999, 2001). Photosynthesis creates an increased partial pressure of oxygen (pO_2) within coral tissues (Shashar et al., 1993) and can lead to the production of reactive oxygen species (ROS; singlet oxygen, superoxide radical, hydroxyl radical and hydrogen peroxide) with the absorption of excitation energy from light. Oxygen concentrations in coral tissue as high as 250% oxygen saturation during daytime have been recorded (Kühl et al., 1995). Therefore, there is a high potential for ROS production in coral given their photosynthetically active symbionts and the necessity to occupy clear, shallow waters. "The Oxidative Theory of Coral Bleaching" proposes that when antioxidant defenses (see below) of a coral are overwhelmed by ROS concentrations exceeding some threshold, corals will expel their endosymbiotic algae in a last ditch effort against the oxidative stress the algae induce (Downs et al., 2002). The resulting expulsion of symbionts from host tissues (Gates et al., 1992) probably occurs as a protective mechanism to prevent further oxidative stress damage to the host (Lesser, 1997).

In the coral symbiosis, potentially harmful production of ROS leads to cellular damage or death when not detoxified by several antioxidant systems in the algal symbiont and/or host coral (Lesser, 1996). ROS are kept in check by multiple protective mechanisms in coral and/or algal symbionts that together act as a comprehensive antioxidant system, scavenging harmful ROS in multiple cellular locations including the cytosol and cellular membranes. The association of corals with photosynthetic, oxygen-producing symbionts has led to inherently high levels of antioxidants compared to other non-symbiotic invertebrates (Dykens et al., 1992). Increased superoxide dismutase (SOD) and catalase (CAT) activities in response to stressors such as elevated temperature and UV radiation have been directly measured in some coral animals (Lesser et al., 1994), reflecting the intrinsic ability of reef corals to ameliorate environmental stress (Gates and Edmunds, 1999). Coral animal cellular defenses against UV damage may include mycosporine-like amino acids that act as sunscreens (Shick et al., 1996) in addition to heat-shock proteins, glutathione (GSH), and ubiquitin that act as intracellular protective and repair agents (Sharp et al., 1997). Corals and algal symbionts contain a number of non-enzymatic ROS scavengers such as ascorbate, GSH, tocopherols, carotenoids and other small molecule antioxidants (Lesser, 2006). Additionally, antioxidant enzymes in algal symbionts include ascorbate peroxidase (APX), SOD and CAT. Antioxidant enzymes present in coral host and algae can be induced in response to oxidative stress (Lesser et al., 1990; Lesser, 1997; Levy et al., 2003). When antioxidant defenses are overwhelmed, cellular oxidative damage may be reflected by protein carbonyl production, lipid peroxidation and DNA damage. Oxidative stress can result in cellular damage to symbiotic dinoflagellates resulting in an overall energetic cost to the host due to decreased translocation of photosynthate and/or exposure to highly reactive oxygen radicals (Lesser and Shick, 1989a).

Changes in the resident *Symbiodinium* population can occur by shifts in the frequency of existing *Symbiodinium* or by algal expulsion and repopulation by different *Symbiodinium* strains (Buddemeier and Fautin, 1993; Hoegh-Guldberg et al., 2002; Hoegh-Guldberg, 2004; Venn et al., 2008). However, switching of symbionts is not likely to take place on short time scales as evidence suggests that coral-algal co-evolution likely occurred over thousands of years (Stat et al., 2006). Rapid changes due to climate change threaten coral-algal symbioses as coral animals have long generation times and low population diversity due to asexual reproduction (Stat et al., 2006). Shifts in *Symbiodinium* populations (due to environmental selection, host expulsion or other mechanisms; see Figure 3) within corals represents one method for overcoming slow coral animal evolution relative to the fast pace of environmental change due to mounting environmental stressors, providing the potential for hosts to take on more 'stress tolerant' symbionts. *Symbiodinium* clades A and B were thought to be resistant to bleaching and clade C more susceptible (Rowan et al., 1997) but variation in thermal tolerance and bleaching resistance was discovered to exist within individual clades (LaJeunesse et al., 2003). Stress susceptibility does not lie solely with the symbiont; some susceptible algae may associate with coral families tolerant of stress (LaJeunesse et al., 2003). The overall holobiont involves the host, symbiont and associated bacteria and while much is understood regarding coral bleaching, a great deal remains to be learned about its underlying mechanisms.

1.3 Dimethylsulfoniopropionate (DMSP) and DMSP-lyase

DMSP is a sulfur compound produced by several groups of marine phytoplankton (Keller et al., 1989), macroalgae (de Souza et al., 1996), and higher plants (Dacey and Blough, 1987; Paquet et al., 1994) and is relatively stable in seawater with a half-life of approximately eight years (Dacey and Blough, 1987). Dimethylsulfide (DMS) production from DMSP is associated with marine algae and has been given much study as DMS emissions may play significant roles in the global sulfur cycle. Though anthropogenic sulfur emissions dominate the sulfur flux at 80-90% of the global sulfur cycle input, sea-to-air flux of DMS represents nearly half of the global biogenic sulfur flux to the atmosphere (Andreae, 1990; Kettle and Andreae, 2000). In the oceans, the fate of DMSP is governed by multiple factors including biotic and abiotic processes, both of which contribute to the fact that DMSP is very labile, turning over in less than a day (Figures 4 and 5; Vila et al., 2004; Miller and Belas, 2004).

Most of the DMSP literature has focused on the production of this multifaceted algal metabolite (Keller et al., 1989; Keller and Korjeff-Bellows, 1996; Van Alstyne et al., 2006; Yoch, 2002). DMSP production is found in many species of marine algae and it’s concentration levels are known to differ between species. DMSP levels have also been reported to change in response to environmental factors including salinity, light, temperature and the availability of limiting nutrients (Stefels and van Leeuwe, 1998; Keller et al., 1999; Sunda et al., 2002; Bucciarelli and Sunda, 2003). Among 123 clones of phytoplankton analyzed for intracellular DMSP by Keller et al. (1989), major DMSP production predominantly occurred among the dinoflagellates and Prymnesiophyceae (Table 1). Thus, the discovery of high DMSP concentrations (mM cellular concentrations) in the dominant marine symbiotic dinoflagellates (*Symbiodinium*) was not entirely surprising (Keller et al., 1989; Table 2). Researchers noticing the 'sea smell' (i.e. DMS) from aerially exposed reefs contributed in part to the discovery of DMSP production by zooxanthellae.

Although the particular enzymatic reactions that govern DMSP breakdown are poorly characterized, most are thought to be DMSP-lyases (Stefels et al., 2007; Schäfer et al., 2010). Because DMSP is structurally stable in seawater, biogenic DMSP cleavage via the enzyme DMSP-lyase is one major mechanism by which DMS evolution to the atmosphere occurs, that is, the enzymatic cleavage of DMSP by DMSP-lyases releases the volatile DMS. Enzymes such as DMSP-lyase are important in living cells because they act as biological catalysts (in addition to performing multiple other cellular functions) enabling chemical reactions to occur quickly, thus sustaining life. Like DMSP concentrations, DMSP cleaving enzymes are known to vary among and within species. DMSP cleaving enzymes are known to exist in several (but not all) species of marine phytoplankton (Stefels et al., 2007; Niki et al., 2000; Steinke et al., 1998, 2002a, b; Wolfe et al., 2002), bacteria (de Souza and Yoch, 1995a, b; van der Maarel et al., 1996; González et al., 1999; Yoch, 2002) and macroalgae (Van Alstyne and Houser, 2003). DMSP-lyase activity was recently quantified in cultured zooxanthellae (*Symbiodinium*) and its activities have been shown to differ within and across strains (Yost and Mitchelmore, 2009). These results suggest that the turnover of DMSP and its potential function(s) within coral symbioses may depend, in part, on algal phylotype (Yost and Mitchelmore, 2009). Also, there is no apparent link between DMSP-lyase activity and intracellular DMSP concentrations in most of the zooxanthellae tested to date, an observation made for other marine algae including Prymnesiophyceae species (Niki et al., 2000; Yost and Mitchelmore, 2009).

1.4 DMSP and DMSP-lyase, potential roles in coral symbioses

The potential importance and diverse functionality of DMSP in marine algae is well documented. It has been shown to act as an osmolyte (Kirst, 1990), a cryoprotectant (Karsten et al., 1996), a herbivory deterrent and attractant (DeBose et al., 2008; Wolfe et al., 1997), an antiviral defense mechanism (Evans et al., 2006), a sulfide detoxifier (Havill et al., 1985), and an antioxidant (Sunda et al., 2002). With the exception of the cryoprotectant function, all of the aforementioned potential roles for DMSP are possible in zooxanthellae and coral symbioses. However, of particular significance to corals and their susceptibility to bleaching via oxidative stress mechanisms, is the proposed antioxidant function of DMSP.

DMSP and its enzymatic breakdown products, DMS, acrylate, dimethylsulfoxide (DMSO) and methane sulfinic acid (MSNA) are known to readily scavenge highly reactive hydroxyl radicals and other ROS (Sunda et al., 2002). The enzymatic lysis of DMSP to DMS and acrylate by DMSP-lyase(s) can greatly increase antioxidant protection because DMS and acrylate are 60 and 20 times, respectively, more reactive toward hydroxyl radicals than DMSP itself. In addition, the stepwise oxidation of DMS and DMSP by hydroxyl radicals produces DMSO and MSNA, which are also highly reactive with hydroxyl radicals, producing an antioxidant cascade effect (Sunda et al., 2002). While DMSP and its subsequent conversion products are known antioxidants, the exact mechanisms by which these reactions occur have yet to be described in detail. It is possible that DMSP may scavenge ROS via sulfhydryl groups, similar to the free radical scavenger GSH that also occurs at high intracellular concentrations. DMSP and its reaction products can potentially relieve oxidative stress in multiple cellular compartments (Sunda et al., 2002). For example, DMS and DMSO can neutralize lipid peroxidation in photosynthetic membranes and harmful radicals in chloroplasts, respectively, thus protecting important ROS target sites (Lee and de Mora, 1999). Regardless of its specific role(s), common threads among studies suggest that DMSP is not only multifunctional, but that it plays a dominant protective physiological role in algae and potentially also in the host coral cells, although translocation (or even the presence) of DMSP in the host coral cells has not yet been demonstrated. All coral DMSP reports to date (see below) analyze the whole coral/algal fractions ($DMSP_t$) and do not tease apart the host from the algae, although studies on cultured zooxanthella have indeed been carried out (e.g. see Hill et al., 1995; Broadbent and Jones, 2002).

Recent studies highlighting the potential antioxidant role of the DMSP/DMSP-lyase system in marine algae (Sunda et al., 2002; Sunda et al., 2007) have spurred DMSP-related investigations in corals. Reefs may be substantial sources of DMS generated sulfur aerosol particles, with one emission estimate from a 1000-km^2 reef area on the order of 10^{19} s^{-1} (Bigg and Turvey, 1978), which is comparable to aerosol emissions from land surfaces and forest fires (Jones and Trevena, 2005). Furthermore, DMSP concentrations per symbiont were higher in bleached colonies of the coral *Acropora formosa* than in unbleached corals of this species (see Table 1; Broadbent et al., 2002). Other algal species respond to stressors by increasing DMSP levels or up-regulating DMSP-lyase activity (Sunda et al., 2002). As discussed, conditions of elevated oxidative stress have been described as playing a crucial role in the onset of coral-algal symbiosis breakdown (Downs et al., 2002) and oxidative stress appears to be a key factor linking increases in ocean temperature, solar radiation and anthropogenic stressors to coral bleaching (Brown, 1997; Glynn, 1991, 1993; Lesser, 1996; Hoegh-Guldberg and Smith, 1989; Dunne and Brown, 2001; Shick et al., 1996; Brown, 2000; Owen et al., 2005; Schiedek et al., 2007). To cope with stress, organisms may increase the production of antioxidants and/or antioxidant enzymes. In stressed corals DMS is released at the coral's surface and DMSP is present in coral mucus (although this may reflect released algae in mucus rather than translocation or a combination of both) (Jones and Trevena, 2005; Broadbent and Jones, 2004, 2006). Therefore, it is plausible that there is a link between DMSP production in coral symbionts and oxidative stress conditions impacting coral reef health and survival. In fact, given the much higher levels of DMSP in *Symbiodinium* than most other antioxidant compounds, coupled with it's metabolism into more powerful antioxidants that can cross cellular membranes, DMSP may be a critical component of a corals antioxidant protection.

Of those phyla harboring *Symbiodinium*, cnidarians have the greatest concentrations of DMSP on a fresh mass basis (Van Alstyne and Puglisi, 2007). Only a few studies have quantified DMSP in field-collected stony corals and DMSP concentrations were found to be highly variable among coral species (Jones et al., 1994; Broadbent et al., 2002; Hill et al., 1995, 2000; van Bergeijk and Stal, 2001; Van Alstyne et al., 2006). While it is known that *Symbiodinium* produce DMSP and have DMSP-lyase capabilities, it remains unclear whether coral animals themselves produce DMSP. Additionally, DMSP-lyases have not been detected in the coral host (or any other animal species). Thus, the regulation and exact function(s) of DMSP and DMSP-lyases are not well characterized for any zooxanthellate-symbiosis to date, though the various functions of DMSP and its breakdown products probably play species- and population-specific roles (Van Alstyne and Puglisi, 2007).

While DMSP appears to be a substantial metabolite in zooxanthellae, little is known about the potential functions, symbiont and host partitioning or distributions of DMSP within coral tissues. Research in the symbiotic giant clam that harbors *Symbiodinium* demonstrates the presence of DMSP in host animal tissues that do not contain symbiotic algae (Hill et al., 2000). These results highlight the potential for DMSP translocation from the symbiont to the host. Given that DMSP is a zwitterion that cannot cross biological membranes passively, this data underscores the potential for active transport of DMSP from algal to animal cells, although the cellular transport mechanisms of this potential translocation remain unknown. High intracellular concentrations of DMSP and activities of DMSP-lyases underscore a potential antioxidant role for DMSP/DMSP-lyase in zooxanthellae and potentially in the coral host cells. Does this antioxidant role (or other suggested roles such as antimicrobial and/or antigrazing functions) for the DMSP/DMSP lyase system exist in *Symbiodinium* and/or host corals, and if so does it impact coral ecosystem health and in what ways? The overarching goal of this dissertation research was to ascertain to what extent DMSP and DMSP-lyase activity varied in *Symbiodinium* and their respective host corals under stress free conditions and during exposure to various oxidative stress factors. Methods were developed so that simultaneous examinations of algal and coral animal DMSP concentrations could be attempted to investigate DMSP partitioning (not previously reported in the coral literature) within the symbiosis, where appropriate. These questions were examined using the hypotheses detailed below, alongside which the respective chapters detailing the associated investigations are listed.

1.5 Hypotheses:

H_{1A} (Chapter 2): DMSP concentrations in cultured *Symbiodinium* vary across strains.

H_{1B} (Chapter 2): Potential DMSP-lyase activity is present in cultured *Symbiodinium* and varies across strains.

H_{IIA} (Chapter 3): DMSP-lyase kinetic properties differ among *Symbiodinium* strains.

H_{IIB} (Chapter 3): DMSP concentrations and potential DMSP-lyase activity increases in cultured *Symbiodinium* in response to conditions of elevated oxidative stress.

H_{IIIA} (Chapter 4): Choice of preparation method for analysis of DMSP concentration analyses in *Symbiodinium* and coral host influences the results and interpretation of resultant DMSP data.

H_{IIIB} (Chapters 4, 6): DMSP concentration varies among coral species and *Symbiodinium* strains isolated from those coral species.

H_{IVA} (Chapter 5): DMSP concentrations in *Symbiodinium* and their respective coral hosts change in response to oxidative stress.

H_{VA} (Chapter 6): DMSP concentrations in *Symbiodinium* and coral species vary with water depth and associated changes in light intensity.

1.6 Tables

1.6.1 – DMSP concentrations in marine algae.

(Table 1 in text). Published DMSP concentrations for selected, cultured members of multiple classes of marine algae. DMSP values represent whole culture samples (cells and water) taken during mid-exponential growth (Keller et al., 1989; Keller 1988, 1989).

Class	Genus/Species	[DMSP] mmol $L_{cell\ volume}^{-1}$
Bacillariophyceae (centric diatoms)	*Thalassiosira pseudonana*	17
Chlorophyceae (chlorophytes)	*Chlorella capsulata*	25
Prasinophyceae (prasinophytes)	*Tetraselmis levis*	33
Prymnesiophyceae (prymnesiophytes/coccolithophores)	*Emiliania huxleyi*	166
Dinophyceae (dinoflagellates)	*Amphidinium carterae*	377
	Gymnodinium nelsoni	30
	Prorocentrum minimum	111
	Pyrocystis noctiluca	0.01
	Symbiodinium microadriaticum	345

1.6.2 – DMSP concentrations in coral and *Symbiodinium*.

(Table 2 in text). Published DMSP concentrations in coral (coral host and *Symbiodinium* spp. algae) and *Symbiodinium* spp. (zooxanthellae) cell cultures.

Coral Species	[DMSP] fmol $cell^{-1}$
Pocillopora damicornis[a]	89; 179
Pocillopora sp.[b]	117
Pocillopora meandrina[c]	80 ± 22
Acropora formosa[a]	235; 641
Acropora formosa (unbleached;bleached)[a]	171; 436
Acropora palifera[a]	2831; 3831
Acropora pulchra[a]	40
Acropora cerealis[c]	950 ± 615
Acropora digitifera[c]	417 ± 162
Acropora valida[c]	425 ± 132
Lobophytum sp.[a]	43
Favites sp.[a]	21
Montipora sp.[b]	73
Porites sp.[b]	77
Porites cylindrica[c]	107 ± 39
Porites rus, decumbent[c]	88 ± 28
Porites rus, upright[c]	69 ± 24
Heliopora coerulea[c]	310 ± 119

Leptastrea purpure[a]	211 ± 230
Pavona decusata[c]	43 ± 19
Psammocora digitata[c]	49 ± 19
Zooxanthellae freshly isolated from *Lobophytum compactum*[a]	285
Zooxanthellae freshly isolated from *Montipora verrucosa*[b]	61-66
Zooxanthellae freshly isolated from *Porites compressa*[b]	48
Zooxanthellae freshly isolated from *Zoanthus* sp.[a]	164
Zooxanthellae freshly isolated from *Tridacna gigas*[d]	310
Zooxanthellae CCMP strain 421 cultured from sea water [e]	201 ± 139
Zooxanthellae CCMP strain 828 cultured from sea water [e]	123 ± 61
Zooxanthellae CCMP strain 829 cultured from *Tridacna crocea* [e]	81 ± 43
Zooxanthellae CCMP strain 830 cultured from *Aiptasia pallida* [e]	34
Zooxanthellae CCMP strain 1633 cultured from *Aiptasia puchella* [e]	330 ± 193

[a] Broadbent et al. (2002); Mean ± SD.
[b] Hill et al. (1995)
[c] Van Alstyne et al. (2006); Mean ± SD.
[d] Jones et al. (1994)
[e] Yost and Mitchelmore (2009); Mean ± SD.

1.7 Figures

Figure 1.7.1 – Coral symbiosis carbon and nutrient flux.

(Figure 1 in text). A model of the potential pathways of carbon and nutrient (N, P) flux in a symbiotic reef coral (Muller-Parker and D'Elia, 1997 after Lewis and Smith, 1971).

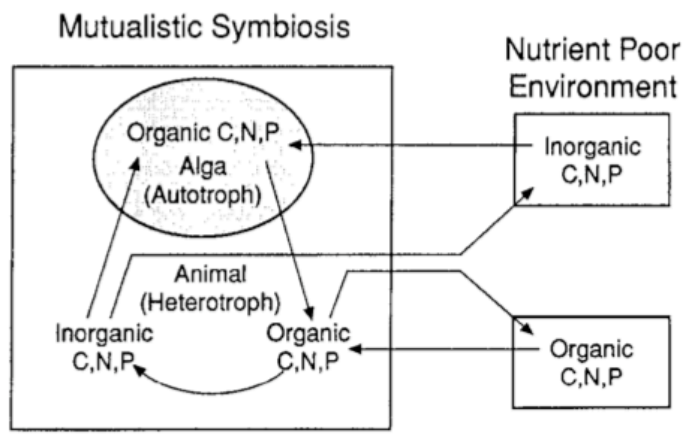


Figure 1.7.2 – Phylogenetic relationships for *Symbiodinium* clades.

(Figure 2a in text), (Pochon et al., 2004; Coffroth and Santos, 2005).

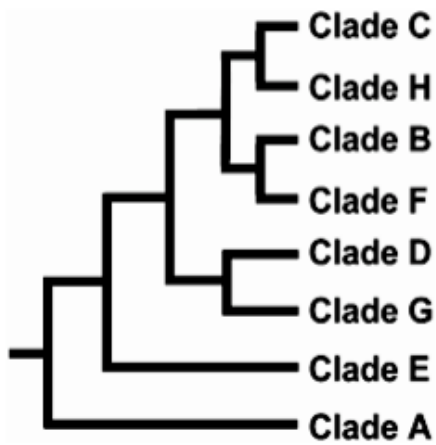


Figure 1.7.3 – *Symbiodinium* within phylotype diversity.

(Figure 2b in text). Example of *Symbiodinium* diversity within phylotype B generated using partial large-subunit rDNA sequences (Savage et al., 2002a).

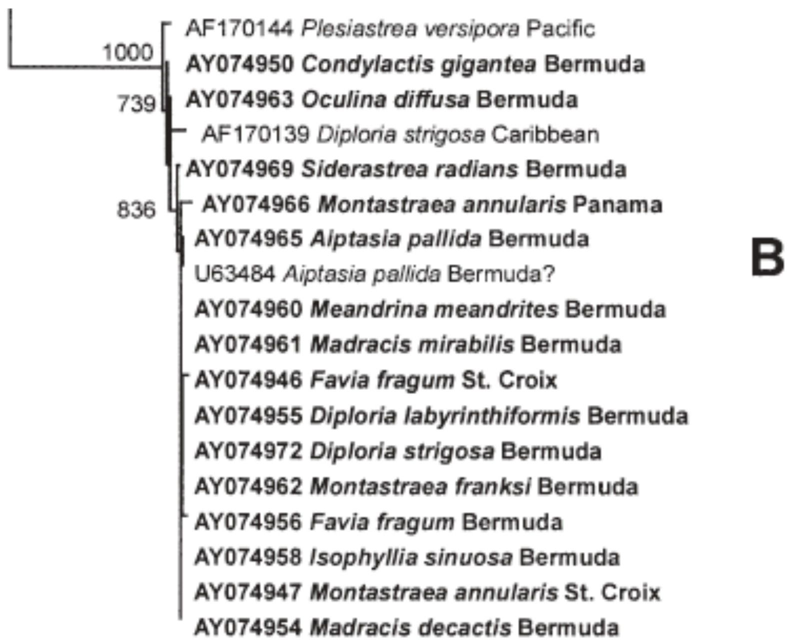


Figure 1.7.4 – Cellular mechanisms of symbiont loss.

(Figure 3 in text). Five different types of cellular mechanisms of symbiont loss from Cnidarian host tissues. Normal host cells (H) are anchored to the acellular mesoglea (M). Symbionts (S) are contained with host vacuoles or symbiosomes (Sy). Figure adapted from Gates et al., 1992.

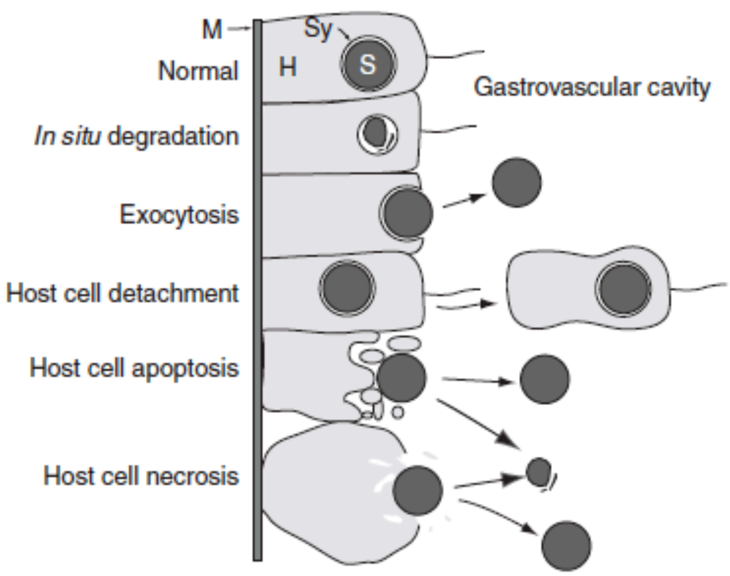


Figure 1.7.5 – Marine biogeochemical cycling of DMSP and DMS.

Figure 4. Marine biogeochemical cycling of DMSP and DMS schematic. "Dominant role of functional groups in the different processes is indicated by colored ellipses: green, phytoplankton; blue, zooplankton; red, bacteria; black, abiotic factors. CCN, cloud-condensation nuclei; DOM, dissolved organic material; DMSO, dimethylsulfoxide; MeSH, methanethiol; MPA, mercaptopropionate; MMPA, methylmercaptopropionate; MSA, methane sulfonic acid" (Stefels et al., 2007).

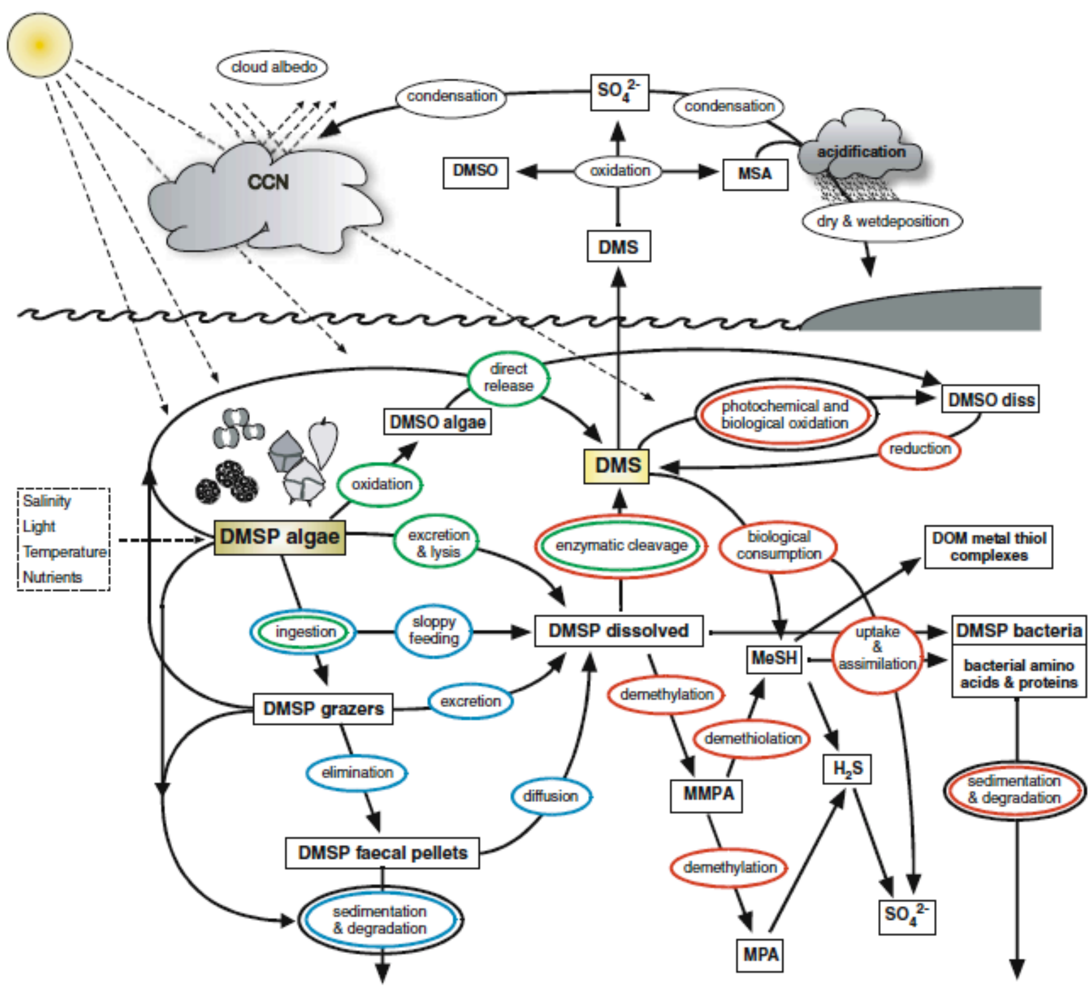
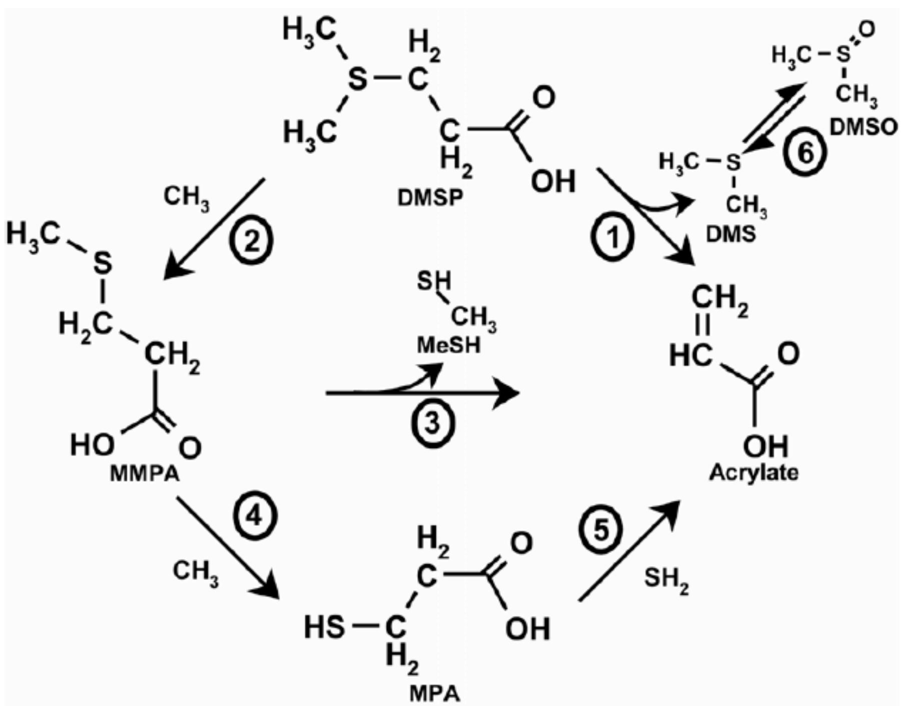


Figure 1.7.6 – Pathways involved in DMSP catabolism.

(Figure 5 in text). Pathways involved in the catabolism of DMSP. Degradation of DMSP may occur by the lyase pathway, hydrolysis of the C3 carbon producing acrylate and DMS (Rxn 1), or by the demethylase pathway, demethylation of the DMS moiety producing MMPA (Rxn 2). MMPA may be further demethylated to MPA (Rxn 4) followed by elimination of hydrogen sulfide (Rxn 5) or demethiolated producing acrylate and MeSH (Rxn 3). In some cases, DMS may be oxidized to DMSO (Rxn 6) (Miller and Belas, 2004).



Chapter 2: Dimethylsulfoniopropionate (DMSP) lyase activity in different strains of the symbiotic alga *Symbiodinium microadriaticum*

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2.1 Introduction

Dimethylsulfoniopropionate (DMSP) is an algal metabolite that is produced by a wide variety of species at different intracellular concentrations (Keller et al. 1989). Marine dinoflagellates, including the coral symbiotic algae of the genus *Symbiodinium*, produce relatively high levels of DMSP (Keller & Korjeff-Bellows 1996, Yoch 2002, Van Alstyne et al. 2006). DMSP and its enzymatic cleavage products have multiple proposed functions at the cellular level, including those of herbivory deterrent (Wolfe et al. 1997), algal osmolyte (Kirst 1996), antioxidant (Sunda et al. 2002), antiviral defense mechanism (Evans et al. 2006), overflow mechanism for excess reduced sulfur (Stefels 2000), methyl donor (Ishida 1968), sulfur storage compound (van Diggelen et al. 1986), foraging cue (Debose et al. 2008) and cryoprotectant in polar algae (Kirst et al. 1991, Karsten et al. 1996). Dimethylsulfide (DMS) is a significant degradation product of DMSP, and is a major source of sulfur to the atmosphere (Kettle & Andreae 2000). DMS is also reputed to affect ocean cloud cover and the radiative climate through formation of aerosols (Charlson et al. 1987).

The enzyme DMSP lyase (dimethylpropiothetin dethiomethylase, EC 4.4.1.3; DL) is responsible for DMSP conversion, producing DMS and other products

(Johnston et al. 2008). This enzyme has been reported in phytoplankton (Steinke et al. 1998), macroalgae (Van Alstyne & Houser 2003), bacteria (de Souza & Yoch 1995b) and fungi (Bacic et al. 1998). As demonstrated by Sunda et al. (2002), DMSP is reactive toward hydroxyl radicals (·OH), but its enzymatic cleavage products, acrylate and DMS, are ~20 and ~60 times more reactive towards ·OH, respectively. Overall, the enzymatic conversion of DMSP forms several potential antioxidant scavengers (Sunda et al. 2002). Though many algal DMSP producers have DL capabilities (DMSP lyase potential activity [DLA]), this is not true for all algal species (Steinke et al. 1996, Niki et al. 2000, van Bergeijk & Stal 2001, Sunda et al. 2002). Since DMSP and DLA potentially mitigate stress, investigations of how these parameters vary across *Symbiodinium* clades may improve understanding of the symbiont-host relationship.

Symbiodinium are the most prominent dinoflagellates in symbioses with marine invertebrates and protists and are commonly found with members of the phyla Cnidaria (i.e. corals, anemones), Platyhelminthes, Mollusca, Porifera and Foraminifera (Trench 1979, Pawlowski et al. 2001). Many symbiont host species have been shown to contain DMSP, including corals (Jones et al. 1994, Hill et al. 1995, Broadbent et al. 2002), anemones (Van Alstyne et al. 2006), flatworms (van Bergeijk & Stal 2001) and clams (Jones et al. 1994, Hill et al. 2000, 2004). Evidence suggests that algal symbionts are responsible for DMSP production in cnidarian species due to a positive correlation between DMSP concentration and symbiotic algal densities (cell number) in cnidarian hosts, although partitioning between host coral and algal symbiont is unknown (Broadbent et al. 2002, Van Alstyne et al. 2006). Significant amounts of DMSP in reef corals suggests that these ecosystems could be significant sources of DMS to the atmosphere (Broadbent and Jones 2004; Jones & Trevena 2005), but it is currently unknown whether *Symbiodinium* have the enzymatic ability to convert DMSP into DMS. With evidence for DMSP variation in *Symbiodinium* from different coral species and bleached versus healthy corals (Hill et al. 1995, Broadbent et al. 2002, Van Alstyne et al. 2006, Jones et al. 2007), but no reports of DLA in *Symbiodinium* or their coral hosts, a characterization of baseline DMSP and DLA levels within and across algal and animal host species is warranted.

This present study investigated five *Symbiodinium microadriaticum* strains to determine (1) whether DLA was detectable in *S. microadriaticum* cultures, (2) whether DLA was primarily associated with the algal fraction of non-axenic cultures and (3) whether *S. microadriaticum* DLA and DMSP were significantly different between the algal strains investigated. *Emiliania huxleyi* strains were analyzed for comparison.

2.2 Materials and Methods

2.2.1 Algal cultures

All experiments were conducted with strains of the dinoflagellate *Symbiodinium microadriaticum* and *Emiliania huxleyi*, purchased from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton (CCMP; Bigelow Laboratory, Maine, USA). Algal strains included CCMP 373 and 374 (*E. huxleyi*;

axenic) and *S. microadriaticum* CCMP 421, 828, 829, 830 and 1633 (Table 1). The cultures were maintained using sterile techniques and grown in sterile K, L1 or f/2-Si media (pH 8.0) according to preference (Bigelow Laboratory). *S. microadriaticum* cultures were not treated with antibiotics to obtain axenicity as our preliminary trials showed that antibiotic treatment negatively affected growth (data not shown). Thus, measures were taken to assess bacterial abundance and potential interference with DLA assays (detailed in 'Bacterial analysis' below). Algal cultures were grown at 26 °C with a 12 h light:12 h dark cycle, without agitation (Rogers & Davis 2006). In common with other studies (Matrai & Keller 1994), our preliminary trials demonstrated that DMSP levels were dependent upon the growth phase; therefore, all algal cultures were sampled during their exponential growth phase, at an average density of 1×10^5 cells ml^{-1}. Each strain was grown in semi-continuous batch culture in 50 ml conical flasks with 30 ml of culture in each, under cool-white fluorescent bulbs (100 $\mu E\ m^{-2}\ s^{-1}$). All cultures were sampled 2 h (± 1 h) into their light periods as DMSP (and DLA) concentrations varied with diel cycle in *S. microadriaticum* (authors' pers. obs.) and have been reported to vary in other algal species as well (Bucciarelli et al. 2007, Jones et al. 2007, Stefels et al. 2007, Sunda et al. 2007). *S. microadriaticum* cultures were genetically verified for clade type (see 'Algal analyses'). Each of the strain replicates was grown separately and analyzed individually.

2.2.2 DMSP and DLA analyses

DMS analysis and calibration

All samples were analyzed with a Hewlett-Packard 5890 Series II gas chromatograph (GC) fitted with a Chromosil 330 packed column (Supelco) and a flame photometric detector (FPD). System temperature settings were 150 °C for the injector, 60 °C for the column oven and 175 °C for the detector. Nitrogen gas was the carrier (60 cm^3 min^{-1}), and air (60 cm^3 min^{-1}) and hydrogen (50 cm^3 min^{-1}) were the flame gases. Data were collected and analyzed using HP ChemStation (Hewlett-Packard). Quantifications were made by headspace analysis following DMSP conversion to DMS by alkaline hydrolysis. Known concentrations of DMSP (purchased from Research Plus) were diluted in sterile water to give working solutions, which were frozen in small aliquots at -80 °C. A standard curve of serial dilutions of DMSP was used to construct a calibration curve (using the square-root values of the peak area), and this linear regression served to convert peak areas from GC headspace measurements to DMS concentrations. Standards and controls ($n = 5$ each) were prepared in parallel to experimental samples by using the same Tris buffer for DLA, incubating at 30 °C for 20 min and using the same total liquid volume (1 ml) in headspace vials. Addition of 5N NaOH to standards (final volume of 1 ml; final concentration of 5N NaOH) occurred prior to heating, and all vials were placed in the dark for at least 4 h for equilibration prior to sampling. Analytical replicates were used only for inter-strain assessment of repeatability. The precision of the DMS analysis was <5 %, and headspace storage trials showed no DMS losses with the analytical methods employed. The detection limit of the GC was 1 nmol DMSP l^{-1}.

Total and particulate DMSP analyses

Total DMSP was determined by placing 0.5 ml of whole culture with 0.5 ml of 10N NaOH into a headspace vial, sealing it and waiting at least 24 h for equilibration (equilibration times were optimized for all procedures; data not shown). Particulate DMSP ($DMSP_p$) was determined using a simple acidification/storage procedure (5 μl 50 % H_2SO_4 ml^{-1} of culture) followed by the total DMSP method (Kiene & Slezak 2006). Appropriate controls (n = 5) were prepared in parallel to sample preparation.

DLA analyses

Our DLA methods were modified from procedures described by Harada et al. (2004) and Steinke et al. (2000) and optimized (pH, exogenous DMSP concentration) for *Symbiodinium microadriaticum*. Briefly, DLA was determined by measuring the production rate of DMS prior to and after the addition of exogenous saturating levels of DMSP to permeabilized cells (using a Tris buffer, 200 mmol l^{-1} Tris containing 500 mmol l^{-1} NaCl; pH 8). Algal cell permeabilization is necessary to allow exogenous DMSP into the cells in order to detect an enzymatic response to saturating DMSP levels. Tests showed that DLA increased in samples with Tris buffer compared to those without, and Tris buffer produced higher DLA than other means of cell disruption (homogenization, varied Tris buffer strength). We found that Tris buffer at pH 8 yielded DLA in *S. microadriaticum* samples greater than those in pH 6 Tris buffer. Appropriate controls (biotic and abiotic in parallel to sample preparation) and standards were run in tandem. Controls consisted of 0.5 ml Tris buffer and 0.5 ml culture medium amended with 5 μl of 1 mol l^{-1} DMSP at t_0. Spot checks of Tris buffer and culture pH before and after DLA analysis were conducted and never deviated significantly from pH 8 (stable at 7.98; pH above 8 results in increased abiotic conversion [any conversion of DMSP not attributed to biological enzymes]). Headspace vials were 6 ml in volume and sealed with Polytetrafluoroethylene (PTFE)/rubber septa (National Scientific).

Algal cells in culture were concentrated by centrifugation at 1310 x *g* for 5 min prior to placement in headspace vials. Concentrating the cells was necessary as the amount of DLA in some cultures was too small and needed to be adjusted according to the limitations of our analytical system (Steinke et al. 2000). Centrifugation has also been used previously for concentrating algal cells in DLA and DMSP/DMS experiments (Steinke et al. 1998, Broadbent et al. 2002). We did not use filters (e.g. GF/F) to capture cells because the filters could not be fully submerged in the small liquid volume of the headspace vials. After centrifugation, algal cells were resuspended in their native media to a final volume of 0.5 ml. Tris buffer was added to samples to permeabilize the cells. Preliminary trials in native media versus those in Tris buffer verified that the buffer was necessary for optimal DLA measurement. Tris buffer (0.5 ml) was added to 0.5 ml algal culture, sealed, incubated in a 30 °C water bath for 20 min and vortexed vigorously for 3 s before sampling at each time point (at 5 min intervals from 10 min prior to, through 30 min after, exogenous DMSP addition).

The temperature chosen for these experiments (30 °C) was previously recommended to enable comparisons among samples and studies (Steinke et al. 2000). At t_0, 5 μl DMSP stock solution (1 mol l^{-1}) was added to give a final concentration of 5 mmol l^{-1}. This amount of DMSP was found to be saturating for lyase-catalyzed DMS production in this system according to preliminary tests. At each time point, 50 μl headspace samples were removed with an Agilent gas-tight syringe (same volume injected for all samples and standards) and injected into the GC for DMS measurement. At least eight headspace samples (-10 min through 30 min) were measured for each vial to yield a rate of DMS increase with time. DMS production was linear for all samples taken, and DLAs for all samples were corrected for abiotic conversion of DMSP by subtraction of DMS production rates measured in control vials. DLA is defined as nanomoles of DMS·per minute and is also reported as DLA:chl *a* (nmol DMS·min^{-1}·μg^{-1}) and DLA:$DMSP_p$ (nmol DMS·min^{-1}·$fmol^{-1}$ DMSP). On a per cell basis, DMSP is reported as femtomoles per cell.

2.2.3 Bacterial analyses

DLA in culture sample filtrates

Algal cultures were sub-sampled prior to centrifugation to determine bacterial contribution to overall DMS production. Culture samples were filtered using Isopore membrane filters (Millipore; 25 mm diameter, 2 μm pore size) and gentle filtration (gravity or <50 mm Hg vacuum; Steinke et al. 2000) to obtain an algal-free culture fraction while allowing unattached bacteria into the filtrate. Filtrate (0.5 ml) was immediately placed in a 6 ml headspace vial, 0.5 ml of Tris buffer was added and

DLA was assessed as described above. Filtrate samples without algae were scaled up to represent the amount of bacteria present in unfiltered (same volume of whole culture as the filtrate) samples to calculate the maximum bacterial DLA contribution to the reported algal DLA measurements. Specifically, the contribution to total potential DLA by bacteria (bacterial contribution) was calculated as follows for each replicate: (1) filtered bacterial counts were divided by unfiltered bacterial counts to determine the ratio of bacteria in the filtered versus unfiltered culture samples, (2) filtered DLA quantities were divided by unfiltered DLA quantities at t = 30 min; each DLA measure was corrected for abiotic conversion by subtracting the amount of DMS measured in controls. Bacterial contributions to total observed DLA (%) were therefore calculated by dividing the above DLA ratio (Point 2) by the above bacterial count ratio (Point 1) and multiplying by 100.

Bacteria enumeration

Prior to centrifugation, culture samples and culture filtrate sub-samples (0.5 ml each) were preserved (Sherr & Sherr 1993), stained with 4'-6-diamidino-2-phenylindole (DAPI, final concentration 20 μg ml^{-1}) and filtered onto 0.8 μm polycarbonate filters (after diluting for cell density) for bacterial enumeration. Cells were enumerated by counting 10 to 20 bacteria grid $field^{-1}$ in 30 random fields $filter^{-1}$ (1000-fold magnification) with an epifluorescence microscope (Kemp et al. 1993). Cell numbers in individual grids were averaged, and the number of cells per milliliter of culture was calculated.

2.2.4 Algal analyses

Symbiodinium microadriaticum were enumerated by haemocytometer using an epifluorescence microscope prior to and after centrifugation. Ten grid squares were counted for each sample and averaged to calculate the total number of algal cells per milliliter. Algal cell sizes were determined using a microscope, haemocytometer and eyepiece graticule. Chl *a* concentrations were measured fluorometrically with a Trilogy Laboratory Fluorometer (Turner Designs). Briefly, 1 ml aliquots of unconcentrated culture were filtered through Whatman GF/F glass fiber filters and extracted in 90 % acetone for 24 h at 4 °C (Parsons et al. 1984). *S. microadriaticum* genetic diversity was assessed using standard RFLP methods to verify *Symbiodinium* clades (Table 1; Rowan & Powers 1991). Algal DNA was extracted using the CTAB/phenol extraction methods as detailed by Coffroth et al. (1992) and Goulet & Coffroth (1997) and amplified with PCR using a 'universal' primer (ss5) and the zooxanthella-biased primer, ss3Z (Rowan & Powers 1991). Samples were subsequently digested with *Taq I* following the protocol of Goulet & Coffroth (2004) and visualized by ultraviolet light after ethidium bromide staining of the product in a 2 % agarose gel.

