Function and Metabolism of Dimethylsulfoniopropionate by Higher Plants and Associated Rhizosphere Bacteria

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Abstract

Dimethylsulfoniopropionate (DMSP) is one of the Earth's most abundant organosulfur compounds, with more than 8 billion tonnes produced annually by marine microorganisms and some plants as an antistress compound. Comparatively few higher plants were known to be high producers, including Sugarcane, Sea Daisies and Cordgrasses. Through sampling a large variety of wild plants, we found that all plants produce DMSP, albeit at low levels, including the model organism *Arabidopsis thaliana.* Additionally, DMSP production is increased in response to salt stress, suggesting it functions as an osmolyte in higher plants. DMSP is known to be produced through the methylation pathway, confirmed when *MMT-* homozygous knockout *A. thaliana* produced significantly less DMSP compared to their wildtype counterparts, and were significantly more stressed in response to salt. Furthermore, when released into the environment, this DMSP is a major carbon and sulfur source for marine microbes through catabolism that yields the climate-active gases and signalling molecules dimethylsulfide (DMS) via DMSP lyase enzymes and methanethiol (MeSH) via DMSP demethylation. *Spartina anglica*, an invasive cordgrass, is one of Earth's highest accumulators of DMSP and is responsible for the far higher DMSP levels per unit area in saltmarsh environments compared to surface seawaters. *Spartina*, which does not cleave DMSP itself, is proposed to feed this nutrient to its holobiome in return for vitamins, hormones and/or antibiotic activities. Here we conducted stable-isotope probing experiments with ^{13}C-DMSP to study DMSP-mediated interactions between *Spartina* and its holobiome. This work identified the abundant and important group of marine bacteria, the *Roseobacters*, as major degraders of DMSP in the *Spartina* rhizosphere via their DMSP demethylation *DmdA* and DMSP lyase *DddL* enzymes. Cultivation-dependent work supported *Roseobacters*, well known to catabolise DMSP and interact with algae, as key bacteria assimilating DMSP in the *Spartina* rhizosphere, potentially interacting with the plant, and liberating significant amounts of DMS and MeSH.

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अनन्तप्रकाशजीवनबुद्धाय आत्मानं समर्पयामि

Declaration

I, Mellieha Grace Evangeline Allen, hereby declare that this thesis is a record of my own work from October 2019- December 2023 and is of my own composition. It has not been submitted for the application of a degree at any other university or institute. Results obtained by other colleagues has been acknowledged in the relevant chapter. All quotations are clearly distinguished by quotation marks and all sources of information are specifically acknowledged with citations.

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Abbreviations

·OH	hydroxyl radical
α	alpha
β	beta
μl	microlitre
μM	micromoles per litre or micromolar
16S rRNA	ribosomal RNA in bacteria and archaea
3-HP	3-hydroxypropionate
ABC	ATP binding cassette
AdoHCY	S-adenosyl-L-homocysteine
AdoMet	S-adenosine-methionine
ALDH	aldehyde dehydrogenase
APS	adenosine phosphosulfate
aq	aqueous
BADH	betaine aldehyde dehydrogenase
BCCT	betaine choline carnitine transport
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
C	carbon
Ca	calcium
CCN	cloud condensation nuclei
CH_4	methane
CH_3OH	methanol
CH_3SH	methanethiol
CH_3-THF	methyltetrahydrofuran
Cl	chlorine
CLAW	Robert **C**harlson, James **L**ovelock, Meinrat **A**ndreae and Stephen **W**atson hypothesis
CoA	Coenzyme A
CO	carbon monoxide
CO_2	carbon dioxide
Col-0	*Arabidopsis thaliana* wildtype Colombia

Abbreviations

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CsCl	caesium chloride
Cu^{2+}	copper ions
DART-TOMFS	Direct Analysis in Real Time – Time of Flight Mass Spectrometry
DDH	diaminopimelate dehydrogenase
DMS	dimethyl sulfide
DMSO	dimethylsulfoxide
DMSOB	4-dimethylsufonio-2-oxobutyrate
DMSHB	4-dimethylsulfonio-2-hydroxybutyrate
DMSP	dimethylsulfoniopropionate
DMSP-a	3-dimethylsulfoniopropylamine
DMSP-ald	3-dimethylsulfoniopropionaldehyde
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
DOX	DMSP-amine Oxidase
EDTA	ethylendiamine-tetraacetic acid
FABMS	Fast-Atom Bombardment and Mass Spectroscopy
Fe	iron
FH_4	tetrahydrofolate
fmol	femtomole per litre or femtomolar
FW	Fresh Weight
g	gram
GBT	glycine betaine
GC	gas chromatography
GSH	glutathionine
H_2	hydrogen
H_2S	hydrogen sulfide
H_2SO_4	sulfuric acid
h	hour
HGT	horizontal gene transfer

Abbreviations

HMT	homocysteine S-methyltransferase
HPLC	High Performance Liquid Chromatography
HSO_3^-	hydrogen sulfite
IMG	Integrated Microbial Genomes
IPTG	isopropyl β-D-1-thiogalactopyranoside
JGI	Joint Genome Institute
K^+	potassium ions
Kbp	Kilo Base Pairs
L	litre
LB	Luria Broth
m	meter
M	molar (concentration)
MAG	Metagenome Assembled Genome
MaS	malonate semialdehyde
MBM	minimal basal media
MeSH	methanethiol
Met	methionine
Mg	magnesium
mg	milligram
mins	minutes
mM	millimolar
mm	millimeter
MMETSP	Marine Microbial Eukaryote Transcriptome Sequencing Project
MMPA	methyl mercaptopropionate
MMT	methionine methyltransferase
Mn	manganese
MTA	methylthioacryloyl
MTHB	4-methylthio-2-hydroxybutyrate
MTOB	4-methylthio-2-oxobutyrate
MTPA	3-methylthiopropanamine

