Latitudinal study of the role of dimethylsulphoniopropionate in marine microbial foodwebs

By

Eva Fernandez Fernandez

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“Caminante no hay camino. Se hace camino al andar”

“Traveller, there are no paths. Paths are made by walking”

Antonio Machado // Spanish poet
& Australian aboriginal saying

Certificate of authorship

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Collection permits

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Table of Contents

List of Figures

List of Tables

Glossary of Terms

3HP	3-hydroxypropionate
ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
ANSTO	Australian Nuclear Science and Technology Organisation
CE	capillary electrophoresis
CCN	cloud condensation nuclei
Chl a	Chlorophyll a
CLAW	Charlson-Lovelock-Andreae-Warren
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DLA	DMSP lyase activity
DMS	dimethyl sulphide
DMSHB	4-dimethylsulfonio-2-hydroxybutyrate
DMSO	Dimethyl sulfoxide
DMSP	dimethylsulfoniopropionate
DMSPd	dissolved DMSP
DMSPp	Particulate DMSP
DMSPt	Total DMSP
EA	Elemental analysis
F_v/F_m	potential quantum yield
FPD	flame photometric detector
GBR	The Great Barrier Reef
GBT	Glycine Betaine Transporter
GC	gas chromatography
GFF	glass fiber filter
GOS	Global Ocean Sampling
HPLC	High performance liquid chromatography
IAEA	Atomic Energy Agency (IAEA)

IMOS	Integrated Observing System
IR	Inside Reef
IRMS	Isotope ratio mass spectrometry
MC	Monte Carlo
MeSH	Methanethiol
MHM	MTHB methyltransferase
MMPA	methylmercaptopropionate
MS	mass spectrometry
MSNA	methane sulphinic acid
MTHB	4-methylthio-2-hydroxybutyrate
MTOB	4.methylthio-2-oxobutyrate
MTPA	3-methylthiopropylamine
MTA-CoA	methylthioacryloyl-CoA
nMDS	non-parametric multi-dimensional scaling
NMR	nuclear magnetic resonance
OR	Outside Reef
OTU	Operational taxonomic unit
PAM	pulse-amplitude-modulation
PCO	Principal Coordinates Analysis (PCO)
PCR	polymerase chain reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PH	Port Hacking
ROS	Reactive oxygen species
RV	Research vessel
SDS	sodium dodecyl sulfate
SMM	S-methyl-L-methionine
SO	Southern Ocean
SST	Sea surface temperature
TD	thermal desorption
UPLC	ultra-performance liquid chromatography

UV	Ultra violet
VPDB	Vienna Pee Dee Belemnite (VPDB)

Abstract

Dimethylsulphoniopropionate (DMSP) is a sulphur compound produced by some species of phytoplankton, coral and bacteria. It acts as a cryoprotectant, compatible osmolyte and antioxidant, and can provide high value nutrients for the whole marine microbial community. Nevertheless, research on DMSP has focused largely on its bacterial degradation to dimethylsulfide (DMS), a climatically active gas that potentially regulates local climate through an increase in cloud albedo. Therefore, other aspects of DMSP cycling, like DMSP utilisation by the marine microbial community, and especially by phytoplankton, are poorly understood. Marine sulphur dynamics, including DMSP production, cycling and DMS flux vary geographically across latitudinal space with different oceanographic characteristics resulting in different DMSP concentrations and microbial communities.

This thesis aimed to improve our understanding of the utilisation of DMSP by marine microbial communities from different oceanographic regions –the tropics (both coral-influenced and oligotrophic open waters), off shore temperate seas of mixed water masses, and late summer polar coastal waters— by investigating (1) the uptake of DMSP by different fractions of the marine microbial community, and (2) by identifying microbes that benefit from the presence of DMSP. This was achieved through a series of field-based studies that incubated natural oceanic waters enriched with DMSP over different time frames. Short incubations of 6-8 h were conducted to determine rapid DMSP uptake by the community following the progression of DMSP concentrations over time. The size separation of the community by serial filtration allowed for the quantification of DMSP that had been taken up by the microbes from the different size classes of the community. Longer incubations of up to 144 h were important for establishing longer-term responses, such as metabolism or fate of DMSP enrichment within the microbial community and DMSP-induced community shifts. Using sequencing information over time, we were also able to ascertain whether DMSP enrichment lead to any changes in DMSP metabolism and marine microbial structure.

Overall, the findings of this thesis challenge the idea that prokaryotes are the major DMSP sinks in the marine environment. Moreover, this thesis shows that phytoplankton uptake of DMSP is a common characteristic across different environments, with diatoms being one of the predominant sinks in the ocean. It has also shown that DMSP supposes an ecological

advantage to many bacteria and some phytoplankton taxa, highlighting the need for more research on DMSP degradation pathways in both bacteria and phytoplankton.

Chapter 1:

Introduction

Chapter 1: Introduction

1.1. Dimethylsulphoniopropionate (DMSP) plays a crucial role in marine food webs, atmospheric fluxes and regional weather

Since the industrial revolution in the 1850s, the constant increase of human activities has been accompanied by an exponential rise in atmospheric carbon dioxide and other greenhouse gases, which have accelerated global warming (Canadell et al., 2007) and, has resulted in what is known as anthropogenic climate change (Hofmann et al., 2009). The consequences of anthropogenic climate change include a significant increase in surface temperatures, resulting in shifts in regional and global climates (Hansen et al., 2006; IPCC, 2014) and substantial changes on ocean ecosystems including variations in sea surface temperature (SST), ultra-violet (UV) radiation, pH, salinity and nutrient limitation (Brierley and Kingsford, 2009; Harley et al., 2006; Meier, 2002). Unlike greenhouse gases, the climatically active trace sulfur gas dimethylsulfide (DMS), which is primarily emitted from the oceans (Kettle and Andreae, 2000), forms low level clouds which have the potential to decrease sea surface temperatures and therefore slow down the effects of climate change (Charlson et al., 1987).

1.1.1. The CLAW hypothesis

The influence of DMS on climate starts as soon as it is released to the atmosphere and oxidised to sulphate aerosols (Koga and Tanaka, 1996). These aerosols contribute to cloud condensation nuclei (CCN), soluble particles whose diameter is > 0.05 µm, by nucleation of new particles in the free troposphere and by growing smaller particles in the marine boundary layer. This increase in CCN density increases the reflection coefficient of clouds (cloud albedo) and thus decreases the Earth's incoming radiation, which results in a decrease on a regional scale of sea surface temperatures (Welsh, 2000).

In 1987, it was hypothesised that phytoplankton utilise the cloud formation capacity of DMS to control surrounding environmental conditions, such as high sunlight and temperature, via increasing its production. This phenomenon, known as the **CLAW hypothesis** (after the first initial of the authors: **C**harlson, **L**ovelock, **A**ndreae and **W**arren) is suggested to be a self-regulated feedback mechanism that links global biosphere and climate. Therefore, when the sea surface temperature decreases, a decrease in biogenic DMS production follows (Charlson et al., 1987). Moreover, it was also hypothesised that the current global warming could

potentially increase the production of biogenic marine DMS, which could counteract some of the warming effects of greenhouse gases through the increase of cloud albedo (Charlson et al., 1987). Nowadays, however, we know that the sulfur cycle, as well as cloud formation mechanisms, are more complex than initially proposed and many questions remain unanswered. The relationship between sulfate aerosols and cloud albedo have been confirmed in waters of the Southern Ocean (McCoy et al., 2015), but feedback effects of climate on the DMS cycle seem to be small when based on climate models (Gunson et al., 2006; Vallina et al., 2007; Wang et al., 2018) to the point that some authors argue the validity of the hypothesis (Quinn and Bates, 2011). Though the CLAW hypothesis may be oversimplified, to truly understand the role of DMS cycling on climate regulation and whether or not DMS may contribute to mitigating climate change; it is necessary first to have a complete understanding of the mechanisms driving the sulfur cycle.

1.1.2. The sulfur cycle

The sulfur cycle involves the transport and transformation of sulfur compounds between living organisms and the ocean-atmosphere-land system. One of the largest reservoirs of sulfur on Earth is the ocean, which is responsible for more than 60% of the global non-anthropogenic biogenic sulfur flux and which is mostly (>90%) in the form of DMS gas, with a total flux of 0.58 Tg S/yr (Andreae, 1990; Kelly and Smith, 1990). The total flux of oceanic DMS equates to one-third of the global anthropogenic sulfur emissions, but due to the shorter lifetime of anthropogenic sulfur with respect to DMS sulfur; DMS represents almost half (40%) of the total sulfur burden of the atmosphere (Simó, 2001). Atmospheric DMS, however, represents no more than 10% of the total oceanic DMS pool (Simó et al., 1999). In order to fully understand the ocean-atmosphere flux of DMS and the diversion of sulfur compounds from this flux, we are required to understand the sources and sinks of sulfur in marine environments, as well as the processes regulating them. Many of these processes occur in the photic zone where the primary source of sulfur is present as dissolved sulfate that is assimilated by photosynthetic organisms, phytoplankton and macroalgae, into the sulphur-based amino acids cysteine and methionine, and ultimately recycled as dimethylsulfoniopropionate (DMSP) (Stefels, 2000).

Phytoplankton-derived DMSP is released into the ocean through different mechanisms such as decomposition, exudation and/or viral attack. A fraction of this phytoplankton-derived

DMSP is consumed by the zooplankton community via grazing; fish, turtles and birds, through feeding, also consume DMSP and hence this sulfur fraction accumulates in oceanic sediment due to the faecal and detrital material from such organisms (Dacey et al., 1994). More importantly, bacterial decomposition of phytoplankton that contain DMSP, produces dissolved DMSP that is consumed by bacteria (Malmstrom et al., 2004; Raina et al., 2017; Ruiz-González et al., 2012c). Both, phytoplankton and bacteria transform part of their intracellular DMSP into dimethylsulfide (DMS) using DMSP lyases via an enzymatic cleavage pathway (Curson et al., 2012; Kiene et al., 2000; Yoch, 2002) and assimilate the rest into proteins through the demethylation/ demethiolation pathway (Kiene et al., 1999; Kiene and Linn, 2000). The DMS released is then assimilated by other bacteria, photo-oxidised to DMSO or ventilated to the atmosphere where it is rapidly transformed to sulfate aerosols (Simó et al., 1999) (Figure 1.1). The DMS that reaches the atmosphere represents only 3% of the total DMSP synthesised by phytoplankton, while the remaining DMSP is taken up by heterotrophic bacteria (10%) and zooplankton (20%), transformed to non-volatile sulfur compounds (30%) or photo-oxidised to DMSO (27%). It has been proposed that the remaining 10% is taken up by non-DMSP producing phytoplankton (Simó, 2001; Vila-Costa et al., 2006b), but some studies suggest that DMSP uptake by phototrophs is negligible (Lavoie et al., 2018) (Figure 1.2).

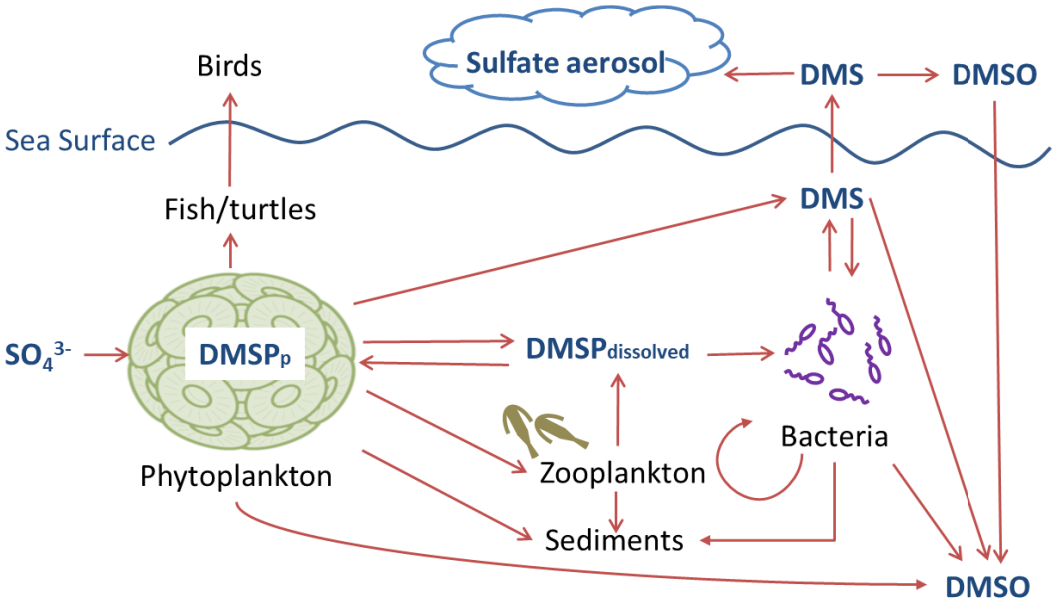

Figure 1. 1. The marine sulphur cycle. *Phytoplankton utilise dissolved sulfate from the environment to synthesise dimethylsulfoniopropionate (DMSP), which reaches the zooplankton community via grazing and the bacterial community through the uptake of the dissolved form. Both phytoplankton and*

bacteria transform part of this DMSP into dimethylsulfide (DMS) by their DMSP lyases and assimilate the other part. The released DMS is then assimilated by other bacteria, photo-oxidised to DMSO or ventilated to the atmosphere where it is rapidly transformed to sulphate aerosols. DMSP is also ingested by fish, turtles and birds through feeding and accumulated in oceanic sediments. Modified from Simó (2001).

1.1.3. The relevance of DMSP in marine food webs

Notwithstanding the importance of DMSP as a precursor of DMS; DMSP itself is a critical chemical in marine environments. It is the most prevalent organic sulfur source in marine food webs and, very few other compounds have as high a mass flux through the microbial food web (representing >50% sulfur flux through bacteria in the North Sea (Zubkov et al., 2001)). Intracellularly, DMSP satisfies up to 71% of the sulfur and 15% of the carbon needs of the phytoplankton cell and up to 95% S and 15% C needs of the bacterial cell (Zubkov et al., 2001; Matrai P.A & Keller M.D 1994; Simó et al. 2002). Moreover, DMSP facilitates a tritrophic mutualism between marine primary producers and top predators through its catalysis to DMS (Savoca and Nevitt, 2014). When primary consumers feed on phytoplankton, the released DMS acts as a foraging cue for marine top predators, which feed on primary consumers reducing grazing pressure on phytoplankton (Savoca, 2018; Savoca and Nevitt, 2014). The presence of top predators also contributes to iron availability and other nutrients that limit phytoplankton growth via excretion, due to the high Fe content in their aeces (Lavery et al., 2014; Ratnarajah et al., 2014; Wing et al., 2014)

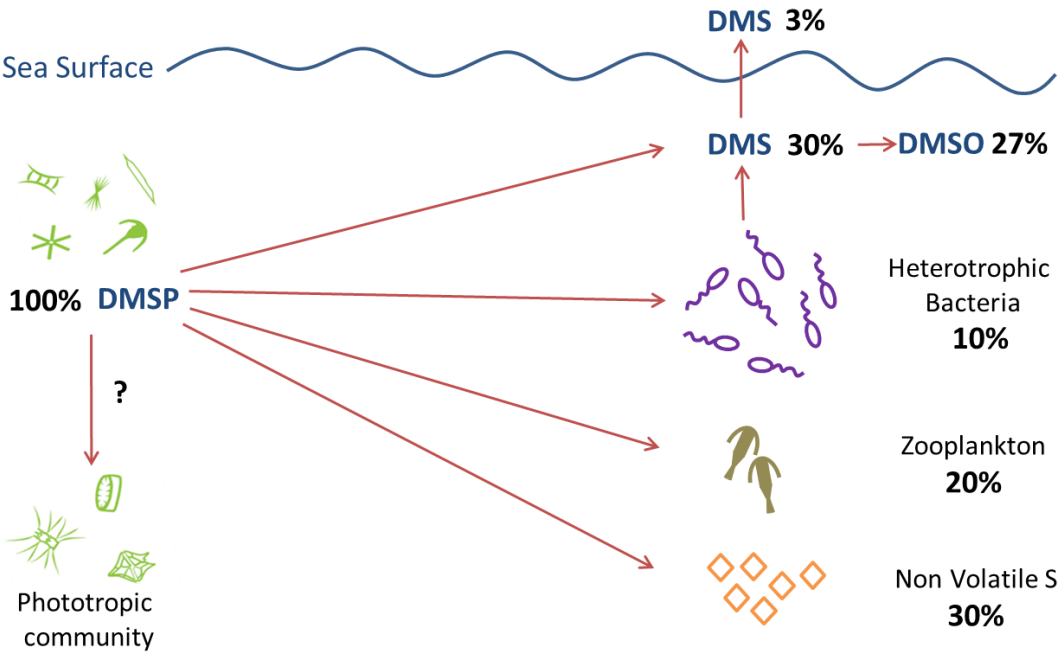

Figure 1. 2 DMSP distribution in the marine microbial food web. *All percentages are referred to 100% of DMSP production. Modified from Vila-Costa et al. (2006)*

1.1.4. Distribution of DMSP in world oceans

Dimethylsulfoniopropionate in the oceans is ubiquitous, but its distribution and abundance is variable (Table 1.1). Its concentration ranges from 5 to 50 nM when dissolved, and higher than 100 nM in phytoplankton blooms (Yoch, 2002); and it is more concentrated in temperate and polar seas than in oligotrophic regions (Kiene et al., 2007; Slezak et al., 2007; Yang et al., 2011; Jones et al., 2007). In many areas of the open ocean, high concentrations of DMSP occur in late spring and summer due to the reduced vertical mixing and high microbial activity typical of stratified waters (Simo & Pedros-Alio 1999, Vila-Costa, Kiene & Simo 2008); while low levels of DMSP commonly occur during late winter and early spring, coinciding with high surface mixing and primary production, and surface waters dominated by blooms of low-DMSP producing diatoms (Dacey et al., 1998). A similar pattern has been found for DMS, and it is known as the 'DMS summer paradox', where the highest annual concentrations of DMS in surface waters occur at latitudes between 10-70º in summer when surface chlorophyll a levels are lowest (Simó et al., 1999). In this thesis we investigated the role of DMSP in three oceanographically and ecologically distinct regions, the oligotrophic tropical waters, DMSP-rich Antarctic waters and a coastal spring bloom in temperate waters.

Table 1.1 DMSP total concentrations (DMSPd + DMSPp) for different seas at different times of the year, including the concentrations measured in this thesis.

Location	Time period	DMSPt (nM)	Reference
Ross Sea (Antarctica)	Oct-Dec	8.5-35.4	(Kiene et al., 2007)
Prydz Bay (Antarctica)	Dec-Feb	8-334*	(Trevena and Jones, 2006)
East China Sea and Yellow Sea	June-July	13.98-44.93	(Yang et al., 2011)
East China Sea and Yellow Sea	Jan-Feb	6.90-17.98	(Yang et al., 2011)
Gulf of Mexico	July-Sept	25.2-42.5	(Slezak et al., 2007)
Mediterranean Sea	August	~24	(Slezak et al., 2007)
Saragasso Sea	April-July	10.5-12.3	(Slezak et al., 2007)
Coral sea	All year	5.9-20.7*	(Jones et al., 2007)
Coral Sea	October	12.34-26.06	Chapter 3 of this thesis
Prydz Bay (Antarctica)	February	62.69-68.03	Chapter 4 of this thesis
Tasman sea	March	16.39	Chapter 5 of this thesis

*The value is the sum of DMSPp and DMSPd

1.2. Dimethylsulfoniopropionate inside the cell: biosynthesis, uptake, degradation and function

1.2.1. Biosynthesis of DMSP

Dimethylsulfoniopropionate biosynthesis occurs mainly in algae such as phytoplankton, where its concentration varies from species to species. It is well established that phytoplankton from the classes Prymnesiophyceae (haptophytes) and Dinophyceae (dinoflagellates) are the greatest producers of DMSP (Keller et al., 1989), whereas diatoms (except for some polar diatoms), green algae and cyanobacteria are generally considered low or non-producers (Belviso et al., 2001). DMSP is also produced by corals that contain Symbiodinium species (Broadbent et al., 2002; Jones et al., 1994; Raina et al., 2013), higher plants (Hanson et al., 1994) and many alphaproteobacteria (Curson et al., 2017) (Table 1.2), and its synthetic production differs for different organisms with three alternative biochemical pathways identified so far (Figure 1.3): A transamination pathway found in marine algae, phytoplankton, corals and alphaproteobacteria (Gage et al., 1997), a

methylation pathway found in angiosperms (Rhodes et al., 1997), and a decarboxylation pathway found in a dinoflagellate (Uchida et al., 1996).

Table 1.2 Concentrations of DMSPp measured in different organisms and blooms.

Organism	DMSP (pmol/cell)	Reference
Higher plants		
Salt marsh grasses (from *Spartina* genus)	4 – 70[a]	(Otte et al., 2004)
Sugar canes (*Saccharum spp.)*	Up to 6[a]	(Otte et al., 2004)
Pacific strand plants (*Wollastonia biflora*)	12-30[a]	(Otte et al., 2004)
Phytoplankton		
Cultured dinoflagellates	0.00011-14.7	(Keller 1989)
Cultured diatoms	0.0006-0.257	(Keller 1989)
Cultured chlorophytes	0.00015-0.012	(Keller 1989)
Cultured haptophytes	0.000373-0.148	(Keller 1989)
Cutured cryptophytes	0.0213[b]	(Keller 1989)
Cutured rhodophyta	0.00231[b]	(Keller 1989)
Cultured cyanobacteria	7.45-10.4x10^{-6}	(Keller 1989)
Natural bloom of *Phaeocystis spp.*	Up to 1650[c]	(Duyl et al., 1998)
	Up to 1740[c]	(Speeckaert et al., 2018)
Natural bloom of *Akashiwo sanguinea*	Average of 1,432 [c] (up to 4,240 [c])	(Kiene et al., 2019)
Natural bloom of *Emiliania huxleyi*	Up to 72[c]	(Levasseur et al., 1996)
Alphaproteobacteria	9.6	(Curson et al., 2017)
Corals	0.15-0.27	(Broadbent et al., 2002)
	0.021-3.831	(Broadbent et al., 2002)
Zooxanthellae (cultured)	0.048-0.285	(Broadbent et al., 2002)

[a] µmol/g dry weight [b] only 1 specie analysed [c] nmol dm^{-3}

For phytoplankton, the third step in the transamination pathway - catalysed by MHM enzyme (4-methylthio-2-hydro- xybutyrate methyltransferase)- is the key step to the biosynthesis of

DMSP (Figure 1.3), as non-producers don't show any MHM activity but do show activity for the enzymes catalysing the earlier and later steps. The MHM enzyme is encoded by the dsyB gene, which is the only identified gene so far for any of the DMSP biosynthesis pathways and is predicted to be present in up to 0.5% of bacteria sampled in the Tara Oceans and Global Ocean Sampling (GOS) marine metagenomes (Curson et al., 2017). The transcription of dsyB has been shown to upregulate under increased salinity, nitrogen limitation and lower temperatures, which are some of the environmental stressors already predicted to stimulate DMSP synthesis in marine phytoplankton (Stefels et al., 1996; Zhuang et al., 2011). An increase in intracellular DMSP has been observed with decreasing temperature (Karsten et al., 1996), increasing temperature (Jones and King, 2015), increasing irradiance or day length (Karsten et al., 1991, 1990), and decreasing nitrogen availability (Kettles et al., 2014; Turner et al., 1988). However, few species have been tested and often the response to the same stressor differs amongst them; like increased salinity, which has been shown to result in an increase in DMSP concentration for some taxa, while having no effect for others (Stefels et al., 1996; Zhuang et al., 2011). The effects that environmental stressors have on intracellular DMSP concentrations suggest that phytoplankton benefit from this compound, possibly as a means to acclimate to new conditions and that DMSP may play a relevant role in cell survival and adaptation.

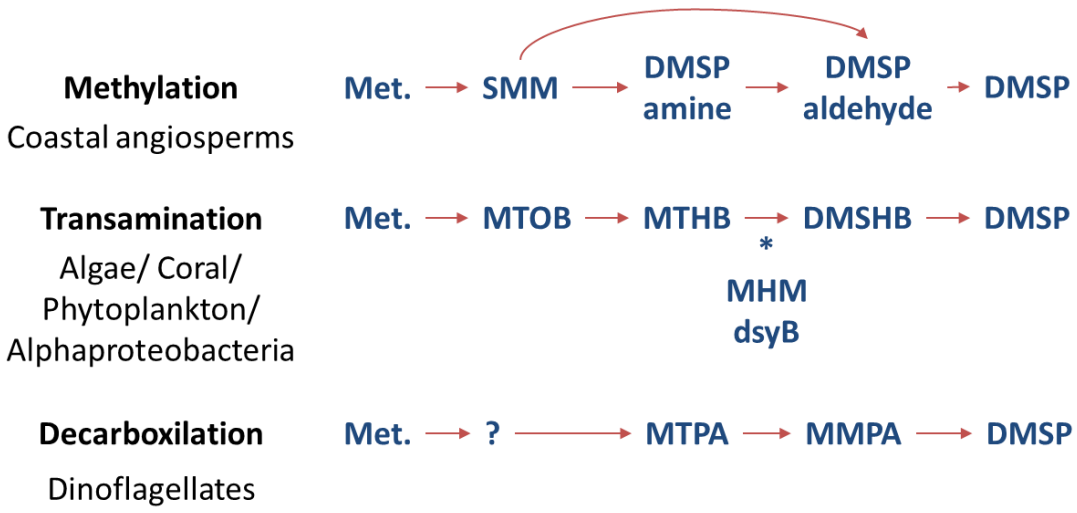

Figure 1. 3 Proposed DMSP biosynthetic pathways. *Met, methionine; MTOB, 4.methylthio-2-oxobutyrate; MTHB, 4-methylthio-2-hydroxybutyrate; DMSHB, 4-dimethylsulfonio-2-hydroxybutyrate; SMM, S-methyl-L-methionine; MTPA, 3-methylthiopropylamine; MMPA, methylmercaptopropionate; MHM, MTHB methyltransferase; dsyB. Modified from Curson et al. (2017)*

1.2.2. Suggested roles for DMSP inside the cell

The primary role of DMSP in marine communities -as a major carbon and sulfur source- is well established, yet complete understanding of its physiological functions inside the cell remain unclear. So far, proposed functions include 1) an antioxidant (Deschaseaux et al., 2014; Sunda et al., 2002), 2) an osmoprotectant (Dickson & Kirst 1986), 3) an N overflow (Colmer and Läuchli, 1996) and 4) a cyroprotectant (Karsten et al., 1996). However, all of these functions are based on changes on DMSP production levels in response to altered environmental conditions, where many other solutes, compounds or cellular processes may also be interacting. To date, no singular functional role of DMSP has been validated, as it would require creating a mutant with a knockout gene to establish an appropriate control group. However, now that the genes for DMSP synthesis have been identified, creating such a mutant may soon be possible.

In its role as an antioxidant, observed increases in DMSP concentrations under oxidative stress may provide some support (Deschaseaux et al., 2014; Haworth et al., 2017; Jones and King, 2015; Karsten et al., 1990; Stefels and van Leeuwe, 1998). DMSP may react with the harmful reactive oxygen species (ROS) alleviating ROS stress, but its degradation products such as DMS, DMSO, acrylate and methane sulphinic acid (MSNA) constitute an antioxidant system that could be far more effective at scavenging ROS than other well-recognised antioxidants, such as ascorbate and glutathione and, to some extent, this antioxidant system can be regulated by enzymatic cleavage of DMSP (Sunda et al. 2002). However, studies on corals show variable results. Broadbent et al.(2002) compared the concentrations of intracellular DMSP in colonies of *A. Formosa* of different thermal bleaching thresholds, and found higher DMSP concentrations in the colonies with higher susceptibility to bleaching. Other studies have observed decreasing DMSP and DMSO concentrations for stressed *Symbiodinium* cells (Gardner et al., 2017; McLenon and Ditullio, 2012), and *Scleractinian* corals with thermally tolerant zooxanthellae (e.g. clade D zooxanthellae) presented constant DMSP concentrations, while in corals with less thermally tolerant zooxanthellae, DMSP concentrations varied in different directions (Jones and King, 2015), demonstrating that the link between DMSP and antioxidant quenching exists, but requires further investigation.

Osmoregulation is the most well-established primary function of DMSP, as it has the characteristics of a compatible solute, compounds responsible for keeping cell water level in the required range for the cellular functions to take place and are not toxic or inhibitory to

biological processes at high concentrations (Csonka, 1989). Like other compatible solutes, DMSP is unable to cross the cell membrane without a transporter, as it does not carry a net electrical charge, and instead, represent a reserve of nutrient and energy, offering protection against other stressors and facilitating a positive turgor pressure necessary for cell growth (Welsh, 2000). Under osmotic stress, intracellular DMSP concentrations increase with salinity for some species while remaining constant in others and, rapid adaptations to hyper- and hypo-osmotic pulses have barely been studied and no consistent trend has been observed (Dickson & Kirst 1986; Diggelen, Rozema & Dickson 1986; Karsten, Kirst & Wiencke 1992; Stefels, Gieskes & Dijkhuizen 1996). There are currently two models to explain how DMSP acts as an osmoprotectant, each expounding the often divergent responses on DMSP concentrations to salinity stress. In the first model, intracellular DMSP concentration increases as salinity increases, protecting the cell from shrinkage. In the second model, intracellular DMSP concentration remains constant with salinity, but moves within the cell. Under hyposalinity stress, DMSP may be present in both the cytoplasm and the vacuole, but when salinity increases, sodium chloride crosses the cell membrane and accumulates in the vacuole while DMSP from the vacuole flows to the cytoplasm and balances the osmotic potential between the compartments (Otte et al., 2004). These two models provide explanations for the divergent responses observed in DMSP producing microalgae, but further work to verify these processes in needed.

DMSP may also play an essential role under nitrogen (N) depletion. Under such conditions, DMSP is synthesised without the uptake of sulfate (Colmer and Läuchli, 1996; Kettles et al., 2014; Turner et al., 1988), instead using intracellular stores of methionine and cysteine. Under these circumstances of low N, cell growth is unbalanced, and methionine and cysteine are not being used to produce proteins, accumulating inside the cell. Stefels (2000) hypothesised that by synthesising DMSP, the cell is able to satisfy the N requirement, while returning cellular methionine and cysteine to low levels, which balance the growth mechanism again. However, in some cases, DMSP concentration does not increase under nitrogen starvation (Keller et al. 1999), highlighting the need to better understand how DMSP is synthesised and utilised in different species.

The suggestion that DMSP may play an essential role as a cryoprotectant stems from the observations that polar diatoms are the only diatoms to produce substantial amounts of DMSP and, that changes in DMSP production has been observed with decreasing

temperature (Karsten et al., 1996, 1992; Kirst et al., 1991; Lee et al., 2001). However, further work isolating DMSP over other known dominant cryoprotectants, such as proline, is needed to confirm any cryoprotective cellular function.

Despite the numerous cellular functions that have been attributed to DMSP, it is still unclear if it plays an active role in the cellular adjustment or instead, it is produced as a side effect of a stressor. Therefore, its main role inside the cell and so, the reason why phytoplankton produces DMSP remains unknown (Welsh, 2000). Moreover, many studies show the widespread ability of heterotrophic bacteria to take up and utilise DMSP (Vila et al. 2004; Malmstrom et al., 2004), which suggests that DMSP may also play an important physiological role for other microbes (Kiene et al., 2000), highlighting the need for further study on DMSP uptake and utilisation by marine microorganisms.

1.2.3. DMSP uptake mechanism

As DMSP is a zwitterion, a molecule that has a separate positive and negative charge, it cannot cross cell membranes and needs to be taken up actively by a transporter. Based on inhibitor studies, it is suggested that bacterial cells use the glycine betaine transporter (GBT) to take DMSP up. The GBT transports molecules with a quaternary ammonium group [R-N $(CH_3)_3$] like glycine betaine, which is the DMSP nitrogen analogue (Kempf and Bremer 1998). Studies with proteobacteria (α, β and γ) show that DMSP can undergo three different uptake mechanisms. In α proteobacteria, DMSP enters the cell due to a binding protein prior to transformation by an intracellular lyase and acrylate is transformed by an extracellular acrylase (Ansede et al., 2001; Ledyard and Dacey 1994). In β proteobacteria, DMSP lyase and acrylase are extracellular and located on the cell surface, while in γ proteobacteria, DMSP is transported inside the cell with an energy-dependent (cyanide-sensitive) transport system before transforming it by an intracellular lyase. Acrylase enzymes are also located intracellularly in γ proteobacteria (de Souza and Yoch, 1995). The three mechanisms can

generally be induced by DMSP, DMSO and acrylate and are inhibited by homocysteine (Yoch, 2002) (Figure 1.4).

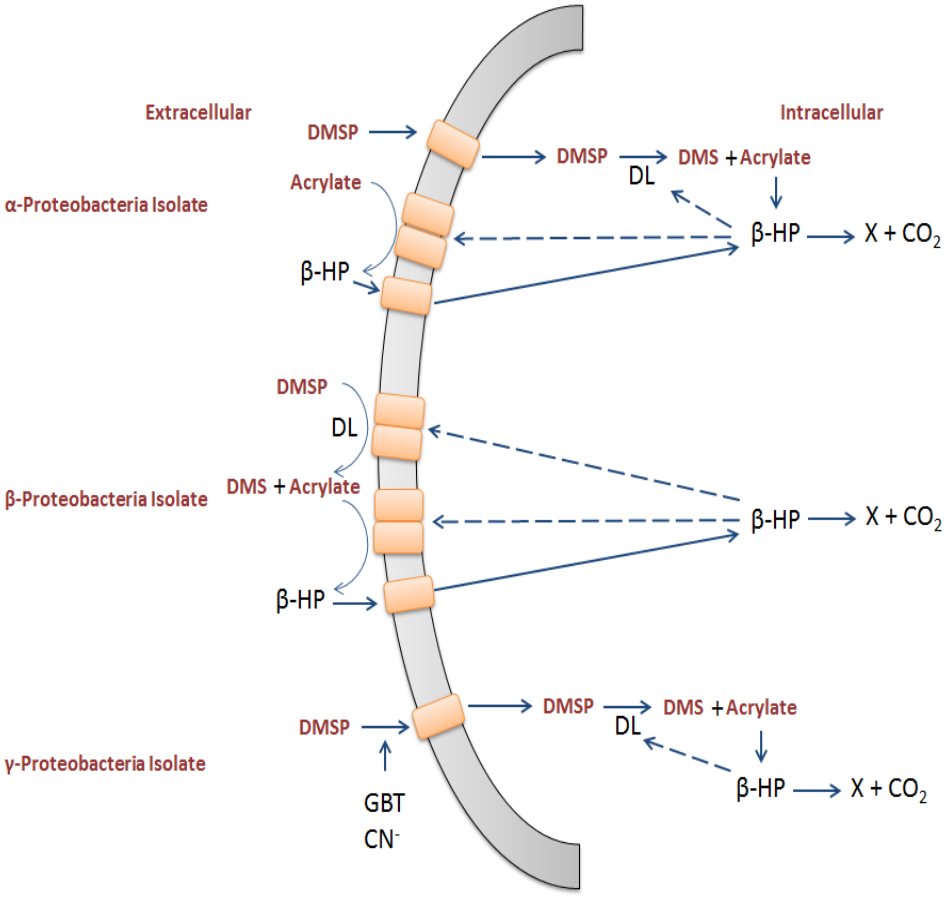

Figure 1. 4 Schematic comparing the uptake and metabolism for DMSP and acrylate in an α, β and γ proteobacteria. *In α proteobacteria, DMSP uptake is carried out by a binding protein prior production of DMS by an intracellular DMSP lyase. Concerning acrylase, it hydroxylates both extracellular and cytosolic acrylate which means that it is located intra and extracellularly or as a single transmembrane acrylase, which can hydroxylate both*(Ansede et al., 2001; Ledyard and Dacey, 1994). *Some β-HP is leaked, but most of it is decarboxylated to an unknown compound. In β proteobacteria, DMSP lyase and acrylase are extracellular and located on the cell surface, but there is a binding protein that allows the transport of β-HP into the cytosol; γ proteobacteria uptake the DMSP with an energy-dependent (cyanide-sensitive) transport system, whereas DMSP lyase and acrylase are located intracellularly. The three mechanisms can generally be induced by DMSP, DMSO and acrylate and are inhibited by homocysteine (Abbreviations: DL, DMSP lyase; GBT, Glycine Betaine Transporter) Modified from Yoch (2002).*

No studies exist on DMSP uptake mechanisms for the phototrophic community, and it is yet not known whether phototrophs that don't produce DMSP represent a major DMSP sink

(Lavoie et al., 2018; Vila-Costa et al., 2006b). Vila-Costa et al. (2006) estimated that non DMSP producing phytoplankton species could take up as much DMSP as heterotrophic bacteria, but a study with cultures of Antarctic species refuted this premise and suggested that phytoplankton do not take up significant amounts of DMSP (Lavoie et al., 2018). More recently however, a lab experiment with the temperate diatom species *Thalassiosira weissflogii* demonstrated that DMSP was taken up rapidly and consistently, and to intracellular concentrations equal to many prolific producers (Petrou and Nielsen, 2018). These data suggest that more species need to be tested to know if DMSP uptake is a prevelant trait among phytoplankton or whether it is only applicable to a few species and, uptake experiments in natural environments would also need to be conducted to take into account spatiotemporal variations which influence population composition and environmental conditions. This thesis aims to fill some of these knowledge gaps by looking at DMSP uptake in three different natural oceanic communities.

1.2.4. Transformations of DMSP inside the heterotrophic cell: The bacterial switch

Once DMSP has been taken up by the heterotrophic cell, it can degrade via two different pathways (Kiene et al., 2000) (Figure 1.5). About 70% of intracellular DMSP degrades through the demethylation/ demethiolation pathway in which methanethiol (MeSH) and acrylate, but not DMS, are produced (Reisch et al., 2011; Vallina and Simó, 2007). The alternative pathway is via cleavage and involves several steps, each of which is mediated by a separate lyase (Yoch, 2002) and produces DMS and acrylate.

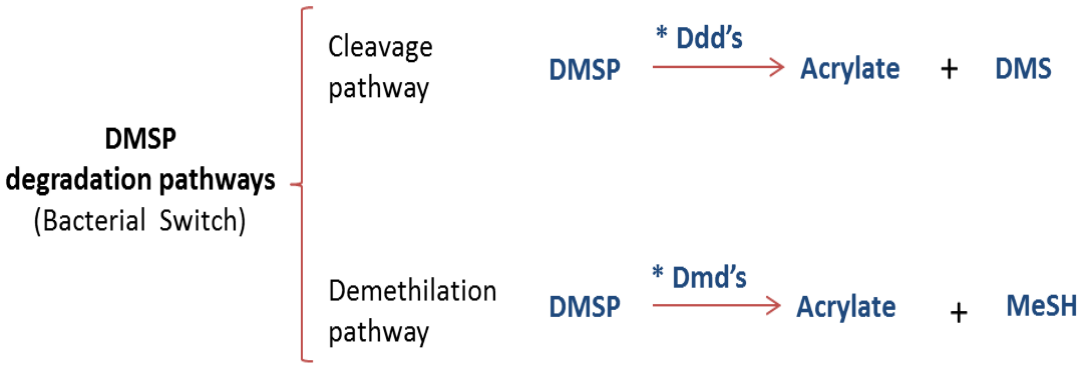

Figure 1. 5 DMSP degradation pathways. *There are two degradation pathways for DMSP, in the first pathway, DMSP undergoes cleavage and degrade to acrylate and DMS and is carried out by Ddd´s genes while on the second pathway, the Dmd´s genes demethylate DMSP and the degradation products are acrylate and MeSH.*

For the bacteria that can utilise both pathways, the preferred conditions for DMSP to degrade through one or the other are still unknown and the sudden shift to favouring one over the other is referred to as the bacterial switch (Simó, 2001). The main bacterial switch regulator is suggested to be the sulfur product obtained from each pathway, that is MeSH or DMS (Kiene et al., 2000). Methanethiol (MeSH) is the sulfur compound obtained in the demethylation/ demethiolation pathway and is used to produce energy or proteins, while DMS is the compound derived from the cleavage pathway and instead of being used by the cell, diffuses away. Therefore, it is hypothesised that DMSP will degrade preferentially through demethylation when there is a need to produce protein for bacterial growth and once that sulfur demand is satisfied, the bacteria will switch to the lyase pathway (Kiene et al., 1999). However, a recent publication demonstrated that in the marine bacterium *pelagibacter*, both pathways happen simultaneously and it is the amount of DMSP going through each of them what changes as the sulfur demand of the cell is satisfied (Sun et al., 2016). While the demethylation pathway has not been observed in phytoplankton, DMSP lyase enzymes have been demonstrated in *Emiliania huxleyi* (Wolfe and Steinke, 1996) and *Phaeocystis* spp. (Van Boekel & Stefels 1993). Even though phototrophs are the primary producers of DMSP, more research into DMSP degradation pathways inside the phototrophic cell is needed.

1.2.5. DMSP catabolic enzymes

In the demethylation pathway, four enzymes (DmdA, DmdB, DmdC, DmdD) are responsible for the process to convert DMSP to MeSH and each of them catabolizes a different step; while in the cleavage pathway, several enzymes, known as DMSP lyases, can cleave DMSP to produce DMS: the bacterial enzyme DddD can cleave DMSP to DMS and 3-hydroxypropionate (3HP) and 6 different bacterial enzymes (DddP, DddQ, DddL, DddW, DddY, DddK) and one algal enzyme (Alma1) can transform DMSP to DMS and acrylate, that degrades further to 3HP and further to Acetyl-CoA (Figure 1.6)(Alcolombri et al., 2015; Curson et al., 2011). However, DmdA and DddP are the only enzymes that have evolved specifically for DMSP metabolism, and so, catabolizing DMSP may be their major role (Bullock et al., 2017). The crystal structure of the enzyme *DddP*, which is one of the six bacterial enzymes able to catabolise the first step of the cleavage pathway, has been elucidated and suggests that its active site has evolved from the M24 peptidase that hydrolyses C-N bonds to favour lysis of C-S bonds and has a smaller cavity, ideal to bind DMSP specifically (Wang et al., 2015). The first step of the

demethylation pathway is solely catalysed by the enzyme *dmdA,* which initially evolved from a glycine cleavage T-protein to contain a binding site with a high affinity for DMSP (Reisch et al., 2008; Schuller et al., 2012). This first step removes a methyl group from the DMSP molecule and therefore eliminates the possibility of DMSP to degrade to DMS, and the molecule undergoes the whole demethylation pathway.

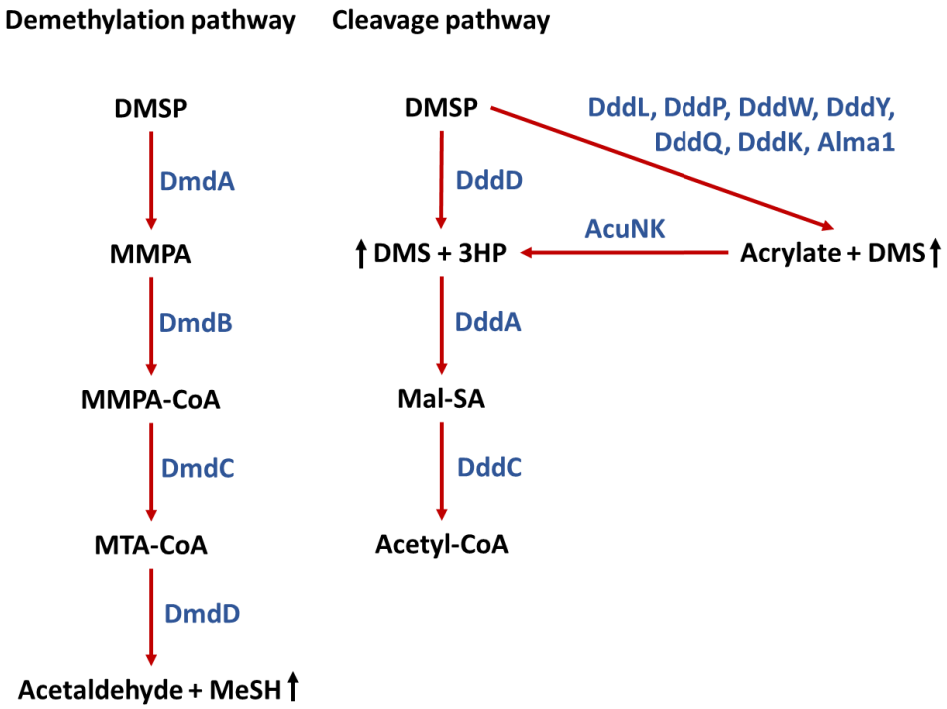

Figure 1. 6. Biochemical pathways for Dimethylsulphoniopropionate (DMSP) degradation*. Enzymes involved in each pathway are shown in blue. Black arrows pointing up indicate volatile compounds. In the demethylation pathway, DMSP is catabolized by DmdA to methylmercaptopropionate (MMPA) which is catabolized to acetaldehyde and methanethiol (MeSH) via the intermediates MMPA-CoA and methylthioacryloyl-CoA (MTA-CoA). DMSP lyases DddL, DddP, DddW, DddY, DddQ, DddK and Alma1, convert DMSP to acrylate and release dimethylsulfide (DMS), and acrylate is converted to 3-hydroxypropionate (3HP) by AcuNK. The DMSP lyase DddD cleaves DMSP to 3HP and DMS. Then, 3HP is converted by DddA to malonate semi-aldehyde (Mal-SA) which is converted to acetyl-CoA by DddC. Modified from* (Curson et al., 2011)

Moreover, DmdA and DddP are generally the most abundant genes for each of the DMSP degrading pathways (Curson et al., 2011). The Global Ocean Sampling Expedition determined that dmdA is the most abundant DMSP degrading gene in the oceans being present in 30% of the total cells and in all the five protein clades: A, B, C, D and E; plus some homologs that are still unclassified (Howard et al., 2008a). The lyase gene *dddP*, which is present in 6% of

cells (all *ddd* genes together are present in less than 10% of bacterial cells), is the most abundant lyase enzyme together with DddK and occurs mostly in the Roseobacter and SAR116 clades (Curson et al., 2011; Nowinski et al., 2019; Todd et al., 2009). The diversity of marine bacteria taxa that contain *dmdA* (Roseobacter, pelagibacter (SAR11), Gammaproteobacteria, SAR116) points to its essential ecological role in natural marine communities (Howard et al., 2008a).

In this thesis, we focused on the lyase gene DddP, as it is one the most abundant lyase gene, and clade D and subclade A1 from the demethylation gene dmdA, as they belong to the abundant marine bacteria SAR11 and Roseobacter, respectively. Research on the key genes DmdA and DddP, together with sequence and biogeochemical data will allow us to draw a more detailed biochemical flux map. However, more studies in enzyme stability and gene diversity, abundance and expression among others are still needed in order to fully understand how DMSP cycling works in natural environments.

1.3. Thesis objective and overview

This project studied the role of DMSP in microbial communities from natural oceanic waters from three different environments across a latitudinal gradient from polar, through temperate and tropical oceans. It took advantage of a marine science voyage on the *RV Investigator* to the Great Barrier Reef, an expedition to the Antarctic continent and coastal sampling of temperate waters from Port Hacking oceanographic station off the coast of Sydney, Australia. This research project used a combination of chemical, biological and genomic analyses to increase our understanding of the sulfur cycle. Specifically, the role of phytoplankton in DMSP uptake and the response of the microbial community to an increase in DMSP concentrations, and how it varies in latitudinally diverse waters.

For all locations, incubation experiments on natural seawater spiked with DMSP were conducted and the evolution of sulfur compounds measured over time. The microbial populations were characterised as was their ability to cleave DMSP and the relative abundance of DMSP metabolising genes recorded. These studies investigated the sulfur dynamics and transfer of DMSP throughout the communities. To date, most studies on microbial utilisation of DMSP have focused on heterotrophic bacteria, due to their ability to produce the climatically active gas DMS. However, the phototrophic community have the

potential to divert available dissolved DMSP from atmospheric fluxes by incorporating it into their cells, as it have been estimated to assimilate the same amount of DMSP as heterotrophic bacteria, and therefore maintain it into the marine environment. The peculiarity of this research is that looked into the whole microbial community, including heterotrophs and phototrophs.

This research was spatially and temporally broad, covering latitudes from below the Antarctic Polar front (<65°S) to the tropical waters of the coral sea (18º S). Being complex and dynamic oceanic systems, characteristics such as nutrient concentrations, temperature, salinity, solar radiation and microbial composition varied extensively and, this variability was also reflected in the sulfur dynamics. Therefore, this thesis had two overall aims. The first aim was to identify which members of the phototrophic community take up and assimilate DMSP and determine what fraction each functional group contributes to diverting the phytoplankton-produced DMSP from atmospheric fluxes of DMS to the food web. The second aim was to investigate how the microbial community composition is shaped under the influence of DMSP. **Chapter 2** provides detailed materials and methods for analyses that are repeated throughout the experimental chapters (**Chapters 3, 4 and 5**) of this thesis and will be referred to in each chapter where applicable.

In **Chapter 3**, we studied the distribution and retention of DMSP in the microbial communities of the Great Barrier Reef and investigated the differences between two sites, one coastal site located inside the reef and one site located outside the reef, a representation of the oligotrophic ocean of the tropics. The experiments were conducted on board the *RV Investigator* and, DMSP was used to spike the samples to trace the uptake of the dissolved DMSP in three size fractions; large eukaryotes, small eukaryotes, prokaryotes and heterotrophic bacteria. The effects of DMSP on microbial composition was also studied, by following the concentrations of sulfur compounds, as well as community structure and metabolism for over five days.