2.2.5 Statistical analyses

Prior to analyses, assumptions of normality and homogeneity were tested and data were transformed as necessary. Regression analyses were used to assess DLA among strains and the relationship between DMS concentration and cell number. Analysis of variance (ANOVA) was used to assess whether DLA:chl *a* measures differed among strains, filtrates, controls, or over time. ANOVA was also used to assess $DMSP_p$ and total DMSP (particulate and dissolved; $DMSP_t$) values per cell. All statistical analyses were conducted using Minitab© V. 10 (Minitab, Ver. 2000), with $\alpha = 0.05$ for all tests.

2.3 Results

2.3.1 Algal DMSP

For each of the *Symbiodinium microadriaticum* cultures investigated, $DMSP_t$ closely paralleled $DMSP_p$ measurements and were not significantly different ($p > 0.05$; Table 2). *S. microadriaticum* $DMSP_t$ per cell and $DMSP_p$ per cell varied according to strain with CCMP 1633 having the greatest and CCMP 830 the least DMSP per cell. $DMSP_t$ and $DMSP_p$ per cell values for CCMP 1633 were significantly different from those for *Emiliania huxleyi* CCMP 373 and 374 ($p < 0.05$), but not from those for other *S. microadriaticum* investigated. Average cell diameters (µm) for *S. microadriaticum* strains were (average ± SD; n = 30 $strain^{-1}$) as follows: CCMP 421 (10.28 ± 1.08), 828 (10.69 ± 1.11), 829 (10.67 ± 1.22), 830 (10.74 ± 1.12) and 1633 (9.72 ± 0.82); and for *E. huxleyi* strains were as follows: CCMP 373 (5.08 ± 0.63) and 374 (5.03 ± 0.66). Only CCMP 1633 was statistically different in cell size ($p < 0.05$) among the various *S. microadriaticum* strains. Both *E. huxleyi* cultures were found to have less $DMSP_p$ when compared with *S. microadriaticum* cultures.

2.3.2 Algal DLA

In all strains, DMS evolution prior to exogenous DMSP addition (Fig. 1) was not statistically different from that in controls ($p > 0.05$). DLA was greatest in strain CCMP 829 and was undetectable in CCMP 830. Additionally, when normalized to cell number or chl *a* (fmol cell^{-1}, nmol·min^{-1}·µg^{-1}), DLA for strains CCMP 373, 421, 828 and 1633 were markedly different from strain CCMP 829 ($p < 0.01$), but not from each other ($p > 0.05$), and CCMP 374 was statistically different from all other strains ($p < 0.01$) (Fig. 2, Table 2). DLA:chl *a* for *Emiliania huxleyi* CCMP 373 was greater than that of CCMP 374, but less than all *Symbiodinium microadriaticum* investigated (except CCMP 830; Table 2). DLA:DMSP$_p$ per cell averages were highest in strains 829 and 373 (0.06 and 0.02 nmol DMS·min^{-1}·fmol DMSP^{-1}, respectively) compared to other strains (Fig. 2). DLA:DMSP$_p$ per cell for strain CCMP 829 was statistically higher than that for all other strains in Fig. 2 ($p < 0.01$). CCMP 374 DLA measures ($n = 2$) are not presented in Fig. 2 because few replicates had measureable activity. Overall, CCMP 829 demonstrated the greatest DLA, whereas CCMP 421, 828 and 1633 had intermediate rates, with CCMP 830 having no detectable activity. Within strains, DMSP$_p$ concentrations were correlated with DLA. For each CCMP strain, r^2 values (in parentheses) were as follows: 373 (0.90), 374 (0.86), 421 (0.87), 828 (0.66), 829 (0.53) and 1633 (0.72). However, DMSP$_p$ concentrations were not correlated with DLA between strains (those strains with high DMSP did not necessarily have high DLA).

2.3.3 Bacterial contribution to DLA

After correction for the quantity of bacteria in the whole culture versus that in the 2 μm filtered sample, the calculated bacterial contribution to total DLA was found to be consistently low (Table 2). Bacterial DLA was greatest for strain CCMP 421 (approximately 15 % of the total) and averaged <5 % across all other strains (excluding CCMP 830 for which no DLA was detected). Therefore, the majority of DLA was associated with the algal component in all cultures examined. In bacterial filtrates, no algal contamination was detected in slide preparations using a fluorescence microscope. Furthermore, no algal cells were detected in the filtrate during bacterial enumeration (DAPI), whereas algal cells were observed in DAPI-stained whole culture samples. It was noted that algal cells in whole cultures did not appear to have attached bacteria as bacteria were evenly distributed and not concentrated around the dinoflagellate cells. Preliminary data (not shown) indicated minimal quantities of DMSP in the filtrate fractions of algal cell cultures examined for total DMSP. These data indicate a lack of algal contamination in filtrates.

2.4 Discussion

Our results demonstrate that four of the five symbiotic dinoflagellate strains of the genus *Symbiodinium* examined in the present study are capable of performing the enzymatic lysis of DMSP to DMS, indicating DLA in these algae. *S. microadriaticum* strains demonstrated a range of DLA when grown in culture and exposed to exogenous DMSP addition. One of the *S. microadriaticum* strains in the present study (CCMP 830) did not demonstrate DLA, suggesting that DL is not a universal enzyme in this species or that DLA was not detected within our analytical capabilities. Furthermore, DLA was primarily associated with the algae (not bacteria) in the non-axenic cultures investigated.

Symbiodinium microadriaticum strains exhibiting DLA averaged DMS production rates of 0.6 to 5.3 nmol·min^{-1}·µg^{-1} chl *a*. The highest average DLA:chl *a* DLA in this study, 5.3 nmol·min^{-1}·µg^{-1}, occurred in CCMP 829. This strain was originally isolated from South Pacific clams and DMSP concentrations in certain tissues of *Tridacna* sp. are some of the highest recorded in animal tissues to date (Hill et al. 2000). DLA for all algal lines closely parallel those reported by Harada et al. (2004) for particle associated DLA in or near waters from the Gulf of Maine (DLA:chl *a* = 0.5-7.9 nmol·min^{-1}·µg^{-1}). In their study, DLA:chl *a* rates ranged from <5 to 53 nmol·min^{-1}·µg^{-1}, with the highest rate (53) occurring at an oligotrophic sampling site in the Sargasso Sea dominated by prymnesiophytes and dinoflagellates. It appears that differences in DLA normalized to chl *a* may be due to several factors including species composition. Other studies have shown a relationship between high DMSP:chl *a* and DLA:chl *a* and have attributed this, in part, to nutrient limitation (Sunda et al. 2007). It is not likely that nutrient limitation played a role in our experiments, though nutrient quantities were not specifically addressed. More detailed experiments addressing the role of nutrient limitation and enzyme kinetics in *Symbiodinium* are needed.

DLA differences were detected between the strains investigated, though we did not specifically address enzyme turnover rates. Our finding that DLA was not detectable in CCMP 830 is consistent with observations of Niki et al. (2000), who reported no DLA for 2 DMSP-producing Prymnesiophyceae species. The genus *Symbiodinium* encompasses 8 divergent clades (A to H) (for example, see Pochon et al. 2004, Coffroth & Santos 2005), and it is generally accepted that these clades are composed of several lineages representing species complexes (Santos 2004). *S. microadriaticum* strains (CCMP 830 and 1633) are the same algal clade (i.e. Clade B), but were isolated from different host origins (Atlantic and Pacific Oceans, respectively) and have very different DLA potentials, suggesting intra-clade DLA variation within a species (Table 2). *Symbiodinium* clades are known to have different susceptibilities to light and thermal stress (Rowan 2004), which may, in part, explain a corals' sensitivity to bleaching. We hypothesize that if DMSP has an antioxidant role in *Symbiodinium*, DMSP production and DLA in these algae might be expected to correlate with one of the primary mechanisms involved in coral bleaching, namely, damage to Photosystem II in the symbionts (Iglesias-Prieto et al. 1992, Lesser & Farrell 2004). Susceptibility to Photosystem II damage may be mitigated if DMSP and its enzymatic cleavage products serve to alleviate conditions of oxidative stress by scavenging harmful reactive oxygen species (ROS; Sunda et al. 2002).

Because bacteria contributed <5 to 20 % of the total DLA, the whole culture DLA values can be primarily attributed to the algal cells. Steinke et al. (2002a) reached a similar conclusion, finding that >95 % of DLA was found in the particle fraction >2 μm. Bacterial DLA was greatest for CCMP 421, and this result is unexplained by the number of bacteria present in the filtrate. All cultures had similar ratios of bacteria to algae (approximately 5:1 on a per cell basis, respectively), and the amount of bacteria in the filtrate versus that in the total culture sample was also similar among strains (76.9 ± 16.6 %). Bacterial DLA may in fact be overestimated due to algal cell rupture via filtration through polycarbonate filters, allowing algal contents into the experimental filtrate.

While the use of axenic cultures allows a more direct analysis of DLA in the algal component alone, we found that *Symbiodinium microadriaticum* treated with antibiotics had lower growth rates (data not shown). Additionally, checking cultures for axenicity by using standard plating techniques will miss a substantial portion of bacteria (Kogure et al. 1979). Though the bacteria in the present study were not genetically characterized, DLA could be over- or underestimated if a significant fraction of DMSP-utilizing bacteria (e.g. *Roseobacters)* were present in cultures. This is due to the finding that bacteria, in addition to the DL pathway, can also metabolize DMSP via the demethylation/demethiolation pathway, which would reduce the DMS quantities evolved (Taylor & Gilchrist 1991). We do not suspect that demethylation/demethiolation played a substantial role in our experiments, as no methanethiol was detected. Alternatively, bacterial DLA may increase the quantity of DMS detected, an important factor that we have addressed in the present study and estimate to be small. It is also recognized that the possibility of algal-attached bacteria cannot be excluded and may in fact be partially responsible for some of the observed

DLA. However, during bacterial enumeration of whole culture samples, bacteria were not observed to be more numerous in close proximity to algal cells.

Our data demonstrate DLA variability within and across *Symbiodinium microadriaticum* strains, providing only the first step in an effort to further elucidate DLA regulation in the algae involved in numerous symbioses. DLA variability within strains could be attributed to several factors. According to Steinke et al. (2007) in their study of *Emiliania huxleyi*, *in vivo* DLA may vary during the course of a day due to enzyme turnover, and individual strains of algae may respond differently to exogenous substrate (DMSP) additions. Our finding that CCMP 373 has greater DLA than CCMP 374 is in agreement with other reports (Steinke et al. 1998), though differences in experimental conditions preclude direct comparison with previously reported rates (Steinke et al. 1998, 2000, 2007). Additionally, *E. huxleyi* DLAs in the present study were used to demonstrate the validity of the given assay for the detection and optimization of DLA in *S. microadriaticum*; the assay was not optimized for *E. huxleyi* DLA measurements. While several assay parameters differed when compared to those in previous studies, our *E. huxleyi* data are in agreement with published DLA measurements when calculations incorporate differences in activity associated with pH. For example, using data presented by Steinke et al. (1998), the calculated DLA:$DMSP_p$ (nmol DMS$\cdot$min$^{-1}\cdot$fmol^{-1} DMSP) for CCMP 373 is 0.27 at an optimal pH of 6, but, at pH 8, would be approximately 10 % of that value based on the reported pH trials. Additional sources of variability may include shifts in enzyme affinity during growth, culture conditions and individual variability associated with enzyme assay parameters. Given these observations, the present study demonstrates that some *S. microadriaticum* have DL capabilities and that DLAs are distinguishable between algal strains at the given concentration of exogenous DMSP addition.

Symbiodinium are known to contain substantial quantities of DMSP (Jones et al. 1994, Hill et al. 1995, Broadbent et al. 2002, Broadbent and Jones 2004), with cultured zooxanthellae having 179 fmol cell^{-1} DMSP (Keller et al. 1989). Our $DMSP_p$ measurements for *S. microadriaticum* strains (34 to 330 fmol cell^{-1}) are in agreement with those from previous studies (Jones et al. 1994, Hill et al. 1995, Broadbent et al. 2002). Several studies support the hypothesis that DMSP and DLA are produced at varying levels by different organisms and that DMSP and DLA vary between strains of the same species (Steinke & Kirst 1996, Steinke et al. 1996, 2002a, b, Wolfe et al. 1997, Niki et al. 2000). Our comparisons of per cell DMSP between the 5 strains of *S. microadriaticum* tested revealed differences, so further investigations of DMSP levels within *Symbiodinium* are warranted. We found no apparent correlation between intracellular DMSP concentrations and DLA between strains, though strain CCMP 830 had no detectable DLA and also had the lowest DMSP concentrations of the *S. microadriaticum* strains investigated.

Corals (host and algal cells) may be exposed to conditions of elevated oxidative stress when harmful ROS are not scavenged or detoxified by antioxidants. Because corals contain photosynthetic, oxygen-producing algae, high levels of antioxidant enzymes (and free radical scavengers) are found in host (and algal) tissues

(Lesser & Shick 1989, Dykens et al. 1992, Downs et al. 2002). With proposed antioxidant functions, DMSP, and more importantly its enzymatic cleavage products via DLA, may play significant roles in alleviating conditions of oxidative stress on reefs. To investigate this potential antioxidant role we are exploring the effects of various oxidative stressors on DMSP levels and DLA in isolated *Symbiodinium microadriaticum*. Our studies herein have shown that coral symbiotic algae contain DLA, and further studies are directed towards determining if cnidarian hosts, in common with other symbiotic host species, contain DLA.

2.5 Tables

Table 2.5.1 – Algal strain characteristics.

(Table 1 in text). *Symbiodinium microadriaticum* and *Emiliania huxleyi*. CCMP algal strain characteristics and clade identification. All data (except for clade) from Provasoli-Guillard Center for the Culture of Marine Phytoplankton, Bigelow Laboratory, Maine, USA.

CCMP strain	Species	Collection location (very approximate)	Ocean	Isolated from	Clade
373	*E. huxleyi*	Sargasso Sea	North Atlantic	Sea water	-
374	*E. huxleyi*	Gulf of ME, USA	North Atlantic	Sea water	-
421	*S. microadriaticum*	Wellington, NZ	South Pacific	Sea water	E
828	*S. microadriaticum*	Florida Keys, FL, USA	North Atlantic	Sea water	A
829	*S. microadriaticum*	Great Barrier Reef, Australia	South Pacific	*Tridacna crocea* (bivalve)	A
830	*S. microadriaticum*	Bermuda Biological Station, Bermuda	North Atlantic	*Aiptasia pallida* (sea anemone)	B
1633	*S. microadriaticum*	Hawaii, USA	North Pacific	*Aiptasia puchella* (sea anemone)	B

Table 2.5.2 – DMSP and DLA.

(Table 2 in text). *Symbiodinium microadriaticum* and *Emiliania huxleyi*. Comparison of $DMSP_p$ and $DMSP_t$ (particulate and total dimethylsulfoniopropionate; fmol cell^{-1}) and DMSP lyase potential activity (DLA; nmol min^{-1} fmol DMSP^{-1}; nmol min^{-1} µg chl *a*$^{-1}$) in cultured strains of *E. huxleyi* (CCMP 373 and 374) and *S. microadriaticum* (CCMP 421, 828, 829, 830 and 1633). Bacterial DLA in *S. microadriaticum* is also shown. Averages ± SD are presented. Sample number is indicated in parentheses. ND: not detected; -: not tested.

CCMP strain	$DMSP_p$ (fmol cell^{-1})	$DMSP_t$ (fmol cell^{-1})	DLA:$DMSP_p$ (nmol·min^{-1}·fmol DMSP^{-1})	DLA:Chl a (nmol·min^{-1}·µg^{-1})	Bacterial DLA (% of total DLA)
373	7.6 ± 2.3 (5)	-	0.1 ± 0.01 (5)	0.2 ± 0.1 (4)	-
374	4.7 ± 2.1 (6)	-	0.007 ± 0.0002 (2)	0.006 ± 0.005 (2)	-
421	201.0 ± 138.9 (3)	199.0 ± 151.4 (3)	3.2 ± 2.7 (5)	0.6 ± 0.5 (5)	15.2 ± 6.7 (4)
828	122.6 ± 60.7 (3)	105.5 ± 51.6 (4)	4.7 ± 1.6 (4)	0.8 ± 0.3 (4)	1.4 ± 0.8 (3)
829	81.4 ± 42.9 (3)	85.4 ± 38.4 (4)	27.3 ± 16.7 (5)	5.3 ± 0.9 (5)	2.2 ± 0.5 (3)
830	33.8 (1)	43.0 ± 14.5 (4)	ND (4)	ND (4)	-
1633	329.9 ± 193.2 (3)	347.9 ± 201.9 (4)	6.5 ± 2.7 (4)	0.7 ± 0.4 (4)	1.0 (1)

2.6 Figures

Figure 2.6.1 – DLA rates.

(Figure 1 in text). *Symbiodinium microadriaticum* and *Emiliania huxleyi*. Dimethylsulfide (DMS; nmol) versus time (min) for (A) *E. huxleyi* strain CCMP 373, n = 4, and the following strains of *S. microadriaticum*: (B) CCMP 421, n = 5; (C) CCMP 828, n = 4; (D) CCMP 829, n = 5; (E) CCMP 830, n = 4; and (F) CCMP 1633, n = 4. Arrows indicate addition of dimethylsulfoniopropionate (DMSP) at t_0. Note different y-axis scales. Symbols indicate averages ± SE.

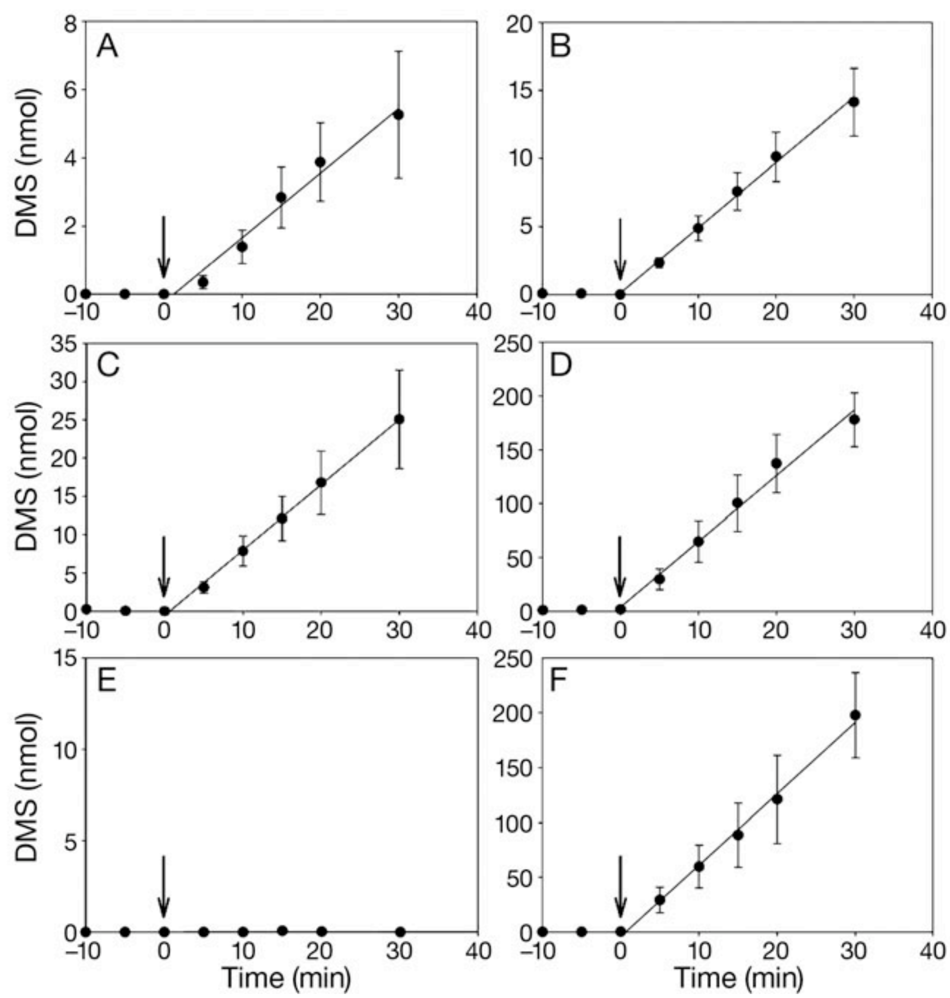
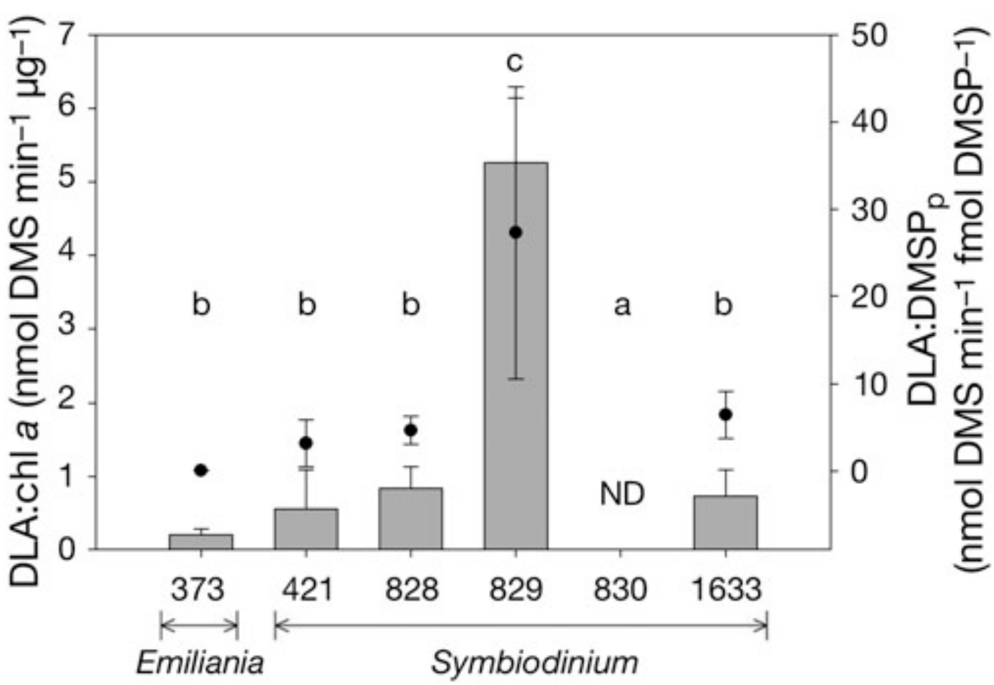


Figure 2.6.2 – DLA:chl *a* and DLA:DMSP$_p$.

(Figure 2 in text). *Symbiodinium microadriaticum* and *Emiliania huxleyi*. Dimethylsulfoniopropionate (DMSP) lyase activity (DLA):chlorophyll *a* (chl *a*) (nmol min^{-1} μg^{-1}; shaded bars) and DLA:DMSP$_p$ (particulate DMSP) (nmol DMS min^{-1} fmol DMSP^{-1}; ●) means for each strain. Error bars are SD; different letters indicate significant differences (ANOVA, $p < 0.01$) and apply to both data sets; ND: not detected. DMS:chl *a* - CCMP 373, n = 4; CCMP 421, n = 5; CCMP 828, n = 4; CCMP 829, n = 5; CCMP 830, n = 4; and 1633, n = 4. Note: CCMP 374 is not depicted (see 'Results').



Chapter 3: Substrate kinetics of DMSP-lyases in various cultured *Symbiodinium* strains

This chapter has been submitted to the Journal of Phycology

3.1 Introduction

Symbiotic dinoflagellates of the genus *Symbiodinium* (Freudenthal) are the predominant marine symbionts in multiple classes of marine animals and in nearly all shallow-water reef corals (Trench, 1979; Pawlowski et al., 2001). Also known as "zooxanthellae", these endosymbionts produce the algal metabolite dimethylsulfoniopropionate (DMSP), a sulfur compound shown to have multiple functions in marine algae (Havill et al., 1985; Kirst, 1990; Karsten et al., 1996; Wolfe et al., 1997; Sunda et al., 2002; Evans et al., 2006; DeBose et al., 2008). Zooxanthellae also have DMSP-lyase capabilities that enable them to enzymatically convert DMSP to the volatile gas dimethylsulfide (DMS) and other products (Yost and Mitchelmore, 2009). Recent reports highlighting the potential antioxidant role of the DMSP/DMSP-lyase system in marine algae (Sunda et al., 2002) have spurred DMSP-related investigations in corals.

DMSP cleaving enzymes are known to exist in several species of marine phytoplankton (Stefels et al., 2007; Niki et al., 2000; Steinke et al., 1998, 2002a, b; Wolfe et al., 2002), bacteria (de Souza and Yoch, 1995a, b; van der Maarel et al., 1996; González et al., 1999; Yoch, 2002) and macroalgae (Van Alstyne and Houser, 2003). Although the particular enzymatic reactions that govern DMSP breakdown are poorly characterized, most are thought to be DMSP-lyases, and for simplicity we will refer to them as DMSP-lyases in the present paper (Stefels et al., 2007; Schäfer et al., 2010). DMSP-lyase activities in cultured zooxanthellae vary within and across strains suggesting that the turnover of DMSP and its potential function(s) within coral symbioses may depend, in part, on algal phylotype (Yost and Mitchelmore, 2009). Also, there is no apparent link between DMSP-lyase activity and intracellular DMSP concentrations in most of the zooxanthellae tested to date, an observation made for other marine algae including Prymnesiophyceae species (Niki et al., 2000; Yost and Mitchelmore, 2009).

Oxidative stress appears to be a key factor linking increases in ocean temperature, solar radiation and anthropogenic stressors to coral bleaching (stress related loss of symbiotic algae and associated decrease in algal photosynthetic pigments) (Brown, 1997; Glynn, 1991, 1993; Lesser, 1996; Hoegh-Guldberg and Smith, 1989; Dunne and Brown, 2001; Shick et al., 1996; Brown, 2000; Owen et al., 2005; Schiedek et al., 2007). DMSP acts as an antioxidant by reacting with highly toxic hydroxyl radicals (Sunda et al., 2002). The enzymatic lysis of DMSP to DMS and acrylate can greatly increase antioxidant protection because DMS and acrylate are 60 and 20 times more reactive toward hydroxyl radicals than DMSP itself. In addition, the stepwise oxidation of DMS and DMSP by hydroxyl radicals produces dimethyl sulfoxide (DMSO) and methane sulfinic acid, which are also highly reactive with hydroxyl radicals, producing an antioxidant cascade effect (Sunda et al., 2002). DMSP and its reaction products can potentially relieve oxidative stress in multiple cellular compartments (Sunda et al., 2002). Also, DMS and DMSO can neutralize lipid peroxidation in photosynthetic membranes and harmful radicals in chloroplasts, respectively, thus protecting important reactive oxygen species (ROS) target sites (Lee and de Mora, 1999b). High intracellular concentrations of DMSP and activities of DMSP-lyases (Yost and Mitchelmore 2009) underscore a potential antioxidant role for DMSP/DMSP-lyase in zooxanthellae. The complexities of the DMSP/DMSP-lyase system are not well characterized in marine algae and there is considerable variation in DMSP levels and DMSP-lyase potential activities within different *Symbiodinium* strains (Yost and Mitchelmore, 2009). Consequently, an examination of potential differences in the kinetic parameters of DMSP-lyase enzymes in *Symbiodinium* is warranted.

Most of the DMSP-lyase literature is focused on cultured phytoplankton species, natural microbial assemblages, seaweeds, and cultured bacteria. These studies indicate a wide range of V_{max} (maximal velocity of DMS production) and K_m (half-saturation constant) values (Stefels et al., 2007), as determined from Michaelis-Menten kinetic analyses of measured enzymatic production rates of DMS as a function of DMSP (substrate) concentration. The intricate, endosymbiotic nature of coral symbioses presents a unique challenge regarding the study of DMSP-lyases, making the use of cultured zooxanthellae optimal for enzyme kinetic studies.

To investigate DMSP-lyases in zooxanthellae, we used five strains of cultured *Symbiodinium* to determine optimal assay parameters (buffer strength, pH and temperature). Enzyme kinetic properties were determined for both CCMP 829 and

CCMP1633, zooxanthellae strains isolated from different host species (the clam *Tridacna crocea* and the sea anemone *Aiptasia puchella*, respectively). These strains were chosen as they differ in their algal classification (genetic phylotype) and have been previously shown to differ in their intracellular DMSP concentrations and DMSP-lyase activities (Yost and Mitchelmore, 2009).

3.2 Materials and Methods

3.2.1 Culture growth conditions

Symbiodinium microadriaticum were purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbor, USA) and included strains CCMP 421, 828, 829, 830 and 1633. The cultures were maintained using sterile techniques and were grown in their optimal growth media at pH 8.0 (K medium for CCMP 828, 829 and 830 and L1 medium for CCMP 421 and 1633) (Bigelow Laboratory, Maine). *Symbiodinium* cultures were not treated with antibiotic to obtain axenicity as it negatively affected growth in previous experiments (Yost and Mitchelmore, 2009). Algal cultures were grown at 26 °C with a 12:12 h light:dark cycle, without agitation (Rogers & Davis, 2006). Each strain was grown in semi-continuous batch culture in 100 mL conical flasks with 50 mL of culture in each, under light (100 $\mu E\ m^{-2}\ s^{-1}$) from cool-white fluorescent bulbs. All cultures were sampled one to three hours after the beginning of the light period. Each of the strain replicates was grown separately and analyzed individually.

3.2.2 Measurements of algal parameters

For each sampling, cultures were gently mixed to prepare a homogeneous suspension of cells (Rogers and Davis, 2006). Several culture aliquots per strain were taken for measurement of cell concentrations, mean cell sizes, and chlorophyll-*a* (chl-*a*) concentrations. To measure cell concentrations, algal cells were diluted with sterile artificial seawater and immediately counted by haemocytometer using an epifluorescence microscope (samples were preserved in 5% buffered formalin). Ten replicate counts per sample were averaged and corrected for dilution to calculate the total number of algal cells per mL of culture. For chl-*a* analysis, one mL culture aliquots were filtered through Whatman GF/F filters and extracted in 90% acetone for 24 h at 4 °C (Parsons et al., 1984). To calculate cell volume, algal cell dimensions were determined using a microscope and eyepiece graticule (samples were preserved in 5% buffered formalin; n = 30 per sample). Phylotype (strain) analysis of zooxanthellae was determined by length heteroplasmy in domain V of the chloroplast large subunit (cp23S)-rDNA (Santos et al., 2003).

3.2.3 Measurements of DMSP and DMS production in DMSP-lyase assays

Particulate DMSP ($DMSP_{p;}$ algae only), total DMSP ($DMSP_t$; whole culture), and DMSP-lyase potential activity measurements were conducted in 6 mL septum vials, each with a final liquid volume of 2 mL. For enzyme assays, DMS production was assumed to be equal to DMSP-lyase activity. $DMSP_t$ was determined by mixing 1 mL of culture with 1 mL of 10 N NaOH; $DMSP_p$ was determined by filtering 1 mL of culture through a GF/F filter and immediately placing the filter in 2 mL of 5 N

NaOH. After sample addition the septum vials were immediately sealed and incubated in the dark for 24 h to allow complete base hydrolysis of DMSP to DMS. The released DMS was analyzed with a Hewlett-Packard 5890 Series II gas chromatograph (GC) fitted with a Chromosil 330 packed column (Supelco, Bellefonte, PA), and flame photometric detector (FPD). System temperature settings were: injector 150 °C, column oven 60 °C and detector 175 °C. Nitrogen gas was the carrier (60 cm^3 min^{-1}) and air (60 cm^3 min^{-1}) and hydrogen (50 cm^3 min^{-1}) were the flame gases. Data was collected and analyzed using HP ChemStation (Hewlett – Packard, Palo Alto, CA). Quantifications were made by headspace analysis of DMS following DMSP conversion to DMS by alkaline hydrolysis. Known concentrations of DMSP (purchased from Research Plus Inc., Bayonne, NJ) were diluted in sterile water to give standard solutions, which were frozen in small aliquots at -80 °C. Multiple standard curves were constructed using serial dilutions of DMSP to ensure that the sample analysis was directly calibrated by the standard curve preparations. The square-root of the peak area was regressed against DMS(P) concentration to generate linear calibration curves. The DMSP standards were prepared in identical proportions of buffers and prepared solutions to those used in experimental samples. The precision of DMS analysis varied <5% and headspace storage trials showed no losses occurred with the analytical methods employed. Detection limit of the GC was 1 nmol DMS.

3.2.4 Optimization experiments

Our *in vivo* DMSP-lyase assay methods measure *potential* lyase activity and were modified from procedures described by Harada et al. (2004) and Steinke et al. (2000); see also Yost and Mitchelmore (2009). Briefly, algal cells (retained on a GF/F filter after gentle gravity filtration) were placed in Tris buffer to permeabilize the algal cells and allow external DMSP to diffuse into the cells. The septum vials containing the buffer and cells was then sealed and the production rate of DMS was measured by gas chromatography prior to and after the exogenous addition of DMSP. To determine the optimal temperature for enzyme reaction, DMSP lyase activity measurements were made at temperatures of 26, 28, 30, 32 and 37 °C. Temperatures varied little (± 1 °C) in each experimental vial throughout the experiments. To determine the optimal strength of buffer for enzyme activity measurements, we used 1X Tris buffer (containing 200 mmol L^{-1} Tris and 500 mmol L^{-1} NaCl) and two other buffers containing 2 times and 2.6 times the 1X buffer concentration of Tris and NaCl. To optimize pH, we examined enzyme activity in 1X Tris buffer at pH 7.0 or 8.0. PH values measured in assay media before and just after each enzyme activity analysis showed minimal variation (± 0.02). The temperature in these latter two experiments was 30 °C and DMSP had a final concentration of 5 mM in 2 mL of buffer in all optimization experiments. Controls consisting of GF/F filtered abiotic media were run in tandem with the experimental samples, and the control values were subtracted from the assay values for algal cells.

3.2.5 Enzyme kinetics experiments

Cultures of CCMP 829 and 1633 were inoculated from stationary phase cultures into fresh media at an initial density of approximately 10^5 cells mL^{-1}. Culture growth was then monitored on days 1, 3, 5, 7, 10 and 12. To ensure sufficient DMS evolution from culture samples (based on the limitations of our analytical system), 2 mL of algal culture was gently filtered (GF/F; gravity) and the filters were placed immediately into 1X Tris buffer at pH 8 in septum vials. The vials were sealed and incubated at 28 °C near the optimum temperature for enzyme kinetic rates. All headspace vials were incubated in the dark for the duration of the experiment, including 20 mins prior to the addition of DMSP. Headspace vials were sampled just before DMSP addition and every 20 min thereafter for an hour. DMSP substrate concentrations were 0.2, 1, 2.5 and 5 mM final concentration in 2 mL of buffer. There were 6 replicates per strain for each substrate concentration and for controls. DMSP-lyase enzyme kinetic properties were determined on day 7 and day 10 for each algal strain batch culture. Maximum velocities (V_{max}) and half saturation constants (K_m) of lyase enzymes were calculated using Lineweaver-Burk, linearized inverse plots of the Michaelis Menten equation.

3.2.6 Statistical analyses

All data were checked for normality and homogeneity of variances prior to statistical analysis and data were transformed as necessary. Analysis of variance (ANOVA) with Tukey's post-hoc tests was used to identify significant differences between buffer strengths, buffer temperatures, $DMSP_p$ and $DMSP_t$ concentrations within algal strains. T-tests were used to compare pH differences. Enzyme kinetics data were analyzed using regression and ANOVA to identify significant differences between days 7 and 10 within algal strains. T-tests were used to compare differences in V_{max} within and between strain types for days 7 and 10. All statistical analyses were conducted using Minitab© v. 10 (Minitab Inc. 2000), with $\alpha = 0.05$ for all tests.

3.3 Results

3.3.1 Optimization experiments

Zooxanthellae (*Symbiodinium*) cultures incubated in 1X Tris buffer at pH 8 (200 mmol L^{-1} Tris and 500 mmol L^{-1} NaCl) showed the highest enzymatic activity levels compared to those in 2X or 2.6X Tris buffer or culture media alone (ANOVA $P < 0.05$) (Fig. 1a, media results are not shown in panel a as all values were < 2.5 % of 1X Tris buffer values.). Enzymatic activity levels were also highest for cultures in Tris buffer at pH 8 compared to pH 7. Average percentage activities at pH 7 relative to those at pH 8 were 3.6% for CCMP 421, 25.8% for CCMP 829, and 11.3% for CCMP 1633 (t-tests $P < 0.05$) (Fig. 1b). In temperature effect experiments, DMSP-lyase activity of *Symbiodinium* cultures increased with increasing temperature up to 30-32 °C, but activity declined at the highest temperature (37 °C) (Fig. 1c).

3.3.2 Enzyme kinetics experiments

Mean volume per cell declined through day 7 and increased thereafter (Fig. 2a). On a cell volume basis, chl-*a* (g $L_{\text{cell volume}}^{-1}$) remained relatively stable through day 7 and increased in strain 1633 from day 7 to 10 (Fig. 2b). Mean algal cell number and chl-*a* per L of algal culture (μg $L_{culture}^{-1}$) increased through day 12, but specific rates of increase declined substantially after day 7 due to the onset of nutrient or CO_2 limitation of growth in the batch cultures (Fig. 3).

The enzyme kinetic properties for DMSP-lyases in CCMP 829 and CCMP 1633 were investigated on days 7 and 10 in 1X Tris buffer at pH 8 and 28 °C. Substrate enzyme kinetic experiments showed maximum DMSP-lyase activity at DMSP concentrations ≥ 1mM (Fig. 4, Table 1). The V_{max} for strain CCMP 829 increased 3-fold from day 7 to day 10 with a K_m increase of 0.063 to 0.124 mM during the same period (Fig. 4a, Table 1). By comparison, CCMP 1633 V_{max} levels were only slightly elevated on day 10 versus day 7 while the K_m declined by half, from 0.043 to 0.020 between day 7 and day 10 (Fig. 4b, Table 1). Regression analyses of linearized Lineweaver–Burk plots were significant for both CCMP 829 (day 10) and CCMP 1633 (day 7) (ANOVA $P < 0.05$). No statistically significant differences were detected among K_m values for strains CCMP 829 and 1633 on days 7 and 10 (ANOVA $P > 0.05$). However, V_{max} values were significantly different between the two strains for days 7 and 10 (t-test $P < 0.01$) and between days 7 and 10 for strain CCMP 829 (t-test $P < 0.01$) and CCMP 1633 (t-test $P < 0.05$).

Intracellular DMSP concentrations decreased from day one through day 7 for both strains and then increased thereafter with the decrease in specific growth rate of the algae. For, strain CCMP 829, these variations in cellular DMSP were not statistically significant, however, an increase in total culture DMSP per unit of cell volume ($DMSP_t$) from day 7 to day 10 and a subsequent decrease in $DMSP_t$ from day 10 to day 12 were statistically significant (ANOVA $P < 0.001$) (Fig. 5a). For CCMP 1633 the cellular DMSP concentration was significantly higher on day one than on days 7 and 10, but the small increases in $DMSP_p$ and $DMSP_t$ per liter of cell volume from day 7 through 12 were not statistically significant (ANOVA $P > 0.05$) (Fig. 5b).

3.4 Discussion

In all *Symbiodinium* strains tested, DMSP-lyase activities were substantially higher at pH 8 (the pH of seawater) than at pH 7, consistent with the reported intracellular pH of 7.62 in zooxanthellae (Leggat et al., 1999). Although the specific location of DMSP-lyases is unknown in zooxanthellae, both isozyme types have been demonstrated in *Emiliania huxleyi* and *Phaeocystis* (Steinke et al., 1998) and intracellular and extracellular lyase locations are not mutually exclusive. However, the very low lyase activity of cells placed in seawater relative to that for cells placed in Tris buffer suggests an intracellular location for the lyase enzyme. The high concentration of Tris renders the cells permeable to DMSP, allowing DMSP to diffuse into the interior of cells (Harada et al. 2004), but this would not have occurred in the cells placed in seawater media alone. An intracellular and possibly chloroplastic location of DMSP-lyases has also been proposed in conjunction with the hypothesized antioxidant role of the DMSP/DMSP lyase system (Sunda et al., 2002). Multiple possible cellular and sub-cellular locations for DMSP-lyases in zooxanthellae could facilitate the efficient functioning of the DMSP/DMSP-lyase system in scavenging toxic free radicals.

Phytoplankton DMSP-lyase K_m values can vary greatly, ranging from micromolar to millimolar concentrations (for a review see Stefels et al., 2007). The half saturation constants (K_m) of DMSP-lyases in strains CCMP 829 and 1633 (range: 0.020 - 0.124 mM DMSP) are two orders of magnitude lower than those (8-21 mM) recently reported by Steinke et al. (2007) for two strains of the coccolithophore *Emiliania huxleyi*, indicating higher lyase affinities for DMSP in zooxanthellae. DMSP-lyases from other *Emiliania* and *Phaeocystis* strains show K_m values in the submillimolar to millimolar range (0.012 – 5.5 mM) (Stefels and van Boekel, 1993; Stefels and Dijkhuizen, 1996; Steinke et al., 1998). The differences in K_m values suggest the existence of many different DMSP-lyase enzymes with different substrate affinities.

We observed up to a three-fold increase in the V_{max} of the DMSP-lyase enzyme system between day 7 and day 10. These changes indicate that the enzymes are not constitutive, but respond to cellular cues or environmental variables in at least some cases. Similarly, Steinke et al. (2007) demonstrated that the *in vivo* DMSP-lyase activity in *E. huxleyi* varied within a 24 h period and the enzyme kinetic parameters for the zooxanthellae strains investigated here have been observed to change within a day (Yost, unpublished data). The ability of cells to alter rapidly the cellular content or affinity of DMSP-lyase enzymes may be a critical response under conditions of oxidative stress, where the conversion of DMSP into DMS and acrylate can greatly increase a cell's ability to scavenge ROS such as hydroxyl radicals and singlet oxygen (Sunda et al., 2002).

The observed three-fold increase in the V_{max} of DMSP-lyase in CCMP 829 may have occurred in response to conditions of oxidative stress. The increase in V_{max} occurred in conjunction with the onset of decreasing growth rate between days 7 and 10, which likely resulted from limitation by either nutrients or CO_2 with increasing cell density in the batch cultures (Bucciarelli and Sunda, 2003; Sunda et al., 2002, 2007). Indeed, increased DMS production and/or DMSP-lyase activity have been found to occur in response to CO_2 and nutrient limitation, which both cause oxidative stress in algae (Nguyen et al., 1988; Sunda et al., 2002, 2007). In CCMP 829, particulate (i.e., cellular) and total DMSP per unit of cell volume also increased in conjunction with the increased potential activity of DMSP-lyase enzymes from day 7 to 10, again consistent with an up-regulation of the DMSP/DMSP lyase enzyme system with the onset of potential oxidative stress effects associated with either CO_2 or nutrient limitation. CCMP 1633 showed a similar response, but there was only a slight increase in cellular DMSP and DMSP-lyase potential activity from day 7 to 10, a response that could become more pronounced with increased levels of CO_2 or nutrient limitation. Such temporal patterns have also been seen for DMS to cell volume ratios in batch cultures of *E. huxleyi* under nitrogen limitation (Sunda et al., 2007) and in cellular DMSP concentrations in *Thalassiosira pseudonana* under nitrogen, phosphorus, silicon, and CO_2 limitation (Bucciarelli and Sunda, 2003). The reported $DMSP_p$ values in the current study are approximately an order of magnitude to several fold less than those reported previously for these strains (CCMP 829, 127 mmol $L_{cell\ volume}^{-1}$; CCMP 1633, 686 mmol $L_{cell\ volume}^{-1}$; Yost and Mitchelmore, 2009).

As demonstrated herein, cell volume changes over the growth phase and DMSP-lyase activity impact $DMSP_p$ concentrations and may in part explain the observed differences. In the current study, cultured algae were sampled more frequently and over a larger portion of their growth cycle and had higher cell densities (~days 5-12) and larger cell sizes (all days) compared to our previous study.

The growth of zooxanthellae is known to be nitrogen limited *in hospite* (Cook et al., 1988; Muscatine et al., 1989; Falkowski et al., 1993), a condition that may be exacerbated under environmentally stressful conditions. Nitrogen limitation can act as an oxidative co-stressor and is known to significantly increase the inhibition of photosynthesis by UV-radiation in dinoflagellates (Litchman et al., 2002). In addition, both DMSP and DMS have been proposed to function as an overflow mechanism for excess photosynthetically produced reducing equivalents (NADPH) and ATP during conditions of unbalanced growth, which further protects cells from oxidative stress (Stefels, 2000). Thus, during periods of nutrient limitation, increased DMSP production and/or DMSP-lyase activity could potentially protect cells from oxidative stress both by limiting ROS production and by scavenging ROS once they are formed (Stefels, 2000; Sunda and Hardison, 2008). Previous reports suggest that DMSP may have a role in stress resistance in nitrogen-poor, sulfate-rich environments such as nutrient poor seawater (Hanson and Burnet, 1994; Le Raudulier et al., 1996). Zooxanthellae produce several nitrogen-containing antioxidant enzymes (including ascorbate peroxidase, superoxide dismutase, catalase and glutathione reductase; for a review see Lesser, 2006). Thus, the production of DMSP from methionine under nitrogen-limited conditions could provide nitrogen-free antioxidants (Sunda et al., 2007) in nitrogen-poor marine environments.