Abbreviations

MPA	3-mercaptoproprionate
MS	Mass Spectrometry
MS-Agar	Murashige and Skoog Medium
MW	molecular weight
N	nitrogen
Na^+	sodium ion
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NaOH	sodium hydroxide
NBP	nucleotide binding protein
NCBI	National Center for Biotechnology Information
nm	nanometers
nM	nanomolar
NMR	Nuclear Magnetic Resonance
NO_3	nitrate
O_2	oxygen
PCR	polymerase chain reaction
PGPR	Plant Growth Promoting Rhizobacteria
PSU	practical salinity units
pmol	picomole
POP2	pyridoxal phosphate (PLP)-dependent transferase
rcf	relative centrifugal force
R.O	Reverse Osmosis
ROS	Reactive Oxygen Species
RT	reverse transcription
S	sulfur
S^{2-}	sulfide
SAM	S-adenosyl methionine
SAH	S-adenosyl-L-homocysteine
SBP	substrate binding protein

Abbreviations

SDC	S-methylmethionine decarboxylase
SDS	sodium dodecyl sulfate
SIP	Stable Isotope Probing
SMCSO	S-methyl-L-cysteine-sulfoxide
SMM	S-methylmethionine
Sn	tin
SO_2	sulfur dioxide
SO_3^{2-}	sulfite
SO_4^{2-}	sulfate
S.O.C	Super Optimal broth with Catabolite repression
SOS	Salt-Overly Sensitive (Pathway)
SULTR	Sulfate Transport Proteins
TAE	tris-acetate-EDTA
TAIR	The Arabidopsis Information Resource
TBE	tris-borate-EDTA
TLC	Thin-Layer Chromatography
TMP	transmembrane protein
Tris-HCL	tris(hydroxymethyl)aminomethane hydrochloride
UEA	University of East Anglia
UV	ultraviolet
V	voltage
VOC	Volatile Organic Compound
W	Watts
XANES	X-ray Absorption Near Edge Structure
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YTSS	yeast tryptone sea salts
Zn	zinc

Chapter 1 - Introduction

Biogeochemical Cycling Systems Overview

Biogeochemical cycling is the global movement of elements between organic and inorganic reservoirs and is essential for life (Brusseau, 2019). The most widely understood cycles are those of the elements that make up the basic components of organic life and are therefore the most abundant in biological systems: hydrogen, oxygen, carbon, nitrogen, sulfur and phosphorus (Emsley, 2001).

These biogeochemical cycles share fundamental factors. Firstly, the movement of elements between different systems of the Earth, known as reservoirs. The major reservoirs are the atmosphere, hydrosphere (oceans and water courses), biosphere (organic matter), pedosphere (the soil) and the lithosphere (outer crust) (Selley, 2005). Secondly, the movement (or flux) of elements between these states through the anabolism and catabolism of different molecules, is driven by the constant movement of matter towards thermodynamic equilibrium (Fig. 1.1) as well as energy from solar radiation (Brusseau, 2019). Finally, biogeochemical cycles do not exist within a closed system. They are deeply interlinked and act as feedback loops for each other, which regulate the flux between reservoirs (Selley, 2005).