In **Chapter 4**, similar experiments were conducted at Davis station, Antarctica, to study the effects of DMSP enrichment on Antarctic marine microbial composition and how it is distributed and metabolised through the polar marine microbial food web. In this study, we used longer incubations (8 and 144h) to allow enough time for the polar microbes to take up

and assimilate DMSP. Knowing that polar communities often contain large cells and chains, the size fractionation of the community included an size class to isolate larger diatoms, which often dominate the waters of this region.

Chapter 5, explores the uptake of DMSP by a natural marine microbial community from temperate waters. Samples were collected from the Port Hacking oceanographic research station 8 km off the coast of New South Wales, Australia. In this study, whole community incubations were conducted to look at DMSP uptake and cycling, as well as smaller scale studies on the microbial community that was fractionated into three groups (large eukaryotes (>8µm), small eukaryotes (>3µm), prokaryotes and heterotrophic bacteria (0.22µm)) prior to the incubation with DMSP. This allowed for a closer look at DMSP uptake rates of each fraction in isolation.

The final chapter in this thesis (**Chapter 6**) provides a general discussion, summarising the key findings and highlighting the contributions this thesis has made to the overall understanding of the effect of DMSP on natural marine microbial communities. In addition, it provides new information on potential DMSP sinks in waters across a latitudinal gradient, demonstrating a potentially important role of diatoms in DMSP cycling and discusses directions for future research.

Chapter 2:

General methods

Chapter 2: General methods

This thesis studied the role of DMSP in microbial communities from natural oceanic waters using intra- and extra- cellular dimethylsulfoniopropionate (DMSP) quantification, DMSP lyase activity, the relative abundance of two key DMSP degrading genes and the microbial composition of the community. This chapter discusses the techniques chosen to run the analyses and the general methodologies employed throughout the thesis. Other details specific to each chapter, such as sample collections, experimental designs and statistical analyses are described within the relevant chapters.

2.1. Analysis of sulfur compounds

Since the discovery of DMSP, a wide variety of techniques have been used to quantify the sulfur molecule: High performance liquid chromatography with UV detection (HPLC-UV) (Colmer et al., 2000), gas chromatography with flame photometric detector (GC-FPD) (Turner et al., 1990), nuclear magnetic resonance (NMR) (Ansede et al., 2001), ultra-performance liquid chromatography with mass spectrometry (UPLC-MS) (Wiesemeier and Pohnert, 2007), gas chromatography with mass spectrometry (GC-MS) (Niki et al., 2004) and capillary electrophoresis with UV detection (CE-UV) (Zhang et al. 2001) among others. However, the GC-FPD method is the most widely used due to its specificity for sulfurous compounds and the use of a concentration technique such as a cryotrap system as used by Simó et al. (1996), which allowed the quantification of naturally occurring low levels of DMSP. Thus, this technique was adopted throughout this thesis for quantification of the different forms of DMSP in water samples (i.e. DMSP total (DMSPt), dissolved (DMSPd), particulate (DMSPp) and DMSP lyase activity (DLA)).

In the GC-FPD method, the sample is injected into the instrument in gas form either by direct injection with the help of a needle or indirectly via a cryotap system connected to a 6-port valve, and a stream of He transports it through the column, where the various components contained in the sample are separated based on their affinity to the stationary phase of the column. As every component elutes out of the column and into the Hydrogen/Air flame of the FPD detector, they combust and emit chemiluminescence. Using an optical filter that only allows light of 394 nm and amplification by a photo-multiplier, only the emission from excited

sulphur molecules (S_2*) is detected andtransformed to a measurable electric signal (Wardencki and Zygmunt, 1991).

To be able to quantify DMSP using GC-FPD, it is necessary to enzymatically cleave the DMSP contained in the samples to the gas DMS before analysis. For that purpose, we followed a variation of the cold alkali treatment proposed by Dacey (1987) in which DMSP is lysed to DMS by the action of OH^- ions at pH>13. So, 100 mg of NaOH was added to 2 mL of sample in a 14 mL glass vial (or 2 mL of MilliQ water containing the sample filter for DMSPp) that was immediately capped with a rubber septum, crimp capped and left to react for at least 6 h before GC-FPD analysis. If the DMS concentrations in the samples were high (>500 pmol), like in the case of DLA, 500 µL of DMS were collected by syringe from the headspace of the sample and manually injected into the instrument through the injection port. For all other samples, volatiles were quantitatively removed by purging the sample with a flow of He (60 mL/min) for 4 min. and the gas cryo-trapped in a loop of PTFE tubing in liquid nitrogen. After that time, the trapped gas was desorbed by placing the loop in hot water (>50°C) while the six port valve, which connects the purge to the trap or the trap to the GC was positioned into injection mode and so, DMS was transported and injected into the GC column (Figure 2.1). Then, DMS was detected by the sulphur specific detector in the GC, which was set up in the log mode (Log response vs. Log concentration) in order to get a linear response, as the emission intensity of sulphur is proportional to the square of the sulfur concentration.

and its peak area integrated. The peak area integration against a calibration curve allowed for direct quantitation of DMS concentration and so, indirect quantitation of DMSP for both injection modes (Simó et al., 1996). Each calibration curve was made of fresh DMSP standards prepared from DMSP chloride crystals (Sigma-Aldrich) and lysed to DMS the same way as samples and loaded into the GC with the same injection mode as the samples. All calibrations were accepted at R^2>0.99 (Figure S.1).

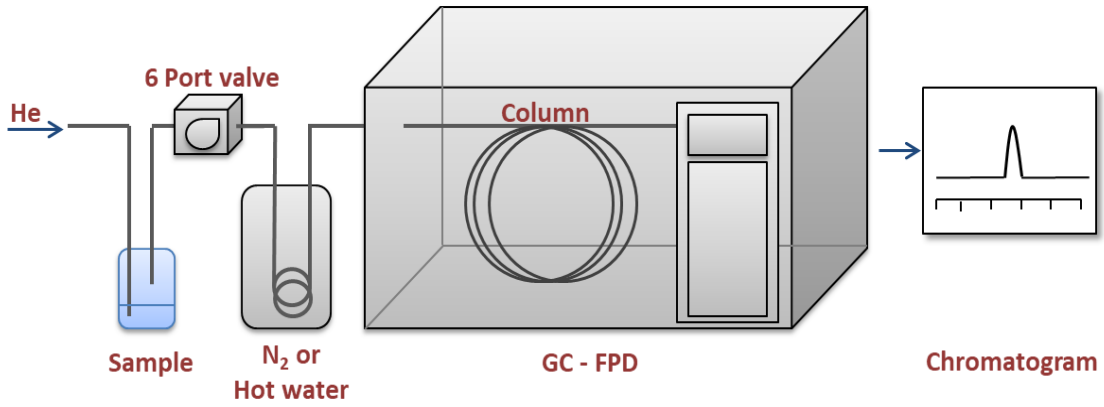

Figure 2. 1 Schematic representation of the purge and trap system. Samples are contained in air-tight serum bottles and sparged with a flow of high purity He *to extract the volatiles, which concentrate in the cold trap of liquid nitrogen. After purging, the volatiles are released using a hot water bath, and injected into the GC via the 6 port valve, which connects the purge and the trap or the trap and the GC. Volatiles are transported and separated through the column based on their affinity to the stationery phase, and reach the FPD, where they combust and emit chemiluminescence, which is detected and transformed to a chromatogram that contains our peak of interest.*

A Shimadzu, GC 2010 Plus (Kyoto, Japan) gas chromatograph was used at the University of Technology Sydney for DMSP quantification. The GC was coupled to a 30m x 0.32 mm x 5 µm DB-1 capillary column (Agilent) and a sulfur-specific FPD. The operational settings for both, direct injection and purge and trap methods are summarized in table 2.1.

Table.2.1. Operational settings of the GC-FPD for direct injection and purge and trap methods.

	Cryotrapping	Direct Injection
Helium flow rate (mL/min)	60	60
Air flow rate (mL/min)	150	150
Total flow rate (mL/min)	12.8	22
Column flow rate (mL/min)	2.1	3.66
Injection temp (°C)	120	120
Column temp (°C)	110	110
FPD temp (°C)	130	160

A subsampling strategy for the collection of sulfur compounds was followed as each compound had to be collected separately (Figure 2.2). For total DMSP (DMSPt), 2 mL of sample was transferred into a 14 mL glass vial prior to hydrolysis with NaOH. For collection

of dissolved DMSP (DMSPd), 3.5mL of water sample were gravity filtered through a GF/F filter (nominal pore size 0.7 µm)as proposed by Kiene & Slezak (2006a) and 2 mL of the filtrate transferred into a 14 mL glass vial prior alkali hydrolysis. The filter was then placed facedown into another 14 mL glass vial containing 2 mL of MilliQ water, andhydrolysed with NaOH for particulate DMSP (DMSPp) measurements. Once all DMSP was hydrolysed to DMS, all DMSP fractions (total, dissolved and particulate) were analysed by GC-FPD using the purge and trap system previously described.

For determination of phytoplanktonic DMSP lyase activity (DLAp), 600 mL of sample was filtered through a GF/C filter (nominal pore size 1.2 µm), and 300 mL of the phytoplankton-free sample filtrate was then filtered through a 0.22 µm polycarbonate filter for bacterial DLA (DLAb). Both filters were placed into cryotubes and snap frozen with liquid nitrogen and stored in -80ºC until analysis. For the analysis of DLA, each filter was defrosted and placed in a 14 mL glass vial with 1 mL of Tris buffer (pH=8) and capped with a rubber stopper, vortexed for 10 s and incubated for 20 min (incubated in iced water for Antarctic samples) prior the addition of DMSP in close to substrate-saturated amounts (5 mM final concentration) and crimped immediately. The vial was then vortexed for 10 s and 5 sequential injections of headspace (500 µL for phytoplankton and 100 µL for bacteria) were loaded into the GC-FPD by direct injection throughout 30 min with approximately one injection every 5 min and the exact time of injections were recorded (Harada et al., 2004; Niki et al., 2000; Yost and Mitchelmore, 2009). A blank containing an empty filter in 1 mL of Tris buffer (pH=8) was run following the same procedure as for the samples to account for any background DMS production. For each sample and the blanks, DMS concentrations at each time point were calculated against a calibration curve (R^2>0.99) made of fresh DMS standards prepared from DMSP chloride crystals (Sigma-Aldrich) that were lysed to DMS with NaOH (Dacey & Blough 1987) and direct injected into the GC. The rate of DMSP lysed to DMS was then calculated by the slope of the linear increase of DMS concentration over the ~30 min of the analysis (Steinke et al., 2000).

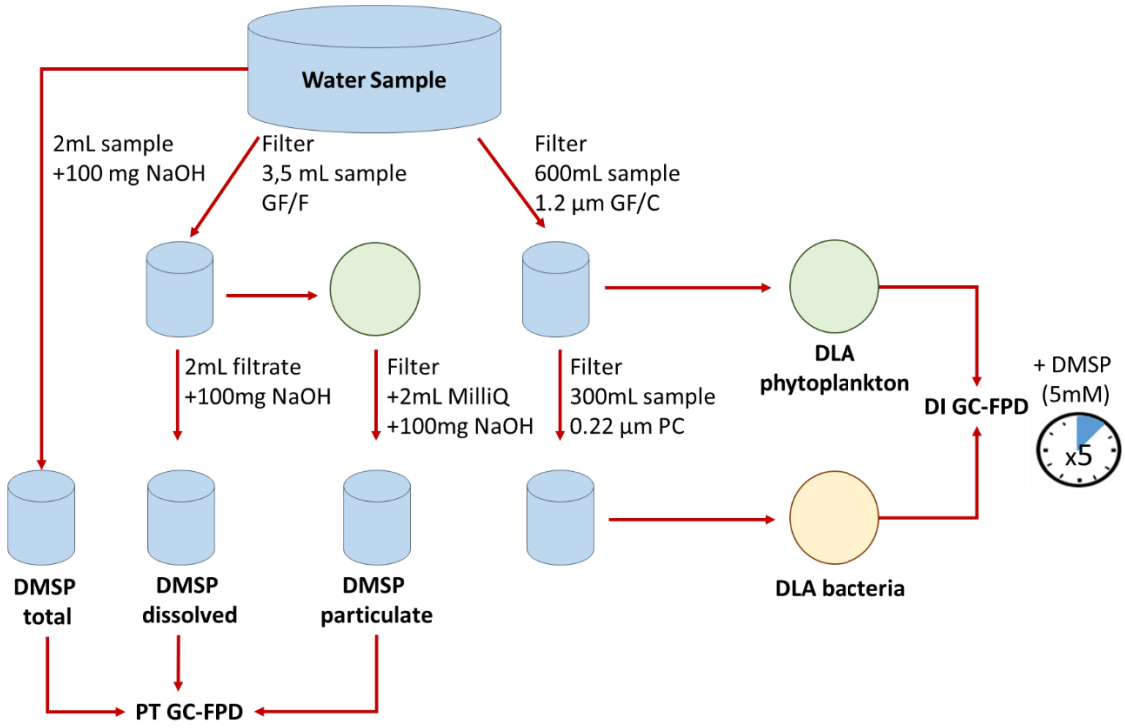

***Figure 2. 2 Water sampling scheme**. A subsample of 2 mL was transferred into a 14mL glass vial prior lysis with 100mg NaOH to collect DMSPt. Then, 3.5mL of water sample were gravity filtered through a GFF and 2mL of the filtrate were transferred into a 14mL glass vial prior adding 100mg of NaOH for collection of DMSPd, while the filter was placed in a 14mL glass vial containing 2mL of MilliQ water and 100mg of NaOH for collection of DMSPp. DMSP total, dissolved and particulate were analysed by GC-FPD using purge and trap. A subsample for phytoplanktonic lyase activity (DLAp) was collected by filtering 600mL of sample into a GF/C filter and 300mL of the same filtrate were filtered through a 0.22µm polycarbonate filter for DLAb. After adding 5mM of DMSP to the filters, 5 consecutive injections were loaded every 5 min by direct injection in the GC-FPD.*

2.2. Calculation of the relative abundances of DMSP degrading genes

Different genes encode for different proteins that are capable of different reactions, such as the capability to degrade DMSP through demethylation or to cleave DMSP into DMS and acrylate. Therefore, to understand the fate of DMSP in marine environments, the relative abundance of the most representative DMSP degrading gene for each pathway was measured.

The polymerase chain reaction technique (PCR) allows for the amplification of specific sequences of DNA by using oligonucleotide primers that cyclically bind into denaturated DNA (single stranded DNA), and are extended by the activity of the DNA polymerase, resulting in exponential amplification of the PCR product molecule (amplicon) to a detectable amount, no matter the initial concentration (Steffan and Atlas, 1991). When used in conjunction with

quantitative PCR (qPCR), a fluorescent reporter dye such as SYBR green, which binds to the double-stranded DNA product, we can quantify the starting material against a calibration curve, as the dye produces measurable fluorescence when bounded to the amplicons (Pabinger et al., 2014).

Two genes responsible for the demethylation pathway, DmdA/A1 and DmdA/Dall and one gene responsible for the cleavage of DMSP, DddP, plus the normalising gene 16S were analysed using qPCR. Samples and standards (1 µL) were mixed with 4 µL of master mix containing 1.1 µL of nuclease-free water (Ambion), 0.2 µL of the respective Reverse Primer (10 µM), 0.2 µL of the respective Forward Primer (10 µM) and 2.5 µL of iTaq Universal SyBR Green Supermix (BioRad). The conditions for the qPCR reaction were as follow: 95°C for 5 min; followed by 40 cycles of 95°C for 30 s, primer-specific annealing temperature for 30 s, 72°C for 30 s; and then 95°C for 1 min, 65 °C for 5 s and 95°C for 1 min (Figure S.2). All runs had R≥0.999 and efficiency between 97%-100% (Figure S.3). The qPCR conditions were optimised for each primer, and the primers and annealing temperatures for each gene are summarised in Table 2.2. Well plates were prepared using the Eppendorf epMotion 5075 liquid handling robot and run in Bio-Rad CFX thermocycler, final data were obtained using Bio-Rad CFX manager 3.1 software. In chapter 3 and 4, qPCR was used to quantify DMSP degrading genes in initial waters and in all time points of the long incubation experiments. Technical triplicates of biological triplicates were analyzed, yielding nine reactions per sample and, results were normalised against the sample´s concentration of genomic bacterial 16S rRNA gene to evaluate the ratios of bacteria within the marine bacterial community that possess DMSP degrading genes.

2.3. Community diversity and composition

For chapters 3 and 4, prokaryote and eukaryote community diversity and composition were characterised in initial seawater samples and at each time point to determine, from the microbes present in the water, which were more likely to participate in DMSP metabolism by either producing it or degrading it.

Table 2.2. Primers and annealing temperatures for the analysed genes.

Gene	Annealing Temperature (°C)	Primers	Primer sequence (5'- 3`)	References
dmdA/A1	54	A/1-spFP	ATGGTGATTTGCTTCAGTTTCT	(Varaljay et al., 2010)
		A/1-spRP	CCCTGCTTTGACCAACC	
dddP	41	dddP_874F	AAYGAAATWGTTGCCTTTGA	(Levine et al., 2012)
		dddP_971R	GCATDGCRTAAATCATATC	
dmdA/Dall	42	D/all-spFP	TATTGGTATAGCTATGAT	(Varaljay et al., 2010)
		D/all-spRP	TAAATAAAAGGTAAATCGC	
16S	53	BACT-1369F	CGGTGAATACGTTCYCGG	(Suzuki et al., 2000)
		PROK-1492R	GGWTACCTTGTTACGGACTT	

To determine the community composition, the extracted DNA was sequenced by Illumina sequencing, which identifies each DNA base from each amplified read, by its unique fluorescent signal. First, samples were collected by filtering 2 L of water through 0.22 µm polycarbonate filters using a peristaltic pump, and the filters were snap frozen until processing. Then, DNA from the samples was extracted, samples of chapter 3 had DNA extracted from the filters using the PowerWater DNA Isolation Kit from MO BIO following the manufacturer's directions. Samples of chapter 4 had their DNA extracted using a phenol-chloroform extraction protocol modified from (Schauer et al., 2000). Briefly, filters were placed in a 5 mL centrifuged vials with 1.8 mL of Lysis buffer, lysozyme (1 mg/ml) and glass beads and vortexed for 10 min, then incubated at 37°C for 1 h. Afterwards, proteinase K (0.2 mg/ml) and sodium dodecyl sulfate (SDS, 1%) were added and the filters vortexed before incubated at 55°C for 1 h. The lysate was recovered from the filter and extracted with an equal amount of phenol–chloroform–isoamyl alcohol (25:24:1, pH 8) and a second time with an equal amount of chloroform-isoamyl alcohol (24:1). The aqueous phase was recovered, and DNA precipitated with 100 µL of sodium acetate (3M). An equal volume of isopropanol was added, and the tube mixed gently. The supernatant was disposed after centrifugation at 20,000 g at 4ºC for 10 min, and DNA washed with ethanol 70% before recovering in 20 µL of MilliQ water. The phenol-chloroform extraction protocol was chosen as it provides high yields, excellent purity and reproducibility as compared to other DNA extraction protocols (Schauer et al., 2000).

Then, 20 µl aliquots ≥10 ng/µl DNA were used for community analysis (AGRFA, Melbourne, VIC, Australia). For the diversity profiling of genomic DNA (16S), samples were amplified using 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) primers and sequenced with Illumina MiSeq platform (Illumina, California, USA) with 30000 reads/sample. For 18S, Next-Generation Sequencing with Eukaryotic V4 Primers (Forward >TAReuk454FWD1: CCAGCASCYGCGGTAATTCC and Reverse >V4_rev_Piredda: ACTTTCGTTCTTGATYRATGA) were used with a thermocycling profile starting at 94° C for 3 min.; then 30 cycles of: 30s at 94°C, 60s at 57°C, 90s at 72°C; and a final 10min at 72°C. The amplicon size is between 87 to 186 bp. Gene sequencing reads were analysed using the QIIME2 (v2018.2.0) pipeline (Bolyen et al. 2018). Raw fastq files were imported using the 'qiime tools import' command. Sequences were then trimmed and denoised using DADA2, which also detects and removes chimeras (Callahan et al., 2016). Taxonomy were then assigned on the rep-set-dada2 output at the single nucleotide level using the sklearn qiime feature classifier (Pedregosa et al., 2011) against the Silva v132 database (Gurevich et al., 2013).The dataset was further cleaned by removing sequence variants (ZOTUs) identified as chloroplasts, mitochondria, and ZOTUs (abundance cut-off of 0.03%). Sequences were then rarefied to the same depth to remove the effect of sampling effort upon analysis. Barplots and all statistical analysis was performed on the rarefied data.

2.4. Analysis of ^{13}C-DMSP

Stable isotopes of a chemical element can be used as tracers if deliberately added to a system, due to their low natural abundance compared to the standard for that element. In order to track the uptake of DMSP by different size fractions of marine microbes of natural environments, we added isotopically labelled DMSP in which both methyl groups contained ^{13}C instead of ^{12}C, which is naturally present at 1.1% abundance, to a closed system containing the natural waters under study (IMOS, 2019).

2.4.1. Elemental analysis isotope ratio mass spectrometry (EA-IRMS)

The technique EA-IRMS is a technique widely used in all areas of environmental sciences to measure natural variations of stable isotope abundances of the same element to characterise the biological, geographical or physical history of the sample (Muccio and Jackson, 2009). Isotope ratio mass spectrometry has been used to determine the source of a water mass and to estimate the proportions from each source (Weldeab et al., 2007), to infer sea turtles and

whales foraging areas by the analysis of the isotopic composition of commensal barnacle shells (Pearson et al., 2019), to determine diets of aquatic animals (Roberts and Britton, 2018) among many others.

The isotope ratio mass spectrometry (IRMS) technique was chosen as a first option to analyse the ^{13}C enrichment in the samples due to the high acceptance and usage of this technique within the scientific community, with the elemental analyser (EA) inlet system as the most commonly used for bulk isotopic analysis. Moreover, the sampling procedure requirements are simple, and there was the possibility to send the samples to be analysed externally. In order to run a sample through the mass spectrometer detector of the EA-IRMS, it is critical that the sample enters in gas form and that only a single chemical enters at a given time. Therefore, samples are combusted and transformed to CO_2, H_2, N_2 and SO_2 gases and separated through gas chromatography prior to entering the detector. Samples were run externally at ANSTO (Australian Nuclear Science and Technology Organisation) using an established on-line combustion, continuous-flow IRMS method based on the method by Anders Ohlsson & Håkan Wallmark (1999), where the crushed and dried samples were weighed into tin capsules and introduced sequentially into an elemental analyser (Thermo Fisher Flash 2000 HT EA) using an autosampler. Each sample was combusted into CO_2 in a combustion furnace (silvered cobaltous/ic oxide, chromium oxide, quartz chips and quartz wool) at 1020ºC before being transferred with a helium carrier gas (100 mL/min) into a reduction furnace (copper) at 600ºC, where any excess nitrous oxides were converted into N_2, and excess O_2 was removed. The analyte gases were passed through a water trap before the CO_2 and N_2 were separated by a GC column at 40ºC. The gases were then transferred to a Thermo Fisher Conflo IV and into a Thermo Fisher Delta V Plus isotope ratio mass spectrometer for $\delta^{13}C$ measurements.

The data reported relative to the International Atomic Energy Agency (IAEA) secondary standards that have been certified relative to Vienna Pee Dee Belemnite (VPDB) for carbon and air for nitrogen. A two point calibration was employed to normalise the data, utilising standards that bracket the samples being analysed. Two quality control references were also included in each run. Results were accurate to 1% for C % and +/- 0.3 permil for $\delta^{13}C$. The results (Table S.1) showed an inconsistent variation of $\delta^{13}C$ among samples and quantification was not possible due to a high variation on sample weight. The percentage

calculation is very dependent on the mass of sample, and so, it is essential to know exactly the amount of sample in each filter by weighing the filters before and after filtration. Still, our samples presented high variations in weight among replicates and samples, and some blanks were heavier than the samples. The weight differences were possibly due to the difficulty of storing the filters without disturbing their shape. A complete removal of water by pressing absorbent filter paper onto the filters was not achieved, and the GFF filters were bloated when stored twice-folded into 1.5 mL cryovials. When the filters were recovered for analysis, their shape had been severely compromised. Moreover, the 5 mm^2 subsample per filter required for the analysis was possibly not representative of the sample as the material on the filter was not uniformly distributed, and the amount of material contained in each subsample was possibly too little to be quantified. Therefore, following these trials, EA-IRMS was considered not suitable for our samples and other possibilities were explored.

2.4.1. Thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS)

The next technique considered to analyse the enrichment of ^{13}C-DMSP in the samples was thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS), in which samples were desorbed from a stainless steel tube coated with a membrane capable of retaining the targeted compound in gas form, and reported successfully at quantifying DMS in ice melt ponds in the Canadian Arctic Archipelago (Gourdal et al., 2017). The requirements of the technique included the target compound to be in gas form, which in our case could be achieved by alkali hydrolysis of DMSP as per GC-FPD, and the existence of a membrane which could quantitatively retain the gas, achieved by the high sulfur affinity of Tenax-TA polymer.

Therefore, the analysis of the samples started with the transformation of DMSP to DMS by cold alkali hydrolysis (Dacey and Blough, 1987), in which filters were placed in a gas tight vial containing 6 mL of MilliQ water and NaOH (1 M). After hydrolysis, the samples were purged for 30 min with a flow of air of 200 mL/min and the gas was trapped in a Teflon-lined stainless steel tube coated with Tenax-TA polymer (Markes International) which was mounted downstream of the Purge and Trap system (Shooter et al., 1992). Purging was always performed no more than 2 weeks before analysis and samples preserved at low temperature (-80ºC). Purged samples were run for 15 min at an oven temperature of 40°C and an equilibration time of 0.25 min. in a 6978 GC coupled to a 7000B Triple-Quad MS (Agilent),

and mass spectra collected in full scan (m/z 12-18, 26-49, 56-66) with the masses 40, 38, 43 and 58 removed for interfering with DMS, and in selected ion monitoring (m/z 62,63,64) modes. A multiple regression of the relative ion abundances was created to predict the amount of labelling. The regression equation had a good linear relationship (R^2>0.90) and the results were significant ($p<0.05$) (Table S.2). However, when used to predict % of labelling from standards with known amount of labelling, the coefficient of variance was between 17-200%, depending on % labelling, and therefore did not allow for a proper discrimination between isotope enrichment.

Great challenges were faced at analysing ^{13}C-DMSP at concentration levels similar to what are found in natural marine environments for non isotopically labelled DMSP, and unfortunately, no results were obtained. As such, no ^{13}C-DMSP data will be presented in this thesis. However, if further experiments were to be conducted, the preferred technique of analysis, which was not available for this thesis, would be thermal desorption GC-IRMS, which combines the quantitatively removal of DMS from the samples through a purge and trap system with the increased sensitivity of IRMS (Kawashima, 2015; von Eckstaedt et al., 2011; Zhuang et al., 2017). Moreover, increasing the mass difference between the 2 DMS compounds (labelled and non labelled) would increase separation of peaks in the chromatogram, facilitating accurate measurement of isotope enrichment. Using deuterium instead of ^{13}C would have increased the total atomic mass difference of DMS from 2 to 6.

2.5. Cell enumeration: Flow cytometry Analysis & Phytoplankton identification by light microscopy

To quantify the number of cells present in the samples we used two different techniques, flow cytometry to count heterotrophic bacteria and picoeukaryotes and light microscopy to count and identify phytoplankton cells. Prior to flow cytometric enumeration, frozen samples were quickly thawed by placing the frozen tubes in hot water (>50ºC). Populations of *Prochlorococcus*, *Synechococcus* and picoeukaryotes were discriminated using side scatter (SSC) and red and orange fluorescence (Marie et al., 1997) using a Beckman Coulter Inc flow cytometer (Indianapolis, USA). Samples for bacterial analysis were stained with SYBR Green I nucleic acid stain (1:10000 final dilution; Invitrogen) and populations were discriminated according to green fluorescence and side scatter properties (Gasol et al., 2000; Seymour et al., 2007). Data were analysed using CytExpert software (Beckman Coulter) (Figure S.4). For

identification and enumeration of phytoplankton genus, a nanoplankton chamber fashioned after the Palmer-Maloney counting chamber holding exactly 0.060 ml of sample was used with a light compound microscope (Nikon eclipse ci-L) (Palmer and Maloney, 1954). All cells within the chamber were counted and identified to genus or species, where possible.

2.6. Nutrients

In chapter 3, nutrients were carried out on board by CSIRO Marine and Atmospheric Research (CMAR) according to Cowley et al. (1999). In chapter 4, nutrients were measured at UTS with a microcolorimetric method following the procedure by Ringuet et al. (2010) for silicate and phosphate, and by Schnetger & Lehners (2014) for NOx. Between 180-200 µL of each sample, with technical triplicates, were mixed with a series of reagents according to the nutrient to be analysed to colour the sample proportionally to their nutrient content and, their absorbance measured at a nutrient specific wavelengths (810 nm for silicate, 880 nm for phosphate and 540nm for NOx) in Synergy 4 Biotek multiplate reader. A minimum of seven standards of Na_2SiF_6 (sigma-aldrich) for silicate, oven-dried KH_2PO_4 (sigma-aldrich) for phosphate and KNO_3 (sigma-aldrich) for NOx and a blank (artificial seawater (Subow, 1931)), all in triplicates, were treated and analysed the same way as samples and, the samples absorbance against the calibration curve allowed for direct nutrient quantification. All calibration curves had a R^2>0.996 (Figure S.5). In chapter 5, nutrients were analysed by IMOS and data obtained from the Integrated Observing System website (IMOS, 2019).

2.7. Photophysiological condition of algae

Chlorophyll *a* fluorescence was measured in live samples to determine the photosynthetic condition of the cells by using Pulse Amplitude Modulation (PAM) fluorometry (Water PAM, Walz GmbH, Effeltrich, Germany), which is a rapid, non-invasive, non-destructive and highly sensitive technique (Kolber and Falkowski, 1993). Subsamples were adapted to dark for 10 min prior to the application of a saturating pulse of light and minimum (F_0) and maximum (F_M) fluorescence were recorded. Maximum quantum yield of photosystem II (PSII) (F_v/F_M), which is the quantum efficiency if all PSII centres were open, was calculated with the following equation (Maxwell and Johnson, 2000):

$$\frac{\mathrm{Fv}}{\mathrm{FM}} = \frac{\mathrm{FM}-\mathrm{F0}}{\mathrm{FM}} \qquad \textit{Equation 2.1}$$

2.8. **Plankton biomass quantification**

A widely used proxy to quantify the density and biomass of microalgae in an ecosystem consists of measuring the concentration of chlorophyll *a* (Johan et al., 2014). In chapter 5, the amount of chlorophyll *a* of each biological replicate of each sample was determined with a non-acidification fluorometric method (Welschmeyer, 1994) and used to normalise the amount of DMSP to phytoplankton biomass. Subsamples of 300 mL were filtered through a GF/F filter and the pigments extracted in 3 mL of 90% acetone and stored in the dark at 4ºC until analysis. No more than 24 h after extraction, aliquots of samples and a blank (90% acetone) were taken and measured in a fluorometer (Turner trilogy, CA USA) at an excitation wavelength of 430 nm and an emission wavelength of 670 nm, and after substraction of the blank fluorescence, Chl *a* concentrations were calculated according to equation 2.2 where Fo is the fluorescence signal of sample, v is the extract volume in litres and V is the volume filtered in litres.

$$Chl\ a = \frac{(F0\ x\ v)}{V} \qquad \textbf{\textit{Equation 2.2}}$$

Chapter 3:

DMSP uptake and assimilation by natural microbial communities of The Great Barrier Reef (GBR), Australia

Chapter 3: DMSP uptake and assimilation by natural microbial communities of the Great Barrier Reef (GBR), Australia

3.1 Abstract

Dimethylsulfoniopropionate (DMSP) is a key organic sulphur compound found in all marine environments. It is generally believed that once released into the water column by DMSP-producing phytoplankton, DMSP is primarily metabolised by heterotrophic bacterioplankton. However, it has been estimated that phytoplankton may take up as much DMSP as hetertrophic bacteria and recent research has shown phytoplankton can accumulate as much as 87 mM $cell^{-1}$ (Petrou and Nielsen, 2018). In this study, we examined the uptake of DMSP by natural marine microbial communities within the waters of the Great Barrier Reef (GBR), Australia. By incubating natural seawater communities of the GBR with DMSP, we aimed to quantify the uptake of DMSP by different fractions of the microbial community (i.e. >8 µm, 3-8 µm, <3 µm) and to evaluate how microbial composition and DMSP degrading gene abundances are influenced by dissolved DMSP levels. Our results showed accumulation of DMSP in all size fractions of the microbial community, with the largest fraction (>8 µm) forming the dominant sink with an increase of DMSPp of 44% and 115% for the inner and outer reef site, respectively. Through increased abundance with DMSP enrichment, we proposed that the capability to degrade DMSP provides an ecological advantage to many bacteria, such as *Synechococcales, Alphaproteobacteria, Rickettsiales, Rhodobacterales* and *Flavobacteriales* and some diatoms which may catabolise DMSP via a yet unknown pathway. These results support the hypothesis that phototrophs take up DMSP and propose that the biggest members of the community (>8 µm), such as diatoms, are relevant DMSP sinks in the GBR, leading to a need to study the possible roles of DMSP in the non-DMSP producing phototrophic cell, as well as their contribution to the reef sulfur cycle.

3.2 Introduction

Dimethylsulfoniopropionate (DMSP) is an important and ubiquitous organic sulphur compound in the marine environment (Zubkov et al., 2001). Its cleavage by bacterioplankton to the climatically active gas dimethylsulfide (DMS), has been suggested to contribute to

climate regulation through the increase of cloud albedo, which reduces incoming solar radiation, lowering seawater temperatures (Charlson et al., 1987). In marine ecosystems, DMSP plays a key role in cell metabolism satisfying up to 71% of the sulphur and 15% of the carbon demands of phytoplankton (Matrai & Keller 1994; Simó et al. 2002), and up to 95% of the sulphur and 15% of the carbon demands of some marine bacteria (Zubkov et al., 2001).

Phytoplankton are the dominant producers of DMSP in marine environments, although intracellular concentrations vary from species to species, ranging from extremely low in cyanobacteria and the majority of diatoms to ~300 pg DMSP $cell^{-1}$ in haptophytes and dinoflagellates, both of which are considered the greatest DMSP producers (Keller, Bellows & Guillard 1989, Belviso, Claustre & Marty 2001). Due to the widespread distribution and high variations of DMSP concentrations among phytoplankton species, several cellular and ecological roles have been attributed to the compound, including antioxidant (Sunda et al., 2002), osmoregulatory (Dickson and Kirst, 1986), cryoprotectant (Karsten et al., 1996), chemoattractant (Seymour et al. 2010), grazing deterrent (Strom et al. 2003; Wolfe et al., 1997) and bacteriocidal (Saha et al. 2012) roles.

Intracellular DMSP or particulate DMSP (DMSPp) is transported from the synthesising cell into the external seawater environment via exudation, viral lysis or cell senescence (Hill et al., 1998; Matrai and Keller, 1993). Once in the water column, dissolved DMSP is readily taken up by the bacterial community (Malmstrom et al. 2004; Raina et al. 2017; Ruiz-González et al. 2012). Inside the bacterial cell, DMSP can degrade via two different pathways, one involving demethylation of the compound and the other cleaving DMSP into various by-products (Curson et al., 2011). In the demethylation pathway, which is catalyzed by the DmdA gene (Reisch et al. 2011), DMSP is transformed to methanethiol (MeSH), which is utilised for cellular energy and protein production (Kiene et al., 1999; Kiene and Linn, 2000). In the cleavage pathway, which is catabolized by the Ddd genes (Curson et al. 2011), DMSP is split into DMS (Kiene, Linn & Bruton 2000; Yoch 2002) and acrylate, or other carbon-containing molecules depending on the Ddd derivative. It has been hypothesised, in what has been termed the bacterial switch, that bacteria will preferentially utilise the demethylation pathway when there is a need to produce protein for growth, and once that demand is satisfied, they will switch to using the lyase pathway to catabolize the remaining DMSP, resulting in DMS production (Kiene, Linn & Bruton 2000). Metagenomic surveys have

determined that DmdA, which catabolizes the first demethylation step, is the most abundant DMSP degrading gene in the oceans and is present in 37-58% of all bacterioplankton cells (Howard et al. 2006, 2008). In contrast, Ddd genes are present in less than 10% of bacterioplankton cells, with the most abundant lyase gene (DddP) present in only 6% of bacterioplankton cells (Todd et al. 2009). This large discrepancy in the abundance of the dominant genes for DMSP degrading pathways has led to an estimation that 80% of DMSP degrades through demethylation (Curson et al. 2011). Moreover, DmdA is not only the most abundant DMSP degrading gene, but is also widespread among marine bacteria including Roseobacter, SAR11, Gammaproteobacteria and SAR116; while DddP occurs mostly in Roseobacter and SAR116 clades. Nevertheless, the abundant SAR11 can cleave as much as 59% of DMSP uptake while simultaneously demethylating DMSP via its DddK lyase (Sun et al., 2016).

In 2001, Simó et. al proposed that in addition to bacterioplankton, non-DMSP producing phytoplankton may also take up DMSP. A follow-up study in which surface seawater was incubated with ^{35}S-DMSP and *Prochlorococcus*, *Synechococcus,* heterotrophic bacteria and picoeukaryote populations sorted using flow cytometry revealed assimilation of DMSP sulphur by all groups, with dinoflagellates, cryptophytes and diatoms captured within the picoeukaryote group (Vila-Costa et al. 2006). More recent studies support significant DMSP uptake by non-DMSP producing phytoplankton (Petrou and Nielsen, 2018; Ruiz-González et al., 2012c; Spielmeyer et al., 2011). To date, however, the proportion and magnitude of DMSP uptake by phytoplankton in natural communities remains unclear, and no genes from the demethylation pathway have yet been found in phytoplankton (Alcolombri et al., 2015). One lyase gene, Alma1, has been found in the haptophyte *Emiliania huxleyi* (Wolfe and Steinke, 1996), and lyase activity has been measured in several phytoplankton species, including *Phaeocystis* spp. (Boekel and Stefels, 1993), *Heterocapsa triquetra, Scrippsiella trochoidea* (Niki et al., 2000) and *Symbiodinium* (Yost and Mitchelmore, 2009), all of which are high DMSP producers. Identifying and describing the uptake and possible catabolisation of DMSP by non-DMSP producing phytoplankton species would increase our understanding on DMS production, sea-air DMS fluxes and climate regulation mechanisms, which are still not completely understood (Malin, 2006; Simó, 2004, 2001), and will improve cloud albedo models (McCoy et al., 2015) and climate projections (Wang et al., 2018), which currently only account for few phytoplankton functional groups known to be critical to the production of

DMS. However, given the limited information on DMSP degradation by phytoplankton species, it is still largely unknown how or whether most phytoplankton catabolise DMSP. Molecular and physiological work focussing on non-DMSP producing phytoplankton species will be important to understand the role of DMSP in the broader phytoplankton community. Similarly, studies on the uptake of DMSP in natural phytoplankton communities will provide new information on DMSP cycling and its contribution to climate regulation.

In this study, we examined the uptake of DMSP by natural microbial communities of the Coral Sea area surrounding the Great Barrier Reef (GBR) in Australia, characterised by high levels of light and solar radiation, low nutrient concentrations and waters dominated by the cyanobacteria *Synechococcus*, *Prochlorococcus* and *Trichodesmium* (Blondeau-Patissier et al., 2018; Partensky et al., 1999; Revelante et al., 1982; Westberry and Siegel, 2006). The distributions of these taxa is defined by nutrient availability, whereby *Synechococcus* are more abundant in coastal waters (Charpy et al., 2012) while *Prochlorococcus* dominate the more oligotrophic, nutrient-depleted areas of the open ocean (Campbell et al., 1994) and *Trichodesmium* blooms seasonally in regionally distinct patterns (Blondeau-Patissier et al., 2018; Revelante et al., 1982). Except for Trichodesmium , cyanobacteria are not considered DMSP producers, but they have been shown to take up DMSP (Vila-Costa et al., 2006b), possibly representing a major DMSP sink (Lei et al., 2017; McParland and Levine, 2019). Despite the inability of most cyanobacteria to produce DMSP and their prominence in reef waters, coral reefs are considered hotspots of DMS and DMSP production (Broadbent and Jones, 2006; Broadbent et al., 2002; Jones et al., 2007, 1994). This is mainly attributed to reef-building corals that harbour symbiotic dinoflagellates from the family *Symbiodiniumaceae*, considered the most prolific of all DMSP producers (up to 3,831 fmol DMSP cell $^{-1}$ (Broadbent, Jones & Jones 2002)) and that may play an important role on combating high temperature stress protecting corals from bleaching depending on the zooxanthellae clade hosted (Jones and King, 2015). Moving away from shallow reef waters, however, DMSP levels in surrounding deeper waters are generally low, more likely due to the dilution of shallow GBR waters with continental slope waters, giving rise to the question of the fate and function of DMSP in tropical ecosystems. It is evident that reef-building corals form an important DMSP source for shallow oligotrophic waters of tropical oceans, and it is known that coral mucus and coral-reef sediment pore waters accumulate high amounts of

DMSP (Broadbent and Jones, 2004; Deschaseaux et al., 2016). However, much less is known about the microbial DMSP sinks in these systems.

To better understand microbial DMSP sinks and sources within the oligrotrophic waters of the GBR, we investigated DMSP uptake among natural microbial communities from two distinct reef locations. We primarily aimed to quantify and compare the uptake of DMSP by the eukaryotic and prokaryotic populations using a size fractionation approach and to evaluate DMSP-induced changes in microbial composition over time.

3.3. Experimental procedure and data analysis

This study was conducted during an oceanographic voyage on the *RV Investigator* in October 2016. Two sites within the Great Barrier Reef were chosen for the study: a southern open ocean site (outer reef site) (152º50´, 21º44´) and a more northern site (146º56´, 18º35´) located between the coast and the reefs (inner reef site) (Figure 3.1). The inner reef site was located at~ 13km from the closest reefs of John Brewer reef, rib reef and Fore and Aft reef, the outer reef was located at ~ 25km from the closest reef of Bills reef.

At each site, two separate incubation experiments were conducted, a short-term study (7h) to quantify DMSP uptake by different size fractions of the microbial community, and a longer (120 h) experiment to investigate the influence of DMSP on community structure and gene regulation. At both sites, surface seawater samples (5 m) were collected with Niskin bottles from CTD casts and subsampling procedures and analyses of sulfur compounds, genes and microbial composition were performed as described in the general methods section (section 2.1).

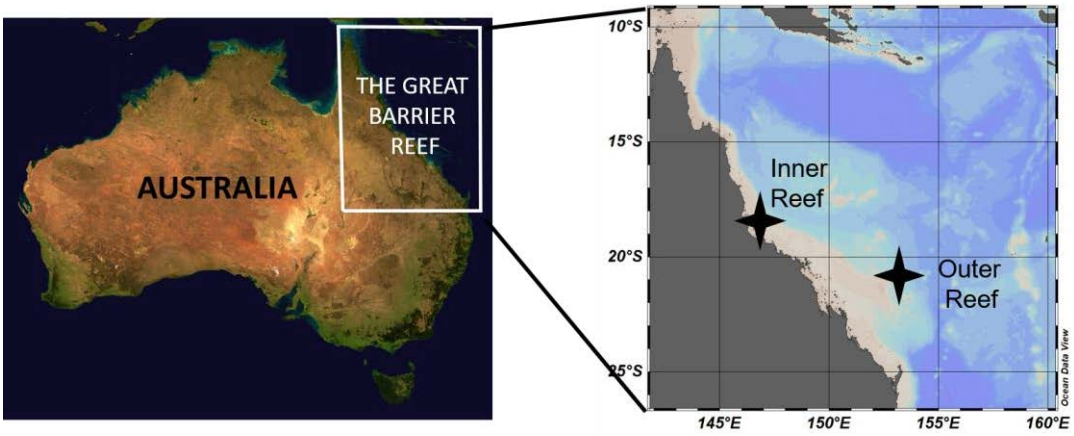

Figure 3.1. Location of sampling sites of initial water for experiments 1 and 2. *Map of Australia showing location of the Great Barrier Reef, with magnified inset image showing location of two sampling sites generated with Ocean data View software.*

3.3.1 Experiment 1: Quantification of rapid DMSP uptake by different fractions of the microbial community

In order to study the uptake of DMSP by the microbial community of the Great Barrier Reef, 28 L of seawater was collected and transferred to 4 L-polycarbonate bottles (3 controls, 1 fixed sample, 3 samples treated with 10 nM DMSP) with no headspace. For the fixed sample, glutaraldehyde (final concentration 1%) was added and left for 1 h before DMSP (10 nM final concentration) was added to control for any passive uptake of DMSP by cells. After DMSP addition, the bottles were closed with screw caps and shaken gently to dissolve the added compound. All flasks were incubated for up to 7 h in an on-deck flow-through incubator to keep the temperature of the water as near to *in situ* water temperature as possible. At four time points (T0, T2, T4 and T7 h) each bottle was subsampled in triplicate for DMSP total and dissolved (DMSPt, DMSPd) as indicated in Figure 3.2 and kept until processing at -80ºC after acidification with H_2SO_4 (0.5%, pH ~1.1) (Del Valle et al. 2011).

After 7 h, two litres from each replicate were size-fractionated via serial filtration using 8 µm, 3 µm and 0.22 µm polycarbonate filters for analysis of particulate DMSP (DMSPp) and the filters were snap frozen and kept at -80ºC until analysis, when the samples were defrosted and hydrolysed prior GC-FPD analysis (described in section 2.1.1). During the first filtration, subsamples (800 µL) from each size fraction were taken, fixed with glutaraldehyde (final concentration 1%) and snap frozen for bacterial abundance. The flow chart of the experiment is shown in figure 3.2.

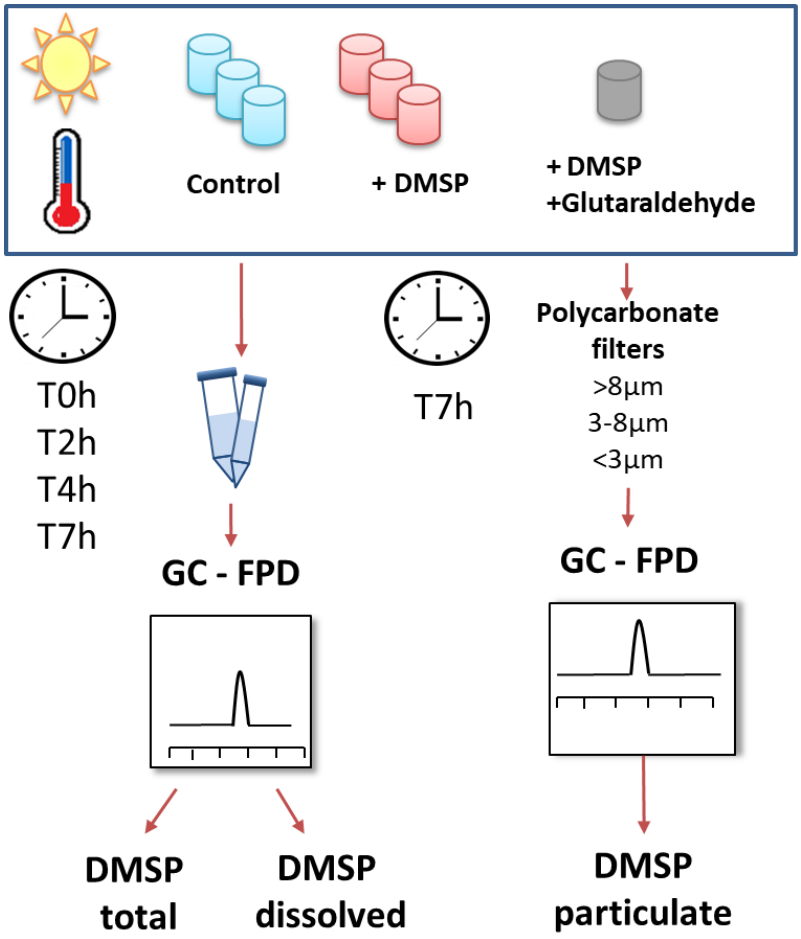

Figure 3.2. Experiment 1 flow chart. *In order to study the microbial uptake of DMSP, initial seawater was split in 3 control, 13 fixed (1% Glutaraldehyde) and 3 DMSP amended samples with no headspace. At every time point, each bottle was subsampled for analysis of DMSP total and dissolved by GC-FPD. In the last time point, each replicate was split and size-fractionated via serial filtration using polycarbonate filters for analysis of particulate DMSP (DMSPp) by GC-FPD.*

3.3.2. Experiment 2: Effect of DMSP on community structure and gene regulation (long-term study)

To study the microbial community changes caused by the addition of DMSP, 24 L of water were divided across 12 x 2 L polycarbonate bottles with no headspace, which includes six controls and six samples treated with DMSP (10 nM final concentration) and bottles were incubated in an on-deck flow-through incubator until destructive sampling at three time points (T24, T72 and T120 h).