Several lines of evidence suggest that in addition to protection from oxidative stress, the DMSP/DMSP-lyase system may also function as an activated chemical defense mechanism against grazing in phytoplankton (Wolfe et al., 1997; Wolfe, 2000; Fredrickson and Strom, 2009) and macroalgae (Van Alstyne et al., 2001). We note that grazing deterrent mechanisms are often substantially up-regulated under nutrient limitation both in individual species (Graneli and Johansson, 2003; Sunda et al., 2006) and in natural phytoplankton communities (Strom and Fredrickson, 2008). Thus the increased V_{max} of DMSP-lyases and increased DMSP to cell volume ratios under nutrient or CO_2 limitation in strain CCMP 829 could also reflect an up-regulation of antigrazing protection. The potential antigrazing function of the DMSP/DMSP-lyase system, however, awaits further investigation in zooxanthellae and intact coral symbioses.

It appears that nutrient limitation and high solar UV exposure select for phytoplankton species with high DMSP concentrations and activities of DMSP-lyase enzymes in thermally stratified ocean waters (Sunda et al., 2007). Though this may explain in part why zooxanthellae have high intracellular concentrations of DMSP and high DMSP-lyase potential activities, the complete understanding of how corals fit into the larger sulfur cycle remains enigmatic. In corals, bleached colonies of *Acropora formosa* show significantly greater DMSP concentrations per zooxanthellae

(436 fmol cell^{-1}) than unbleached colonies (171 fmol cell^{-1}) suggesting that coral reefs are potentially significant sources of DMS, especially when corals are faced with environmental stresses (Broadbent et al., 2002). Jones et al. (2007) reported significant DMS and DMSP correlations with sea surface temperatures up to 30 °C and Sunda et al. (2005) postulated that an observed increase in DMSP:chl-*a* in dinoflagellates represented a physiological increase in intracellular DMSP in response to solar-induced oxidative stress. If DMSP has an antioxidant function in zooxanthellae, one might expect a variety of complex responses typical of other antioxidant systems that are dependent upon the type of stress and its duration (i.e. acute versus chronic exposures). For example, glutathione is a well-known cellular antioxidant and is often initially depleted but later elevated due to an up-regulation in its production in response to oxidative stress (Sies, 1999). Similar behavior may occur in the DMSP/DMSP lyase system with exposure to oxidative stress.

Our investigations of DMSP-lyases in cultured *Symbiodinium* provide a basis for further standardized enzymatic comparisons that could incorporate effects of additional environmental stressors and an expanded spectrum of symbionts. The increased incidence and severity of coral bleaching events is predicted to continue (Hoegh-Guldberg, 1999; Sheppard, 2003) and further investigations of the DMSP/DMSP-lyase system are needed to elucidate the role(s) of this enzyme system in the health and survival of coral-symbioses. Both intracellular DMSP concentrations and DMSP-lyase activity vary among zooxanthellae algae (Keller et al., 1989; Broadbent et al., 2002; Broadbent and Jones, 2006; Hill et al., 1995; Van

Alstyne et al., 2006, 2009; Yost and Mitchelmore, 2009), highlighting the possibility for differential DMSP/DMSP-lyase responses to changing environmental conditions. With multiple environmental stressors influencing zooxanthellae physiology, the proposed antioxidant cascade stemming from the conversion of DMSP to DMS via DMSP-lyase (Sunda et al., 2002) may be of particular importance to coral symbioses, complimenting the other ROS scavengers of algae and host.

3.5 Table

Table 3.5.1 – *Symbiodinium* CCMP 829 and 1633 DMSP-lyase kinetic parameters.

(Table 1 in text). Kinetic parameters for DMSP-lyase activities in *Symbiodinium* strains CCMP 829 and CCMP 1633. Assay conditions were Tris buffer (200 mmol L^{-1} Tris and 500 mmol L^{-1} NaCl), pH 8 at 28 °C.

Symbiodinium strain	Day of experiment	Regression analysis (R^2; P; n)	K_m DMSP [mM]	V_{max} (nmol DMS $L_{cell\ volume}^{-1}$ min^{-1})
CCMP 829	7	0.71; >0.05; 6	0.063	188.8
	10	0.91; <0.05; 6	0.124	589.6
CCMP 1633	7	0.95; <0.05; 6	0.043	86.9
	10	0.89; >0.05; 6	0.020	97.1

3.6 Figures

Figure 3.6.1 – DMSP-lyase assay optimization.

(Figure 1a, b, c in text). Effect of Tris buffer concentration (a), pH (b), and temperature (c) on DMSP-lyase potential activity (nmol DMS $L_{cell\ volume}^{-1}$ min^{-1}) in cultured *Symbiodinium* strains. DMSP substrate addition was 5 mM (final concentration). Error bars are SE, n = 3, except for 37 °C in panel c where n = 1. Asterisks (*) indicate statistically significant differences between treatments. Results in culture media alone (no buffer) are not shown in panel (a) as all values were < 2.5 % of 1X Tris buffer values.

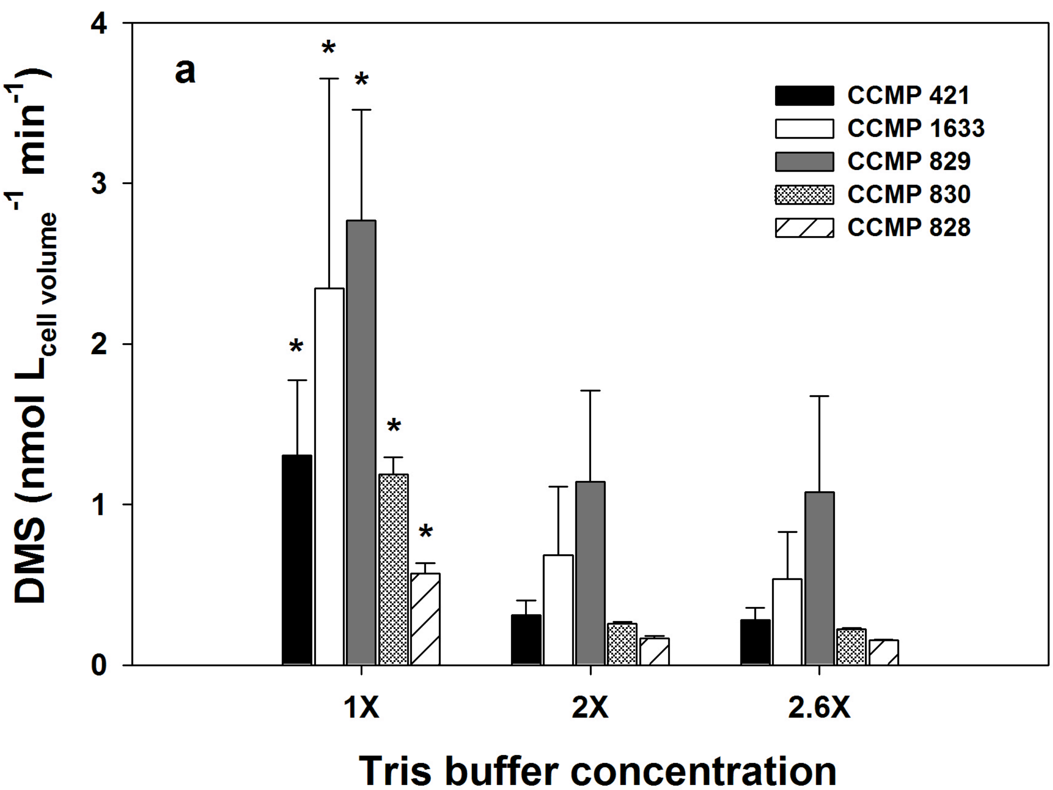


Figure 3.6.1 – continued.

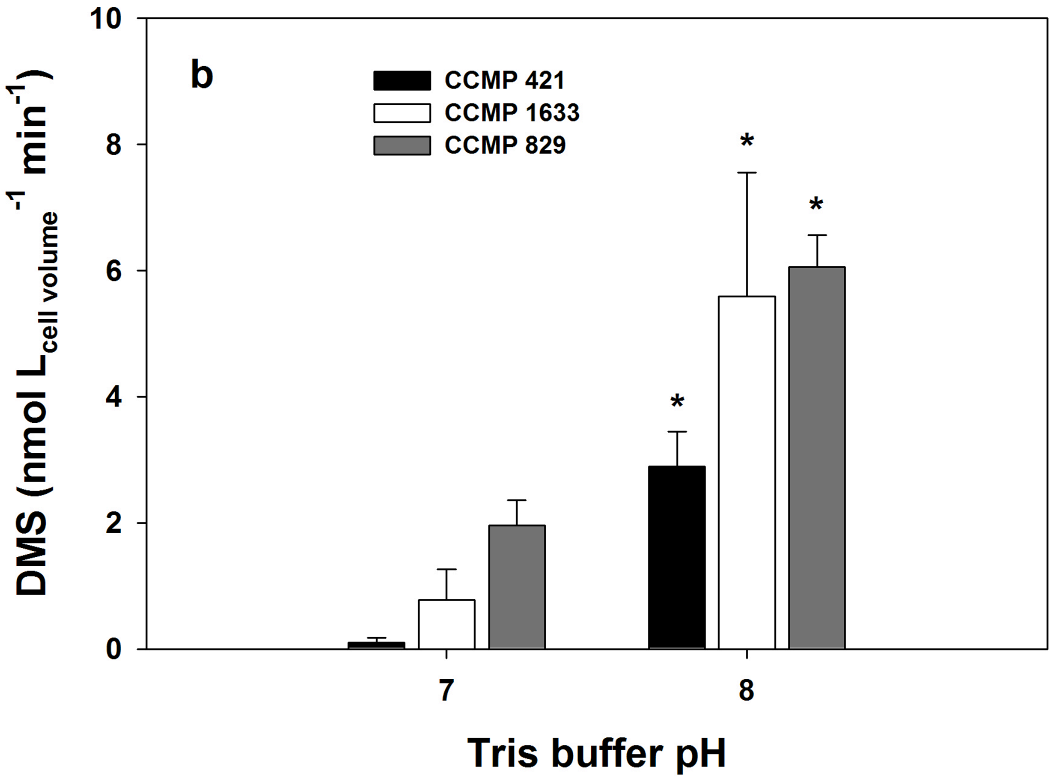


Figure 3.6.1 – continued.

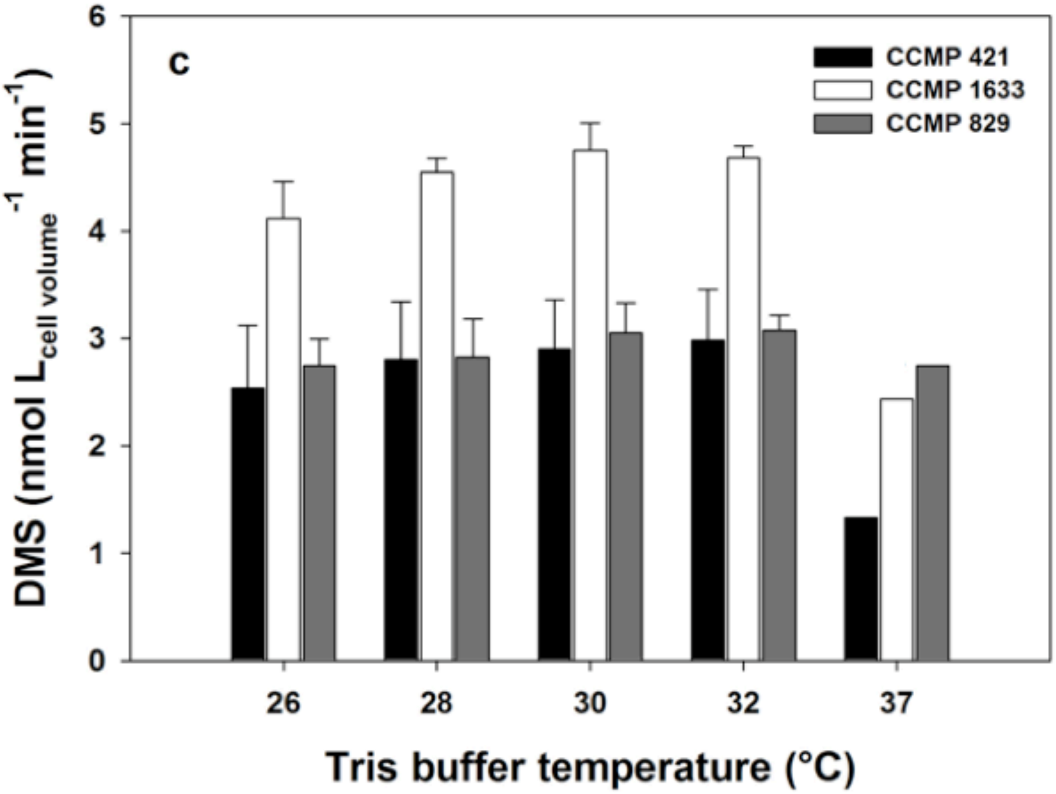


Figure 3.6.2 – *Symbiodinium* CCMP 829 and 1633 cell volume and chl-*a*.

(Figure 2a, b in text). Mean volume per cell (µL) (a) and cell chl-*a* (g $L_{cell\ volume}^{-1}$) (b) for *Symbiodinium* strains CCMP 829 and CCMP 1633. Vertical dashed lines indicate sampling days, 7 and 10. Error bars are SE (some error bars hidden by symbols), n = 6.

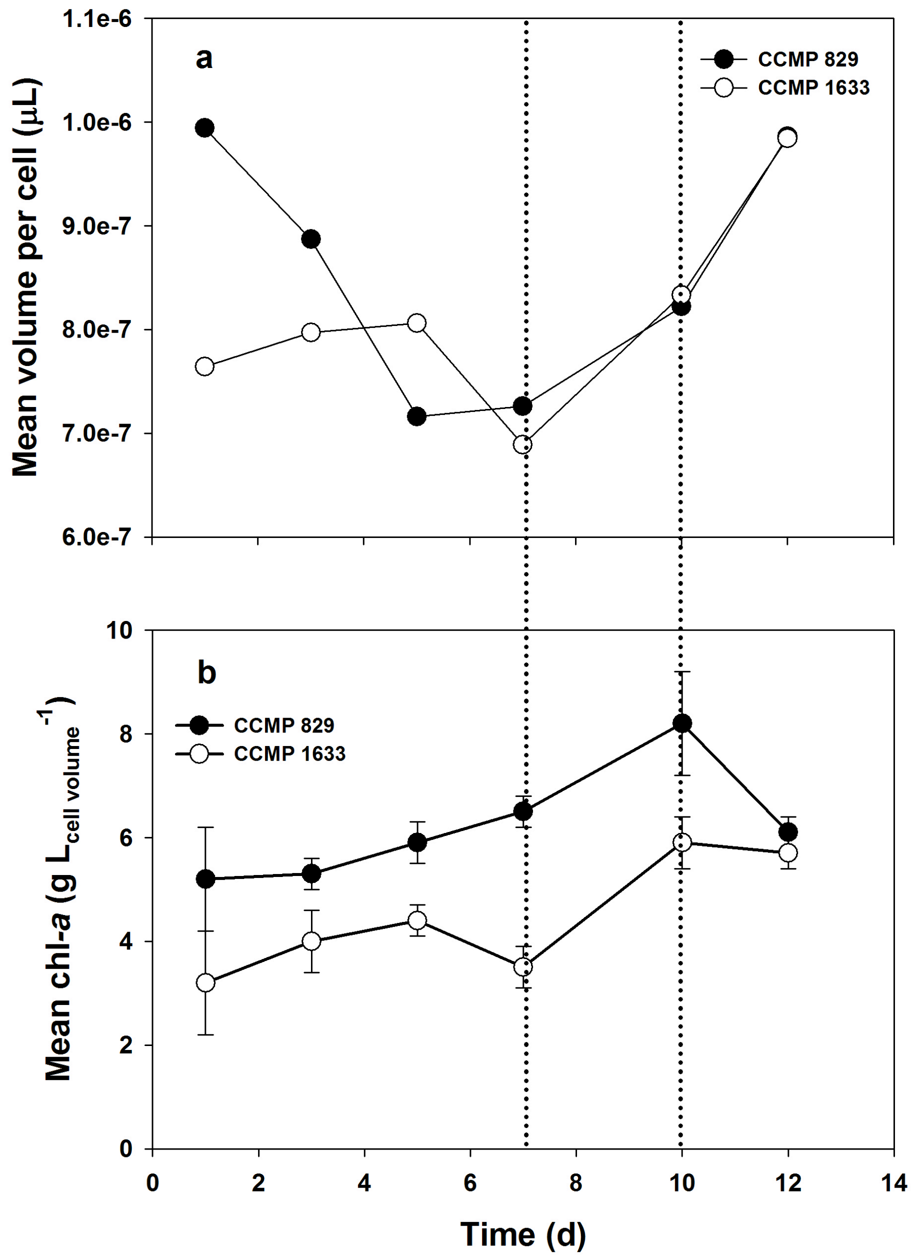


Figure 3.6.3 – *Symbiodinium* CCMP 829 and 1633 cell density and chl-*a*.

(Figure 3a, b in text). Concentration of cells (cells mL^{-1}) (a) and chl-*a* (μg L $culture^{-1}$) (b) for *Symbiodinium* strains CCMP 829 and CCMP 1633. Vertical dashed lines indicate sampling days, 7 and 10. Error bars are SE (some error bars hidden by symbols), n = 6.

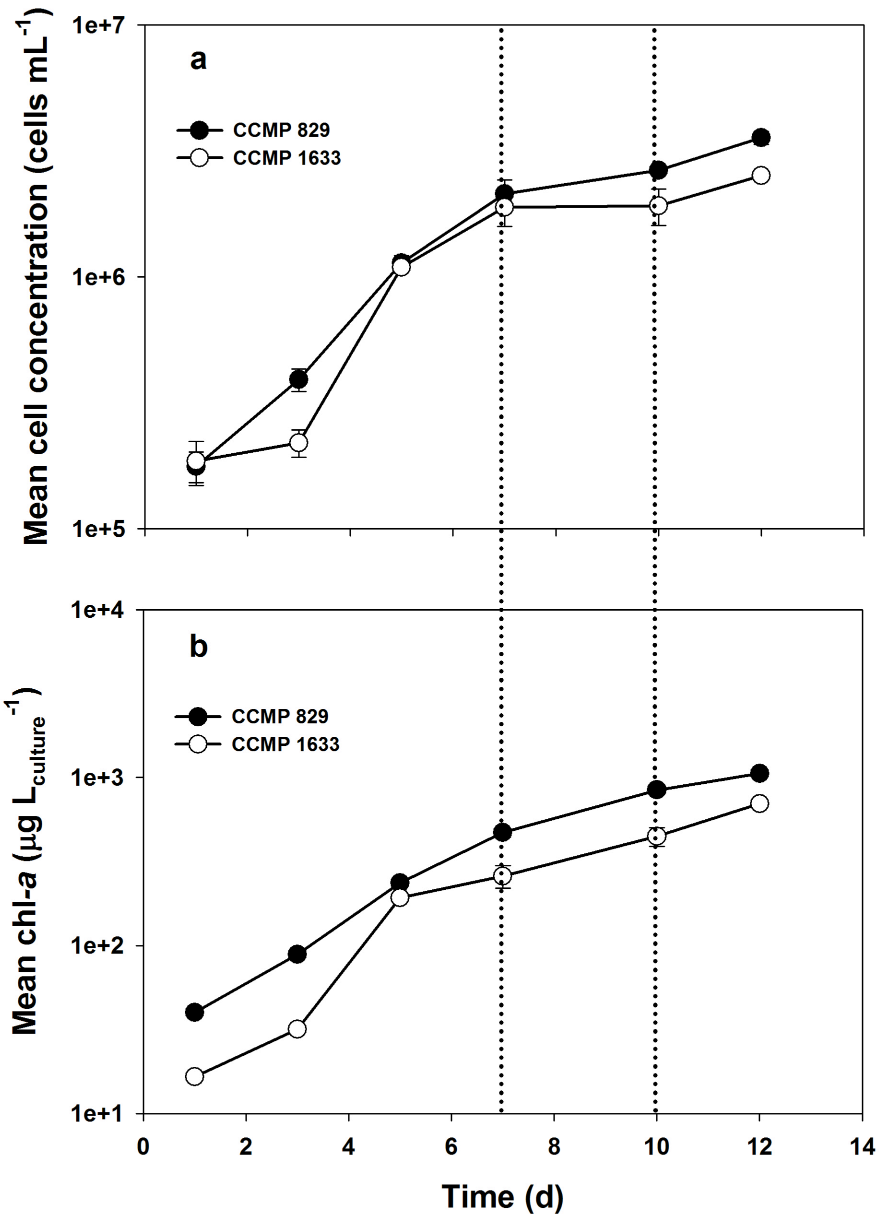


Figure 3.6.4 – *Symbiodinium* CCMP 829 and 1633 DMSP-lyase potential activity. (Figure 4a, b in text). DMSP-lyase potential activity (nmol DMS $L_{cell\ volume}^{-1}$ min^{-1}) (DLA) for *Symbiodinium* strains CCMP 829 (a) and CCMP 1633 (b) versus DMSP concentration for days 7 and 10. Error bars are SE, n = 6.

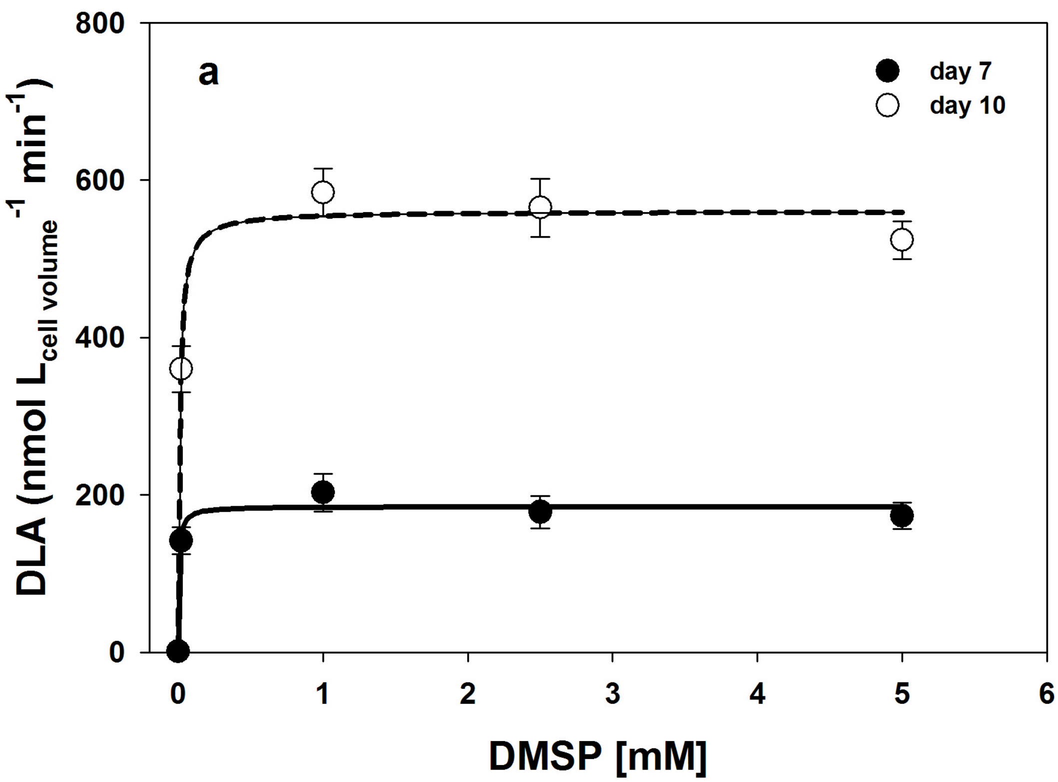


Figure 3.6.4 – continued.

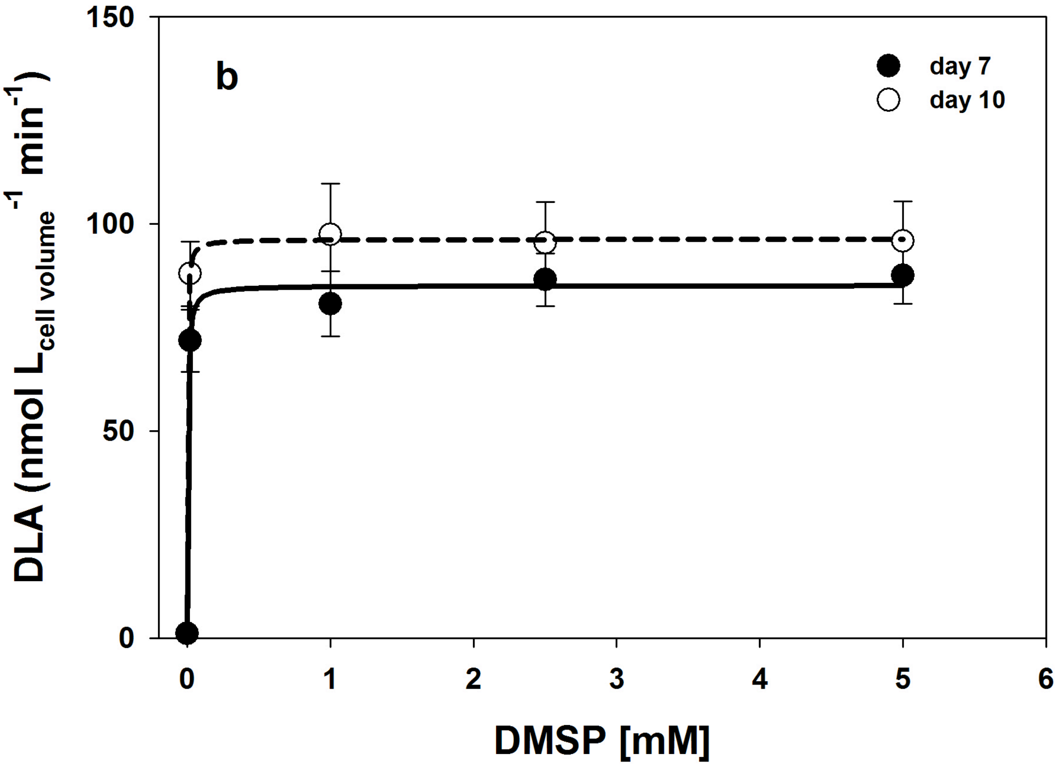


Figure 3.6.5 – *Symbiodinium* CCMP 829 and 1633 $DMSP_p$ and $DMSP_t$.

(Figure 5a, b in text). $DMSP_p$ and $DMSP_t$ concentrations normalized to cell volume (mmol L cell volume $^{-1}$) for *Symbiodinium* strains CCMP 829 (a) and CCMP 1633 (b) versus time (d). Error bars are SE, n = 6. Letters indicate significant statistical differences between days for $DMSP_p$ or $DMSP_t$ to cell volume ratios.

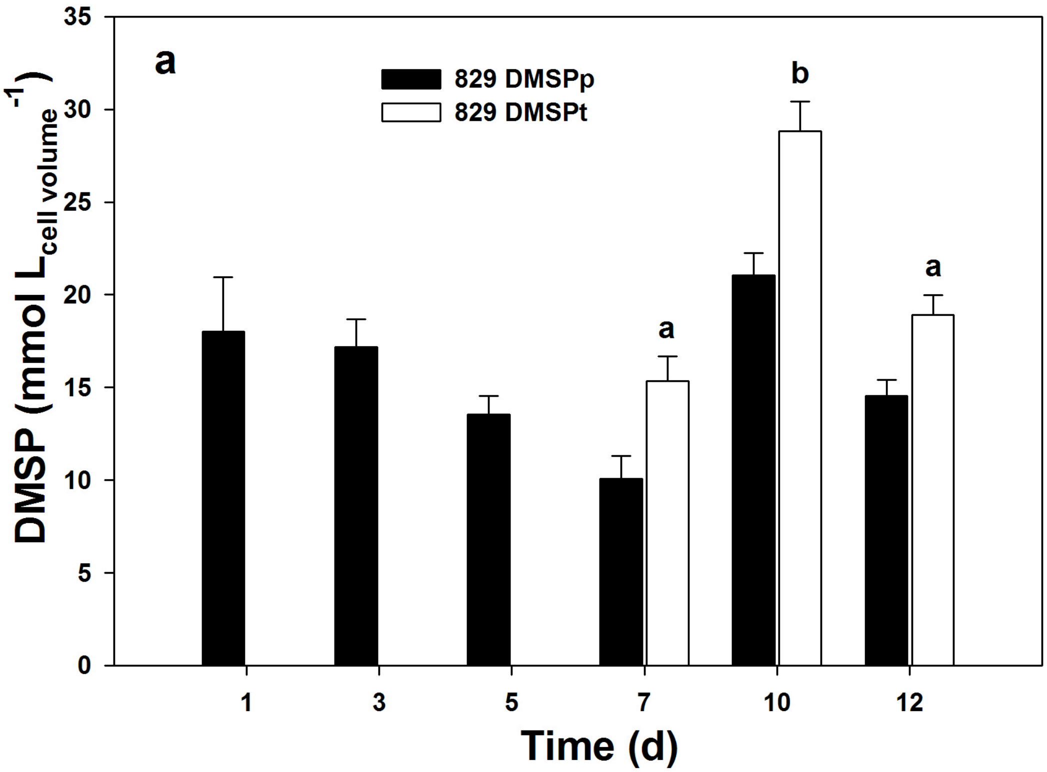
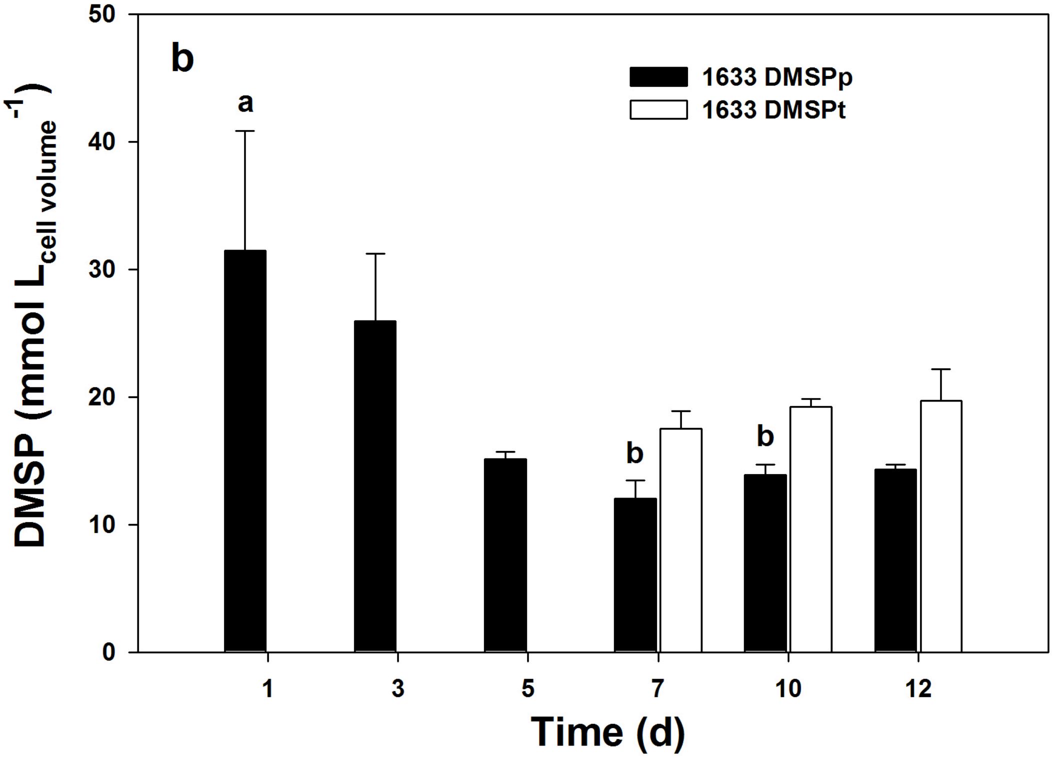


Figure 3.6.5 – continued.



Chapter 4: Determination of total and particulate DMSP concentrations in four scleractinian corals: a comparison of methods

This chapter has been submitted to the Journal of Experimental Marine Biology and Ecology

4.1 Introduction

Hermatypic corals form the foundation of some of the world's largest biological structures, coral reef ecosystems. Recent investigations indicate that reefs may impact local climate through the release of the volatile gas dimethylsulfide (DMS) (Jones and Trevena, 2005; Broadbent and Jones, 2006). Corals harbor dinoflagellate symbionts, commonly known as zooxanthellae, that produce the algal metabolite dimethylsulfoniopropionate (DMSP). DMSP may be cleaved enzymatically by DMSP-lyases, releasing DMS. DMSP-lyases are found in many but not all algal species (Niki et al., 2000; van Bergeijk and Stal, 2001; Sunda et al., 2002), and DMSP-lyase activity was recently quantified in cultured zooxanthellae (*Symbiodinium* spp., Yost and Mitchelmore, 2009). Different clades of zooxanthellae (*Symbiodinium* spp.) have been shown to differ in their DMSP-lyase activity and baseline levels of DMSP production and DMSP concentrations are highly variable among coral species (Hill et al., 1995; Broadbent et al., 2002; Van Alstyne et al., 2006; Yost and Mitchelmore, 2009). It remains unclear whether coral animals themselves produce DMSP, and DMSP-lyases have not been detected in the coral host (or any other animal species). Thus, the regulation and exact function(s) of DMSP and DMSP-lyases are not well characterized for any zooxanthellate-symbiosis to date, though the various functions of DMSP and its breakdown products probably play species- and population-specific roles (Van Alstyne and Puglisi, 2007).

Most DMSP research has focused on open ocean water column measurements of algal DMSP-producers (Kiene et al., 1996; Yoch, 2002). Methods used for such quantifications are rarely directly applicable to teasing apart an intact symbiosis as evidenced by the many methods used thus far for quantifying intracellular DMSP from isolated symbionts (zooxanthellae; $DMSP_p$ (particulate)) or from intact symbioses (coral and zooxanthellae; $DMSP_t$ (total)) (for a summary of published methods see Table 1). Non-standardized methods limit ones ability to index DMSP to relevant endpoints in a complex symbiosis and compare findings across studies. The diversity of methods may prevent cross-comparisons and obscure the inherent variability of DMSP production and turnover (via DMSP-lyases) within a given species and specific symbiotic partner. It may be methodologically simplest to report total DMSP values by simply placing an intact fragment into a liquid that dissociates the coral tissue from the skeleton. However, progress towards understanding the physiological responses of symbiont/host and the function(s) of DMSP in the symbiosis would require a partitioned investigation, especially in studies investigating potential DMSP changes due to stress. However, hard corals pose huge methodological challenges in attempts to achieve this. First, tissue must be separated from the skeleton and secondly, the symbiotic partners must be teased apart. For example, removing the coral tissue via airbrushing is one of the two main methods that have been used in coral DMSP research, although only $DMSP_t$ values were reported. We take this method a step further by separating the symbionts before analysis.

Choice of the appropriate DMSP normalization indice(s) (e.g. cell volume, chl-*a*) is still under debate in the DMSP literature. However, an often-overlooked additional aspect is that DMSP studies are further complicated by physiological normalization indices that show dynamic responses to stress, therefore, limiting the choices available (see Yost et al., 2010). Further complicating DMSP analyses in coral is the fact that coral tissue isolation procedures can preclude many of these normalization indices commonly used in the algal field.

Ideally, all DMSP analyses should be conducted on material freshly collected and immediately analyzed, however this is not typically feasible logistically. Even if corals are promptly evaluated, specific preparation methods can substantially impact the results (see Table 1). Sample preparation involves two main aspects: first, separation of living tissue from the coral skeleton (i.e. coral and algal isolation) and second, the choice of analytical technique and therefore sample manipulation via the use of chemicals (e.g. NaOH, MeOH, specified below). Typically, corals are either airbrushed or water-piked with seawater to obtain a homogenate, or are examined intact (i.e. directly placed in an extractant solution). Following sample isolation, one of two main preparation techniques has been used for DMSP analysis. Methanol has been used to extract and store DMSP (DMSP is methanol-soluble) samples and is usually followed by alkaline hydrolysis (with NaOH) before analysis. Alternatively, DMSP samples have been immediately placed in NaOH prior to analysis. Direct comparisons of DMSP methods have been reported in a few studies (see Table 1). However, while these reports use the two main tissue separation techniques (airbrushing or intact in extractant), they only include $DMSP_t$ values. Also, there are no comparisons to date for $DMSP_p$ or $DMSP_t$ methodologies from intact coral samples, limiting a full evaluation of DMSP responses in corals. Such evaluations are critical as both aspects of sample preparation (sample isolation and preparation technique) effectively limit which normalization indices (e.g. coral surface area, algal cell size) can be used to analyze data and draw conclusions (see for example Edmunds and Gates, 2002).

The few studies that have specifically quantified DMSP in coral symbioses are summarized in Table 1. Hill et al. (1995) quantified DMSP in Hawaiian corals, comparing fractions of homogenized tissue retained on a glass fiber filter with whole pieces of coral placed in methanol for extraction of DMSP. Results from this study indicated significant differences between the two methods, with DMSP values 1.4 - 2.8 times higher in methanol-extracted samples compared to those in the filtered extract. The authors attributed this difference primarily to the loss of DMSP due to tissue homogenization procedure and secondarily to the possible presence of endolithic algae, but noted also that this conclusion was the most parsimonious interpretation of their data. The apparent difference in DMSP values may also be attributed to the sample preparation, as their filtered samples would exclude DMSP in the host tissues, resulting in lower DMSP values (i.e. essentially $DMSP_p$ only). A direct comparison of the total homogenate with the intact fraction was not carried out. Despite such evident methodological differences, coral DMSP has since been quantified using coral tissue homogenates after removal from their calcium carbonate skeletons, as this allows for the measurement of multiple indices that are used to normalize DMSP concentrations (Broadbent et al., 2002).

DMSP method development is likely to continue as new questions and species are explored. Although some zooxanthellae are known for substantial DMSP production, the role(s) of DMSP in coral-algal symbioses remains obscure, highlighting the need for improved sampling methods for its quantification. This paper is aimed at investigating in detail the two major coral DMSP preparation methods. In addition, to address several known methodological hurdles, we focused on issues pertaining to the separation of coral and algae, from the skeleton using multiple DMSP extraction techniques, isolation of the symbiotic partners and several normalization indices in prominent reef building corals. Our efforts to investigate the preparation methods in detail were aimed at quantifying the potential loss of DMSP due to these methods. We also sought to determine if the airbrushing technique could be used for measuring DMSP in corals, as it allows for multiple normalization indices and thus more information compared to the intact fragment analyses. Several potential preparation methods for quantifying the partitioning of $DMSP_t$ and $DMSP_p$ were examined in four, field-collected species: *Montastraea cavernosa*, *Madracis mirabilis*, *Montastraea franksi* and *Porites asteroides*. These corals are significant reef builders and were also chosen for their variability in growth form (boulder-like, branching, massive and encrusting, respectively), their abundance in coral reef communities and their endosymbionts of varying clades.

4.2 Materials and Methods

4.2.1 Study site and coral handling

Corals were collected in Bermuda (Location: Three Hill Shoals (32° 25' 30.7", 064° 42' 19.0"); Depth: 3.3 ± 1.1 m; Date: October 26, 2007; Temperature: 24.4 °C) and kept in ambient seawater (up to 2 hours) until processing at the Bermuda Institute for Ocean Science (BIOS) station. All corals were doubly wrapped in aluminum foil, flash frozen in liquid nitrogen, shipped on dry ice and immediately stored at -80 °C until analysis of DMSP and associated indices, which were conducted at the Chesapeake Biological Laboratory. Corals were analyzed after storage at -80 °C because this type of handling was shown to be an appropriate technique in other cnidarians, where DMSP concentrations in the frozen cnidarian tissues did not statistically differ from the extracted, freshly collected cnidarian tissues (Hill et al., 2004; Van Alstyne et al., 2006). Additionally, storage at -80 °C allows for logistically feasible coral collection, limited coral handling, processing and preparation times and minimal DMSP loss during airbrushing (due to very small amounts of homogenate froth).

4.2.2 Summary of methodology

The experimental procedure used is summarized schematically in Figure 1. Coral sub-fragments were analyzed after removal of coral tissue from the skeleton (to form a homogenate) or after the intact sub-fragment was placed in absolute MeOH. Sub-fragments processed to obtain homogenates were further divided between 2 treatments: (1) absolute MeOH (for parallel comparison between airbrushing and intact sub-fragments) or (2) NaOH. Specific methods for DMSP and multiple indices follow below.

4.2.3 Intact coral sub-fragments

Whole coral sub-fragments were placed directly (completely submerged) in 4 mL absolute MeOH and equilibrated for 24 h. Subsequently, 1 mL of the MeOH extract was mixed with 1 mL 10 N NaOH in a headspace vial and equilibrated for an additional 24h for subsequent $DMSP_t$ analysis, following its base hydrolysis to DMS. Another aliquot of the MeOH extract was analyzed for chlorophyll-*a* (chl-*a*). Following MeOH extraction, coral sub-fragments were dried and measured for surface area (Marsh, 1970) and polyp number indices. Coral skeletons were devoid of tissue after submersion in MeOH indicating that extraction from coral sub-fragments was complete or nearly completed. There was no indication that saturation of MeOH in sample vials occurred based on the knowledge that much higher DMSP concentrations than were measured in the samples readily dissolve in MeOH (Yost pers. obs., Hill et al., 2000). No $DMSP_p$ or algal indices (i.e. cell number or volume), other than chl-*a* analysis, were conducted as *intact* sub-fragments preclude such quantifications. Intact fragment analysis not only limits the normalization indices that can be carried out, but also only determines total DMSP levels; no data on DMSP partitioning between the algae and host can be obtained.

4.2.4 Airbrushed coral sub-fragment

We used a slight modification of the coral homogenization technique as detailed in Broadbent et al. (2002), which was used previously to obtain DMSP and DMS quantities in corals, *Symbiodinium* spp. and macroalgae. Individual coral fragments were removed from -80 ºC freezer storage and immediately airbrushed (Szmant and Gassmann, 1990) using sterile artificial seawater to obtain a homogenate that was collected into a 0.5 L plastic bag. Total homogenate volumes were measured with a volumetric cylinder. We did not encounter a froth layer on top of the homogenate as reported by Hill et al. (1995). Therefore, a homogenizer was not used, though the sample was well mixed and homogeneous after airbrushing. Sub-samples were immediately removed using sterile pipettes for multiple endpoints including $DMSP_t$ and $DMSP_p$ analyses, total protein, chl-*a*, algal cell counts, genetic typing (clades) and cell volumes. Coral sub-fragments were dried and measured for surface area (Marsh, 1970) and polyp number indices.

Total homogenate aliquots were used for analysis of $DMSP_t$, chl-*a*, zooxanthellae size and number and total protein content. One mL of total homogenate was added to one mL 10 N NaOH, in a headspace vial, to measure $DMSP_t$. To isolate the algal component from the sub-fragment homogenate, each sample was processed as follows: one mL of homogenate was passed through a GF/F filter at very low pressure (<25 mm Hg; Steinke et al., 2000) and placed immediately in a headspace vial containing 2 mL 5N NaOH, for $DMSP_p$ analysis. Filtration effects were not observed during sample preparation in pilot experiments (DMS did not increase in filtrates with an increasing number of cells filtered), indicating that there was minimal particulate DMS contribution (if any) to $DMSP_t$ concentrations due to filtration.

4.2.5 DMSP analyses

To analyze DMSP, the molecule was first converted to DMS by base hydrolysis. All DMS analyses were conducted after a 24 h headspace equilibration period following NaOH addition to a 5M final concentration. During the equilibration period, all samples were stored in the dark at room temperature. Samples were analyzed for DMS with a Hewlett-Packard 5890 Series II gas chromatograph (GC) fitted with a Chromosil 330 packed column (Supelco, Bellefonte, PA), and flame photometric detector (FPD). System temperature settings were: injector 150 °C, column oven 60 °C and detector 175 °C. Nitrogen gas was the carrier (60 cm^3 min^{-1}) and air (60 cm^3 min^{-1}) and hydrogen (50 cm^3 min^{-1}) were flame gases. Data were collected and analyzed using HP ChemStation (Hewlett – Packard, Palo Alto, CA). Quantifications were made by headspace analysis of DMS. Known concentrations of DMSP (purchased from Research Plus Inc., Bayonne, NJ) were diluted in sterile water to give working solutions, which were frozen in small aliquots at -80 °C. Multiple standard curves (4) of serial dilutions of DMSP were used to construct the calibration curves (using the square-root of the peak area) and linear regressions served to convert peak areas from GC headspace measurements to DMS concentrations. The standards were prepared in identical proportions of buffers and preparation solutions to those used in experimental samples. The same total liquid volume (2 mL) was used in all headspace vials. The precision of DMS analysis varied <5% and headspace storage trials showed no losses occurred with the analytical methods employed. Detection limit of the GC was 1 nmol DMS.

4.2.6 Algal endpoints

Using a haemocytometer and epifluorescent microscope, algal cell counts were conducted immediately using total homogenate aliquots diluted with sterile artificial seawater. Algal cell sizes (to calculate cell volume) were determined using a microscope, eyepiece graticule and calibrated ocular micrometer (samples were preserved in 5% buffered formalin; n=30 per sample). Cell volumes were calculated assuming that zooxanthellae are spherical; dividing cells were excluded from sizing measurements.

Chl-*a* was measured to serve as an index of zooxanthellae biomass. Briefly, 1 mL of each whole tissue homogenate was filtered through a Whatman GF/F glass fiber filter and extracted in 90% acetone for 24 h at 4 °C (Parsons et al., 1984) before being measured fluorometrically with a Trilogy Laboratory Fluorometer (Turner Designs, Sunnyvale, CA). Alternatively, for chl-*a* samples extracted in MeOH, whole tissue homogenate samples (both unfiltered and filtered as above) or intact sub-fragments were measured fluorometrically after calibration of chl-*a* standards in absolute MeOH (Holm-Hansen and Riemann, 1978). To ensure that all samples were measured in the linear portion of the calibration curve, additional dilutions were carried out in absolute MeOH (or 90% acetone for the method above), if necessary.

Phylogenetic (clade) analysis of zooxanthellae was determined by length heteroplasmy in domain V of chloroplast large subunit (cp23S)-rDNA (Santos et al., 2003).