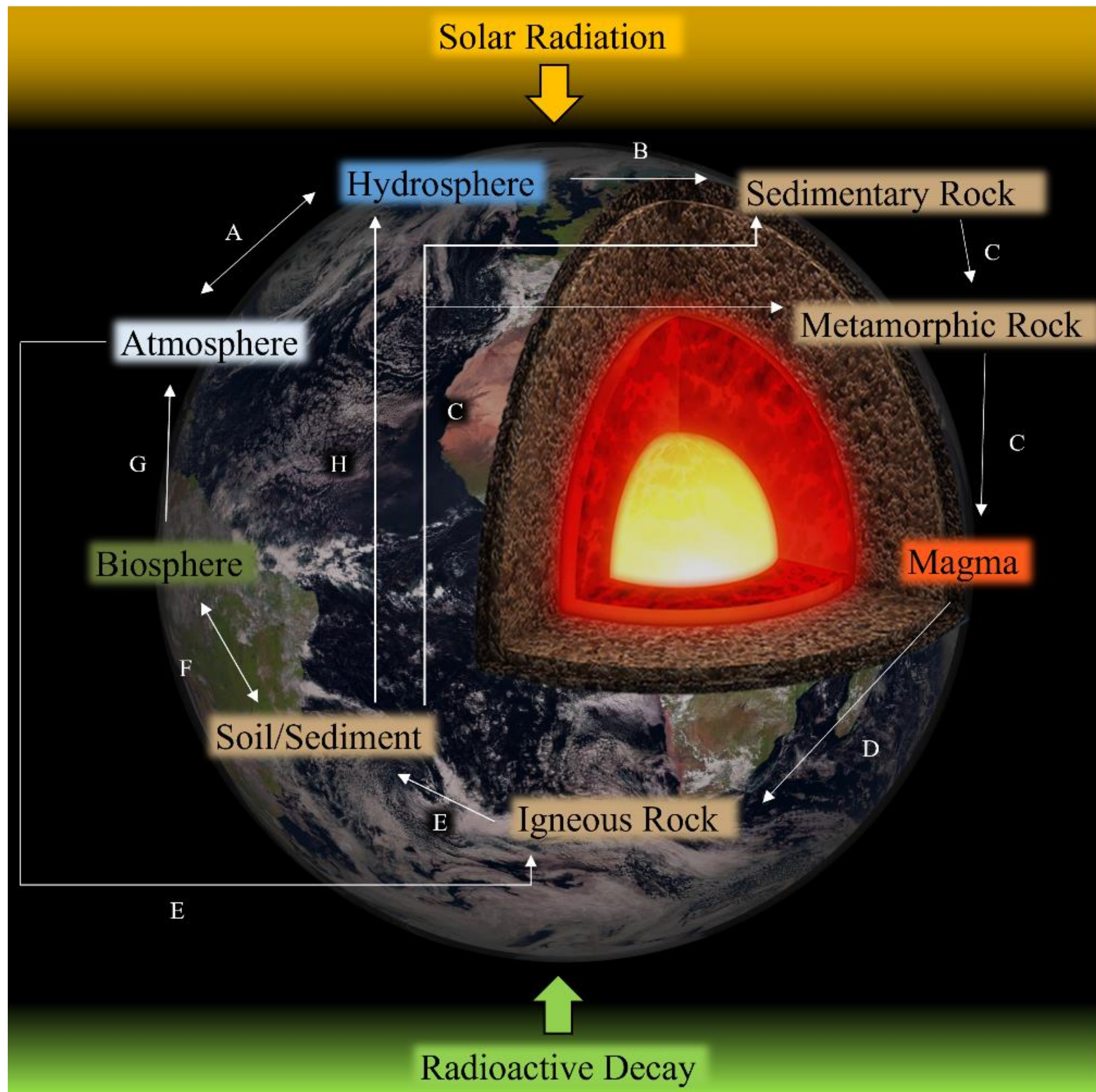


Figure 1.1: A simplified diagram showing the flux of matter between the different reservoirs. Processes shown are A: Evaporation and Precipitation B: Aquifer Recharge C: Heat and Pressure D: Melting E: Weathering F: Nutrient Cycling G: Evapotranspiration H: Ion Exchange. Adapted from Jacobson *et al.*, 2000. Background image licensed from © EUMETSAT/ESA.

Feedback loops are well known within the context of bodily homeostasis, for example, increased blood glucose is detected by beta cells in the pancreas, causing the liver to cease glucose production (Koeslag *et al.,* 2003). This is an example of a negative feedback loop, in which the output of one system reduces the output of another and are critical to preventing tipping points in which positive feedback loops – where the output of one system increases the output of another – from accelerating the formation of products to a point that it disrupts the flux of other systems (Selley, 2005). Another example is the *Trp* operon, ubiquitous in bacteria, that is repressed when the product – tryptophan – is plentiful (Oxender *et al.,* 1979). A key example of positive feedback in

biogeochemical cycling is that of increased CO_2 in the atmosphere from volcanic eruptions or emissions from human activity. This causes heat energy from solar radiation to become trapped in the atmosphere, warming the planet and increasing the rate of evaporation. Water vapour is itself a climate warming gas, so increased water vapour causes a warmer atmosphere and so on, *ad infinitum,* unless a negative feedback loop counteracts it (Friedlingstein, 2015). Another example of a positive feedback loop is the increase in ground cover by plants in response to temperature. In the arctic zones, seasonally increasing ground temperatures cause more shrubs to grow, which insulates the ground and further warms it, perpetuating a cycle until counteracted by another factor (Sturm *et al.,* 2005).

The importance of biogeochemical cycles for life on Earth, therefore, cannot be overstated. As such, research that seeks to understand these cycles and their interplay is critical to understanding the impacts of change to these systems and predicting the future effects of such changes. Plants and microbes both rely on these systems for survival, as well as maintain the existence of these cycles. For example, the role of plants as sources of oxygen and sinks for carbon through photosynthesis is well characterised (Austen & Zanne, 2015). Additionally, microbes such iron-oxidising bacteria are essential for inorganic cycling within the lithosphere (Andrews *et al.,* 2013). The work focuses on the biogeochemical cycling of sulfur, and the roles of plants and microbes therein.

The Sulfur Cycle

Importance of Sulfur in Biological Systems

As previously stated, sulfur is one of the crucial elements in the basic molecules of biological systems. Sulfur is an abundant, non-metallic element with an atomic number of sixteen and in its native form, found as octamer rings (Fig. 1.2).

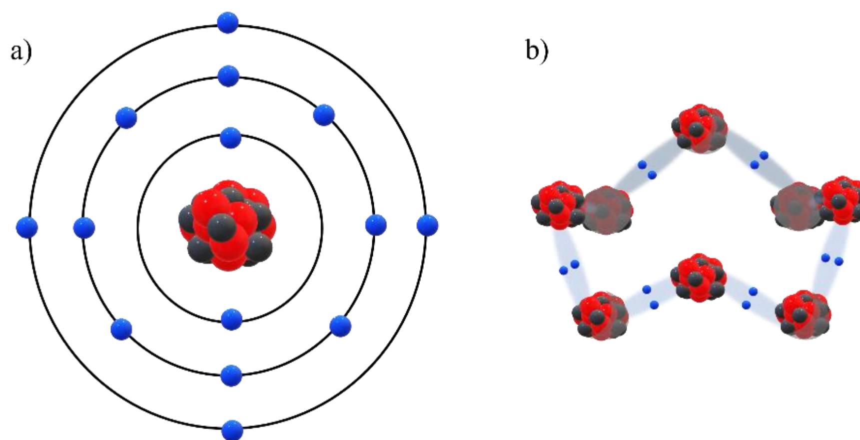
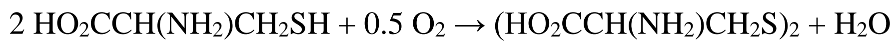


Figure 1.2: a) The atomic structure of sulfur. The nucleus contains 16 protons (red) and 16 neutrons (grey), with 16 electrons (blue) arranged in their valence shells. B) The characteristic octatomic crystals of native cyclo-S_8 (note that not all electrons are shown). Diagrams not to scale.

Sulfur is typically found in deposits of calcium sulfate ($CaSO_4$) or iron sulfate ($FeSO_4$), as well as in its pure form, as the result of volcanic activity – due to its high melting point of 115.21 °C (Kleine & Hurlbut, 1985). These deposits have been of considerable interest throughout history, due to their many uses. The flammable nature of pure sulfur was known to early Greek and Chinese civilisations, leading to its early designation as brimstone and use in crude incendiary devices as gunpowder (Kutney, 2007). The earliest Arabo-Latin written practices of alchemy, that led to the modern discipline of chemistry, were centred around the purification of sulfates (Newman, 2014; Balīnūs, circa 750-850 C.E.) and it's likely that much of the mythology surrounding the ability to create gold from base metals derived from the purification of sulfates to its native, yellow form (Ragai, 1992). The production of sulfuric acid (H_2SO_4) from sulfur dioxide (SO_2) and water – known as the Bell process - was also a driver of the industrial revolution in Europe, as H_2SO_4 is a key component of oil and fertiliser refining processes (Kutney, 2007).