At each time point, subsamples for DMSP total and dissolved were taken as indicated in figure 3.2 prior filtration of the samples (2 controls and 2 samples treated with DMSP) through a 0.22 µm polycarbonate filters, and the filters snapped frozen and kept at -80ºC. DNA from

the filters was extracted using the "Power water DNA isolation kit" (MO BIO Laboratories, Inc.) following manufacturer's instructions and the DNA concentration was quantified using a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific) and stored at -20°C until required. Resulting DNA was split into two fractions; one fraction was used for the analysis of 16S and 18S of the whole community as described in section 2.3, and the second fraction was used for the quantification of DMSP degrading genes by real time Polymerase Chain Reaction (qPCR) as described in section 2.2. The flow chart of the experiment is shown in figure 3.3.

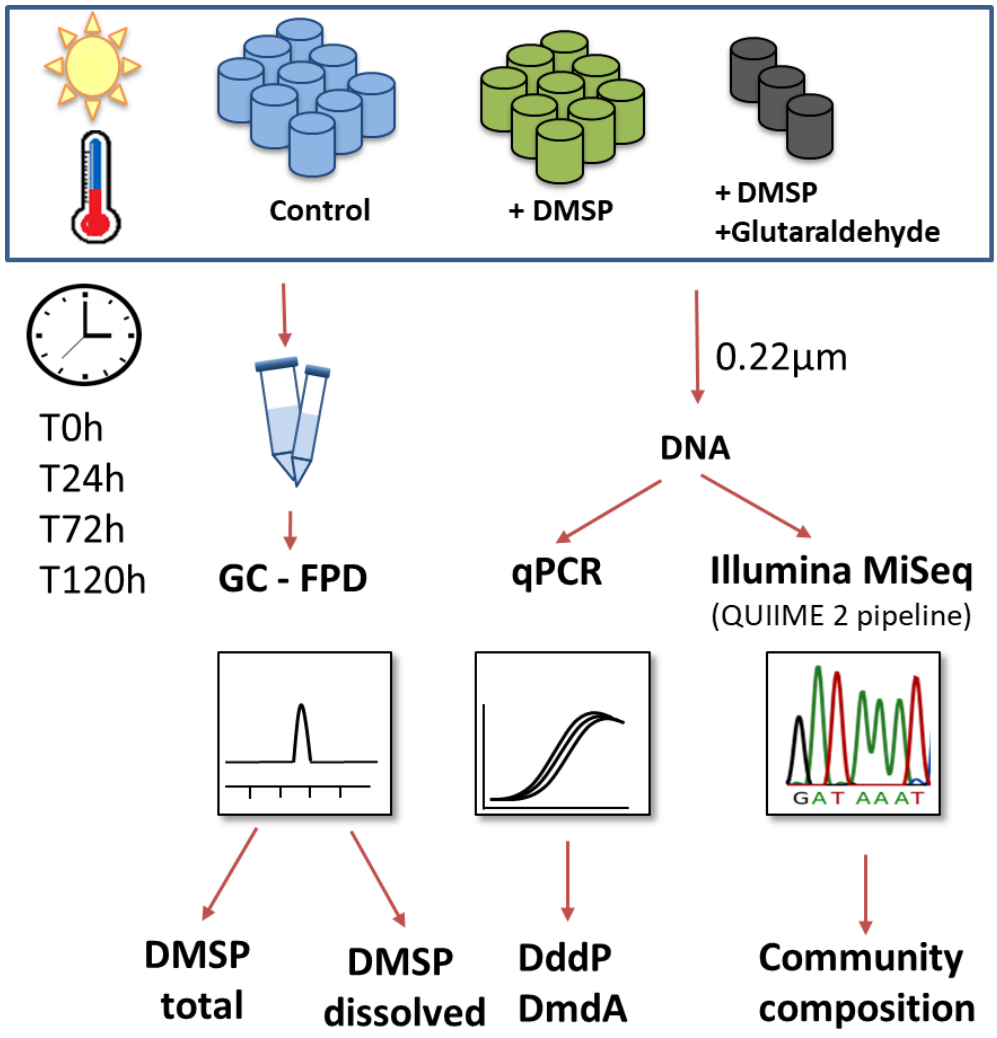

Figure 3.3. Experiment 2 flow chart. *To study the microbial community changes caused by the addition of DMSP, initial natural waters were divided into 9 controls, 9 DMSP enriched, and 3 fixed (1% Glutaraldehyde) +DMSP samples. At each time point, subsamples for DMSP total and dissolved were taken and samples filtered. DNA from the filters was extracted and used for the analysis of 16S and 18S of the whole community.*

3.3.3. Statistical analysis

After having ascertained homogeneity of variance using Levene's test, a one-way Analysis of Variance (ANOVA) was used to analyse differences between sites for the initial water masses for each experiment, differences between treatments for DMSPp data and differences between sites for dmdA/A1, dmdA/Dall and DddP gene abundance data from experiment 1. These analyses were performed using the statistical package SPSS (IBM Statistics v.24). Permutational Multivariate Analysis of Variance (PERMANOVA) with pair-wise comparisons was used to determine significant changes or differences in DMSPt and DMSPd concentrations over time and between treatments, in a nested design for experiment 1 and 2, and to analyse the differences between treatments over time for the relative abundance of dmdA/A1, dmdA/Dall and DddP genes data in experiment 2.

Differences in community composition between treatments and time were characterized using PERMANOVA, non-parametric multi-dimensional scaling (nMDS) and "Analysis of Similarities" (ANOSIM). Discriminatory microbes were identified using a Two-way crossed analyses of Similarity Percentages (SIMPER) with time and treatment variables as factors (Bray–Curtis similarity matrix) (Clarke, 1993). High contributors of microbes were selected from the treatment pair-wise comparisons at the final time point (120 h) to explain a minimum of 70 % of treatment dissimilarity. These analyses were performed in PRIMER v6 statistical package (Clarke and Gorley, 2006) with PERMANOVA+ module (Anderson, 2005).

Correlations between DMSP degrading genes and bacteria concentrations (including all time points – 0 h, 24 h, 72 h and 120 h and treatments (controls and DMSP-enriched)) using the statistical package SPSS (IBM Statistics v.24) were analysed using Pearson correlation coefficients and only considered when the correlation was highly significant at the 99.999% level ($r < 0.001$) and significant at the 95% level ($P < 0.05$). Using the Primer v6 statistical package (Clarke and Gorley, 2006), a Principal Coordinates Analysis (PCO) using Bray-Curtis similarity matrices were used to examine the effects of increasing DMSP levels on the bacterial community composition and overlying vectors of the relative abundance of dmdA/A1, dmdA/Dall and DddP genes were represented. Relative abundance data for both, genes and bacterial composition were fourth root transformed *a priori* to meet assumptions of multivariate homogeneity and to reduce the influence of highly abundant species. Phylogenetic trees using presence/absence of genera of the microbes present in at least two

of the replicates identified by 18S were constructed for identification of the eukaryotic community using R (Team, 2013) with phytools package (Revell, 2012). Due to missing phylogenic information for many 18S sequences, only genera for which the taxonomy could be verified in WoRMS (World Register Marine Species; marinespecies.org) were included in the trees.

3.4. Results

3.4.1 Characteristics of initial water masses

The physicochemical characteristics of the initial water masses were similar across both reef sites and experiments (Table 3.1). Nutrient concentrations were generally low (Figure 3.4), with nitrates (NOx) consistently below the detection limit at both sites and nitrite and ammonium detected in only one of the experiments of the outer reef (Figure 3.4). Phosphate concentrations were similar at both sites, ranging from 0.038 to 0.050 µM in the inner reef, and 0.049 to 0.066 µM in the outer reef. Silicate concentrations were higher in the outer reef site (0.798 and 0.882 µM) compared with the inner reef site (0.623 and 0.500 µM).

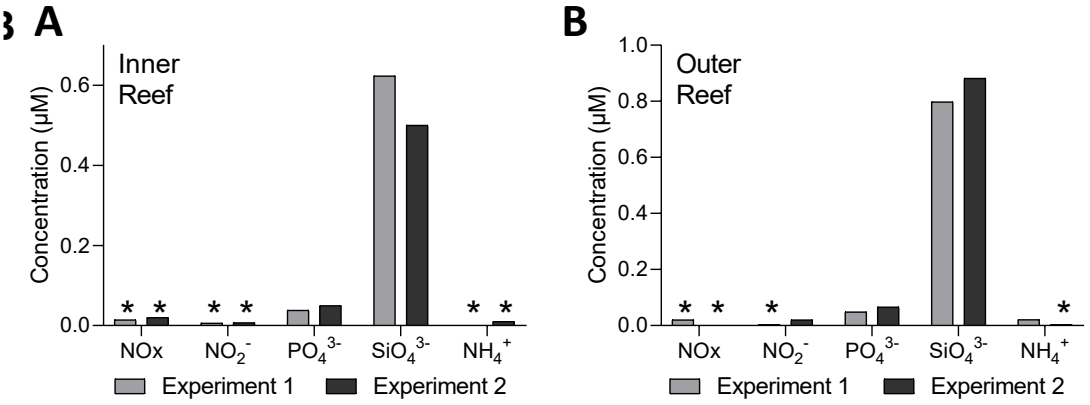

Figure 3.4. Nutrients concentrations of initial water for experiments 1 and 2. *Concentrations of nitrates (NOx), nitrite (NO_2^-), phosphate (PO_4^{3-}), silicate (SiO_4^{3-}) and ammonium (NH_4^+) for experiment 1 (light grey bars) and 2 (dark grey bars) at the initial sampling time point for (A) the inner reef site and (B) the outer reef site. Data obtained with one single replicate. *Below detection limit.*

Initial water temperatures were between 26.3 and 27.1 ºC for the two sites, while salinity was constant (~35.4) and dimethylsulfide (DMS) was ~0.9 nM across both sites except for the inner reef site for experiment 1, where it was below the detection limit. Likewise, DMSPt was ~15 nM across both sites except for inner reef site for experiment 1 that had 26 nM.

Phytoplankton and bacterial DMSP lyase activity (DLAp and DLAb, respectively) did not show significant differences between sites (F=1.097, P=0.343). Rates of DLAp ranged between 0.04-0.06 µM DMS min^{-1} for both sites and experiments except the inner reef site during experiment 2, had much higher DLAp rates(0.62 µM DMS min^{-1}) , while bacterial DLA rates (DLAb) ranged between 0.01 and 0.23 µM DMS min^{-1} and like DLAp, had the highest rate (0.66 µM DMS min^{-1}) at the inner reef site during experiment 2.

Picoeukaryotes were significantly more abundant inside the reef (1566 and 1416 cells ml^{-1}) than outside the reef, with values of 616 and 716 cells mL^{-1} (F= 14.126, P=0.02). *Synechococcus* abundance was also significantly higher inside the reef (2.65 and 1.73 cells mL^{-1}) than outside the reef (0.2 and 0.93 cells mL^{-1}) (F=36.29, P=0.04). *Prochlorococcus* abundances were not significantly different between sites during experiment 1, but were significantly higher outside the reef during experiment 2 (F=46.377, P=0.002). Heterotrophic bacterial abundances were ~$4x10^6$ cells ml^{-1} for every sample except for the outer reef sample of experiment 2, which had a significantly lower abundance of only $2.37x10^6$ cells ml^{-1} (F=19.056, P=0.002).

Table 3.1. Characteristics of initial water masses. *The sampling depth, temperature, salinity and DMS data from the initial water sampled at both sites and experiments. DMSP lyase activity for the phytoplankton fraction (DLAp) and the bacterial fraction (DLAb) measured as DMS in µM min^{-1}. Microbial abundance (cells mL^{-1}) measured using flow cytometry. Data represent mean ± SD (n=3). Where no SD is shown, data represent single measurements. [a] Represents mean ± SD (n=2), * denote statistical differences at P<0.05 between sites for each experiment.*

	Inner Reef		**Outer Reef**	
	Experiment 1	**Experiment 2**	**Experiment 1**	**Experiment 2**
Depth (m)	4	3.4	5	5
Temperature (°C)	26.3	27.1	26.7	26.5
Salinity (psu)	35.4	35.4	35.4	35.5
DMS (nM)	b.d.l.	0.9	0.86	1
DMSPt (nM)	26.06	17.36	12.34	17.35
DLAp (µM DMS min^{-1})	0.06±0.001[a]	0.62±0.21[a]	0.07±0.003[a]	0.04
DLAb (µM DMS min^{-1})	0.017±0.013[a]	0.66±0.20[a]	0.23±0.060[a]	0.01
Picoeukaryotes (cells ml^{-1})	1566.67± 361.71*	1416.67 ± 57.74*	616.67 ± 246.64*	716.67 ±144.34*
***Synechococcus* (cells $x10^3$ ml^{-1})**	2.65 ± 0.71*	1.73 ± 0.03*	0.200 ± 0.14*	0.93 ± 0.02*
***Prochlorococcus* (cells $x10^5$ ml^{-1})**	6.56 ±2.7	2.11 ± 0.15*	2.29 ± 0.06	5.59 ± 0.53*

Heterotrophic Bacteria (cells x10^6 ml^{-1})	4.12	±0.48	4.72±0.45*	4.73±0.11	2.37±0.22*

3.4.2 Experiment 1: Quantification of DMSP uptake by different fractions of the microbial community

3.4.2.1. Analysis of sulfur compounds: DMSPt, DMSPd, and DMSPp

Inside the reef, total DMSP (Pseudo-F= 27.111, P_{MC}=0.001) and dissolved DMSP (Pseudo-F= 18.047, P_{MC}=0.001) concentrations were significantly different between the controls and DMSP-enriched samples, ensuring a successful enrichment. During 7h of incubation, the concentration of DMSPt decreased significantly from 26.06 to 14.76 nM in the control (t=14.628, P_{MC}=0.001) and from 33.23 to 20.28 nM in the DMSP enriched samples (t= 6,862, P_{MC}=0.003), representing a loss of initial DMSPt of ~43% in both treatments (Figure 3.5A). Initial concentrations of DMSPd were 4.69 and 15.61 nM for control and +DMSP samples, respectively (Figure 3.5B). Even though control samples presented an abnormally high value of 10.88 nM at 4h due to filtration artifacts, mainly leaks of unfiltered water reaching the filtrate their DMSPd concentration at the final time point was only slightly higher (6 nM) than at the outset (t =0). The values for the DMSP-enriched samples remained constant over the first four hours of the experiment, but declined significantly (F= 4,8996, P_{MC}=0.009) to 10.79 nM in the last 2 h (Figure 3.5B). There was no change in DMSPt and DMSPd concentrations over time for the fixed samples.

Particulate DMSP content varied according to size fraction in the inner reef samples, whereby the largest fraction (>8 µm) contained significantly higher DMSPp than the medium fraction (>3 µm), which had higher DMSP content than the prokaryotic / heterotrophic bacterial (<3 µm) fraction (Figure 3.5C). The fractions that contained mostly phototrophic microorganisms (8 and 3 µm), significantly increased their DMSP concentration from 1.69 to 2.44 nM (F=19.849, P=0.008) and from 0.80 to 1.14 nM (F= 18.124, P=0.001) respectively when enriched with DMSP, while there was no effect of DMSP in the smallest fraction. There was no change in DMSPp in fixed cells with enrichment. DMSP lyase activity rates of the phytoplankton (0.06 µM min^{-1}) were three-times higher than those of the bacterial fraction (0.017 µM min^{-1}) (F=108.765, P=0.009) (Figure 3.5D).

Significant differences in total (Pseudo-F= 26.558, P_{MC}=0.001) and dissolved (Pseudo-F= 16.217, P_{MC}=0.001) DMSP concentrations between controls and DMSP-enriched samples occurred for the outer reef site. Total DMSP concentrations from the outer reef declined significantly over time from 12.34 to 8.70 nM in the control (t=3.17, P_{MC}=0.02) and from 22.62 to 17.63 nM in the DMSP-enriched samples (F=4.66, P_{MC}=0.007), representing a 29.5% and 22% loss of DMSPt over 7 h, respectively (Figure 3.5E). Dissolved DMSP, however, remained constant in both the control (~6 nM) and DMSP enriched samples (~16 nM) over the same period (Figure 3.5F). There was no change in DMSPt concentrations in the fixed samples.

In contrast with the inner reef, particulate DMSP was consistent across all three fractions in the absence of enrichment (Figure 3.5G). For the outer reef site, the largest (>8 µm) and smallest (<3 µm) fractions significantly increased their intracellular DMSP from 0.52 to 1.12 nM (F=48.935, P=0.01) and from 0.68 to 1.16 nM (F=10.632, P=0.031) respectively when samples were enriched with DMSP. For fixed cells, DMSPp remained low in all fractions. The total concentrations of DMSPp in the whole samples were 1.66 nM for the control and 2.34 nM for the amended samples. Also different to the inner reef site, the DLAp rates (0.07 µM DMS min^{-1}) from the outer reef were lower than the bacterial fraction, which had rates of 0.23 µM DMS min^{-1} but the differences were not significant (F=12.603, P=0.071) (Figure 3.5H).

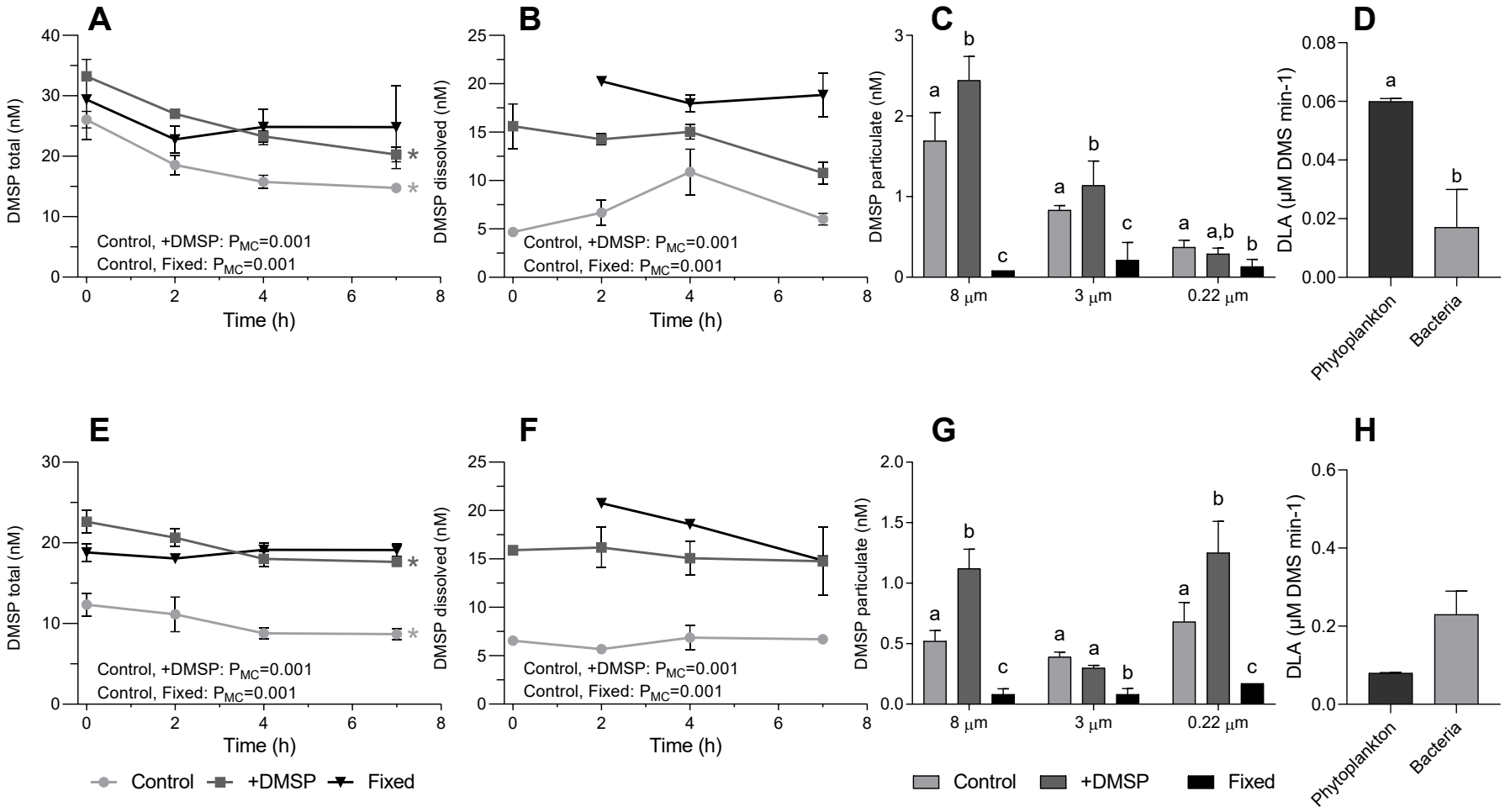

Figure 3. 5. DMSP concentrations and DMSP lyase activity over 7 h during experiment 1. *(A,E) Time course of DMSPt (B,F) Time course of DMSPd (C,G) DMSPp retained in 8 µm, 3 µm and 0.2 µm filters after 7 h and (D,H) DLA rates of initial community. Inner reef site (top) and outer reef site (bottom). Data represent the mean ± standard deviation (n=3). Results from Permanova statistical tests are shown in the top right corner of each plot for treatment factor if P<0.05, and with an asterisk next to the final time point for time factor if initial and final concentrations are different at P<0.05. Lowercase letters above bars denote statistical differences at P<0.05*

3.4.2.2. Relative gene abundance and microbial composition

Of the DMSP catabolising genes tested, the one with the highest relative abundance was DmdA/Dall, which was present in 8.6 ± 2.1 % of bacterial cells for the inner reef site and 6.74 ± 0.47 % of cells from the outer reef site (Figure 3.6A). The common lyase gene DddP was statistically significant more abundant in the inner reef site (0.67 ± 0.04 %) than the outer reef site (F=54.291, P=0.018), where it was present in only 0.36 ± 0.05 % of the total bacterial population (Figure 3.6B). As with DddP, the abundance of the gene DmdA/A1 was statistically significant more abundant in the inner reef (F= 18.97, P=0.049) than the outer reef (Figure 3.6C).

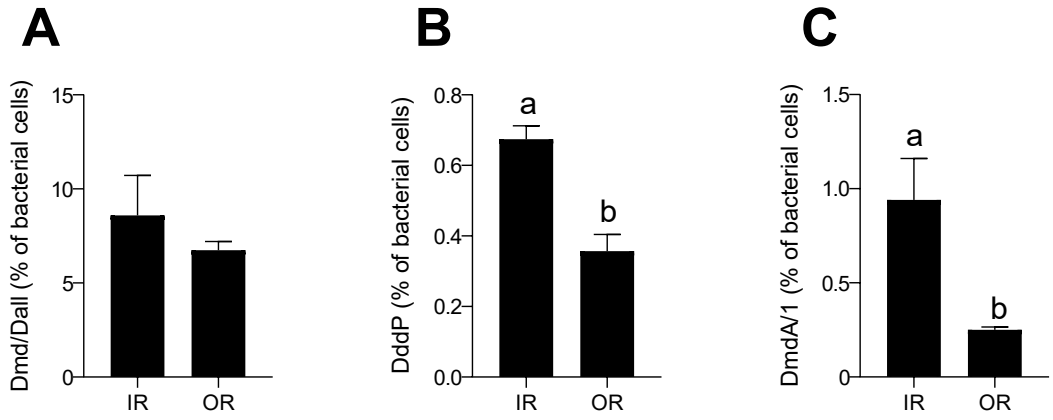

Figure 3. 6. Relative abundance of DMSP degradation genes. *Percentage of (A) DmdA/Dall, (B) DddP (p<0.01) and (C) DmdA/A1 (p<0.01) for initial waters for inside the reef site (IR) and outside the reef site (OR) for experiment 1. Data represent mean +/- SD (n=2). Lower case letters denote significant differences at P<0.05.*

Prokaryotic diversity was similar at both sites with seven different orders present at >1% of relative abundance (Figure 3.7A). Both sites were dominated by *Prochlorococcus* (*Subsection I)* (~36%) and *SAR11* (27.6% and 38%), which together represented more than 60% of the relative abundance for each site. *Flavobacteriales* (12% and 5%), *Rhodobacterales* (~5%), *Oceanospirillales (*~5%*), Acidimicrobiales (*~3%*), and Rickettsiales* (including *SAR116*) *(*~2%*)* were also present. Eukaryotic diversity was similar between experimental sites (Figure 3.7.B) with dinoflagellates as the dominant group and diatoms slightly more abundant for the inner reef site (Figure 3.7.B). Due to multiple copy numbers of 18S in some of the eukaryotes present in our samples, a representation of the relative abundance of the microorganisms conforming the eukaryotic community was not possible. Instead, a phylogenetic tree was constructed indicating the phytoplankton genera present in the samples (Figure 3.8 & 3.9).

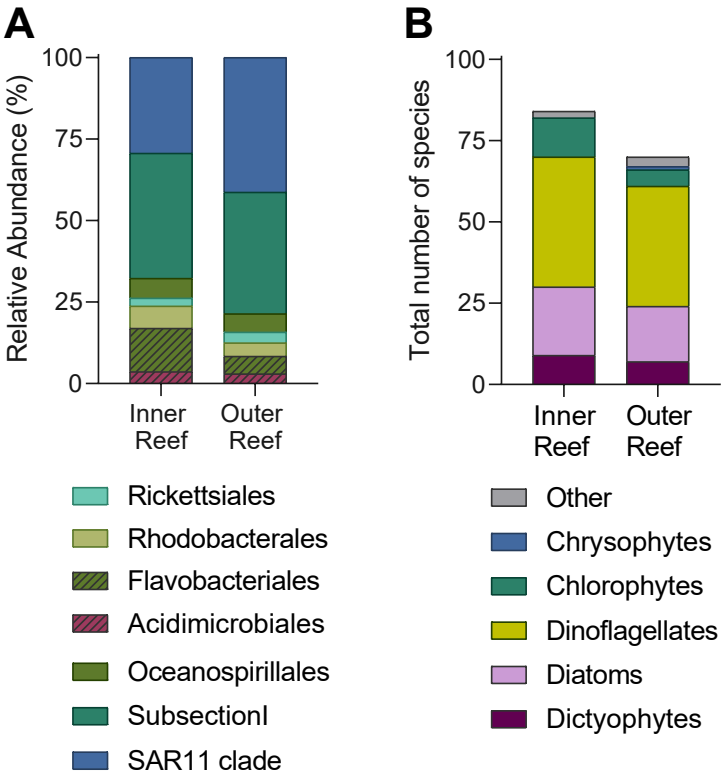

Figure 3. 7. Bacterial and phytoplankton composition. *Community (16S and 18S) composition of initial waters for both sites for experiment 1. (A) Bacterial community at the 4thtaxonomic rank using relative abundance and. The graphs only display results with >1% of relative abundance for clarity purposes. (B) Total number of species of phytoplankton present at both sites split in major groups.*

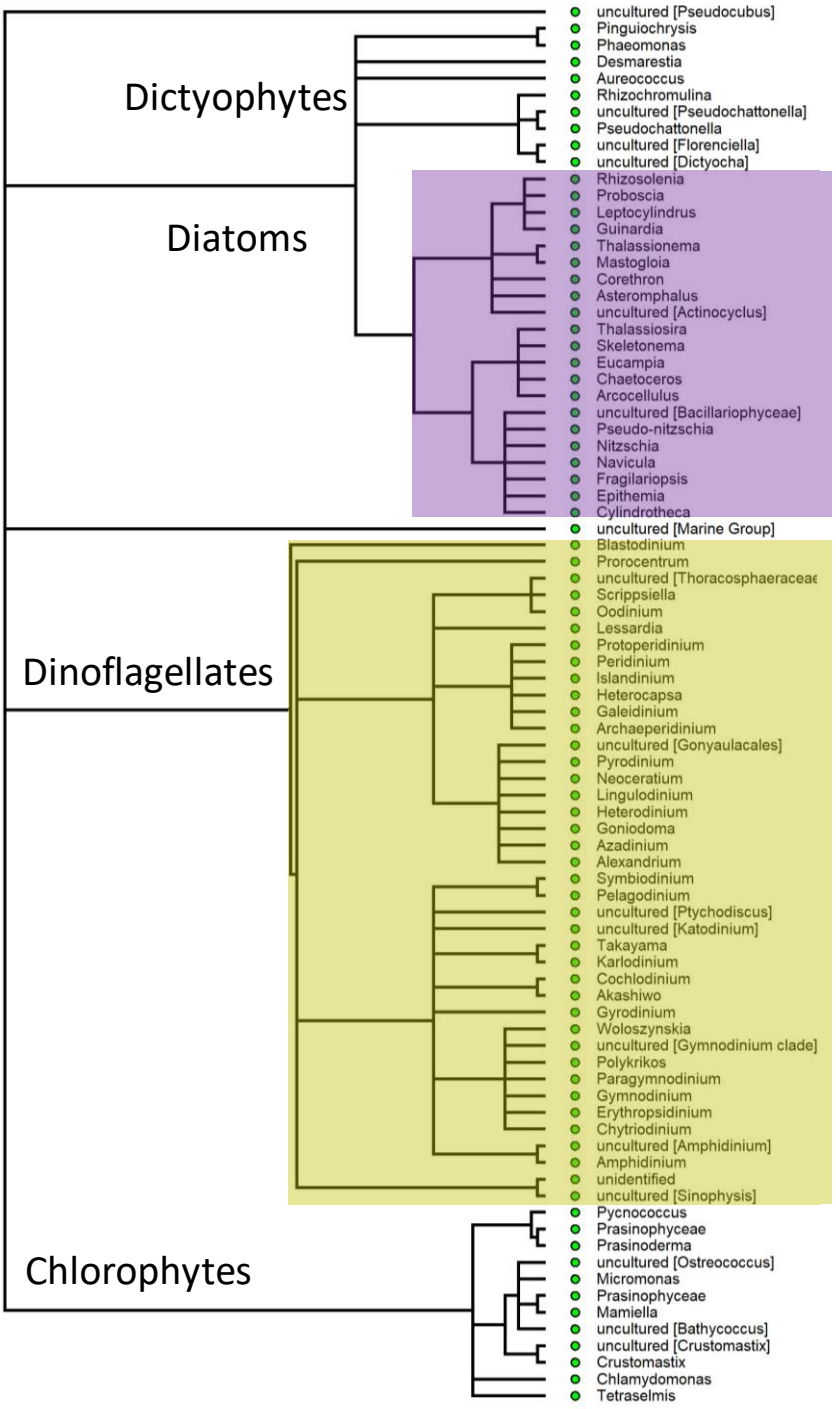

***Figure 3.8. P*hylogenetic tree of eukaryotic (18S) community for the inner reef site.** Potential DMSP sinks are highlighted in purple and likely DMSP producers in yellow. Green dots indicate genus was present at the initial time.

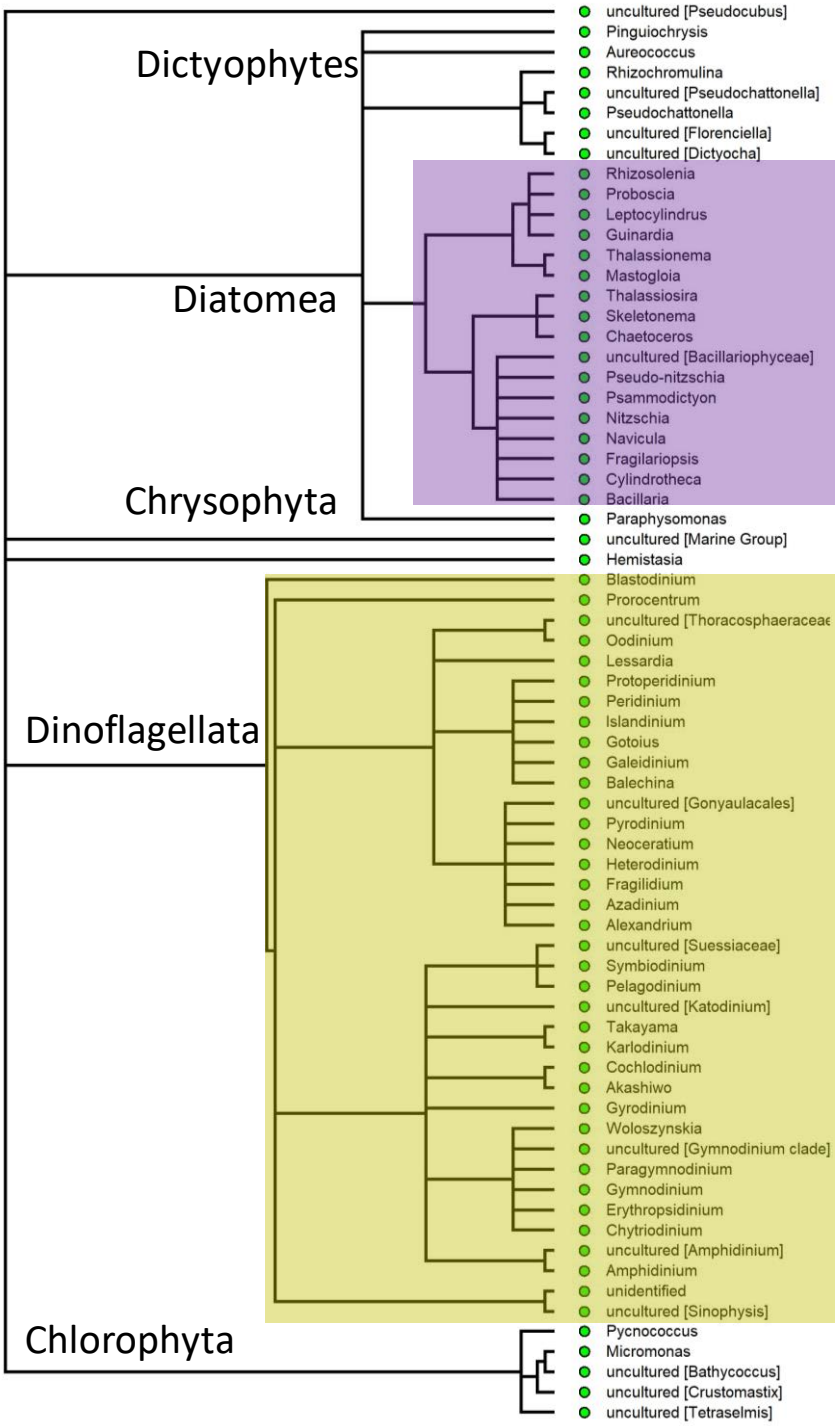

***Figure 3. 9. P*hylogenetic tree of eukaryotic (18S) community for the outer reef site.** Potential DMSP sinks are highlighted in purple and potential DMSP producers in yellow. Green dots indicate genus was present at the initial time.

3.4.3. Experiment 2: Dynamic changes in microbial community composition and DMSP catabolising gene abundance

3.4.3.1. Analysis of sulfur compounds: DMSPt, DMSPd and DMSPp

In order to evaluate the effect of DMSP-enrichment on DMSP catabolising gene abundance and microbial taxa in natural sea waters of the GBR, we incubated water samples with trace levels of DMSP (final concentration 10 nM). For the inner reef site, significant differences between controls and DMSP-enriched samples occurred for DMSPt (Pseudo-F=5.4189, P_{MC}=0.016) and DMSPd (Pseudo-F=6.37, P_{MC}=0.016). DMSPt declined significantly from 17.36 to 10.64 nM in the control (t=12.15, P_{MC}=0.006) and 25.72 to 13.54 nM in the DMSP-enriched incubations (t=9.71, P_{MC}=0.002). The greatest decline in DMSP concentration occurred within the first 24 h, with a disappearance rate of 5.04 nM day^{-1} for the controls and 12.24 nM day^{-1} for DMSP-enriched samples, equating to almost half of the total DMSP (Figure 3.10A). Concentrations of DMSPt remained constant for the remaining four days.

For control samples, DMSPd concentrations did not change over time and remained constant at ~2 nM. However, DMSP-enriched samples showed a rapid and significant decline in the first 24 h (t=5.14, P_{MC}=0.001), followed by a steady, slow continuous decrease over the remaining 96 h (Figure 3.10B). For the +DMSP samples, a total of 80 % of initial DMSPd was lost over the 120 h, with a loss rate of 8.64 nM day^{-1} for the first 24 h and 0.47 nM day^{-1} for the next 96 h. Particulate DMSP, calculated by subtracting DMSPd from DMSPt (Figure 3.10C) remained constant at ~12 nM for the DMSP-enriched samples, while declining significantly from 14 nM to 9 nM in the controls (F=18.521, P_{MC}=0.043) with a loss rate of 1.07 nM day^{-1}. Phytoplankton DLA was 0.62 µM DMS min^{-1}, and bacterial DLA rates were similar at 0.66 µM DMS min^{-1} (Figure 3.10D).

Outside the reef, significant differences between treatments occurred for DMSPt (Pseudo-F=3.89, P_{MC}=0.003), which declined significantly from 17.35 to 7.08 nM in the control (t=6.33, P_{MC}=0.006) and from 33.38 to 6.9 nM in the DMSP-enriched samples (t= 10.27, P_{MC}=0.002) (Figure 3.10E). As with the inner reef, the greatest decline happened during the first 24 h at a loss rate of 9.36 nM day^{-1} for the controls and 19.92 nM day^{-1} for DMSP enriched samples. A similar pattern was detected for DMSPd, which declined from 9.33 to 2.50 (t=2.38, P_{MC}=0.001) and from 22.91 to 1.94 nM (t=9.81, P_{MC}=0.001) for controls and DMSP enriched samples, respectively (Figure 3.10F). As per DMSPt, the highest loss rate happened during

the first 24 h with 7.736 nM day^{-1} (controls) and 18.557 nM day^{-1} (+DMSP). For control samples, this meant 6.8 nM DMSPd was lost in 120 h equating to approximately 73 % loss of the initial DMSPd, whereas for DMSP-amended samples, it equated to a total loss of >20 nM DMSPd in 120 h and ~91 % loss of initial DMSPd. The calculated DMSPp (Figure 3.10G) remained constant at ~6 nM for the controls and significantly diminish from 10.463 nM to 4.94 nM for the DMSP-amended samples ($F=0.32$, $P_{MC}=0.001$) at a rate of 1.10 nM day^{-1}. In contrast to the inner reef site, phytoplankton lyase activity (0.04 µM DMS min^{-1}) was four times higher than bacterial DLA (0.011 µM DMS min^{-1}) (Figure 3.10H).

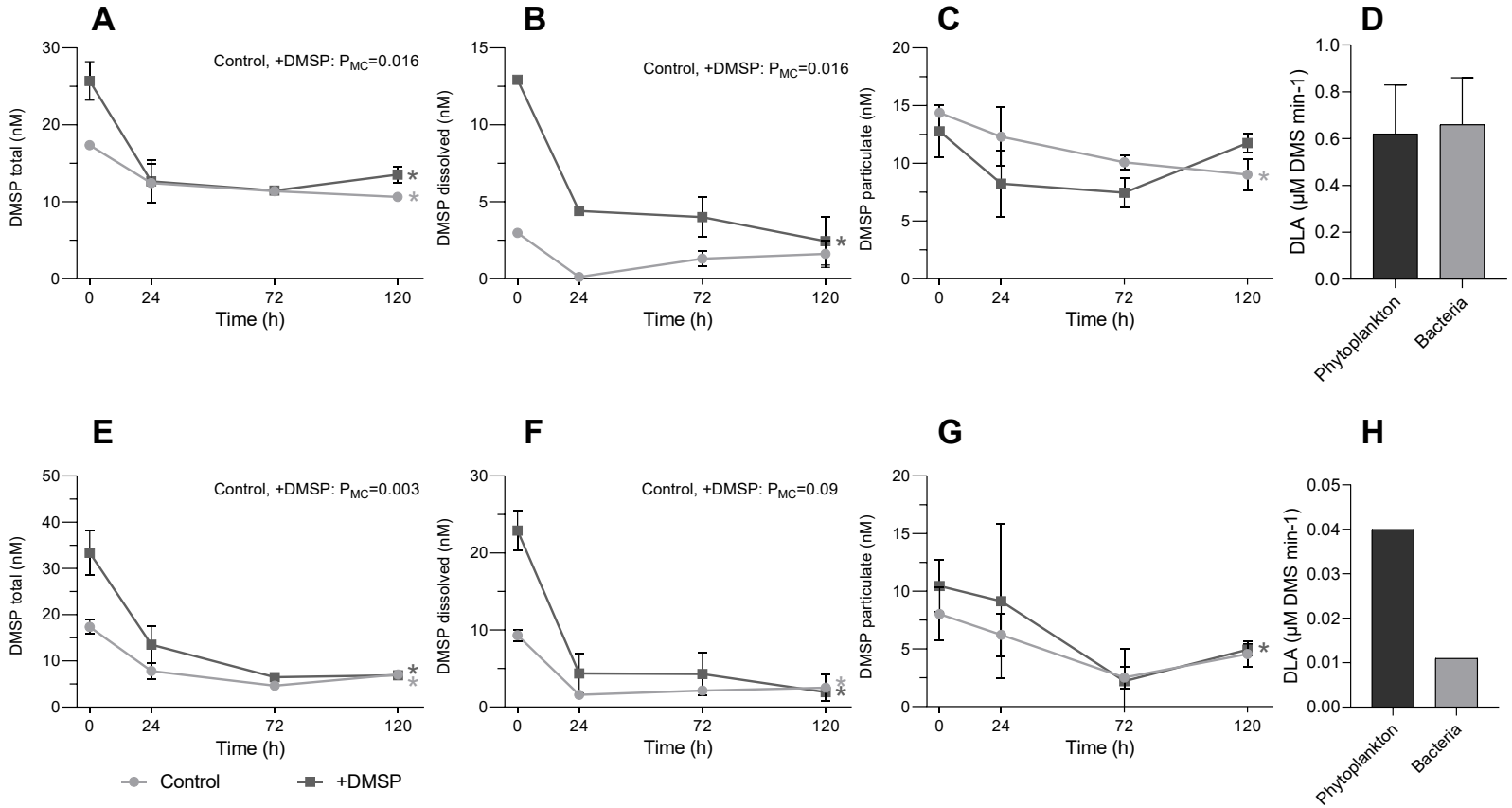

Figure 3. 10. DMSP concentrations and DMSP lyase activity during experiment 2. *(A,E) Time course of DMSPt (B,F) Time course of DMSPd (C,G) Time course of DMSPp. Inner reef site (top) and outer reef site (bottom). Data represent mean ± standard deviation (n=3). H One replicate only. Results from Permanova statistical tests are shown in the top right corner of each plot for treatment factor if P<0.05, and with an asterisk next to the final time point for time factor if initial and final concentrations are different at P<0.05.*

3.4.3.2 Gene regulation/abundance

Inside the reef, DMSP degrading genes DmdA/A1 (Pseudo-F=10.82, P_{MC}=0.02) and DmdA/Dall (Pseudo-F=5.09, P_{MC}=0.014) were significantly higher for control samples compared to DMSP-enriched samples. The demethylation gene DmdA/A1 while low, significantly increased its abundance from 0.61% to 1.7% (t=9.39, P_{MC}=0.003) for controls and to 1.2% (F=6.44, P_{MC}=0.003) for DMSP-enriched samples (Figure 3.11A). The cleavage gene DddP increased significantly from 0.41 % to 1.1 % (t=3.87, P_{MC}=0.013) in controls and to 0.9 % (t=7.46, P_{MC}=0.036) for DMSP-enriched samples (Figure 3.11B). The demethylation gene DmdA/Dall occurred in highest relative abundance (Figure 3.11C); initially present in ~4% of bacterial cells, but significantly increasing to 12% (t=1.26, P_{MC}=0.002) in controls and 10 % (t=25.89, P_{MC}=0.002) in DMSP enriched samples over 120 h.

In the outside reef samples, a different pattern was observed, with overall significantly higher relative abundance of all three genes in the DMSP-enriched samples (DmdA/A1 (Pseudo-F=8.77, P_{MC}=0.001), DddP (Pseudo-F=0.55, P_{MC}=0.009) and DmdA/Dall (Pseudo-F=13.26, P_{MC}=0.001) (Figure 3.11). The DmdA/A1 gene decreased significantly from 0.34% to 0.061% (t=6.72, P_{MC}=0.003) in controls and to 0.17% (t=3.79, P_{MC}=0.002) in DMSP-enriched samples (Figure 3.11D). Similarly, DddP decreased significantly from 0.24% to 0.04% (t=1.53, P_{MC}=0.001) for controls and to 0.07% (t=7.28, P_{MC}=0.006) for amended samples (Figure 3.11.E). The gene DmA/Dall increased from 5.1% to 5.8% (t=3.88, P_{MC}=0.029) for controls and 8.2% (t=2.04, P_{MC}=0.011) for DMSP-enriched samples (Figure 3.11F).

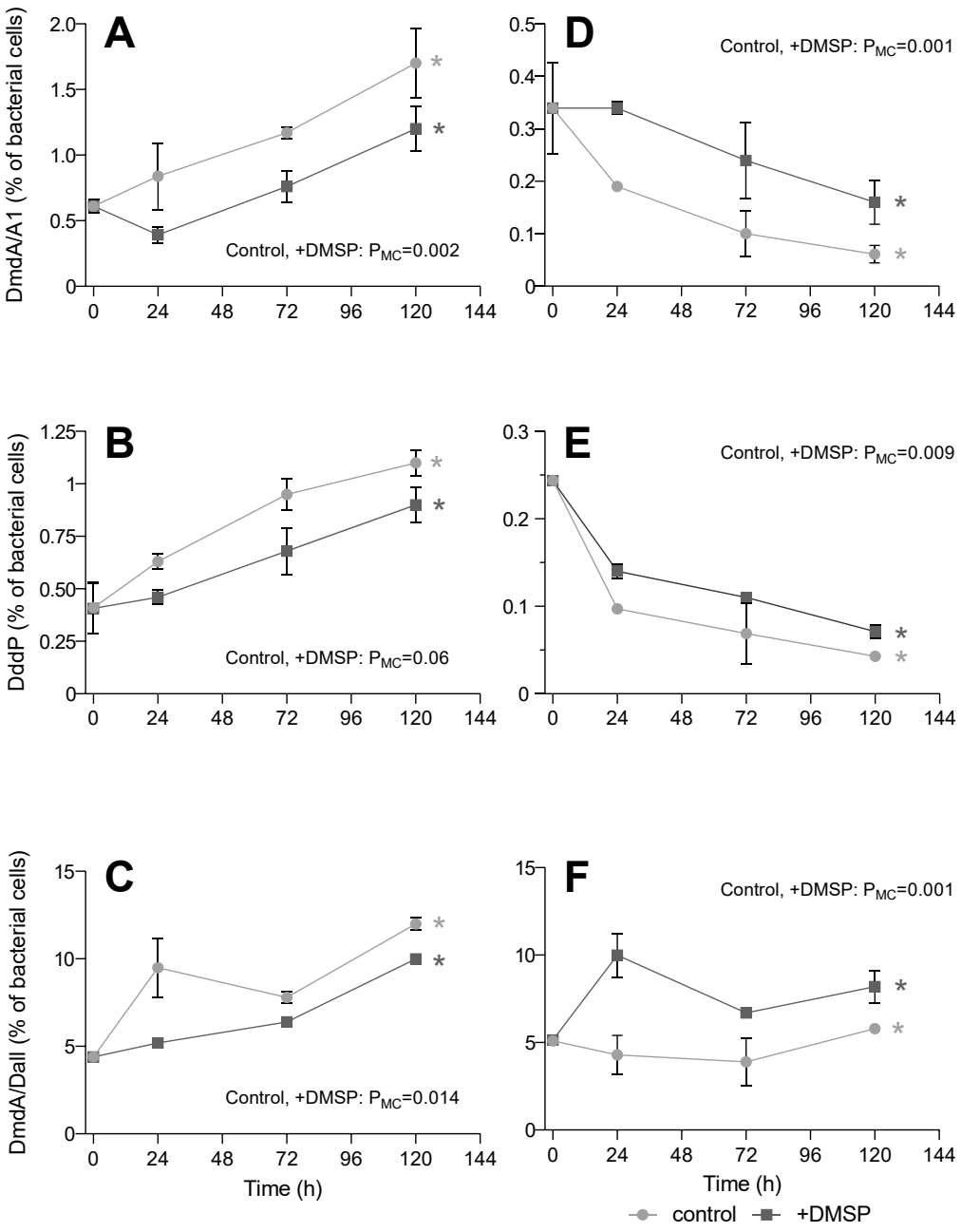

Figure 3. 11. Time course of the relative abundance of DMSP degrading genes from both experimental sites. *(A) DmdA/A1 inside the reef, (B) DddP inside the reef, (C) DmdA/DalI inside the reef, (D) DmdA/A1 outside the reef, (E) DddP outside the reef and (F) DmdA/DalI outside the reef. Error bars indicate ± standard deviation and not shown when smaller than symbol. Results from Permanova statistical tests are shown for each plot and with an asterisk next to the final time point for time factor if initial and final concentrations are different at P<0.05.*

3.4.3.3. The influence of DMSP on 16S and 18S diversity

The bacterial community inhabiting the inner reef site was dominated by *Prochlorococcus* (*Subsection I)* (39%) and SAR11 clade (34%) (Figure 3.12A). Other bacteria at >1% relative abundance at the 4th order level included *Acidimicrobiales* (2%), *Flavobacteriales* (8%), *Rhodobacterales* (5%), *Rickettsiales* (2%) and *Oceanospirillales* (4.5%). After 24 h, the bacterial assemblage changed from the initial community composition but there were no significant differences between treatments. The initially dominant groups *Subsection I* and *SAR 11* declined in relative abundance to <1%, while overall diversity increased with the appearance of *Synechococcales* as a major group (40% and 44%), along with unclassified *Alphaproteobacteria (4.5%), Kiloniellales (~2%),* and *Alteromonadales (~2%).* The relative abundance increased for *Rickettsiales* (~18%) (F=0.21, $P(control)_{MC}$=0.025, t=0.81, $P(DMSP)_{MC}$=0.002) while in the case of the control samples, *Flavobacteriales* decreased to 4.5% (t=109, P_{MC}=0.022). After 72 h, there were only small variations in bacterial composition, with the addition of *Myxococcales* (~2%) in both treatments and *Stramenopiles* in DMSP-enriched samples (1.2%). *Flavobacteriales* increased for control samples (11%) (t=4.90, P_{MC}=0.044) and *Oceanospirillales* for DMSP-enriched samples (5.5%) (t=3.41, P_{MC}=0.024), while *Synechococcales* diminished for both treatments (23%, 26%) (t=17.35, $P(control)_{MC}$=0.002 and t=0.82, $P(DMSP)_{MC}$=0.035). After 120 h, there were differences between the treatments (t=12.47, P_{MC}=0.012), where the control samples had a greater relative abundance of *Rhodobacterales* (50% vs 8%) (t=2.70, P_{MC}=0.004) and contained *Sphingomonadales* for the first time at >1%, while treated samples were dominated by *Rickettsiales* (35% vs 18%) and *Synechococcales* (24% vs 6%) (t=1.70, $P(DMSP)_{MC}$=0.018). Similarly, *unclassified Alphaproteobacteria* (8% and 3%) (t=0.67, P_{MC}=0.033) were in higher abundance in the DMSP-enriched samples. The nMDS plots supported these differences, illustrating a clear separation of bacterial composition between initial waters, the control samples at 120 h and the remaining samples (Figure 3.12B). This clustering was principally driven by the dominant bacteria within these samples for initial waters (*SAR 11* clade and *Subsection I),* and for the controls at 120 h, was driven by *Rhodobacterales (*dominant group*), Alteromonadales and Sphingomonadales.* To improve clarity of separation, the nMDS was then plotted without the initial water samples and the controls at 120 h (creating a nMDS subset), it became evident that the samples were grouped by time with treatments within each time plotted together (Figure 3.12C).