4.2.7 Additional Indices

The surface areas and polyp numbers of the coral skeletons, used for either the intact or homogenate coral endpoints, were determined using the aluminum foil (Marsh, 1970) and polyp count techniques (Broadbent et al., 2002). Homogenate subsamples for protein analysis were frozen, thawed and quantified for total protein by the BCA assay (Pierce Chemical). Bovine serum albumin was used to construct the standard curve.

4.2.8 Statistical analyses

All data were checked for normality and homogeneity of variances prior to statistical analysis and data were transformed as necessary. All data were analyzed using analysis of variance (ANOVA) with Tukey's post-hoc tests to identify significant differences between methodological approaches. All statistical analyses were conducted using Minitab© v. 10 (Minitab Inc. 2000), with $\alpha = 0.05$ for all tests.

4.3 Results

With the exception of *M. cavernosa*, there were no significant differences between $DMSP_t$ for intact corals in MeOH or airbrushed coral homogenates in NaOH or MeOH ($P < 0.05$). There was a clear pattern that showed higher $DMSP_t$ than

$DMSP_p$ values when data were normalized to either coral surface area or per algal cell (Figures 2, 3). Both $DMSP_t$ and $DMSP_p$ were observed in every coral species using both NaOH and MeOH techniques. Higher $DMSP_t$ than $DMSP_p$ concentrations were observed in all of the airbrushed coral sub-fragments regardless of preparation method (Table 2). $DMSP_t$ was typically 2 - 3X greater than $DMSP_p$ with a range of 2.3 to 9.6X.

Zooxanthellae (*Symbiodinium* spp.) showed variability in cell diameter, volume and clade type according to coral species, with the exception of *M. cavernosa* and *M. mirabilis* both harboring clade C180 (Table 3). No significant differences were detected for $DMSP_p$ homogenates in NaOH or MeOH normalized to surface area ($P > 0.05$, Figure 2b, Table 4). $DMSP_p$ per cell varied among species ($P < 0.05$) but was only significantly different between the NaOH and MeOH techniques for *Madracis mirabilis* ($P < 0.01$, Figure 3b).

Considerable $DMSP_t$ variability was observed in *M. cavernosa* corals according to extraction method (Table 4). $DMSP_t$ was significantly higher in *M. cavernosa* homogenates in MeOH compared to intact sub-fragments in MeOH ($P < 0.05$, Figure 2a). $DMSP_t$ and $DMSP_p$ concentrations (nmol cm^{-2}) obtained from MeOH extraction of coral homogenates were consistently higher in *M. cavernosa* than in *P. astreoides* or *M. franksi* corals (Figure 2a).

DMSP normalized to multiple indices resulted in significant differences between preparation techniques, but no clear trend emerged among the indices investigated. $DMSP_p$ values normalized per cell or per cell volume showed similar trends. By contrast, $DMSP_t$ levels normalized per cell resulted in substantially higher values than $DMSP_p$ levels on a per cell basis (Figure 3). Protein and chl-*a* indices resulted in the most numerous significant differences when normalizing DMSP (Table 5). Chl-*a* samples extracted in acetone or MeOH showed similar results when normalized to multiple indices. However, intact sub-fragments in MeOH showed lower chl-*a* extraction efficiencies and unfiltered homogenate samples in MeOH had the lowest chl-*a* concentrations. Significant differences in chl-*a* concentrations emerged for filtered homogenate samples in MeOH for both *M. mirabilis* and *M. franksi* ($P < 0.05$, Table 5).

4.4 Discussion

Our results indicate that $DMSP_t$ concentrations were recovered effectively by placing airbrushed coral sub-fragment homogenates in NaOH or MeOH - or by placing intact sub-fragments in MeOH and subsequently measuring DMS via headspace analysis after the addition of strong base and sufficient equilibration time. This pattern appears to be consistent for $DMSP_p$ values as well and for both $DMSP_p$ and $DMSP_t$ across the four coral species we investigated, indicating that the two main methodologies for separating coral tissues from their skeletons are comparable regarding DMSP measurements. Also, the higher $DMSP_t$ than $DMSP_p$ values in this study may indicate that the coral animal contained DMSP within its tissues in addition to the $DMSP_p$ contained within the algae residing within the animal cells. However, it is also possible that the freezing/thawing of coral fragments and/or airbrushing may have damaged the algal cells and allowed DMSP to leak from the cells into the surrounding tissues and coral homogenate, the extent of which remains unknown. Therefore, the present $DMSP_p$ measurements may give only minimum symbiont DMSP concentrations (i.e. underestimations of $DMSP_p$ values) while the $DMSP_t$ values normalized to appropriate algal indices (e.g. chl-*a*) give DMSP concentrations that are equal to or greater than the true algal cellular values. High intracellular levels of DMSP within the symbiotic algae could contribute to $DMSP_t$ values if the majority of these cells were damaged during processing, a result that was not observed in this study, though it is possible that cells were leaky without being ruptured.

Our results are similar to those reported by Hill et al. (1995), who reported that intact coral pieces in MeOH yielded DMSP values that were 1.4 - 2.8 times greater than filtered (comparable to our $DMSP_p$ samples) coral homogenates in MeOH, indicating that algal DMSP may have also been lost in their sample preparations. Additionally, three potential methodological factors (in addition to coral species differences) may explain why our homogenate $DMSP_p$ to $DMSP_t$ ratios are comparatively greater than those reported by Hill et al. (1995). In contrast to our methods, Hill et al. (1995) (1) used a glass-plunger homogenizer after obtaining their blastate sample, (2) 'frothy material' was decanted before filtration and (3) filtration occurred under an unknown suction; each of these factors potentially reduces the amount of $DMSP_p$ in their filtered coral homogenate samples.

Additionally, the lower $DMSP_t$ values for *M. cavernosa* intact coral sub-fragments compared to airbrushed fragments may be partially explained by differences in coral morphology and the available surface area for MeOH extraction. For example, *M. cavernosa* has a ‘deeper’ tissue layer depth relative to the other corals investigated and we observed considerable variability in several homogenate (MeOH) endpoints for this coral. However, upon visual inspection of the coral sub-fragments after MeOH extraction, there was no appearance of residual coral tissue, suggesting that there may be tissue matrix complications using MeOH extraction. *M. cavernosa* coral homogenates extracted in MeOH showed higher $DMSP_t$ and $DMSP_p$ values than intact coral sub-fragments in MeOH or homogenates in NaOH. Additionally, for parallel homogenate samples in NaOH or MeOH, our data indicate that DMSP is about 2X more effectively extracted in MeOH from such complex mixtures. While the exact explanation for our results awaits definitive explanation, the higher DMSP values may have occurred because DMSP is MeOH-soluble and MeOH is an effective extractant of DMSP/DMS from a complex mixture of macromolecules in the tissue homogenate as mentioned above (*M. cavernosa* has a complex morphology and tissue layer compared to the other corals investigated). Also, the homogenate may provide more ‘access’ to algal cells and coral tissues through increased surface area contact with MeOH. In addition, the complex homogenate mixture may have affected other indices, again highlighting the importance of DMSP normalization. For instance, the homogenate matrix may have impeded chl-*a* extraction, a possible explanation for our low homogenate chl-*a* results.

These findings highlight the importance of our methodological comparisons, bringing into focus the limitations for several techniques on a per-species basis. In addition, the choice of preparation method also limits the potential indices that can be used for normalizing DMSP concentrations. For example, placing whole coral fragments in strong base precludes direct $DMSP_p$ analyses, total protein content measurements, zooxanthellae counts and sizing (using the techniques specified above), and the comparison of identical methods for chl-*a* quantification. Likewise, algal cells in homogenates are ruptured when exposed to NaOH preventing accurate enumeration by epifluorescent microscope (e.g. average cell loss of 49% at 0.5 M and 98% at 5M NaOH versus control; Yost unpublished data). Such effects underscore the need for methods that allow for algal cell enumeration and other coral and algal indices. However, many indices used to normalize DMSP concentrations can change in response to inherent variabilities (e.g. depth, location, algal and/or coral species) and/or environmental stress (e.g. temperature, UV, pollutants; see Yost et al., 2010). Furthermore, $DMSP_t$ levels normalized to algal cell number cannot accurately reflect DMSP partitioning between symbiont and host as $DMSP_p$ concentrations may be masked by total (coral *and* algal) DMSP levels and potential DMSP-lyase activity. By comparison, a coral homogenate allows for multiple indices for DMSP normalization, $DMSP_p$ quantification (demonstrated in this study) and feasibility despite some

DMSP loss (via DMS, which can be minimized) due to sample preparation. It is again noted here that methods used for separating symbiont from host may have primarily impacted $DMSP_p$ values herein, although also plausible is DMSP translocation to the host tissues as indeed evidenced in symbiotic clams (Hill et al., 2000). We deduce from these considerations that normalizing $DMSP_t$ and $DMSP_p$ data per coral surface area, with parallel measures of $DMSP_p$ quantifications per cell and/or cell volume, provides a comprehensive and informative approach to DMSP investigations in coral. Furthermore, we emphasize the value of quantifying multiple indices that are informative in their own right, while considering with caution the use of DMSP normalization indices that may change in response to multiple factors.

Each of the four coral species investigated revealed higher $DMSP_t$ concentrations than $DMSP_p$ concentrations when normalized to zooxanthellae number or coral surface area. Evidence suggests that zooxanthellae solely produce DMSP in cnidarian-algal symbioses and invertebrate tissue concentrations of DMSP are well correlated with symbiont density (Hill et al., 2000; Van Alstyne and Puglisi, 2007; Van Alstyne et al., 2009). If translocation is occurring, it can be inferred that the amount of DMSP in coral tissues is due to DMSP translocation and/or DMS diffusion from zooxanthellae, as DMSP is a zwitterion and cannot readily cross membranes, whereas DMS can readily diffuse across membranes. For coral sub-fragments placed directly in MeOH, Hill et al. (1995) reported mean $DMSP_t$ concentrations (nmol cm^{-2}) ranging from 126 to 290 for *Montipora verrucosa*, *Pocillopora damicornis* and *Porites compressa*. Similar to these findings, our sub-fragments in MeOH had average $DMSP_t$ levels (nmol cm^{-2}) that ranged from 150-183 for *Montastraea cavernosa*, *Porites astreoides* and *Montastraea franksi*. By comparison, reports of $DMSP_p$ levels from intact corals are limited and $DMSP_p$ values are often reported from cultured symbionts as an alternative. $DMSP_p$ values reported per zooxanthella from hard corals range from less than one hundred to more than three thousand (Hill et al., 1995; Broadbent et al., 2002; Van Alstyne et al., 2006). Often $DMSP_t$ values per algal cell are reported as measures of $DMSP_p$ levels, an assumption that may over estimate $DMSP_p$ if our findings that $DMSP_t$ levels exceed $DMSP_p$ levels due to translocation. $DMSP_p$ concentrations could be critical measures to miss when considering the variability of DMSP production and DMSP-lyase capabilities among zooxanthellae clades (Yost and Mitchelmore, 2009). Additionally, the function(s) of DMSP in coral-algal symbioses remain unclear and there are inherent methodological challenges associated with the separation of the symbiotic partners, highlighting the need for further investigations to determine possible DMSP partitioning within the symbiosis.

There is a growing interest in the roles of DMSP in multiple disciplines and an increase in investigations focused on the potential antioxidant role of DMSP in systems under stress (Bucciarelli and Sunda, 2003; Sunda et al., 2002, 2005; Ross and Van Alstyne, 2007; Husband and Kiene, 2007; Harada et al., 2009). These studies emphasize the importance of methodologies that allow for cross-comparisons, especially for investigations in the toxicological arena where normalization indices may be responding concurrently. Advancing our understanding of DMSP in cnidarian-algal symbioses first requires a recognition of the trade-offs between the best DMSP methodologies and normalization indices and what is reasonably feasible regarding sufficient coral sampling.

This is the first study to investigate the two prominent methodological techniques for the quantification of DMSP in coral symbioses in addition to teasing apart the relative partitioning of DMSP between coral and symbiont. Our efforts to improve DMSP measurements in several conspicuous hard corals emphasize three major points. First, DMSP was effectively quantified from airbrushed coral sub-fragment tissue homogenates in NaOH or MeOH - or from intact sub-fragments in MeOH that were subsequently measured via DMS headspace analysis after the addition of strong base and sufficient equilibration time. Second, DMSP normalization indices should be used with caution with careful consideration given to indices that demonstrate variability. Third, our data suggests that DMSP levels vary between host and symbiont, suggesting translocation from symbiont to host or possible methodological complications that effectively limit our interpretation of $DMSP_p$ concentrations, highlighting the need for further investigations focused on the quantification of both $DMSP_t$ and $DMSP_p$ from intact corals. Finally, continued research efforts focused on the response of DMSP levels, in addition to potential DMSP-lyase capabilities, need to be addressed in both symbiont and host, as the exact roles and functions of this environmentally relevant and enigmatic compound remain obscure in corals.

4.5 Tables

Table 4.5.1 – DMSP preparation methods.

(Table 1 in text). Comparison of preparation methods used in the literature for quantifying DMSP levels in symbiotic organisms and cultured symbionts.

Indice	Sample type for DMSP analysis	Sample preparation	Indices used to normalize DMSP	Reference
$DMSP_t$ (total, host and algae)	Intact hard coral fragment	100% MeOH followed by alkaline hydrolysis (0.5 or 1mL in 25mL 2N KOH)	pheopigment^{-1}, coral surface area (cm^{-2})	Hill et al. 1995
	Homogenate from frozen coral pieces	Subsamples immediately analyzed after alkaline hydrolysis with NaOH (conc. not specified)	algal cell^{-1}, algal cell volume (cm^{-3}), coral surface area (cm^{-2}), water column (m^{-2}, m^{-3})	Broadbent et al. 2002
	Coral mucus	Acidified with HCl to pH < 2 followed by alkaline hydrolysis (10N NaOH, vol.s not specified)	none	Broadbent and Jones 2004
	Unfiltered seawater samples from live coral nubbins in chambers	alkaline hydrolysis (10 mL sample in 2 mL 10M NaOH)	Number of coral nubbins or polyps	Broadbent and Jones 2006
	Fresh soft coral tentacles	alkaline hydrolysis (4N NaOH)	Coral fresh and dry mass, algal cell^{-1}	Van Alstyne et al. 2006
	"	Dried O/N at 60 °C; alkaline hydrolysis (4N NaOH)	Coral fresh tissue mass	"
	"	Frozen at -20 °C for 1 wk; alkaline hydrolysis (4N NaOH)	"	"
	"	Frozen at -80 °C for 1 wk; alkaline hydrolysis (4N NaOH)	"	"
	"	100% MeOH for 1wk	"	"

	Seawater; unfiltered seawater samples from live coral nubbins in chambers	alkaline hydrolysis (concentrated NaOH, vol.s and conc.s not specified)	polyp	Jones et al. 2007
	Aiptasia (anemones) or clipped tentacles (*A. elegantissima*)	alkaline hydrolysis (4N NaOH)	Anemone fresh mass, algal cell^{-1}	Van Alstyne et al. 2009
$DMSP_p$ (algae only)	Cultured zooxanthellae: whole culture, gravity-filtered culture and filtrate samples	alkaline hydrolysis (5N NaOH)	algal cell^{-1}, algal cell volume (cm^{-3})	Keller et al. 1989
	Homogenate from hard coral fragment	Filtered (A/E, GF/F), 100% MeOH followed by alkaline hydrolysis (0.5 or 1mL sample in MeOH in 25mL 2N KOH)	protein^{-1}, pheopigment^{-1}, algal cell^{-1}	Hill et al. 1995
	Cultured zooxanthellae	Filtered (A/E, GF/F), 100% MeOH followed by alkaline hydrolysis (0.5 or 1mL in 25mL 2N KOH)	algal cell^{-1}	Hill et al. 1995
	Cultured zooxanthellae spp.	Not explicitly stated	algal cell^{-1}, water column (m^{-3})	Broadbent et al. 2002
	Seawater; unfiltered and filtered seawater samples from live coral nubbins in chambers	alkaline hydrolysis (10 mL sample in 2 mL 10M NaOH)	Calculated $DMSP_p$ from the difference between unfiltered and filtered samples	Broadbent and Jones 2006
	unfiltered and filtered seawater samples from live coral nubbins in chambers	alkaline hydrolysis (concentrated NaOH, vol.s and conc.s not specified)	Calculated $DMSP_p$ from the difference between unfiltered and filtered samples	Jones et al. 2007
	Cultured zooxanthellae spp. (whole culture samples)	Low gravity filtration (GF/F), Alkaline hydrolysis (5N NaOH)	algal cell^{-1}	Yost and Mitchelmore 2009

Table 4.5.2 – $DMSP_t$ to $DMSP_p$ ratios according to preparation method.

(Table 2 in text). Ratio of $DMSP_t$ to $DMSP_p$ concentrations for airbrushed coral sub-fragments placed in NaOH or MeOH.

Coral Species	**NaOH**	**MeOH**
M. cavernosa	3.0 ± 0.5	2.6 ± 0.2
M. mirabilis	2.9 ± 0.3	9.6 ± 2.4
P. astreoides	3.2 ± 0.2	4.6 ± 0.8
M. franksi	2.3 ± 0.5	3.2 ± 0.3

Results are means ± SE (n=5)

Table 4.5.3 – *Symbiodinium* genotypes.

(Table 3 in text). *Symbiodinium* cp23s-rDNA genotype (clade), cell diameter and cell volume.

Coral Species	Algal Clade	Cell diameter (μm)	Cell volume (μL) 10^6 cells^{-1}
M. cavernosa	C180	13.5 ± 0.6	1.30 ± 7.04 x 10^{-4}
M. mirabilis	C180	10.3 ± 0.5	0.58 ± 3.88 x 10^{-4}
P. astreoides	A194	12.6 ± 0.6	1.06 ± 7.02 x 10^{-4}
M. franksi	B184	11.9 ± 0.6	0.88 ± 7.28 x 10^{-4}

Results expressed as means (± SD; n=5)

Table 4.5.4 – DMSP normalizations.

(Table 4 in text). DMSP levels (nmol except where indicated for cell^{-1}) normalized to multiple indices.

M. cavernosa	(mg protein^{-1})	(polyp^{-1})	(µg chl-*a* $^{-1}$)	(µl^{-1} cell vol)
intact sub-fragment				
$DMSP_t$ (MeOH)	NP	227.5±51.0	27.2±3.0	NP
airbrushed sub-fragment				
$DMSP_t$ (NaOH)	114.2±9.8 †	217.8±37.1	20.2±2.3 †	157.1±31.7
$DMSP_t$ (MeOH)	254.4±49.4	506.9±144.7	61.4±13.4	391.6±168.2
$DMSP_p$ (NaOH)	44.2±9.3	87.9±23.1	7.5±1.4 †	58.3±12.5
$DMSP_p$ (MeOH)	101.5±25.3	203.8±69.1	24.5±6.3	163.2±72.2
M. mirabilis				
intact sub-fragment				
$DMSP_t$ (MeOH)	NP	NP	17.4±2.2	NP
airbrushed sub-fragment				
$DMSP_t$ (NaOH)	62.0±3.5 †	NP	11.3±0.4 # †	42.1±3.1
$DMSP_t$ (MeOH)	77.1±4.9	NP	40.4±3.9 *	52.4±4.5
$DMSP_p$ (NaOH)	22.5±2.9 †	NP	4.0±0.3	15.4±2.2 †
$DMSP_p$ (MeOH)	9.5±1.5	NP	5.1±0.9	6.5±1.1
P. astreoides				
intact sub-fragment				
$DMSP_t$ (MeOH)	NP	NP	48.5±18.9	NP
airbrushed sub-fragment				
$DMSP_t$ (NaOH)	55.7±8.8	NP	24.2±2.7 †	825.2±299.6
$DMSP_t$ (MeOH)	81.4±13.1	NP	44.9±3.8	1215.8±438.0
$DMSP_p$ (NaOH)	18.1±4.1	NP	7.7±1.1	277.4±110.3
$DMSP_p$ (MeOH)	18.7±3.1	NP	11.4±2.6	277.0±74.0

M. franksi				
intact sub-fragment				
$DMSP_t$ (MeOH)	NP	55.2±8.1	11.4±1.9	NP
airbrushed sub-fragment				
$DMSP_t$ (NaOH)	103.0±8.8	41.3±4.1	15.0±1.8 †	106.0±14.7
$DMSP_t$ (MeOH)	128.8±15.5	51.2±5.6	34.0±4.7 *	131.7±19.5
$DMSP_p$ (NaOH)	56.9±17.1	22.3±5.7	7.5±1.7	56.2±15.1
$DMSP_p$ (MeOH)	40.6±2.3	16.4±1.5	10.6±0.6	41.1±2.0

Results are expressed as means ± SE.

[a] NP indicates not possible to measure using the described methods

[b] * indicates significant statistical difference between airbrushed and intact samples in MeOH

[c] # indicates significant statistical difference between airbrushed in NaOH and intact samples in MeOH

[d] † indicates significant statistical difference between airbrushed samples in NaOH or MeOH

Table 4.5.5 – Chlorophyll-*a* concentrations.

(Table 5 in text). Chlorophyll-*a* concentrations for intact and airbrushed coral sub-fragments extracted with either acetone or absolute MeOH.

Coral species		homogenate GF/F (MeOH)	homogenate GF/F (acetone)	homogenate unfiltered (MeOH)	intact sub-fragment (MeOH)
M. cavernosa	pg $cell^{-1}$	7.8±1.5	10.2±1.5	2.3±0.6	NP
	µg µl cell $vol.^{-1}$	6.0±1.1	7.8±1.1	1.8±0.5	NP
	µg cm^{-2}	7.4±0.9	10.9±2.2	2.1±0.3	5.8±1.0
	µg mg $protein^{-1}$	4.4±0.5	6.0±0.9	1.2±0.1	1.7±0.4
M. mirabilis	pg $cell^{-1}$	0.8±0.0*	2.2±0.2	0.1±0.0	NP
	µg µl cell $vol.^{-1}$	1.3±0.1*	3.8±0.3	0.2±0.0	NP
	µg cm^{-2}	NP	NP	NP	NP
	µg mg $protein^{-1}$	1.9±0.1*	5.5±0.4	0.2±0.0	1.5±0.2
P. astreoides	pg $cell^{-1}$	4.5±0.6	5.8±0.7	0.5±0.2	NP
	µg µl cell $vol.^{-1}$	28.8±11.3	37.0±14.7	4.0±2.6	NP
	µg cm^{-2}	5.0±1.0	6.5±1.4	0.7±0.3	5.3±1.1
	µg mg $protein^{-1}$	1.9±0.4	2.5±0.6	0.3±0.1	0.6±0.1
M. franksi	pg $cell^{-1}$	3.5±0.3*	6.4±0.9	0.4±0.1	NP
	µg µl cell $vol.^{-1}$	3.9±0.3*	7.3±1.0	0.5±0.1	NP
	µg cm^{-2}	6.0±1.2	11.1±2.4	15.5±2.4	0.7±0.1
	µg mg $protein^{-1}$	3.9±0.3*	7.3±1.0	3.3±1.0	0.5±0.0

Results are expressed as means ± SEM (n=5 for all measures).
[a] NP indicates not possible to measure using the described methods
[b] * indicates significant difference between homogenate GF/F samples in MeOH or acetone.

4.6 Figures

Figure 4.6.1 – Schematic of coral preparation and processing.

(Figure 1 in text). Schematic representation of the preparation and processing of coral fragments and the respective normalization indices used for data analyses. Double arrows indicate parallel analyses between methods.

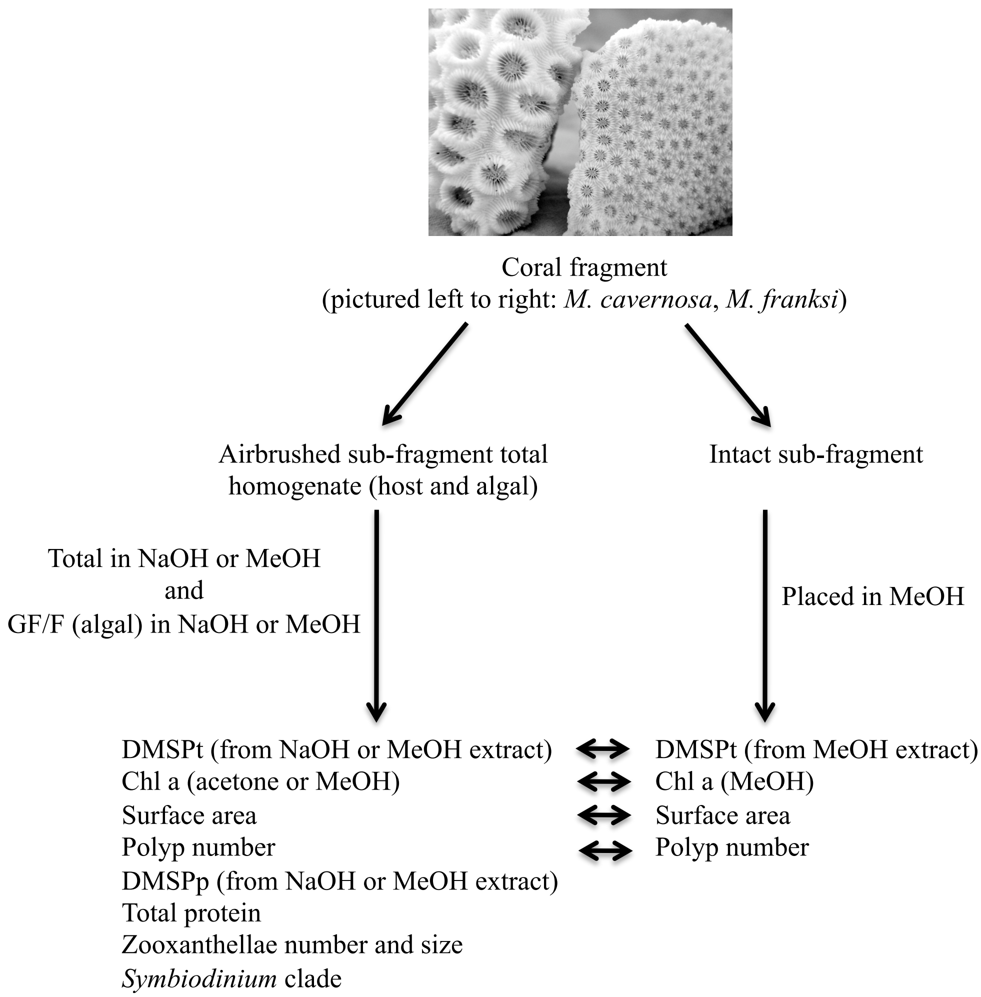


Figure 4.6.2 – Coral sub-fragment and homogenate $DMSP_t$ and $DMSP_p$.

(Figure 2a, b in text). Coral sub-fragment intact (2a) and homogenate (2b) $DMSP_t$ and $DMSP_p$ (nmol cm^{-2}) for *Montastraea cavernosa*, *Porites astreoides* and *Montastraea franksi*. *Madracis mirabilis* not shown (surface area not measured).

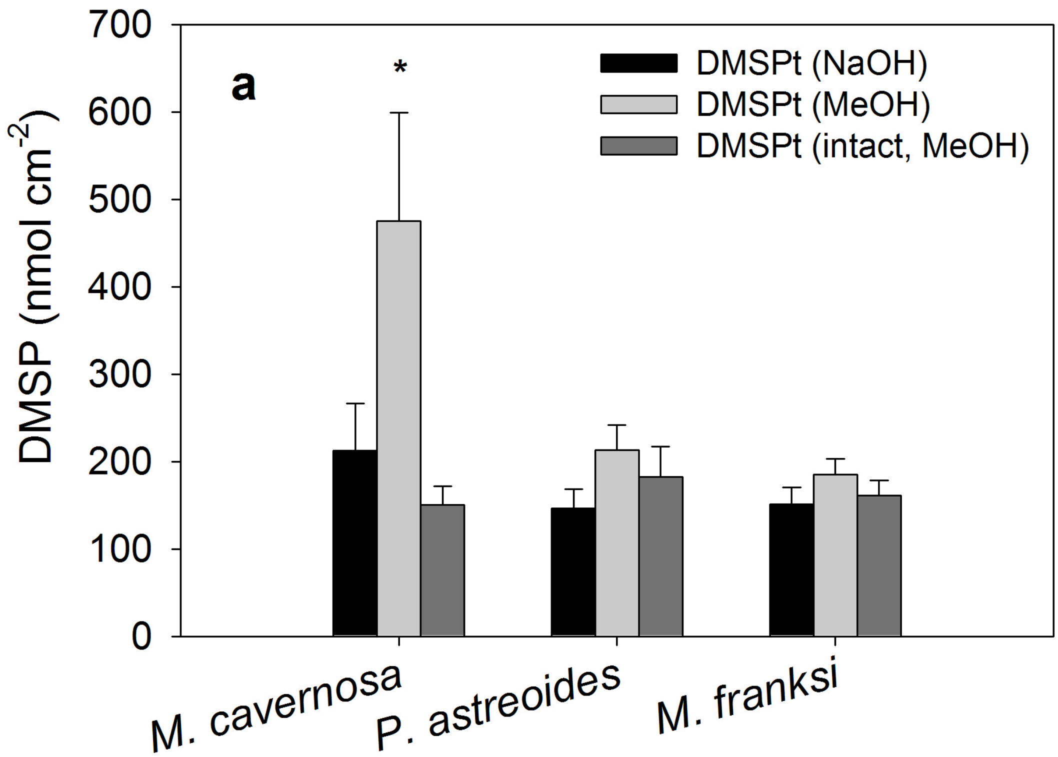


Figure 4.6.2 – continued.

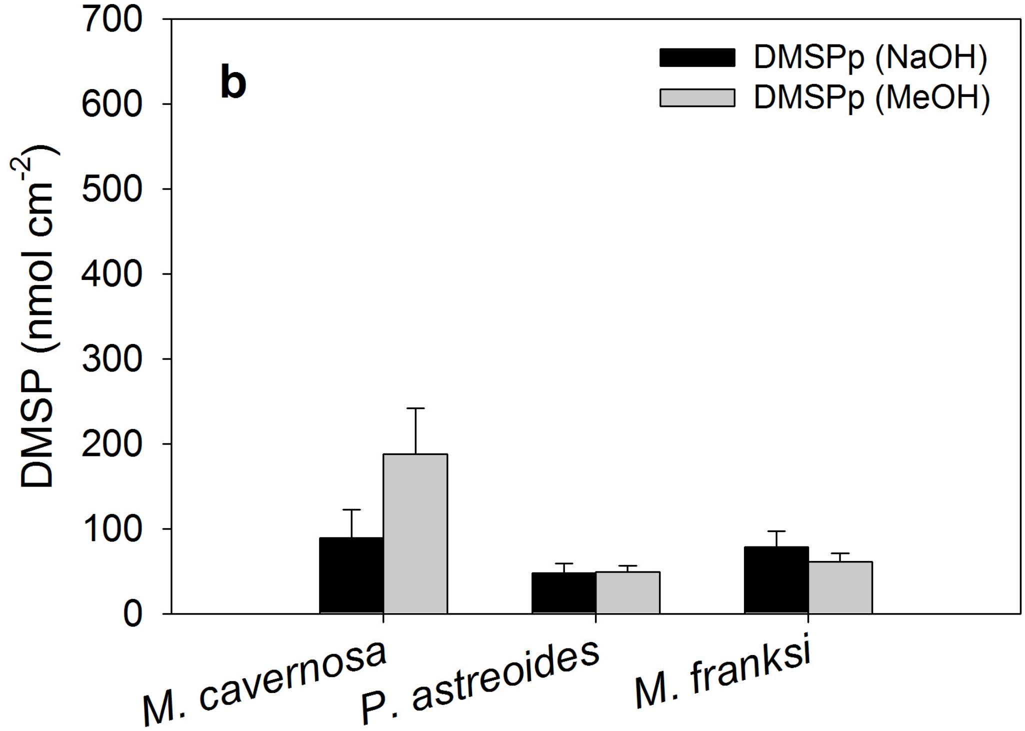


Figure 4.6.3 – Airbrushed coral sub-fragment $DMSP_t$ and $DMSP_p$.

(Figure 3a, b in text). Airbrushed coral sub-fragment homogenate $DMSP_t$ and $DMSP_p$ (nmol cell^{-1}) for *Montastraea cavernosa*, *Madracis mirabilis*, *Porties astreoides* and *Montastraea franksi*. Please note different y-axis scales.

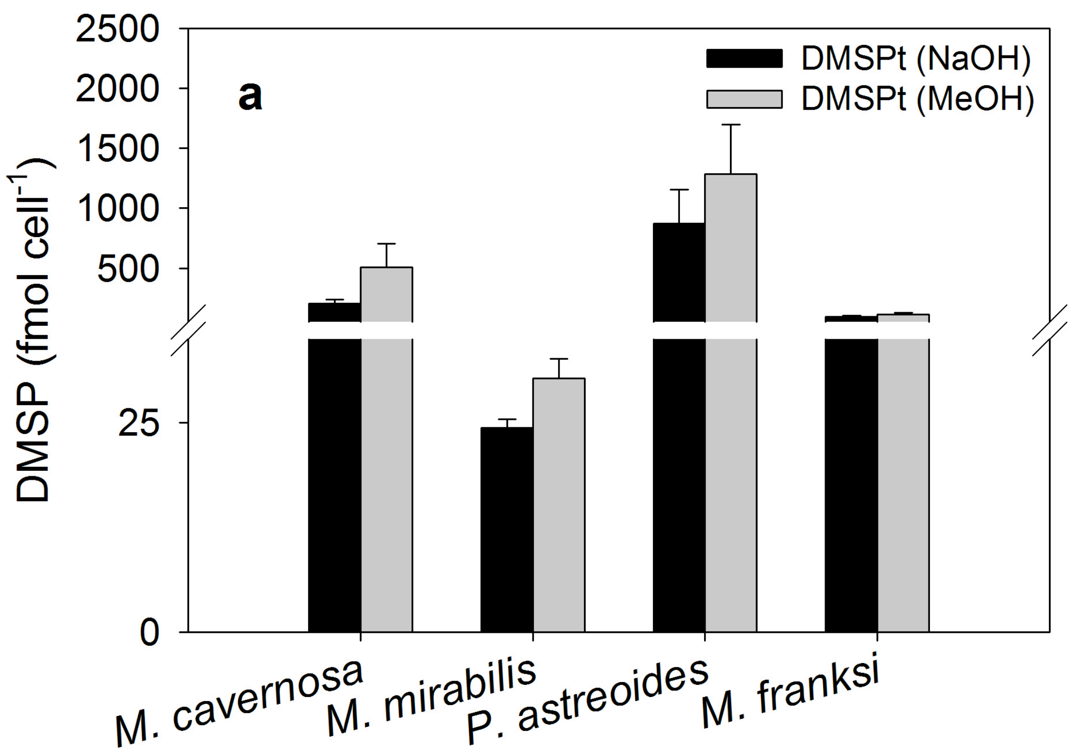
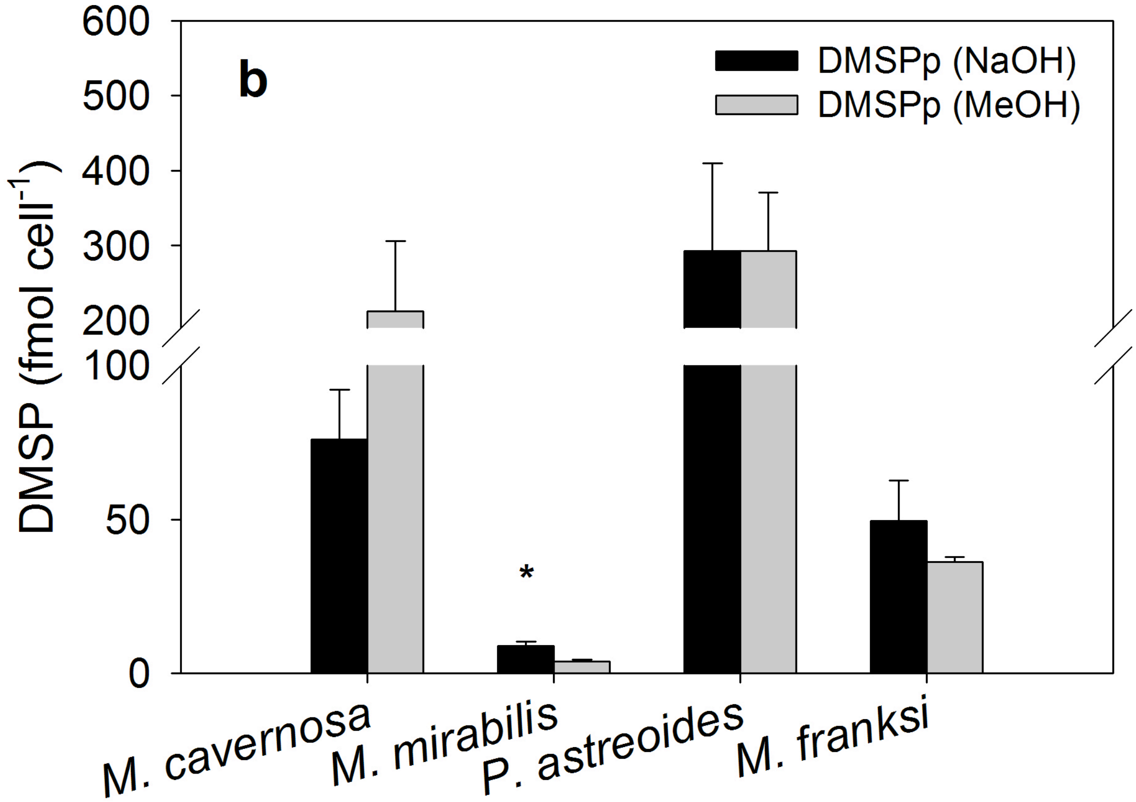


Figure 4.6.3 – continued.



Chapter 5: Alterations in dimethylsulfoniopropionate (DMSP) levels in the coral *Montastraea franksi* in response to copper exposure

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5.1 Introduction

Symbiotic cnidarians routinely experience hyperoxic conditions within their tissues due to the photosynthesis of the endosymbiotic dinoflagellate microalgae (*Symbiodinium* spp.) (Dykens and Shick, 1982; Kühl et al., 1995). To mitigate against potentially damaging reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (• OH), and singlet oxygen ($^1O_2$), corals possess various constitutive and/or inducible enzymatic and non-enzymatic antioxidant systems, including superoxide dismutases, glutathione, xanthophylls and ascorbate peroxidases (for a review see Lesser, 2006). However, inherent physiological stress in coral symbioses can be compounded by additional exogenous oxidative stressors. For example, environmental pollutants can exacerbate conditions of oxidative stress in corals, compromising their ability to cope with and recover from imposed stressors by overwhelming their antioxidant protective system (Downs et al., 2002).

Symbiodinium spp. and many other species of marine algae, produce high intracellular levels of the osmolyte dimethylsulfoniopropionate (DMSP) (Keller et al., 1989; Keller and Korjeff-Bellows, 1996; Van Alstyne et al., 2006; Yoch, 2002). It has recently been discovered in marine algae that DMSP and its enzymatic breakdown products also play a significant role in the scavenging of cellular ROS (Sunda et al., 2002). Since damage by ROS has been implicated in the physiological mechanism underlying coral bleaching - the stress related dissociation of the coral-algal symbiosis which occurs during exposure to elevated water temperatures and a suite of other stressors (see for example Lesser et al., 1990) - it is not surprising that the role of DMSP in corals is of growing interest.

In marine algae, DMSP has also been shown to be an osmolyte (Kirst, 1990), a cryoprotectant (Karsten et al., 1996), a herbivory deterrent and attractant (DeBose et al., 2008; Wolfe et al., 1997), an antiviral defense mechanism (Evans et al., 2006) and involved in sulfide detoxification (Havill et al., 1985). However, DMSP and its enzymatic breakdown products, dimethylsulfide (DMS), acrylate, dimethylsulfoxide (DMSO) and methane sulfinic acid (MSNA) can readily scavenge hydroxyl radicals and other ROS (Sunda et al., 2002). Significant in this regard is the enzymatic cleavage of DMSP by DMSP lyase (DL), as the cleavage products, acrylate and DMSO are 60 and 20 times, respectively, more effective in scavenging hydroxyl radicals than DMSP itself (Sunda et al., 2002). Additionally, this suite of antioxidant compounds can scavenge ROS in multiple cellular compartments as it is composed of water- or lipid-soluble scavengers of the extremely harmful hydroxyl radical and other ROS. DMS and DMSO can neutralize lipid peroxidation in photosynthetic membranes and harmful radicals in chloroplasts, respectively, thus targeting important ROS sites (Lee and de Mora, 1999b).

In corals, the presence and role(s) of DMSP have only recently been investigated (see for example: Broadbent et al., 2002; Broadbent and Jones, 2004, 2006; Hill et al., 1995; Jones et al., 2007; Jones and Trevena, 2005; Van Alstyne et al., 2006, 2009; Yost and Mitchelmore, 2009). It has been established that the symbiotic dinoflagellates of corals produce DMSP and have DL capabilities, but so far DL has not been shown to exist in corals or other host species harboring symbiotic dinoflagellates (Hill et al., 2004). It is known that there is a considerable variation in DMSP levels within zooxanthellae clades (Yost and Mitchelmore, 2009) and it is plausible that the translocation of DMSP from symbiont to host occurs (Hill et al., 2004), highlighting the need for particulate DMSP (algal cells only; $DMSP_p$) quantification in addition to total DMSP (algal and host cells; $DMSP_t$) measures. Elevated levels of DMS above stressed reefs have led authors to suggest the involvement of DMSP in a corals stress response (Jones et al., 2007).

Support for the antioxidant role of DMS/DMSP comes from experiments showing that oxidative stressors such as solar ultraviolet radiation, CO_2 limitation, Fe limitation, H_2O_2 and high Cu^{2+} (ions) substantially increased cellular DMSP and/or its lysis to DMS (Sunda et al., 2002, 2005). Copper was used in these experiments as it is known to cause oxidative stress by catalyzing the formation of the hydroxyl radical by Haber-Weiss and Fenton reactions, from the photosynthetic by-product, hydrogen peroxide (Abalde et al., 1995; Okamoto et al., 2001). The hydroxyl radical is the most powerful oxidizing radical likely to arise in biological systems, and is capable of reacting with practically every biological molecule (Buettner, 1993), resulting in damage to lipids, proteins and nucleic acids (Gutteridge and Wilkins, 1983).

The response of corals and their symbiotic algae to copper is well studied from ecotoxicological experiments. For example, copper reduces fertilization success (i.e. EC_{50} values ranging from 14.5 to 39.7 μg L^{-1}) (Heyward, 1988; Negri and Heyward, 2001; Reichelt-Brushett and Harrison, 1999, 2005). Sublethal loss of symbiotic dinoflagellates and bleaching of tissues has been reported in adult corals exposed to concentrations of 10 - 60 μg L^{-1} Cu between 2 and 10 days (Evans, 1977; Jones, 1997, 2004); however, not all studies report sublethal loss of symbiotic dinoflagellates (for example Grant et al., 2003). Several studies have reported copper threshold levels of sublethal stress between 20 and 50 μg L^{-1} in different species of cultured dinoflagellates (Goh and Chou, 1997; Mandelli, 1969) and reduced specific growth rates at 40 μg L^{-1} (Goh and Chou, 1997). Alterations in chlorophyll *a* (chl-*a*) concentrations in *Symbiodinium* spp. and other algae have also been reported in response to copper stress (e.g. see Brown, 2000; Edmunds et al., 2003; Fitt et al., 1993; Gleason and Wellington, 1993; Grant et al., 2003; Nystrom et al., 2001; Sunda et al., 2002). Corals also respond to alleviate copper toxicity by up-regulating cellular defense mechanisms, including antioxidants (Gilbert and Guzman, 2001; Grant et al., 2003; Mitchelmore et al., 2007; Morgan et al., 2001, 2005; Morgan and Snell, 2006; Venn et al., 2009b).

To further understand the function(s) and regulation of DMSP within the coral-algal symbiosis we quantified the response of intact, field-collected coral (*Montastraea franksi*) to 0-50 μg L^{-1} Cu. DMSP concentrations in air-brushed coral fragments (host and algae; total DMSP ($DMSP_t$)) and in the zooxanthellae isolated from these corals (particulate DMSP ($DMSP_p$)) were quantified together with numerous other algal and coral cellular and biochemical indices. These indices were measured to monitor potential bleaching effects and/or to serve as normalization factors for DMSP concentrations. DL was not assessed due to the logistical limitations.

5.2 Materials and Methods

5.2.1 Collection and handling

Five large (50-60 cm diameter) colonies of *M. franksi* were collected from 5 m depth from offshore patch reefs (32º25°30.14°N, 64º42°27.15°W) in the Bermuda lagoon and transported back to the Bermuda Institute of Ocean Sciences (BIOS) submerged within large (100 L) high density polyethylene (HDPE) containers (fish tote containers). Small fragments (3-5 cm diameter) were then cut from the corals using a hammer and chisel and the bases of the fragments leveled off using a carbide-tipped circular cut-off wheel mounted to a high speed rotary tool. Each explant was numbered with a small (3 mm diamater) glue-on, flexible polyethylene shellfish tag (Hallprint, Victor Harbour, Australia) attached to the base of the fragment using gel-type cyanoacrylate glue (Loctite Quik Set, Superglue 404). Explants were left for 14

days to recover from the handling and preparation procedures in flowing seawater under one layer of 50% neutral density shade-cloth before experimentation.
Corals were then exposed to different concentrations of copper in specially constructed all-Teflon dosing chambers. In these chambers, seawater was pumped at a rate of 500 mL/min (i.e. recirculating with a 20 min total replenishment time) by a Valcor Teflon metering pump (Valcor Engineering Corporation, Springfield, NJ, USA) from a 50 L fluoride impregnated, HDPE rectangular carboy (a 'reservoir')(Fisher Scientific, Agawam, MA, USA) into a 10 L fluoride impregnated, HDPE 10 L Jerrican (a 'dosing chamber')(Fisher Scientific, Agawam, MA, USA) located on the upper level of a four foot, two shelf, polyethylene utility cart. Seawater then gravity feeds back into the reservoir on the corresponding lower level of the cart. Four dosing chambers (and associated reservoirs) could be fitted onto a single cart and there were replicate carts, providing 16 replicates, all-Teflon dosing chambers for experimental exposures.