Sulfur is extremely important in biological systems. It constitutes approximately 1% of the biomass of any organism as a key component of proteins - within the amino acids cysteine and methionine – as well as acting as a cofactor (Sievert, 2007). Cysteine and methionine are chiral zwitterions in water (Fig. 1.3). Cysteine is crucial for protein tertiary and quaternary structure stability due to its sulfur containing side chain (thiol) forming strong covalent disulphide bonds with each other (Brosnan & Brosnan, 2006). This occurs through an oxidation reaction:



$$2\ HO_2CCH(NH_2)CH_2SH + 0.5\ O_2 \rightarrow (HO_2CCH(NH_2)CH_2S)_2 + H_2O$$

The resulting disulfide bonds, or bridges, hold different sections of the protein secondary together to form the tertiary structure and are a critical part of the formation of proteins with hydrophobic cores (Sevier & Kaiser, 2002). Methionine is the initiating amino acid for protein primary structures in eukaryotes (*N*-formyl methionine in prokaryotes) because its hydrophobic side chain allows transfer RNA (tRNA) and Eukaryotic Initiation Factor 2 (*eIF-2*) to associate in the correct conformation (Drabkin & Rajbhandary, 1998). This hydrophobic side chain is also a key component of hydrophobic cores (Brosnan & Brosnan, 2006).

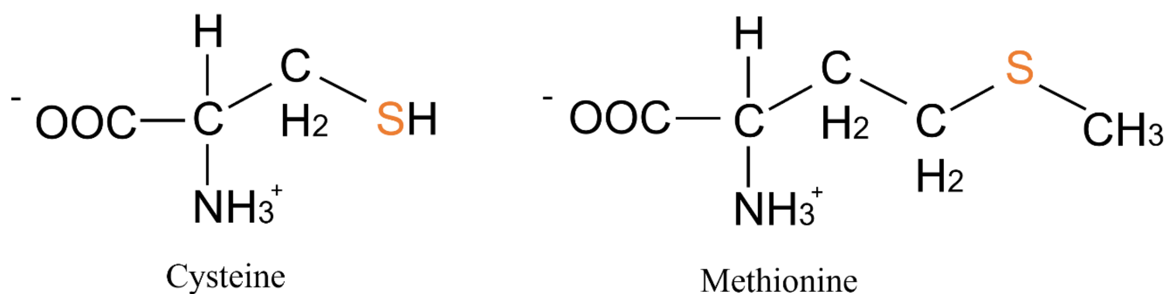


Fig. 1.3: Condensed structural formula diagrams of the amino acids cysteine and methionine. Consistent with all amino acids are the negatively charged carbonyl group, the positively charged amino group and chirality (excepting glycine). The key difference is in the R group, with cysteine having a thiol group bonded to the α carbon, whereas methionine has a methylated sulfur bonded to the β carbon.

Additionally, sulfur is a crucial cofactor in the movement of electrons through the electron transport chain in mitochondria. Iron-sulfur (Fe-S) clusters are a single iron atom, surrounded by four sulfur-containing cysteine residues (Read *et al.,* 2021). These are found in complexes I, II and III of the electron transport chain, located in the mitochondrial matrix, where they are known as Rieske proteins (Rieske *et al.,* 1964). Here, they form 'electron-tunnelling chains' that pass electrons in single file from $FADH_2$ to NADH, and then to Ubiquinone (Read *et al.,* 2021). Fe-S also function as intracellular oxygen sensors in aerobic organisms, as they are highly sensitive to changes in O_2, leading to changes in oxidation state that can trigger cellular cascades (Crack & Le Brun, 2021).

Another important role is that of intracellular signalling compounds. These are mostly derived from secondary sulfur metabolism - the compounds downstream of methionine

and cysteine (Chan *et al.,* 2019). In plants, glucosinolates trigger pathways to respond to reactive oxygen species and brassinosteroids mediate growth enhancement pathways (Chan *et al.,* 2019). In bacteria, cellular sulfur regulates sulfur-metabolism genes and upregulates quorum sensing sensitivity (Xu *et al.,* 2021). Furthermore, the characteristic smell associated with sulfur-based compounds is a useful signalling molecule to macrofauna, such as the highly potent thiols produced by members of the Skunk family (*Mephitidae*) to ward off predators (Wood, 1999).

Thus, the metabolic acquisition of sulfur is very important to biological systems. It is rarely taken up in its elemental form, but prokaryotes such as bacteria and archaea can use inorganic sulfates (SO_4^{2-}) through a series of enzymatically driven redox reactions known as assimilation (Sievert, 2007). Inorganic sulfate derives from the release of SO_2 from sedimentary rocks by natural weathering, as well as industrial processes (Schäfer *et al.* 2010). SO_2 oxidises to SO_4^{2-} when in contact with air, which is deposited in the oceans and back to earth as acid rain (H_2SO_4) or as aerosols, known respectively as wet and dry deposition (Marchetto, 2021).

Consequently, the high importance of sulfate for eukaryotes such as plants drives a large industry of sulfur-based fertilisers (Jordan & Ensminger, 1959). Sulfur-deficient soils are characterised by decreased aerial and root growth, withered and yellow leaves (chlorosis) and poor immunity against pathogens (Jordan & Ensminger, 1959). This is due to the high metabolic demand for sulfur by plants to generate cysteine and methionine, as well as the growth-regulating vitamins biotin and thiamine (Begley *et al.,* 1999) and defence compounds such as glycosides (Tabatadze *et al.,* 2007). The global demand for sulfur is an estimated 246 million tonnes, with increases predicted due to increasingly fertiliser-intense agricultural practices to meet rising food demands (Maslin *et al.,* 2022).