For the outer reef site, the prokaryotic community structure closely resembled that of the initial inner reef waters (Figure 3.12D), where it was dominated by *SAR11* clade (39 %) and *Prochlorococcus (Subsection I)* (32 %), with the same bacterial orders present at >1 % relative abundance: *Acidimicrobiales* (5 %), *Flavobacteriales* (6 %), *Rhodobacterales* (4 %), *Rickettsiales* (3 %) and *Oceanospirillales* (5 %) (Figure 3.12.D). As with the inner reef, the outer reef community also underwent the greatest change in community composition within the first 24 h (t=3.09, P_{MC}=0.031), where *SAR11* and *Subsection I* declined to <1% relative abundance. However, the enrichment of DMSP seemed to have a greater effect on the community structure of the outer reef, where after 24 h, the controls were equally dominated by *Rhodobacterales* (26 %), *Rickettsiales* (28 %) and *Synechococcales* (23 %), while DMSP-enriched communities were dominated by *Rhodobacterales* (54 %) *with Rickettsiales* at 16 % and *Synechococcales at 18 %.* Other orders present at >1% relative abundance in both treatment of the outer reef waters at this time were *other Alphaproteobacteria* at ~4 %, *Sphingomonadales* at 2% (controls) and 1% (DMSP), *Alteromonadales 5 %* (controls) and 2 % (DMSP) and *Oceanospirillales* at 6% (controls) and 2 % (+DMSP). *Flavobacteriales were only present in the control samples (2 %).* After 72 h, treatments were more abundant in *Alteromonadales* (1.08% vs. 3.2%) (t=13.93, P_{MC}=0.028). *Acidimicrobiales* (7 %) were only present in control samples while *Sphingomonadales* were present only in DMSP-enriched samples at 1.4% (Figure 3.12.B). At the final time point (120 h), treatments were significantly different (t=4.15, P_{MC}=0.008) and DMSP-enriched samples were richer than controls in other *alphaproteobacteria* (4.5% vs 3%) (Pseudo-F=11.19, P_{MC}=0.045) and *Acidimicrobiales* (1.4%), while *Flavobacteriales* (10.7%) were only present in DMSP-enriched samples. The nMDS plots separated the initial water samples from the remaining samples by the dominant bacteria (*SAR 11* clade and *Subsection I)* (Figure 3.12D). While the nMDS subset excluding the initial water samples grouped the samples by time, and only at the final time (120 h), treatments grouped separately. Controls at 120 h were characterised by *Sphingomonadales* while +DMSP samples were characterised by *Synechococcales* (Figure 3.12F).

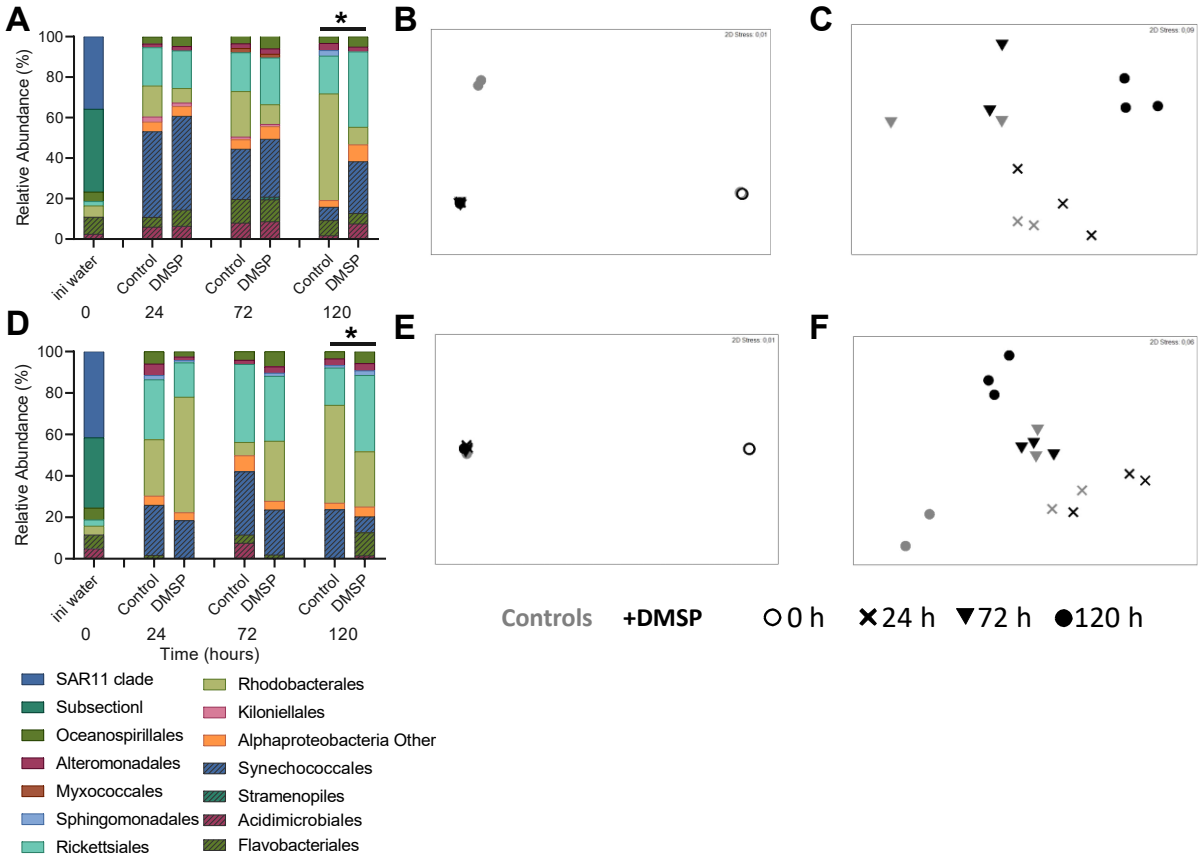

Figure 3.12. Prokaryotic (16S) composition and nMDS plots for each experimental site. *(A) Bacterial community at the 4th order level (B) nMDS plot with all replicates and samples (C) nMDS subset (D) Bacterial community at the 4th order level (E) nMDS plot with all replicates and samples and (F) nMDS subset. Inner reef site (top) and outer reef site (bottom). Bacterial community graphs only display results with >1% of relative abundance for clarity purposes. nMDS plots are based on lower triangular resemblance calculated with S17 Bray-Curtis similarity matrices. * denote statistical differences at P<0.05 between treatments for each time point.*

The initial eukaryotic community in the inner site consisted of eight genera of diatoms, 37 dinoflagellates and 23 belonging to other groups (Figure 3.13.A). The biodiversity of both treatments diminished over time with proportional decrease for each group to a final number of genera of no diatoms for control samples and six for +DMSP samples, 23 and 21 dinoflagellates and, 9 and 16 other genera for control and +DMSP samples respectively. Dinoflagellate diversity clearly dominated the final community in control samples while +DMSP samples sustained greater taxonomic diversity, suggesting that DMSP may play a relevant role for non-DMSP producing species. A nMDS plot of all replicates for both treatments and all time points, grouped the control samples at 144 h apart from the rest (Global R: 0,585, P=0.002) (Figure 3.13.B) confirming an effect of DMSP on eukaryotic composition. As per experiment 1, a phylogenetic tree showing presence and absence of phytoplankton genera was preferred over relative abundance to display the eukaryotic microbial composition for both treatments over time (Figure 3.13 C and D). After 24 h, control samples were more biodiverse than +DMSP samples due to a higher number of dinoflagellates, but the diatoms *Eucampia* and *Nitzschia* were only present in the +DMSP samples. After 48 h, control samples harboured higher number of dinoflagellates and other groups but had the same number of diatoms than +DMSP samples. However, *Leptocylindrus* was only present in +DMSP samples while *Thalassiossira* was only present in the controls. At the last time point of 120 h, +DMSP samples had greater overall diversity and harboured the diatoms *Skeletonema*, *Rhizosolenia*, *Leptocylindrus*, *Eucampia*, *Chaetoceros* and *Nitzschia*. Other groups that were only present in +DMSP samples at any time point were *Ceratoperidinium*, MAST-10, MAST-4E, MAST-9C, Dino-Group-II-Clade-28 and *Halosphaera*.

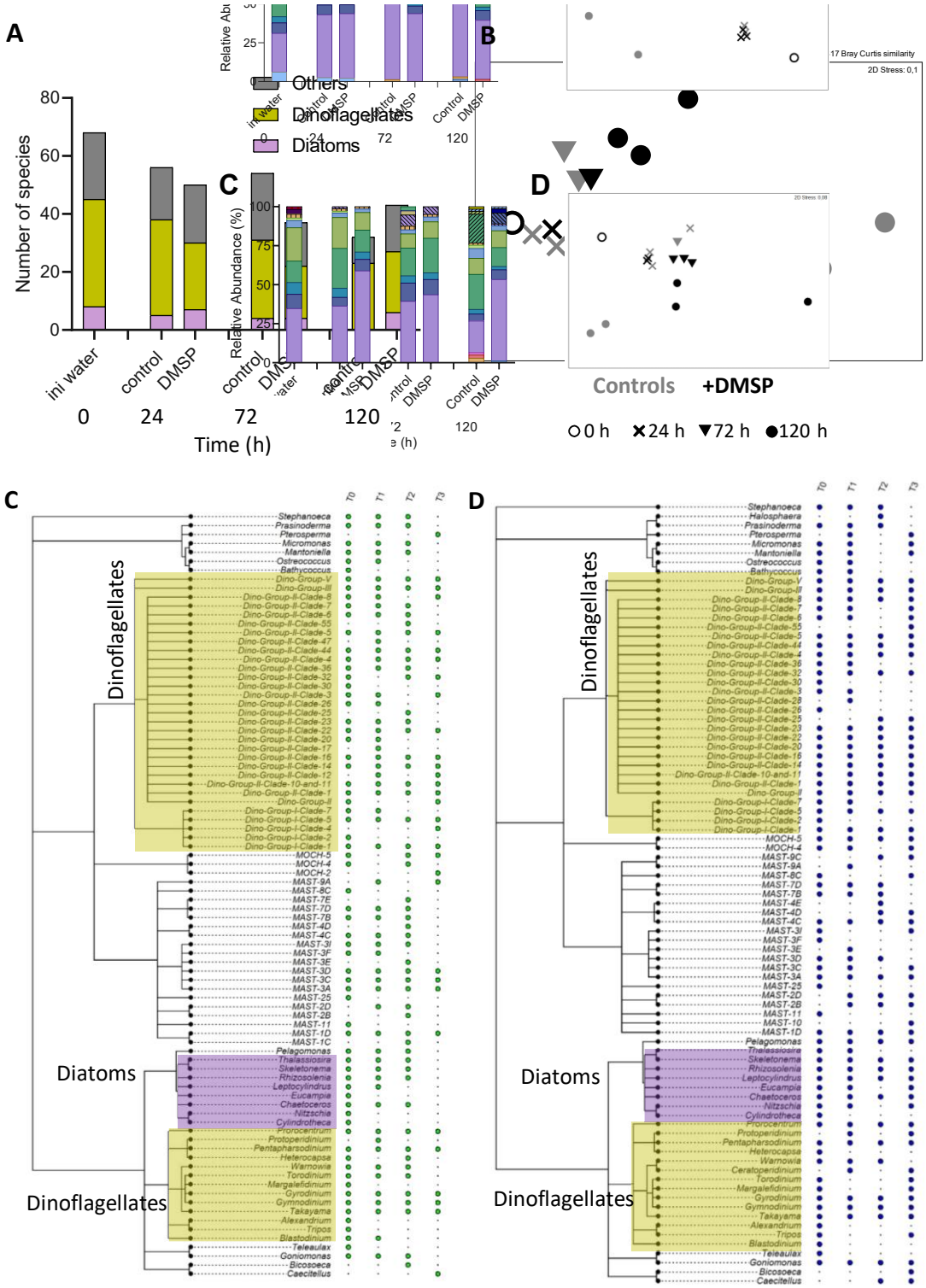

Figure 3.13. Eukaryotic biodiversity, nMDS subset plot and phylogenetic trees for eukaryotic (18S) composition during experiment 2 for the inner reef site. *(A)Eukaryotic biodiversity for initial water, control and treated samples during experiment 2, (B) nMDS subset plot for all time point for 18S data, (C) phylogenetic tree of presence (green circles)/ absence (dots) of eukaryotic genera for control samples for each time point T0 (0h), T1(24h), T2(72h), T3(120h), and (D) phylogenetic tree of presence (blue circles)/ absence (dots) of eukaryotic genera for +DMSP samples for each time point T0 (0h), T1(24h), T2(72h), T3(120h).*

The initial eukaryotic community in the outer site consisted of 12 genera of diatoms, 27 dinoflagellates and 23 belonging to other groups (Figure 3.14.A). The biodiversity of both treatments diminished over time, and +DMSP samples were slightly more diverse than controls except for the last time point when controls were highly abundant harbouring 63 different taxa. Dinoflagellate diversity dominated in both treatments over the course of the study except for the control samples at 120 h, when dinoflagellates, diatoms and other groups were similarly diverse. Unlike the inner reef, it is not clear that DMSP supposed an ecological advantage to any group of phytoplankton. A nMDS plot of all replicates for both treatments and all time points, did not differentiate the samples except for the control samples at 144 h that were closer to the initial samples (Global R: 0,604, P=0.001) (Figure 3.14.B). The phylogenetic tree showed the differences in biodiversity between both treatments in more detail (Figure 3.14 C and D). After 24 h, +DMSP samples were more biodiverse than controls due to a higher number of dinoflagellates and unlike the inner reef, had less taxa of diatoms with *Guinardia* present only in +DMSP samples and *Skeletonema*, *Epithemia* and *Proboscia* only in controls. After 48 h, +DMSP samples harboured more number of dinoflagellates, diatoms and other groups, and the diatoms *Nitzschia* and *Mastoglia* were only present in the +DMSP samples. At the last time point of 120 h, control samples were more biodiverse and harboured the diatoms *Rhizosolenia*, *Leptocylindrus*, *Proboscia*, *Dactyliosolen*, *Navicula*, *Eucampia*, *Arcocellulus* and *Psammodictyon*. Other groups that were only present in +DMSP samples at any time point were *Haplozoon, Ceratocorys, Pyrodinium, Scrippsiella*.

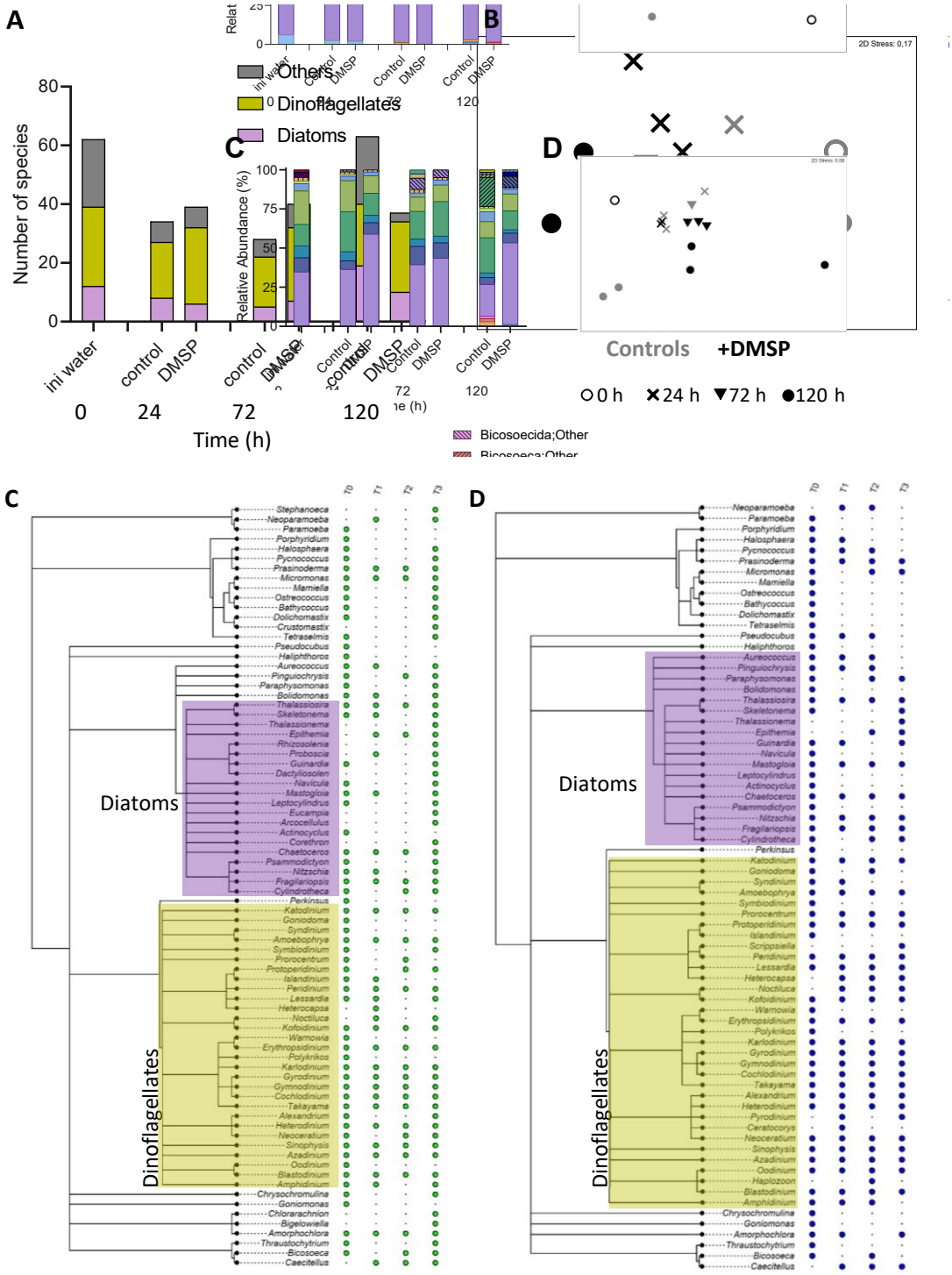

Figure 3.14. Eukaryotic biodiversity, nMDS subset plot and phylogenetic trees for eukaryotic (18S) composition during experiment 2 for the outer reef site. *(A)Eukaryotic biodiversity for initial water, control and treated samples during experiment 2, (B) nMDS subset plot for all time point for 18S data, (C) phylogenetic tree of presence (green circles)/ absence (dots) of eukaryotic genera for control samples for each time point T0 (0h), T1(24h), T2(72h), T3(120h), and (D) phylogenetic tree of presence (blue circles)/ absence (dots) of eukaryotic genera for +DMSP samples for each time point T0 (0h), T1(24h), T2(72h), T3(120h*

In order to explain 70 % of the treatment dissimilarity at the final time point (120 h), 6 and 5 bacterial taxa, and more than 20 phytoplankton taxa were used (Table 3.2). The four greatest contributors to bacterial composition dissimilarity were common to both sites (*Rhodobacterales, Synechococcales, Sphingomonadales, Acidimicrobiales*), and together contributed to explain more than 50 % of the treatments dissimilarity. Moreover, these bacterial groups were also identified in the nMDS as drivers for treatment characterisation at 120 h (Table 3.2 A). Dinoflagellates contributed more than diatoms to treatment dissimilarity (Figure 3.2.B) but only one dinoflagellate, *Pelagodinium*, was amogst the four greatest contributors.

Table 3. 2. Major microbial groups and its contribution to treatment dissimilarity at the final time point (120h). *(A) Bacterial groups for each site (B) Phytoplankton groups. High contributors were selected from the treatment pair-wise comparisons at the final time point (120h) to explain a minimum of 70% of treatment dissimilarity. Yellow colour indicates dinoflagellate, Purple color indicates diatoms. Contrib% is the contribution of each group. Cum.% is the accumulative contribution. Dissimilarity between treatments is indicated in the first row for each site.*

A

Inner reef (dissimilarity = 11.83)			Outer Reef (dissimilarity = 15.07)		
Bacteria	**Contrib%**	**Cum.%**	**Bacteria**	**Contrib%**	**Cum.%**
Rhodobacterales	20,01	20,01	**Rhodobacterales**	19,71	19,71
Synechococcales	13,61	33,62	**Synechococcales**	19,58	39,29
Sphingomonadales	12,09	45,71	**Acidimicrobiales**	15,50	54,79
Acidimicrobiales	10,63	56,35	**Sphingomonadales**	12,80	67,59
Rickettsiales	8,94	65,29	**Flavobacteriales**	11,35	78,95
Kiloniellales	7,88	73,18			

B

(dissimilarity = 33.37)	Control	DMSP				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
uncultured [Ebria]	0,38	0,13	1,37	0,96	4,1	4,1
Chytriodinium	0,54	0,63	1,34	0,84	4,02	8,13
Pelagodinium	0,62	0,63	1,29	0,85	3,87	11,99
Cryothecomonas	0,38	0,75	1,2	0,82	3,6	15,59
Cochlodinium	0,31	0,5	1,19	0,89	3,56	19,16
Blastodinium	0,62	0,5	1,09	0,77	3,25	22,41
Erythropsidinium	0,69	0,75	1,08	0,77	3,22	25,63
Micromonas	0,15	0,5	1,07	0,88	3,21	28,84
Cylindrotheca	0,46	0,75	1,03	0,76	3,09	31,93
Scrippsiella	0,85	0,75	1,01	0,61	3,04	34,96
Paraphysomonas	0,85	0,75	1,01	0,61	3,04	38
uncultured [Mediophyceae]	0,92	0,75	1,01	0,61	3,04	41,03
uncultured [Sinophysis]	1	0,75	0,99	0,55	2,98	44,01
Karlodinium	1	0,75	0,99	0,55	2,98	46,98
Protoperidinium	0,85	0,75	0,99	0,55	2,98	49,96
Corethron	1	0,75	0,99	0,55	2,98	52,93
Islandinium	0,85	0,88	0,95	0,54	2,85	55,78
Chaetoceros	0,92	0,88	0,95	0,54	2,85	58,63
Thalassiosira	0,85	0,88	0,92	0,58	2,77	61,4
Eucampia	0,46	0,25	0,9	0,64	2,71	64,1
Navicula	0,38	0,38	0,77	0,58	2,31	66,41
uncultured [Katodinium]	0,23	0,13	0,77	0,67	2,3	68,71
Asteromphalus	0,23	0,38	0,76	0,74	2,28	70,99

3.4.3.4. Correlation analysis and Principal Component Analysis

In the inner reef, three bacteria groups were significantly correlated with at least one DMSP catabolising gene (Figure 3.15 A-D). *Synechococcales* were negatively correlated with DmdA/A1 (r=-0.8928) (Figure 3.15.A) and DddP (r=-0.9009) genes (Figure 3.15.B), while *Rhodobacterales* were positively correlated with DmdA/A1 (r=0.8072) (Figure 3.15.C) and DddP (r=0.7003) (Figure 3.15.D). In the outer reef site, other *Alphaproteobacteria* were negatively correlated with DmdA/A1 (r=0.8098) (Figure 3.15.E) and *Rickettsiales* with DddP (r=-0.8231) (Figure 3.15.F).

Principal Component Ordination on the effect of DMSP on bacterial community composition for the inner reef site, revealed the variation (PC1 87.1 %) of the samples was mostly driven by the bacterial composition of the initial waters (Figure 3.16A). The second principal component (PC2) explained very little of the variation (7.5 %). Together, these data show the total change was driven by the bacterial composition of the controls at the final time point (120 h) (Figure 3.16A). The overlaying vectors showed negative correlations between DmdA/A1 and DddP with PC1 and positive correlation with PC2.

In the outer reef site, PC1 explained 86.7% of the variation of the samples and like in the inner reef site, this variability was driven by the difference in the bacterial composition of the initial waters (Figure 3.16C). There was only minimal effect along PC2 (6.6% variation). Excluding the large variability driven by the differences in the initial community composition, the majority of the variability is explained by the control samples at 24 h and 120 h (Figure 3.16B). The overlaying vectors showed a positive correlation of PC1 with DmdA/A1 and DddP, and a negative correlation with DmdA/Dall. There was an even correlation of each gene with PC2.

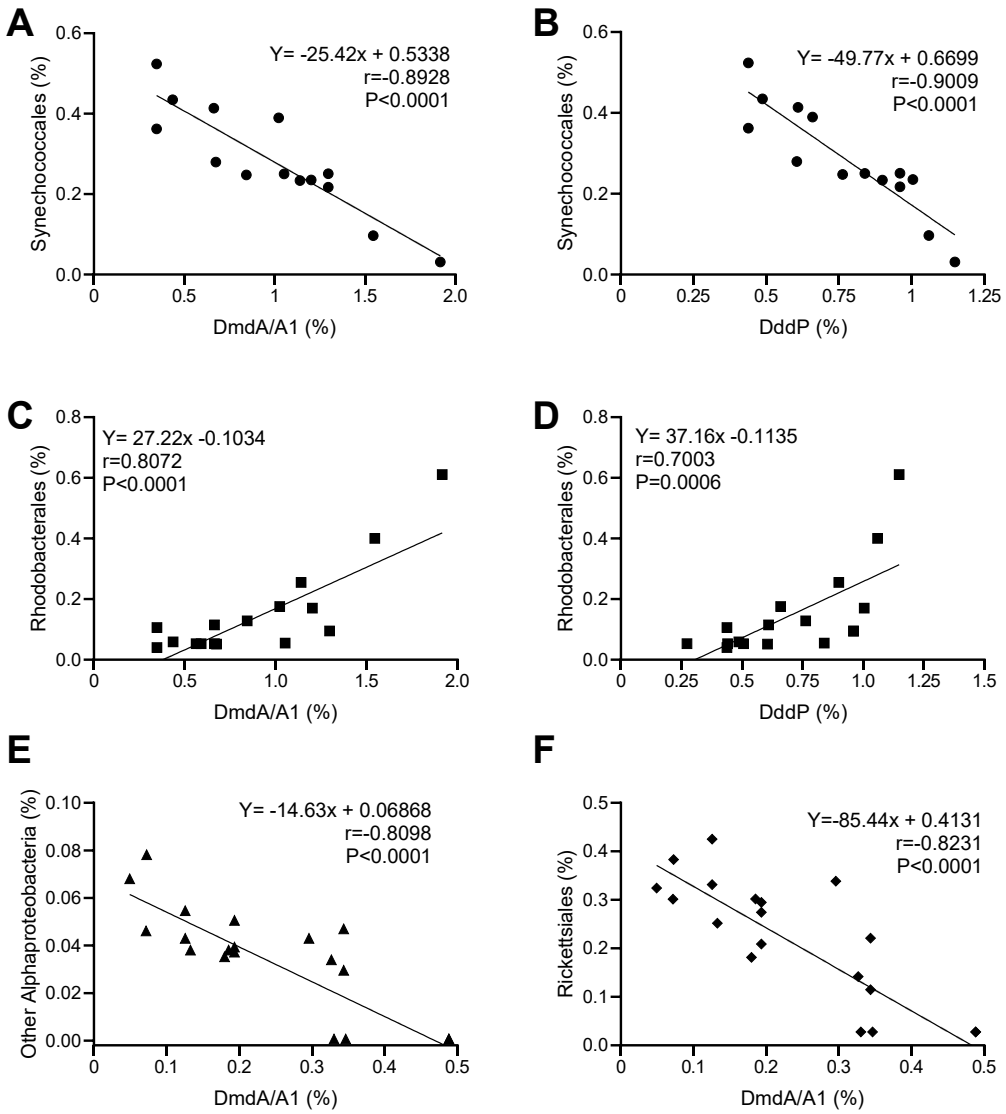

Figure 3.15. Correlations of the relative abundance of DMSP-degrading genes and bacterial abundances for both sites. *Only significant correlations (P<0.05) with a strong Pearson's correlation coefficient (r >0.7) are shown. (A) the relative abundance of dmdA/A1 and Synechococcales for the inner site, (B) the relative abundance of DddP and Synechococcales for the inner site, (C) the relative abundance of dmdA/A1 and Rhodobacterales for the inner site, (D) the relative abundance of DddP and Rhodobacterales for the inner site, (E) the relative abundance of dmdA/A1 and other Alphaproteobacteria for the outer site and (F) the relative abundance of Rickettsiales and DmdA/A1 for the outer site.*

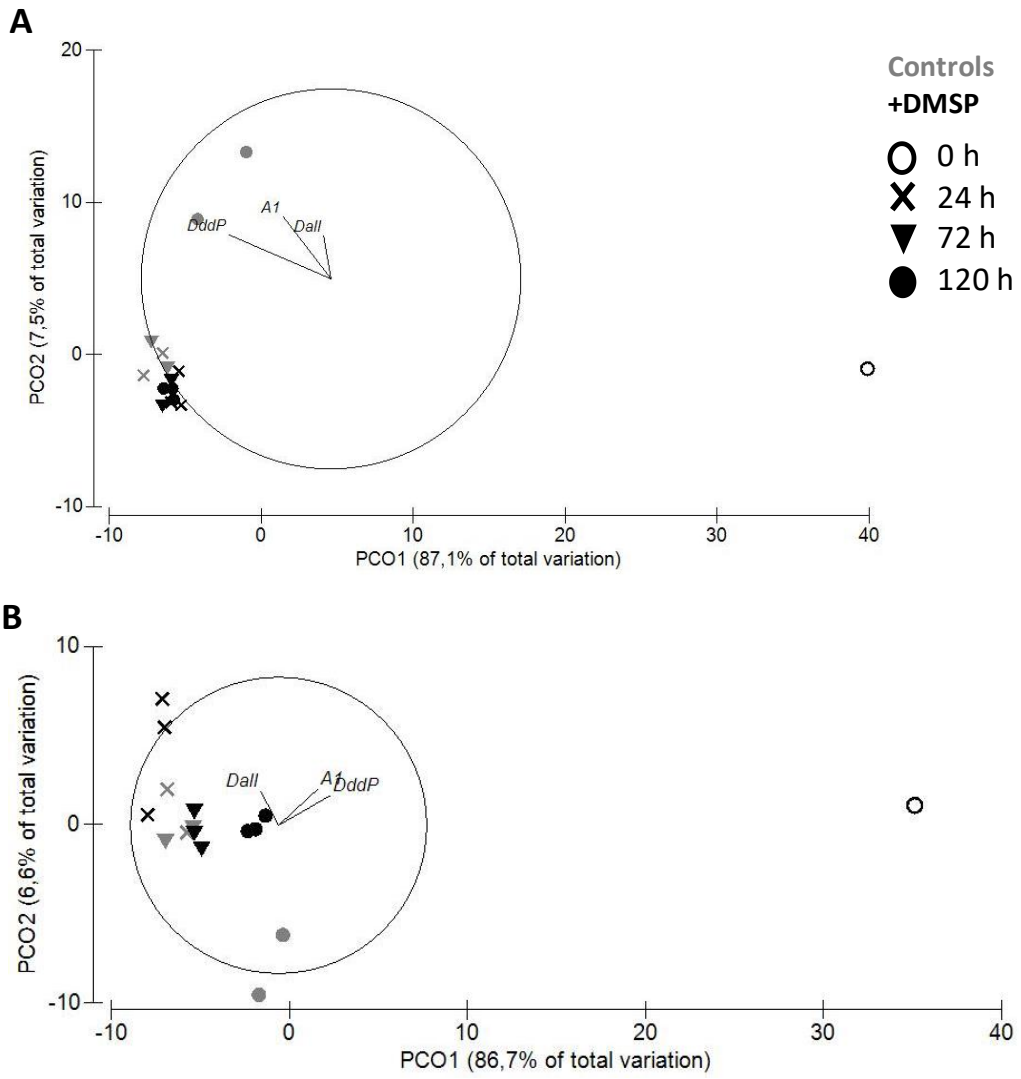

Figure 3.16. Principal Component Ordination (PCO) of bacteria composition for each site. *(A) PCO for the inner site and (B) PCO for the outer site. Ordination used Bray-Curtis similarity matrices and overlaying vectors of the DMSP degrading genes indicate direction of the parameter effect in the ordination plot. Samples are coloured by treatments with different shape for time.*

3.5. Discussion

Dimethylsulfoniopropionate (DMSP), which is predominantly produced by marine phytoplankton, is taken up by marine heterotrophic bacteria to satisfy their sulfur and carbon demands (Simó et al., 2002; Zubkov et al., 2001). While there is considerable evidence on bacterial uptake and processing of DMSP (Howard et al., 2008a), little research exists on the uptake of DMSP by phytoplankton (Petrou and Nielsen, 2018; Vila-Costa et al., 2006). This study investigated whether all members of the marine microbial community take up available DMSP from the environment and the effect that increasing DMSP availability has on the abundance of DMSP degradation genes and microbial community composition in

natural sea waters of the Great Barrier Reef in Australia. We found that short term responses included a decrease in DMSPt and an accumulation of DMSPp in all size fractions of the community (8 µm, 3 µm, 0.22 µm), suggesting that phytoplankton, like bacteria, take up dissolved DMSP with the largest fraction (>8 µm) forming the dominant sink with an increase of DMSPp of up to 115%. Longer term incubations revealed further differences between sites with community responses and DMSP metabolism, suggesting regionally specific differences in DMSP requirement, uptake and utilisation strategies with community composition and environmental conditions (i.e. nutrient availability due to upwellings). These data support the hypothesis that phytoplankton take up bioavailable dissolved DMSP and provide evidence that dominant members of the microbial community (Synechococcales, Alphaproteobacteria, Rickettsiales, Rhodobacterales, Flavobacteriales, diatoms) may act as important DMSP sinks in coral reef waters.

3.5.1. Quantification of DMSP uptake by different fractions of the microbial community

The seawater characteristics of both sites were typical of tropical waters, with nutrients and temperature values within ranges of previous studies made on the GBR (Andrews, 1983; Crosbie and Furnas, 2001; Muslim and Jones, 2003), where the East Australian Current (EAC), which flows poleward on the eastern side of the reefs, pumps deep, cold, saline, nutrient-rich water up the slope to the shelf break (Andrews and Gentien, 1982). DMS/P concentrations were within ranges of values previously found in the GBR with DMS siting at the lower range. Jones et al. (2007) measured average DMS concentrations of 2.0 (nd-54) nM and 1.3 (nd-3.9) nM in the reefs of Orpheus and Magnetic islands respectively, and Broadbent and Jones (2006) of 3.8 (0.36-35) nM in One tree reef. The lower values measured in this study are probably due to the location of the sampling points and time of collection. Jones et al. (2007) found that high DMS values occurred over the reef flat in the day light (between 08:00 and 20:00 hours) during the summer months (December to February), while this study happened in October and samples were collected before sunrise and not in a reef (inner reef site was located at~ 13km from the closest reefs of John Brewer reef, rib reef and Fore and Aft reef, outer reef was located at ~ 25km from Bills reef).

Similarly, the communities were dominated by the cyanobacteria *Prochlorococcus* and *Synechococcus* with typical abundances for warm oligotrophic areas, where *Prochlorococcus* is 100 times more abundant than *Synechococcus* due to its adaptability to poor nutrient

waters in contrast to the nutrient-rich and well-lit waters required by *Synechococcu*s (Campbell et al., 1994; Partensky et al., 1999). Because *Synechococcus* and *Prochlorococcus* are ubiquitous and abundant, and given their propensity to take up DMSP (Vila-Costa et al., 2006b), these two important taxa may also play an important role in DMSP cycling.

3.5.1.1. Analysis of sulfur compounds

The uptake of DMSP by different fractions of the marine microbial community of the GBR determined over 7 h (experiment 1) explains only part of the DMSP transfer. There was a loss of DMSPt, for both sites and treatments, while DMSPd mostly remained constant and DMSPp increased (Figure 3.5), indicating that DMSP was escaping from the system. DMSP could escape from the system either by bacterioplankton conversion to DMS, DMSO and MeSH or by photo chemical oxidation to DMSO (Brimblecombe and Shooter, 1986; Visscher et al., 1992; Zeyer et al., 1987). As DMSPp increased with time, we suggest that a significant fraction of available DMSPd was taken up and transformed, either to satisfy the sulfur demand in form of MeSH, to cleave into DMS and be lost to the atmosphere or to oxidise to DMSO to protect the cells from ROS (Kiene et al., 2000; Sunda et al., 2002). In the inner reef, it was shown that DMSP-enrichment led to DMSP uptake in the large (8 µm) and medium (3 µm) eukaryotic fractions, but not in the smallest fraction, consisting primarily of prokaryotes and heterotrophic bacteria (<3 µm and >0.22 µm). This contrasted with the outer reef incubations, where the largest and smallest fractions took up most of the excess DMSP (Figure 3.5G). These data support the hypothesis that phytoplankton as well as bacteria take up DMSP (Malmstrom et al., 2005; Petrou and Nielsen, 2018; Ruiz-González et al., 2012c; Spielmeyer et al., 2011) and provide evidence that larger members of the microbial community can act as important DMSP sinks in coral reef waters. The differences in DMSP uptake between sites (**Figure 3. 5**) may be due to differences in microbial composition, among other factors, as not all phytoplankton are capable of taking up DMSP (Lavoie et al., 2018). Unlike bacteria, little is known about DMSP degradation inside the phototrophic cell, and only one lyase gene and no demethylation genes have yet been found (Alcolombri et al., 2015). Information on how phytoplankton may utilise DMSP would have implications for understanding DMS fluxes to the atmosphere over the GBR, which currently are stimated to be > 0.6 Gmol S per annum (Graham Jones et al., 2018), and any phytoplankton and bacterial contribution to climate regulation (Charlson et al., 1987).

3.5.1.2. Relative gene abundance

The most abundant DMSP degrading gene at both the inner and outer reef sites was the demethylation gene DmdA, clade D (Figure 3.6) and belongs to the ubiquitous marine bacteria *SAR11* (Curson et al., 2011), which was predominant at both reef sites. The DmdA gene is responsible for the first step of the demethylation pathway (Howard et al., 2008a) and so, it is likely that the disappearance of DMSP from the incubations over 7 h for both reef sites was through MeSH production, with ~95% of the bacterial cells capable of utilising DMSP via demethylation (Figure 3.6), a slightly higher percentage compared to the 80 % previously predicted (Curson et al. 2011).

Similar relative abundances of the DMSP degradation genes have been reported in the North Pacific subtropical gyre (NPSG) (Varaljay et al., 2012). However, the relative abundances obtained by qPCR in this study were lower than the values reported by metagenomic surveys. In the Global Ocean Sampling Expedition (GOS), DmdA has been reported to be present in 37-58 % of marine bacterioplankton (Howard et al., 2008a, 2006) and DddP, although not always present, in abundances of 2-10 % (Todd et al. 2009). In this study, DmdA was present at ~10 % and DddP 0.3-0.6 % (Figure 3.6). Discrepancies between the two methods (qPCR and metagenomics) have been found previously for both the North Pacific subtropical gyre study and other studies (Cui et al., 2015; Liu et al., 2018) and are assigned to two main reasons. First, the selection of 16S rRNA gene as the normalising gene could cause an underestimation of the relative abundance since some bacterial genomes have multiple copies of 16S rRNA (Biers et al., 2009; Cui et al., 2015). Second, the highly conserved nucleotide sequences of the primers used in qPCR leave many sequences out of the analysis. For example, in the case of the demethylation gene DmdA, primers only target about half of known DmdA sequences (Varaljay et al., 2010), and additionally, we only encountered four of the 14 subclades: A1, D1, D2 and D3. Aside from the limitations of qPCR, our results on gene abundance are in good accordance with the metagenomic estimates, where DmdA subclade D was the predominant gene with an abundance one order of magnitude higher than that of Clade A1 and DddP, proportions that have been reported previously and may be a common characteristic of marine environments (Howard et al. 2008; Levine et al. 2012; Liu et al. 2018; Todd et al. 2009; Varaljay et al. 2012).

3.5.1.3. Microbial composition

Many dinoflagellates were present in the samples and were likely the main source of DMSP (Keller et al., 1989) (Figure 3.7B) and concentrations of DMSP were higher inside the reef, suggesting a greater absolute abundance of DMSP-producing cells, including coral zooxanthellae, in the inner location (Broadbent and Jones, 2006; Broadbent et al., 2002; Jones et al., 2007). Moreover, the increased concentration of DMSP in the inner reef could be due to DMSP production by the coral host (Raina et al., 2013) , and potential contribution by bacteria from the order of *Rhodobacterales,* which harboured the DMSP-synthesis gene dsyB (Curson et al., 2017) and were more abundant at the inner reef site.

We propose that non-DMSP producing dinoflagellates and big diatoms were the main taxa responsible for the uptake of DMSP in the 8 µm fraction, as they were highly diverse in both locations where most DMSPp was concentrated into the 8 µm fraction. Small diatoms from the class of *Mediophyceae* (*Bacillariophyta*) were possibly responsible for the uptake of DMSP in the 3 µm fraction from the inner reef site. For the 0.22 µm size fraction, DMSP uptake was likely dominated by *Prochlorococcus* (*Subsection I*) (34%) and *SAR11* (~39%). Previous work has shown that cyanobacteria take up DMSP (Vila-Costa et al., 2006b) yet are not believed to be significant DMSP catabolisers, as only one sequenced cyanobacterial strain, *Synechococcus sp*. KORDI-100 has been found to have a DMSP lyase gene (Lei et al., 2017). Furthermore, in co-cultures of the two dominant groups, *Prochlorococcus* was able to meet *SAR11*'s requirement for organic carbon, but not reduced sulfur and so, it is likely that these two organisms compete for DMSP (Becker et al., 2019). While *Prochlorococcus* and *SAR11* were present at both experimental sites, DMSP only accumulated in the small fraction of the outer reef, where *Prochlorococcus* made up 97 % of the cyanobacterial community, compared to 54 % in the inner reef (Table S.2.B), strongly implicating its dominant role in DMSP uptake.

Of the prokaryotic taxa, the group most likely to demethylate DMSP was *SAR11* and Roseobacters (*Rhodobacterales),* both of which are known to harbour *dmdA* genes widely among their taxa (Howard et al., 2008a). However, as *SAR11* (29 and 41%) dominated the community and were much more abundant than *Rhodobacterales* (7 and 4%) for both sides of the reef, *SAR11* is assumed to be the greatest potential DMSP consumer in the surface waters of the GBR, commensurate with findings for the Sargasso Sea and North Atlantic

Ocean (Rex R Malmstrom et al., 2004), Tropical and Subtropical Pacific Ocean (Cui et al., 2015) and East China sea (Liu et al., 2018).

SAR86 clade (>90% of O*ceanospirillales*) (Table S.2.D), which lacks the enzymes required for sulphate uptake or assimilatory reduction (Dupont et al., 2012), and *SAR 116* clade (87% IR and 67% OR of Rickettsiales) (Table S.2.A), could also be potential DMSP demethylators in the GBR. Howard et. al. (2008) found that dmdA clades B and C counts significantly correlated with SAR11, SAR86 and SAR116, and suggested that the latter two, may be source organisms for some DmdA clusters without phylogenetic anchor sequences. These clades have no culture representatives, and thus there are few available hints as to their biogeochemical roles. However, more research based on the genomic data of SAR86 may be soon be possible due to the recently generated partial SAR86 genome assemblies (Dupont et al., 2012). If SAR86 and SAR116 contain DmdA genes, they would be global DMSP catabolisers as they are highly abundant and ubiquitous in surface sea waters of different environments (González et al., 2000; Malmstrom et al., 2007; Schattenhofer et al., 2009).

The cleavage pathway was likely carried out by the abundant *SAR11* through its DddK lyase (Sun et al., 2016). However, bacteria with known DddP genes were also present in minor abundances, including *SAR116* (~67% of all *Rickettsiales*) (Choi et al., 2015) (Table S.2.A), and *Sulfitobacter*, *Ruegeria* and *Roseovarius* (*Rhodobacterales*) (Liu et al., 2018) (Table S.2.C). As some *Actinobacteria* cleave DMSP to DMS, it is possible that *Acidimicrobiales* also contributed to the DMS pool (Liu et al. 2018). It is impossible to rule out that some bacterioplankton taxa lacking DMSP degradation genes may take up DMSP for other reasons, such as for osmolytic benefit, as shown in a previous study, in which coastal seawater filtrates containing mostly bacteria, diminished their MeSH production and retained up to 54% of their intracellular DMSP when under osmotic stress (Motard-Côté and Kiene, 2015), or for its properties as a predator deterrent (Strom et al., 2003). It is also possible however, that they may fulfil their reduced sulfur needs via yet unknown mechanisms of acquisition with yet unidentified genes.

3.5.2. Dynamic changes in microbial community composition and DMSP degradation genes abundance

Longer-term incubations (experiment 2) designed to follow DMSP-induced community changes showed that both DMSPt and DMSPd concentrations decreased over time in both control and DMSP-enriched treatments in waters from both sites, indicating that initially DMSP was taken up from the solution, which is in accordance with experiment 1, and then lost within 24 h via production of DMS or MeSH as a result of microbial activity (Kiene, Linn & Bruton 2000).

3.5.2.1. Analysis of sulfur compounds

The calculated particulate DMSP did not show any intracellular accumulation, supporting the idea that the DMSP that was being taken up by the cells within the first few hours (as seen with Experiment 1) and was being utilised, regardless of DMSP availability, which is in accordance with the results of Kiene & Linn (2000), who found that added S^{35}-DMSPd was transformed into several products over time scales of minutes to 1 day. Moreover, DMSP consumption rates (Inner Reef: 5.04 (controls) and 12.24 (+DMSP) nM day^{-1}, Outer Reef: 9.36 (controls) and 19.92 (+DMSP) nM day^{-1}) were also within regular values, which generally range between 7- 80 nM d^{-1} but can be up to 730 nM d^{-1} (Simó et al. 2000; Simó & Pedrós-Alió 1999). Further research in which DMSP is added several times after the first 24 h, would help to elucidate to what extent DMSP would continue being degraded and whether it would benefit different organisms or instead, if the bacterial sulfur demand would be fully satisfied and no further DMSP demethylation would occur.

Kiene et al. (1999) proposed that bacteria preferentially utilise DMSP using the demethylation when there is a need to produce protein for bacterial growth, but once that sulfur demand is satisfied, the bacteria will use the lyase pathway. In our study, the high DMSP lyase activities for the inner reef suggest that DMSP concentrations may have exceeded the necessary levels to cover the sulfur demands of the bacterioplankton community and therefore much of the excess was cleaved to DMS. In contrast, outside the reef, it is likely that most of the added DMSP was utilised to meet the sulfur demands of the bacterioplankton community, which expressed lower lyase rates. These data suggest that the inner reef site probably had high inputs of organic carbon and sulfur and highly efficient

nutrient recycling systems, typical of reef ecosystems, while the outer reef site was more representative of the oligotrophic open ocean.

Even though DMSP lyase activity has been poorly studied in phytoplankton, it has been demonstrated in *Emiliania huxleyi* (Wolfe and Steinke, 1996), *Phaeocystis* spp. (Van Boekel & Stefels 1993) and different *Symbiodinium* strains (Yost and Mitchelmore, 2009, 2012), high DMSP producers. Therefore, we hypothesise that the DLAp in the GBR is mostly done by the high DMSP producers *Gymnodiniphycidae* and *Peridiniphycidae,* which include zooxanthellae algae and were present in both sites.