During experimentation, all fragments were held on a 10 cm square Teflon, 4 mm thick polytetrafluoroethylene (PTFE) platform inserted into the center of each of the dosing chambers. The height of the platform was adjusted using PTFE Teflon screws attached to the corners of the platform and adjusted so the corals' surface was ~2-3 cm below the water surface. All experiments were conducted outdoor under natural sunlight. To provide cover against rainfall, a layer of 3 mm UV transparent acrylic sheet (Acrylite® OP-4, Cyro Industries Rockaway, NJ, USA) was placed over the four dosing chambers. This acrylic sheet transmits a high percentage of light in the UV-A (315-400 nm) and UV-B (280-315 nm) regions. Photosynthetically Active Radiation (PAR, 400-700 nm) is slightly attenuated (<10%) using the 3 mm thick sheet, but irradiance was further reduced to ~50% of ambient using neutral density filters (Lee Filters, Burbank, CA, USA). During experiments PAR light levels were recorded over 10 min intervals with a LI-190SA Quantum Sensor and a LI-1400 data logger (Licor, Lincoln, NE, USA).

To achieve temperature regulation within the dosing chambers, de-ionized cooling water from water/heater units (6000 Series, Heater/Chiller unit, Polyscience, Niles, IL, USA) was pumped via 1/2 inch commercial garden hosing through a 4-way manifold into tygon high purity tubing and then into 2 m long coiled, 3/8 inch (internal diameter) Perfluoroalkoxy (PFA) cooling coils which were inserted into each of the dosing chambers on each cart. All sunlight-exposed tubing associated with the cooling system was surrounded by 3/8 inch thick, polyethylene pipe and tube insulation. Temperatures within the dosing chambers were regulated at 25 ± 0.2 °C using this system.

Coral explants were exposed to one of the four copper sulfate concentrations (i.e. treatments) including <1 $\mu g\ L^{-1}$ (i.e. a control or ambient seawater), or 5, or 10 or 50 $\mu g\ L^{-1}$ copper (as Cu_2SO_4) for 48 h. This range of copper concentrations was chosen based on environmentally relevant copper concentrations that are often much higher in estuaries and coastal waters, areas close to anthropogenic pollution sources where concentrations as high as 29.2 $\mu g\ L^{-1}$ Cu have been reported (Sadiq, 1992).

Actual copper concentrations (50 μg L^{-1}: 30 ± 7; 10 μg L^{-1}: 8 ± 4; 5 μg L^{-1} and control below detection limit) were determined via ICP-AAS by Jodi Schwarz (Vassar College, NY). Each treatment was replicated four times i.e. 4 treatments × 4 replicates = 16 individual dosing chambers. At the start of the experiment, one fragment from each of the five colonies was placed in each of the 16 chambers. Water changes and re-dosing occurred at 24 h. All experiments were started at 09:00 h and ended with samples being wrapped in two layers of aluminum foil and flash frozen in liquid nitrogen. Corals were then shipped on dry ice and stored at -80 °C (for up to 4 months) until analysis of DMSP (and associated indices) at the Chesapeake Biological Laboratory, Solomons, MD.

5.2.2 Homogenized coral fragment

We used a modification of the coral homogenization technique described in Broadbent et al. (2002), to measure DMSP and DMS. Individual coral fragments were removed from -80 °C storage and immediately airbrushed (Szmant and Gassman, 1990) using sterile artificial seawater to obtain a homogenate that was collected in a 0.5 L plastic bag. Total homogenate volumes were measured with a volumetric cylinder. We did not encounter a froth layer on top of the homogenate in these samples as reported by Hill et al. (1995), therefore a homogenizer was not used, although the sample was well mixed and homogeneous after airbrushing. Sub-samples were immediately removed using sterile transfer pipettes for multiple indices including $DMSP_t$ and $DMSP_p$ analyses, total protein, chl-*a*, algal counts and algal size (diameter). The remaining coral skeleton was saved for surface area measurements (Marsh, 1970).

5.2.3 Particulate and total DMSP homogenate analyses

To isolate the algal component ($DMSP_p$) from the whole filtrate, 1 mL of homogenate was passed through a Whatman GF/F filter at low pressure (<25 mm Hg) and placed immediately in a headspace vial containing 2 mL 5N NaOH, for $DMSP_p$ analysis. Loss of $DMSP_p$ due to the filtration was virtually minimal as preliminary tests showed no increase in filtrate DMSP when filtering larger numbers of algae (Yost, unpublished results). For $DMSP_t$ analysis, 1 mL of unfiltered homogenate was added to 1 mL 10N NaOH in a headspace vial.

5.2.4 Algal indices

For chl-*a* analysis, 1 mL homogenate aliquots were filtered through Whatman GF/F filters and extracted in 90% acetone for 24 h at 4 °C (Parsons et al., 1984). Unfiltered homogenate aliquots were used for analysis of zooxanthellae size, number and total protein content. Homogenate sub-samples for protein analysis were frozen, thawed and quantified for total protein by the BCA assay (Pierce Chemical). Bovine serum albumin was used to construct a standard curve. Algal cells, diluted with sterile artificial seawater, were immediately enumerated by haemocytometer using an epifluorescence microscope. Thereafter, 10 replicate counts per sample were averaged and corrected for dilution to calculate the total number of algal cells per mL of homogenate. Algal cell sizes (to calculate cell volume) were determined using a microscope and eyepiece graticule (samples were preserved in 5% buffered formalin; *n*=30 per sample). Phylogenetic (clade) analysis of zooxanthellae was determined by length heteroplasmy in domain V of chloroplast large subunit (cp23S)-rDNA (Santos et al., 2003).

5.2.5 DMSP analyses and DMS calibration

All analyses were conducted after a 24 h headspace equilibration period following NaOH addition. During the equilibration period all samples were stored in the dark at room temperature. Samples were analyzed with a Hewlett-Packard 5890 Series II gas chromatograph (GC) fitted with a Chromosil 330 packed column (Supelco, Bellefonte, PA), and flame photometric detector (FPD). System temperature settings were: injector 150 °C, column oven 60 °C and detector 175 °C. Nitrogen gas was the carrier (60 cm^3 min^{-1}) and air (60 cm^3 min^{-1}) and hydrogen (50 cm^3 min^{-1}) were flame gases. Data were collected and analyzed using HP ChemStation (Hewlett-Packard, Palo Alto, CA). Quantifications were made by headspace analysis following DMSP conversion to DMS by alkaline hydrolysis. Known concentrations of DMSP (purchased from Research Plus Inc., Bayonne, NJ) were diluted in sterile water to give working solutions, which were frozen in small aliquots at -80 °C. Multiple standard curves of serial dilutions of DMSP were used to construct calibration curves (using the square-root of the peak area) and these linear regressions served to convert peak areas from GC headspace measurements to DMS concentrations. The standards were prepared in identical proportions of buffers and preparation solutions to those used in experimental samples (ASW, NaOH). The same total liquid volume (2 mL) was used in all headspace vials. The precision of DMS analysis varied <5% and headspace storage trials showed no losses occurred with the analytical methods employed. Detection limit of the GC was 1 nmol DMS.

5.2.6 Statistical analyses

After testing assumptions of normality and homogeneity, analysis of variance (ANOVA) was used to assess whether DMSP ($DMSP_p$ and $DMSP_t$), chl-*a*, algal cell number and cell size, total protein and coral surface area indices differed among controls and corals exposed to copper. Pearson's correlations were used to determine correlation between indices (pairwise: chl-*a*, algal cell number, total protein and coral surface area). All statistical analyses were conducted using Minitab© v. 10 (Minitab Inc. 2000).

5.3 Results

Symbiotic dinoflagellate densities (cell numbers per cm^2) declined with increasing copper concentration and were significantly different from control treatments at 50 $\mu g\ L^{-1}$ Cu after 48 h exposures ($p < 0.05$) (Fig. 1).

Chl-*a* concentrations, normalized either to surface area (cm^{-2}), to algal cell number ($cell^{-1}$) or to total protein all showed similar patterns of response to copper with significantly less chl-*a* at 5 and 10 $\mu g\ L^{-1}$ Cu (but not at 50 $\mu g\ L^{-1}$) compared to controls ($p < 0.05$) (Table 1). Total protein levels normalized to surface area (cm^{-2}) also showed a similar trend, although they were only significantly different compared to controls ($p < 0.05$) at 10 $\mu g\ L^{-1}$ Cu (Fig. 2).

During this experiment we found that some of our normalizing indices alone were responding to Cu addition, complicating our DMSP concentration analyses. To investigate this further, correlations were performed between multiple indices to determine how these indices were responding to Cu (Table 2). Chl-*a*, symbiotic dinoflagellate density, total protein and coral surface area were significantly correlated between all copper exposures with the exception of correlations that included protein indices at 5 μg L^{-1} Cu (Table 2).

Because chl-*a* and protein concentrations changed in response to copper, they were not included as indices for DMSP. Yet, in the DMSP literature, chl-*a* is a commonly used index. In addition, as algal cell numbers decreased with copper exposure, indexing $DMSP_t$ levels to algal cell number alone could be misleading. There was no effect of copper exposure on the genetic type of dominant *Symbiodinium* spp. present, as all algal samples analyzed were clade B184. Algal cell volumes were not significantly different among treatments (calculated average cell volume for controls was 7.4 μl/10^7 cells).

$DMSP_t$ concentrations were significantly greater than $DMSP_p$ concentrations for all copper treatments and controls when indexed to coral surface area or algal cell number (average of 6x greater for $DMSP_t$) showing that a large amount of the DMSP is present in host tissues. $DMSP_t$ levels (nmol cm^{-2}) declined with increasing concentrations of copper and were significantly less for corals exposed to 50 μg L^{-1} Cu ($p < 0.05$) (Fig. 3). However, on an algal basis, $DMSP_t$ (fmol $cell^{-1}$), although lower with copper exposures, did not differ significantly at any of the copper exposures compared with the controls ($p > 0.05$) (Fig. 4).

$DMSP_p$ concentrations (nmol cm^{-2}) were significantly less at 5 and 10 $\mu g\ L^{-1}$ Cu ($p < 0.05$), but unlike $DMSP_t$ were not significantly different compared with controls at 50 $\mu g\ L^{-1}$ Cu ($p > 0.05$) (Fig. 3). Similar patterns emerged for $DMSP_p$ concentrations when normalized to algal cell number instead of surface area. $DMSP_p$ (fmol $cell^{-1}$) was significantly lower at 5 and 10 $\mu g\ L^{-1}$ Cu exposures compared to controls ($p < 0.05$) (Fig. 4).

5.4 Discussion

This study is the first to demonstrate significant changes in DMSP concentrations in corals following exposure to an oxidative stressor, copper, and is the first study to examine the relationship between particulate algal DMSP ($DMSP_p$) and total DMSP ($DMSP_t$); which is the most commonly reported measure in studies of DMSP and corals. Copper exposure resulted in up-regulation of various antioxidant genes (Schwarz, unpublished data) and induction of DNA damage in host cells (Mitchelmore, unpublished data) consistent with oxidative stress conditions. We found that exposure of the hard coral *M. franksi* to elevated levels of copper caused significant changes in DMSP, chl-*a*, algal cell number and total protein levels.

Corals and other invertebrates in symbioses with zooxanthellae have significant body burdens of DMSP (Hill et al., 2000; Van Alstyne and Puglisi, 2007).

Recent evidence that DMSP is solely produced by zooxanthellae in cnidarian-algal symbioses (Van Alsytne et al., 2009) and that DMSP is present in tissues lacking symbionts in other invertebrates (Hill et al., 2000, 2004) suggests translocation of DMSP. Indeed, we found that $DMSP_t$ concentrations were greater than $DMSP_p$ concentrations, evidence that supports this potential DMSP translocation. The significance of DMSP in host tissues is yet unknown and may depend, in part, on whether host coral cells have DL. If host corals have DL, then DMSP, through its conversion to subsequently more powerful antioxidants as shown by Sunda et al. (2002), could be used to protect host tissues from excessive oxidative stress (as could DMSP itself). Alternatively, high concentrations of DMSP in host tissues could perturb host protein/enzyme functions, a condition that could be ameliorated by host DL (i.e. similar to other detoxifying enzymes) (Karsten et al., 1996; Nishiguchi and Somero, 1992). However, to date DL has only been described in the algal component (Yost and Mitchelmore, 2009).

DMSP and/or DL levels have been shown to respond to oxidative stressors, including copper, in marine algae (Sunda et al., 2002). Several antioxidant functions in corals resulting from *Symbiodinum* spp. DMSP and DL activities have been recently inferred (Jones et al., 2007; Yost and Mitchelmore, 2009). Given that DMSP is at mM concentrations within the symbiotic algal cells, DMSP may be a critical and multifaceted component in a coral's overall antioxidant defense mechanism. If DMSP is being used as an antioxidant under conditions of oxidative stress, levels may decrease following initial or chronic exposures as DMSP and its conversion products are being used as ROS scavengers. Indeed, we found that levels of $DMSP_p$ decreased following exposure to 5 and 10 μg L^{-1} copper. Conversely, in response to excess ROS, higher intracellular concentrations of DMSP may result from an up-regulation (increased production) of DMSP. For example, alterations in intracellular concentrations of DMSP have been linked to oxidative stressors including high Cu^{2+} (ions) in marine algae (Sunda et al., 2002, 2005). This potential up-regulation could explain why levels of $DMSP_p$ did not differ from control levels at the highest copper dose. The threshold for the up-regulation of DMSP in response to oxidative stress is unknown and may reflect ROS levels, or occur once a low, threshold level of DMSP is reached. Therefore, changes in DMSP levels in corals may be complex and biphasic, particularly given the nature of the symbiotic relationship, as DMSP (and its conversion products) can be diffusively or actively passed from the algae to the host cells.

Current protocols for DMSP in corals measure $DMSP_t$, which may limit the physiological significance of DMSPs response to stressors in coral-algal symbioses. Complex DMSP responses and physiological implications were shown in our investigation of multiple indices and the teasing apart of $DMSP_t$ and $DMSP_p$; responses which would have been missed using current protocols. Corals showed a decrease in $DMSP_t$ concentrations per cm^2 and loss of algal cells with increasing concentrations of copper, although only significant at the highest dose. When $DMSP_t$ levels were normalized to algal cell number, no significant reduction in $DMSP_t$ at the highest copper dose was evident. Therefore, results based solely on $DMSP_t$ levels normalized to coral surface area (commonly used in the coral field) may simply reflect algal cell loss. Indeed, the loss of algae between controls and high copper dose averages 17%, mirroring the average 23% loss of $DMSP_t$. Analyzing intact coral fragments for $DMSP_t$ analyses would preclude accurate quantification of algal cell numbers so that these indice correlations could not be made.

A markedly different response to copper was observed in the algal cells alone. $DMSP_p$ levels, per surface area or per algal cell, both decreased an average of 70% at the lower two copper doses compared to controls. These decreases may be due to a decrease in intracellular DMSP pools (as previously discussed) and/or an increase in DL, where the up-regulation of the enzyme could be accomplished through increased DL biosynthesis or changes in DL affinity for the DMSP substrate under conditions of oxidative stress. However, there was no reduction in $DMSP_p$ concentrations at 50 $\mu g\ L^{-1}$ Cu, i.e. the exposure at which $DMSP_t$ (on a per surface area basis) and algal cell contents were significantly reduced. This indicates that, even with fewer zooxanthellae at the highest copper exposure, DMSP concentrations in the remaining algae likely increased or at least were maintained (via increased production) at the original control levels. Fig. 4 demonstrates only a slight $DMSP_p$ increase (i.e. 19.6-21.1 fmol $cell^{-1}$), although elevated production will be missed if the algae are translocating DMSP to the host, which in turn is being used to maintain the steady levels seen in the $DMSP_t$ response (on a per algal basis). These results suggest that $DMSP_p$ levels are responsive to copper exposure.

Interestingly, similar trends emerged for $DMSP_p$ (nmol cm^{-2}, fmol $cell^{-1}$), chl-*a* and protein concentrations, all of which were reduced at 5 and 10 μg L^{-1} Cu but returned to control levels at 50 μg L^{-1} Cu. It is known that bleaching can reduce algal number and/or chl-*a* concentrations (Gleason and Wellington, 1993; Porter et al., 1989). Also, copper exposure has been reported to cause reductions in photosynthetic pigments (chl-*a*)/efficiency as well as algal cell loss (bleaching) and even increases in total carotenoids, implying oxidative damage to lipids (Droppa and Horvath, 1990; Jones, 1997; Sunda et al., 2002; Grant et al., 2003). However, chl-*a* concentrations are known to increase on a per cell basis in bleached corals and *M. franksi* specifically (Fitt et al., 1993; Edmunds et al., 2003). Nystrom et al. (2001) reported no copper (11 μg L^{-1}) effects on acutely exposed corals, though corals pre-exposed to increased levels of copper had significant reductions in gross primary production. Others report no decrease in chl-*a* (Brown, 2000; Grant et al., 2003) and no decrease in photosynthetic capacity in corals chronically exposed to copper (Grant et al., 2003), though excess copper usually decreases photosynthesis (Droppa and Horvath, 1990). A parallel increase in $DMSP_p$ at 50 μg L^{-1} Cu and an increase in chl-*a* (relative to the lower copper doses) may indicate that DMSP and its conversion products are acting as a protective mechanism, scavenging ROS at the site of production in zooxanthellae, a hypothesis worthy of further investigation. Additionally, whether up- or down-regulation (or loss) of chl-*a* is occurring, these data demonstrate that this commonly used algal DMSP indice may not be appropriate when investigating levels in response to 'natural' or anthropogenic stressors.

The reduction of total protein at 5 and 10 μg L^{-1} Cu does not appear to be explained by zooxanthellae loss, as protein concentrations were higher (respectively) at 50 μg L^{-1} Cu, the only exposure at which significant algal loss was recorded. There are several plausible explanations for the observed changes in total protein. Proteins can be up- or down-regulated, damaged due to ROS, or degraded. The DMSP pool may be controlled by the availability of methionine, via protein degradation (a process known to occur in times of increased stress, i.e. as evidenced by elevated heat shock proteins and ubiquitin levels in stressed corals; Downs et al., 2000, 2002), therefore liberating this key amino acid and providing the necessary building blocks for increased DMSP synthesis.

One point of concern raised in this study is the appropriateness of algal indices that are commonly used to normalize DMSP concentrations in corals (see for example, Broadbent et al., 2002; Edmunds and Gates, 2002; Hill et al., 1995, 2000; Van Alstyne et al., 2006; Yost and Mitchelmore, 2009). Our data clearly show that these indices change in response to oxidative stress and cannot be used when investigating DMSP-related questions in a toxicological arena. This includes investigations of stressors (e.g. solar irradiance, temperature or chemical pollutants) that are known to alter these parameters. It is also noted that indices such as coral surface area can limit the interpretation of DMSP concentrations as, for example, DMSP concentrations per surface area may decrease in some cases simply as a result of algal cell loss. Furthermore, our analysis of $DMSP_p$ and $DMSP_t$ levels demonstrates that algal physiological responses (i.e. use (loss) or alternately up-regulation of $DMSP_p$) may be masked by DMSP levels in coral host cells ($DMSP_t$).

5.4.1 Conclusion

In conclusion, *M. franksi* corals exposed to environmentally relevant concentrations of copper exhibited changes in both $DMSP_t$ and $DMSP_p$ concentrations in response to increased physiological stress. Additionally, common indices used to normalize DMSP concentrations in coral changed significantly (chl-*a*, protein, algal cell number) with copper exposure, highlighting the importance of normalizing DMSP to indices that remain unchanged throughout the duration of toxicological studies (e.g. coral surface area). Common DMSP normalization indices may not be operable in coral DMSP studies due to the complex nature of the algal-animal symbiosis. The added complexity of toxicological research also limits the available DMSP normalization indices because the indices themselves often respond to stress. These data make a strong case for further investigations that aim to quantify both symbiont and host DMSP concentrations in an effort to better understand the role and function of DMSP in corals. Our data for $DMSP_p$ and chl-*a* at the highest copper dose suggest that DMSP may serve as a key antioxidant in zooxanthellae (and potentially their host coral), ameliorating the effects of ROS production in response to copper exposure. Finally, higher $DMSP_t$ versus $DMSP_p$ concentrations are suggestive of DMSP translocation from symbiont to host, but further experiments are needed to substantiate this. While the underlying physiological function(s) and regulation of DMSP in corals remains undetermined, we present evidence that DMSP may contribute to the inherent antioxidant systems present in zooxanthellae, thus serving a supportive antioxidant role.

5.5 Tables

Table 5.5.1 – Impact of copper on chlorophyll *a*.

(Table 1 in text). Impact of copper exposure on chlorophyll *a* indices in *M. franksi* (mean ± SE).

Copper conc. (μg L^{-1})	n	Chlorophyll a (pg)		
		algal $cell^{-1}$	surface area (cm^{-2})	total protein (mg^{-1})
<1	20	6.7±0.5	17.0±1.5	7.5±0.9
5	20	4.7±0.2*	11.7±0.7*	11.3±1.6*
10	20	5.0±0.3*	11.8±1.2*	11.1±1.3*
50	20	7.3±0.9	13.3±0.7	6.5±0.5

* $p<0.05$ compared to controls.

Table 5.5.2 – Impact of copper on symbiosis indices.

(Table 2 in text). Impact of copper exposure on symbiosis indices in *M. franksi* (Pearson's correlation coefficient; all *p*-values<0.001 except where indicated; *n*=20 per indice).

	Copper exposure ($\mu g\ L^{-1}$)			
Indices	<1	5	10	50
Chl-*a* / algal cell	0.83	0.90	0.93	0.75
Chl-*a* / total protein	0.68	0.07 *	0.78	0.81
Chl-*a* / surface area	0.80	0.86	0.61†	0.88
Algal cells / total protein	0.81	-0.03 *	0.78	0.71
Algal cells / surface area	0.87	0.84	0.68	0.78
Total protein / surface area	0.88	0.00 *	0.36*	0.79

* *p*>0.05.
† *p*<0.01.

5.6 Figures

Figure 5.6.1 – Impact of copper on zooxanthellae density.

(Figure 1 in text). *Montastraea franksi* symbiotic dinoflagellate density ($10^6\times$) per coral surface area (cm^2) in corals exposed to <1 (control), 5, 10 and 50 μg L^{-1} Cu for 48 h. Data are $\bar{x}$ ± SE (n =20); asterisk (*) indicates significant difference compared to control ($p < 0.05$).

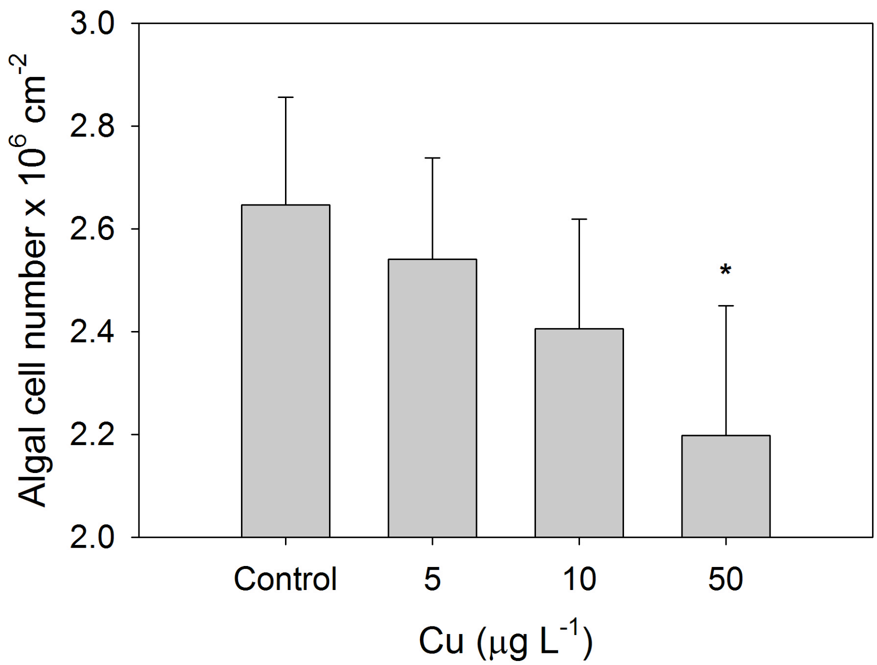


Figure 5.6.2 – Impact of copper on total protein.

(Figure 2 in text). *Montastraea franksi* total protein (mg) per coral surface area (cm^2) in corals exposed to <1 (control), 5, 10 and 50 µg L^{-1} Cu for 48 h. Data are $\bar{x}$ ± SE (n=20); asterisk (*) indicates significant difference compared to control ($p < 0.05$).

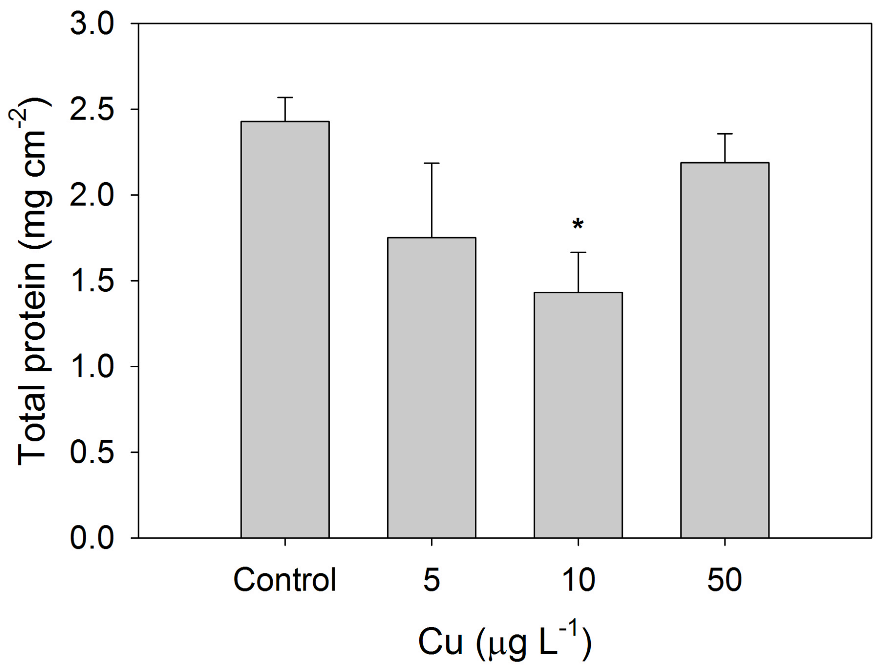


Figure 5.6.3 – Impact of copper on $DMSP_t$ and $DMSP_p$ (nmol cm^{-2}).

(Figure 3 in text). *Montastraea franksi* DMSP (nmol) per coral surface area (cm^2) for control, 5, 10 and 50 µg L^{-1} Cu exposures. Data are $\bar{x}$ ± SE (n=20); asterisks (*) indicates significant difference compared to control ($p < 0.05$).

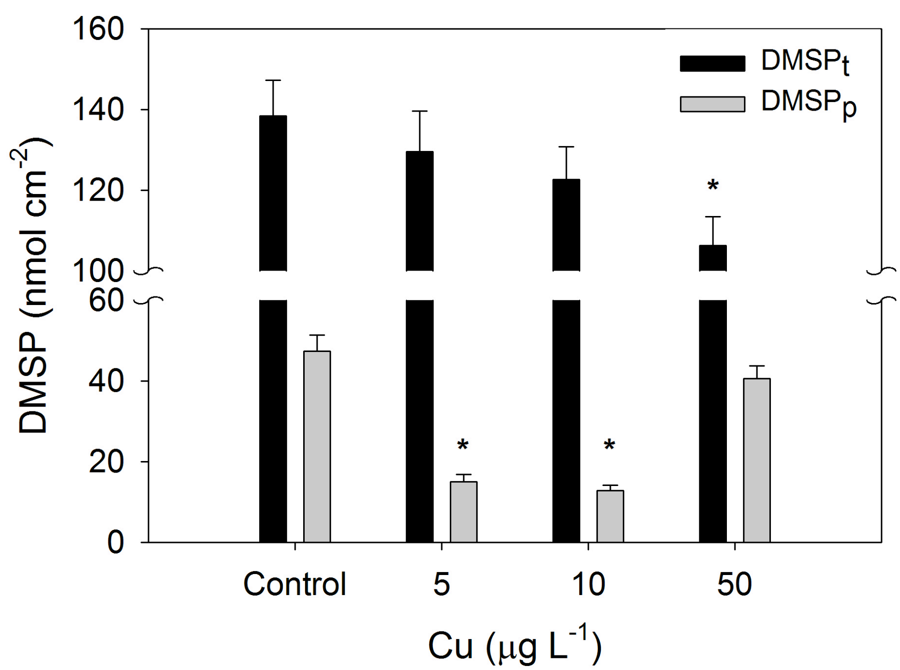
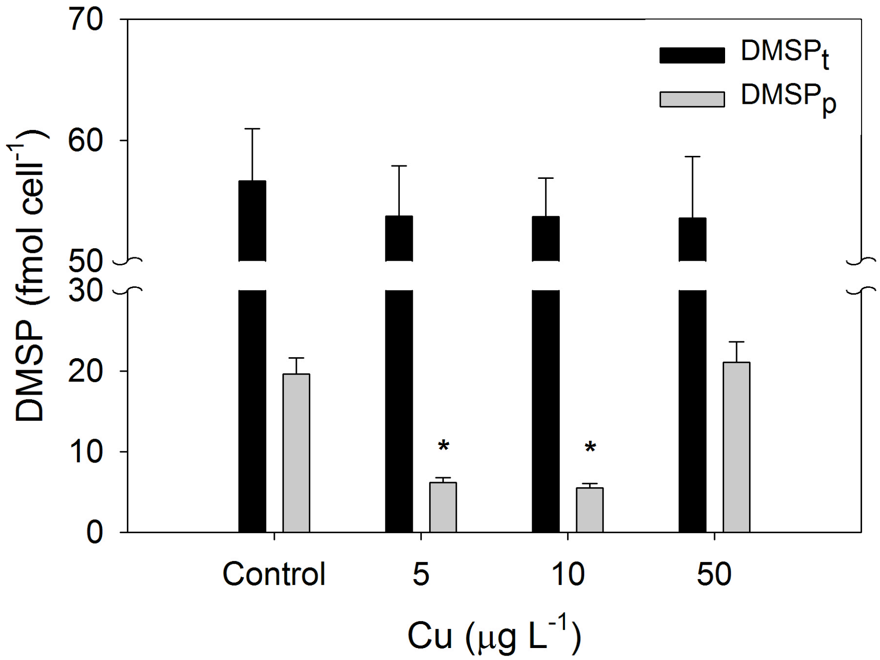


Figure 5.6.4 – Impact of copper on $DMSP_t$ and $DMSP_p$ (fmol cell^{-1}).

(Figure 4 in text). *Montastraea franksi* DMSP (fmol) per algal cell for control, 5, 10 and 50 µg L^{-1} Cu exposures. Data are $\bar{x}$ ± SE (n=20); asterisks (*) indicates significant difference compared to control ($p < 0.05$).



Chapter 6: Quantification of total and particulate DMSP in five Bermudian corals across a depth gradient

This chapter to be submitted to Coral Reefs

6.1 Introduction

Global climate change brings coral reefs into the spotlight, highlighting their potential demise if they cannot physiologically adapt given the current rates of environmental change (Hoegh-Guldburg, 1999; Hoegh-Guldburg et al., 2007). The number and severity of bleaching events (stress related dissociation of the coral-algal symbiosis) is predicted to increase (Hoegh-Guldberg, 1999; Sheppard, 2003), driven by increases in ocean temperature (Brown, 1997; Glynn, 1991, 1993) and hastened by solar stress (Hoegh-Guldberg and Smith, 1989; Dunne and Brown, 2001; Shick et al., 1996) and anthropogenic stressors (Brown, 2000; Owen et al., 2005; Schiedek et al., 2007). It is well known that corals respond differently to stressors. For example, *Acropora* corals have been subject to increased rates of disease following bleaching events, subsequently leading to high rates of mortality (Muller et al., 2008; Rogers et al., 2008). Additionally, corals have shown depth-dependent responses to stressors such as light and temperature (Richier et al., 2008; Downs et al., 2002).

Coral and algae have multiple cellular defense mechanisms to combat physiological stress, with both partners ultimately contributing to the sensitivity or resilience of the integrated symbiosis. Corals typically inhabit warm, high light, shallow locations that facilitate photosynthesis by their photosynthetic algal symbionts. They are thus susceptible to environmental and anthropogenic variables within these habitats (e.g. elevated temperature, UV-light), that may individually or synergistically induce physiological stress in algae and/or host. Coral symbionts fix large quantities of carbon dioxide via photosynthesis, but high light levels can overwhelm their intracellular oxygen-handling capacity, resulting in oxidative stress. Both coral and algae have high levels of antioxidants (Lesser, 2006) to combat daily periods of hyperoxic conditions due to symbiont photosynthesis (Dykens and Shick 1992; Kühl et al., 1995), but these radical scavenging systems can be overwhelmed by synergistic effects of other stressors (temperature, UV-light, pollution, disease), compromising cellular integrity (Downs et al., 2002). For example, generation and accumulation of reactive oxygen species (ROS) often occurs as a result of the impairment of photosynthesis by high temperature and solar irradiance and it has been stated that oxidative stress may play a pivotal role in coral bleaching (Downs et al., 2002). Corals show general declines in the activity of antioxidants such as superoxide dismutase (SOD) with increasing depth, a response also seen for SOD, catalase and ascorbate peroxidase in symbiotic zooxanthellae, suggesting a simultaneous reduction in antioxidant power with a decreased potential for photooxidative stress (Shick et al., 2004). In contrast, mean concentrations of the antioxidants GSH, SOD and small heat-shock proteins have been shown to be significantly higher with increased depth (Downs et al., 2002).

Historically, distributions and diversity of corals in Castle Harbor Bermuda (near our study site) were more extensive than they are currently, primarily due to anthropogenic disturbances that have reduced water quality (Dryer and Logan, 1978;

Flood et al., 2005). A coral's sensitivity to bleaching may be attributed to host or symbiont characteristics and the debate over studies indicating that corals may respond to environmental stress by switching their compliment of symbionts (Buddemeier and Fautin, 1993) has led to numerous investigations focusing on physiology of symbionts (Goulet et al., 2005; Ulstrup et al., 2007; Winters et al., 2009). Evidence suggests that tolerance to environmental stress among genetically distinct algal partners may explain variability in bleaching patterns (Fitt and Warner, 1995; Rowan et al., 1997), although stress tolerance of the host corals themselves cannot be discounted (Brown et al., 2002; Bhagooli and Hidaka, 2003). Near Bermuda, the *Diploria-Montastraea-Porites* species assemblage dominates coral cover (Venn et al., 2009a). Typically associated with these corals are algal phylotypes that exhibit a range of physiological/ecological traits. For example, *Symbiodinium* phylotypes B and C have been described as sun or shade specialists, respectively, and phylotype E has been described as a stress tolerant symbiont (Rowan 1998, Toller et al. 2001a, b). Savage (2001) showed that each of the three dominant *Symbiodinium* phylotypes in Bermudian corals (A, B, C) varied significantly in their photosynthetic responses to irradiance. However, evidence supports within-phylotype variation for several ecological characteristics including thermal tolerance and photosynthetic response to irradiance for *Symbiodinium* phylotypes (Warner et al. 1999, Iglesias-Prieto & Trench 1994).

Coral sensitivity may be partially explained by tolerances of symbionts that are linked to oxidative stress and known antioxidant responses of both host and symbiont with water depth. This study aimed to quantify the algal generated metabolite DMSP in several common hard corals across a depth gradient. *Symbiodinium* produce high intracellular levels of dimethylsulfoniopropionate (DMSP; at mM cellular concentrations), a multifaceted algal metabolite that is also found in many other species of marine algae (Keller et al., 1989; Keller and Korjeff-Bellows, 1996; Van Alstyne et al., 2006; Yoch, 2002). The potential importance and diverse functionality of DMSP in marine algae is well documented (Havill et al., 1985; van Diggelen et al., 1986; Kirst, 1990; Karsten et al., 1996; Wolfe et al., 1997; Stefels, 2000; Sunda et al., 2002; Evans et al., 2006; DeBose et al., 2008). Sunda et al. (2002) proposed that DMSP, along with its conversion products, might form an antioxidant cascade that is more effective at scavenging highly damaging hydroxyl radicals than the well-known antioxidants ascorbate and glutathione. For example, through DMSP-lyase conversion, DMSP and its enzymatic breakdown products can scavenge ROS in multiple cellular compartments and photosynthetic membranes where harmful radicals are generated (Sunda et al., 2002; Lee and de Mora, 1999a, b). This is particularly relevant given the very high cellular concentrations of DMSP reported in algal cells, and recently also in coral host tissues (see Yost et al., 2010). Environmental factors including solar UV radiation, light intensity, and availability of limiting nutrients have been shown to influence intracellular DMSP levels, often resulting in increased $DMSP_p$ concentrations or DMSP lysis (via DMSP-lyase), indicating that DMSP is in fact responsive to such stressors (Bucciarelli and Sunda, 2003; Sunda et al., 2002; Sunda et al., 2005). Regardless of its specific role(s), findings common to many studies suggest that DMSP is not only multifunctional, but that it plays a dominant protective physiological role in algae.

Recent reports indicate that substantial amounts of DMSP and dimethylsulfide (DMS, a DMSP conversion product and volatile gas) production are associated with coral reefs, especially when corals are faced with environmental stressors, suggesting that these ecosystems are important ecological sources of such compounds (Broadbent et al., 2002). For example, bleached colonies of *Acropora formosa* show significantly greater DMSP concentrations per zooxanthellae (436 fmol $cell^{-1}$) than unbleached colonies (171 fmol $cell^{-1}$) and Jones et al. (2007) reported significant DMS and DMSP correlations with sea surface temperatures up to 30 °C. Yost et al. (2010) showed that levels of DMSP in the coral *Montastraea franksi* significantly changed in response to copper-induced oxidative stress. Furthermore, Sunda et al. (2005) postulated that an observed increase in DMSP:chl-*a* in dinoflagellates represented a physiological increase in intracellular DMSP in response to solar-induced oxidative stress. These reports are of importance when considering the potential role of DMSP in coral symbioses because they signify a potential link between DMSP production and function. Cellular damage by ROS has been implicated in the physiological mechanism underlying coral bleaching (see for example Lesser et al., 1990, Downs et al., 2002) and both DMSP production and DMSP-lyase enzymatic activity appear to vary among zooxanthellae types (Yost and Mitchelmore, 2009). Thus, while an antioxidant role for DMSP is not the only potential function for this compound in coral symbioses, it is a plausible and potentially extremely important one.

While DMSP in zooxanthellae appears to be a substantial metabolite, little is known about the potential functions, quantities, partitioning (between symbiont and host) or distributions of DMSP within corals. Only a few studies have quantified DMSP in field-collected stony corals (Jones et al., 1994; Broadbent et al., 2002; Hill et al., 1995; Van Alstyne et al., 2006). These studies suggest that there is great variability in DMSP concentrations among coral species and Van Alsytne et al. (2006) reported that the amount of DMSP within cnidarians is correlated with symbiont density. However, only total (algae and host) DMSP values have been included in field-collected coral assessments, which are limited to Australia, USA (Hawaii) and Guam (Jones et al., 1994; Broadbent et al., 2002; Hill et al., 1995; Van Alstyne et al., 2006). Further investigations of DMSP levels in the algae and host would potentially aid in further understanding its biochemical and physiological significance and role(s) in the symbiosis. Responses may be missed using $DMSP_t$ measures of the intact symbiosis alone (as shown in Yost et al., 2010).

Furthermore, there have been no studies characterizing how DMSP in corals (unlike with other antioxidants and biological endpoints) might vary with water depth. The current study considers how differences in the coral species (*Porites astreoides*, *Montastraea franksi*, *Montastraea cavernosa*, *Diploria strigosa*, *Diploria labyrinthiformis*), algal phylotypes (A, B and C) and an environmental depth gradient might influence DMSP concentrations in both algae ($DMSP_p$) and host ($DMSP_t$). DMSP concentrations along with multiple normalization indices (some of which are known to change in response to stressors and/or are limited by methodological complexities, Yost and Mitchelmore, submitted) were measured to provide further insight into the production and accumulation of DMSP within zooxanthellate corals.

6.2 Materials and Methods

Coral samples were collected along an approximately 1,000 m transect just southeast of Castle Harbor, Bermuda at depths of 4, 12, 18 and 24 m (4 m: 32°20’20.33”N, 64°39’44.39”W; 12 m: 32°20’10.82”N, 64°39’38.70”W; 18 m: 32°20’1.25”N, 64°39’24.30”W; 24 m: 32°19’51.60”N, 64°39’25.42”W) in June, 2009. The exact collection sites were not monitored for incident radiation; however, long-term studies in Bermuda indicate 90% of surface irradiance penetrating to 5 m, 40% to 10 m and 20% to 15 m (R. Smith pers. comm. in Savage et al., 2002b). Mean water temperatures (°C) in Bermuda for sites similar to those sampled were as follows (T. Noyes, pers. comm.): 5 m, 25.6°; 12 m, 25.0°; 16 m, 24.3°; all temperatures varied < 1°.

Coral explants were removed from parent colonies using a hammer and chisel and kept in individual plastic bags with ambient seawater on ice. Corals were transported back to the Bermuda Institute of Ocean Sciences where they were wrapped in two layers of aluminum foil and flash frozen in liquid nitrogen. Corals were then shipped on dry ice and stored at -80 °C (for up to 1 month) until analysis of DMSP (and associated indices) was conducted at the Chesapeake Biological Laboratory, Solomons, MD. Corals were analyzed after storage at -80 °C because this type of handling was shown to be an appropriate technique in other cnidarians, where DMSP concentrations in the frozen cnidarian tissues did not statistically differ from the extracted, freshly collected cnidarian tissues (Hill et al. 2004; Van Alstyne et al. 2006; Yost and Mitchelmore, 2009; Yost et al., 2010). Additionally, storage at -80 °C allows for logistically feasible coral collection, limited coral handling, processing and preparation times and minimal DMSP loss during airbrushing (due to very small amounts of homogenate froth).

6.2.1 Airbrushed coral sub-fragment

A slight modification of the coral homogenization technique as detailed in Broadbent et al. (2002) was used (see also Yost et al., 2010), which had been used previously to obtain DMSP and DMS quantities in corals, *Symbiodinium* spp. and macroalgae. This technique was chosen as it allows for comparisons between host *and* algal DMSP concentrations and expansion of the normalization indices that could be used. We previously found that similar $DMSP_t$ values were obtained for corals prepared for DMSP analyses by this method in comparison to intact coral procedures (Yost and Mitchelmore, submitted). Individual coral fragments were removed from -80 °C storage and immediately airbrushed while still frozen (Szmant and Gassman, 1990) using sterile artificial seawater to obtain a homogenate that was collected into a 0.5 L plastic bag. Total homogenate volumes were measured with a volumetric cylinder. No froth layer on top of the homogenate was encountered as reported by Hill et al. (1995). Therefore a homogenizer was not used, though the sample was well mixed and homogeneous after airbrushing. Sub-samples were immediately removed using sterile pipettes for multiple endpoints including $DMSP_t$ and $DMSP_p$ analyses, total protein, chl-*a*, algal cell counts, genetic typing (clades) and cell volumes. Coral sub-fragments were dried and measured for surface area (Marsh, 1970) and polyp number indices.

Total homogenate aliquots were used for analysis of $DMSP_t$, chl-*a*, zooxanthellae diameter and number and total protein content. One mL of total homogenate was added to two mL 100% MeOH (HPLC grade) in a headspace vial, to measure $DMSP_t$. To isolate the algal component from the sub-fragment homogenate, each sample was processed as follows. One mL of homogenate was passed through a GF/F filter at very low pressure (<25 mm Hg; Steinke et al. 2000) and the filter was placed immediately in a headspace vial containing 2 mL 100% MeOH, for $DMSP_p$ analysis. After 24 h, 1 mL of each respective MeOH extract sample was mixed with 1 mL 10 N NaOH in a headspace vial and incubated for 24 h to quantitatively hydrolyze DMSP to DMS. Filtration effects were not observed during sample preparation in pilot experiments (DMS did not increase in filtrates with an increasing number of cells filtered), indicating that there was no loss of DMSP from the algal cells during filtration.

6.2.2 DMSP analyses and DMS calibration

DMSP was quantified by headspace analysis of DMS following DMSP conversion to DMS by alkaline hydrolysis. All analyses were conducted after a 24 h equilibration period following NaOH (5M final) addition. During the equilibration period, all samples were stored in the dark at room temperature. Samples were analyzed for DMS concentration in the headspace with a Hewlett-Packard 5890 Series II gas chromatograph (GC) fitted with a Chromosil 330 packed column (Supelco, Bellefonte, PA), and flame photometric detector (FPD). System temperature settings were: injector 150 °C, column oven 60 °C and detector 175 °C. Nitrogen gas was the carrier (60 cm^3 min^{-1}) and air (60 cm^3 min^{-1}) and hydrogen (50 cm^3 min^{-1}) were flame gases. Data were collected and analyzed using HP ChemStation (Hewlett – Packard, Palo Alto, CA). Known concentrations of DMSP (purchased from Research Plus Inc., Bayonne, NJ) were diluted in sterile water to give working solutions, which were frozen in small aliquots at -80 °C. Two different standard curves (one each paralleling $DMSP_p$ or $DMSP_t$ procedures) of serial dilutions of DMSP were used to construct the calibration curves (using the square-root of the peak area) and linear regressions served to convert peak areas from GC headspace measurements to DMS concentrations. The standards were prepared in identical proportions of buffers and preparation solutions to those used in experimental samples. The same total liquid volume (2 mL) was used in all headspace vials. The precision of DMS analysis varied <5% and headspace storage trials showed no losses occurred with the analytical methods employed. Detection limit of the GC was 1 nmol DMS.