Once deposited, prokaryotes take up SO_4^{2-}, which induces a series of reduction reactions that convert SO_4^{2-} through intermediates adenosine phosphosulfate (APS) and sulfite (SO_3^{2-}) to sulfide (S^{2-}) (Sievert, 2007). This constitutes an evolutionary advantage for these prokaryotes, as they can derive energy from oxidative phosphorylation that uses the oxygen released from SO_4^{2-} reduction (Le Faou *et al.,* 1989). The resulting S^{2-} reduces either the amino acid serine or *O*-acetylserine to produce cysteine and water or acetate respectively (Soda, 1987). Cysteine undergoes a series of enzyme-driven transsulfurylation reactions with methane thiol (CH_3SH) to produce methionine (Soda,

1987) (Fig 1.4). Methionine is a precursor for the molecule of interest in this study: 3-dimethylsulfoniopropionate.

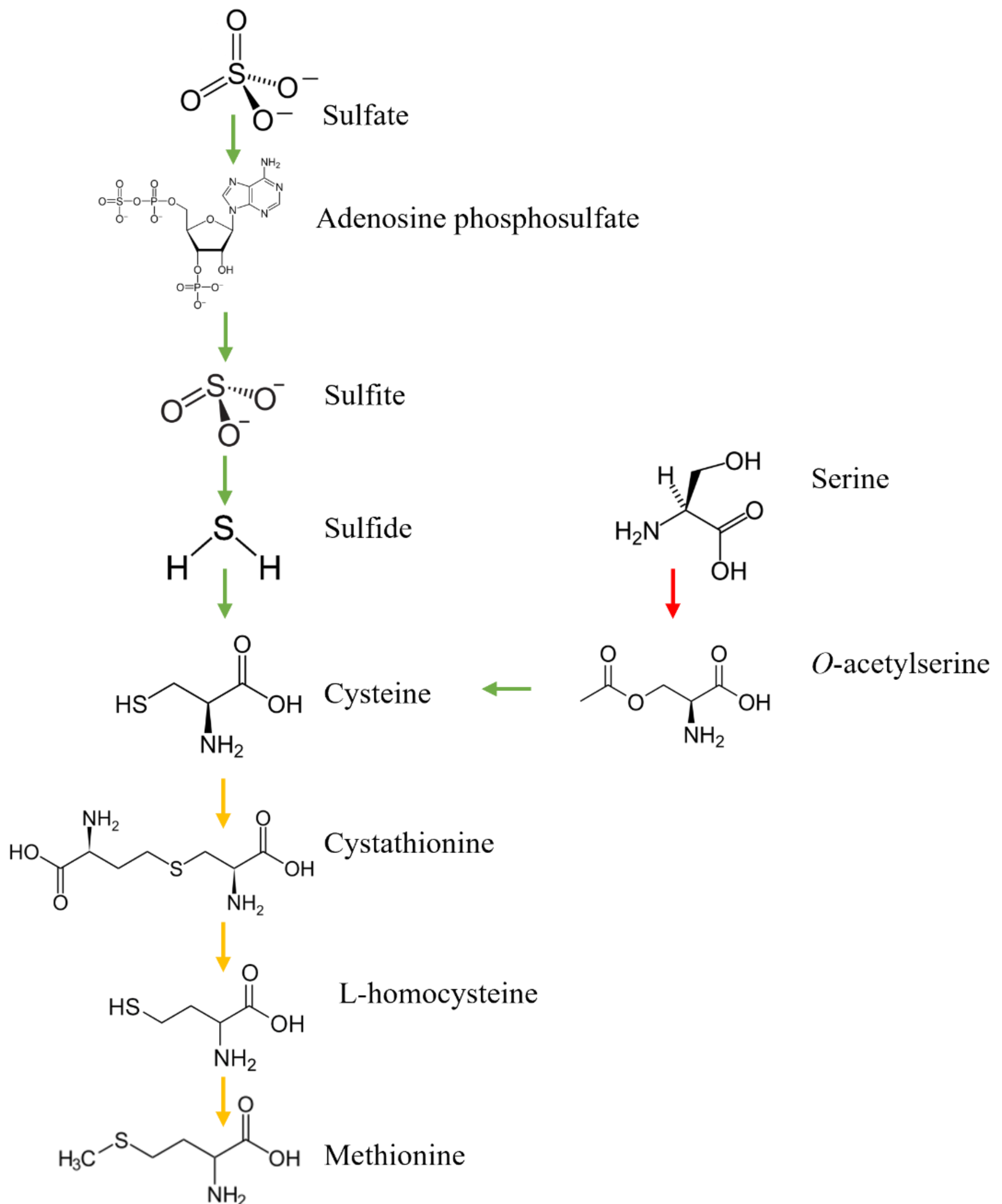


Fig. 1.4: The biochemical assimilation of sulfur by bacteria, converting environmental free sulfate to amino acids cysteine and methionine, via the intermediates shown as skeletal diagrams. Reactions are grouped as follows: red arrows – oxidation, green arrows – reduction

and yellow arrows – transulfurylation. Adapted from Wawrzyńska *et al.*, 2015 and Kettles *et al.*, 2014.

Dimethylsulfoniopropionate

3 - dimethylsulfoniopropionate (DMSP) is a tertiary sulfonium compound; a zwitterion (Fig. 1.5) that is one of the most abundant organosulfur compounds found in the marine environment, with annual production in the ocean estimated at 2.0 petagrams (Ksionzek *et al.,* 2016). It was first identified in the red algae *Polysiphonia fastigiata* in 1948 (Challenger & Simpson, 1948).