3.5.2.2. Relative gene abundance

As with the short-term experiments, the relative gene abundances obtained by qPCR for the 120 h incubation experiments were lower than the percentages reported by metagenomic surveys, likely because of the fact that we only analysed four of the 14 DmdA subclades and one of eight cleavage genes (Cui et al., 2015; Varaljay et al., 2010). Yet, congruent with previous studies, our results still found that DmdA subclade D was the dominant gene, with an abundance one order of magnitude higher than Clade A1 and DddP (Howard et al. 2008; Levine et al. 2012; Liu et al. 2018; Todd et al. 2009; Varaljay et al. 2012). The gene DmdA (A1+Dall) was the dominant DMSP degradation gene during the entire experiment (120 h) with similar relative abundances at both sites at the outset, when most of the DMSP was lost from the system, suggesting that most of the DMSP was degraded through demethylation (Kiene et al. 1999; Kiene & Linn 2000). The relative abundances of the different DMSP degradation genes over time showed variable behaviours for the two locations. In the inner reef site, all degradation genes increased in relative abundance with time, suggesting that sulfur demands were met quickly, after which bacterial growth and cleavage were able to take over. However, increased availability of DMSP did not seem to benefit the DMSP-utilising bacteria in terms of relative abundance within the microbial community, possibly because the DMSP concentration present inside the reef was enough to cover the sulfur and carbon demands of the bacterioplankton community, and therefore the excess DMSP was cleaved, consistent with the high lyase activity measured inside the reef. In the outer reef site, the relative abundance of genes were higher for the DMSP amended samples compared to the controls, indicating that the microbial community from the outer reef benefited from the DMSP enrichment. This is largely in accordance with the low DMSP lyase levels found in

this site, supporting the idea that the DMSP concentrations in the oligotrophic waters outside the reef were not enough to satisfy the sulphur and carbon demands of the microbial community. Moreover, we propose that microorganisms competed for this rich and scarce compound and that the microorganisms containing DmdA/Dall may be the major beneficiaries, as DmdA/Dall relative abundance increased in contrast to the decline in the relative abundance of DddP and A1.

3.5.2.3. 16S and 18S diversity

Consistent with experiment 1, the major DMSP producers in the initial waters for both reef sites were most likely dinoflagellates, while *SAR11* was the main taxa responsible for DMSP demethylation (Howard et al. 2008a; Lei, Alcolombri & Tawfik 2017; Malmstrom et al. 2004). During incubations, the significant drop in the relative abundance of *SAR11* to <1% after 24 h indicates that there was an issue with bottle effects, with the abundant organisms disappearing within the first few hours for both treatments, which is likely to significantly impact the results. Similarly, we saw a significant decrease in *Prochlorococcus* (*Subsection I)* abundance after 24 h, which we also associate to maintenance difficulties.

For the inner reef, the greater abundance of *Synechococcales* in the DMSP-enriched samples at the last time point indicates that this group likely benefits from DMSP and may have been one of the contributors to the demethylation and cleavage of the DMSP. The ability for cyanobacteria to take up DMSP has been previously reported (Vila-Costa et al., 2006b), but further research may be needed to verify its ability to utilise DMSP, as so far, only *Synechococcus sp*. KORDI-100 has been found to possess DMSP lyase activity (Lei et al., 2017). *Rickettsiales and Acidimicrobiales* were also abundant in the DMSP-enriched samples. Many of the DMSP catabolic enzymes are widespread among α-*Proteobacteria* (Reisch et al., 2011), and therefore this group may be responsible for the demethylation of DMSP in the inner reef, while *Rickettsiales,* which contain DddP genes (Choi et al., 2015), may be responsible for DMSP cleavage. To our knowledge, there is no evidence that *Acidimicrobiales* contain DMSP degradation genes. Therefore, it is unclear what advantage the DMSP may bring to this group, perhaps, they utilise DMSP in one of its cellular roles, such as its antioxidant role, to protect themselves against the high sea surface temperatures and solar radiation levels of the GBR, as has been suggested for corals (Jones et al., 2014, 2007). Alternatively, they may possess orthologs of known DMSP-degrading genes that have not been correctly assigned or

contain DMSP degradation genes yet to be found. Perhaps, in favouring the other DMSP utilisers, DMSP addition disrupts the competitive hierarchy of the microbial community allowing these taxa to proliferate, which would also explain why *Rhodobacterales*, despite being one of the taxa with more organisms containing DmdA, seemed to not benefit from the enrichment with DMSP.

In contrast to the inner reef, the bacterial community outside the reef showed that *Synechococcales* were inhibited by an increase of DMSP, probably due to the high competition for the substrate. *Rhodobacterales* was the first bacterial order to benefit from an increase in DMSP. However, over time, there was a succession of bacteria benefitting from DMSP, with *Oceanospirillales* and S*phingomonadales* dominating after 72 h and *Rickettsiales* and *Flavobacteriales* after 120 h. Except for *Rickettsiales*, which harbour DddP genes (Choi et al., 2015) and *Rhodobacterales* that contain DmdA (Howard et al., 2008b), there is no evidence that any of the other taxa contain DMSP degradation genes. However, Pinhassi et al. (2005) and Vila-Costa et al., (2010) have suggested that *Flavobacteriales* may be relevant taxa to consider when addressing the role of bacterioplankton community dynamics in controlling DMSP cycling, after observing an increase in their abundance (or transcripts abundance) during a DMSP-enriched experiment in coastal Gulf of Mexico and Bermuda Atlantic Time-series Study (BATS) station respectively, and so, they may be also a relevant taxa to reef sulphur cycling. Similarly, Howard et al. (2008) suggested that *SAR86* clade (O*ceanospirillales*), may be source organisms for some DmdA clusters without phylogenetic anchor sequences.

The ordination of the samples by nMDS (Figure 3.12 B,C,E,F) support our previous results and indicate that DMSP enrichment has an effect on the bacterial community of the GBR for both sites of the reef as samples of different treatments are grouped separately for the last time point, and the PCO (Figure 3.16) supports that the relative abundance of DmdA/A1 and DddP are important drivers in the inner site, while its abundance seem to not be as relevant for the outer reef. The bacterial taxa that correlated with the analysed genes DmdA/A1 and DddP, in the inner reef site, *Synechococcales* (negatively) and *Rhodobacterales* (positively) (Figure 3.7A-D), also responded to the addition of DMSP, which was advantageous for *Synechococcales* and inhibitory for *Rhodobacterales* latter (Figure 3.12.A), and contributed the most to treatment dissimilarity (Table 3.2A). *Rhodobacterales* have been previously

found to positively correlate with relative abundances of DddP and DmdA (Cui et al., 2015) and are one of the major groups responsible for DMSP demethylation (Howard et al. 2008). In the outer reef site, two known DMSP catabolisers negatively correlated with DMSP degradation genes, *Rickettsiales* and α-*Proteobacteria.* These results may mean that these five bacterial taxa are relevant for DMSP cycling in GBR waters. A major limitation to studying DMSP cycling by taxa and gene abundance is the lack of consideration of the different genes turnover rates and Km (Michaelis constant), which differ between species and are yet to be described, and may be relevant for different community composition and water masses (Reisch et al., 2008).

In these reef waters, DMSP had a much smaller effect on the eukaryotic communities with treatments being significantly different only after 120 h (Figure 3.13 & 3.14) and no clear group of taxa identified to explain treatment dissimilarity (Table 3.2B). However, diatoms were able to benefit from the presence of DMSP at least for the inner site. These results suggest that heterotrophic bacteria may be the major DMSP beneficiaries in the waters surrounding the GBR, but as few phytoplankton taxa, mostly diatoms, benefited from DMSP in the inner site, we hypothesise that there may be other DMSP utilisation pathways in phytoplankton yet to be discovered.

3.6. Conclusions

In this study we evaluated the uptake of DMSP by different fractions of the marine microbial community and revealed that both bacteria and phytoplankton from natural reef waters take up DMSP over short time scales. Moreover, it was shown that DMSP distribution among individuals of different size classes differs among reef environments. In the inner reef environment of the GBR, which harbours microbial communities typical of coastal habitats, the main sinks for DMSP were the largest (mostly A*moebophira* and *Syndiniales*) and mid-sized (*Mediophyceae* (*Bacillariophyta*), *Coscinodiscophytina* and *Mamiellales*) microorganisms, while in the open ocean, DMSP uptake was evenly distributed between the largest (A*moebophira* and *Syndiniales*) and smallest groups (mostly *Prochlorococcus* with a contribution of *SAR11*). By investigating the main DMSP catabolising genes we were able to show that in both the inner and outer GBR ~95% of bacterial cells that utilise DMSP have the capacity to degrade it through demethylation, with *SAR11* presenting itself as potentially the greatest DMSP consumer in the surface waters of the GBR.

The effect of DMSP on the composition of marine microbial populations of the GBR demonstrated that microorganisms that took up and utilised DMSP, regulated DMSP demethylation and cleavage so as to maintain intracellular DMSP levels regardless of DMSP availability. Moreover, the ability to degrade DMSP supposes an ecological advantage to many bacteria such as *Synechococcales,* members of the *alphaproteobacteria, Rickettsiales, Rhodobacterales and Flavobacteriales*. However, the major group of DMSP consumers changed with location and time, depending on the presence of other competitors, as well as the sulfur needs of the community. By examining patterns in the eukaryotic community, as defined by sequencing of the 18S rRNA gene over time, we showed that DMSP-enrichment affected the community structure of eukaryotes, with DMSP-related growth in diatoms, suggesting that perhaps there may be other DMSP utilisation pathways in phytoplankton yet to be discovered.

The results of our study show that DMSP enrichment, can influence the marine microbial community structure, and in combination with environmental factors such as nutrient-rich currents or SST, influence the way DMSP is metabolised, ultimately affecting DMS emissions over the Great Barrier Reef. In general, communities dominated by non-DMSP producing species like diatoms will metabolise DMSP to produce proteins, decreasing seawater DMS concentrations, while communities dominated by DMSP producers will increase them. Hence, DMS fluxes to the atmosphere may increase if DMSP producers predominate and decrease when non-DMSP producers do so. Therefore, increasing knowledge on the distribution of DMSP among the community, including phototrophs, and how microbial populations and DMSP degradation genes change in response to DMSP concentrations, will contribute to a better understanding of the marine DMSP cycle in reef environments.

Chapter 4:

DMSP uptake and assimilation by microbial communities of Antarctic waters

Chapter 4: Dissolved DMSP uptake and assimilation by microbial communities of Antarctic waters

4.1. Abstract

In order to adapt to the extreme environmental variability that is the hallmark of the Antarctic marine ecosystem, polar phytoplankton have evolved different strategies, such as the capability to synthesise high amounts of the biogenic sulphur compound dimethylsulfoniopropionate (DMSP), which is believed to act as a cryoprotectant and osmolyte in the cell. In the marine environment, dissolved DMSP is readily metabolised by heterotrophic bacterioplankton, but recent research has shown phototrophs to take up dissolved DMSP to concentrations up to 17.7 fmol cell^{-1}. In this study, we looked at the uptake of DMSPd in the natural marine microbial communities of Antarctic coastal waters off Davis station in East Antarctica. By incubating the natural seawater with DMSP, we aimed to quantify the uptake of DMSPd by different fractions of the microbial community and to evaluate whether dissolved DMSP enrichment leads to changes in the microbial composition. Our results showed rapid uptake of DMSPd by all size fractions (14 µm, 8 µm, 3 µm and 0.22 µm) of the community following a size-dependent gradient, but that the greatest proportional increase occurred in the smallest fraction. Over six days, we saw an overall decrease of $DMSP_t$ from 13.26 to 4.41 µM and dissolved DMSP from 7.34 to 1.45 µM under DMSP-enrichment, suggesting high rates of DMS production by the community, likely achieved by *Rhodobacterales* group during the first 48 h. There was however, no major changes in the composition of the microbial communities as a result of DMSP enrichment. These results indicate that phytoplankton from Antarctic waters of all size classes can incorporate significant amounts of DMSPd and suggest that much of the available DMSP is likely cleaved into DMS as calculated DMSPp and microbial composition remain unchanged. This favoritism for the cleavage pathway over the demethylation of DMSP could be one reason to explain the high DMS fluxes to the atmosphere often associated with polar waters and should be further investigated.

4.2. Introduction

The Southern Ocean (SO), which represents 20 % of the global ocean area, is one of the most productive oceans on earth, with annual primary production exceeding 1.7×10^9 Tg C yr^{-1} in waters south of 50 ºS (Priddle et al., 1998). A significant amount of the production of organic matter (Chl a) in the Southern Ocean is often confined to the melting ice edge, which experiences extreme changes between seasons. In winter, sea ice (both pack and fast ice) cover large areas of ocean around Antarctica and much of the phytoplankton community becomes trapped inside the ice matrix, where concentrations of salt and dissolved iron are high, light is limited, diffusion rates of dissolved gases is low and there is poor exchange of inorganic nutrients (Arrigo et al., 2014; Thomas et al., 2001; Wang et al., 2014). While in summer, phytoplankton are released as the ice melts, providing surface water conditions characterised by high levels of solar radiation and high nutrient concentrations due to mixing with deep-water and results in fast DMSP production (Dierssen et al., 2002; Vance et al., 2013).

In order to adapt to these annual freeze and thaw cycles, phytoplankton from polar environments have evolved different strategies that help maintain membrane fluidity under freezing temperatures (Morgan-Kiss et al., 2006) and internal osmotic balance during salinity shifts (Krell et al., 2007), as well as strategies to modify their microenvironment (Bayer-Giraldi et al., 2010; Janech et al., 2006; Krembs and Deming, 2008; Raymond et al., 2009). One of these adaptation mechanisms is the capability to synthesise large amounts of DMSP (up to 2910 nmol DMSP /L sea ice (Trevena and Jones, 2006). Antarctic sea ice contains very large but variable concentrations of DMSP (Curran et al., 1998; Gambaro et al., 2004; Kirst et al., 1991; Lee et al., 2001; Turner et al., 1995) that can range from less than 5 to around 1660 nM and averages of the order of 200 nM (Trevena, 2003). Dimethylsulfoniopropionate (DMSP) is believed to act as a cryoprotectant by stabilizing enzymes, and as an osmoregulator by accumulation in the cytoplasm to maintain osmotic pressure (Otte et al., 2004; Stefels, 2000). While culture-based studies have well documented increases in DMSP concentration with a decline in temperature (Karsten et al., 1992; Van Rijssel and Gieskes, 2002), there are no consistent trends in relation to salinity (Colmer and Läuchli, 1996; Dickson and Kirst, 1987, 1986; Diggelen et al., 1986; Edwards et al., 1987; Karsten et al., 1992; Stefels et al., 1996), and therefore DMSP may play varying biological roles in sea ice algae.

Phytoplankton-derived DMSP is released into the ocean through different mechanisms such as exudation or viral attack (Dacey et al., 1994) and is available to the bacterial community in dissolved form for uptake (Rex R. Malmstrom et al., 2004; Raina et al., 2017; Ruiz-González et al., 2012c). Once inside the bacterial cell, it can degrade via two processes: the demethylation pathway that is catabolized by the dmd genes (Reisch, Moran & Whitman 2011), during which DMSP is transformed to methanethiol (MeSH) to be utilised for energy and protein production (Kiene et al. 1999; Ronald P Kiene & Linn 2000); and the cleavage pathway that is catabolized by the Ddd genes (Curson et al. 2011), where DMSP is cleaved to DMS that diffuses from the cell (Kiene, Linn & Bruton 2000; Yoch 2002) and as a volatile compound, readily escapes to the atmosphere, where it may contribute to low level cloud formation and climate regulation (Charlson et al., 1987). It has been hypothesised that the mechanism utilised to degrade DMSP depends on the bacterial sulfur demand, and that bacteria will preferentially degrade DMSP through demethylation to produce protein for bacterial growth, and hence, meet the sulfur demand before adopting the cleavage pathway to generate DMS (Kiene et al., 1999).

For a long time, it was thought that phytoplankton only produce DMSP and do not take it up, as no genes from the demethylation pathway have been found and to date, only one lyase gene has been identified in phytoplankton (Alcolombri et al., 2015). However, one study suggested that phytoplankton may take up equal amounts of DMSP as bacterioplankton, after observing assimilation of DMSP sulfur by *Prochlorococcus*, *Synechococcus,* heterotrophic bacteria and picoeukaryotes in surface seawater from various locations incubated with ^{35}S-DMSP (Vila-Costa et al., 2006b). Follow on studies have shown DMSP uptake in the diatom *Thalassiosira weissflogii* (Petrou and Nielsen, 2018), the haptophyte *Emiliana huxleyi* (Spielmeyer et al., 2011) and the polar diatom *Thalassiosira gravida* (Lavoie et al., 2018) .

To explore how DMSP, produced by few phytoplankton species, might be utilised by the rest of the Antarctic marine microbial community, we investigated the uptake of DMSP by natural phytoplankton and bacteria from near shore waters off Davis station in East Antarctica. First, we aimed to quantify the uptake of DMSP by the autotrophic and heterotrophic populations by incubating samples with DMSP and size fractionating the community to differentiate between possible DMSP sinks. Secondly, we intended to evaluate DMSP-induced changes in

microbial composition over time using diversity profiling of genomic DNA. Through these studies we aimed to increase our knowledge on DMSP sinks and sources in Antarctic surface sea waters and gain better insight of the key marine microbes responsible for DMSP degradation in Antarctic marine summer waters.

4.3. Experimental procedure

The study took place during an expedition to Davis Station in East Antarctica in February 2017 (Figure 4.1). Two separate incubation experiments were conducted on seawaters of *Prydz Bay* collected from the same site (77°51'E, 68°32'S), sampled five days apart. 1) A short-term study to quantify DMSP uptake by different size fractions of the Antarctic marine microbial community, and 2) a longer-term (144 h) experiment to investigate the influence of DMSP on community structure.

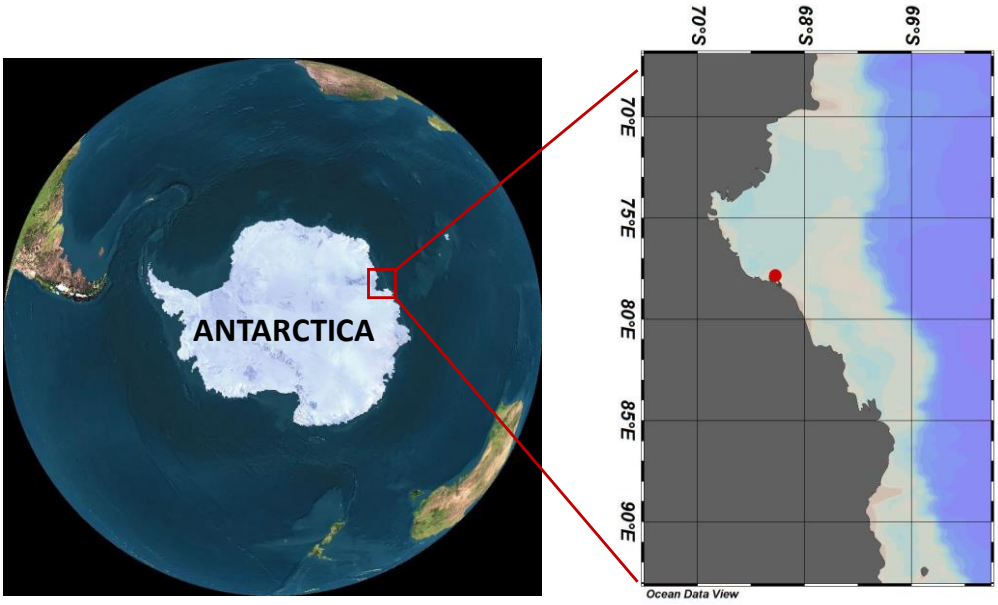

Figure 4. 1. Location of sampling site of initial water masses for experiments 1 and 2. *Map of Antarctica showing location of Davis station in East Antarctica with magnified inset image showing location of sampling site, generated with Ocean Data View software* (Schlitzer, 2016).

4.3.1. Experiment 1: Quantification of rapid DMSP uptake by different fractions of the Antarctic microbial community

To study the uptake of DMSP by the Antarctic microbial community, 52 litres of seawater were transferred to polycarbonate bottles (4 controls, 1 fixed sample, and 4 treated with DMSP (10 nM)) with no headspace. Glutaraldehyde (final concentration 1%) and DMSP was

added to the fixed sample to control for any passive uptake of DMSP by cells. After DMSP addition, the bottles were closed with screw caps and shaken gently to dissolve the added compound. All bottles were incubated for up to 8 h inside a fridge to maintain original temperature (0°C) and low light (40 µmol photons m^{-2} s^{-1}).

At four time points (T0, T2, T5 and T8 h) each bottle was subsampled as indicated in figure 2.2 for DMSP total and dissolved in triplicate and preserved with HCl (1.6%, pH < 1) and kept at -80ºC until processing, procedure that have been proved to maintain DMSP concentrations within the analytical precision (Curran et al., 1998; Del Valle et al., 2011; Simó et al., 1996). After 8 h, two litres from each bottle were size-fractionated via serial filtration using 14 µm, 8 µm, 3 µm and 0.22 µm polycarbonate filters for analysis of particulate DMSP (DMSPp) and the filters were preserved with 6 mL of HCl (1.6%, pH < 1) and kept at -80ºC until analysis, when samples were defrosted and hydrolysed prior to GC-FPD analysis. During the first filtration, subsamples (800 µL) from each size fraction were taken, fixed with glutaraldehyde (final concentration 1%) and snap frozen for community composition using for flow cytometry. The flow chart of the experiment is shown in Figure 4.2. Filter concentrated samples of the original seawater were also taken for phytoplankton counts and identification via light microscopy.

4.3.2. Experiment 2: Effect of DMSP on Antarctic community structure and gene regulation and the Identification of microorganisms that assimilate DMSP into their biomass

To identify the active microorganisms that assimilate DMSP into their cellular biomass, 42 L of water were divided across 21 x 2L polycarbonate bottles with no headspace which includes 9 controls and 9 samples treated with DMSP (7 µM) and 3 fixed samples treated with DMSP (7 µM). Flasks were incubated at 0ºC under 55 umol photons m^{-2} s^{-1} of light until destructive sampling at three time points (T48 h, T96 h, and T6 days).

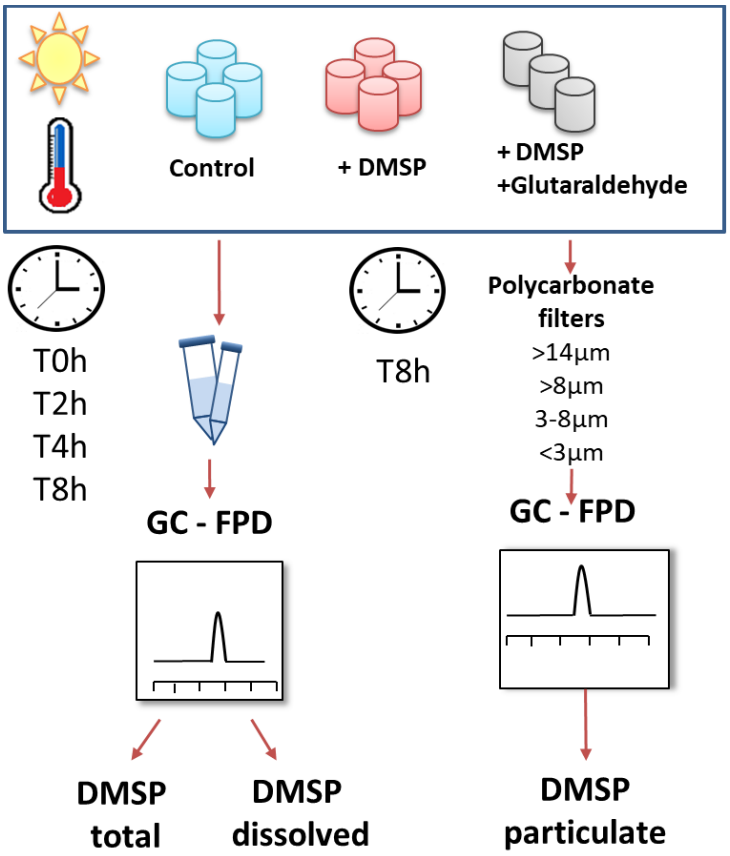

Figure 4.2. Experiment 1 flow chart. *In order to study the microbial uptake of DMSP, initial seawater was split in 4 control, 3 fixed (1% Glutaraldehyde) and 4 DMSP amended samples with no headspace. At every time point, each bottle was subsampled for analysis of DMSP total and dissolved by GC-FPD. In the last time point, each replicate was split and size-fractionated via serial filtration using polycarbonate filters for analysis of particulate DMSP (DMSPp) by GC-FPD.*

At each time point, subsamples for DMSP total and dissolved were taken prior to filtration of the samples for community DNA (3 controls, 3samples treated with DMSP (7 µM) and 1 fixed sample treated with DMSP (7 µM)) through a 3 µm and 0.22 µm polycarbonate filters to obtain two size fractions and the filters snap frozen and kept in -80ºC until analysis. DNA from the filters was extracted using the phenol-chloroform extraction protocol described in section 2.1.3.

The resulting DNA was split into two samples; one was used for the analysis of 16S and 18S of the whole community, a second was used for the quantification of DMSP degrading genes by real time Polymerase Chain Reaction (qPCR) of the free-living bacteria (0.22µm) and particle-associated bacteria (3 µm). The flow chart of the experiment is shown in figure 4.3.

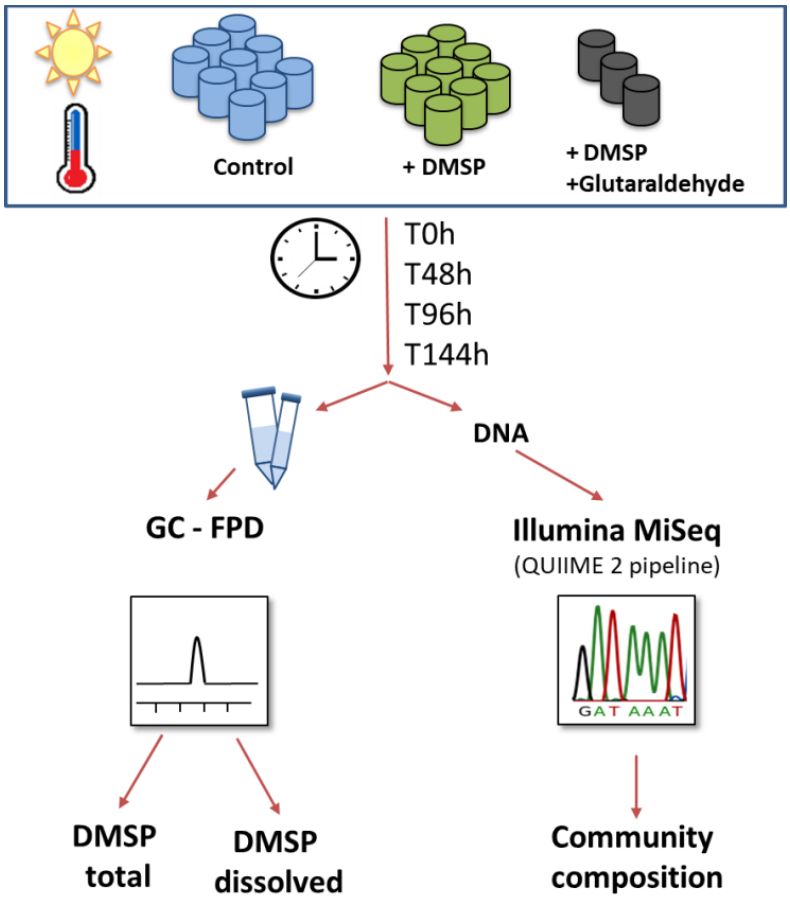

Figure 4.3. Experiment 2 flow chart. *To study the microbial community changes caused by the addition of DMSP, initial natural waters were divided into 9 controls, 9 DMSP enriched, and 3 fixed (1% Glutaraldehyde) +DMSP samples. At each time point, subsamples for DMSP total and dissolved were taken and samples filtered. DNA from the filters was extracted and used for the analysis of 16S and 18S of the whole community.*

4.3.3. Statistical analysis

After having ascertained homogeneity of variance using Levene's test, a one-way Analysis of Variance (ANOVA) was used to analyse differences in initial nutrient concentrations between experiments and between the treatments for the final time point for experiment 2. ANOVA was also used to analyse differences between treatments for DMSPp data from experiment 1. These analyses were performed using the statistical package SPSS (IBM Statistics v.24). Permutational Multivariate Analysis of Variance (PERMANOVA) with pair-wise comparisons was used to determine significant changes or differences in DMSPt, DMSPd and F_V/F_M values over time and between treatments, in a nested design for experiments 1 and 2. Differences in community composition between treatments and time were characterized using PERMANOVA, non-parametric multi-dimensional scaling (nMDS) and Analysis of Similarities (ANOSIM). Discriminatory microbes were identified using a two-way crossed Analysis of

Similarity Percentages (SIMPER) with time and treatment variables as factors (Bray–Curtis similarity matrix) (Clarke, 1993). High contributors were selected from the treatment pair-wise comparisons at the final time point (144 h) to explain a minimum of 70 % of treatment dissimilarity. These analyses were performed in PRIMER v6 statistical package (Clarke and Gorley, 2006) with PERMANOVA+ module (Anderson, 2005) using fourth root transformed data of microbial relative abundance (16S and 18S) to meet assumptions of multivariate homogeneity and to reduce the influence of highly abundant species. Phylogenetic trees using presence/absence of genera present in at least two of the replicates identified by 18S were constructed for analysis of the eukaryotic community using R (Team, 2013) with phytools package (Revell, 2012). Due to missing phylogenic information for many 18S sequences, only genera for which the taxonomy could be verified in WoRMS (World Register Marine Species; marinespecies.org) were included in the trees.

4.4. Results

4.4.1. Characteristics of initial water masses

The water masses of both experiments had very similar characteristics at the time of collection (Table 4.1). Both experiments were run with surface seawater collected at 5 m and had an initial temperature of 0.5 ºC. Water samples for experiment 1 had a salinity of 34 psu and a pH of 8.03 while samples for experiment 2 salinity was higher at 37 psu and pH was lower at 7.93 (Table 4.1). Seawater DMSPt concentrations were high for both experiments with 60 nM and 38 nM DMSP, respectively.

Table 4. 2 Characteristics of initial water masses. *The sampling depth, temperature, salinity and pH data from the initial water sampled for both experiments. Data represent mean ± SD (n=3). Where no SD is shown, data represent single measurements.*

	Experiment 1	**Experiment 2**
Depth (m)	4.8	4.0
Temperature (°C)	0.5	0.5
Salinity (psu)	34±0	37±1.15
pH	8.03±0.02	7.93±0.01
DMSPt (nM)	60	68

Water samples of experiment 1 had higher values of phosphate (1.38 µM phosphate) (F= 101.049, P=0.01) and silicate (66.98 µM silicate) (F=25.189, P=0.07) than water samples of experiment 2, with 0.81 µM of phosphate and 42.91 µM of silicate, respectively (Figure 4.4). However, no difference in NOx concentrations were observed.

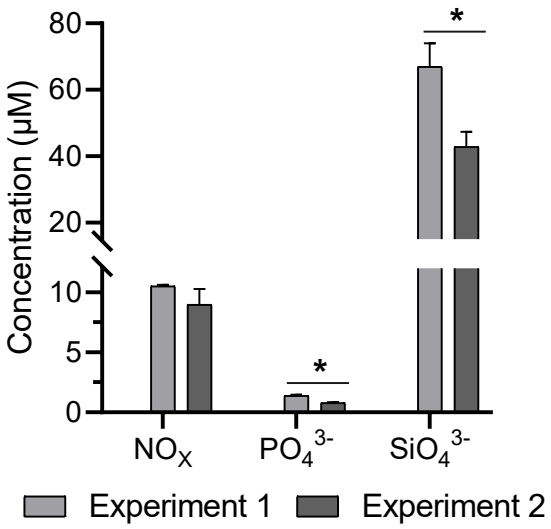

Figure 4. 4. Nutrient concentrations of initial water masses for experiments 1 and 2. *Concentrations of nitrates (NOx), Phosphate (PO_4^{3-}) and Silicate (SiO_4^{3-}) for both surface water samples at the initial sampling time for experiment 1 (light grey) and experiment 2 (dark grey). * Denotes statistical differences at P<0.05. Data represent the mean ± standard deviation (n=3).*

The phytoplankton community was taxonomically diverse and dominated by large diatoms in both samples. An example of dominant diatom taxa identified by light microscopy in the larger (14 µm) fraction were: *Corethron*, which is characterised by its cylindrical cells with dome-shaped valves containing spines, and are generally solitary (Figure 4.5A); the centric diatom *Asteromphalus* (Figure 4.5B); the chain-forming diatom *Thalassiosira*, characterised by its discoid cells (Figure 4.5C); and *Chaetoceros*, which usually form chains but can be found in single cells and is characterised by long setae emerging from the corners of the cells (Figure 4.5D).

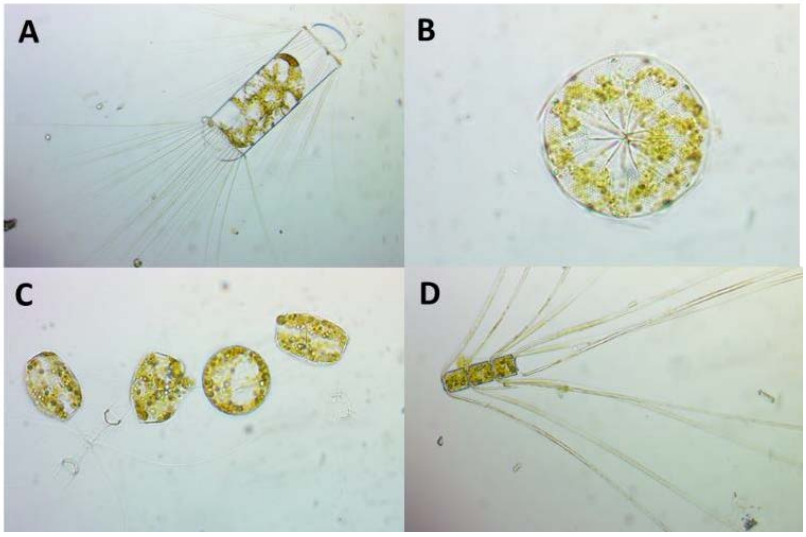

Figure 4.5. Example of common large (20 µm) Phytoplankton taxa identified in initial water samples for both experiments. *(A) Corethron, (B) Asteromphalus, (C) Thalassiosira, (D) Chaetoceros*

4.4.2. Experiment 1: Quantification of DMSP uptake by different fractions of the microbial community

4.4.2.1. Analysis of sulfur compounds: DMSPt, DMSPd and DMSPp

The concentrations of DMSPt, while significantly different between treatments (Pseudo-F: 28.88, P_{MC}=0.001), remained constant through time (Figure 4.6.A). Control samples ranged between 60 and 73 nM, samples enriched with DMSP ranged between 72 and 82 nM, and fixed samples between 51 and 59 nM. The concentrations of DMSPd also showed significant differences between treatments (Pseudo-F: 20.35, P_{MC}=0.001), diminishing significantly from 30.90 to 25.20 nM in the control (t= 4.19, 0.039, P_{MC}=0.003), from 30.16 to 14.50 nM in the DMSP enriched samples (t=5.10, P_{MC}=0.001). At the final time point, control samples had lost 22.65% of the initial DMSPd, while +DMSP samples had lost almost half of the dissolved DMSP (48.67%). The rates of disappearance of DMSPd were 91.52 nM day^{-1} for controls and 100.17 nM day^{-1} for +DMSP samples during the first four hours, after that time, DMSP concentrations in both controls and +DMSP remained stable.

Particulate DMSP, calculated by subtracting DMSPd from DMSPt (Figure 4.6.C), showed an increase over time from ~42 nM to 60 (t=4.71, P_{MC}=0.002) and 68 nM (t= 11.1, P_{MC}=0.001) for control and +DMSP samples respectively. The calculated uptake rates were 51.56 and 76.42 nMd^{-1} for controls and +DMSP samples, respectively (Figure 4.6.C). Particulate DMSP content measured at the final time point, followed a size gradient, where the largest eukaryotic

fraction (> 14 µm) contained significantly higher DMSPp than the medium sized eukaryotes (> 8 µm), which had higher DMSP content than the smaller eukaryotes (> 3 µm), which in turn, contained more DMSPp than the prokaryotic / heterotrophic bacterial (0.22 µm) fraction (Figure 4.6.D-E). When comparing treatments, all size fractions significantly increased their DMSPp concentration, with DMSP enrichment resulting in greater particulate DMSP within the fraction; the largest eukaryotes increased their DMSPp from 13.77 to 25.29 nM ($F=54.29$, $P=0.001$), the medium eukaryotes from 1.11 to 2.17 nM ($F=18.20$, $P=0.001$), the smallest eukaryotes from 0.51 to 0.96 nM ($F= 29.57$, $P=0.004$), and the prokaryotes and heterotrophic bacteria from 0.17 to 0.40 nM ($F=9.11$, $P=0.009$). The amount of DMSP taken up by each fraction also followed a size gradient, with the largest eukaryotic fraction (> 14 µm) taking up 11.52 µM of DMSP, the > 8 µm fraction 1.06 µM, the > 3 µm fraction 0.45 µM and the > 0.22 µm fraction 0.23 µM of DMSP. However, when calculated as the percentage of DMSP taken up, this pattern was not retained, instead the smallest fraction (> 0.22 µm) had the highest proportional uptake of DMSP of 135.29% compared with its control DMSP concentration. The eukaryotic size fractions took up similar percentages, with an increase of DMSP of 83.66% for the 14 µm fraction, 95.49% for the 8 µm fraction, 88.23% for the 3 µm fraction. DMSPp in fixed cells with enrichment was low across all fractions.

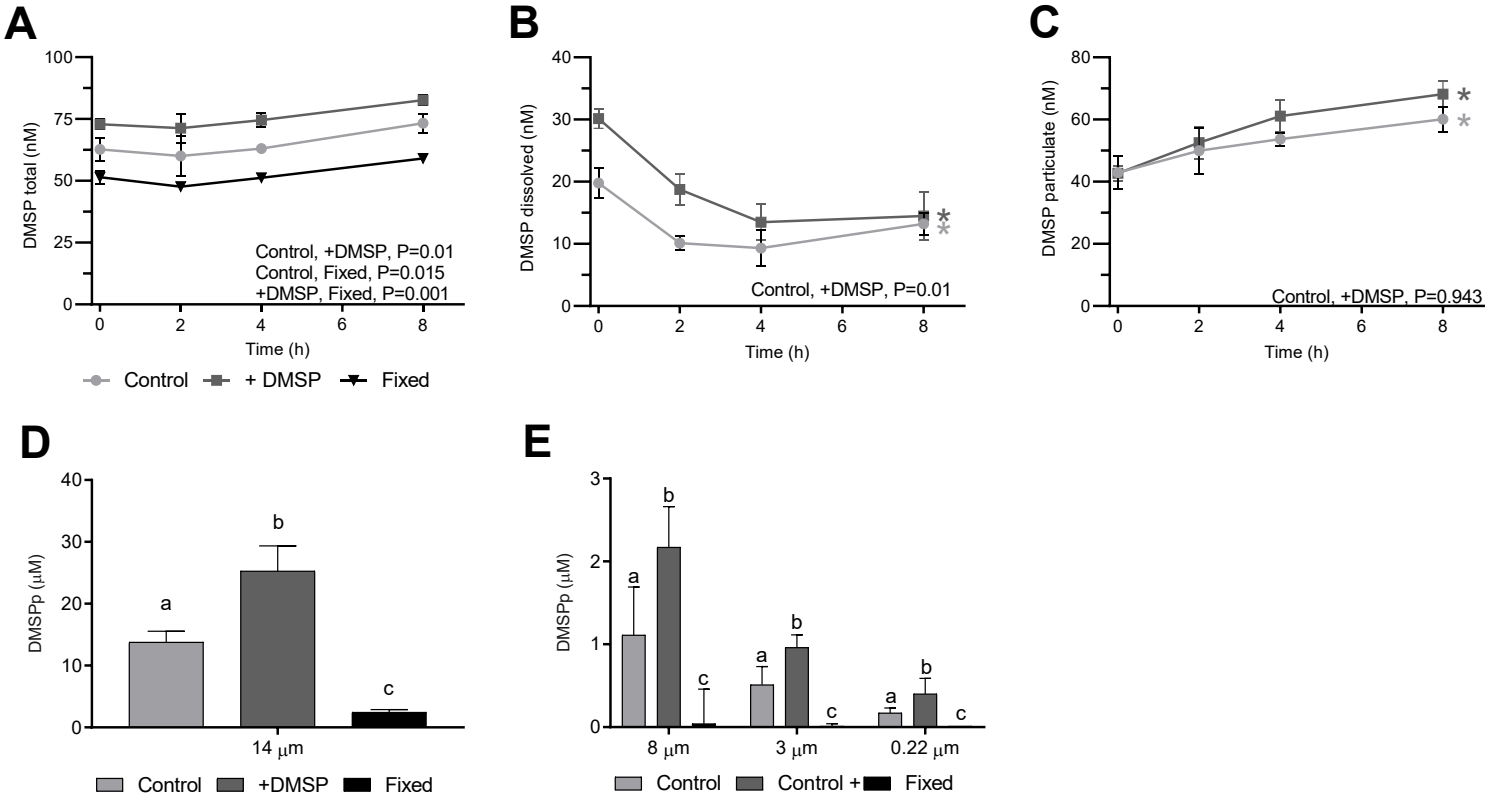

Figure 4. 6. DMSP concentrations over 8 h during experiment 1. *(A) Time course of DMSPt (B) Time course of DMSPd (C) Time course of calculated DMSPp (D) DMSPp retained in 14 µm filters after 8h and (E) DMSPp retained in 8 µm, 3 µm and 0.2 µm filters after 8 h. Data represent the mean ± standard deviation (n=4 and n=3 for fixed samples). Results from Permanova statistical tests are shown in the top right corner of each plot for treatment factor, * denotes statistical difference at P<0.05 between concentrations of final time point and initial time point. Lowercase letters above bars denote statistical differences at P<0.05. Error bars not visible when smaller than symbol.*

4.4.2.2. Relative gene abundance and microbial composition

Of the DMSP catabolising genes tested, the most abundant one was DmdA/Dall, which formed 90 % of the total genes (Figure 4.7.A). The common lyase gene DddP was present at 8 % and the gene DmdA/A1 at 1 % (Figure 4.7.A).

The microbial community consisted of more than 30 bacterial OTUs and 50 phytoplankton OTUs. However, prokaryotic biodiversity only presented three different taxa at the *4thtaxonomic rank* at >1 % of relative abundance and was dominated by *Rhodobacterales* (80 %) with contributions of *Flavobacteriales* (18 %) and *SAR 11 clade* (1.6 %) (Figure 4.5.B). The *Flavobacteriales* group was dominated by *Polaribacter 1* (70.8 %) and harboured *Polaribacter* 2 (7.3 %), *Ulvibacter* (1.2 %), uncultured *Flavobacteriaceae* (14.4 %), and uncultured *Cytophaga* sp. (6.4%). The *Rhodobacterales* were dominated by *Loktanella* (43 %) and *Sulfitobacter* (54 %), as well as 3 % of Other *Rhodobacteraceae*, and *Candidatus Pelagibacter* was present at 8% of SAR11 clade (Figure 4.7.B).

Due to multiple copy numbers of 18S in some of the eukaryotes present in our samples, a representation of the relative abundance of the microorganisms forming the eukaryotic community was not possible. Instead, a phylogenetic tree was constructed indicating the phytoplankton genera present in the samples (Figure 4.7.C), which consisted of 18 diatom taxa and 12 dinoflagellate genera, among others. Considering the known intracellular DMSP content of these groups, dinoflagellates, which generally contain high amounts of intracellular DMSP, were the potential DMSP producers, while diatoms, which usually contain low amounts of intracellular DMSP, were potentially DMSP sinks.

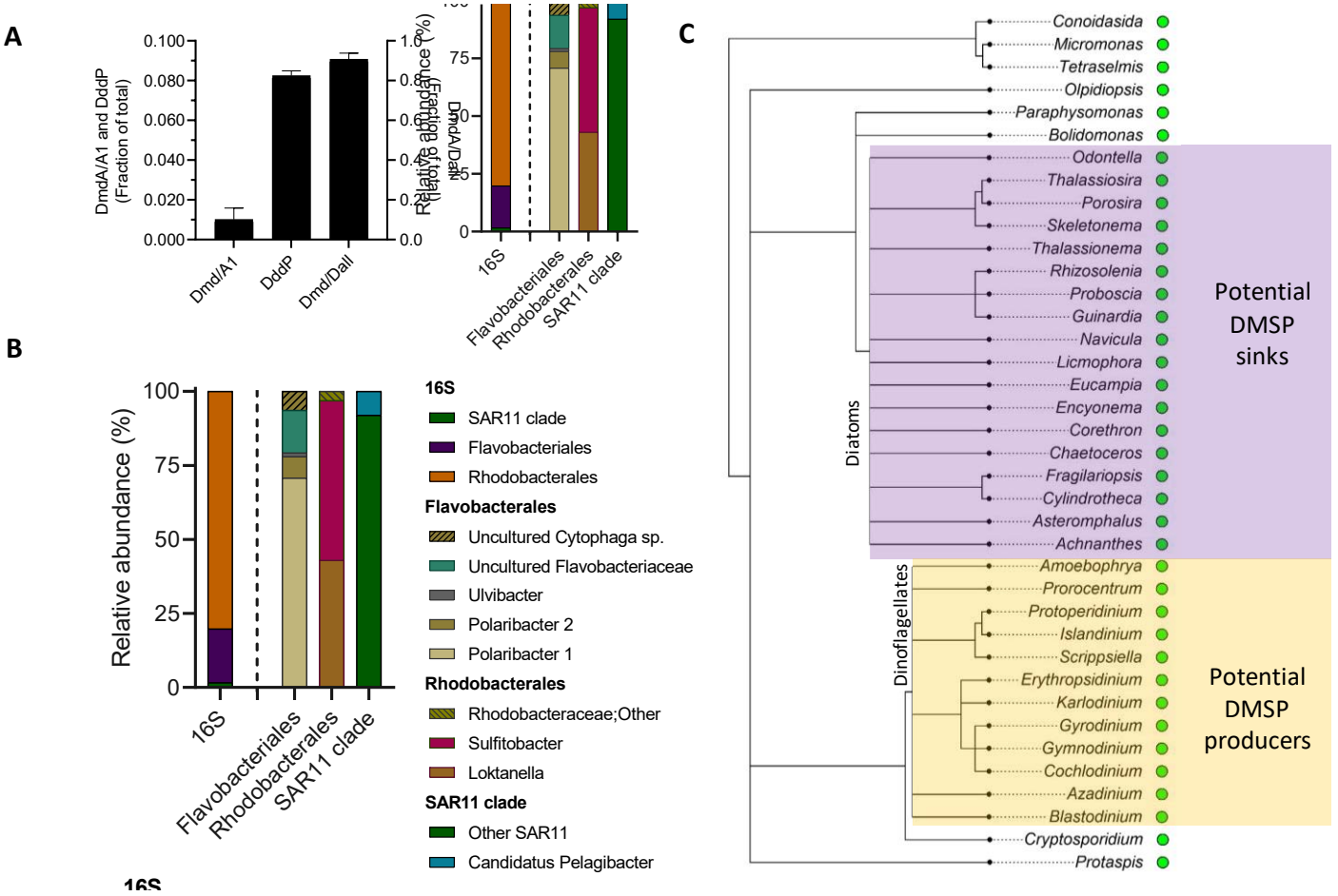

Figure 4.7. Abundance of DMSP degradation genes and microbial community composition. *(A) Data represent fraction of total for DmdA/A1, DmdA/Dall and DddP for initial experimental waters. Mean +/- SD (n=2). DmdA/A1 and DddP represented in left Y axis, DmdA/Dall represented in right Y axis (B) Bacterial community (16S) at the 5thtaxonomic rank using relative abundance and Flavobacteriales, Rhodobacterales and SAR11 clade composition. Data represent mean (n=4). The graphs only display results with >1% of relative abundance for clarity purposes. (C) phylogenetic tree of eukaryotic (18S) community at the genus level. Potential DMSP sinks are highlighted in purple and potential DMSP producers in yellow. Green dots indicate genus was present at the initial time.*

4.4.3. Experiment 2: Identification of microorganisms that assimilate DMSP into their biomass

4.4.3.1. Nutrients concentrations and Fv/Fm values

Nutrient concentrations at the initial time point were 8.98 µM for NOx, 0.81 µM for phosphate and 42.91 µM for silicate (Figure 4.8.A), and decreased significantly over time to a final NOx concentration of 1 µM in control and 0.54 µM in +DMSP (F= 431.53, P=0.001). For phosphate, concentrations declined to 0.35 and 0.18 µM in controls and +DMSP samples, respectively (F=108.648, P=0.001), while silicate declined to 27 and 16 µM of silicate in controls and +DMSP samples, respectively (F=34.588, P=0.001). F_V/F_M significantly increased during the first 48 h from 0.53 to 0.66 and 0.61 for controls and +DMSP samples, respectively (t= 6.26, P_{MC}=0.001), and remained constant for the rest of the experiment at ~0.6, with no differences between treatments (Figure 4.8.B).