6.2.3 Algal indices

Using a haemocytometer and epifluorescent microscope, algal cell counts were conducted immediately using total homogenate aliquots diluted with sterile artificial seawater. Algal cell sizes (to calculate cell volume) were determined using a microscope, eyepiece graticule and calibrated ocular micrometer (samples were preserved in 5% buffered formalin; n=30 per sample). Cell volumes were calculated assuming that zooxanthellae are spherical; dividing cells were excluded from sizing measurements.

Chl-*a* was measured to serve as an index of zooxanthellae density. Briefly, 1 mL of each whole tissue homogenate was filtered through a Whatman GF/F glass fiber filter and extracted in 90% acetone for 24 h at 4 °C (Parsons et al. 1984) before being measured fluorometrically with a Trilogy Laboratory Fluorometer (Turner Designs, Sunnyvale, CA). Alternatively, for chl-*a* samples extracted in methanol, whole tissue homogenate samples (both unfiltered and filtered as above) or intact sub-fragments were measured fluorometrically after calibration of chl-*a* standards in absolute methanol (Holm-Hansen and Riemann 1978). To ensure that all samples were measured in the linear portion of the calibration curve, additional dilutions were carried out in absolute methanol (or 90% acetone for the method above), if necessary. Phylogenetic (clade) analysis of zooxanthellae was determined by length heteroplasmy in domain V of chloroplast large subunit (cp23S)-rDNA (Santos et al. 2003).

6.2.4 Additional indices

The surface areas and polyp numbers of the coral skeletons, used for either the intact or homogenate coral endpoints, were determined using the aluminum foil

(Marsh, 1970) and polyp count techniques (Broadbent et al., 2002). Homogenate subsamples for protein analysis were frozen, thawed and quantified for total protein by the BCA assay (Pierce Chemical). Bovine serum albumin was used to construct a standard curve.

6.2.5 Statistical Analyses

All data were checked for normality and homogeneity of variances prior to statistical analysis and data were log transformed as necessary. All data were analyzed using analysis of variance (ANOVA) with Tukey's post-hoc tests to identify significant differences within a coral species for $DMSP_p$ or $DMSP_t$ concentrations normalized to coral surface area, protein and zooxanthellae cell volume as well as $DMSP_p$ or $DMSP_t$ concentrations versus *Symbiodinium* phylotype. T-tests were used to assess differences within a coral species for zooxanthellae cell density, zooxanthellae cell volume and zooxanthellae chl-*a* values. Pearson's correlations were used for within-species correlations between depth and $DMSP_p$ or $DMSP_t$ normalized to zooxanthellae cell volume, (per) zooxanthellae cell, zooxanthellae chl-*a*, coral surface area and protein. All statistical analyses were conducted using Minitab© v. 10 (Minitab Inc. 2000), with $\alpha = 0.05$ for all tests.

6.3 Results

In each of the coral species analyzed (Table 1), $DMSP_t$ concentrations were consistently higher than $DMSP_p$ concentrations. $DMSP_p$ and $DMSP_t$ concentrations normalized to coral surface area, protein (coral biomass) or zooxanthellae cell volume were significantly different with increasing water depth for most coral species investigated. *D. strigosa* showed a pattern of $DMSP_p$ and $DMSP_t$ decline with increasing depth (Fig. 1, 2). *D. labyrinthiformis* was the only coral species to show significant declines in $DMSP_p$ and $DMSP_t$ values from 4 to 12 m, with significant increases in both $DMSP_p$ and $DMSP_t$ up to 24 m (Fig. 1, 2). *M. cavernosa* $DMSP_p$ and $DMSP_t$ significantly decreased with increasing depth when normalized to coral surface area (Fig. 1). However, there were no significant differences in either $DMSP_p$ or $DMSP_t$ when these values were normalized to coral protein (biomass) (Fig. 2). *M. franksi* showed the least amount of variability for $DMSP_p$ and $DMSP_t$ concentrations across depths (Fig. 1, 2). However, $DMSP_p$ per cm^{-2} significantly decreased with increasing depth for *M. franksi* (Fig. 1). *P. astreoides* $DMSP_p$ and $DMSP_t$ per cm^{-2} significantly decreased from 4 to 12 m (Fig. 1). $DMSP_t$ remained unchanged thereafter up to 24 m (Fig. 1). $DMSP_p$ per *P. astreoides* biomass also showed a significant decrease from 4 to 12 m, with subsequent significant increases at 18 and 24 m.

D. labyrinthiformis $DMSP_p$ and $DMSP_t$ per zooxanthellae cell volume showed significant increases between 4 and 24 m (Fig. 3). *D. strigosa* $DMSP_p$ per zooxanthellae cell volume was significantly higher at 18 m versus 4 and 12 m and showed a significant decrease at 24 m (Fig. 3).

Within a coral species, zooxanthellae densities showed no significant statistical differences with increasing depth (Table 2a.) Zooxanthellae cell volumes showed a general pattern of decline (all species) across the four depths, and, for each coral species were significantly different in 12 of the 20 comparisons (Table 2b). *M. cavernosa* had the highest cell volume among the corals investigated (Table 2b). Chl-*a* per cell did not vary within a species with increasing water depth, except in 2 of the 4 comparisons (4 or 12 m versus 24 m) for *D. strigosa* (Table 2c). *Symbiodinium* phylotype remained unchanged across the depth gradient, with *D. labyrinthiformis*, *D. strigosa* and *M. franksi* hosting phylotype B, *M. cavernosa* hosting phylotype C and *P. astreoides* hosting phylotype A (Table 2d).

The impact of the depth gradient on $DMSP_p$ and $DMSP_t$ concentrations normalized to multiple indices was significant for at least two comparisons per coral species (Table 3). Most correlations were negative, except for *D. labyrinthiformis* correlations (Table 3). Both $DMSP_p$ and $DMSP_t$ values per cm^{-2} showed significant, negative correlations across the depth range in *D. strigosa* and *M. cavernosa* (Table 3). This relationship also occurred in *M. franksi* for $DMSP_p$ values and in *P. astreoides* for $DMSP_t$ values (Table 3). $DMSP_p$ and $DMSP_t$ concentrations indexed to chl-*a* showed significant and negative correlations with increasing water depth for *D. strigosa* and *M. franksi* (Table 3). *D. strigosa* was the only coral species showing a significant, negative correlation between $DMSP_p$ per coral biomass with increasing depth (Table 3). *M. cavernosa* was the only coral species showing a significant, negative correlation between $DMSP_t$ per zooxanthellae and increasing depth (Table 3). In *D. labyrinthiformis*, both $DMSP_p$ and $DMSP_t$ per zooxanthellae cell volume showed significant, positive correlations across depth (Table 3). *P. astreoides* also showed a significant, positive correlation for $DMSP_p$ per zooxanthellae cell volume with bathymetric decline (Table 3).

Among algal phylotypes, $DMSP_p$ and $DMSP_t$ concentrations per zooxanthellae cell were significantly different between phylotypes B and C at a depth of 4 m (Table 4). Additionally, $DMSP_t$ concentrations per zooxanthellae cell were significantly different between phylotypes B and C at a depth of 12 m (Table 4). However, these differences were not found when DMSP concentrations were normalized to zooxanthellae cell volume (ANOVA, $P > 0.05$), except that phylotypes B and C were significantly different at a depth of 18 m on a $DMSP_t$ per zooxanthellae cell volume basis (Table 4).

6.4 Discussion

The purpose of this study was to examine the distribution of $DMSP_p$ and $DMSP_t$ concentrations across a depth gradient in dominant, Bermudian reef-building corals. DMSP was detected in all coral species sampled, providing further insight into the production and accumulation of DMSP within zooxanthellate corals. The explanations for DMSP production, regulation and function in such cnidarian symbioses remain unclear, but the results of this study indicate that differences in DMSP patterns across a depth gradient occur among coral species and *Symbiodinium* phylotypes, between host and symbiont and also vary according to the DMSP normalization indices used.

$DMSP_t$ concentrations were consistently greater than $DMSP_p$ concentrations in all of the coral species investigated, indicating a possible translocation of DMSP from symbiont to host. However, it is also possible that the freezing/thawing of coral fragments and/or airbrushing may have damaged the algal cells and allowed DMSP to leak from the cells into the surrounding tissues and coral homogenate, the extent of which remains unknown. Therefore, the present $DMSP_p$ measurements may give only minimum symbiont DMSP concentrations while the $DMSP_t$ values normalized to appropriate algal indices (e.g. chl-*a*) give DMSP concentrations that are equal to or greater than the true algal cellular values. Also, we have previously shown that $DMSP_t$ values for airbrushed versus intact coral fragments were comparable indicating no significant $DMSP_t$ losses between the two major preparation techniques (Yost and Mitchelmore, submitted).

$DMSP_t$ values among coral species (53-172 fmol cell^{-1}) are in agreement with other studies that demonstrated $DMSP_t$ concentrations ranging from 21 to 2831 (fmol cell^{-1}) in multiple species of stony corals (Hill et al., 1995, Broadbent et al., 2002; Van Alstyne et al., 2006; Yost and Mitchelmore, 2010 (methods ms)). Additionally, $DMSP_t$ values normalized to algal cell volume coincide with the majority of concentrations reported by Broadbent et al. (2002) and Yost and Mitchelmore (2010) that typically ranged from less than 100 to 419 mmol $L_{cell\ volume}^{-1}$, although a few of their reported values were exceptionally high (1193-7590 mmol $L_{cell\ volume}^{-1}$). $DMSP_p$ and $DMSP_t$ concentrations generally increased or decreased in parallel and showed significant variations within the coral species across depth. These results were most apparent when DMSP values were normalized to coral surface area or total protein. Interspecifically, *D. labyrinthiformis* was notably different from the other corals investigated, showing the same general pattern of $DMSP_p$ and $DMSP_t$ increase (per coral surface area or biomass) with bathymetric decline when normalized to surface area or biomass. However, DMSP patterns were not consistent for all species when normalized to different indices. For example, DMSP concentrations in *M. cavernosa* showed significant differences across depths when normalized to coral surface area, but this was not the case when DMSP values were normalized to coral biomass.

It is interesting to note that intra-species $DMSP_t$ values were strikingly similar among the corals investigated, despite ranging in morphology from boulder-like and massive to encrusting. For example, *P. astreoides* has a very thin layer of biomass and typically grows in a hemi-spherical, encrusting-like shape (Sterrer, 1986) compared to *M. cavernosa* that has a larger biomass and a boulder-like form, yet their respective DMSP concentrations and partitioning of DMSP between symbiont and host were very similar. These results suggest that the accumulation of DMSP in host tissues may not simply be a function of tissue depth or coral topography and that both of these factors potentially influence the interpretation of DMSP concentrations within a coral species. Nonetheless, both coral surface area and biomass indices may be informative for various DMSP investigations relating to, for example, mass-transfer limited processes or coral biomass, respectively (Edmunds and Gates, 2002).

Cell volume and chl-*a* are common indices for DMSP normalizations in the DMSP field, but such parameters can change in response to environmental factors or stressors. Significant changes in zooxanthellae cell volume were important when normalizing DMSP values in the present study. Cell volumes in *M. cavernosa* were highest among the zooxanthellae investigated and all algal cell sizes generally decreased with increasing depth. These findings suggest that, in addition to the common indice of algal cell number, $DMSP_p$ concentrations should also be reported on a cell volume basis to account not only for natural variation, but also to account for other factors (potential oxidative stressors such as increased temperature, UV-light, pollution) that might influence cell volume and thereby $DMSP_p$ values. For example, Sunda et al. (2002) showed that increased $DMSP_p$ concentrations increased cell sizes in *Emiliania huxleyi* under CO_2 limitation, a known oxidative stressor. In contrast to the change in zooxanthellae cell volumes with depth, we did not find that zooxanthellae cell density varied significantly within a coral species in the present study. Savage et al. (2002b) found significantly lower zooxanthellae densities in Bermudian *M. franksi* corals from 4 to 8 m, but there are also reports of shallow water symbioses (within 4 m) with higher zooxanthellae densities compared to those at greater depths (Li et al., 2008). The lack of zooxanthellae density change in this study resulted in more significant correlations for DMSP concentrations across depth when normalized to cell volume rather than cell density. These results again highlight the need for multiple DMSP normalization indices and more specifically, the data herein illustrate that normalizing DMSP to cell number alone may prevent one from determining significant DMSP changes that also influence cell size.

The *Symbiodinium* phylotype results show no intra-colony variation in symbiont type and are in agreement with those reported by Savage et al. (2002a) for the same five coral species in Bermuda. Among the phylotypes investigated, DMSP concentrations per zooxanthellae showed significant differences between phylotype B and C symbionts ($DMSP_p$) and their respective hosts ($DMSP_t$). Within-phylotype variation occurred between *D. labyrinthiformis* and *D. strigosa* corals that both host phylotype B algae. Both of these brain-like corals have similar depth distributions, occurring over a range of 1-30 m (Logan, 1988). However, as noted previously, *D. labyrinthiformis* showed a pattern of increasing $DMSP_p$ and $DMSP_t$ values with depth, whereas *D. strigosa* showed the opposite pattern. *Symbiodinium* phylotypes have been described as invasive/opportunistic (phylotype A), sun or shade specialists (phylotype B and C, respectively) and stress tolerant (phylotype E) (Rowan 1998, Toller et al. 2001a, b). However, evidence supports within-phylotype variation for several important ecological characteristics including thermal tolerance and photosynthetic response to irradiance (Warner et al. 1999, Iglesias-Prieto & Trench 1994), which are likely to change across a depth gradient. Of particular significance to this study is the reported statistically significant variation in photosynthetic response within phylotypes A, B and C symbionts isolated from Bermudian symbioses (Savage, 2001). At 4 m depths, where total irradiance is expected to be highest, *M. cavernosa*, which rarely bleaches (Japp, 1979, 1985) and *P. astreoides*, that hosts the bleaching resistant A algal phylotype (Rowan 1998; Knowlton and Rohwer, 2003) both showed the highest $DMSP_t$ values (mmol $_{\text{Lcell volume}}{}^{-1}$) among the coral species investigated. Because they inhabit high light environments, both coral and algae have constitutive and inducible antioxidant systems to combat periods of oxidative stress due to, for example, stressful light conditions. Still, corals are known to differentially respond to such stressors. For example, the bleaching-resistant *P. astreoides* showed activated photoprotective xanthophyll cycling in response to elevated temperature/irradiance (Venn et al., 2006). Additionally, high $DMSP_t$ concentrations have been linked to conditions of oxidative stress in colonies of the stony coral *Acropora formosa*, with bleached colonies showing significantly higher DMSP levels compared to unbleached colonies (Broadbent et al., 2002). Furthermore, *M. franksi* corals under copper-induced oxidative stress conditions showed significant $DMSP_p$ decreases (fmol $cell^{-1}$ and nmol cm^{-2}) at low copper exposure (5 and 10 µg L^{-1}) but $DMSP_p$ levels at 50 µg L^{-1} were not different from controls, unlike $DMSP_t$ concentrations that significantly decreased (nmol cm^{-1}) or remained unchanged (fmol $cell^{-1}$) (Yost et al., 2010). Thus, given the variable production and accumulation of DMSP concentrations in algae and host, the significance of high $DMSP_t$ levels in *M. cavernosa* and *P. astreoides* inhabiting shallow waters (4 m) remains unknown. Van Alstyne et al. (2006) suggested that the high DMSP concentrations found in *Acropora* spp. may be explained by environmental induction of DMSP production or genetic differences (coral) or differences in the types of *Symbiodinium* within the corals. Although the genetic relatedness among corals was not assessed in this study, *Symbiodinium* phylotypes showed depth-dependent responses in DMSP production and accumulation. This suggests that symbiont, host and the environment contribute to the complexities of $DMSP_p$ and $DMSP_t$ concentrations within the integrated symbiosis.

The observed differences in DMSP concentrations between phylotypes B and C suggest that $DMSP_p$ may be differentially produced, used (i.e. converted by DMSP-lyase) or accumulated by different *Symbiodinium* phylotypes. In addition, differences in $DMSP_t$ concentrations may be reflective of host coral characteristics (potential DMSP accumulation/conversion), but further research is needed to substantiate these possibilities. It is likely that both host and symbiont ultimately contribute to the physiological concentrations of DMSP.

6.5 Tables

Table 6.5.1 – Coral sample sizes.

(Table 1 in text). Sample sizes for *Diploria labyrinthiformis*, *Diploria strigosa*, *Montastraea cavernosa*, *Montastraea franksi* and *Porites astreoides* collected at 4, 12, 18 and 24 m.

	Depth (m)			
Coral species	4	12	18	24
D. labyrinthiformis	5	6	11	10
D. strigosa	5	5	7	5
M. cavernosa	10	10	10	10
M. franksi	8	10	10	10
P. astreoides	10	10	11	10

Table 6.5.2 – Impact of depth gradient on zooxanthellae density, cell volume, chl-*a* and phylotype.

(Table 2a-d in text). *Diploria labyrinthiformis*, *Diploria strigosa*, *Montastraea cavernosa*, *Montastraea franksi* and *Porites astreoides*. Impact of depth gradient (m) on zooxanthellae cell density (cells x 10^6 cm^{-2}) (panel a), zooxanthellae cell volume (μL) (panel b), zooxanthellae chl-*a* (pg $cell^{-1}$) (panel c) and zooxanthellae (*Symbiodinium*) phylotype (panel d).

a. zooxanthellae cells density (cells x 10^6 cm^{-2}). Data are mean ± SD.

	Depth (m)			
Coral species	4	12	18	24
D. labyrinthiformis	2.47 ± 0.67	2.10 ± 1.15	2.10 ± 0.75	2.17 ± 1.16
D. strigosa	3.93 ± 1.07	5.25 ± 2.90	2.31 ± 1.11	2.69 ± 1.17
M. cavernosa	2.31 ± 1.15	1.85 ± 0.69	2.39 ± 1.74	1.66 ± 0.86
M. franksi	4.59 ± 1.25	2.53 ± 0.87	3.00 ± 2.25	3.24 ± 1.48
P. astreoides	2.11 ± 0.49	1.82 ± 0.39	1.92 ± 0.67	3.24 ± 1.48

[a] No significant statistical differences were detected across depths within a coral species.

Table 6.5.2 – continued.

b. zooxanthellae cell volume (μL). Data are mean ± SD. All comparisons were significantly different (t-test, $P < 0.05$) except where indicated.

Coral species	Depth (m)			
	4	12	18	24
D. labyrinthiformis (δ)	$7.96 \pm 7.14 \times 10^{-10}$	$6.27 \pm 6.73 \times 10^{-10}$	$5.99 \pm 8.33 \times 10^{-10}$	$5.65 \pm 7.65 \times 10^{-10}$
D. strigosa (†)	$7.52 \pm 8.26 \times 10^{-10}$	$5.96 \pm 6.03 \times 10^{-10}$	$5.38 \pm 7.66 \times 10^{-10}$	$5.80 \pm 6.91 \times 10^{-10}$
M. cavernosa (* ‡)	$10.9 \pm 1.24 \times 10^{-9}$	$10.4 \pm 1.22 \times 10^{-9}$	$9.73 \pm 1.15 \times 10^{-9}$	$9.40 \pm 1.02 \times 10^{-9}$
M. franksi (‡)	$6.13 \pm 6.33 \times 10^{-10}$	$6.57 \pm 8.10 \times 10^{-10}$	$5.19 \pm 6.20 \times 10^{-10}$	$5.21 \pm 7.25 \times 10^{-10}$
P. astreoides (δ † ‡)	$9.22 \pm 7.90 \times 10^{-10}$	$6.62 \pm 1.08 \times 10^{-9}$	$6.75 \pm 9.94 \times 10^{-10}$	$6.65 \pm 1.14 \times 10^{-9}$

[a] All means are μL x 10^{-7}.
[b] For each sample (see Table 1), n = 30 cells sized.
[c] Symbols indicate no significant statistical differences (t-test, $P > 0.05$) between pairwise depths (m) within a coral species: 4/12 = *; 12/18 = δ; 12/26 = †; 18/26 = ‡.

Table 6.5.2 – continued.

c. zooxanthellae chl-*a* (pg $cell^{-1}$). Data are mean ± SD.

Coral species	Depth (m) 4	12	18	24
D. labyrinthiformis	5.8 ± 2.1	3.5 ± 1.8	4.4 ± 1.5	6.2 ± 2.3
D. strigosa († **)	3.7 ± 0.5	3.7 ± 2.2	2.5 ± 1.3	4.1 ± 1.3
M. cavernosa	3.5 ± 1.1	3.9 ± 0.8	4.1 ± 1.8	4.7 ± 2.6
M. franksi	3.5 ± 2.4	2.9 ± 0.9	7.9 ± 2.7	4.7 ± 2.2
P. astreoides	4.0 ± 3.1	7.7 ± 4.9	2.4 ± 1.2	2.3 ± 0.5

[a] Symbols indicate significant statistical differences between pairwise depths (m) within a coral species:12/26 = † (t-test, P < 0.000; 4/26 = ** (t-test, P < 0.05).

Table 6.5.2 – continued.

d. zooxanthellae (*Symbiodinium*) phylotype. Sample size is indicated in ( ).

	Depth (m)			
Coral species	4	12	18	24
D. labyrinthiformis	B (3)	B (2)	B (4)	B (2)
D. strigosa	B (5)	B (5)	B (4)	B (5)
M. cavernosa	C (5)	C (3)	C (5)	C (5)
M. franksi	B (5)	B (5)	B (5)	B (5)
P. astreoides	A (3)	A (3)	A (4)	A (4)

Table 6.5.3 – Impact of depth gradient on $DMSP_t$ and $DMSP_p$.

Table 3. Impact of depth gradient (m) on $DMSP_t$ and $DMSP_p$ concentrations in *Diploria labyrinthiformis*, *Diploria strigosa*, *Montastraea cavernosa*, *Montastraea franksi* and *Porites astreoides* normalized to multiple indices. Pearson's correlation coefficient, P-value; sample sizes are indicated in Table 1.

Indices	*D. labyrinthiformis*	*D. strigosa*	*M. cavernosa*	*M. franksi*	*P. astreoides*
$DMSP_p$ (mmol $L_{cell\ volume}^{-1}$)	0.489, 0.005 *	0.088, 0.697	-0.061, 0.710	0.146, 0.381	0.331, 0.035 *
$DMSP_t$ (mmol $L_{cell\ volume}^{-1}$)	0.532, 0.002 *	0.162, 0.472	-0.191, 0.239	0.234, 0.158	0.198, 0.214
$DMSP_p$ (fmol $cell^{-1}$)	0.256, 0.157	-0.129, 0.567	-0.219, 0.174	0.079, 0.636	0.023, 0.884
$DMSP_t$ (fmol $cell^{-1}$)	0.292, 0.105	0.026, 0.907	-0.340, 0.032 *	0.178, 0.286	-0.180, 0.260
$DMSP_p$ (nmol µg chl-a^{-1})	-0.052, 0.778	-0.536, 0.010 *	0.094, 0.566	-0.341, 0.036 *	-0.110, 0.493
$DMSP_t$ (nmol µg chl-a^{-1})	-0.015, 0.937	-0.441, 0.040 *	0.004, 0.983	-0.460, 0.005 *	-0.288, 0.068
$DMSP_p$ (nmol cm^{-2})	0.180, 0.325	-0.560, 0.007 *	-0.453, 0.003 *	-0.451, 0.004 *	-0.174, 0.276
$DMSP_t$ (nmol cm^{-2})	0.188, 0.304	-0.436, 0.043 *	-0.640, 0.000 *	-0.209, 0.209	-0.453, 0.003 *
$DMSP_p$ (nmol mg $protein^{-1}$)	0.164, 0.369	-0.606, 0.003 *	0.026, 0.876	-0.276, 0.093	-0.103, 0.521
$DMSP_t$ (nmol mg $protein^{-1}$)	0.176, 0.335	-0.403, 0.063	-0.067, 0.683	-0.089, 0.596	0.292, 0.064

[a] * Indicates significant statistical difference.

Table 6.5.4 – *Symbiodinium* phylotype comparisons for $DMSP_p$, $DMSP_t$ across depth.

(Table 4 in text). *Symbiodinium* phylotype comparisons for $DMSP_p$ and $DMSP_t$ (fmol $cell^{-1}$) and $DMSP_t$ (mmol $L_{cell\ volume}^{-1}$) concentrations with statistically significant differences for a given depth (m). Data are mean ± SD.

Indice	*Symbiodinium* phylotype	n	Depth
			4 m
$DMSP_p$ (fmol $cell^{-1}$) * †	*A*	10	46.16 ± 29.27
	B	18	24.32 ± 7.66
	C	10	62.1 ± 43.5
$DMSP_t$ (fmol $cell^{-1}$) * †	*A*	10	131.3 ± 52.5
	B	18	67.92 ± 18.96
	C	10	171.6 ± 68.3
			12 m
$DMSP_t$ (fmol $cell^{-1}$) * δ	*A*	10	99.4 ± 44.8
	B	21	80.0 ± 50.6
	C	10	139.0 ± 54.1
			18 m
$DMSP_t$ (mmol $L_{cell\ volume}^{-1}$) * δ	*A*	11	149.3 ± 49.0
	B	28	195.2 ± 106.5
	C	10	105.9 ± 42.3

[a] * Indicates significant statistical difference between phylotypes B and C.
[b] † Indicates ANOVA, $P < 0.01$.
[c] δ Indicates ANOVA, $P < 0.05$

6.6 Figures

Figure 6.6.1 – Coral $DMSP_p$ and $DMSP_t$ across depth (nmol cm^{-2}).

(Figure 1 in text). *Diploria labyrinthiformis*, *Diploria strigosa*, *Montastraea cavernosa*, *Montastraea franksi*, and *Porites astreoides* $DMSP_p$ (gray bars) and $DMSP_t$ (black bars) concentrations normalized to surface area (nmol cm^{-2}) across depth (m). Letters indicate significant statistical difference within $DMSP_p$ or $DMSP_t$ concentrations (ANOVA, $P < 0.05$). Data are means ± SE.

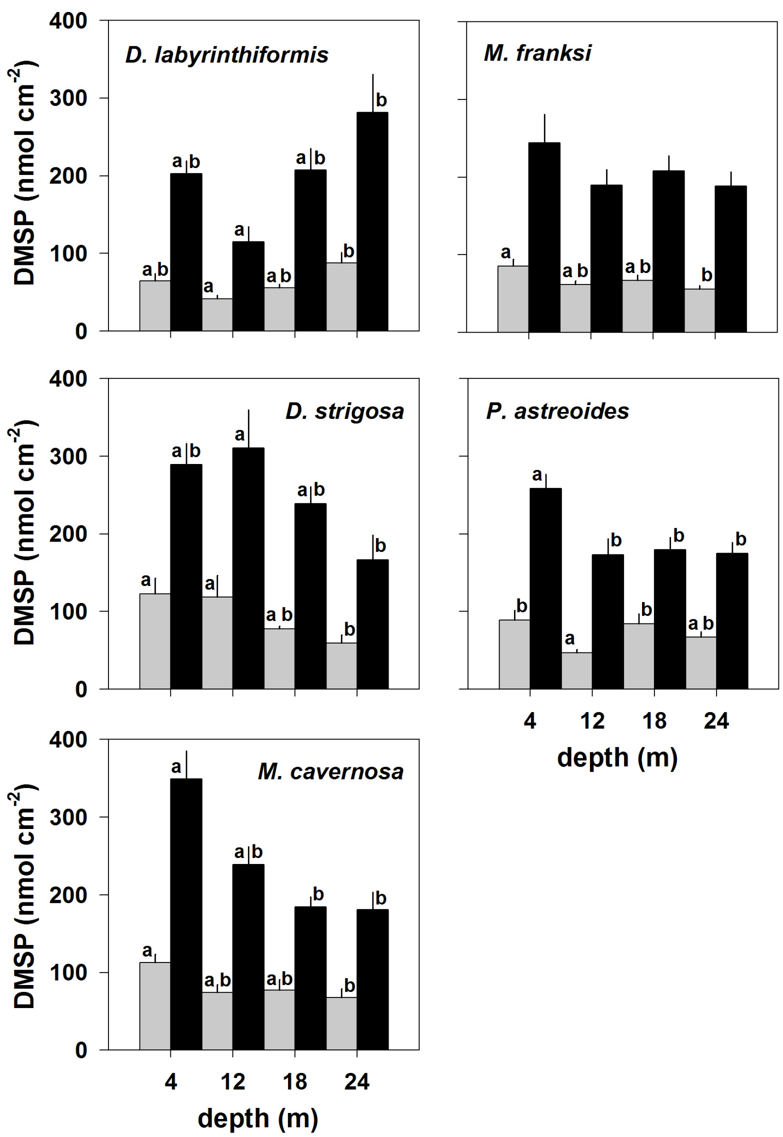


Figure 6.6.2 – Coral $DMSP_p$ and $DMSP_t$ across depth (nmol mg protein^{-1}). (Figure 2 in text). *Diploria labyrinthiformis*, *Diploria strigosa*, *Montastraea cavernosa*, *Montastraea franksi*, and *Porites astreoides* $DMSP_p$ (gray bars) and $DMSP_t$ (black bars) concentrations normalized to protein (nmol mg protein^{-1}) across depth (m). Letters indicate significant statistical difference within $DMSP_p$ or $DMSP_t$ concentrations (ANOVA, $P < 0.05$). Data are means ± SE.

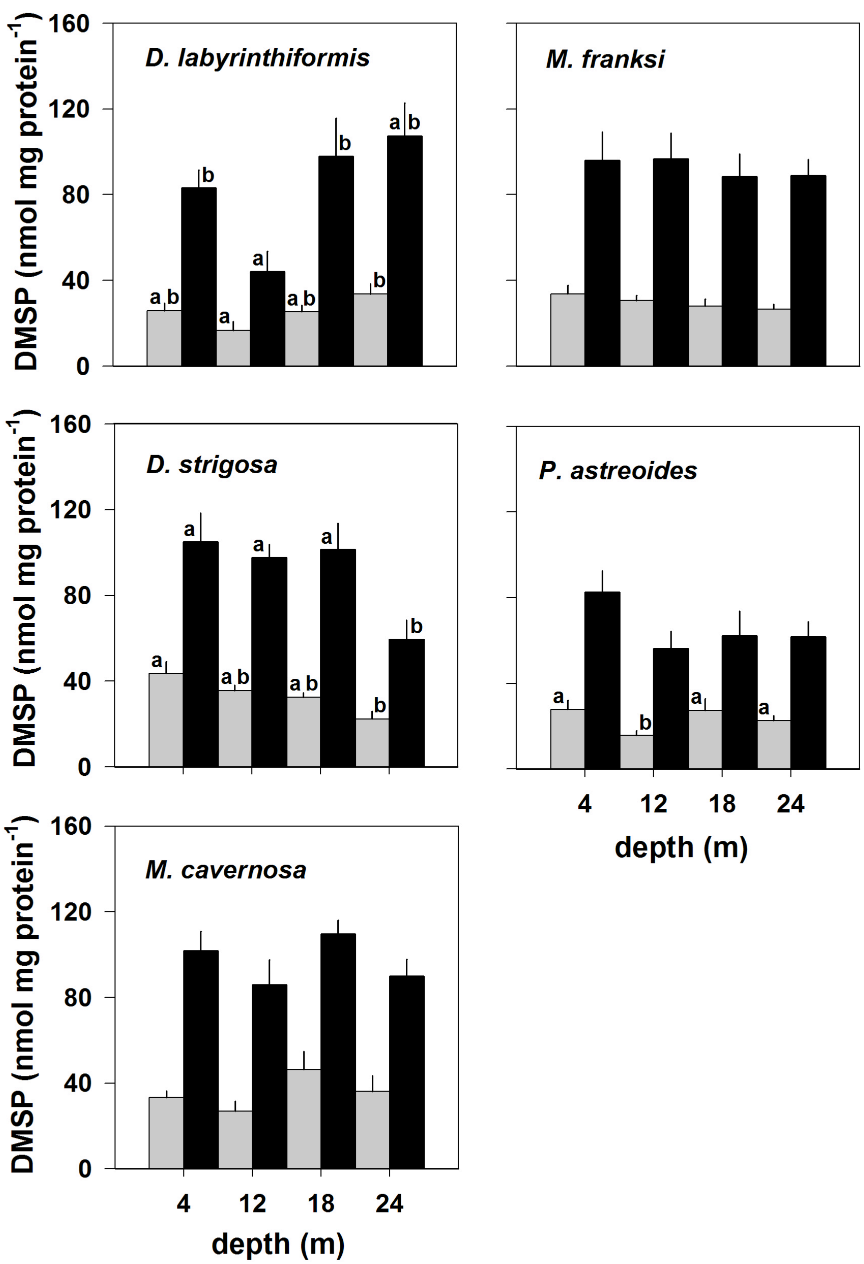
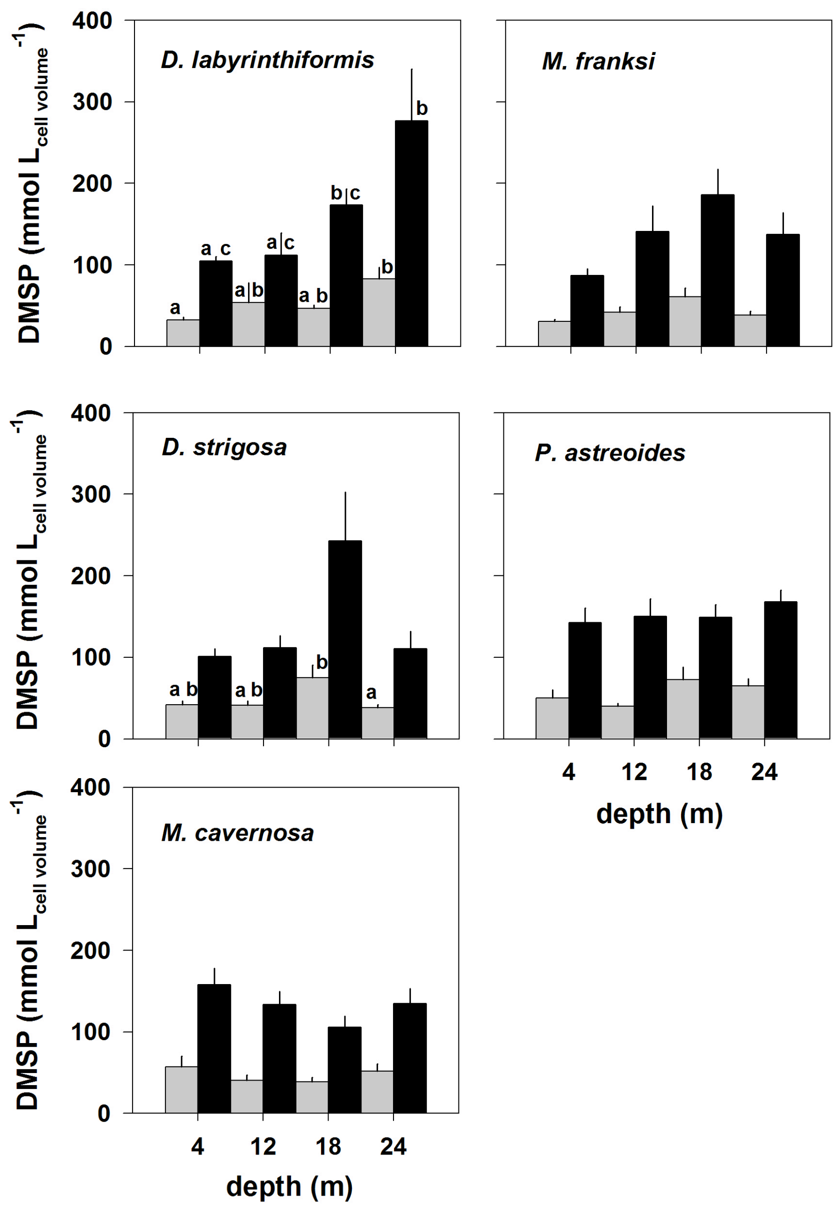


Figure 6.6.3 – Coral $DMSP_p$ and $DMSP_t$ across depth (mmol $L_{cell\ volume}^{-1}$).

(Figure 3 in text). *Diploria labyrinthiformis*, *Diploria strigosa*, *Montastraea cavernosa*, *Montastraea franksi*, and *Porites astreoides* $DMSP_p$ (gray bars) and $DMSP_t$ (black bars) concentrations normalized to zooxanthellae cell volume (mmol $L_{cell\ volume}^{-1}$) across depth (m). Letters indicate significant statistical difference within $DMSP_p$ or $DMSP_t$ concentrations (ANOVA, P < 0.05). Data are means ± SE.



Chapter 7: Discussion

7.1 DMSP and DMSP-lyase in coral-algal symbioses

To further our understanding of the production, potential turnover and function(s) of DMSP in coral-algal symbioses, laboratory and field investigations used multiple approaches from various research fields (coral symbioses, DMSP/DMSP-lyase studies and toxicology) to examine several factors that potentially influence the variable DMSP levels observed in coral symbioses. The interdisciplinary nature of this research was challenging from a methodological and interpretive perspective. Highlights from this research include the discovery and characterization of DMSP-lyases in *Symbiodinium*, and in-depth investigations of DMSP levels for several stony corals. Zooxanthellae/host coral DMSP partitioning and the response of the DMSP/DMSP-lyase system to conditions of oxidative stress were also investigated in this research and provided insight into the possible role(s) for DMSP in coral-algal symbioses. Given the complexities of the coral-algal system, methodological and indexing limitations for DMSP analyses were apparent, and furthermore complicated when placed in a toxicological context. The results described in chapters 2-6 are important because they address the inherent complexities of the coral holobiont and provide solutions for overcoming the difficulties associated with the lack of standardized indices and methods for measuring DMSP in algae, coral host and integrated symbioses.

Initial laboratory experiments were conducted using cultured *Symbiodinium* to examine whether DMSP concentrations varied across strains (strain is synonymous with clade and phylotype as used herein) and whether potential DMSP-lyase activity was present and, if so, if it was variable between these strains. Additionally, the potential contribution of bacterial DMSP-lyase activity was addressed because non-axenic algal cultures were used as *Symbiodinium* did not grow well otherwise. We did purchase axenic *Symbiodinium* strains which when tested did contain bacteria, highlighting the need for verification of axenicity when axenic cultures are stated to have been used.

DMSP-lyase activity rates were assessed and $DMSP_p$ (algae only) and $DMSP_t$ (algae and culture media) levels were also measured. Multiple indices are commonly used in the DMSP literature to normalize DMSP levels, although there is no consensus regarding which are the best to use. Therefore, we used multiple indices, including algal clade, chl-*a*, algal and bacterial cell numbers and algal diameter to normalize DMSP-lyase rates and DMSP concentrations. Results presented in chapter 2 found: 1) four of the five *Symbiodinium* strains examined showed *in vivo* potential DMSP-lyase activity 2) *Symbiodinium* strains demonstrated a range of potential DMSP-lyase activity when grown in culture and exposed to exogenous DMSP addition 3) potential DMSP-lyase activity was primarily associated with the algae (not bacteria) in the non-axenic cultures investigated 4) $DMSP_p$ concentrations for *Symbiodinium* strains was variable among strains and 5) there was no apparent correlation with $DMSP_p$ concentrations and potential DMSP-lyase activity between *Symbiodinium* strains.

The results highlight the discovery, for the first time, of DMSP-lyase activity in *Symbiodinium* and have been published (Yost and Mitchelmore, 2009). The data suggest that *Symbiodinium* are not only among the highest DMSP producers, but that they can enzymatically cleave DMSP, which may facilitate the proposed antioxidant function of DMSP in *Symbiodinium*. In addition, variable DMSP-lyase activity and DMSP production are suggestive of inter- and intra-clade variation. Thus, variable production and turnover of DMSP in symbionts may impact DMS(P) concentrations in corals, potentially serving multiple other functional roles such as a herbivory deterrent/attractant (DeBose et al., 2008; Wolfe et al., 1997) or antiviral defense mechanism (Evans et al., 2006).

The research in chapter 2 illustrated that the DMSP/DMSP-lyase system in *Symbiodinium* was variable and complex. As the results were also the first to show DMSP-lyase activity in *Symbiodinium* and because no previous studies had attempted to define the enzymatic qualities (i.e. affinities, not just presence of) DMSP-lyases in *Symbiodinium*, it followed that an examination of potential differences in the kinetic parameters of DMSP-lyase enzymes in *Symbiodinium* was warranted. To investigate DMSP-lyases in zooxanthellae, five strains of cultured *Symbiodinium* were used to determine optimal enzymatic assay parameters (buffer strength, pH and temperature). Furthermore, enzyme kinetic properties (K_m, V_{max}) were determined for two of the five strains. These strains were chosen because they were isolated from different host species, initially showed different DMSP-lyase rates and $DMSP_p$ concentrations and were known to be different genetic clades of *Symbiodinium*. Laboratory experiments evaluated DMSP-lyase activity rates, $DMSP_p$ and $DMSP_t$ values, chl-*a*, algal cell volume, diameter, number and *Symbiodinium* clade. Results presented in chapter 3 found: 1) in all *Symbiodinium* strains tested, DMSP-lyase activities were substantially higher in Tris buffer (200 mmol L^{-1} Tris and 500 mmol L^{-1} NaCl) at pH 8 and DMSP-lyase activity increased with increasing temperature up to 30-32 °C 2) the half saturation constants (K_m) of DMSP-lyases in strains CCMP 829 and 1633 ranged from 0.020 - 0.124 mM DMSP 3) the DMSP-lyase enzyme system showed up to a three-fold increase between day 7 and day 10 in CCMP 829 and 4) in CCMP 829, $DMSP_p$ and $DMSP_t$ per unit of cell volume also increased in conjunction with the increased potential activity of DMSP-lyase enzymes from day 7 to 10.

The Tris buffer results suggest an intracellular location for DMSP-lyase in *Symbiodinium* because cells placed in seawater showed very low lyase activity compared to those placed in the cell permeabilizing Tris buffer. Further optimization parameters indicated that *Symbiodinium* lyases functioned optimally near the known cellular pH and typical temperature regimes for these algae (pH 8, 28-30 °C). The half saturation constants (K_m) for *Symbiodinium* were lower than those for other algal DMSP-lyases indicating higher lyase affinities for DMSP in zooxanthellae. Also, the differences in K_m values among strains suggest the existence of different DMSP-lyase enzymes with different substrate affinities. Changes in enzyme kinetic parameters and DMSP concentrations suggest that the enzymes are constitutive and responsive to cues or environmental variables (such as conditions of oxidative stress effects associated with either CO_2 or nutrient limitation) in at least some cases. These findings are important because they are indicative of a dynamic enzyme system capable of responding to oxidative stress conditions in *Symbiodinium*, thus potentially mitigating cellular ROS like other well-recognized antioxidants. Furthermore, these investigations of DMSP-lyases in cultured *Symbiodinium* provide a basis for further standardized enzymatic comparisons that could incorporate effects of additional environmental stressors (i.e. those associated with coral bleaching, temperature and UV-light) and an expanded spectrum of symbionts (from multiple phylotypes and sub-phylotypes).

The DMSP and DMSP-lyase results from the isolated symbiont investigations found in the chapters 2 and 3 led to questions regarding the partitioning of DMSP concentrations (symbiont versus coral and symbiont) within field-collected corals. To date, all coral DMSP measurements have been made on the whole coral holobiont ($DMSP_t$) using two main preparation methods to remove the coral tissue from it's underlying skeleton (i.e. airbrush/water-pik and dissolution of tissues from intact coral fragments). Additionally, in some investigations the freshly isolated algae ($DMSP_p$) have also been quantified. To date no studies have looked at parallel measures of DMSP in the coral host and algal partners. Additionally, numerous methods to prepare corals for DMSP analyses have been used, which may impact the interpretation of the generated data. As methods for quantifying DMSP from isolated symbionts or intact symbioses were not standardized, the aim of this study was to improve DMSP measurements in the coral field so that cross comparisons between studies could more readily be made. Issues pertaining to the separation of host and symbiont, multiple DMSP preparation and extraction techniques and several normalization indices in four reef building coral species were investigated.

Measurements of $DMSP_t$ and $DMSP_p$ levels together with common DMSP indices including, chl-*a*, zooxanthellae number and diameter, total protein, coral surface area and polyp number. Results presented in chapter 4 found: 1) $DMSP_t$ values were consistent between the 2 main preparation methods, no significant loss of $DMSP_t$ was observed in airbrushed corals in comparison with coral intact fragments 2) $DMSP_t$ and $DMSP_p$ concentrations were recovered effectively by placing airbrushed coral sub-fragment homogenates in NaOH or MeOH - or by placing intact sub-fragments in MeOH 3) All corals had higher $DMSP_t$ than $DMSP_p$ concentrations (typically 2-3X) when analyzed using the same preparation method and 4) DMSP values varied among the corals on a per-species bases and according to the type of normalization indice used.

This was the first study to investigate the two prominent methodological techniques for the quantification of DMSP in coral symbioses in addition to expanding this research by teasing apart the relative partitioning of DMSP between coral and symbiont. These efforts to improve DMSP measurements in several conspicuous hard corals emphasized that DMSP normalization indices are limited according to the choice of preparation method used. Of central importance was the finding that DMSP appears to be present in the coral host tissues and that DMSP levels vary between host and symbiont, suggesting potential translocation from symbiont to host and highlighting the need for the quantification of both $DMSP_t$ and $DMSP_p$ from intact corals. Consistently higher $DMSP_t$ versus $DMSP_p$ concentrations for the corals investigated throughout the different studies appears to be reflective of physiological phenomena and further efforts should be directed towards corroborating this substantial finding. The insights presented in this investigation are central to any attempts that aim to understand the complex nature of DMSP in cnidarian-algal symbioses.