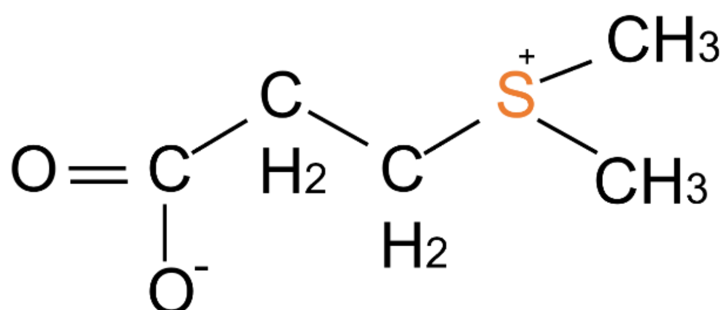


Figure. 1.5: The Lewis Structure for DMSP, showing the positive charge at the methyl-sulfur end and the negative charge at the carboxylic acid end of the molecule.

DMSP is a major contributor to the global sulfur cycle from the marine environment due to the sheer amount of sulfur it contains globally and its microbial catabolism (see below). It was generally thought that the largest proportion of DMSP production was in phytoplankton-rich surface waters (Asher *et al.*, 2017), but more recent studies indicate that deeper benthic waters and oceanic sediments, such as saltmarshes, contain large amounts of DMSP (Cheng *et al.,* 2023; Curson *et al.,* 2018). Indeed, there are orders of magnitude more DMSP in diverse marine sediments per unit volume than oceans, especially in salt marshes potentially due to the abundance of *Spartina* grasses that are high DMSP producers (Dacey *et al.,* 1987; Steudler & Peterson, 1984; Williams *et al.,* 2019).

DMSP is the major progenitor of the volatile, climate-active gases dimethylsulfide (DMS) and methanethiol (MeSH), whose oxidation products form cloud condensation nuclei (CCN) – micrometer sized particles that provide a surface in the atmosphere for

water to condense on and form vapour. DMS is produced by the enzymatic hydrolysis of the bond between the sulfur ion and the carbon backbone in DMSP, through DMSP lyase enzymes (Fig. 1.6). Some of this DMS is then released to the atmosphere, whilst most is assimilated by marine microorganisms or further oxidised to dimethyl sulfoxide (DMSO). Thus, DMSO is both a source and a sink for DMS. DMS can be generated by the reduction of DMSO through algal and bacterial anaerobic respiration, (Zindler-Schlundt *et al.,* 2015). Of these fates, an estimated 90% is assimilated by marine microorganisms. (Archer *et al*., 2002; Kiene & Bates, 1990; Zubkov *et al*., 2012). MeSH is produced from the demethylation of DMSP to the intermediate methylmercaptopropionate (MMPA), which is then reduced to MeSH and formaldehyde (Kiene and Taylor, 1988; Taylor and Gilchrist, 1991). MeSH is extremely volatile, and is considered likely to contribute to CCN, albeit in a lower proportion to DMS (Kiene and Taylor, 1988; Taylor and Gilchrist, 1991).

The oxidation and hydrolysis of the cleaved sulfur dioxide (SO_2) from DMSO to sulfate (SO_4^{2-}) and sulfuric acid (H_2SO_4) allows the sulfur cycle to continue by the formation of acid rain (Sievert, 2007). The similarity in seasonal concentrations of oceanic DMS and atmospheric sulfate suggest that DMS is the major contributor of sulfate particles to acid rain, estimated at 40% of all atmospheric sulfate particles being contributed by DMS catabolism (Nguyen *et al.,* 1992). Overall, the contribution of DMSP to the global sulfur cycle is vast, with an estimate of 10% to global sulfur flux (Simó & Pedrós-Alió, 1999).