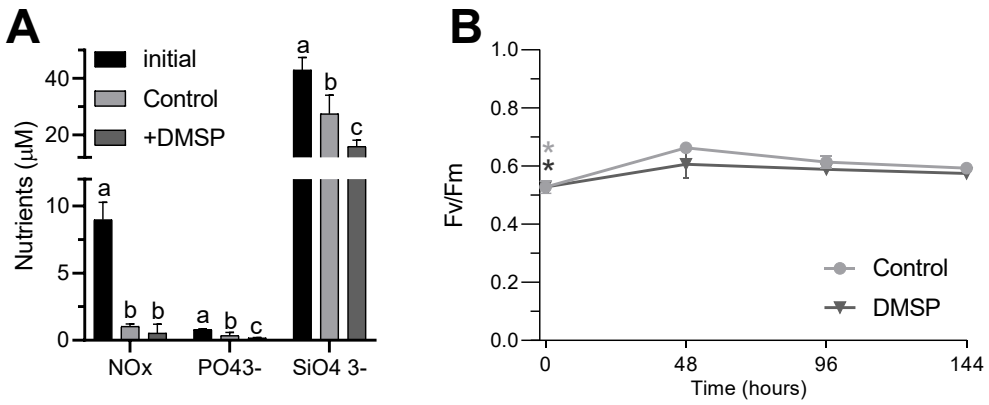

Figure 4. 8. Nutrient concentrations and maximum quantum yield of PSII (Fv/Fm) during experiment 2. *(A) Concentrations of nitrates (NOx), Phosphate (PO_4^{3-}) and silicate (SiO_4^{3-}) for experimental waters at initial time (black bars) and final time for controls (light grey bars) and +DMSP (dark grey bars) samples. (B) Maximum quantum yield of PSII calculated as $F_V/F_M = (F_M - F_O)/F_M$ for controls (light grey dots), and +DMSP (dark grey triangles) samples throughout the experiment. Data represent mean ± SD (n=3). Error bars not shown if smaller than symbol. Lowercase letters above bars denote statistical differences at P<0.05. * Above error bars indicates statistical differences at P<0.05 between time points.*

4.4.3.2. Analysis of sulfur compounds: DMSPt, DMSPd

To quantify the amount of DMSP retained in Antarctic natural microbial communities, we enriched water samples with dissolved DMSP (final concentration 7 µM) and incubated them for 144 h. As expected, significant differences between controls and DMSP-enriched samples occurred for all DMSP concentrations, total DMSP (Pseudo-F= 28,313, P_{MC}=0.01), dissolved DMSP (Pseudo-F= 21,144, P_{MC}=0.01) and particulate DMSP (Pseudo-F= 114.82, P_{MC}=0.01), calculated by subtracting DMSPd from DMSPt (Figure 4.9).

Total DMSP increased from 68 to 77 nM DMSP in the control samples, while significantly declining from 13.26 to 4.41 µM at a rate of 1.48 µM day^{-1} in the DMSP-enriched incubations (t= 3.73, P_{MC}=0.017) (Figure 4.9.A). After 144 h, more than 60 % of the initial DMSPt was lost in the +DMSP samples. For control samples, DMSPd concentrations did not change over time, remaining constant at ~12 nM (Figure 4.9.B). In contrast, DMSP-enriched samples showed a continuous and significant decline (t=4.28, P_{MC}=0.007) from 7.34 µM at the initial time point to 1.45 µM after 144 h (Figure 4.9.B). For the +DMSP samples, a total of 80 % of initial DMSPd was lost over the 144 h, with a loss rate of 0.98 µM day^{-1}. Particulate DMSP, calculated by subtracting DMSPd from DMSPt (Figure 4.9.C) increased from 54.86 to 85.39 nM for the control samples at a rate of 5.09 nM day^{-1}, while declining from 5.97 µM to 2.96 µM in the +DMSP samples with a loss rate of 0.50 µM day^{-1}.

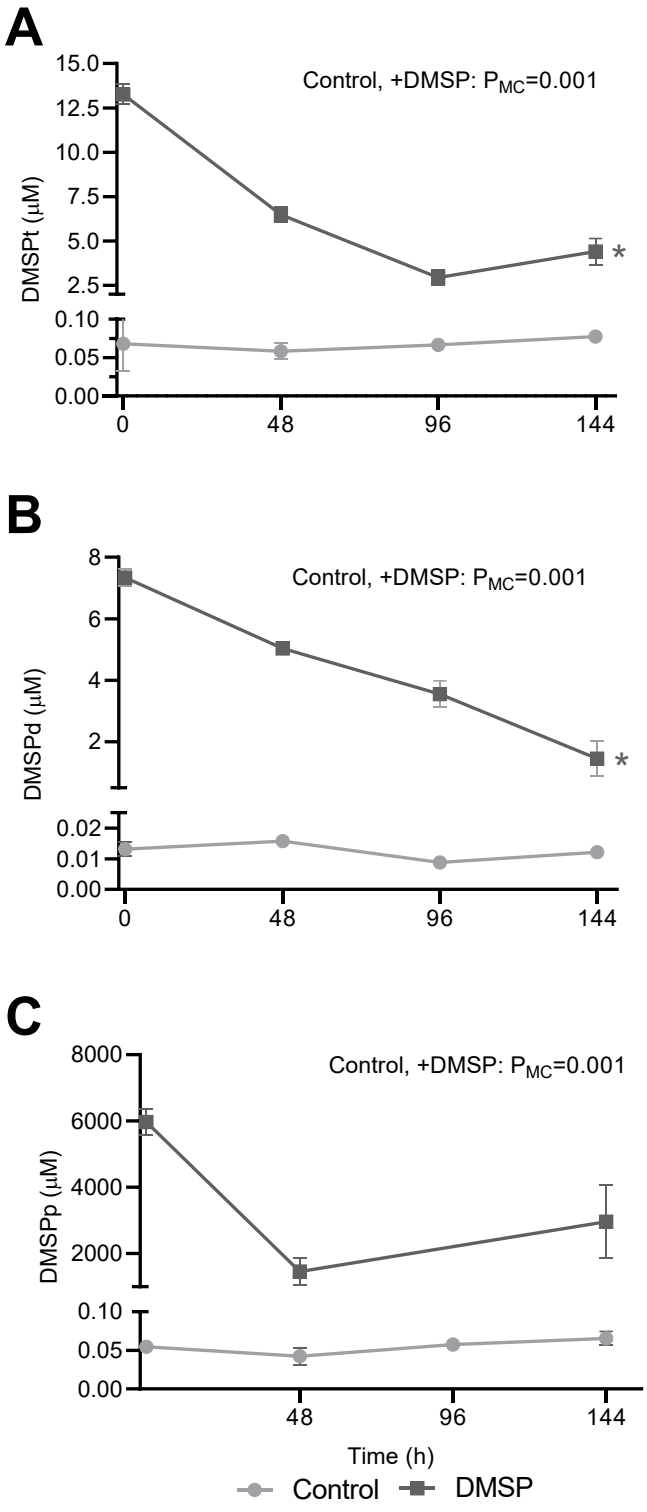

Figure 4.9. DMSP concentrations during experiment 2. *(A) Time course of DMSPt, (B) time course of DMSPd, (C) time course of DMSPp. Data represent mean ± standard deviation (n=4). Results from Permanova statistical tests are shown in the top right corner of each plot for treatment factor if P<0.05. * Denotes differences between time initial and time final at a significance level of P<0.05. Note: break in y axes.*

4.4.3.3. Gene abundance

As with experiment 1, the most abundant gene was DmdA/Dall, which made up 99.9 % of the total genes detected (Figure 4.10). The common lyase gene DddP was present at 0.1 % and the gene DmdA/A1 at 0.0001 % (Figure 4.10).

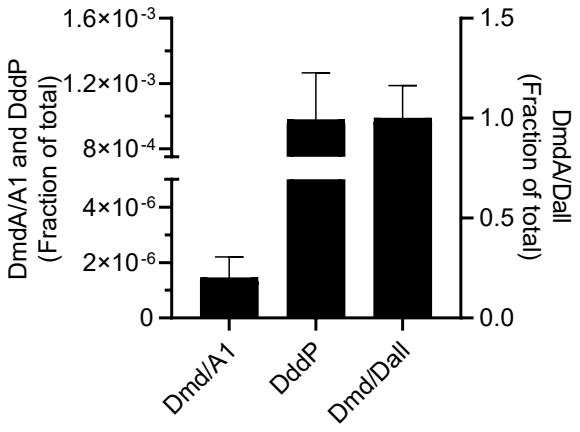

Figure 4.10. Abundance of DMSP degradation genes. *Abundance of DmdA/A1, DmdA/Dall and DddP for initial waters of experiment 2. Data represent mean +/- SD (n=2). DmdA/A1 and DddP genes represented in the Y right axis and DmdA/Dall in the Y right axis. Note: break in left y axis.*

4.4.3.4. Community shift 16S and 18S

Although additions of DMSP were artificially high (final concentration 7 µM DMSP), we chose these conditions so that they could promote the growth of indigenous DMSP utilising microbes and thus facilitate the identification of discriminatory taxa. The bacterial community as determined by 16S, changed significantly within the first 24 h (Global R =0.506, P=0.001), but showed no treatment effect (Figure 4.11.A and B). The initial coastal community was dominated by *Rhodobacterales* that was present at a relative abundance of 72.73 % (Figure 4.11.A). Other bacterial taxa at >1 % relative abundance included *Flavobacteriales* (21.83%), *SAR11 clade* (2.68%), *Cellvibrionales* (1.71%) and *Thiotrichales* (1.05%). After 48 h, the initial community composition changed (t=2.6, P_{MC}=0.02), but remained similar between treatments. The dominant group in the initial samples, *Rhodobacterales,* declined in relative abundance to 19.92 and 13.89% for controls and +DMSP samples, respectively (Figure 4.11.A), while the other dominant taxa, *Flavobacteriales*, *SAR11 clade*, *Cellvibrionales* and *Thiotrichales,* all decreased to <1% relative abundance. However, overall biodiversity increased with the appearance of *Cytophagales* (32.49 and 40.74%), *Rhodothermales* (~1.70%), *Kordiimonadales (*29.06 and 31.77%),

Sva0853 (~4.5%) and *Enterobacteriales* (11.80 and 7.52%). After 96 h, there were only small variations in bacterial composition, none of which were significant, and no differences between treatments. After 144 h, control and +DMSP enriched samples remained without significant changes, except for the appearance of *Oceanospirillales* in +DMSP samples at a relative abundance of 1.5 %. However, when looking at higher taxonomic levels, differences between treatments appeared for this time point. *Enterobacteriales* were conformed only of *Yersinia* for the +DMSP samples, while the controls also contained 12.7% of *Escherichia coli. Sva0853* was only conformed of SAR 324 for control samples while it was only present at 34.67% for +DMSP samples that also contained 15.3% of S25_1238 and 50% of unidentified microorganisms. *Cytophagales* did not change significantly between treatments and was dominated by *Flammeovirgaceae* with 92% relative abundance and Cytophagaceae at 6.5%. *Rhodobacterales* also did not change significantly between treatments and was composed by ~30% *Loktanella*, ~21% *Octadecabacter* and ~48% *Phaeobacter*. The nMDS plot supported these similarities between treatments and time points, and only the initial waters were clearly grouped and separated from the rest of the samples (Global R: 0.506, P=0.001) (Figure 4.11.B).

The initial eukaryotic community consisted of 12 genera of diatoms, 13 dinoflagellates and 1 chrysophyte (Figure 4.12.A). The biodiversity of control samples substantially diminished from 26 to 19 genera after 48 h with the major loss occurring within diatoms, and remained constant after that time. For +DMSP samples, biodiversity progressively diminished over time to 15 genera at 144 h also with the major loss occurring within diatoms. After 48 h, control samples harboured 19 different genera of which 5 were diatoms and 12 dinoflagellates, while +DMSP samples diminished its number of diatoms to 8 genera and increased the dinoflagellates to 16. After 96 h, control samples lost 1 dinoflagellate genus and +DMSP samples decreased their biodiversity from 26 to 21 different genera after the disappearance of 4 dinoflagellates and 2 diatoms. After 144 h, both treatments harboured only 5 diatom genera, and controls were more biodiverse than +DMSP samples with 12 dinoflagellates compared to just 9 dinoflagellates in the +DMSP samples. A subset of the nMDS plot after removing 4 different replicates, did not show any grouping of samples by treatment or time except for samples at the initial time point (Global R: 0.646, P=0.019) (Figure 4.12.B). As per experiment 1, a phylogenetic tree showing presence and absence of phytoplankton genera was preferred over relative abundance to display the eukaryotic microbial composition for

both treatments over time (Figure 4.12 C and D). After 48 h (T1), +DMSP samples were more diverse than control samples with the presence of *Asteromphalus*, *Actinocyclus*, *Cylindroteca, Prorocentrum*, *Peridinium*, *Heterocapsa, Cochlodinium* and *Cryothecomonas.* After 96 h (T2), all these genera except for *Cylindroteca sp. and Cryothecomonas sp.* had disappeared from +DMSP samples, and *Asteroplanus*, *Blastodinium* and *Micromonas* became present, while *Eucampia* was only present in the control samples. After 144 h, *Blastodinium, Gymnodinium* and *Erythropsidinium* were present only in controls, while the other 15 genera were present in both treatments.

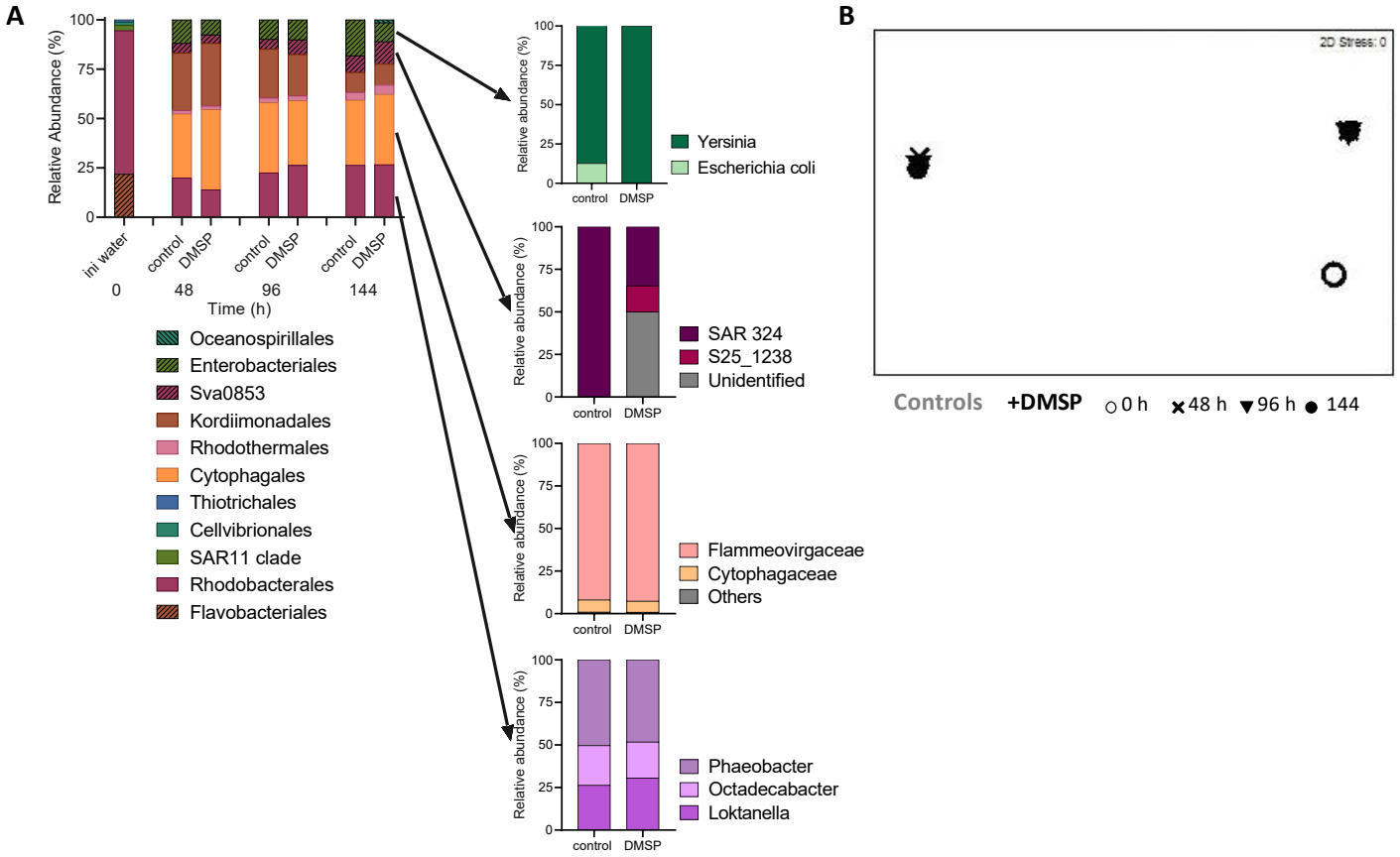

Figure 4.11. Prokaryotic (16S) composition and nMDS plot during experiment 2*. (A) Bacterial community at the 4th taxonomic level for each time point and treatment only displaying results with >1% of relative abundance with subsets displaying the composition of Rhodobacterales, Cytophagales, Sva0853 and Enterobacteriales at the final time point for both treatments. (B) nMDS plot with all replicates and time point for 16S data. nMDS plots are based on resemblance calculated with S17 Bray-Curtis similarity matrices.*

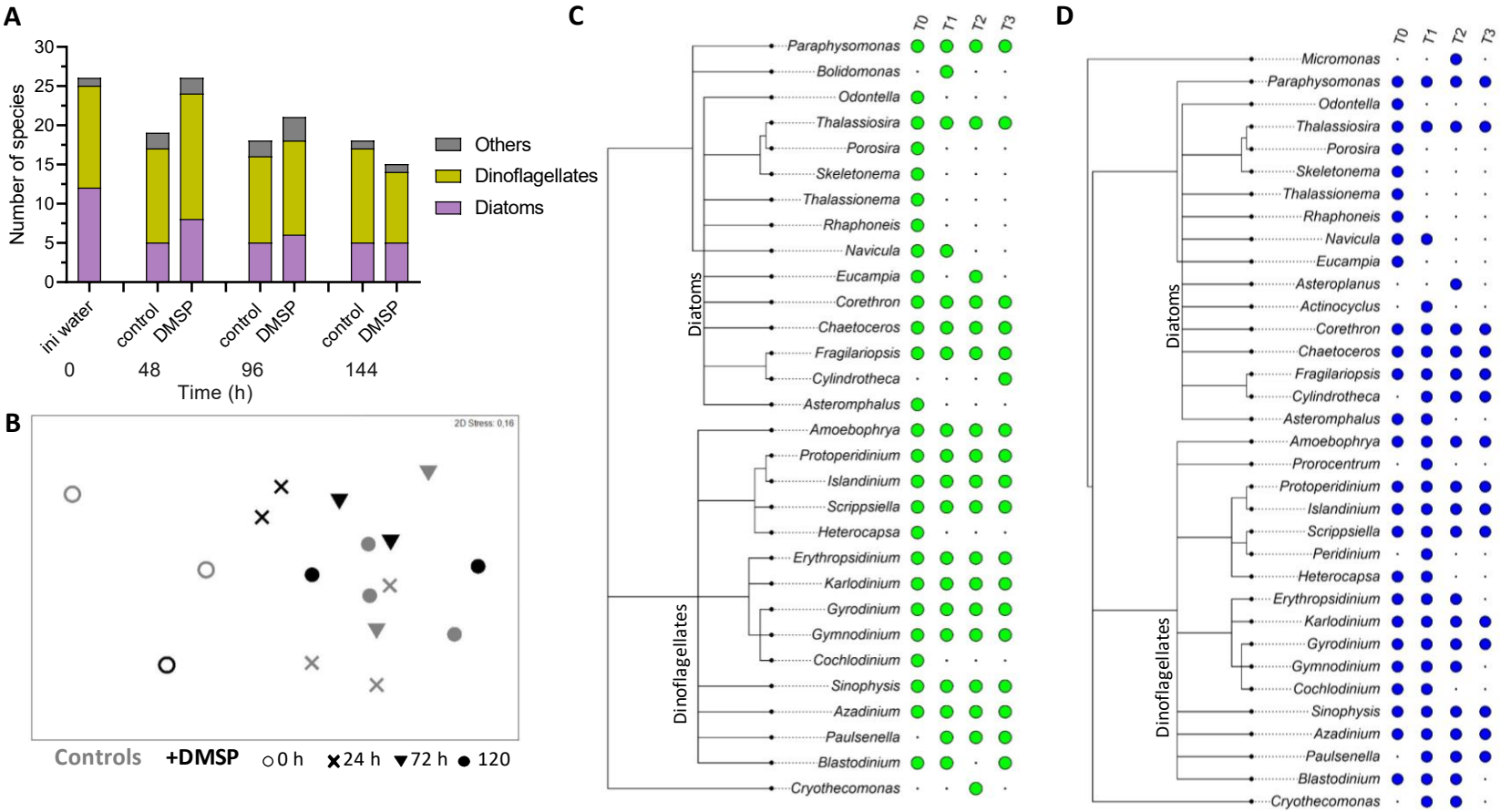

Figure 4.12. Eukaryotic biodiversity, nMDS subset plot and phylogenetic trees for eukaryotic (18S) composition during experiment 2. *(A)Eukaryotic biodiversity for initial water, control and treated samples during experiment 2, (B) nMDS subset plot for all time point for 18S data after removing 4 replicates, (C) phylogenetic tree of presence (green circles)/ absence (dots) of eukaryotic genera for control samples for each time point T0 (0h), T1(48h), T2(96h), T3(144h), and (D) phylogenetic tree of presence (blue circles)/ absence (dots) of eukaryotic genera for +DMSP samples for each time point T0 (0h), T1(48h), T2(96h), T3(144h).*

4.4.3.5. Correlation and Analysis of Similarity Percentages (SIMPER)

Even though we used much higher levels of DMSP in our experiment than found in natural polar environments, only few bacterial taxa seemed to benefit from the addition of DMSP contrasting with several phytoplankton taxa that benefited from its presence, and only *Rhodobacterales* significantly correlated with DMSP total concentrations (r=0.8741) (Figure 4.13). Treatments showed low percent dissimilarity with values of 16.87% for prokaryotes and 29.95% for eukaryotes (Table 4.2). The prokaryotic taxa that contributed the most to treatment dissimilarity were *Cytophagales*, *Kordiimonadales* and *Enterobacteriales* that belong to bacteroidetes, alpha- and gamma- proteobacteria respectively, and together contributed 63.02% of treatment dissimilarity and, on average were more abundant in the controls (Table 4.2.A). Dinoflagellates were the phytoplankton group that contributed the most to treatment dissimilarity with 11 out of 21 genera needed to describe 71% of treatment dissimilarity, and like the prokaryotic community, were more abundant in the controls (Table 4.2.B).

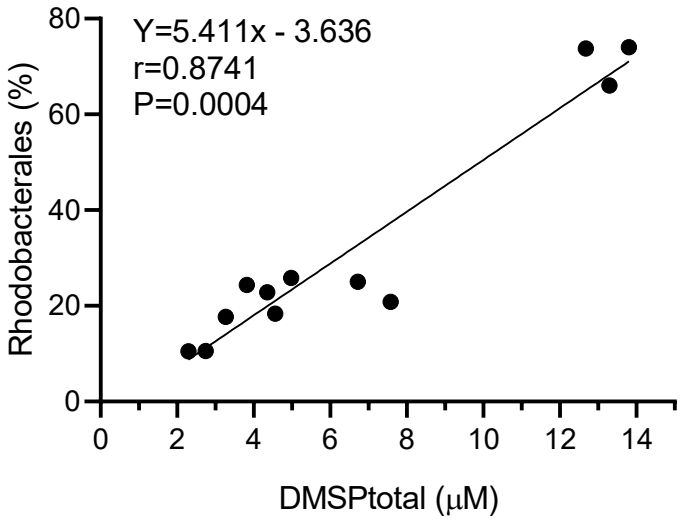

Figure 4.13. *Correlation between DMSP total concentrations and Rhodobacterales relative abundance.*

Table 4.2. Major microbial taxa and their contribution to treatment dissimilarity. *(A) Prokaryotic taxa (B) Eukaryotic taxa. All taxa present at >1% relative abundance were used for treatment pair-wise comparisons.Yellow shadow indicates dinoflagellates.Pink shadow indicates diatoms. Av.Abund is the average abundance of the respective taxa for a specific treatment, Av.Diss is the average dissimilarity between treatments, Diss/SD is the ratio (dissimilarity/standard deviation), Contrib% is the contribution of each group. Cum.% is the cumulative contribution of taxa. Dissimilarity between treatments is indicated in the first row.*

A (16.87% diss)	**Control Av.Abund**	**DMSP Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Cytophagales	0,75	0,55	3,92	0,63	23,23	23,23
Kordiimonadales	0,65	0,49	3,48	0,65	20,63	43,86
Enterobacteriales	0,56	0,41	3,23	0,73	19,15	63,02
Sva0853	0,48	0,37	2,63	0,66	15,61	78,62
Rhodothermales	0,38	0,30	2,07	0,65	12,24	90,87

B (29.95% diss)	**control Av.Abund**	**DMSP Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Gymnodinium	0,77	0,56	1,42	0,86	4,74	4,74
Blastodinium	0,62	0,67	1,36	0,93	4,53	9,27
Cryothecomonas	0,38	0,78	1,34	0,91	4,46	13,73
Cochlodinium	0,31	0,44	1,24	0,92	4,15	17,88
Erythropsidinium	0,69	0,78	1,07	0,75	3,57	21,45
Bolidomonas	0,38	0,22	1,03	0,67	3,44	24,90
Protoperidinium	0,85	0,67	1,02	0,58	3,42	28,31
Cylindrotheca	0,46	0,67	1,02	0,74	3,40	31,72
Scrippsiella	0,85	0,78	1,01	0,60	3,36	35,08
Paraphysomonas	0,85	0,78	1,01	0,60	3,36	38,44
Paulsenella	0,69	0,67	0,99	0,60	3,29	41,73
Micromonas	0,15	0,44	0,98	0,77	3,27	45,00
Islandinium	0,85	0,89	0,95	0,55	3,16	48,16
Porosira	0,38	0,11	0,89	0,78	2,97	51,13
Sinophysis	1,00	0,78	0,88	0,50	2,93	54,06
Karlodinium	1,00	0,78	0,88	0,50	2,93	57,00
Corethron	1,00	0,78	0,88	0,50	2,93	59,93
Chaetoceros	0,92	0,89	0,85	0,50	2,83	62,76
Thalassiosira	0,85	0,89	0,84	0,53	2,80	65,56
Katodinium	0,23	0,11	0,82	0,66	2,73	68,29
Eucampia	0,46	0,33	0,81	0,58	2,70	71,00
Asteromphalus	0,23	0,44	0,80	0,72	2,67	73,67
Navicula	0,38	0,44	0,80	0,58	2,66	76,33
Acanthamoeba	0,23	0,11	0,78	0,65	2,61	78,94

Asteroplanus	0,08	0,22	0,61	0,56	2,03	80,97
Heterocapsa	0,23	0,22	0,60	0,51	2,01	82,97
Rhaphoneis	0,15	0,11	0,56	0,43	1,88	84,86
Fragilariopsis	1,00	0,89	0,46	0,34	1,54	86,39
Cryptosporidium	0,08	0,11	0,45	0,45	1,49	87,88
Peridinium	0,00	0,22	0,44	0,51	1,48	89,35
Prorocentrum	0,08	0,22	0,43	0,44	1,45	90,81

4.5. Discussion

Dimethylsulfoniopropionate (DMSP), which is abundant in Antarctic waters, is taken up by marine heterotrophic bacteria and some phytoplankton species. While there is considerable evidence of bacterial uptake and processing of DMSP, research on DMSP uptake by phytoplankton has been limited to a few lab culture studies and one oceanographic one. This study investigated whether members of the Antarctic coastal phytoplankton community take up available DMSP from the environment and the effect that increasing DMSP availability has on the microbial community composition in natural sea waters of East Antarctica. We found that short term responses included a decrease in DMSPd and an accumulation of DMSPp for all fractions of the community (14 µm, 8 µm, 3 µm, 0.22 µm,) in a size-dependent manner, indicating that not only bacteria but also phytoplankton take up DMSP, and that phytoplankton form a significant sink for dissolved DMSP. Longer-term incubations showed that the concentrations of DMSP total and dissolved disappeared with time, with minimal effect on the microbial composition, suggesting that the dominant process occurring with DMSP enrichment was the cleavage of DMSP to DMS by the microbial community.

4.5.1. Quantification of DMSP uptake by different fractions of the microbial community

The seawater characteristics of both experiments were typical of Antarctic waters, with temperature, salinity and pH values within ranges of previous Antarctic studies (Davidson & Biology 1992; Perrin & Lu 1987). Similarly, the major nutrients (nitrate, phosphate and silicate) were present at high concentrations, indicating that phytoplankton growth was not limited by macronutrients (Ehnert and McRoy, 2007; Martin and Fitzwater, 1988; Sedwick et al., 2000; Takeda, 1998). The differences in pH and nutrient between the initial waters from the two sampling time points, indicates increased production during the five days between samples, with the reduction in silicate suggestive of a diatom bloom during the second sample. Large diatoms (>14 µm), such as the ones identified in this study (*Corethron,*

Asteromphalus, Thalassiosira and Chaetoceros), often dominate Antarctic coastal waters (Almandoz et al., 2008; Annett et al., 2010; Kopczyńska et al., 2007a; Roden et al., 2013; Yang et al., 2013). However, they are generally considered to be non-producers of DMSP (Keller, 1989; Lyon and Mock, 2014; McParland and Levine, 2019). Given the absence of the haptophyte *Phaeocystis antarctica*, a species considered one of the main DMSP-producer in polar waters (Boekel and Stefels, 1993), indicates that it was most likely the presence of large dinoflagellates that contributed to DMSP production in this study, supporting the very high initial surface water concentrations of around 60 nM.

The uptake of DMSP by the different fractions of the microbial community determined over 8 h (experiment 1) showed constant values of DMSPt , but a rapid loss of DMSPd (Figure 4.4. A-C), indicating that the majority of the DMSP was taken up from the surrounding sea water by the cells and accumulated in the cells as particulate DMSP (Figure 4.4. D-E). These data support the hypothesis that phytoplankton may take up DMSP (Malmstrom et al., 2005; Petrou and Nielsen, 2018; Ruiz-González et al., 2012c; Spielmeyer et al., 2011; Vila-Costa et al., 2006b) and provide evidence that not only bacteria, but also larger members of the microbial community act as important DMSP sinks in Antarctic waters. As expected, the largest fraction (>14 μm), composed of dinoflagellates and diatoms, contained the most particulate DMSP, reflecting the significance of dinoflagellates as prolific producers of DMSP. Interestingly however, the increase in DMSPp with enrichment indicates that some polar diatoms despite not being able to synthesise DMSP (Lyon and Mock, 2014), may still utilise it for their own cellular regulation (cryoprotection and osmoregulation) by taking it up from the environment. Comparable results were found in a study from Arctic and Antarctic waters where the sulfur from S^{35}-DMSP was detected by microautoradiography in pennate and centric diatoms, as well as dinoflagellates (Ruiz-González et al., 2012b).

Many strategies have been described in polar phytoplankton to cope with the extremely cold temperatures and dramatic salinity changes (Morgan-Kiss et al., 2006). Some of these strategies include an increase in polyunsaturated fatty acids (PUFAs), which maintain membrane fluidity at freezing temperatures (Morgan-Kiss et al., 2006); production of ice-binding proteins that prevent ice recrystallization (Bayer-Giraldi et al., 2010; Janech et al., 2006; Raymond et al., 2009); production of exopolymeric substances such as glycoproteins that modify external microenvironments and maintain salinity levels (Krembs and Deming,

2008); and synthesis of specific amino acids that maintain internal osmotic balance (Krell et al., 2007). Moreover, as Stefels (2000) concludes, the main role of DMSP is probably not to maintain the osmotic balance, as its intracellular concentrations change too slowly against salinity shifts, and synthesis of multiple osmolytes and antioxidants, not only DMSP, have been observed during salinity acclimation in *Fragilariopsis cylindrus* (CCMP 1102) (Lyon et al. 2011). Therefore, all Antarctic microbial fractions, including the large diatoms, while possessing the capability to take up DMSP may not necessarily utilise it. More research on DMSP inside the phototrophic cell must be pursued to reveal the principal role of DMSP inside the cell, as well as reveal potential DMSP degradation pathways and genes in phototrophs.

The microbial community composition was typical of Antarctic waters with strong presence of diatoms and dinoflagellates in the eukaryotic assemblage (Kopczyńska et al., 2007b), as well as common dominant bacteria such as *Flavobacteriales, Rhodobacterales and SAR11* (Bowman and Nichols, 2005, 2002; Malmstrom et al., 2007; Straza et al., 2010). The main DMSP producers were likely the dinoflagellates (Keller et al., 1989; McParland and Levine, 2019), plus the prokaryotic *Rhodobacterales,* which was present at an abundance of 80 % in the 16S fraction, and is known to harbour the DMSP-synthesis gene dsyB (Curson et al., 2017). Dinoflagellates are considered the most prolific producers of DMSP in the marine environment (Keller et al., 1989), so their presence in these water samples would account for the high levels of DMSP measured in this study. We propose that the large diatoms, including those identified by light microscopy, *Corethron*, *Asteromphalus*, *Thalassiosira* and *Chaetoceros,* were the major DMSP sinks in the biggest fraction (>14 µm), while smaller diatoms may be the potential DMSP sinks for the >8 and >3 µm. *Sulfitobacter* and *Loktanella (Rhodobacterales)* were the predominant sink for the smallest fraction (>0.22 µm) and belong to the recognized DMSP catabolising clade of Roseobacters (Reisch et al., 2011; Zeng et al., 2016). These results challenge the idea that prokaryotes are the major DMSP sinks in the ocean. Instead, the increase in particulate DMSP in all fractions with DMSP-enrichment demonstrates the phylogenetic diversity of potential DMSP sinks in the marine environment, and the need to look at both sinks and sources to gain a complete understanding of the marine sulfur cycle.

The dominance of the gene DmdA (clade D) is consistent with metagenomics surveys that have determined it the most abundant DMSP degrading gene in the ocean (Howard et al. 2006, 2008) and the order of magnitude lower abundance of DddP and DmdA/A1 match values reported previously (Howard et al. 2008; Levine et al. 2012; Liu et al. 2018; Todd et al. 2009; Varaljay et al. 2012) as discussed in previous chapters. Due to the high relative abundance of *Loktanella* and *Sulfitobacter,* both of which belong to the *Roseobacter clade* (80%) and have a widespread ability to demethylate DMSP, we hypothesise that these groups represent the greatest potential DMSP consumers in the surface waters of Antarctica at the time of sampling. *Roseobacters* have also been found to play an important role in DMSP catabolism in surface waters of Kongsfjorden, in the Arctic, during summer (Zeng et al., 2016). Both results are in accordance with findings by Howard et al. (2006), who after searching for DmdA homologs in natural bacterioplankton communities, suggested that *Roseobacters (mostly Rhodobacteraceae)* may dominate demethylation in coastal regions and during phytoplankton blooms (high DMSP concentration scenarios). Therefore, most probably, the demethylation of DMSP was the predominant pathway and once the sulfur demand was met (i.e. in control samples), the rest of the DMSP was taken up by other sources, instead of being cleaved into DMS.

It is possible that, despite lacking evidence, *Flavobacteriales* (which had a relative abundance of 18 %) may contain some DMSP degradation genes, as they have been observed to increase transcript abundance during DMSP-enriched experiments (Pinhassi et al., 2005; Vila-Costa et al., 2010). However, our knowledge on DMSP degradation genes is still in its infancy and new genes are continually being discovered (Lei et al., 2017; Todd et al., 2011, 2009) in bacteria, phytoplankton (Alcolombri et al. 2015) and Antarctic environments (Delmont et al., 2015). Therefore, it is impossible to rule out that some bacterioplankton taxa lacking DMSP degradation genes may take up DMSP for any of its many other assigned roles (Kiene et al., 2000; Kirst et al., 1991; Strom et al., 2003; Sunda et al., 2002).

4.5.2. Dynamic changes in microbial community composition and DMSP degradation genes abundance

Over a longer period of incubation we saw a strong decline in DMSP (DMSPt and DMSPd) concentrations in the DMSP-enriched treatment confirming that the DMSP that was likely

taken up by the cells in the first 24 h, (based on rapid uptake in experiment 1), was catabolised into DMS or MeSH over the following days. This considerable DMSP loss observed over 144 h in the DMSP-enriched environment can be explained by the quick turnover of DMSP in seawater, which can reach 129 nM d^{-1} (Ronald P Kiene and Linn, 2000; Ledyard et al., 1996) and even higher values during a phytoplankton bloom (Duyl et al., 1998). Incubations of 20-31 h of oceanic and coastal waters with ^{35}S-DMSP have shown that DMSP was mostly metabolized by microorganisms with only a minor fraction accumulated into the biomass, and that high concentrations of DMSP enriched the volatiles such as DMS (Kiene & Linn, 2000). Therefore, the consistent and high abundance of *Roseobacter* found over the whole duration of the experiment could explain the high DMSP loss rates in this study.

As with experiment 1, the main DMSP producers were likely the dinoflagellates along with the prokaryotic *Rhodobacterales,* which also synthesises DMSP (Curson et al., 2017; Keller et al., 1989; McParland and Levine, 2019), while *Rhodobacterales,* the major group harbouring DmdA genes (Curson et al., 2011; Howard et al., 2006; Newton et al., 2010; Todd et al., 2009), were probably responsible for the high proportion of DmdA genes measured at the outset and are complicit in the large loss of DMSP from the system within the first 48 h.

After 48 h, we saw a significant decrease in the relative abundance of key taxa like *SAR11, Flavobacteriales* and *Rhodobacterales*, while *Cytophagales* (Bacteroidetes), *Rhodothermales* (Bacteroidetes), K*ordiimonadales (alphaproteobacteria)* and *Enterobacteriales (gammaproteobacteria)* among others, increased. A study of the distribution patterns of *Rhodobacter, Bacteroidetes* and *SAR11* of the Southern Ocean observed that the growth and distribution of these taxa were especially susceptible to environmental change (Tada et al., 2013), making it possible that the initial manipulation of the seawater, including the removal of grazers, had a significant effect on the community composition in this study. The implications of this shift would include a major contribution on DMSP cycling by the species that became dominant after the initial manipulation of the samples, as well as a lower contribution of the species that decreased in abundance. And so, after 48 h, *Rhodobacterales* had decreased in abundance and ceased being the dominant DMSP cataboliser, and it's interesting to note that the rate of DMSP loss was reduced after 48 h, correlating with the loss of *Rhodobacterales* from the community. With the relative decline in *Rhodobacterales*, there was a concomitant increase in the abundance of *Cytophagales*, K*ordiimonadales* and

Enterobacteriales and may have significantly contributed to continued DMSP degradation, as *m*any of the DMSP catabolising genes are widespread among α-proteobacteria and γ-proteobacteria (Howard et al., 2008a; Moran et al., 2012; Reisch et al., 2011). Furthermore, field studies have shown that both, α- and γ- proteobacteria consume DMSP (Vila‐Costa et al., 2007) and in the case of *Cytophagales*, despite not harbouring any known DMSP-related genes, has previously been observed to increase in abundance as a response to a DMSP enrichment in the ice-covered Arctic Ocean (Maria Vila-Costa et al., 2008) as well as in other oceans (Ruiz-González et al., 2012a; M Vila-Costa et al., 2008; Vila-Costa et al., 2014, 2010).

The general lack of change in the microbial community composition with DMSP enrichment indicates that the bacterial sulfur demand was likely satisfied under the high DMSP ambient conditions, and that the additional DMSP was thus cleaved to DMS (Kiene et al., 1999). However, some differences within the Sva0853 and *Enterobacteriales* orders were observed at the last time point (144 h). Sva0853, which was solely formed by SAR 324 in the control samples, increased in biodiversity for the +DMSP samples with the appearance of S25_1238 and other unidentified families. High concentrations of DMSP may benefit some Sva0853 families allowing them to compete with the chemolithotroph SAR 324, which needs inorganic chemicals to obtain energy (Swan et al., 2011). *Enterobacteriales* however, decreased in biodiversity for +DMSP samples losing the presence of E.coli. Despite no general community differences between treatments, taxa such as *Cytophagales, Kordiimonadales* and *Enterobacteriales* together, which described 67.02% of the dissimilarity, support the idea that these taxa may be relevant in DMSP cycling in Antarctic coastal waters.

Like in the prokaryotes, the initial eukaryotic community also suffered the biggest change in the first 48 h, but were not negatively affected by the incubations, as evidenced by the high F_V/F_M. DMSP delayed the loss of biodiversity (Figure 4.9.A) and benefited the occurrence of two diatoms, two dinoflagellates and one chlorophyte (Figure 4.9.C-B), suggesting that DMSP may benefit these phytoplankton taxa. However, as the majority of the DMSP was removed in the first 48 h, as supported by the rapid uptake measured in experiment 1, it is more likely that most of the DMSP was cleaved to DMS by those with lyases, whose S demand was already satisfied.

The high abundance of DMSP degradation genes, together with the loss of DMSPt and DMSPd over time, provide evidence that DMSP was being utilised effectively in the surface waters of Prydz Bay. However, the minimal changes in the microbial community after enrichment with DMSP, suggest that the sulfur demand of both, phytoplankton and bacteria, were met by the natural concentrations of DMSP, and that the cleavage pathway was preferred over the demethylation one *(*Kiene et al., 1999*)*. These results are in accordance with the high levels of DMS measured from the Antarctic seawaters (Aumont et al., 2002; Kettle et al., 1999; Turner et al., 1995). If we want to reveal which microorganisms are capable of utilising DMSP in a sulfur rich environment, it would be useful to run experiments using cultures of Antarctic species and grow them in S-depleted medium to guarantee the occurrence of the demethylation pathway. Furthermore, the discovery of new DSMP degradation genes and information on the organisms harbouring them would be key to understand the fate of DMSP in Antarctic waters.

4.6. Conclusions

In this study we evaluated the uptake of DMSP by different fractions of the marine microbial community and revealed that both, bacteria and phytoplankton from natural Antarctic waters take up dissolved DMSP, including the larger eukaryotes. In the coastal waters of Antarctica, we suggest that not only dinoflagellates produce DMSP, but that *Sulfitobacter* and *Loktanella (Rhodobacterales),* may make a relevant contribution due to their high abundance, and assert that the main sinks for DMSP were diatoms and *Sulfitobacter* and *Loktanella*. By investigating the main DMSP catabolising genes and the microbial composition, we were able to show that demethylation is the dominant DMSP degradation pathway in Antarctica and *Sulfitobacter* and *Loktanella* its greatest DMSP consumers.

Through these experiments, we were able to demonstrate that the effect of DMSP enrichment on the composition of marine microbial populations of Antarctica was minimal, but established that cells were able to take up DMSP, and rather than store it, most likely catabolised it. It was shown that the bacterial and phytoplankton sulfur demands were satisfied with DMSP concentrations naturally found in this environment, as the added DMSP samples did not change the microbial community, and that added DMSP was likely to have been preferentially cleaved to DMS, as it was not used for bacterial growth, and it was

asserted that *Roseobacters*, *Cytophagales, Kordiimonadales, Enterobacteriales* may be relevant catabolisers of DMSP in Antarctic coastal waters.

The results of our study show that all fractions of the microbial community can take up DMSP, playing a role in DMSP cycling. Moreover, under conditions of high DMSP concentrations (such as bloom scenarios and melting sea ice), the Antarctic microbial community is likely to preferentially cleave the extra DMSP, resulting in high DMS concentrations and fluxes to the atmosphere, providing supporting evidence for the DMS hotspots often found in Antarctic waters. These data have added to the increasing pool of knowledge on the role of the community composition and the bacterial switch in determining DMSP cycling and DMS fluxes from our oceans, supporting the hypothesis that the bacterial switch is regulated by the microbial S-demand, and will preferentially demethylate DMSP until this demand is satisfied, after that, DMSP will be cleaved to DMS.

Chapter 5:

Revealing the sulfur cyclers on Port Hacking natural sea waters (NSW, Australia)

Chapter 5: Revealing the sulfur cyclers on Port Hacking natural sea waters (NSW, Australia)

5.1 Abstract

Dimethylsulfoniopropionate is produced by some species of phytoplankton and plays a key role in cellular responses to environmental change. Moreover, it is utilised by heterotrophic bacteria for cell growth, satisfying 95% of the sulfur and 15% of the carbon needs of the cell. Recently, the uptake of DMSP up to intracellular concentrations of 87mM by non DMSP producing phytoplankton species have been demonstrated, highlighting that many knowledge gaps exists regarding DMSP distribution through the microbial food web and its utilisation by different microorganisms. In this study, we traced the uptake and distribution of DMSP through a natural temperate microbial community collected from Port Hacking oceanographic station, located on the east coast of Australia off Sydney, during a *Trichodesmium* bloom. Our results showed that microbes from all size fractions of the community formed DMSP sinks, and that the largest fraction (>8 µm) was the dominant sink, with an increase of DMSPp by 138% with enrichment. Using microscopy, we identified three diatoms from the genera *Asteromphalus*, *Cylindrotheca* and *Chaetoceros* as the most likely phytoplankton taxa responsible for the uptake and storage of DMSP from this temperate community. These findings confirm the role of large phytoplankton taxa as potentially important players in the cycling of DMSP in coastal waters of Australia and emphasise the need to better understand the fate of accumulated DMSP and its significance in cellular metabolism.

5.2 Introduction

The temperate southeast coast of Australia is flanked by the East Australian Current (EAC), which originates in the Coral Sea and brings warm, oligotrophic waters south towards the Tasman Sea (Ridgway and Dunn, 2003). The EAC separates from the coast at 31-32ºS (Cetina-Heredia et al., 2014) where the majority of the water mass moves east towards New Zealand, while the remainder continues downstream , breaking down into warm and cold core eddies (Figure 1.A). This complex seasonal interplay of oceanographic features at the tail end of the EAC results in waters off the coast of Sydney region being highly dynamic in nature with a mix of EAC and Tasman Sea influences, as well as episodic upwelling events.

One of the long-term monitoring coastal stations in Australia is at Port Hacking (PH). It is located on the temperate east coast of Australia at approximately 30 km south of Sydney and about 8 km offshore and has a complex current structure due to a variety of oceanographic processes which, apart from the collision of the Tasman Sea and EAC with their associated eddies, include the northward propagating coastal trapped waves, local wind-driven currents operating over small distances and internal tides and waves of relatively high frequency (Lee and Pritchard, 1996; Middleton et al., 1996; Rendell and Pritchard, 1996). However, PH's shelf waters are usually dominated by the activity of the EAC, which uplifts cold and nutrient-rich waters (Rendell and Pritchard, 1996; Ridgway and Dunn, 2003), and is characterised by thermal stratification for most of the year (Figure 1.B) (Hahn et al., 1977)). Over the last few decades, the EAC has strengthened and moved poleward, resulting in higher temperatures, salinity, phosphate and chlorophyl *a*, and lower silicate and dissolved oxygen concentrations becoming more typical at PH station (Figure 1.B) (Thompson et al., 2009). Changes in phytoplankton composition have also been observed with an emerging dominance of tropical phytoplankton species such as *Trichodesmium erythraeum* and *Bacteriastrum spp*. (Ajani et al., 2014a, 2014b).

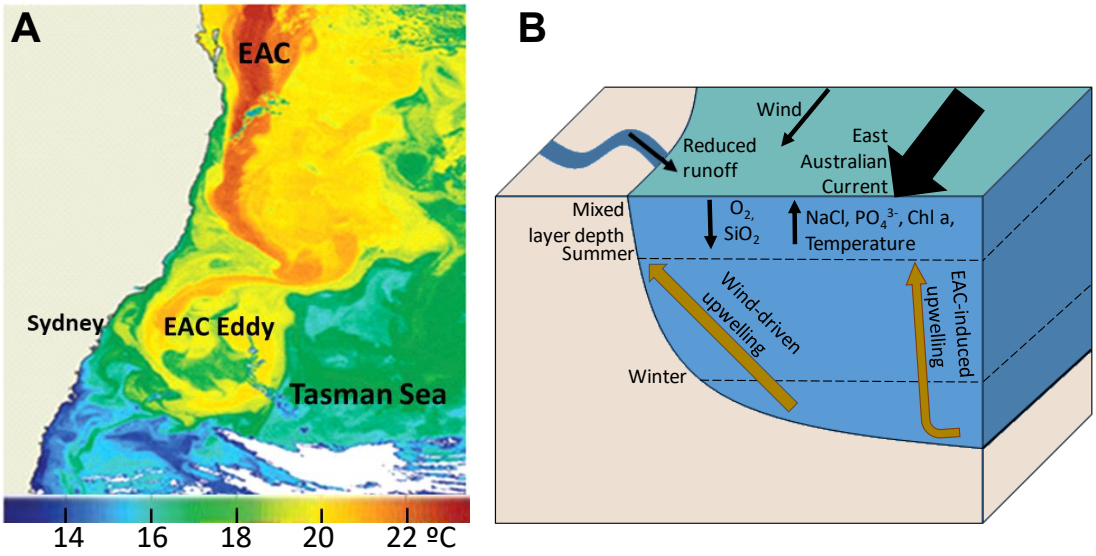

Figure 5.1. East Australian current (EAC) and oceanographic properties of Port Hacking station. *(A) Satellite image of sea surface temperature along the east coast of Australia showing the position of the EAC, the EAC separation and an Eddy relative to the Tasman Sea (modified from NOAA 11 TMS 45S 29 Sep, 1991 1615z, 1999 CSIRO). (B) Conceptualization of the oceanic properties and long – term trends at Port Hacking station (modified from Thompson et al. 2009)*

Phytoplankton species composition have started to shift in response to climate change (Winder and Sommer, 2012). However, phytoplankton have evolved many physiological strategies to deal with the constantly changing environment of the ocean (Behrenfeld et al., 2008; Mackey et al., 2008). One of these strategies is the capability to synthesise high amounts of DMSP, which protects the cells from osmotic and oxidative stress among others (Dickson and Kirst, 1986; Stefels et al., 1996; Sunda et al., 2002). However, not all phytoplankton species are capable of producing DMSP (Keller et al., 1989). Haptophytes and dinoflagellates are amongst the highest producers of DMSP (Keller et al., 1989), whereas diatoms, green algae and cyanobacteria are generally considered low or non-producers (Belviso et al., 2001). Recent work has shown that some species of *Trichodesmium* produce DMSP (Bucciarelli et al., 2013a; McParland and Levine, 2019), so their contribution to the sulfur cycle should not be overlooked.