With a better understanding of the methodological techniques necessary to measure DMSP concentrations in coral symbioses, laboratory techniques were expanded to include the field studies presented in chapters 5 and 6. Symbiotic corals routinely experience hyperoxic conditions within their tissues due to the photosynthesis of their endosymbiotic dinoflagellates. Because *Symbiodinium* are known to produce high intracellular levels of DMSP and DMSP and its enzymatic breakdown products have been shown to play a significant role in the scavenging of cellular ROS in other marine algae (Sunda et. al, 2002), chapter 5 investigations focused on the response of DMSP to oxidative stress. To further understand the function(s) and regulation of DMSP within the coral-algal symbiosis we quantified the response of intact, field-collected corals (*Montastraea franksi*) exposed to 0-50 μg L^{-1} Cu using short-term mesocosm experiments. DMSP concentrations in air-brushed coral fragments ($DMSP_t$) and in the zooxanthellae isolated from these corals ($DMSP_p$) were quantified together with numerous other algal and coral cellular and biochemical endpoints. These endpoints were measured to monitor potential bleaching effects and/or to serve as DMSP indices to determine the most reliable normalization indices for DMSP concentrations in corals exposed to copper. Results presented in chapter 5 found: 1) Levels of $DMSP_t$ were on average 6X higher than particulate $DMSP_p$ concentrations 2) Significant changes in levels of $DMSP_t$ and $DMSP_p$ occurred in *M. franksi* following 48 h of copper exposure where $DMSP_t$ and $DMSP_p$ levels decreased with copper dose, although at the highest copper dose $DMSP_p$ levels increased, a result not observed for $DMSP_t$ 3) This observed differential response to copper between $DMSP_t$ and $DMSP_p$ demonstrated that physiological changes may have been overlooked if the conclusions were based upon $DMSP_t$ levels alone (the common measure used in coral studies to date) and 4) chl-*a* and algal cell numbers decreased in response to elevated copper.

This study was the first to demonstrate significant changes in $DMSP_p$ and $DMSP_t$ concentrations in corals following exposure to a known oxidative stressor, copper. A key conclusion drawn from the results was that the use of common indices for normalizing DMSP might not always be appropriate, especially in toxicological studies where such indices may be directly impacted by the stressor(s) investigated. This has implications not only for the coral host/*Symbiodinium* DMSP field, but also for the DMSP field as a whole where normalization indices are likely to vary among species, studies, temporally and spatially. Additionally, corals exposed to environmentally relevant concentrations of copper exhibited changes in both $DMSP_t$ and $DMSP_p$ concentrations in response to increased physiological stress, suggesting as in chapter 3, the plausible translocation of DMSP from *Symbiodinium* to the coral host, though other mechanisms or processes (DMSP-lyase in host tissues, up- or down-regulation of the DMSP/DMSP-lyase system) may also influence these concentrations. While DMSP and its subsequent conversion products are known antioxidants, the exact mechanisms by which these reactions occur have yet to be described in detail. It is possible that DMSP may scavenge ROS via sulfhydryl groups, similar to the free radical scavenger GSH that also occurs at high intracellular concentrations.

The anomalous response of the symbiosis (especially $DMSP_p$ values) to 50 μg L^{-1} Cu is an interesting finding considering the responses seen at 5 and 10 μg L^{-1} Cu, although a U-shaped toxicant response curve is not uncommon in toxicological evaluations. Further possible explanations for the observed algal DMSP concentrations (besides DMSP up-regulation at 50 μg L^{-1} itself) may be related to the possible up-regulation of cellular antioxidants (i.e. APX, GSH) resulting in less cellular stress and possibly less DMSP turnover in cells, damage to DMSP-lyases and/or the production of DMSP that exceeded DMSP-lyase activity. To assess these possibilities, quantification of cellular antioxidants and DMSP-lyase activity are necessary measures. It is also possible that different coral and symbiont species will respond differently based on individual tolerances to copper stress. Finally, investigations focused on the possible interaction of DMSP and Cu^{2+} would be useful for more fully understanding the interactions between Cu^{2+} and DMSP within algal and coral host cells. To this end, mass balance experiments involving Cu and isolated

Symbiodinium cells could serve to elucidate how exogenous Cu additions are might be sequestered by cells and how this affects intra- and extra-cellular DMSP concentrations. Therefore, to fully understand the physiological significance of the dynamic DMSP system it is imperative that these detailed partitioning studies are carried out. As evidenced by our $DMSP_t$ data, responses can be missed using this all-encompassing endpoint. However, by teasing out the $DMSP_p$ contribution, a clear response to physiological stress (i.e. oxidative stress in this case) became apparent. Finally, the data suggest an antioxidant role for DMSP in corals where the DMSP/DMSP-lyase system may contribute to the inherent antioxidant systems present in zooxanthellae and/or coral animals, thus serving a supportive antioxidant role.

There are a few previous studies that have quantified DMSP in field-collected stony corals, but these reports were limited in scope as they did not include a partitioned investigation of DMSP values for the corals investigated and were limited to Australia, USA (Hawaii) and Guam (Jones et al., 1994; Broadbent et al., 2002; Hill et al., 1995; Van Alstyne et al., 2006). These studies showed great variability in DMSP concentrations among coral species. What drives such DMSP differences among corals remains enigmatic; there are several potential factors that may contribute to variable DMSP concentrations including coral species, algal phylotype, geographic location and/or environmental variables. In an effort to address and expand what is known about DMSP in corals, multiple corals were collected from Bermuda. To investigate if DMSP levels are reflective of the coral species and/or some environmental cues, DMSP levels (within algal symbionts and their coral hosts) were quantified in five prominent Bermudian coral species along a depth gradient. Alongside DMSP concentrations, chl-*a*, coral surface area, total protein, zooxanthellae number, diameter and *Symbiodinium* phylotype were analyzed. The work presented in chapter 6 showed: 1) $DMSP_t$ concentrations were consistently greater than $DMSP_p$ concentrations in all of the coral species investigated (2-3X) 2) At the inter-species level, *Diploria labyrinthiformis* was unique, showing $DMSP_p$ and $DMSP_t$ increases (per coral surface area or biomass) with bathymetric decline 3) At 4 m depths, *Montastraea cavernosa*, which rarely bleaches and *Porites astreoides*, that hosts the bleaching resistant phylotype A symbiont, showed the highest $DMSP_t$ values (mmol $_{\text{Lcell volume}}{}^{-1}$) among the coral species investigated 4) DMSP concentrations per zooxanthellae showed significant differences between phylotype B and C symbionts ($DMSP_p$) and their respective hosts ($DMSP_t$) and 5) within-phylotype variation occurred between *D. labyrinthiformis* and *D. strigosa* corals that both host phylotype B algae.

This was the first comparative field campaign to investigate $DMSP_p$ and $DMSP_t$ concentrations across a depth gradient in several conspicuous stony corals from the West Atlantic, specifically, Bermuda at 32 °N (June, 2009). DMSP patterns suggested that symbiont, host and the depth at which corals are found contribute to the complexities of $DMSP_p$ and $DMSP_t$ concentrations within the integrated symbiosis. As found in chapters 3, 4 and 5, data in chapter 6 also indicates the possible translocation of DMSP from symbiont to host. Again, results here suggest that multiple indices for indexing DMSP concentrations are necessary to encompass the complexities of the DMSP and coral fields. In a broader context, the DMSP patterns among coral species and their respective symbiont phylotypes were congruent with known host and symbiont tolerances to oxidative stress (detailed above). While the greater DMSP/DMSP-lyase system awaits further investigation to substantiate the intricacies of these findings, it is noteworthy to recognize that DMSP is a substantial compound in corals and its production and accumulation involves, at least, symbiont, host coral and environmental cues.

Taken together, the results of these studies underscore the inherent variability in, and complexities of, the DMSP/DMSP-lyase system in one of the world's most important and fascinating biological systems, coral-algal symbioses. Of fundamental importance is the understanding that these investigations were focused towards elucidating the extent to which DMSP and DMSP-lyase activity varied in *Symbiodinium* and their respective host corals under conditions of oxidative stress versus no induced stress (natural or artificial). To this end, this research shows that *Symbiodinium* of varying clades from cultured and field-collected corals differentially produce DMSP and are capable of DMSP-lyase activity that, like DMSP, varies under conditions of no induced stress. Additionally, the DMSP/DMSP-lyase system is responsive to oxidative stressors, namely copper, nutrient or CO_2 stress and possibly variables associated with a depth gradient (light and temperature), which indicates that DMSP may serve protective role(s) in the symbiosis.

Inherent complexities were encountered in attempts to isolate coral tissues from their respective skeletons. Using the two known preparations for such coral processing revealed that differences between methods were not significant and that airbrushing corals allowed for a potential partitioned investigation of coral DMSP, though further scrutiny of this technique is warranted. Initially, airbrushing was considered substandard as mechanical stress and cell rupture were thought to contribute to DMSP loss via DMS. However, the comparative results suggest that DMSP recovered from intact fragments or airbrushed fragments were similar, indicating minimal loss of DMSP due to algal rupture. Chapters four, five and six include methodologies that aimed to separate symbionts from their respective host corals in efforts to more fully understand how DMSP might be partitioned between the symbiotic partners. The discussion below pertains to the fact that airbrushing may have damaged the algal cells and allowed DMSP to leak from the cells into the surrounding tissues and coral homogenate, a possibility that exists, but the extent of which remains unknown. However, we present and discuss multiple lines of evidence supporting the possibility that DMSP from algal symbionts could be accumulating in coral hosts. In addition to the arguments already presented (e.g. DMSP translocation in clams), *Symbiodinium* are known to transfer upwards of 95% of their photosynthetic products to their hosts and it is plausible that DMSP may also be transferred to the host coral. While the possibility of translocation of DMSP from symbiont to host is plausible and discussed, there are also other possibilities that may explain the higher $DMSP_t$ versus $DMSP_p$ concentrations reported.

Preliminary data using isolated algal cells indicated no loss of DMSP after cells were frozen, thawed and analyzed for $DMSP_p$. $DMSP_t$ concentrations would not likely be substantially affected by such considerations. There were no apparent DMSP losses associated with the duration of freezing (weeks to months) for coral fragments kept at -80 °C. Cultured *Symbiodinium* cells appeared resistant to physical damage when filtered using GF/F filters, as there was no increase in DMSP in the filtrates with increasing volumes of algae filtered. DMSP-lyase assays showed no DMSP 'leakage' prior to substrate addition suggesting that the algal cells needed to be permeabilized, but that DMSP was not readily escaping the cells. Also, using a fluorescent microscope, cell and chl-*a* debris in homogenates was minimal with the majority of algal cells remaining intact. Given the 2-3X difference in $DMSP_t$ versus $DMSP_p$, a majority of algal cells would have had to have been compromised. Further studies addressing *Symbiodinium* leakage could measure specific cellular metabolites such as the dinoflagellate-specific pigment, peridinin. However, while isolated symbionts are good models for understanding *Symbiodinium*, it should be noted that they likely differ from symbionts within intact symbioses with corals.

If airbrushing and/or the thawing of coral tissues during the airbrushing process has a significant affect on how 'leaky' *Symbiodinium* cells may become, one might expect to see higher $DMSP_t$ to $DMSP_p$ ratios in those corals that required longer processing times (e.g. *M. cavernosa* processing required more time than *P. astreoides*; all processing times were <10 min.). This was not observed. A consistent ratio of $DMSP_t$ to $DMSP_p$ was observed across studies conducted at different times of the year. This consistency was also observed with the several species of hard corals used in the studies. Within a coral species, $DMSP_t$ to $DMSP_p$ ratios should be similar if coral morphology and therefore processing time and associated thawing are not affecting symbiont integrity and DMSP leakage, but these ratios changed across depth gradients. In the copper study, the higher ratios of $DMSP_t$ to $DMSP_p$ are probably, in part, reflective of the study design, where corals were not immediately processed, but were acclimated for a period of time before the mesocosm experiment and subsequent processing. Significant differences within $DMSP_t$ and $DMSP_p$ concentrations in response to copper (chapter five) did not necessarily coincide; for example, higher DMSP concentrations in symbionts were not reflected in the coral host. If substantial DMSP leakage from symbionts was occurring it is likely that these observed differences in $DMSP_p$ and $DMSP_t$ would not have been observed. Additionally, significant $DMSP_p$ values were observed between symbiont phylotypes in chapter six.

Finally, while $DMSP_p$ values in field samples may be conservative if there are methodological affects on symbionts, one cannot rule out the possibility that symbionts *in hospite* might have $DMSP_p$ values that fall on the lower end of the known values of DMSP in dinoflagellates. $DMSP_p$ values for *Symbiodinium* from field studies herein were in agreement with $DMSP_p$ values measured in cultured *Symbiodinium*. It is also possible that $DMSP_p$ concentrations may be underestimating algal DMSP if the airbrushing technique or effects of freezing/thawing corals compromised the integrity of symbiont cellular membranes.

Given the methodological limitations and their associated uncertainties, it may be that it is not currently possible to accurately determine cellular DMSP concentrations of endosymbiotic dinoflagellates. It may be that most or all of the DMSP in corals was present in the algal cells. Also, it is still unknown if corals have DMSP-lyase activity, which could also influence the concentrations of DMSP in coral tissues. Therefore, the present $DMSP_p$ measurements may give only minimum symbiont DMSP concentrations while the $DMSP_t$ values normalized to appropriate algal indices (e.g. chl-*a*) give DMSP concentrations that are equal to or greater than the true algal cellular values. In total, uncertainties regarding the partitioned values of DMSP in the symbiosis do not detract from the reports of $DMSP_t$, which are varied according to species, across water depths and in response to stress.

Efforts focused on the partitioning of DMSP between symbiont and coral host have furthered our understanding of DMSP production and accumulation in corals. The results indicate that DMSP concentrations in host corals vary, suggesting the potential translocation of DMSP from symbiont to host. Across experiments and methodologies, $DMSP_t$ concentrations were found to be higher than $DMSP_p$ concentrations (typically 2-3X). Variable DMSP accumulation among coral species may be due to complex factors such as symbiont type and environmental cues. The discoveries and insights presented in chapters 2-6 underscore the need for multiple DMSP normalization indices due to the unique characteristics of coral species (topography) and symbionts (phylotype, cell size). Furthermore, because the investigations herein were conducted at multiple scales of resolution, this research highlights the ongoing need for understanding the cellular processes and mechanisms that drive the DMSP/DMSP-lyase system in corals and are central to any attempts to understand the stability of coral symbioses.

7.2 Overall significance of this work to coral ecology and future research directions

Important questions regarding the ability of coral reefs to meet current and future challenges of increasing environmental stress requires knowledge of coral biology, the flexibility, functioning and specificity of corals and the symbioses they form (Stat et al., 2006). The focus of this dissertation was to ascertain to what extent DMSP and DMSP- lyase activity varied in *Symbiodinium* and their respective host corals under conditions of oxidative stress versus no induced stress (natural or artificial). Taken together, the findings detailed in the discussion above indicate that the DMSP/DMSP-lyase system in cnidarian-algal symbioses is complex and that DMSP patterns are reflective of symbiont, host and environmental attributes that together contribute to the complexities of $DMSP_p$ and $DMSP_t$ concentrations within integrated symbioses.

As much of this dissertation research was on the leading edge of what is currently known about DMSP and DMSP-lyase in cnidarian-algal symbioses, many additional questions have also been raised due to the investigations presented. The challenges met in an attempt to cross and merge the disciplines of DMSP, coral and toxicology research were numerous yet enriching. This dissertation research has changed our understanding of DMSP and DMSP-lyase in cnidarian-algal symbioses and thus serves the important role of providing the platform and fuel for future investigations. The extent and function of DMSP translocation from *Symbiodinium* to the host coral remains unclear, as does the extent of DMSP-lyase function(s) in the symbiosis. Does DMSP play multiple role(s) in *Symbiodinium* and if so, how important are these roles respectively? What are the subsequent effects of such role(s) on cnidarian host cells? If present, why does DMSP-lyase exist in host tissues? Do other species of corals, in different environmental regimes than those already investigated, contain different amounts of DMSP and/or DMSP-lyase? What are the primary factors that drive these differences? Under environmental regimes that promote coral bleaching, does DMSP serve an antioxidant role in *Symbiodinium* and does this relate to the susceptibility/resilience of the algae and/or the coral host to symbiosis breakdown? In other words, does DMSP serve to alleviate conditions of oxidative stress in coral-algal symbioses and to what extent does this occur? Is this a more important protective mechanism than any (or even combinations of) of the traditional antioxidant systems in corals? DMSP isolation from *Symbiodinium* and other algae and/or labeled DMSP/DMSP-lyase (^{35}S) studies to further investigate the production and fate of DMSP in symbiont and host coral would be beneficial tools for use in future efforts that are needed to specifically describe the physiological production, turnover and cellular movement of this enigmatic compound.

While the specific function(s) of the DMSP/DMSP-lyase system in corals are still under investigation, DMSP production and turnover from coral reefs has already been shown to have impacts on local ecosystems and climate. As discussed in chapter one, conditions of elevated oxidative stress, elicited by multiple potential stressors, have been described as playing a crucial role in the onset of coral-algal symbiosis breakdown (Downs et al., 2002). To cope with stress, organisms may increase the production of antioxidants and/or antioxidant enzymes. In stressed corals, DMS is released at the coral's surface and DMSP is present in coral mucus (Broadbent and Jones, 2004). Therefore, it is plausible that there is a link between DMSP production in coral symbionts and oxidative stress conditions impacting coral reefs. DMSP occurs at mM concentrations within *Symbiodinium* and this dissertation research has shown that an antioxidant cascade stemming from DMSP conversion is possible for coral symbionts. Thus, the dynamic DMSP/DMSP-lyase system could play a supportive or major antioxidant role (or antiherbivory role via acrylate, or multiple functional roles) in the symbiosis, contributing to ecosystem-level patterns of coral sensitivity if various phylotypes produce and enzymatically turnover DMSP as supported by the data presented herein.

The production of DMSP by coral symbionts may in fact be linked to other significant reef ecosystem processes. For example, bacteria are known to play a critical role in the global sulfur cycle and are an integral part of the coral holobiont. Potentially beneficial (e.g. *Roseobacters*) or harmful (e.g. *Vibrio* spp.) bacteria associated with corals may serve to protect (via the production of antimicrobial compounds) or harm (via disease) integrated symbioses thus potentially impacting reef health at ecosystem levels (Jean-Baptiste et. al, 2009). As DMSP is a known energy source for bacteria, DMSP production by *Symbiodinium* and its conversion by DMSP-lyases may also play a role in shaping bacterial communities associated with reef corals. Beyond the coral holobiont, DMSP production on reefs may deter or attract other organisms in the coral reef community, influencing larger food web interactions.

Reefs may be substantial sources of DMS aerosol particles, with one emission estimate from a 1000-km reef area on the order of 10^{19} s^{-1} (Bigg and Turvey, 1978), which is comparable to aerosol emissions from land surfaces and forest fires (Jones and Trevena, 2005). This dissertation research supports the potential for high amounts of DMS release above reefs as *Symbiodinium* are now known to have DMSP-lyase activity and, coupled with high levels of DMSP in corals, coral reefs may arguably contribute substantially to the processes involved in the greater sulfur cycle. Thus, corals may serve as significant sources of DMS production, especially under conditions of oxidative stress (Jones and Trevena, 2005; Broadbent and Jones, 2006). Measurements of DMSP herein indicate that multiple species of reef-building corals could potentially contribute substantial amounts of DMSP and thus DMS to the water column, impacting local climates through increased cloud formation. Additionally, with global distributions, high rates of DMSP production and the immediate and ongoing threat of global climate impacts threatening to cause repeated bleaching, DMS evolution from stressed reefs may have an increasing effect on local climates.

Coral reefs provide many invaluable ecosystem services associated with world fisheries, coastline protection and sea-scape level processes involved with their common, adjacent ecosystems, namely mangroves and seagrasses (Sheppard, 2006). However, global climate change brings coral reefs into the spotlight, highlighting their potential demise if they cannot physiologically adapt given the current rates of environmental change (Hoegh-Guldburg, 1999; Hoegh-Guldburg et al., 2007). Major environmental shifts, namely altered ocean temperatures (increased temperatures and duration of warming) and pH (i.e. decreases) due to the addition of carbon dioxide and other greenhouse gases to the atmosphere, threaten corals because they already exist at their critical upper temperature limits and are vulnerable to low-pH induced carbonate skeleton dissolution. Indeed, the impact of climate change may be the primary threat to coral reefs based on the global scale and intensity of its effects (Hughes et al., 2003). The increased number and severity of bleaching events (stress related dissociation of the coral-algal symbiosis) is predicted to continue (Hoegh-Guldberg, 1999; Sheppard, 2003). The potential consequences of reef decline may include, for example, shifts in coral species composition and biodiversity changes and/or community phase shifts, where algae replace corals as the dominant group (Sheppard, 2006). Regardless of the specific outcomes, research focused on understanding the cellular processes and mechanisms involved in "the highly successful but environmentally sensitive phenomenon" (Hoegh-Guldberg et. al, 2007) of cnidarian-algal symbioses are of utmost importance if we intend to preserve and protect that which we cannot create.

Appendices

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8 March 2010

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Dimethylsulfoniopropionate (DMSP) lyase activity in different strains of the symbiotic alga *Symbiodinium microadriaticum*

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ABSTRACT: Dimethylsulfoniopropionate (DMSP) lyases are responsible for the enzymatic conversion of the algal metabolite DMSP into dimethylsulfide (DMS) and other products. DMSP lyase potential activity (DLA) was assayed in 5 cultured strains of *Symbiodinium microadriaticum* to investigate whether DLA was present and whether variations in DLA occurred among strains. *S. microadriaticum* are important primary producers symbiotic with corals and other cnidarians. Four of the 5 tested *S. microadriaticum* strains were capable of performing enzymatic lysis of DMSP, and the levels of DLA varied significantly among the strains. Average DLA normalized to chlorophyll *a* (chl *a*) was significantly higher in strain CCMP 829 (5.3 ± 0.9 nmol DMS · min^{-1} · µg^{-1}) compared with all other strains investigated. DLA was not detected in strain CCMP 830, suggesting that DMSP lyase may not be a universal enzyme in *S. microadriaticum*. DMSP levels also varied between strains, but there was no correlation between cellular DMSP and DLA. Additionally, our data indicate that in non-axenic *S. microadriaticum* strains, the bacterial contribution to DLA was not substantial in any of the algal cultures investigated. These results are supportive of the idea that *S. microadriaticum* contain DLA, that DMSP producers are not necessarily capable of DLA, and that, even within a species, DMSP and DLA levels vary significantly. These findings suggest important differences in the regulation of DMSP and DLA in *S. microadriaticum*, but the reasons for these differences remain to be elucidated.

KEY WORDS: *Symbiodinium* · DMSP lyase · Dimethylsulfoniopropionate · Coral

INTRODUCTION

Dimethylsulfoniopropionate (DMSP) is an algal metabolite that is produced by a wide variety of species at different intracellular concentrations (Keller et al. 1989). Marine dinoflagellates, including the coral symbiotic algae of the genus *Symbiodinium*, produce relatively high levels of DMSP (Keller & Korjeff-Bellows 1996, Yoch 2002, Van Alstyne et al. 2006). DMSP and its enzymatic cleavage products have multiple proposed functions at the cellular level, including those of herbivory deterrent (Wolfe et al. 1997), algal osmolyte (Kirst 1996), antioxidant (Sunda et al. 2002), antiviral defense mechanism (Evans et al. 2006), overflow mechanism for excess reduced sulfur (Stefels 2000), methyl donor (Ishida 1968), sulfur storage compound (van Diggelen et al. 1986), foraging cue (DeBose et al. 2008) and cryoprotectant in polar algae (Kirst et al. 1991, Karsten et al. 1996). Dimethylsulfide (DMS) is a significant degradation product of DMSP, and is a major source of sulfur to the atmosphere (Kettle & Andreae 2000). DMS is also reputed to affect ocean cloud cover and the radiative climate through formation of aerosols (Charlson et al. 1987).

The enzyme DMSP lyase (dimethylpropiothetin dethiomethylase, EC 4.4.1.3; DL) is responsible for DMSP conversion, producing DMS and other products (Johnston et al. 2008). This enzyme has been reported in phytoplankton (Steinke et al. 1998), macroalgae (Van Alstyne & Houser 2003), bacteria (de Souza &

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Yoch 1995) and fungi (Bacic et al. 1998). As demonstrated by Sunda et al. (2002), DMSP is reactive toward hydroxyl radicals (·OH), but its enzymatic cleavage products, acrylate and DMS, are ~20 and ~60 times more reactive towards ·OH, respectively. Overall, the enzymatic conversion of DMSP forms several potential antioxidant scavengers (Sunda et al. 2002). Though many algal DMSP producers have DL capabilities (DMSP lyase potential activity [DLA]), this is not true for all algal species (Steinke et al. 1996, Niki et al. 2000, van Bergeijk & Stal 2001, Sunda et al. 2002). Since DMSP and DLA potentially mitigate stress, investigations of how these parameters vary across *Symbiodinium* clades may improve understanding of the symbiont–host relationship.

Symbiodinium are the most prominent dinoflagellates in symbioses with marine invertebrates and protists and are commonly found with members of the phyla Cnidaria (i.e. corals, anemones), Platyhelminthes, Mollusca, Porifera and Foramnifera (Trench 1979, Pawlowski et al. 2001). Many symbiont host species have been shown to contain DMSP, including corals (Jones et al. 1994, Hill et al. 1995, Broadbent et al. 2002), anemones (Van Alstyne et al. 2006), flatworms (van Bergeijk & Stal 2001) and clams (Jones et al. 1994, Hill et al. 2000, 2004). Evidence suggests that algal symbionts are responsible for DMSP production in cnidarian species due to a positive correlation between DMSP concentration and symbiotic algal densities (cell number) in cnidarian hosts, although partitioning between host corals and algal symbionts is unknown (Broadbent et al. 2002, Van Alstyne et al. 2006). Significant amounts of DMSP in reef corals suggest that these ecosystems could be significant sources of DMS to the atmosphere (Broadbent & Jones 2004, Jones & Trevena 2005), but it is currently unknown whether *Symbiodinium* have the enzymatic ability to convert DMSP into DMS. With evidence for DMSP variation in *Symbiodinium* from different coral species and bleached versus healthy corals (Hill et al. 1995, Broadbent et al. 2002, Van Alstyne et al. 2006, Jones et al. 2007), but no reports of DLA in *Symbiodinium* or their coral hosts, a characterization of baseline DMSP and DLA levels within and across algal and animal host species is warranted.

The present study investigated 5 *Symbiodinium microadriaticum* strains to determine (1) whether DLA was detectable in *S. microadriaticum* cultures, (2) whether DLA was primarily associated with the algal fraction of non-axenic cultures and (3) whether *S. microadriaticum* DLA and DMSP were significantly different between the algal strains investigated. *Emiliania huxleyi* strains were analyzed for comparison.

MATERIALS AND METHODS

Algal cultures. All experiments were conducted with strains of the dinoflagellate *Symbiodinium microadriaticum* and *Emiliania huxleyi*, purchased from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton (CCMP; Bigelow Laboratory, Maine, USA). Algal strains included CCMP 373 and 374 (*E. huxleyi*; axenic) and *S. microadriaticum* CCMP 421, 828, 829, 830 and 1633 (Table 1). The cultures were maintained using sterile techniques and grown in sterile K, L1 or f/2-Si media (pH 8.0) according to preference (Bigelow Laboratory). *S. microadriaticum* cultures were not treated with antibiotics to obtain axenicity as our preliminary trials showed that antibiotic treatment negatively affected growth (data not shown). Thus, measures were taken to assess bacterial abundance and potential interference with DLA assays (detailed in 'Bacterial analyses' below). Algal cultures were grown at 26°C with a 12 h light:12 h dark cycle, without agitation (Rogers & Davis 2006). In common with other studies (Matrai & Keller 1994), our preliminary trials demonstrated that DMSP levels were

Table 1. *Symbiodinium microadriaticum* and *Emiliania huxleyi*. CCMP algal strain characteristics and clade identification. All data (except for clade) from Provasoli-Guillard Center for the Culture of Marine Pytoplankton, Bigelow Laboratory, Maine, USA

CCMP strain	Species	Collection location (very approximate)	Ocean	Isolated from	Clade
373	*E. huxleyi*	Sargasso Sea	North Atlantic	Sea water	–
374	*E. huxleyi*	Gulf of ME, USA	North Atlantic	Sea water	–
421	*S. microadriaticum*	Wellington, NZ	South Pacific	Sea water	E
828	*S. microadriaticum*	Florida Keys, FL, USA	North Atlantic	Sea water	A
829	*S. microadriaticum*	Great Barrier Reef, Australia	South Pacific	*Tridacna crocea* (bivalve)	A
830	*S. microadriaticum*	Bermuda Biological Station, Bermuda	North Atlantic	*Aiptasia pallida* (sea anemone)	B
1633	*S. microadriaticum*	Hawaii, USA	North Pacific	*Aiptasia puchella* (sea anemone)	B

dependent upon the growth phase; therefore, all algal cultures were sampled during their exponential growth phase, at an average density of 1×10^5 cells ml^{-1}. Each strain was grown in semi-continuous batch culture in 50 ml conical flasks with 30 ml of culture in each, under cool-white fluorescent bulbs (100 µE m^{-2} s^{-1}). All cultures were sampled 2 h (±1 h) into their light periods as DMSP (and DLA) concentrations varied with diel cycle in *S. microadriaticum* (authors' pers. obs.) and have been reported to vary in other algal species as well (Bucciarelli et al. 2007, Jones et al. 2007, Stefels et al. 2007, Sunda et al. 2007). *S. microadriaticum* cultures were genetically verified for clade type (see 'Algal analyses'). Each of the strain replicates was grown separately and analyzed individually.

DMSP and DLA analyses. ***DMS analysis and calibration:*** All samples were analyzed with a Hewlett-Packard 5890 Series II gas chromatograph (GC) fitted with a Chromosil 330 packed column (Supelco) and a flame photometric detector (FPD). System temperature settings were 150°C for the injector, 60°C for the column oven and 175°C for the detector. Nitrogen gas was the carrier (60 cm^3 min^{-1}), and air (60 cm^3 min^{-1}) and hydrogen (50 cm^3 min^{-1}) were the flame gases. Data were collected and analyzed using HP ChemStation (Hewlett-Packard). Quantifications were made by headspace analysis following DMSP conversion to DMS by alkaline hydrolysis. Known concentrations of DMSP (purchased from Research Plus) were diluted in sterile water to give working solutions, which were frozen in small aliquots at –80°C. A standard curve for serial dilutions of DMSP was used to construct a calibration curve (using the square-root values of the peak area), and this linear regression served to convert peak areas from GC headspace measurements to DMS concentrations. Standards and controls (n = 5 each) were prepared in parallel to experimental samples by using the same Tris buffer for DLA, incubating at 30°C for 20 min and using the same total liquid volume (1 ml) in headspace vials. Addition of 5 N NaOH to standards (final volume of 1 ml; final concentration of 5 N NaOH) occurred prior to heating, and all vials were placed in the dark for at least 4 h for equilibration prior to sampling. Analytical replicates were used only for inter-strain assessment of repeatability. The precision of the DMS analysis was <5%, and headspace storage trials showed no DMS losses with the analytical methods employed. The detection limit of the GC was 1 nmol DMSP l^{-1}.

Total and particulate DMSP analyses: Total DMSP was determined by placing 0.5 ml of whole culture with 0.5 ml of 10 N NaOH into a headspace vial, sealing it and waiting at least 24 h for equilibration (equilibration times were optimized for all procedures; data not shown). Particulate DMSP ($DMSP_p$) was determined using a simple acidification/storage procedure (5 µl 50% H_2SO_4 ml^{-1} of culture) followed by the total DMSP method (Kiene & Slezak 2006). Appropriate controls (n = 5) were prepared in parallel to sample preparation.

DLA analyses: Our DLA methods were modified from procedures described by Harada et al. (2004) and Steinke et al. (2000) and optimized (pH, exogenous DMSP concentration) for *Symbiodinium microadriaticum*. Briefly, DLA was determined by measuring the production rate of DMS prior to and after the addition of exogenous saturating levels of DMSP to permeabilized cells (using a Tris buffer, 200 mmol l^{-1} Tris containing 500 mmol l^{-1} NaCl; pH 8). Algal cell permeabilization is necessary to allow exogenous DMSP into the cells in order to detect an enzymatic response to saturating DMSP levels. Tests showed that DLA increased in samples with Tris buffer compared to those without, and Tris buffer produced higher DLA than other means of cell disruption (homogenization, varied Tris buffer strength). We found that Tris buffer at pH 8 yielded DLA in *S. microadriaticum* samples greater than those in pH 6 Tris buffer. Appropriate controls (biotic and abiotic in parallel to sample preparation) and standards were run in tandem. Controls consisted of 0.5 ml Tris buffer and 0.5 ml culture medium amended with 5 µl of 1 mol l^{-1} DMSP at t_0. Spot checks of Tris buffer and culture pH before and after DLA analysis were conducted and never deviated significantly from pH 8 (stable at 7.98; pH above 8 results in increased abiotic conversion [any conversion of DMSP not attributed to biological enzymes]). Headspace vials were 6 ml in volume and sealed with polytetrafluoroethylene (PTFE)/rubber septa (National Scientific).

Algal cells in culture were concentrated by centrifugation at 1310 × *g* for 5 min prior to placement in headspace vials. Concentrating the cells was necessary as the amount of DLA in some cultures was too small and needed to be adjusted according to the limitations of our analytical system (Steinke et al. 2000). Centrifugation has also been used previously for concentrating algal cells in DLA and DMSP/DMS experiments (Steinke et al. 1998, Broadbent et al. 2002). We did not use filters (e.g. GF/F) to capture cells because the filters could not be fully submerged in the small liquid volume of the headspace vials. After centrifugation, algal cells were resuspended in their native media to a final volume of 0.5 ml. Tris buffer was added to samples to permeabilize the cells. Preliminary trials in native media versus those in Tris buffer verified that the buffer was necessary for optimal DLA measurement. Tris buffer (0.5 ml) was added to 0.5 ml algal culture, sealed, incubated in a 30°C water bath for 20 min and vortexed vigorously for 3 s before sampling at each time point (at

5 min intervals from 10 min prior to, through 30 min after, exogenous DMSP addition).

The temperature chosen for these experiments (30°C) was previously recommended to enable comparisons among samples and studies (Steinke et al. 2000). At t_0, 5 µl DMSP stock solution (1 mol l^{-1}) was added to give a final concentration of 5 mmol l^{-1}. This amount of DMSP was found to be saturating for lyase-catalyzed DMS production in this system according to preliminary tests. At each time point, 50 µl headspace samples were removed with an Agilent gas-tight syringe (same volume injected for all samples and standards) and injected into the GC for DMS measurement. At least 8 headspace samples (–10 min through 30 min) were measured for each vial to yield a rate of DMS increase with time. DMS production was linear for all samples taken, and DLAs for all samples were corrected for abiotic conversion of DMSP by subtraction of DMS production rates measured in control vials. DLA is defined as nanomoles of DMS·per minute and is also reported as DLA:chl *a* (nmol DMS · min^{-1} · $µg^{-1}$) and DLA:$DMSP_p$ (nmol DMS · min^{-1} · $fmol^{-1}$ DMSP). On a per cell basis, DMSP is reported as femtomoles per cell.

Bacterial analyses. ***DLA in culture sample filtrates:*** Algal cultures were sub-sampled prior to centrifugation to determine bacterial contribution to overall DMS production. Culture samples were filtered using Isopore membrane filters (Millipore; 25 mm diameter, 2 µm pore size) and gentle filtration (gravity or <50 mmHg vacuum; Steinke et al. 2000) to obtain an algal-free culture fraction while allowing unattached bacteria into the filtrate. Filtrate (0.5 ml) was immediately placed in a 6 ml headspace vial, 0.5 ml of Tris buffer was added and DLA was assessed as described above. Filtrate samples without algae were scaled up to represent the amount of bacteria present in unfiltered (same volume of whole culture as the filtrate) samples to calculate the maximum bacterial DLA contribution to the reported algal DLA measurements. Specifically, the contribution to total potential DLA by bacteria (bacterial contribution) was calculated as follows for each replicate: (1) filtered bacterial counts were divided by unfiltered bacterial counts to determine the ratio of bacteria in the filtered versus unfiltered culture samples, and (2) filtered DLA quantities were divided by unfiltered DLA quantities at $t = 30$ min; each DLA measure was corrected for abiotic conversion by subtracting the amount of DMS measured in controls. Bacterial contributions to total observed DLA (%) were therefore calculated by dividing the above DLA ratio (Point 2) by the above bacterial count ratio (Point 1) and multiplying by 100.

Bacteria enumeration: Prior to centrifugation, culture samples and culture filtrate sub-samples (0.5 ml each) were preserved (Sherr & Sherr 1993), stained with 4′-6-diamidino-2-phenylindole (DAPI, final concentration 20 µg ml^{-1}) and filtered onto 0.8 µm polycarbonate filters (after diluting for cell density) for bacterial enumeration. Cells were enumerated by counting 10 to 20 bacteria grid $field^{-1}$ in 30 random fields $filter^{-1}$ (1000-fold magnification) with an epifluorescence microscope (Kemp et al. 1993). Cell numbers in individual grids were averaged, and the numbers of cells per milliliter of culture was calculated.

Algal analyses. *Symbiodinium microadriaticum* were enumerated by hemocytometer using an epifluorescence microscope prior to and after centrifugation. Ten grid squares were counted for each sample and averaged to calculate the total number of algal cells per milliliter. Algal cell sizes were determined using a microscope, hemocytometer and eyepiece graticule. Chl *a* concentrations were measured fluorometrically with a Trilogy Laboratory Fluorometer (Turner Designs). Briefly, 1 ml aliquots of unconcentrated culture were filtered through Whatman GF/F glass fiber filters and extracted in 90% acetone for 24 h at 4°C (Parsons et al. 1984). *S. microadriaticum* genetic diversity was assessed using standard RFLP methods to verify *Symbiodinium* clades (Table 1; Rowan & Powers 1991). Algal DNA was extracted using the CTAB/phenol extraction methods as detailed by Coffroth et al. (1992) and Goulet & Coffroth (1997) and was amplified with PCR using a 'universal' primer (ss5) and the zooxanthella-biased primer ss3Z (Rowan & Powers 1991). Samples were subsequently digested with *Taq*I following the protocol of Goulet & Coffroth (2004) and visualized by ultraviolet light after ethidium bromide staining of the product in a 2% agarose gel.

Statistical analyses. Prior to analyses, assumptions of normality and homogeneity were tested and data were transformed as necessary. Regression analyses were used to assess DLA among strains and the relationship between DMS concentration and cell number. Analysis of variance (ANOVA) was used to assess whether DLA:chl *a* measures differed among strains, filtrates, controls, or over time. ANOVA was also used to assess $DMSP_p$ and total DMSP (particulate and dissolved; $DMSP_t$) values per cell. All statistical analyses were conducted using Minitab V. 10 (Minitab, Ver. 2000), with $\alpha = 0.05$ for all tests.

RESULTS

Algal DMSP

For each of the *Symbiodinium microadriaticum* cultures investigated, $DMSP_t$ closely paralleled $DMSP_p$ measurements and were not significantly different ($p > 0.05$; Table 2). *S. microadriaticum* $DMSP_t$ per cell and $DMSP_p$ per cell varied according to strain with CCMP

Table 2. *Symbiodinium microadriaticum* and *Emiliania huxleyi*. Comparison of $DMSP_p$ and $DMSP_t$ (particulate and total dimethylsulfoniopropionate; fmol cell^{-1}) and DMSP lyase potential activity (DLA; nmol min^{-1} fmol DMSP^{-1}; nmol min^{-1} µg chl a^{-1}) in cultured strains of *E. huxleyi* (CCMP 373 and 374) and *S. microadriaticum* (CCMP 421, 828, 829, 830 and 1633). Bacterial DLA in *S. microadriaticum* is also shown. Averages ± SD are presented. Sample number is indicated in parentheses. ND: not detected; –: not tested

CCMP strain	$DMSP_p$ (fmol cell^{-1})	$DMSP_t$ (fmol cell^{-1})	DLA:$DMSP_p$ (nmol min^{-1} fmol DMSP^{-1})	DLA:chl *a* (nmol min^{-1} µg^{-1})	Bacterial DLA (% of total DLA)
373	7.6 ± 2.3 (5)	–	0.1 ± 0.01 (5)	0.2 ± 0.1 (4)	–
374	4.7 ± 2.1 (6)	–	0.007 ± 0.0002 (2)	0.006 ± 0.005 (2)	–
421	201.0 ± 138.9 (3)	199.0 ± 151.4 (3)	3.2 ± 2.7 (5)	0.6 ± 0.5 (5)	15.2 ± 6.7 (4)
828	122.6 ± 60.7 (3)	105.5 ± 51.6 (4)	4.7 ± 1.6 (4)	0.8 ± 0.3 (4)	1.4 ± 0.8 (3)
829	81.4 ± 42.9 (3)	85.4 ± 38.4 (4)	27.3 ± 16.7 (5)	5.3 ± 0.9 (5)	2.2 ± 0.5 (3)
830	33.8 (1)	43.0 ± 14.5 (4)	ND (4)	ND (4)	–
1633	329.9 ± 193.2 (3)	347.9 ± 201.9 (4)	6.5 ± 2.7 (4)	0.7 ± 0.4 (4)	1.0 (1)

1633 having the greatest and CCMP 830 the least DMSP per cell. $DMSP_t$ and $DMSP_p$ per cell values for CCMP 1633 were significantly different from those for *Emiliania huxleyi* CCMP 373 and 374 ($p < 0.05$), but not from those for other *S. microadriaticum* investigated. Average cell diameters (µm) for *S. microadriaticum* strains were (average ± SD; n = 30 strain^{-1}) as follows: CCMP 421 (10.28 ± 1.08), 828 (10.69 ± 1.11), 829 (10.67 ± 1.22), 830 (10.74 ± 1.12) and 1633 (9.72 ± 0.82); and for *E. huxleyi* strains were as follows: CCMP 373 (5.08 ± 0.63) and 374 (5.03 ± 0.66). Only CCMP 1633 was statistically different in cell size ($p < 0.05$) among the various *S. microadriaticum* strains. Both *E. huxleyi* cultures were found to have less $DMSP_p$ when compared with *S. microadriaticum* cultures.

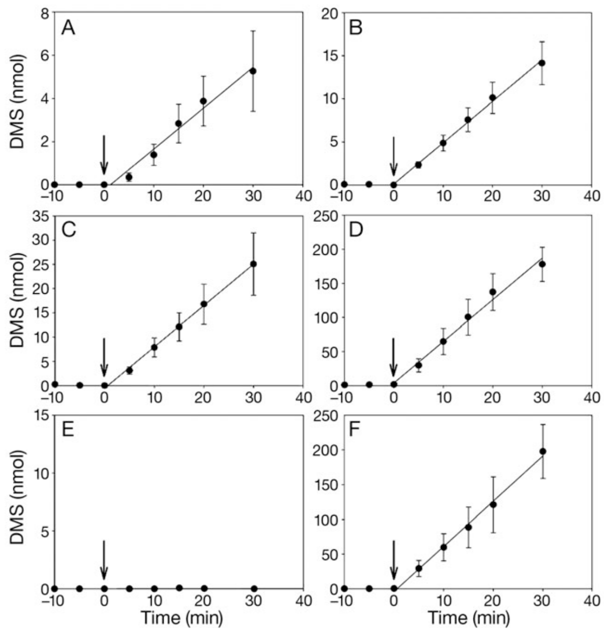

Fig. 1. *Symbiodinium microadriaticum* and *Emiliania huxleyi*. Dimethylsulfide (DMS; nmol) versus time (min) for (A) *E. huxleyi* strain CCMP 373, n = 4, and the following strains of *S. microadriaticum*: (B) CCMP 421, n = 5; (C) CCMP 828, n = 4; (D) CCMP 829, n = 5; (E) CCMP 830, n = 4; and (F) CCMP 1633, n = 4. Arrows indicate addition of dimethylsulfoniopropionate (DMSP) at t_0. Note different *y*-axis scales. Symbols indicate averages ± SE

Algal DLA

In all strains, DMS evolution prior to exogenous DMSP addition (Fig. 1) was not statistically different from that in controls ($p > 0.05$). DLA was greatest in strain CCMP 829 and was undetectable in CCMP 830. Additionally, when normalized to cell number or chl *a* (fmol cell^{-1}, nmol · min^{-1} · µg^{-1}), DLA for strains CCMP 373, 421, 828 and 1633 were markedly different from strain CCMP 829 ($p < 0.01$), but not from each other ($p > 0.05$), and CCMP 374 was statistically different from all other strains ($p < 0.01$) (Fig. 2, Table 2). DLA:chl *a* for *Emiliania huxleyi* CCMP 373 was greater than that of CCMP 374, but less than all *Symbiodinium microadriaticum* investigated (except CCMP 830; Table 2). DLA:$DMSP_p$ per cell averages were highest in strains 829 and 373 (0.06 and 0.02 nmol DMS · min^{-1} · fmol DMSP^{-1}, respectively) compared to other strains (Fig. 2). DLA:$DMSP_p$

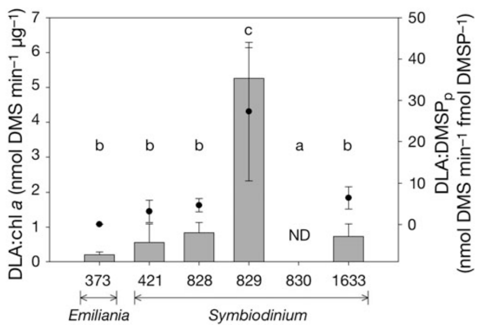

Fig. 2. *Symbiodinium microadriaticum* and *Emiliania huxleyi.* Dimethylsulfoniopropionate (DMSP) lyase activity (DLA): chlorophyll *a* (chl *a*) (nmol min^{-1} µg^{-1}; shaded bars) and DLA:DMSP$_p$ (particulate DMSP) (nmol DMS min^{-1} fmol DMSP^{-1}; ●) means for each strain. Error bars are SD; different letters indicate significant differences (ANOVA, $p < 0.01$) and apply to both data sets; ND: not detected. DMS:chl *a* — CCMP 373, n = 4; CCMP 421, n = 5; CCMP 828, n = 4; CCMP 829, n = 5; CCMP 830, n = 4; and CCMP 1633, n = 4. Note: CCMP 374 is not depicted (see 'Results')

per cell for strain CCMP 829 was statistically higher than that for all other strains in Fig. 2 ($p < 0.01$). CCMP 374 DLA measures (n = 2) are not presented in Fig. 2 because few replicates had measureable activity. Overall, CCMP 829 demonstrated the greatest DLA, whereas CCMP 421, 828 and 1633 had intermediate rates, with CCMP 830 having no detectable activity. Within strains, DMSP$_p$ concentrations were correlated with DLA. For each CCMP strain, r^2 values (in parentheses) were as follows: 373 (0.90), 374 (0.86), 421 (0.87), 828 (0.66), 829 (0.53) and 1633 (0.72). However, DMSP$_p$ concentrations were not correlated with DLA between strains (those strains with high DMSP did not necessarily have high DLA).