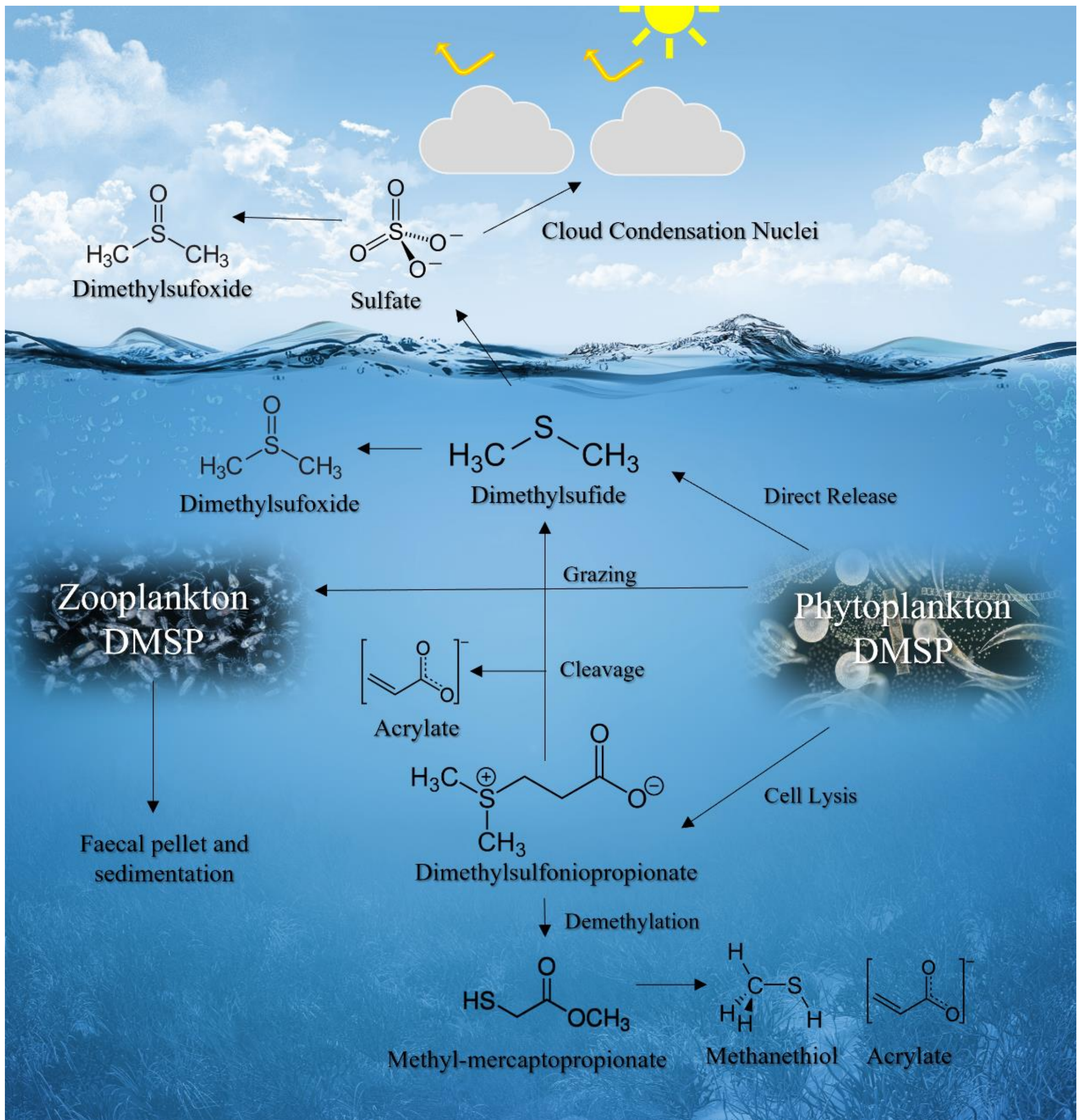


Figure 1.6: The fates of DMSP and the marine sulfur cycle. The mechanisms of cloud condensation nuclei (shown in top right of figure) make up a relatively small proportion of the fate of DMSP. The majority of marine DMSP remains in the ocean, assimilated by bacteria (mid) or cycling between DMS and DMSO (mid-left). Figure adapted from Yoch, 2002. Background Image licensed ©Adobe.Pixelschoen. Phytoplankton: www.secchidisk.org. Zooplankton: www.encyclopaediabrittanica.org/ FLPA/Alamy

Claw Hypothesis and Disputes

The generation of DMS from DMSP was proposed as a negative feedback loop, known as the CLAW hypothesis (Charlson *et al.,* 1987). The CLAW hypothesis proposed that

CCN, derived from DMSP, increase the Earth's albedo, reflecting more solar radiation back to the Earth and causing decreased oceanic temperature. It was proposed that this is a way for DMSP-producers to regulate their environment (Charlson *et al.,* 1987). This hypothesis has been the subject of debate. A further study by one of the principal authors suggested that the positive correlation between the concentrations of DMS produced in the tropical South Atlantic, the atmospheric DMS and the condensation nuclei particles is evidence in favour of the CLAW hypothesis (Andreae *et al.,* 1995).

However, the hypothesis has been criticised for being oversimplified by neglecting other sulfur compounds as CCN, the role of DMSO in the DMS production cycle and the complicated roles and inducers of DMSP in marine organisms (Green & Hatton, 2014). Conversely, the hypothesis has also been criticised as overestimating the importance of the marine microbial component, suggesting that the natural cycle of cloud evaporation is enough to maintain CNN in the atmosphere without being driven by biological DMS production (Shaw *et al.,* 1998). Both criticisms were expanded on in a heavily critical literature review of the CLAW hypothesis (Quinn & Bates, 2011). In addition to supporting these previous arguments against the CLAW hypothesis, the authors also state that microscopic analysis of CCN particles show that the primary component is sea salt. They suggest that the CLAW hypothesis is now redundant (Quinn & Bates, 2011).

One of the principal authors even proposed the ANTI-CLAW hypothesis, stating that it was a positive feedback loop and that ocean warming will lead to decreased numbers of phytoplankton, thus decreased DMS and CNN (Lovelock, 2007). Therefore, although the importance of DMSP in marine sulfur cycling has been established, the CLAW hypothesis as the mechanism of explaining how the cycle is maintained is debateable, at best.

Roles of DMSP

There have been many roles proposed for DMSP. Although each proposed role has evidence to support it, none have been definitively established. The hypothesised roles are mostly based on the relative concentrations of DMSP found in cells grown under different conditions. There are no knockout mutants to confirm these roles and in bacteria where knockouts exist, no phenotypes have been established for mutants producing no DMSP under any tested condition (Curson *et al.,* 2017).

DMSP for osmoregulation/compatible solute

The most probable role for DMSP is as an osmolyte, or compatible solute, of marine microorganisms. Structurally analogous to the known osmolyte glycine betaine (Fig. 1.7), DMSP counters decreases in cellular water potential by rapidly accumulating inside cells from the extracellular environment through synthesis or the activation of channels. This triggers excess positively charged sodium (Na^+) to be excreted through ion channels and prevents water efflux (Kirst, 1989; Van Bergeijk *et al.,* 2010). This is an important compensatory mechanism for microorganisms because they cannot maintain turgor pressure through import of water directly into the cytosol (Kempf & Bremer, 1998).

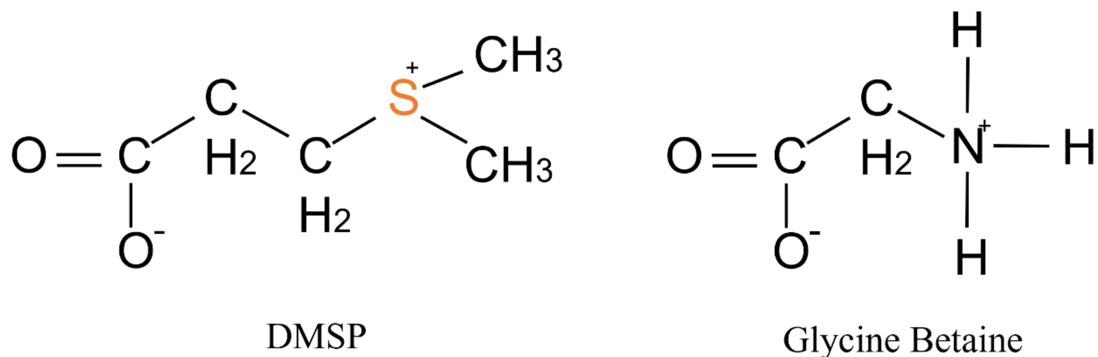


Figure 1.7: The Lewis Structures for DMSP and Glycine Betaine, showing the homologous structure of the carbonyl and adjacent α carbon, but the replacement of the β carbon in DMSP with an amino group. Both molecules have a positive and negative dipole.