Once produced, DMSP is released into the surrounding seawater where it becomes available to the bacterial community. It plays an important role in ocean microbial ecology, satisfying the bacterial sulfur and carbon needs up to 95% and 15%, respectively (Zubkov et al., 2001). Once inside the bacterial cell, DMSP can be metabolised via two means: the demethylation pathway, during which DMSP is transformed to methanethiol (MeSH) to be utilised for energy and protein production (Kiene et al. 1999; Ronald P Kiene & Linn 2000), and the DMSP lyase pathway where DMSP is cleaved to the volatile gas dimethylsulfide (DMS), which is lost by diffusion out of the cell (Kiene, Linn & Bruton 2000; Yoch 2002). It has been hypothesised that bacteria will degrade DMSP preferentially through demethylation to meet the sulfur demand for protein production and bacterial growth, before adopting the cleavage pathway that generates DMS, and so, the DMSP degradation mechanism employed at any given time is regulated by the sulfur demand of the bacteria (Kiene et al., 1999).

The production of DMS by bacteria can influence local climate (Charlson et al., 1987). As soon as DMS is released to the atmosphere, it is oxidised to sulphate aerosols (Koga and Tanaka, 1996) which contribute to cloud condensation nuclei (CCN), which in turn increases the reflection coefficient of the cloud (cloud albedo), and results in a decrease of both solar irradiance and surface temperature on a regional scale (Welsh, 2000). It has been hypothesised that phytoplankton utilise the climatic properties of DMS to protect themselves from solar radiation in a self-regulated feedback mechanism (Charlson et al., 1987). Under

conditions of high UV radiation, phytoplankton increase DMSP production, which results in an increase of DMS and cloud coverage that reduces UV stress on phytoplankton, which then, reduces the production of DMSP. However, feedback effects of climate on the DMS cycle are difficult to quantify, and estimates on these feedbacks have shown the effect to be quite small (Gunson et al., 2006; Vallina et al., 2007; Wang et al., 2018).

While the role of DMSP metabolism and DMS production have been largely attributed to heterotrophic bacterioplankton, DMSP lyase activity has been found in phytoplankton, including the haptophyte *Emiliania huxleyi* (Wolfe and Steinke, 1996) and the prymnesiophyte *Phaeocystis* spp. (Van Boekel & Stefels 1993), demonstrating that phytoplankton may also cleave DMSP to DMS. Moreover, it has been suggested that non DMSP producing phytoplankton species can and do take up DMSP from the surrounding environment, and that this phytoplanktonic uptake can be in similar quantities to the uptake by bacterioplankton (Vila-Costa et al. 2006). A recent lab experiment with the temperate diatom species *Thalassiosira weissflogii* demonstrated that DMSP was taken up rapidly and consistently, with intracellular concentrations saturating at around 83 mM (Petrou and Nielsen, 2018), suggesting that individual phytoplankton species can have a high capacity for rapid uptake and storage of DMSP when available, thereby forming a potential sink and subsequent reservoir for DMSP in the ocean. Taken together, these findings show that phytoplankton may play a larger more complete role in marine sulfur cycling and DMS flux from the ocean, than merely the production of DMSP and DMS.

Using a two-pronged approach, in this chapter we aimed to better understand the sources and sinks of DMSP in Australian temperate waters. Through a whole community DMSP enrichment incubation, we sought to quantify the uptake and degradation of DMSP by the entire microbial community, providing an estimation of the demand for sulfur in these waters. Then, to differentiate the responses between microbial size classes and gain detailed insight into who the key sinks and sources might be, our second approach was to fractionate the community into three size classes and then enrich each fraction with DMSP, with the aim to uncover the dominant sinks and metabolisers in PH waters and determine the potential sulfur demand of each size group.

5.3 Experimental procedure

The study consisted of two separate incubation experiments, a whole water incubation to investigate the uptake of DMSP by the natural marine microbial community of Port Hacking station, and an incubation of the fractioned community to quantify DMSP uptake in each size fraction. Both incubation experiments were conducted with surface sea water (5 m) collected from Port Hacking Station (-34.1192S, 151.2267E) located 8 km offshore from Sydney (Figure 5.2), Australia. Samples were collected during one day in March 2019 and transported back to the lab in dark carboys. Seawater was first filtered through a 210 µm mesh to remove large grazers before being sub-sampled into bottles or size fractionated for incubation experiments.

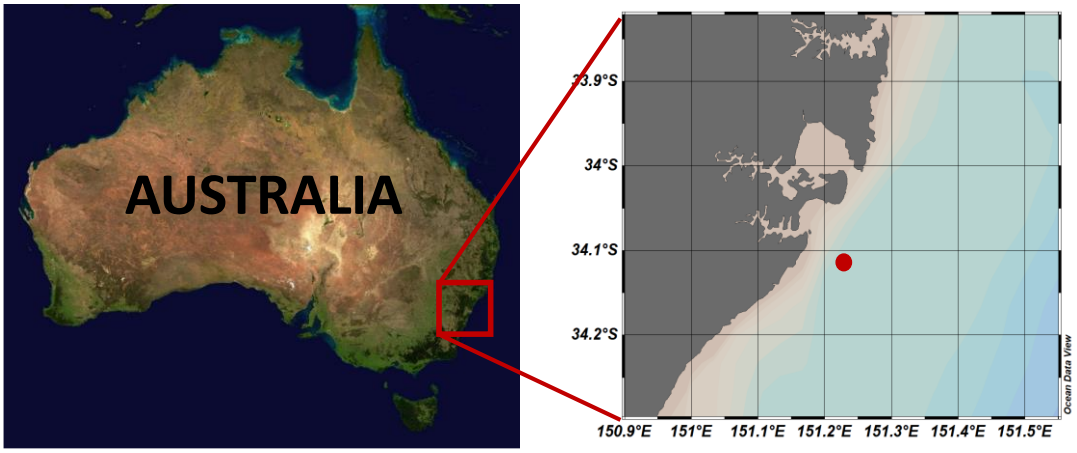

Figure 5.2. Location of sampling site and nutrients concentrations of initial experimental waters*. Map of Australia showing location of Sydney basin, with magnified inset image showing location of sampling site generated with Ocean data View software* (Schlitzer, 2016).

5.3.1. Quantification of rapid DMSP uptake by the Port Hacking marine microbial community

To study the uptake of DMSP by the natural microbial community of Port Hacking waters, 10 L of seawater was transferred to polycarbonate bottles (4 controls, 2 fixed samples and 4 treated with DMSP (20 nM final concentration)) with no headspace. Glutaraldehyde (final concentration 1%) and DMSP was added to the fixed samples to control for any passive uptake of DMSP by cells. Bottles were then closed with screw caps and shaken gently to dissolve the added compound. All bottles were incubated for up to 17 h inside an incubator with a temperature of 22.2°C and light at 100 µE. Each bottle was subsampled in triplicate for DMSP total, dissolved and particulate at time 0 h, 5 h and 17 h plus cell counts by flow cytometry at time 0 h (Figure 5.3). Samples for analysis of DMSP total, dissolved and

particulate were hydrolysed with NaOH and measured after 24 h to ensure complete transformation of DMSP to DMS. All samples were analysed according to the methods described in section 2.1.

5.3.2. Quantification of rapid DMSP uptake by different fractions of the Port Hacking marine microbial community

To study the uptake of DMSP by the different size classes of the microbial community, we collected 20 L of water and size-fractionated via serial filtration using 8 µm, 3 µm, and 0.22 µm polycarbonate filters, washed the filters with F/20 media and resuspended the particulate matter in 400 mL of F/20 media to ensure no nutrient limitation during overnight incubation (22.2°C, 70 r.p.m, ~55 µmol photons m^{-2} s^{-1}, 12:12 h light:dark cycle). The next day, we split the 400 mL of each fraction in 4x 50mL controls and 4x 50mL treated samples with DMSP (100 nM final concentration). At 3 time points (T0, T3 and T6 h) each sample was subsampled for DMSP total, particulate and dissolved in triplicate (Figure 5.3). At time point 6 h, subsamples for Chl *a* were also taken and at time initial 1 mL subsamples were taken and fixed in 1% glutaraldehyde for phytoplankton counts and identification using light microscopy as described in methods section 2.1.

5.3.3. Statistical analysis

Differences in DMSPt, DMSPd and DMSPp values over time and between treatments were analysed using Permutational Multivariate Analysis of Variance (PERMANOVA) in a nested design, with non-parametric multi-dimensional scaling (nMDS) and Analysis of Similarities (ANOSIM) for both incubation experiments. These analyses were performed using PRIMER v6 statistical package (Clarke and Gorley, 2006) with PERMANOVA+ module (Anderson, 2005). Using the statistical package SPSS (IBM Statistics v.24), a one-way Analysis of Variance (ANOVA), after having ascertained homogeneity of variance using Levene's test, was used to analyse differences between treatments for DMSPp:Chl *a* data. Correlations between DMSP concentrations for the whole community and each size fraction were performed using Pearson correlation coefficients and only considered when the correlation was strong ($r > 0.7$) and significant ($P < 0.05$).

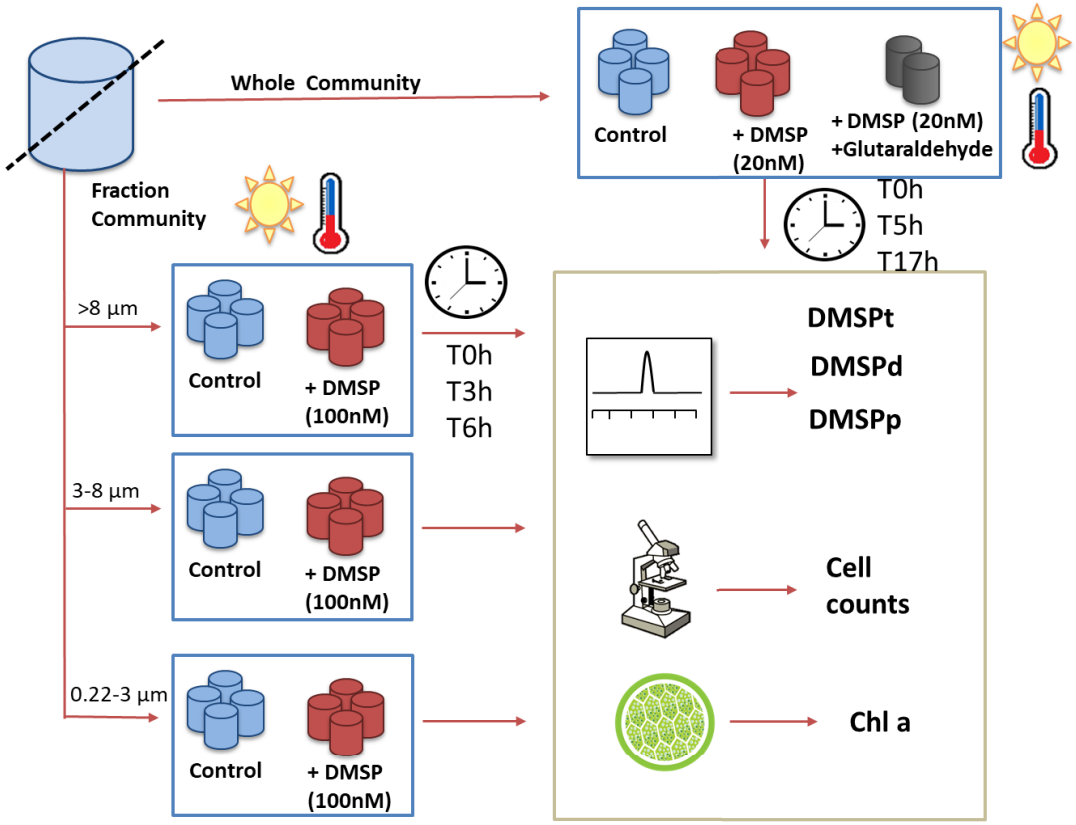

Figure 5.3. Experiment flow chart. *We followed the uptake of DMSP by the whole microbial community and the fractioned community. For the whole community, seawater was split in 4 controls, 2 fixed (1% Glutaraldehyde) and 4 DMSP amended samples, all incubated with no headspace. For the fractionated community, initial water was size fractionated via serial filtration and the filters washed and the particulates resuspended in F/20 media. Each fraction was split in 4 controls and 4 DMSP amended samples. At each time point, each bottle of each experiment was subsampled for DMSP total, dissolved and particulate. For the whole community incubation, samples for cell counts were taken at initial time and for the fractioned community, subsamples for Chl a were taken at time final.*

5.4. Results

5.4.1. Characteristics of initial water masses

The Port Hacking seawater showed low nutrient concentrations, indicative of EAC influenced seawater, with silicate concentrations of 0.5 µM, phosphate and ammonium concentrations of 0.09 µM and no detectable levels of nitrate (Figure 5.4). Dimethylsulfide (DMS) and DMSPt concentrations in the initial water sample were also relatively low (1.51 ± 0.06 nM DMS and 16.37 nM DMSPt) at the time of sampling (Table 5.1). Reflective of nutrient poor waters, flow cytometric counts of the microbial community revealed relatively low abundances of picoeukaryotes, *Synechococcus*, *Prochlorococcus* and heterotrophic bacteria (Table 5.1).

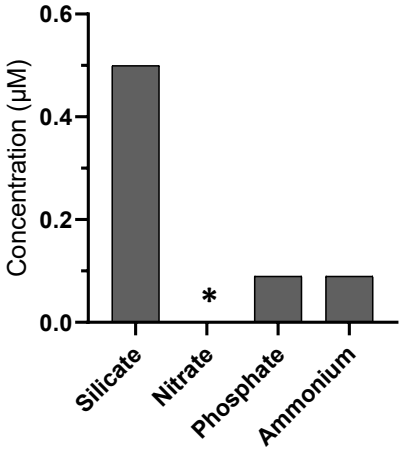

Figure 5.4. Nutrients concentrations of initial experimental waters. *Concentrations of silicate, nitrate, phosphate and ammonium for both water masses at the initial sampling time point. Data obtained by IMOS (2019). *Below detection limit.*

Table 5.2. Sulphur chemistry and flow cytometric counts of microbial community. *DMS, DMSPt and DMSPd concentrations and counts of the initial microbial community from PH waters.*

	Initial water
DMS (nM)	1.51±0.06
DMSPt (nM)	16.37±1.41
Picoeukaryotes (cells x10^4 ml^{-1})	2.01±0.40
***Synechococcus* (cells x10^5 ml^{-1})**	1.35±0.01
***Prochlorococcus* (cells x10^4 ml^{-1})**	4.89±0.58
Heterotrophic Bacteria (cells x10^5 ml^{-1})	1.37±0.34

5.4.2. Whole community incubation experiment

Concentrations of DMSP for the whole community incubation experiment responded differently between control and +DMSP samples (Figure 5.5). The control samples had constant values for all sulfur compounds over the course of the experiment with concentrations of ~15 nM of DMSP total, out of which ~10 nM where DMSPp and ~2 nM DMSPd (Figure 5.5.A). Samples enriched with DMSP showed a significant decrease in DMSP total from 49.23 to 27.89 nM (t=5.4882, P_{MC}=0.003) and DMSPd from 25.06 to 1.65 nM (t=12.31, P_{MC}=0.001), while DMSPp remained constant at ~12 nM (Figure 5.5.B). This decrease in DMSP concentration represented a loss of ~43% of initial DMSPt and ~93% of initial DMSPd in 17 h at a rate of disappearance of 30.13 nM d^{-1} and 33.05 nM d^{-1} for DMSPt

and DMSPd, respectively. As expected, samples fixed with glutaraldehyde showed constant DMSP concentrations over the course of the experiment (Figure 5.5.C).

Non-metric multidimensional scaling using all replicates for all treatments and time points, grouped the samples by treatment (Global R=0.704, P=0.004), and time points in the case of the +DMSP samples (Global R=0.468, P=0.001) (Figure 5.5.D). These data highlight the differences in DMSP total (Pseudo-F=14.413, P_{MC}=0.001) and dissolved (Pseudo-F =12.168, P_{MC}=0.001) between controls and DMSP-enriched samples, and the progression of +DMSP samples over time towards concentrations similar to the controls. Correlation analysis between DMSP concentrations (DMSPt, DMSPd and DMPp) for control and +DMSP treatments shows no relationships in control samples (Figure 5.6.A-C), but a significant and positive correlation (r=0.8171, P= 0.0021) between DMSPd and DMSPt for the +DMSP samples, suggesting the loss of DMSPt is due to a loss in DMSPd (Figure 5.6.D-F).

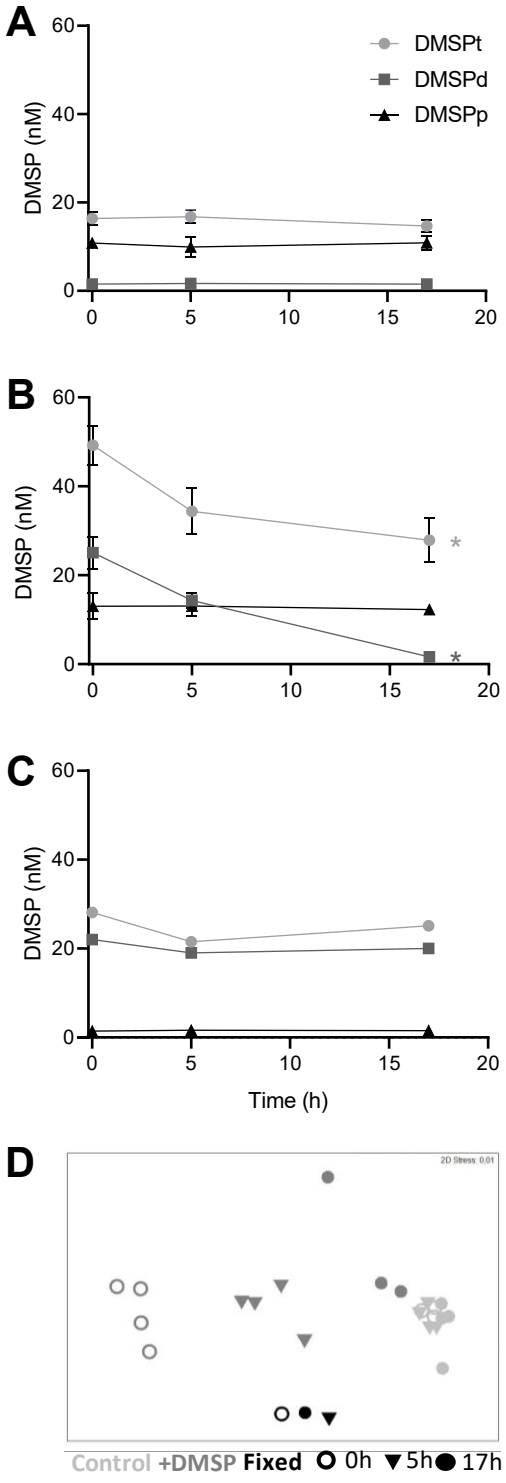

Figure 5.5. DMSP concentrations of the whole marine microbial community over 17 h and nMDS plot. *Concentrations of DMSPt (light grey circles), DMSPd (dark grey squares) and DMSPp (black triangles) over 17h for (A) control samples (B) DMSP enriched samples and (C) fixed samples. * denote statistical differences at P<0.05 between time points for each treatment. (D) nMDS plot with control samples (light grey), +DMSP samples (dark grey) and fixed samples (black) at initial time point (empty circle), after 5h (triangles) and 17h (full circles) including all replicates.*

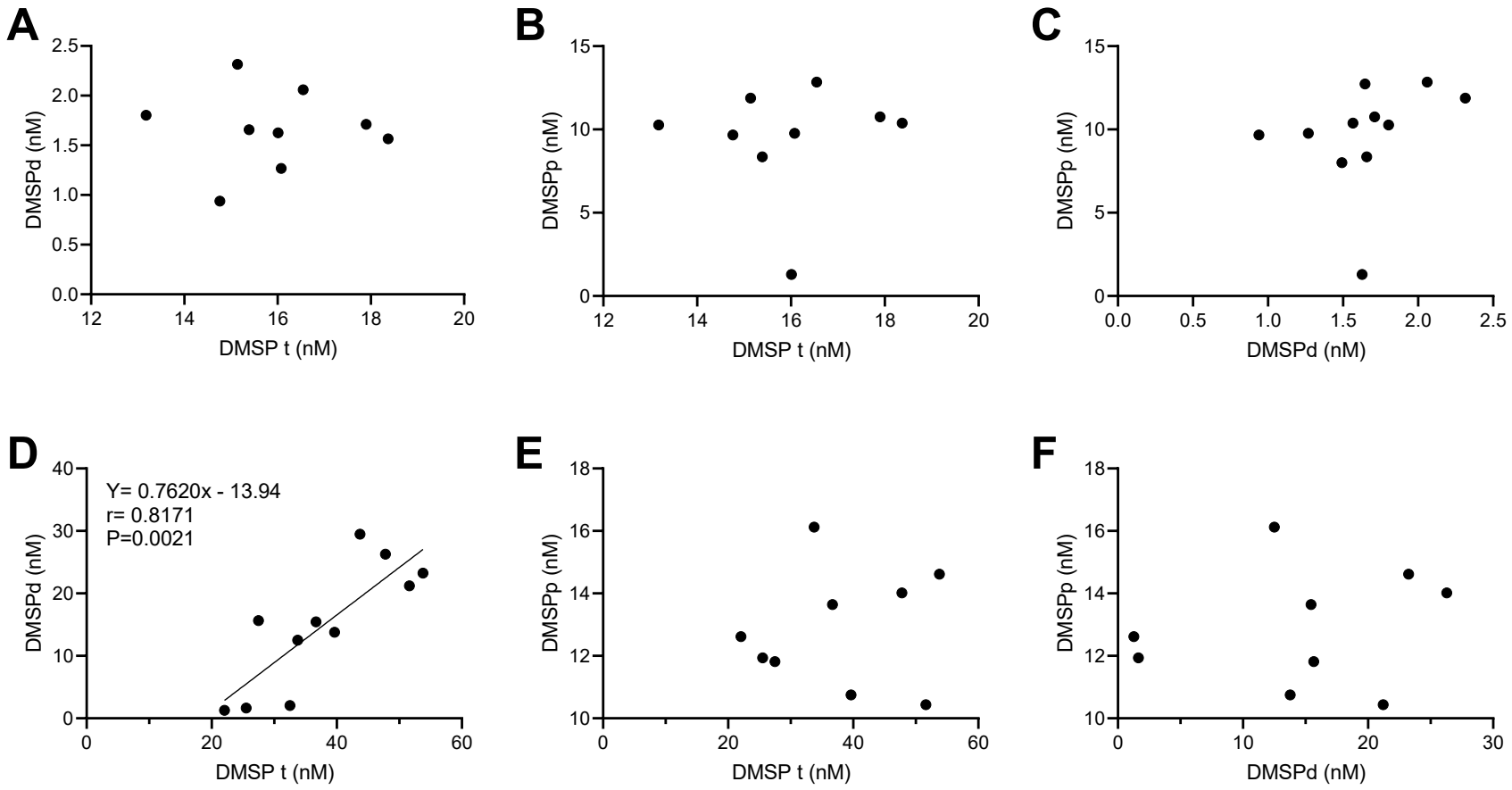

Figure 5. 6. Correlations between the different DMSP concentrations for control and +DMSP samples. *Only significant correlations (P<0.05) with a strong Pearson's correlation coefficient (r >0.7) are shown with a line of best fit. (A) DMSPd vs DMSPt for control samples, (B) DMSPp vs DMSPt for control samples, (C) DMSPp vs DMSPd for control samples, (D) DMSPd vs DMSPt for +DMSP samples, (E) DMSPp vs DMSPt for +DMSP samples, and (F) DMSPp vs DMSPd for +DMSP samples.*

5.4.3. Fractioned community incubation experiment

The phytoplankton community was dominated by the bloom forming cyanobacterium *Trichodesmium* (136,700 colonies L^{-1}) common in EAC waters (Table 5.2; Figure 5.7). Other taxa included the DMSP producers *Phaeocystis* (37,400 ± 5,290 cells L^{-1}) and dinoflagellates from the genus *Gymnodinium* (88,800 ± 2,960 cells L^{-1}). Three diatom taxa were identified in low abundance, from the genera *Asteromphalus*, *Cylindrotheca* and *Chaetoceros* (Table 5.2, figure 5.7).

Table 5.2. Phytoplankton composition of the community. *Phytoplankton was quantified and identified to genus level using light microscopy. * Indicates number of string colonies.*

Phytoplankton genus	DMSP producers	Cells L^{-1}
Trichodesmium	Yes	136,700 ± 7,100*
Phaeocystis	Yes	37,400 ± 5,290
Gymnodinium	Yes	88,800 ± 2,960
Asteromphalus	No	1,000 ± 331
Cylindrotheca	No	28,000 ± 1,900
Chaetoceros	No	7,000 ± 1,300

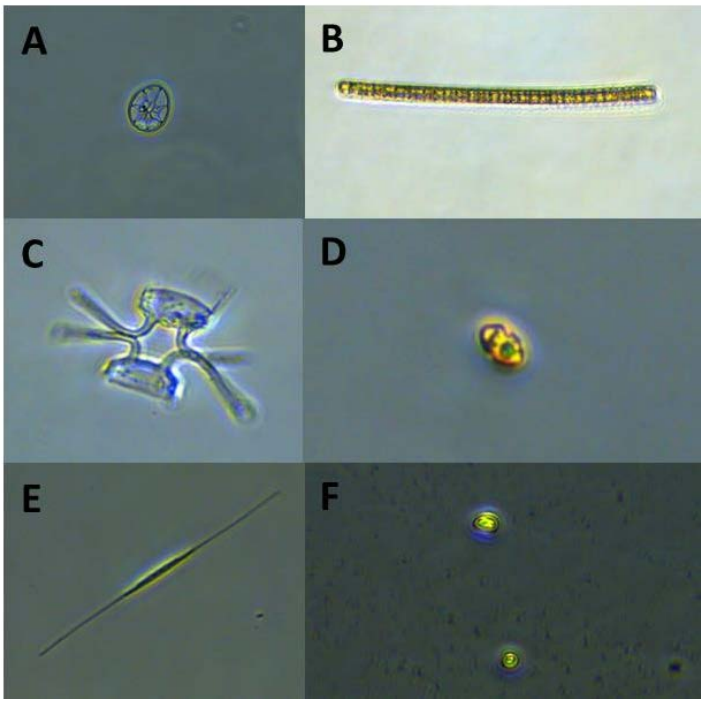

Figure 5.7. Key Phytoplankton taxa identified in initial water samples*. (A) Asteromphalus, (B) Trichodesmium, (C) Chaetoceros, (D) Gymnodinium, (E) Cylindrotheca and (F) Phaeocystis.*

Concentrations of DMSP for the fractionated community incubation experiment showed similar responses across the different fractions with decreasing concentrations of DMSPt and DMSPd and increasing concentrations of DMSPp for the +DMSP samples (Figure 5.8). In the largest fraction (>8µm), DMSPd in the controls decreased significantly from 8.77 to 3.44 nM (t=5.7967, P=0.003), DMSPp increased significantly from 29.14 to 37.51 nM (t=6.3069, P=0.002), while DMSPt remained stable at around 38 nM (Figure 5.8.A). In the +DMSP samples, DMSPd decreased significantly from 142.84 to 89.21 nM (t=5.5153, P=0.003), while DMSPp increased significantly from 29.59 to 73.58 nM (t=3.4614, P=0.016), yet unlike the controls, DMSPt decreased significantly from 142.84 to 89.21 nM (t=3.1152, P=0.019) (Figure 5.8.B). While there was clear separation of the sulfur chemistry between treatments (Global R=1, P=0.001), and some shifts over time within each treatment (Global R=0.8, P=0.001), the overall response pattern was similar in both treatments over time for the large fraction (Figure 5.8.C).

In the control samples of the medium fraction (>3µm), DMSPd decreased significantly from 7.38 to 4.84 nM (t=11.062, P=0.001), while DMSPp and DMSPt remained the same over the 7 h (Figure 5.8.D). In the +DMSP samples DMSPd also decreased from 122.39 to 35.80 nM (t=5.5153, P=0.009), while there was a strong increase in DMSPp from 26.17 to 36.35 nM, and a decline in DMSPt from 150.24 to 55.20 nM (t=3.2491, P=0.02) (Figure 5.8.E). As with the large fraction, samples grouped by treatment (Global R=1, P=0.002) with a strong differentiation in the +DMSP samples at 6 h, reflecting the significant differences in DMSP total, dissolved and particulate between treatments (Figure 5.8.F).

In the smallest fraction (>0.22 µm), there were no significant changes of any DMSP concentrations over time for controls (Figure 5.8.G). However, in the +DMSP samples, DMSP concentrations changed over time (Figure 5.8.H), with a significant decline in DMSPd from 96.23 to 7.72 nM (t=14.659, P=0.001) and DMSP total from 161.27 to 95.99 nM (t=4.8277, P=0.01), while DMSPp increased from 50.19 to 66.43 nM (t=4.3163, P=0.007). There was clear sample grouping by treatment (Global R=0.576, P=0.01) and +DMSP samples were differentiated at 0h from the rest (Figure 5.8.I).

There was a significant loss of DMSPt in all fractions of the community when enriched with DMSP, indicating that all fractions catabolise DMSP to DMS or MeSH, when available in high

concentrations. Loss rates varied within fractions, whereby the >3 µm fraction had the highest DMSPt loss rate of 380 nM day^{-1} compared to 214 and 2612 nM day^{-1} for >8 and >0.22 µm fraction, respectively (Table 5.3). All fractions showed a loss of DMSPd at a similar rate to DMSPt, suggesting that the loss measured from the total pool is the dissolved fraction. The increase in DMSPp in all fractions occurred at a lower rate than the loss of DMSPt and DMSPd. However, the rate of DMSPp does not show the total DMSP uptake rate, as DMSP was being taken up by the cell while being catabolised and DMSPp was increasing over time, it suggests that the uptake of DMSP was faster than its degradation and that the rate of DMSPp is the rate in which DMSP accumulates into the cells.

Particulate DMSP normalised to chlorophyll *a* content showed similar amounts of DMSPp for initial and final time points in controls, and higher amounts in +DMSP samples at the last time point, for each fraction (Figure 5.9). Chlorophyll normalised particulate DMSP increased over 6 h for all three fractions (Figure 5.9.). The percentage of DMSP taken up by each fraction with respect to its initial concentration, followed a size gradient, with the largest fraction (>8 µm) more than doubling its DMSPp concentration expressing a 138 % increase. For the >3 µm fraction, DMSPp increased 55 % and for the >0.22 µm fraction it increased only 22 %.

In the >8 µm +DMSP samples, DMSPd concentrations showed a strong positive correlation with DMSPt ($r=0.8495$, $P=0.0019$) and negative correlation with DMSPp ($r=-0.8638$, $P=0.0027$) (Figure 5.10.A-B), suggesting that the decrease of DMSPt and the increase of DMSPp is solely due to the decrease of DMSPd. Similarly, in the >3 µm +DMSP samples, DMSPt was correlated positively with DMSPd ($r=0.9277$, $P=0.0003$) and negatively with DMSPp ($r=-0.7516$, $P=0.0195$), suggesting that the DMSP disappearing from the system was originally in the dissolved formed and was taken up into the particulate fraction (Figure 5.10.C-D). In the >0.22 µm +DMSP samples, DMSPd was correlated significantly and negatively with DMSPp ($r=0.743$, $P=0.02$), indicating that DMSP disappearing from the solution was taken up into the particulate fraction (Figure 5.10.E).

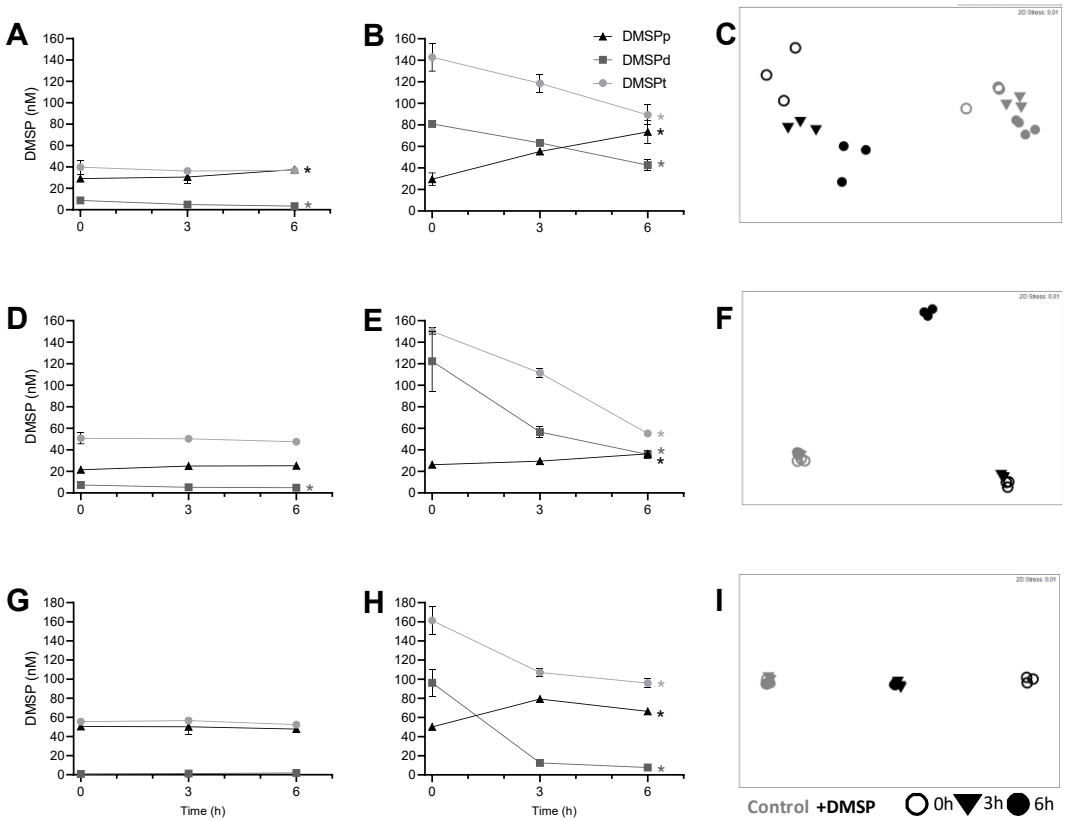

Figure 5.8. DMSP concentrations of fractionated marine microbial community over 6 h and nMDS plots. *(A) DMSP concentrations for > 8 µm fraction control samples, (B) DMSP concentrations for > 8 µm fraction +DMSP samples, (C) nMDS plot for > 8 µm fraction, (D) DMSP concentrations for > 3 µm fraction control samples, (E) DMSP concentrations for > 3 µm fraction +DMSP samples, (F) nMDS plot for > 3 µm fraction, (G) DMSP concentrations for > 0.22 µm fraction control samples, (H) DMSP concentrations for > 0.22 µm fraction +DMSP samples, (I) nMDS plot for > 0.22 µm fraction. DMSP concentrations of DMSPt (light grey circles), DMSPd (dark grey squares) and DMSPp (black triangles). nMDS plots with control samples (light grey), +DMSP samples (dark grey) and fixed samples (black) at initial time point (empty circle), after 3h (triangles) and 6h (full circles) including all replicates. * denotes statistical differences at P<0.05 over time for DMSP concentrations.*

Table 5.3. Percentage and rate of DMSP loss/gain for each size fraction and treatment for DMSPt, DMSPd and DMSPp. Samples with no significant (P<0.05) DMSP loss or gain are indicated with a dash. Samples that lose DMSP over time are indicated with negative values and samples that gain DMSP over time are indicated with positive values.

Fraction (µm)	treatment	DMSP total		DMSP dissolved		DMSP particulate	
		DMSP lost/gained (%)	Loss/gain rate (nM day^{-1})	DMSP lost/gained (%)	Loss/gain rate (nM day^{-1})	DMSP lost/gained (%)	Loss/gain rate (nM day^{-1})
> 8	Control	-	-	- 60.77	+ 21.32	+ 27.69	+ 33.48
> 8	+DMSP	- 37.54	+ 214.52	- 37.55	+ 214.52	+ 148.67	+ 175.96
> 3	Control	-	-	- 34.42	+ 10.16	-	-
> 3	+DMSP	- 63.26	+ 380.16	- 70.75	+ 346.36	+ 38.90	+ 40.72
> 0.22	Control	-	-	-	-	-	-
> 0.22	+DMSP	- 40.48	+ 261.12	- 91.98	+ 354.04	+ 32.36	+ 64.96

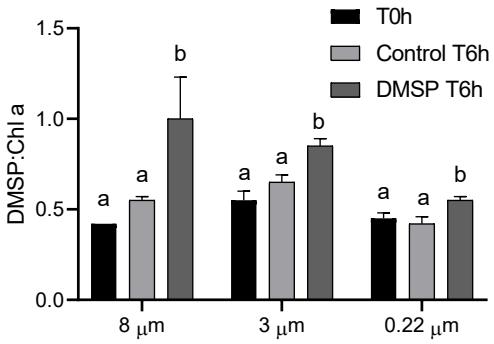

Figure 5.9. DMSPp at the final time point. *DMSPp concentrations normalised to Chlorophyll a for time zero (black bar), control samples at the final time point for control (light grey bars) and +DMSP samples (dark grey bars) at the last time point for each fraction of the community. Data represent the mean ± standard deviation (n=4). Lowercase letters above bars denote statistical differences at P<0.05.*

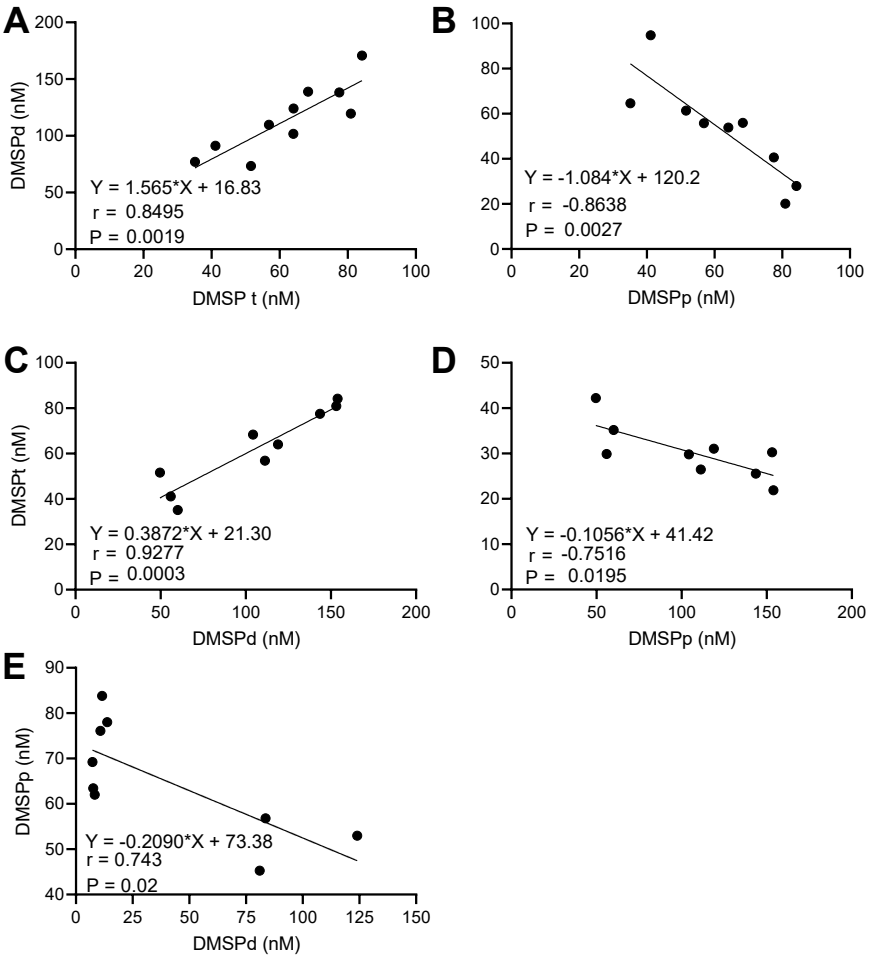

Figure 5.10. Correlations between DMSP concentrations for each size fraction of the community. *Only significant correlations (P<0.05) with a strong Pearson's correlation coefficient (r >0.7) are shown. (A) DMSPd vs DMSPt for >8µm +DMSP samples, (B) DMSPd vs DMSPp for >8µm +DMSP samples, (C) DMSPt vs DMSPd for >3µm +DMSP samples, (D) DMSPd vs DMSPt for >3µm +DMSP samples, and (E) DMSPp vs DMSPd for >0.22 µm +DMSP samples.*

5.5. Discussion

Dimethylsulfoniopropionate (DMSP) is a key chemical compound that underpins many ecological interactions and metabolic processes within marine microbial communities. It is synthesised in high amounts by some species of phytoplankton like dinoflagellates and haptophytes and taken up by heterotrophic bacteria that utilise it for cell growth. The uptake and subsequent degradation of DMSP has been well studied in bacteria, but only a few experiments have studied its uptake and utilisation in phytoplankton. This study investigated the uptake of DMSP by phytoplankton and bacteria in natural sea waters of a temperate location in east Australia. We found that when the whole community was enriched with DMSP, concentrations of DMSPd and DMSPt declined over time, indicating that DMSP was being taken up and utilised by the microbial community. However, studying the microbial community as a whole, revealed only a small part of the DMSP dynamics. Incubations using different size classes of the community (8µm, 3µm, 0.22 µm) showed an increase in DMSPp and a decrease in DMSPt with enrichment, indicating that all fractions were responsible for the uptake and metabolisation of DMSP. These results support the hypothesis that phytoplankton may also take up dissolved DMSP from the environment and provide evidence that they may act as important DMSP sinks in temperate waters.

The influence of the East Australian Current (EAC) on the sampled waters was evident by its low nutrient signal, with values within ranges of previous EAC studies (Doblin et al., 2016; Hassler et al., 2011) and the occurrence of a bloom of the tropical cyanobacterium *Trichodesmium*. *Trichodesmium* sp. is an ecologically important cyanobacterium that dominates in nitrogen-poor waters, fixing atmospheric nitrogen into ammonium, making it bioavailable to other microorganisms (Capone et al., 1997). It has also recently been suggested to be a DMSP producer (Bucciarelli et al., 2013b; Lee et al., 2017) and has been one of the two dominant species at PH in the last decades (Ajani et al., 2014a, 2014b). Therefore, it is likely that *Trichodesmium* will play an increasingly important role in the microbial ecology and seasonal cycling of DMSP in the temperate waters off Port Hacking.

Phytoplankton dynamics are closely linked to climate, and changes on ocean conditions derived from climate change are substantially altering phytoplankton biogeography, abundance and phenology and favoring species best adapted to changing conditions (Winder and Sommer, 2012). However, it is difficult to state if the presence of the tropical *Trichodesmium* sp. is a consequence of climate change due to the high variability in species

abundance (of orders- of-magnitude) over seasonal, inter-annual and inter- decadal time scales (Zingone et al., 2010).

5.5.1. Quantification of DMSP uptake by the whole microbial community

The uptake of DMSP by the whole marine microbial community of PH station determined over 17 h (experiment 1) explains only part of the DMSP transfer. Control samples showed high concentrations of DMSPt with most of it being measured in the particulate fraction (Figure 5.5.A), suggesting that it was mostly inside the DMSP producing species, such as those identified by microscopy *Trichodesmium, Phaeocystis* and *Gymnodinium.* The low levels of DMS in the initial seawater sample, suggest that despite the presence of *Phaeocystis,* which is known to cleave DMSP to DMS (Boekel and Stefels, 1993; Mohapatra et al., 2014), DMSP lyase activity was low. The lack of change in the control incubations demonstrates stability in the production and consumption processes of DMSP by this PH microbial community. This stability could indicate that the sulfur demand of the community was not yet satisfied and that much of the DMSP was retained inside the cells to be used in one of its cellular roles, such as an osmolyte, antioxidant or compatible solute (Stefels, 2000). However, when we enriched the samples with DMSP, there was a decline in DMSPd and DMSPt over time, while DMSPp remained constant (Figure 5.5.B), indicating that the additional available DMSP was taken up and rapidly transformed to DMS or MeSH and not retained within the sink cells. Furthermore, the positive correlation between DMSPt and DMSPd for the +DMSP samples (Figure 5.6) lends additional supports the idea that excess DMSP was cleaved into DMS and lost to the atmosphere (Kiene et al., 2000).

In brief, we can say that the natural communities of Port Hacking were highly influenced by the EAC, showing the typical nutrient and phytoplankton signatures of oligotrophic tropical waters. The relatively high concentration of DMSP in the seawater was due to the high intracellular concentrations of DMSP producing species present. The low levels of DMS however, would indicate that sulfur demands were not yet or only just met, and that for DMS flux to increase in PH, there would need to be an injection of additional DMSPd into the water column. This was confirmed following our incubations, where it was clear that when enriched with DMSP, the microbial community was able to take up the available DMSP and rapidly metabolised the excess, likely resulting in an increased flux of DMS to the atmosphere.

5.5.2. Quantification of DMSP uptake by different fractions of the microbial community

The pre-fractionation approach (experiment 2) allowed us to gain insight into how the DMSP sources and sinks of Port Hacking were distributed within the marine microbial community. As per the whole community incubation (Experiment 1), most of the DMSP in the control samples was retained within the particulate fraction, especially for the >8 µm and <3 µm size classes (Figure 5.8. A,D,G). Similarly, concentrations of DMSPt remained constant over time in all fractions, indicating that the sulfur demand was close to satisfied and DMSP could be retained inside the phytoplankton cells to be used for one of its cellular roles (Stefels, 2000). Unlike the whole community incubation, all fractions when enriched with DMSP increased in DMSPp over time (Figure 5.8. B,E,H), indicating that the degradation of DMSP was slower than its uptake and allowed DMSP to accumulate inside the cell, which has also been observed for natural marine microbial communities of the Great Barrier Reef (Chapter 3 of this thesis) and Antarctica (Chapter 4 of this thesis) plus in some phytoplankton cultures (Levasseur, 2013; Petrou and Nielsen, 2018). This would indicate that DMSP was being taking up by non-producers helping to meet sulfur demands and that the DMSP excess, once sulfur demands were met, was being catabolised to produce DMS.

The cell size dependent accumulation of DMSP observed in this study may be attributable to the higher spatial capacity of larger cells, such as in centric diatoms, which have vacuoles that may act as storage sites for osmolytes and other compounds (Finkel et al., 2016). However, independent of uptake capacity, this study has provided evidence that phototrophs, which are mostly in the >8 and >3 µm fraction, can form major DMSP sinks in the temperate waters off Sydney, and support the hypothesis that autotrophic phytoplankton take up significant amounts of DMSP (Petrou and Nielsen, 2018; Vila-Costa et al., 2006b). Therefore, if phytoplankton are capable of taking up DMSP, it is plausible that they may also have the capacity to metabolise it. However, limited research has been done on DMSP degradation by phytoplankton and to date, only two taxa are known to possess DMSP lyase activity (Boekel and Stefels, 1993; Wolfe and Steinke, 1996), while no evidence of demethylation pathways in phytoplankton exists, leaving the door wide open for further research.

Phytoplankton diversity at PH was not very high, most probably due to the cyanobacterial bloom of *Trichodesmium*, which is known to diminish phytoplankton diversity and biomass (Bockwoldt et al.), but there were six major phytoplankton taxa that were commonly found in all samples (Table 5.2). Of them, three are known DMSP producers, while the other three

(albeit less dominant taxa) are considered non-producers. Of the producers, *Trichodesmium* has the lowest intracellular DMSP concentrations with reported values of 0.05 mM (Bucciarelli et al., 2013b), but given that it was blooming at the time of sampling, its contribution to the DMSP pool was somewhat significant. The dinoflagellates from the genus *Gymnodinium* have reported values of DMSPp between 45.5 - 124.6 mM (Keller, 1989), and given that they were present in relatively high abundance, they were likely the major contributors to the particulate DMSP concentrations. The most prolific DMSP producer present in the seawater was the *Phaeocystis* sp. whose intracellular concentrations of DMSP can range between 121 – 358 mM (Keller, 1989). However, as smaller cells, only present in relatively low abundance and as the only known taxa with lyase capacity (Boekel and Stefels, 1993; Mohapatra et al., 2014), given the low DMS values, likely had minimal influence on the sulfur chemistry at PH at this time. Of the non-DMSP producers, we identified two centric diatoms and one pennate diatom from the genera *Asteromphalus*, *Chaetoceros* and *Cylindrotheca*. We hypothesise that these taxa formed the major DMSP sink for PH waters at the time of the sampling, together with smaller flagellates, and picoeukaryotes that were present at $2.01x10^4$ cells mL^{-1}.