Bacterial contribution to DLA

After correction for the quantity of bacteria in the whole culture versus that in the 2 µm filtered sample, the calculated bacterial contribution to total DLA was found to be consistently low (Table 2). Bacterial DLA was greatest for strain CCMP 421 (approximately 15% of the total) and averaged <5% across all other strains (excluding CCMP 830 for which no DLA was detected). Therefore, the majority of DLA was associated with the algal component in all cultures examined. In bacterial filtrates, no algal contamination was detected in slide preparations using a fluorescence microscope. Furthermore, no algal cells were detected in the filtrate during bacterial enumeration (DAPI), whereas algal cells were observed in DAPI-stained whole culture samples. It was noted that algal cells in whole cultures did not appear to have attached bacteria as bacteria were evenly distributed and not concentrated around the dinoflagellate cells. Preliminary data (not shown) indicated minimal quantities of DMSP in the filtrate fractions of algal cell cultures examined for total DMSP. These data indicate a lack of algal contamination in filtrates.

DISCUSSION

Our results demonstrate that 4 of the 5 symbiotic dinoflagellate strains of the genus *Symbiodinium* examined in the present study are capable of performing the enzymatic lysis of DMSP to DMS, indicating DLA in these algae. *S. microadriaticum* strains demonstrated a range of DLA when grown in culture and exposed to exogenous DMSP addition. One of the *S. microadriaticum* strains in the present study (CCMP 830) did not demonstrate DLA, suggesting that DL is not a universal enzyme in this species or that DLA was not detected within our analytical capabilities. Furthermore, DLA was primarily associated with the algae (not bacteria) in the non-axenic cultures investigated.

Symbiodinium microadriaticum strains exhibiting DLA averaged DMS production rates of 0.6 to 5.3 nmol · min^{-1} · µg^{-1} chl *a*. The highest average DLA:chl *a* DLA in this study, 5.3 nmol · min^{-1} · µg^{-1}, occurred in CCMP 829. This strain was originally isolated from South Pacific clams, and DMSP concentrations in certain tissues of *Tridacna* sp. are some of the highest recorded in animal tissues to date (Hill et al. 2000). DLA for all algal lines closely parallel those reported by Harada et al. (2004) for particle-associated DLA in or near waters from the Gulf of Maine (DLA:chl *a* = 0.5 to 7.9 nmol · min^{-1} · µg^{-1}). In their study, DLA:chl *a* rates ranged from <5 to 53 nmol · min^{-1} · µg^{-1}, with the highest rate (53) occurring at an oligotrophic sampling site in the Sargasso Sea dominated by prymnesiophytes and dinoflagellates. It appears that differences in DLA normalized to chl *a* may be due to several factors, including species composition. Other studies have shown a relationship between high DMSP:chl *a* and DLA:chl *a* and have attributed this, in part, to nutrient limitation (Sunda et al. 2007). It is not likely that nutrient limitation played a role in our experiments, though nutrient quantities were not specifically addressed. More detailed experiments addressing the role of nutrient limitation and enzyme kinetics in *Symbiodinium* are needed.

DLA differences were detected between the strains investigated, though we did not specifically address enzyme turnover rates. Our finding that DLA was not detectable in CCMP 830 is consistent with the observations of Niki et al. (2000), who reported no DLA for 2 DMSP-producing Prymnesiophyceae species. The genus *Symbiodinium* encompasses 8 divergent clades (A to H) (for example, see Pochon et al. 2004, Coffroth & Santos 2005), and it is generally accepted that these clades are composed of several lineages representing species complexes (Santos 2004). *S. microadriaticum* strains (CCMP 830 and 1633) are the same algal clade (i.e. Clade B), but were isolated from different host origins (Atlantic and Pacific Oceans, respectively) and have very different DLA potentials, suggesting intraclade DLA variation within a species (Table 2). *Symbiodinium* clades are known to have different susceptibilities to light and thermal stress (Rowan 2004), which may, in part, explain a corals' sensitivity to bleaching. We hypothesize that if DMSP has an antioxidant role in *Symbiodinium*, DMSP production and DLA in these algae might be expected to correlate with one of the primary mechanisms involved in coral bleaching, namely, damage to Photosystem II in the symbionts (Iglesias-Prieto et al. 1992, Lesser & Farrell 2004). Susceptibility to Photosystem II damage may be mitigated if DMSP and its enzymatic cleavage products serve to alleviate conditions of oxidative stress by scavenging harmful reactive oxygen species (ROS; Sunda et al. 2002).

Because bacteria contributed <5 to 20% of the total DLA, the whole culture DLA values can be primarily attributed to the algal cells. Steinke et al. (2002a) reached a similar conclusion, finding that >95% of DLA was found in the particle fraction >2 µm. Bacterial DLA was greatest for CCMP 421, and this result is unexplained by the number of bacteria present in the filtrate. All cultures had similar ratios of bacteria to algae (approximately 5:1 on a per cell basis, respectively), and the amount of bacteria in the filtrate versus that in the total culture sample was also similar among strains (76.9 ± 16.6%). Bacterial DLA may in fact be overestimated due to algal cell rupture via filtration through polycarbonate filters, allowing algal contents into the experimental filtrate.

While the use of axenic cultures allows a more direct analysis of DLA in the algal component alone, we found that *Symbiodinium microadriaticum* treated with antibiotics had lower growth rates (data not shown). Additionally, checking cultures for axenicity by using standard plating techniques will miss a substantial portion of bacteria (Kogure et al. 1979). Though the bacteria in the present study were not genetically characterized, DLA could be over- or underestimated if a significant fraction of DMSP-utilizing bacteria (e.g. *Roseobacter*) were present in cultures. This is due to the finding that bacteria, in addition to the DL pathway, can also metabolize DMSP via the demethylation/demethiolation pathway, which would reduce the DMS quantities evolved (Taylor & Gilchrist 1991). We do not suspect that demethylation/demethiolation played a substantial role in our experiments, as no methanethiol was detected. Alternatively, bacterial DLA may increase the quantity of DMS detected, an important factor that we have addressed in the present study and estimate to be small. It is also recognized that the possibility of algal-attached bacteria cannot be excluded and may in fact be partially responsible for some of the observed DLA. However, during bacterial enumeration of whole culture samples, bacteria were not observed to be more numerous in close proximity to algal cells.

Our data demonstrate DLA variability within and across *Symbiodinium microadriaticum* strains, providing only the first step in an effort to further elucidate DLA regulation in the algae involved in numerous symbioses. DLA variability within strains could be attributed to several factors. According to Steinke et al. (2007) in their study of *Emiliania huxleyi*, *in vivo* DLA may vary during the course of a day due to enzyme turnover, and individual strains of algae may respond differently to exogenous substrate (DMSP) additions. Our finding that CCMP 373 has greater DLA than CCMP 374 is in agreement with other reports (Steinke et al. 1998), though differences in experimental conditions preclude direct comparison with previously reported rates (Steinke et al. 1998, 2000, 2007). Additionally, *E. huxleyi* DLAs in the present study were used to demonstrate the validity of the given assay for the detection and optimization of DLA in *S. microadriaticum*; the assay was not optimized for *E. huxleyi* DLA measurements. While several assay parameters differed when compared to those in previous studies, our *E. huxleyi* data are in agreement with published DLA measurements when calculations incorporate differences in activity associated with pH. For example, using data presented by Steinke et al. (1998), the calculated DLA:$DMSP_p$ (nmol DMS · min^{-1} · $fmol^{-1}$ DMSP) for CCMP 373 is 0.27 at an optimal pH of 6, but, at pH 8, it would be approximately 10% of that value based on the reported pH trials. Additional sources of variability may include shifts in enzyme affinity during growth, culture conditions and individual variability associated with enzyme assay parameters. Given these observations, the present study demonstrates that some *S. microadriaticum* have DL capabilities and that DLAs are distinguishable between algal strains at the given concentration of exogenous DMSP addition.

Symbiodinium are known to contain substantial quantities of DMSP (Jones et al. 1994, Hill et al. 1995, Broadbent et al. 2002, Broadbent & Jones 2004), with cultured zooxanthellae having 179 fmol $cell^{-1}$ DMSP (Keller et al. 1989). Our $DMSP_p$ measurements for *S. microadriaticum* strains (34 to 330 fmol $cell^{-1}$) are in agreement with those from previous studies (Jones et al. 1994, Hill et al. 1995, Broadbent et al. 2002). Several studies support the hypothesis that DMSP and DLA are produced at varying levels by different organisms and that DMSP and DLA vary between strains of the same species (Steinke & Kirst 1996, Steinke et al. 1996, 2002a,b, Wolfe et al. 1997, Niki et al. 2000). Our comparisons of per cell DMSP between the 5 strains of *S. microadriaticum* tested revealed differences, so further investigations of DMSP levels within *Symbiodinium* are warranted. We found no apparent correlation between intracellular DMSP concentrations and DLA between strains, though strain CCMP 830 had no detectable DLA and also had the lowest DMSP concentrations of the *S. microadriaticum* strains investigated.

Corals (host and algal cells) may be exposed to conditions of elevated oxidative stress when harmful ROS are not scavenged or detoxified by antioxidants. Because corals contain photosynthetic, oxygen-producing algae, high levels of antioxidant enzymes (and free radical scavengers) are found in host (and algal) tissues (Lesser & Shick 1989, Dykens et al. 1992, Downs et al. 2002). With proposed antioxidant functions, DMSP, and more importantly its enzymatic cleavage products via DLA, may play significant roles in alleviating conditions of oxidative stress on reefs. To investigate this potential antioxidant role we are exploring the effects of various oxidative stressors on DMSP levels and DLA in isolated *Symbiodinium microadriaticum*. Our studies herein have shown that coral symbiotic algae contain DLA, and further studies are directed towards determining if cnidarian hosts, in common with other symbiotic host species, contain DLA.

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Alterations in dimethylsulfoniopropionate (DMSP) levels in the coral *Montastraea franksi* in response to copper exposure

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ABSTRACT

Symbiotic corals routinely experience hyperoxic conditions within their tissues due to the photosynthesis of the endosymbiotic dinoflagellate microalgae (*Symbiodinium* spp.). *Symbiodinium* spp. produce high intracellular levels of the osmolyte dimethylsulfoniopropionate (DMSP). It has recently been discovered in marine algae that DMSP and its enzymatic breakdown products also play a significant role in the scavenging of cellular reactive oxygen species (ROS). To examine this potential for DMSP in corals, we exposed the hard coral *Montastraea franksi* to 1, 10 and 50 $\mu g\,L^{-1}$ (ppb) concentrations of the oxidative stressor, copper. Levels of total ($DMSP_t$, all coral tissue) were higher than particulate $DMSP_p$ (algal component only), demonstrating partitioning of DMSP between algal symbionts and coral host. Significant changes in levels of $DMSP_t$ and $DMSP_p$ occurred in *M. franksi* after 48 h, demonstrating a response to copper and indicating a potential antioxidant role for DMSP. $DMSP_t$ and $DMSP_p$ levels decreased with copper dose, although at the highest copper dose $DMSP_p$ levels increased, whereas $DMSP_t$ levels did not. This observed differential response to copper between $DMSP_t$ and $DMSP_p$ demonstrates that physiological changes may be overlooked if conclusions are based upon $DMSP_t$ levels alone, which is a common measure used in coral studies. Decreases in chlorophyll *a* and algal cell numbers in response to elevated copper were also observed. These indices are important physiological indicators and are often used as indices to normalize DMSP levels. Our data suggest that the use of these common indices for normalizing DMSP may not always be appropriate.

1. Introduction

Symbiotic cnidarians routinely experience hyperoxic conditions within their tissues due to the photosynthesis of the endosymbiotic dinoflagellate microalgae (*Symbiodinium* spp.) (Dykens and Shick, 1982; Kühl et al., 1995). To mitigate against potentially damaging reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($^\bullet OH$), and singlet oxygen ($^1O_2$), corals possess various constitutive and/or inducible enzymatic and non-enzymatic antioxidant systems, including superoxide dismutases, glutathione, xanthophylls and ascorbate peroxidases (for a review see Lesser, 2006). However, inherent physiological stress in coral symbioses can be compounded by additional exogenous oxidative stressors. For example, environmental pollutants can exacerbate conditions of oxidative stress in corals, compromising their ability to cope with and recover from imposed stressors by overwhelming their antioxidant protective system (Downs et al., 2002).

Symbiodinium spp. and many other species of marine algae, produce high intracellular levels of the osmolyte dimethylsulfoniopropionate (DMSP) (Keller et al., 1989; Keller and Korjeff-Bellows, 1996; Van Alstyne et al., 2006; Yoch, 2002). It has recently been discovered in marine algae that DMSP and its enzymatic breakdown products also play a significant role in the scavenging of cellular ROS (Sunda et al., 2002). Since damage by ROS has been implicated in the physiological mechanism underlying coral bleaching – the stress related dissociation of the coral–algal symbiosis which occurs during exposure to elevated water temperatures and a suite of other stressors (see for example Lesser et al., 1990) – it is not surprising that the role of DMSP in corals is of growing interest.

In marine algae, DMSP has also been shown to be an osmolyte (Kirst, 1990), a cryoprotectant (Karsten et al., 1996), a herbivory deterrent and attractant (DeBose et al., 2008; Wolfe et al., 1997), an antiviral defense mechanism (Evans et al., 2006) and involved in sulfide detoxification (Havill et al., 1985). However, DMSP and its enzymatic breakdown products, dimethylsulfide (DMS), acrylate, dimethylsulfoxide (DMSO) and methane sulfinic acid (MSNA) can readily scavenge hydroxyl radicals and other ROS (Sunda et

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al., 2002). Significant in this regard is the enzymatic cleavage of DMSP by DMSP lyase (DL), as the cleavage products, acrylate and DMSO are 60 and 20 times, respectively, more effective in scavenging hydroxyl radicals than DMSP itself (Sunda et al., 2002). Additionally, this suite of antioxidant compounds can scavenge ROS in multiple cellular compartments as it is composed of water- or lipid-soluble scavengers of the extremely harmful hydroxyl radical and other ROS. DMS and DMSO can neutralize lipid peroxidation in photosynthetic membranes and harmful radicals in chloroplasts, respectively, thus targeting important ROS sites (Lee and de Mora, 1999).

In corals, the presence and role(s) of DMSP have only recently been investigated (see for example; Broadbent et al., 2002; Broadbent and Jones, 2004, 2006; Hill et al., 1995; Jones et al., 2007; Jones and Trevena, 2005; Van Alstyne et al., 2006, 2009; Yost and Mitchelmore, 2009). It has been established that the symbiotic dinoflagellates of corals produce DMSP and have DL capabilities, but so far DL has not been shown to exist in corals or other host species harboring symbiotic dinoflagellates (Hill et al., 2004). It is known that there is a considerable variation in DMSP levels within zooxanthellae clades (Yost and Mitchelmore, 2009) and it is plausible that the translocation of DMSP from symbiont to host occurs (Hill et al., 2004), highlighting the need for particulate DMSP (algal cells only; $DMSP_p$) quantification in addition to total DMSP (algal and host cells; $DMSP_t$) measures. Elevated levels of DMS above stressed reefs have led authors to suggest the involvement of DMSP in a corals stress response (Jones et al., 2007).

Support for the antioxidant role of DMS/DMSP comes from experiments showing that oxidative stressors such as solar ultraviolet radiation, CO_2 limitation, Fe limitation, H_2O_2 and high Cu^{2+} (ions) substantially increased cellular DMSP and/or its lysis to DMS (Sunda et al., 2002, 2005). Copper was used in these experiments as it is known to cause oxidative stress by catalyzing the formation of the hydroxyl radical by Haber–Weiss and Fenton reactions, from the photosynthetic by-product, hydrogen peroxide (Abalde et al., 1995; Okamoto et al., 2001). The hydroxyl radical is the most powerful oxidizing radical likely to arise in biological systems, and is capable of reacting with practically every biological molecule (Buettner, 1993), resulting in damage to lipids, proteins and nucleic acids (Gutteridge and Wilkins, 1983).

The response of corals and their symbiotic algae to copper is well studied from ecotoxicological experiments. For example, copper reduces fertilization success (i.e. EC_{50} values ranging from 14.5 to 39.7 $\mu g\,L^{-1}$ (Heyward, 1988; Negri and Heyward, 2001; Reichelt-Brushett and Harrison, 1999, 2005). Sublethal loss of symbiotic dinoflagellates and bleaching of tissues has been reported in adult corals exposed to concentrations of 10–60 $\mu g\,L^{-1}$ Cu between 2 and 10 days (Evans, 1977; Jones, 1997, 2004); however, not all studies report sublethal loss of symbiotic dinoflagellates (for example Grant et al., 2003). Several studies have reported copper threshold levels of sublethal stress between 20 and 50 $\mu g\,L^{-1}$ in different species of cultured dinoflagellates (Goh and Chou, 1997; Mandelli, 1969) and reduced specific growth rates at 40 $\mu g\,L^{-1}$ (Goh and Chou, 1997). Alterations in chlorophyll *a* (chl-*a*) concentrations in *Symbiodinium* spp. and other algae have also been reported in response to copper stress (e.g. see Brown, 2000; Edmunds et al., 2003; Fitt et al., 1993; Gleason and Wellington, 1993; Grant et al., 2003; Nystrom et al., 2001; Sunda et al., 2002). Corals also respond to alleviate copper toxicity by up-regulating cellular defense mechanisms, including antioxidants (Gilbert and Guzman, 2001; Grant et al., 2003; Mitchelmore et al., 2007; Morgan et al., 2001, 2005; Morgan and Snell, 2006; Venn et al., 2009).

To further understand the function(s) and regulation of DMSP within the coral–algal symbiosis we quantified the response of intact, field-collected coral (*Montastraea franksi*) to 0–50 $\mu g\,L^{-1}$ Cu. DMSP concentrations in air-brushed coral fragments (host and algae; total DMSP ($DMSP_t$)) and in the zooxanthellae isolated from these corals (particulate DMSP ($DMSP_p$)) were quantified together with numerous other algal and coral cellular and biochemical indices. These indices were measured to monitor potential bleaching effects and/or to serve as normalization factors for DMSP concentrations. DL was not assessed due to the logistical limitations.

2. Materials and methods

2.1. Collection and handling

Five large (50–60 cm diameter) colonies of *M. franksi* were collected from 5 m depth from offshore patch reefs (32°25°30.14°N, 64°42°27.15°W) in the Bermuda lagoon and transported back to the Bermuda Institute of Ocean Sciences (BIOS) submerged within large (100 L) high density polyethylene (HDPE) containers (fish tote containers). Small fragments (3–5 cm diameter) were then cut from the corals using a hammer and chisel and the bases of the fragments leveled off using a carbide-tipped circular cut-off wheel mounted to a high speed rotary tool. Each explant was numbered with a small (3 mm diameter) glue-on, flexible polyethylene shellfish tag (Hallprint, Victor Harbour, Australia) attached to the base of the fragment using gel-type cyanoacrylate glue (Loctite Quik Set, Superglue 404). Explants were left for 14 days to recover from the handling and preparation procedures in flowing seawater under one layer of 50% neutral density shade-cloth before experimentation.

Corals were then exposed to different concentrations of copper in specially constructed all-Teflon dosing chambers. In these chambers, seawater was pumped at a rate of 500 mL/min (i.e. recirculating with a 20 min total replenishment time) by a Valcor Teflon metering pump (Valcor Engineering Corporation, Springfield, NJ, USA) from a 50 L fluoride impregnated, HDPE rectangular carboy (a 'reservoir') (Fisher Scientific, Agawam, MA, USA) into a 10 L fluoride impregnated, HDPE 10 L Jerrican (a 'dosing chamber') (Fisher Scientific, Agawam, MA, USA) located on the upper level of a four foot, two shelf, polyethylene utility cart. Seawater then gravity feeds back into the reservoir on the corresponding lower level of the cart. Four dosing chambers (and associated reservoirs) could be fitted onto a single cart and there were replicate carts, providing 16 replicates, all-Teflon dosing chambers for experimental exposures.

During experimentation, all fragments were held on a 10 cm square Teflon, 4 mm thick polytetrafluoroethylene (PTFE) platform inserted into the centre of each of the dosing chambers. The height of the platform was adjusted using PTFE Teflon screws attached to the corners of the platform and adjusted so the corals' surface was ∼2–3 cm below the water surface. All experiments were conducted outdoor under natural sunlight. To provide cover against rainfall, a layer of 3 mm UV transparent acrylic sheet (Acrylite® OP-4, Cyro Industries Rockaway, NJ, USA) was placed over the four dosing chambers. This acrylic sheet transmits a high percentage of light in the UV-A (315–400 nm) and UV-B (280–315 nm) regions. Photosynthetically Active Radiation (PAR, 400–700 nm) is slightly attenuated (<10%) using the 3 mm thick sheet, but irradiance was further reduced to ∼50% of ambient using neutral density filters (Lee Filters, Burbank, CA, USA). During experiments PAR light levels were recorded over 10 min intervals with a LI-190SA Quantum Sensor and a LI-1400 data logger (Licor Lincoln, NE, USA).

To achieve temperature regulation within the dosing chambers, de-ionized cooling water from water/heater units (6000 Series, Heater/Chiller unit, Polyscience, Niles, IL, USA) was pumped via 1/2 inch commercial garden hosing through a 4-way manifold into tygon high purity tubing and then into 2 m long coiled, 3/8 inch (internal diameter) Perfluoroalkoxy (PFA) cooling coils which were

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inserted into each of the dosing chambers on each cart. All sunlight-exposed tubing associated with the cooling system was surrounded by 3/8 inch thick, polyethylene pipe and tube insulation. Temperatures within the dosing chambers were regulated at 25 ± 0.2 °C using this system.

Coral explants were exposed to one of the four copper sulfate concentrations (i.e. treatments) including <1 μg L^{-1} (i.e. a control or ambient seawater), or 5, or 10 or 50 μg L^{-1} copper (as Cu_2SO_4) for 48 h. This range of copper concentrations was chosen based on environmentally relevant copper concentrations that are often much higher in estuaries and coastal waters, areas close to anthropogenic pollution sources where concentrations as high as 29.2 μg L^{-1} Cu have been reported (Sadiq, 1992). Actual copper concentrations (50 μg L^{-1}: 30 ± 7; 10 μg L^{-1}: 8 ± 4; 5 μg L^{-1} and control below detection limit) were determined via ICP-AAS by Jodi Schwarz (Vassar College, NY). Each treatment was replicated four times, i.e. 4 treatments × 4 replicates = 16 individual dosing chambers. At the start of the experiment, one fragment from each of the five colonies was placed in each of the 16 chambers. Water changes and re-dosing occurred at 24 h. All experiments were started at 09:00 h and ended with samples being wrapped in two layers of aluminum foil and flash frozen in liquid nitrogen. Corals were then shipped on dry ice and stored at −80 °C (for up to 4 months) until analysis of DMSP (and associated indices) at the Chesapeake Biological Laboratory, Solomons, MD.

2.2. Homogenized coral fragment

We used a modification of the coral homogenization technique described in Broadbent et al. (2002), to measure DMSP and DMS. Individual coral fragments were removed from −80 °C storage and immediately airbrushed (Szmant and Gassman, 1990) using sterile artificial seawater to obtain a homogenate that was collected in a 0.5 L plastic bag. Total homogenate volumes were measured with a volumetric cylinder. We did not encounter a froth layer on top of the homogenate in these samples as reported by Hill et al. (1995), therefore a homogenizer was not used, although the sample was well mixed and homogeneous after airbrushing. Sub-samples were immediately removed using sterile transfer pipettes for multiple indices including $DMSP_t$ and $DMSP_p$ analyses, total protein, chl-*a*, algal counts and algal size (diameter). The remaining coral skeleton was saved for surface area measurements (Marsh, 1970).

2.3. Particulate and total DMSP homogenate analyses

To isolate the algal component ($DMSP_p$) from the whole filtrate, 1 mL of homogenate was passed through a Whatman GF/F filter at low pressure (<25 mm Hg) and placed immediately in a headspace vial containing 2 mL 5N NaOH, for $DMSP_p$ analysis. Loss of $DMSP_p$ due to the filtration was virtually minimal as preliminary tests showed no increase in filtrate DMSP when filtering larger numbers of algae (Yost, unpublished results). For $DMSP_t$ analysis, 1 mL of unfiltered homogenate was added to 1 mL 10N NaOH in a headspace vial.

2.4. Algal indices

For chl-*a* analysis, 1 mL homogenate aliquots were filtered through Whatman GF/F filters and extracted in 90% acetone for 24 h at 4 °C (Parsons et al., 1984). Unfiltered homogenate aliquots were used for analysis of zooxanthellae size, number and total protein content. Homogenate sub-samples for protein analysis were frozen, thawed and quantified for total protein by the BCA assay (Pierce Chemical). Bovine serum albumin was used to construct a standard curve. Algal cells, diluted with sterile artificial seawater, were immediately enumerated by haemocytometer using an epifluorescence microscope. Thereafter, 10 replicate counts per sample were averaged and corrected for dilution to calculate the total number of algal cells per mL of homogenate. Algal cell sizes (to calculate cell volume) were determined using a microscope and eyepiece graticule (samples were preserved in 5% buffered formalin; $n = 30$ per sample). Phylogenetic (clade) analysis of zooxanthellae was determined by length heteroplasmy in domain V of chloroplast large subunit (cp23S)-rDNA (Santos et al., 2003).

2.5. DMSP analyses and DMS calibration

All analyses were conducted after a 24 h headspace equilibration period following NaOH addition. During the equilibration period all samples were stored in the dark at room temperature. Samples were analyzed with a Hewlett-Packard 5890 Series II gas chromatograph (GC) fitted with a Chromosil 330 packed column (Supelco, Bellefonte, PA), and flame photometric detector (FPD). System temperature settings were: injector 150 °C, column oven 60 °C and detector 175 °C. Nitrogen gas was the carrier (60 cm^3 min^{-1}) and air (60 cm^3 min^{-1}) and hydrogen (50 cm^3 min^{-1}) were flame gases. Data were collected and analyzed using HP ChemStation (Hewlett-Packard, Palo Alto, CA). Quantifications were made by headspace analysis following DMSP conversion to DMS by alkaline hydrolysis. Known concentrations of DMSP (purchased from Research Plus Inc., Bayonne, NJ) were diluted in sterile water to give working solutions, which were frozen in small aliquots at −80 °C. Multiple standard curves of serial dilutions of DMSP were used to construct calibration curves (using the square-root of the peak area) and these linear regressions served to convert peak areas from GC headspace measurements to DMS concentrations. The standards were prepared in identical proportions of buffers and preparation solutions to those used in experimental samples (ASW, NaOH). The same total liquid volume (2 mL) was used in all headspace vials. The precision of DMS analysis varied <5% and headspace storage trials showed no losses occurred with the analytical methods employed. Detection limit of the GC was 1 nmol DMS.

2.6. Statistical analyses

After testing assumptions of normality and homogeneity, analysis of variance (ANOVA) was used to assess whether DMSP ($DMSP_p$ and $DMSP_t$), chl-*a*, algal cell number and cell size, total protein and coral surface area indices differed among controls and corals exposed to copper. Pearson's correlations were used to determine correlation between indices (pairwise: chl-*a*, algal cell number, total protein and coral surface area). All statistical analyses were conducted using Minitab© v. 10 (Minitab Inc. 2000).

3. Results

Symbiotic dinoflagellate densities (cell numbers per cm^2) declined with increasing copper concentration and were significantly different from control treatments at 50 μg L^{-1} Cu after 48 h exposures ($p < 0.05$) (Fig. 1).

Chl-*a* concentrations, normalized either to surface area (cm^{-2}), to algal cell number (cell^{-1}) or to total protein all showed similar patterns of response to copper with significantly less chl-*a* at 5 and 10 μg L^{-1} Cu (but not at 50 μg L^{-1}) compared to controls ($p < 0.05$) (Table 1). Total protein levels normalized to surface area (cm^{-2}) also showed a similar trend, although they were only significantly different compared to controls ($p < 0.05$) at 10 μg L^{-1} Cu (Fig. 2).

During this experiment we found that some of our normalizing indices alone were responding to Cu addition, complicating our DMSP concentration analyses. To investigate this further, correlations were performed between multiple indices to determine how these indices were responding to Cu (Table 2). Chl-*a*, symbiotic

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Table 1
Impact of copper exposure on chlorophyll *a* indices in *M. franksi* (mean ± SE).

Copper conc. ($\mu g\,L^{-1}$)	*n*	Chlorophyll *a* (pg)		
		Algal cell^{-1}	Surface area (cm^{-2})	Total protein (mg^{-1})
<1	20	6.7 ± 0.5	17.0 ± 1.5	7.5 ± 0.9
5	20	4.7 ± 0.2*	11.7 ± 0.7*	11.3 ± 1.6*
10	20	5.0 ± 0.3*	11.8 ± 1.2*	11.1 ± 1.3*
50	20	7.3 ± 0.9	13.3 ± 0.7	6.5 ± 0.5

* $p < 0.05$ compared to controls.

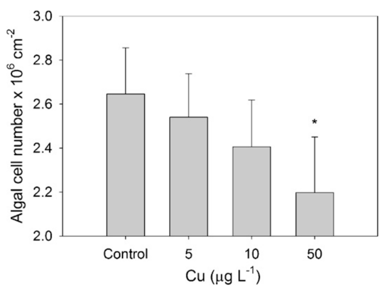

Fig. 1. *Montastraea franksi* symbiotic dinoflagellate density ($10^6\times$) per coral surface area (cm^2) in corals exposed to <1 (control), 5, 10 and 50 $\mu g\,L^{-1}$ Cu for 48 h. Data are $\bar{x} \pm$ SE ($n = 20$); asterisk (*) indicates significant difference compared to control ($p < 0.05$).

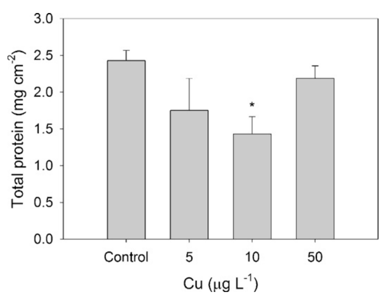

Fig. 2. *Montastraea franksi* total protein (mg) per coral surface area (cm^2) in corals exposed to <1 (control), 5, 10 and 50 $\mu g\,L^{-1}$ Cu for 48 h. Data are $\bar{x} \pm$ SE ($n = 20$); asterisk (*) indicates significant difference compared to control ($p < 0.05$).

dinoflagellate density, total protein and coral surface area were significantly correlated between all copper exposures with the exception of correlations that included protein indices at 5 $\mu g\,L^{-1}$ Cu (Table 2).

Table 2
Impact of copper exposure on symbiosis indices in *M. franksi* (Pearson's correlation coefficient; all *p*-values < 0.001 except where indicated; $n = 20$ per indice).

Indices	Copper exposure ($\mu g\,L^{-1}$)			
	<1	5	10	50
Chl-*a*/algal cell	0.83	0.90	0.93	0.75
Chl-*a*/total protein	0.68	0.07*	0.78	0.81
Chl-*a*/surface area	0.80	0.86	0.61†	0.88
Algal cells/total protein	0.81	−0.03*	0.78	0.71
Algal cells/surface area	0.87	0.84	0.68	0.78
Total protein/surface area	0.88	0.00*	0.36*	0.79

* $p > 0.05$.
† $p < 0.01$.

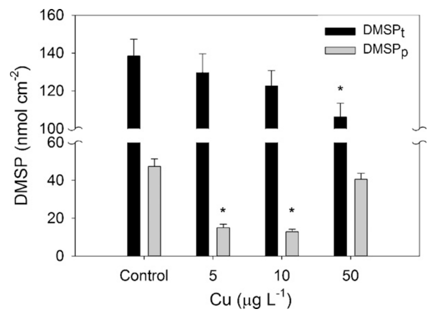

Fig. 3. *Montastraea franksi* DMSP (nmol) per coral surface area (cm^2) for control, 5, 10 and 50 $\mu g\,L^{-1}$ Cu exposures. Data are $\bar{x} \pm$ SE ($n = 20$); asterisks (*) indicate significant difference compared to control ($p < 0.05$).

Because chl-*a* and protein concentrations changed in response to copper, they were not included as indices for DMSP. Yet, in the DMSP literature, chl-*a* is a commonly used index. In addition, as algal cell numbers decreased with copper exposure, indexing $DMSP_t$ levels to algal cell number alone could be misleading.

There was no effect of copper exposure on the genetic type of dominant *Symbiodinium* spp. present, as all algal samples analyzed were clade B184. Algal cell volumes were not significantly different among treatments (calculated average cell volume for controls was 7.4 $\mu l/10^7$ cells).

$DMSP_t$ concentrations were significantly greater than $DMSP_p$ concentrations for all copper treatments and controls when indexed to coral surface area or algal cell number (average of 6× greater for $DMSP_t$) showing that a large amount of the DMSP is present in host tissues. $DMSP_t$ levels ($nmol\,cm^{-2}$) declined with increasing concentrations of copper and were significantly less for corals exposed to 50 $\mu g\,L^{-1}$ Cu ($p < 0.05$) (Fig. 3). However, on an algal basis, $DMSP_t$ ($fmol\,cell^{-1}$), although lower with copper exposures, did not differ significantly at any of the copper exposures compared with the controls ($p > 0.05$) (Fig. 4).

$DMSP_p$ concentrations ($nmol\,cm^{-2}$) were significantly less at 5 and 10 $\mu g\,L^{-1}$ Cu ($p < 0.05$), but unlike $DMSP_t$ were not significantly different compared with controls at 50 $\mu g\,L^{-1}$ Cu ($p > 0.05$) (Fig. 3). Similar patterns emerged for $DMSP_p$ concentrations when normalized to algal cell number instead of surface area. $DMSP_p$ ($fmol\,cell^{-1}$) was significantly lower at 5 and 10 $\mu g\,L^{-1}$ Cu exposures compared to controls ($p < 0.05$) (Fig. 4).

4. Discussion

This study is the first to demonstrate significant changes in DMSP concentrations in corals following exposure to an oxidative stressor, copper, and is the first study to examine the relationship between particulate algal DMSP ($DMSP_p$) and total DMSP ($DMSP_t$); which is the most commonly reported measure in studies of DMSP

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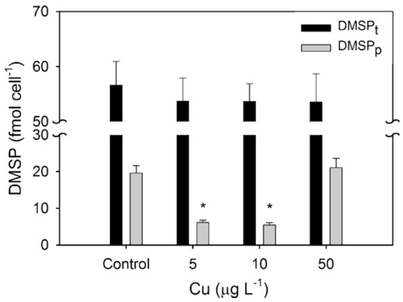

Fig. 4. *Montastraea franksi* DMSP (fmol) per algal cell for control, 5, 10 and 50 $\mu g L^{-1}$ Cu exposures. Data are $\bar{x} \pm$ SE ($n = 20$); asterisks (*) indicate significant difference compared to control ($p < 0.05$).

and corals. Copper exposure resulted in up-regulation of various antioxidant genes (Schwarz, unpublished data) and induction of DNA damage in host cells (Mitchelmore, unpublished data) consistent with oxidative stress conditions. We found that exposure of the hard coral *M. franksi* to elevated levels of copper caused significant changes in DMSP, chl-*a*, algal cell number and total protein levels.

Corals and other invertebrates in symbioses with zooxanthellae have significant body burdens of DMSP (Hill et al., 2000; Van Alstyne and Puglisi, 2007). Recent evidence that DMSP is solely produced by zooxanthellae in cnidarian–algal symbioses (Van Alstyne et al., 2009) and that DMSP is present in tissues lacking symbionts in other invertebrates (Hill et al., 2000, 2004) suggests translocation of DMSP. Indeed, we found that $DMSP_t$ concentrations were greater than $DMSP_p$ concentrations, evidence that supports this potential DMSP translocation. The significance of DMSP in host tissues is yet unknown and may depend, in part, on whether host coral cells have DL. If host corals have DL, then DMSP, through its conversion to subsequently more powerful antioxidants as shown by Sunda et al. (2002), could be used to protect host tissues from excessive oxidative stress (as could DMSP itself). Alternatively, high concentrations of DMSP in host tissues could perturb host protein/enzyme functions, a condition that could be ameliorated by host DL (i.e. similar to other detoxifying enzymes) (Karsten et al., 1996; Nishiguchi and Somero, 1992). However, to date DL has only been described in the algal component (Yost and Mitchelmore, 2009).

DMSP and/or DL levels have been shown to respond to oxidative stressors, including copper, in marine algae (Sunda et al., 2002). Several antioxidant functions in corals resulting from *Symbiodinum* spp. DMSP and DL activities have been recently inferred (Jones et al., 2007; Yost and Mitchelmore, 2009). Given that DMSP is at mM concentrations within the symbiotic algal cells, DMSP may be a critical and multifaceted component in a coral's overall antioxidant defense mechanism. If DMSP is being used as an antioxidant under conditions of oxidative stress, levels may decrease following initial or chronic exposures as DMSP and its conversion products are being used as ROS scavengers. Indeed, we found that levels of $DMSP_p$ decreased following exposure to 5 and 10 $\mu g L^{-1}$ copper. Conversely, in response to excess ROS, higher intracellular concentrations of DMSP may result from an up-regulation (increased production) of DMSP. For example, alterations in intracellular concentrations of DMSP have been linked to oxidative stressors including high Cu^{2+} (ions) in marine algae (Sunda et al., 2002, 2005). This potential up-regulation could explain why levels of $DMSP_p$ did not differ from control levels at the highest copper dose. The threshold for the up-regulation of DMSP in response to oxidative stress is unknown and may reflect ROS levels, or occur once a low, threshold level of DMSP is reached. Therefore, changes in DMSP levels in corals may be complex and biphasic, particularly given the nature of the symbiotic relationship, as DMSP (and its conversion products) can be diffusively or actively passed from the algae to the host cells.

Current protocols for DMSP in corals measure $DMSP_t$, which may limit the physiological significance of DMSPs response to stressors in coral–algal symbioses. Complex DMSP responses and physiological implications were shown in our investigation of multiple indices and the teasing apart of $DMSP_t$ and $DMSP_p$; responses which would have been missed using current protocols. Corals showed a decrease in $DMSP_t$ concentrations per cm^2 and loss of algal cells with increasing concentrations of copper, although only significant at the highest dose. When $DMSP_t$ levels were normalized to algal cell number, no significant reduction in $DMSP_t$ at the highest copper dose was evident. Therefore, results based solely on $DMSP_t$ levels normalized to coral surface area (commonly used in the coral field) may simply reflect algal cell loss. Indeed, the loss of algae between controls and high copper dose averages 17%, mirroring the average 23% loss of $DMSP_t$. Analyzing intact coral fragments for $DMSP_t$ analyses would preclude accurate quantification of algal cell numbers so that these indice correlations could not be made.

A markedly different response to copper was observed in the algal cells alone. $DMSP_p$ levels, per surface area or per algal cell, both decreased an average of 70% at the lower two copper doses compared to controls. These decreases may be due to a decrease in intracellular DMSP pools (as previously discussed) and/or an increase in DL, where the up-regulation of the enzyme could be accomplished through increased DL biosynthesis or changes in DL affinity for the DMSP substrate under conditions of oxidative stress. However, there was no reduction in $DMSP_p$ concentrations at 50 $\mu g L^{-1}$ Cu, i.e. the exposure at which $DMSP_t$ (on a per surface area basis) and algal cell contents were significantly reduced. This indicates that, even with fewer zooxanthellae at the highest copper exposure, DMSP concentrations in the remaining algae likely increased or at least were maintained (via increased production) at the original control levels. Fig. 4 demonstrates only a slight $DMSP_p$ increase (i.e. 19.6–21.1 fmol $cell^{-1}$), although elevated production will be missed if the algae are translocating DMSP to the host, which in turn is being used to maintain the steady levels seen in the $DMSP_t$ response (on a per algal basis). These results suggest that $DMSP_p$ levels are responsive to copper exposure.

Interestingly, similar trends emerged for $DMSP_p$ (nmol cm^{-2}, fmol $cell^{-1}$), chl-*a* and protein concentrations, all of which were reduced at 5 and 10 $\mu g L^{-1}$ Cu but returned to control levels at 50 $\mu g L^{-1}$ Cu. It is known that bleaching can reduce algal number and/or chl-*a* concentrations (Gleason and Wellington, 1993; Porter et al., 1989). Also, copper exposure has been reported to cause reductions in photosynthetic pigments (chl-*a*)/efficiency as well as algal cell loss (bleaching) and even increases in total carotenoids, implying oxidative damage to lipids (Droppa and Horvath, 1990; Jones, 1997; Sunda et al., 2002; Grant et al., 2003). However, chl-*a* concentrations are known to increase on a per cell basis in bleached corals and *M. franksi* specifically (Fitt et al., 1993; Edmunds et al., 2003). Nystrom et al. (2001) reported no copper (11 $\mu g L^{-1}$) effects on acutely exposed corals, though corals pre-exposed to increased levels of copper had significant reductions in gross primary production. Others report no decrease in chl-*a* (Brown, 2000; Grant et al., 2003) and no decrease in photosynthetic capacity in corals chronically exposed to copper (Grant et al., 2003), though excess copper usually decreases photosynthesis (Droppa and Horvath, 1990). A parallel increase in $DMSP_p$ at 50 $\mu g L^{-1}$ Cu and an increase in chl-*a* (relative to the lower copper doses) may indicate that DMSP and its conversion products are acting as a protective mechanism, scavenging ROS at the site of production in zooxanthellae, a hypoth-

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esis worthy of further investigation. Additionally, whether up- or down-regulation (or loss) of chl-*a* is occurring, these data demonstrate that this commonly used algal DMSP indice and may not be appropriate when investigating levels in response to 'natural' or anthropogenic stressors.

The reduction of total protein at 5 and 10 $\mu g\,L^{-1}$ Cu does not appear to be explained by zooxanthellae loss, as protein concentrations were higher (respectively) at 50 $\mu g\,L^{-1}$ Cu, the only exposure at which significant algal loss was recorded. There are several plausible explanations for the observed changes in total protein. Proteins can be up- or down-regulated, damaged due to ROS, or degraded. The DMSP pool may be controlled by the availability of methionine, via protein degradation (a process known to occur in times of increased stress, i.e. as evidenced by elevated heat shock proteins and ubiquitin levels in stressed corals; Downs et al., 2000, 2002), therefore liberating this key amino acid and providing the necessary building blocks for increased DMSP synthesis.

One point of concern raised in this study is the appropriateness of algal indices that are commonly used to normalize DMSP concentrations in corals (see for example, Broadbent et al., 2002; Edmunds and Gates, 2002; Hill et al., 1995, 2000; Van Alstyne et al., 2006; Yost and Mitchelmore, 2009). Our data clearly show that these indices change in response to oxidative stress and cannot be used when investigating DMSP-related questions in a toxicological arena. This includes investigations of stressors (e.g. solar irradiance, temperature or chemical pollutants) that are known to alter these parameters. It is also noted that indices such as coral surface area can limit the interpretation of DMSP concentrations as, for example, DMSP concentrations per surface area may decrease in some cases simply as a result of algal cell loss. Furthermore, our analysis of $DMSP_p$ and $DMSP_t$ levels demonstrates that algal physiological responses (i.e. use (loss) or alternately up-regulation of $DMSP_p$) may be masked by DMSP levels in coral host cells ($DMSP_t$).

5. Conclusion

In conclusion, *M. franksi* corals exposed to environmentally relevant concentrations of copper exhibited changes in both $DMSP_t$ and $DMSP_p$ concentrations in response to increased physiological stress. Additionally, common indices used to normalize DMSP concentrations in coral changed significantly (chl-*a*, protein, algal cell number) with copper exposure, highlighting the importance of normalizing DMSP to indices that remain unchanged throughout the duration of toxicological studies (e.g. coral surface area). Common DMSP normalization indices may not be operable in coral DMSP studies due to the complex nature of the algal–animal symbiosis. The added complexity of toxicological research also limits the available DMSP normalization indices because the indices themselves often respond to stress. These data make a strong case for further investigations that aim to quantify both symbiont and host DMSP concentrations in an effort to better understand the role and function of DMSP in corals. Our data for $DMSP_p$ and chl-*a* at the highest copper dose suggest that DMSP may serve as a key antioxidant in zooxanthellae (and potentially their host coral), ameliorating the effects of ROS production in response to copper exposure. Finally, higher $DMSP_t$ versus $DMSP_p$ concentrations are suggestive of DMSP translocation from symbiont to host, but further experiments are needed to substantiate this. While the underlying physiological function(s) and regulation of DMSP in corals remains undetermined, we present evidence that DMSP may contribute to the inherent antioxidant systems present in zooxanthellae, thus serving a supportive antioxidant role.

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