Generally, cleavage of DMSP to DMS is considered the domain of key bacterioplankton, with many identified DMSP lyases with Km ranging from 0.4 nM to 82 mM (Bullock et al., 2017), and cleavage rates that depend on the taxa present, their sulfur demand and their abundance (Mohapatra et al., 2014; Steinke et al., 2002, 1998). Of the phytoplankton taxa identified, *Phaeocystis* was the only species known to be able to cleave DMSP to DMS, producing up to 3.05 nmol DMS min^{-1} (Van Boekel & Stefels 1993). What is interesting is that our size-sorted results suggested that all fractions cleaved DMSP to DMS although at slightly different rates (Table 5.3). In the +DMSP samples, the DMSP loss rates measured for each fraction were equivalent to those values found during the exponential growth phase of a phytoplankton bloom, while the measurements in the control samples were more similar to those found at a later stage of the bloom (Duyl et al., 1998). The differences in DMSP lyase activity between different phytoplankton and bacterioplankton species may explain these differences (Niki et al., 2000), but in order to identify the members of the community that are capable to cleave DMSP to DMS, and so, the major DMSP metabolisers in the temperate waters of Port Hacking, more research on DMSP degradation by phytoplankton is needed.

5.6. Conclusions

In this study we evaluated the uptake of DMSP by different size fractions of the marine microbial community of a temperate location of the east coast of Australia during a bloom of the cyanobacteria *Trichodesmium.* Our results showed that the microbial community of PH had low sulfur concentrations that potentially satisfied the existing community's sulfur demand, as no changes in DMSP concentrations happened during the experiment. This was supported by the rapid uptake and metabolisation of DMSP when provided in excess, potentially resulting in DMSP conversion to DMS. Taken together, this study has revealed that both, bacteria and phytoplankton take up DMSP in similar quantities over relatively short time scales (6 h), identifying diatoms from the genera *Asteromphalus*, *Chaetoceros* and *Cylindrotheca,* together with smaller picoeukaryotes and bacteria as the major DMSP sinks in this environment. These data have shown that PH waters dominated by *Trichodesmium* are low in sulfur supply for the microbial community and that for sulfur demands of the community to be met, there needs to be additional DMSP production and injection of DMSP into the water column. This study has increased our knowledge on the distribution of DMSP within a coastal microbial community, and uncovered how microbial populations utilise and divert DMSP in response to DMSP enrichment, contributing to a better understanding of the marine DMSP cycle.

Chapter 6:

General conclusions

Chapter 6: General conclusions

The research presented in this thesis builds on the current knowledge of sulfur cycling, specifically, by studying DMSP distribution throughout the marine microbial food webs of different marine environments, it conclusively establishes the importance of all members of the bacterial and phytoplankton community as DMSP sinks. The effect of DMSP on modulating the marine microbial composition was studied with results highlighting that many bacteria, and some phytoplankton taxa, are capable of metabolising DMSP, which suggests an ecological advantage for these taxa, supporting their proliferation and outcompeting other taxa. . In this concluding chapter, I summarise the results of each of the three latitudinally distinct habitats presented and discuss them in relation to one of the two main aims of the thesis. (1) To study the uptake of dissolved DMSP and its distribution throughout marine microbial communities and (2) to investigate how the microbial community composition is shaped under the influence of dissolved DMSP, to deliver a broader perspective of these aims gained by the latitudinal study. Then, I conclude by highlighting the implications of these findings to sulfur cycling research and identify future research directions that would further our understanding of the sulfur cycle, specifically, its role in the marine microbial food web and the chemical landscape of the ocean, as well as its implications for local or regional climate regulation.

6.1 Uptake and distribution of DMSP in marine microbial communities of different latitudes

Marine microbial community composition and their respective activities underpin nutrient cycling in the oceans, playing a key role in global biogeochemistry and the regulation of ocean to atmospheric fluxes of trace gases. Dimethylsulfoniopropionate (DMSP) is a key organic sulphur compound primarily synthesised by phytoplankton that directly links microbial activity with atmospheric sulfur fluxes. Different processes such as viral attack, exudation or autolysis of phytoplankton cells, leads to the release of DMSP from the cell to the environment, where it reaches the microbial foodweb and is taken up by heterotrophic bacteria (Zubkov et al., 2001) either to be utilised for growth or cleaved into DMS, which diffuses out of the cell into seawater and enters the atmosphere by air-sea exchange. There it is oxidised to sulfate aerosols and contributes to cloud condensation nuclei, which will increase cloud albedo, resulting in a decrease in solar irradiance and surface temperature on a regional and global scale (Charlson et al. 1987; Welsh, 2000).

An important study by Vila-Costa et al. (2006) challenged the notion that DMS flux was driven solely by the activity of hetertrophic bacterioplankton and instead suggested that autotrophic phytoplankton taxa may also utilise dissolved pools of DMSP, taking it up directly from the environment. They also proposed that this uptake could even equate to the amount taken up by bacterioplankton, which has been estimated to be ~10% of the total marine DMSP synthesised. Since this study, there have been others showing direct dissolved DMSP uptake by low or non-DMSP producing phytoplankton (Vila-Costa et al 2010; Petrou and Nielsen 2018; Lavoie et al 2018), lending support to the hypothesis that the autotrophic members of the microbial community may also act as important recyclers of organic sulfur in the ocean.

Inspired by these studies, this thesis investigated the uptake of dissolved DMSP by all members of the marine microbial community, including phototrophs, to determine whether or not they form a significant sink of DMSP in natural environments, potentially diverting DMSP from being converted to DMS and decreasing atmospheric DMS fluxes. To gain a more geographically diverse perspective on the uptake of DMSP and its distribution throughout the marine microbial communities, natural seawaters from different latitudes covering the three climatic biomes of earth – tropical, temperate and polar- were investigated. Common to all three locations, we found that all fractions increased their particulate DMSP content when enriched with DMSP, with the largest fractions often containing the greatest increase in DMSPp. These findings suggested diatoms as a key phototrophic sink for DMSP (Figure 6.1) and highlight the need to better understand why these organisms would take up DMSP and what they are able to utilise it for. Specifically, do they have the genes that can metabolise DMSP for growth? Do they utilise it for stress relief or perhaps even degrade it into DMS, thus contributing to DMS flux from the ocean?

Comparing the inner and outer reef sites in Chapter 3, there were some key differences between the dominant sinks for DMSP, with diatoms dominating in the inner reef and SAR11 and *Procholococcus* forming the major sinks for the outer reef. The results suggest that the differences in nutrient concentrations most probably due to the upwelling on the continental shelf of cool nutrient-rich water, microorganism taxa and abundance could influence DMSP uptake and distribution thoughout the different fractions or species of the community. This in turn, will influence the amount of DMSP degraded through each pathway and hence, the DMS fluxes to the atmosphere, affecting cloud albedo and regional climate.

Despite latitude, the waters from Port Hacking had a typically tropical signature (Chapter 5), showing a strong influence of EAC water. The phytoplankton community was dominated by *Trichodesmium*, which meant low phytoplankton diversity and relatively high DMSP concentrations. Within the larger size fractions there was clear separation of the community between producers and temporary sinks, with the presence of three recognized high DMSP producers and a few non-DMSP producing diatoms, again highlighting the role of diatoms as transient pools of DMSP in oceanographic waters.

In conclusion, these results challenge the idea that heterotrophic prokaryotes are the only DMSP sinks in the oceans and support the hypothesis of Vila Costa et. al. (2006) that non DMSP producing phytoplankton species also take up DMSP. For all three latitudes, we have shown that all members of the community, which include big and small eukaryotes and prokaryotes, are important DMSP sinks and that diatoms formed one of the dominant sinks across all three environments (Figure 6.1).

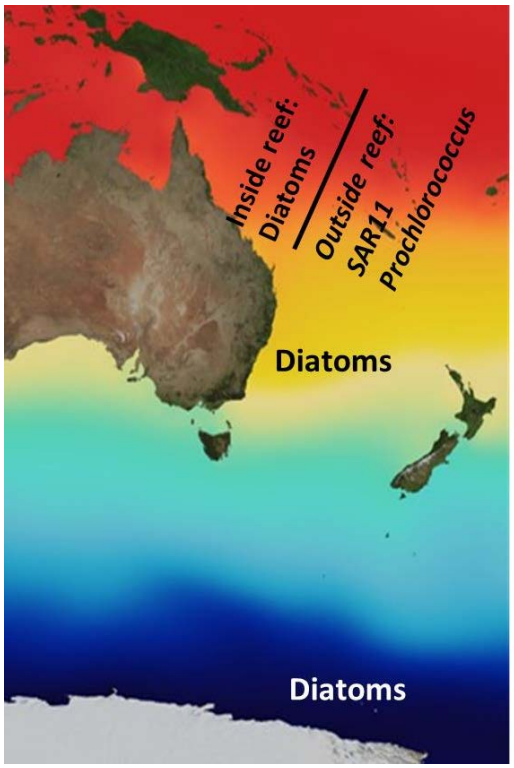

Figure 6.1. Major DMSP sinks for each climatic zone. *Our results of DMSPp showed that the major DMSP sink for the tropical climate differed between reef environments, being diatoms the biggest sink for the inner reef site and SAR11 and Prochlorococcus for the outer reef site. Diatoms were also the biggest DMSP sink for the temperate and polar climate.*

6.2. Effect of DMSP on the composition of marine microbial communities of different latitudes

A complete understanding of the role of DMSP in shaping marine microbial communities is still in its infancy. It is known that the bacterial biomass production increases during phytoplankton blooms and that a wide variety of bacteria benefits from it. However, different bacterioplankton proliferate during different phytoplankton blooms as well as different locations (Campbell et al., 2005; Delmont et al., 2014; Fandino et al., 2001).

There is considerable information available on major DMSP producers in the oceans, including many studies on the coccolithophores in temperate seas, more recently *Trichodesmium* from tropical waters and *Phaeocysistis* blooms in Antarctica. These three taxa are abundant and bloom frequently in their climatic regions studied in this thesis (Delmont et al., 2014; DiTullio et al., 2000; Malin et al., 1993; Matrai and Keller, 1993). Moreover, Coccolithophores and *Phaeocysistis* belong to the few phytoplankton taxa with DMSP lyase activity, releasing copious amounts of DMS during bloom scenarios and are important sources of atmospheric DMS (Wolfe & Steinke 1996, Boekel & Stefels 1993). On the other hand, the N fixing *Trichodesmium* is expected to play an increasingly important role in the cycling of DMSP in temperate waters undergoing climate change driven tropicalisation (Ajani et al., 2014a, 2014b; Bucciarelli et al., 2013b; Lee et al., 2017).

There has also been numerous studies investigating the role of bacterioplankton in the demethylation and cleavage of DMSP. It appears that DMSP is catabolised by a wide variety of bacteria, such as alpha- and gamma- proteobacteria, and that demethylation is the dominant pathway with SAR11 and Roseobacter clades as the major catabolisers (Howard et al., 2008b). Roseobacter has been found to be the major cataboliser in surface waters of Kongsfjorden, in the Arctic (Zeng et al., 2016) and in coastal regions during phytoplankton blooms (Howard et al. 2006), while *SAR11* is the primary DMSP cataboliser in oligotrophic areas such as the Sargasso Sea (Rex R Malmstrom et al., 2004) and the tropical and Subtropical Pacific Ocean (Cui et al., 2015). Other less abundant marine bacteria seems to be responsible for DMSP cleavage. The minor cleavage pathway occurs mostly in the Roseobacter and SAR116 clades (Curson et al., 2011; Todd et al., 2009).

In a study in which natural seawaters of the oligotrophic Sargasso Sea were incubated with DMSP for 30 min, transcripts of many bacteria including *Gammaproteobacteria* and *Bacteroidetes*, which harbour DMSP degradation genes, increased in relative abundance

(Vila-Costa et al., 2010). In another study, in which a phytoplankton bloom was induced in seawater from the coastal Gulf of Mexico, an increase in particulate DMSP was accompanied by an increase in bacterial biomass production and higher abundances of *Roseobacter*, *Bacteroidetes* and *Gammaproteobacteria* (Pinhassi et al., 2005). Inspired by these two studies, we anticipated that DMSP would modify the microbial composition of the seawater. There was the additional expectation that DMSP enrichment would result in an increase in microbes that harbour DMSP degradation genes, compared to the microbes that cannot utilise DMSP.

The work contained within chapters 3, 4 and 5, confirm a strong and rapid decline in DMSP within the first 24 h linked with rapid DMSP uptake, but that over time, DMSP concentrations decline, suggestive of catabolic processes resulting in an overall loss of DMSP from the system. Over longer incubation periods of 120-144h, DMSP concentrations decreased over time for both studied tropical (Chapter 3) and polar (Chapter 4) communities, but showed significant differences in the responses of the microbial communities. While in the tropical communities many bacteria benefited from the presence of DMSP, there were no differences in polar microbial composition. This divergent response was due to the differences in nutrient composition and natural DMSP concentrations between both habitats. In the oligotrophic tropical sea, the bacterial sulfur demand was not satisfied resulting in the bacterial utilisation of DMSP for cell growth via the demethylation pathway, which led to the proliferation of DMSP-degrading bacteria. In contrast, the nutrient and DMSP rich polar waters showed that the bacterial sulfur demand was met and excess DMSP was cleaved to DMS with no effect on microbial composition.

In brief, these findings support the hypothesis that the bacterial switch is regulated by the sulfur demand and showed that DMSP supposes an ecological advantage to some bacteria and phytoplankton taxa, and that the marine bacterioplankton may rely more on DMSP as a source of organic sulfur and carbon under oligotrophic conditions than under nutrient-rich conditions. This work has highlighted the need to look for new DMSP degradation pathways and genes in phytoplankton and continued research into identifying the bacteria that harbour DMSP demethylation and degradation genes.

6.3. Future directions

To identify all the sources and sinks of DMSP that ultimately control its fate in the ocean, there are a number of key areas of study that may help to resolve much of the missing

information. One way to enhance the resolution of data obtained from incubation experiments would be to use DNA stable-isotope probing (DNA-SIP). DNA-SIP is a powerful technique for identifying microorganisms that actively assimilate particular substrates and nutrients into cellular biomass (Dumont and Murrell, 2005; Radajewski et al., 2000). The technique consists on separating the total community DNA into two bands, according to whether it has ^{13}C or not, using caesium chloride (CsCl) buoyant density-gradient centrifugation (Neufeld et al., 2007) and the bands sequenced to provide taxon-specific information (Figure 2.1.3) (Dumont and Murrell, 2005). As such, this technique could be used to identify the incorporation of labelled DMSP into various members of the microbial community of natural seawaters.

Independent of the technique, additional studies across different climatic and oceanographic regions at a different periods of the year would enable us to draw a more detailed map covering the spatial and temporal similarities and differences of the major DMSP sinks and sources responsible for sulphur cycling. Moreover, new studies focusing on non-DMSP producing phytoplankton species are necessary to determine wether or not they play a relevant role in the sulfur cycle. Research would need to cover aspects such as the physiological role of DMSP inside these cells, and identification of new DMSP degradation pathways and genes. Likewise, further studies into bacterial DMSP degradation are needed to elucidate which bacteria are capable of degrading DMSP and what are the genes responsible.

6.4. Concluding remarks

This thesis has tried to address some key knowledge gaps on the distribution of DMSP within three distinct marine microbial communities and its utilisation by the different microbes, providing new insights into the complexities of the marine sulfur cycle. Through a series of incubation experiments it has confirmed the importance of autotrophic phytoplankton as DMSP sinks in water bodies from three distinct latitudes and identified diatoms as a potentially important group to contribute to marine sulfur dynamics. The significant uptake of DMSP by diatoms emphasises the need for further research to determine its utility in the cell. Do diatoms act as temporary storage pools? Does DMSP provide the cell with a physiological advantage? or do diatoms possess a yet unidentified DMSP degradation pathway? Answers to these questions will help to determine whether diatoms make a significant and previously unrecognised contribution to marine sulfur cycling. Similarly, the

identified shifts in bacterial composition with DMSP enrichment that were not directly related to known DMSP degrading taxa, highlights the need for increased knowledge on DMSP degraders in our marine environments.

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Appendix A: Supporting Figures and tables for chapter 1

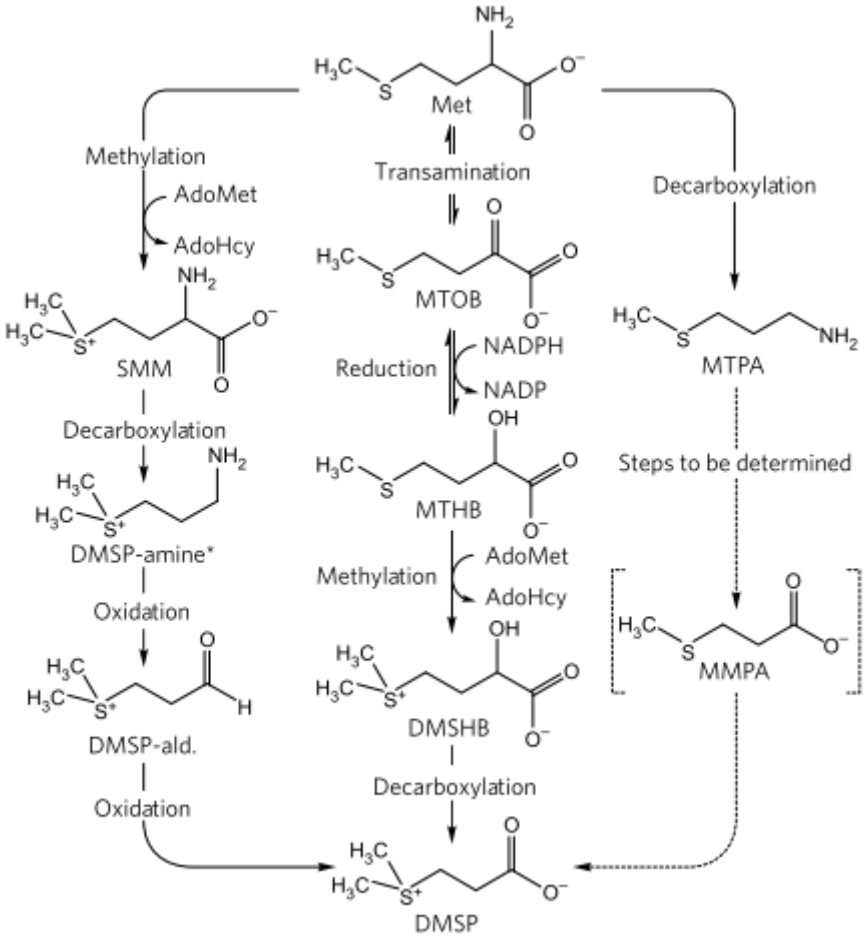

Figure S.1. Structure of enzymes participating in DMSP biosynthetic pathways*. Met, methionine; MTOB, 4.methylthio-2-oxobutyrate; MTHB, 4-methylthio-2-hydroxybutyrate; DMSHB, 4-dimethylsulfonio-2-hydroxybutyrate; SMM, S-methyl-L-methionine; MTPA, 3-methylthiopropylamine; MMPA, methylmercaptopropionate; MHM, MTHB methyltransferase; dsyB. From Curson et al. (2017)*

Appendix B: Supporting Figures and tables for chapter 2

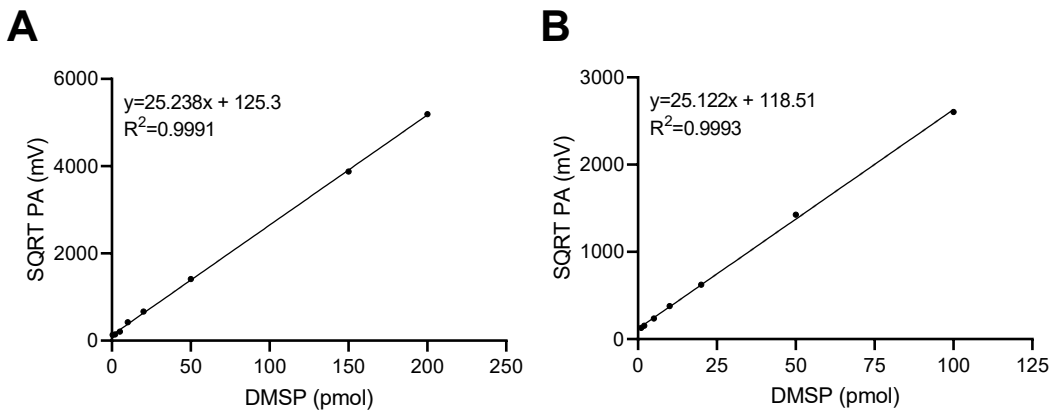

Figure S.1. Example DMS calibrations. *(A) Purge and trap calibration. (B) Direct Injection calibration.*

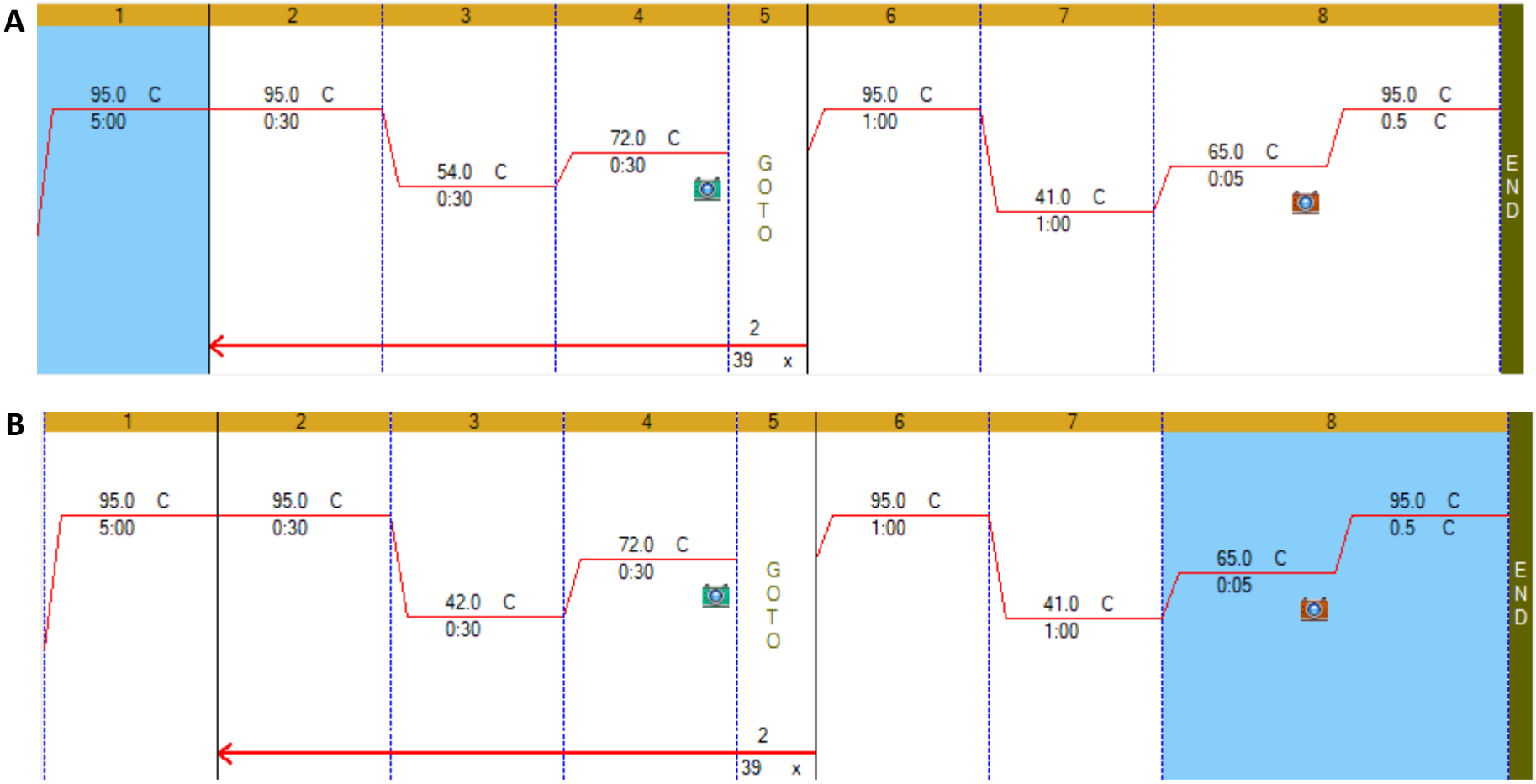

Figure S.2. Conditions for qPCR reaction. *(A) Conditions for 16S and A1 qPCR reaction. (B) conditions for DmdA/Dall and DddP qPCR reaction.*

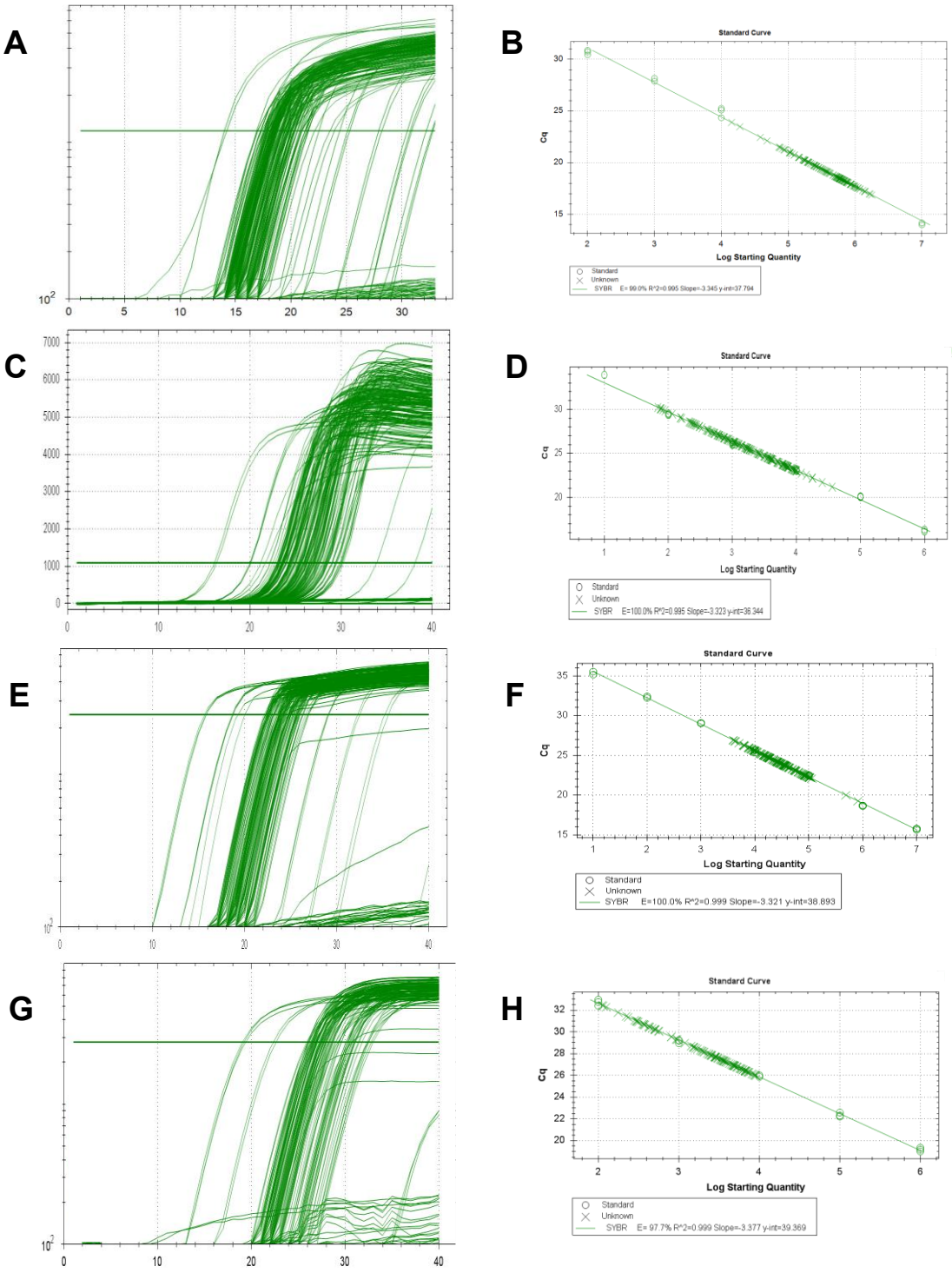

Figure S.3. Amplification and calibration curves of qPCR analysis. *(A) Amplification curve of 16S. (B) Calibration curve 16S. (C) Amplification curve of DmdA/A1. (D) Calibration curve DmdA/A1. (E) Amplification curve of DmdA/DalI. (F) Calibration curve DmdA/DalI. (G) Amplification curve of DddP. (H) Calibration curve DddP.*

Table S.1. Results of ^{13}C-DMSP samples analysed by EA-IRMS at ANSTO. *(A) Values of δ^{13}C and uncertainty for a control (C13), a DMSP enriched sample (D13) and a ^{13}C-DMSP enriched sample (L13). (B) Standard Reference Materials (SRM) used for data normalisation. (C) Quality control checks*

A

LIMS Number	Client Identification	Sample No.	Carbon Data $\delta^{13/12}C_{V\text{-}PDB}$ ‰	Carbon Data $\delta^{13/12}C$ Uncertainty V-PDB ‰	Amount of GFF analysed	Peak Height mV	Comments
2017/0313J-1	B2701	5	-26.0	2.0	1 punch ~ 5mm diameter	125	JvH17Nov30
2017/0313J-6	C13	6	-27.4	0.5	1 punch ~ 5mm diameter	725	JvH17Nov30
2017/0313J-7	D13	7	-25.3	0.3	1 punch ~ 5mm diameter	3052	JvH17Nov30
2017/0313J-8	L13	8	-26.3	0.6	1 punch ~ 5mm diameter	619	JvH17Nov30
2017/0313J-8(2)	L13	8	-25.7	0.3	1 punch ~ 5mm diameter	1148	JvH17Nov30

NOTE: The C% data cannot be quantified as the mass of the GFF punches varies too much i.e. mass of a blank punch is more than mass of sample punch out. C% alculation is based on the mass of samples. The amount of material on the GFF varies too much.

B

Name	Nitrogen %	Nitrogen $\delta_{15/14}N_{AIR}$‰	Carbon %	Carbon $\delta_{13/12}C_{V\text{-}PDB}$‰
IAEA USGS-40 L-Glutamic Acid	9.5 ± 0.3	-4.5 ± 0.1	40.9 ± 0.5	-26.4 ± 0.04
IAEA USGS-41 L-Glutamic Acid	9.8 ± 0.3	47.6 ± 0.2	42 ± 0.6	37.6 ± 0.05
Sercon SC0419 Methionine	9.4 ± 0.1	-2.0 ± 0.1	40.3 ± 0.3	-30.3 ± 0.1

C

RUN NO.	ID	# SAMP	C%	$\delta_{13/12}C$ ‰ $_{V\text{-}PDB}$
	CERTIFIED VALUES for B2160		**44.7 ± 0.2**	**-18.9 ± 0.1**
JvH17Nov30	B2160	1	44.3	-19.2
JvH17Nov30	B2160	2	44.0	-19.1
		AVERAGE	**44.1**	**-19.2**
		S.D.	**0.2**	**0.1**
		Difference from Actual	**0.6**	**0.3**
RUN NO.	ID	# SAMP	C%	$\delta_{13/12}C$ ‰ $_{V\text{-}PDB}$
	CERTIFIED VALUES for AILS-102		**47.4 ± 0.2**	**-22.2 ± 0.04**
JvH17Nov30	AILS-102	1		-22.0
		AVERAGE		**-22.0**
		S.D.		
		Difference from Actual		**-0.2**

Table S. 2. Results of the regression analysis for the equation to predict % DMSP labelled in samples run in TD-GC-MS. *(A) Overall fit of the linear regression equation. (B) Individual components of the sum of the squares (C) Regression coefficients*

Regression Analysis

A

OVERALL FIT	
Multiple R	0.961202
R Square	0.923909
Adjusted R Square	0.917568
Standard Error	14.49774
Observations	14

B

ANOVA				Alpha	0.05	
	df	SS	MS	F	p-value	sig
Regression	1	30625.26	30625.26	145.7066	4.53E-08	yes
Residual	12	2522.214	210.1845			
Total	13	33147.47				

C

	coeff	std err	t stat	p-value	lower	upper
Intercept	-17.9008	6.772597	-2.64312	0.02145	-32.657	-3.14455
16	2.149083	0.178038	12.0709	4.53E-08	1.761171	2.536995

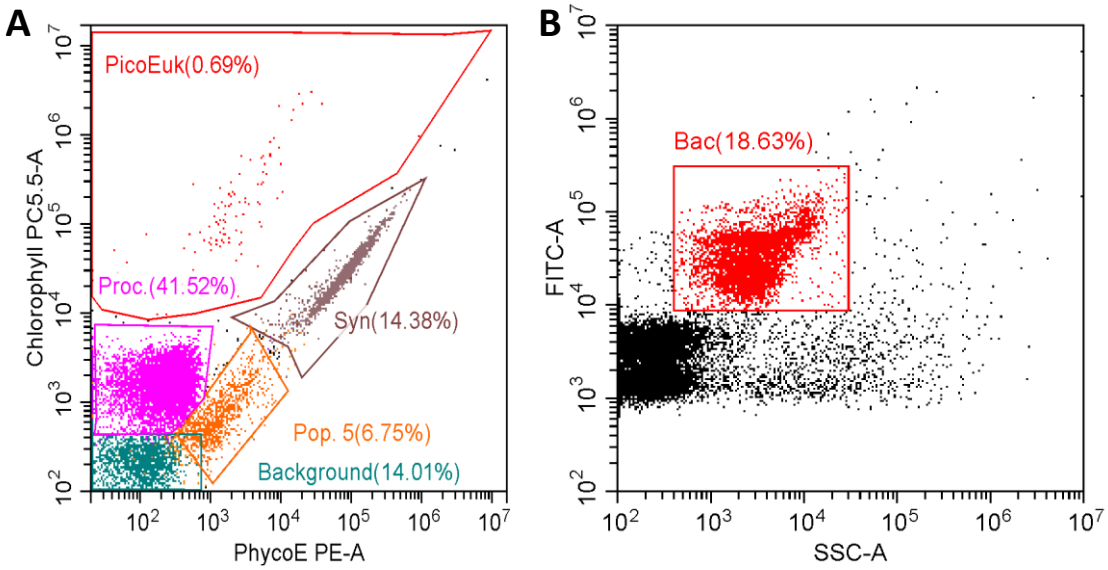

Figure S. 4. Example of Flow cytometry charts. *(A) Dot plot of Chlorophyll a vs phycoerythrin showing picoeukaryotes, Prochlorococcus and Synechococcus. (B) Dot plot of FITC fluorescence vs side scatter showing heterotrophic bacteria stained with SYBR Green.*

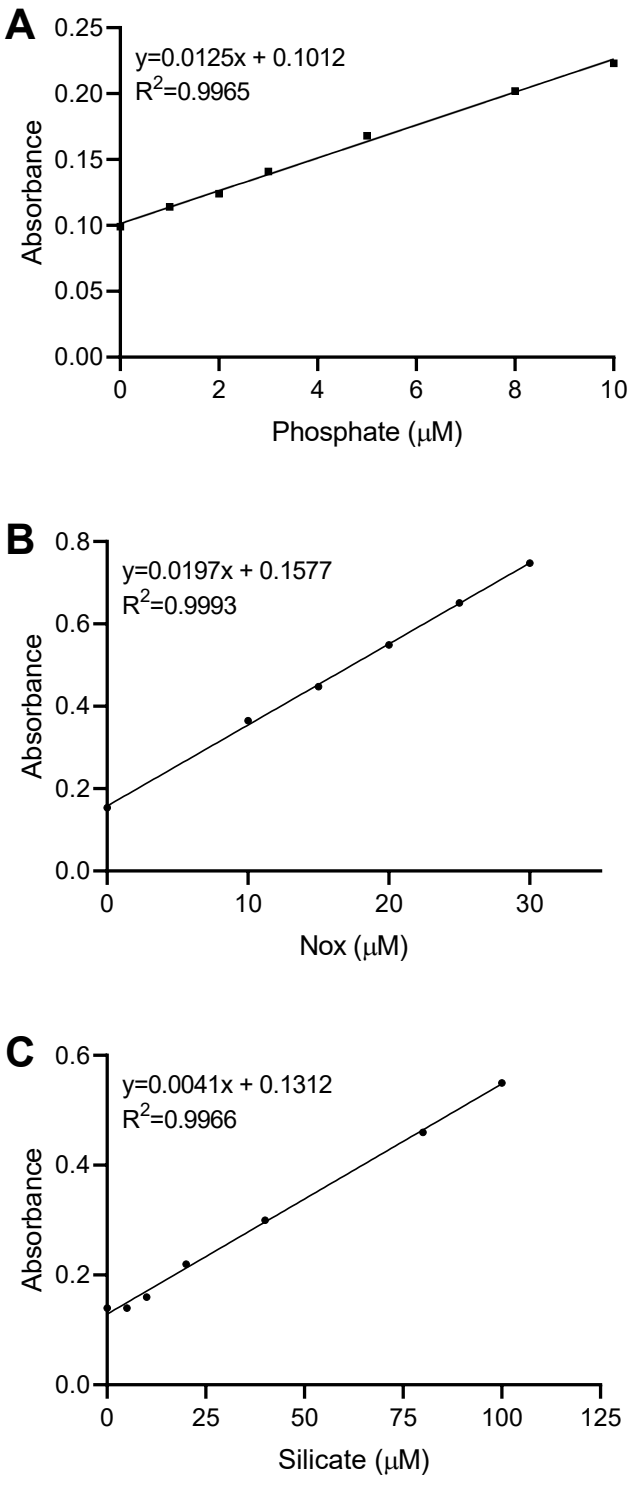

Figure S. 5. Calibration curves for nutrient analysis using a microplate based method. *(A) Calibration curve for phosphate analysis (B) Calibration curve for Nox analysis. (C) Calibration curve for Silicate analysis*

Appendix C: Supporting Figures and tables for chapter 3

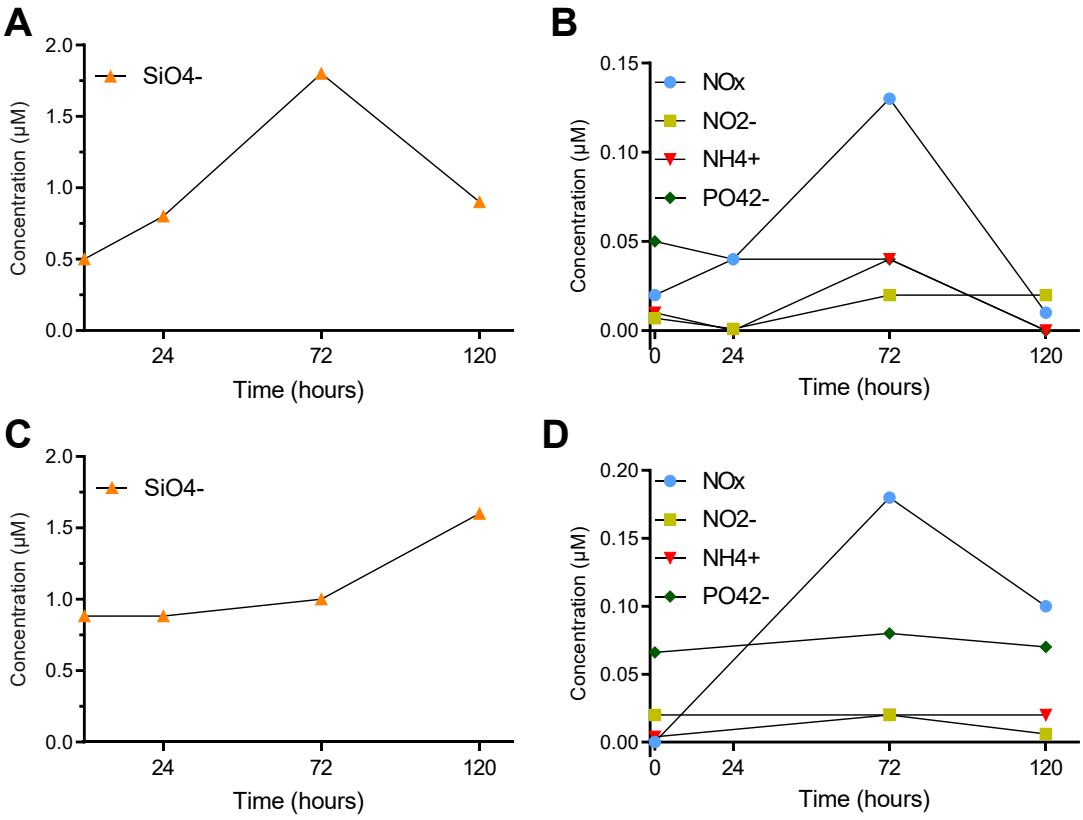

Figure S.6. Nutrients concentrations of natural waters during experiment 2. *Silicate concentration inside the reef (A) and outside the reef (C). Phosphate, ammonium,nitrite and NOx concentrations inside the reef (B) and outside the reef (D).*

Table S. 1 Nutrient concentrations for controls and DMSP enriched samples during experiment 2. *Nutrients concentrations for inside the reef site (A) and outside the reef site (B). * below detection limit*

A

	0h	24h	72h	120h
NOx				
Control	0.02*	0.04	0.13	0.01*
DMSP	0.02*	0.07	0.09	0.03
Nitrite				
Control	0.007*	0.001*	0.02*	0.02*
DMSP	0.007*	0.004*	0.03*	0.01*
Silicate				
Control	0.5	0.8	1.80	0.90
DMSP	0.5	1.6	2.00	0.97
Ammonium				
Control	-0.01*	0.00	0.04	-0.02*
DMSP	-0.01*	0.00	0.02*	-0.03*
Phosphate				
Control	0.05	0.04	0.04	0.02*
DMSP	0.05	0.04	0.07	0.01*

Concentrations in µM * Below detection Limit

B

	0h	24h	72h	120h
NOx				
Control	-0.01*	No data	0.18	0.1
DMSP	-0.01*	No data	0.16	0.057
Nitrite				
Control	0.02*	No data	0.02*	0.006*
DMSP	0.02*	No data	0.02*	0.001*
Silicate				
Control	0.882	No data	1.00	1.600
DMSP	0.882	No data	1.10	1.367
Ammonium				
Control	0.004*	No data	0.02*	0.020*
DMSP	0.004*	No data	0.17	0.073
Phosphate				
Control	0.066	No data	0.08	0.070
DMSP	0.066	No data	0.07	0.067

Concentrations in µM * Below detection Limit

Table S. 2. Relative abundance (%) of bacteria for initial waters. *(A) Rickettsiales, (B) Cyanobacteria, (C) Rhodobacterales, (D) Oceanospirillales*

A	**Experiment 1**		**Experiment 2**	
	IR	**OR**	**IR**	**OR**
LWSR-14	0	0	0.014	0.041
Rickettsiaceae	0	0	0	0.027
S25-593	0.299	0.965	0.408	0.802
SAR116 clade	2.025	2.025	2.025	2.025

B	**Experiment 1**		**Experiment 2**	
	IR	**OR**	**IR**	**OR**
Candidatus Atelocyanobacterium (UCYN-A)	0,05434783	0,08152174	0,06793478	0,06793478
Prochlorococcus	19,6059783	34,0217391	28,6820652	31,3179348
Synechococcus	16,3451087	0,625	10,1902174	0,55706522
Other	0,12228261	0,02717391	0,10869565	0,01358696
Calothrix	0,01358696	0	0	0
Oscillatoria	0	0	0	0,06793478

C	Experiment 1		Experiment 2	
	IR	OR	IR	OR
Ascidiaceihabitans	1.033	0.353	0.679	0.326
Dinoroseobacter	0.000	0.014	0.027	0.000
Donghicola	0.000	0.014	0.000	0.000
Epibacterium	0.000	0.014	0.000	0.014
Jannaschia	0.000	0.027	0.000	0.000
Labrenzia	0.000	0.000	0.014	0.000
Loktanella	0.027	0.000	0.027	0.000
Oceaniovalibus	0.000	0.000	0.027	0.000
Pelagicola	0.000	0.014	0.000	0.000
Roseovarius	0.109	0.014	0.190	0.000
Ruegeria	0.041	0.014	0.000	0.014
Sulfitobacter	0.258	0.122	0.313	0.095
Tropicibacter	0.041	0.068	0.014	0.000
uncultured	4.837	3.071	3.967	3.560
Other	0.122	0.163	0.068	0.054

D	Experiment 1		Experiment 2	
	IR	OR	IR	OR
Alcanivoracaceae	1.4E-04	9.5E-04	0.0E+00	0.0E+00
Halomonadaceae	8.2E-04	0.0E+00	1.4E-04	0.0E+00
OM182 clade	1.1E-03	1.4E-04	6.8E-04	0.0E+00
Oceanospirillaceae	3.4E-03	4.1E-04	1.1E-03	4.1E-04
SAR86 clade	5.2E-02	5.1E-02	4.3E-02	5.4E-02
Other	0.0E+00	2.7E-04	0.0E+00	1.4E-04

Appendix D: Supporting Figures and tables for chapter 4

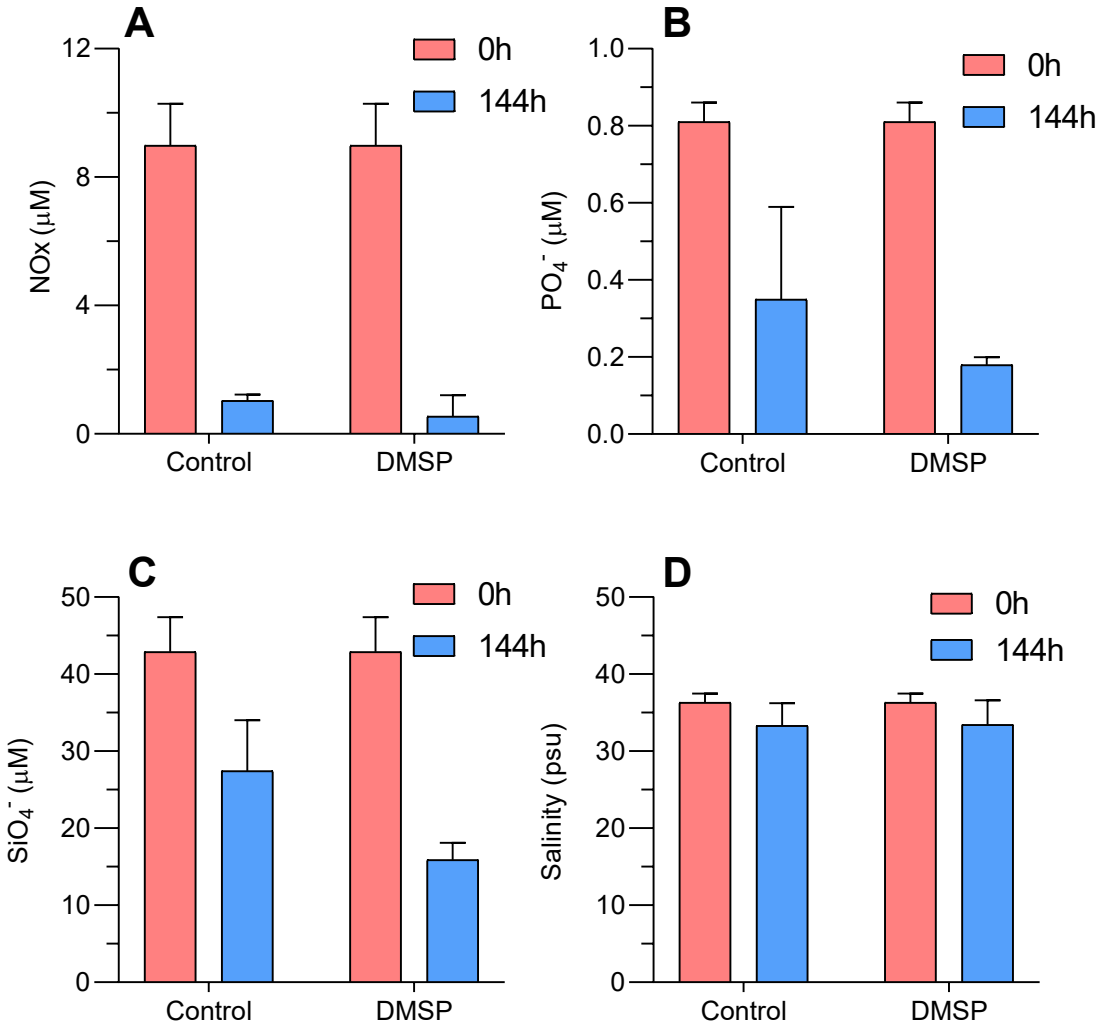

Figure S.7. Nutrients concentrations and salinity for experiment 2. *Concentrations at initial (0h) and final (144h) time for controls and DMSP spiked samples. (A) Nox, (B) Phosphate, (C) Silicate, (D) Salinity.*