The correlation between DMSP and salinity has been demonstrated or suggested in a wide variety of marine organisms. Intracellular DMSP was found to linearly increase with increasing salinities in green macroalgae *Ullotrix spp., Enteromorpha bulba* and *Acrosiphonia arcta* but significantly more so in *Ulva rigida* and *Blidingia minima*

(Karsten *et al.,* 1992). Furthermore, ice-algae collected from the Weddell Sea was found to have significantly higher intracellular concentrations in samples collected from hypersaline ice pockets compared to those in the open water (Kirst *et al.,* 1992).

A variety of *alpha-* and *gamma-proteobacteria* in marine ecosystems have been shown to produce DMSP (Curson *et al.,* 2017; Liao & Seebeck, 2019), and its sheer abundance in marine and coastal systems might indicate its use as an osmoprotectant. In samples of the *Alphaproteobacteria, Labrenzia aggregata,* collected from the Chiangjiang Estuary and the East China Sea, intracellular DMSP concentrations were shown to significantly increase along with salinity (Liu *et al.,* 2021; Sun *et al.,* 2021). Genes associated with DMSP were also found to be transcriptionally upregulated along the salinity gradient, indicative of an osmoprotectant role (Sun *et al.,* 2021). Direct manipulation of salinity also shows similar results. For example, radiolabelled ^{35}S-DMSP was added to sea water samples containing a mixture of marine bacteria. It was found through liquid scintillation counting that bacterial uptake of DMSP increased with salinity (Motard-Coté & Kiene, 2015). However, it is worth noting that the filtration methods may not entirely exclude other marine organisms in this study. Bacterial growth can also be used as a proxy measurement for the effect of DMSP. A study of *Vibrio* species showed that increasing DMSP proportionally increased the growth rate of *Vibrio parahaemolyticus* at 6% NaCl, and the addition of DMSP compared to a non-treated control significantly increased the rate at which exponential phase was reached in saline conditions (Gregory *et al.,* 2021).

Multiple studies have indicated that DMSP functions as an osmoprotectant in diatoms. For example, increasing salt concentrations from 35 practical salinity units (psu) to 70 psu resulted in an 85% increase in intracellular DMSP concentrations in *Fragilariopsis cylindrus* (Lyon *et al.,* 1996) and from 11 psu to 44 psu and increase of more than 5000% in *Cylindrotheca Closterium* (Van Bergeijk *et al.,* 2010). Furthermore, in a study of diatom rich mucus ropes produced by reef-forming corals, the authors showed that these complex assemblages of exudate and diatom species produced up to 120 nmol DMSP (Broadbent & Jones, 2004). The authors concluded that these elevated levels were likely to be the result of diatom activity concentrating in the mucus layers at low tide. An indirect approach is to measure gene expression of those involved in DMSP production. A study of the temperate marine diatom *Thalassiosira pseudonana* showed that SAM-synthetase expression increased by 2.9-fold when salinity was increased,

leading to a 3.6-fold increase in SAM-dependant methyltransferase, the first step of the methylation pathway for DMSP synthesis (Kettle *et al.,* 2014).

There is some tenuous evidence that reef-forming corals have also been shown to produce DMSP to levels suggestive of osmotic functions. A study of Cnidarian corals from the Great Barrier Reef showed that members of the stony coral family, *Acropora*, produced between 371 – 3341 fmol DMSP per photosynthetic cell, determined by methanol extraction (Broadbent *et al.,* 2002). The authors deemed this to be an osmotically significant level, although it is worth noting that no threshold of osmotic significance was stated. A study of dominant osmolytes by HPLC within corals containing endosymbiotic *Symbiodinium spp.* dinoflagellates from the Hawaiian coast, determined that all species contained between 0.19 – 3.18 $mmol.kg^{-1}$ DMSP, which was not detectable in the symbiote-free control (Yancey *et al.,* 2009). However, the authors noted that these were relatively low amounts compared to other osmolytes, such as glycine betaine (32.7 - 68.6 $mmol.kg^{-1}$) and suggested that it might have other primary cellular functions.

Many of the higher plants shown to produce DMSP are halotolerant to varying degrees. Thus, it seems likely that salinity induced DMSP production to function as an osmolyte like its nitrogenous compound glycine betaine. In *M. biflora*, increasing salinity to 400 mol m^{-3} was positively correlated with the concentration of DMSP (Storey *et al.,* 1993). It was further shown that increasing the salinity in *M. biflora* more specifically resulted in increased DMSP accumulation at the chloroplasts, suggesting DMSP functions as an osmolyte to defend photosynthetic processes (Trossat *et al.,* 1998).

The relationship between DMSP and salinity in *Spartina spp.* has less support, with laboratory studies showing that salinity had no effect on DMSP production in *S. alterniflora* but that there was an inverse relationship between nitrogen supply and DMSP concentrations in the leaf tissues (Colmer *et al.,* 1995; Mullholland and Otte, 2000; Stefels, 2000). The authors suggested that this may be because of the significant increase in glycine betaine detected reducing the requirement for DMSP as on osmolyte. This would be contrary to studies in marine phytoplankton, which found that DMSP concentrations were significantly higher the glycine betaine such that a trade-off between the two osmolytes is not supported (Keller *et al.,* 1999). However, as glycine betaine is a more dominant osmolyte in plants than DMSP (Pareek *et al.*, 2009), it is possible that a reciprocal relationship between glycine betaine and DMSP may exist in

plants. However, one of the studies also found that DMSP concentrations were significantly increased in the root tissues in response to increased nitrogen (Mullholland and Otte, 2000). The authors speculate that DMSP is translocated from the leaf tissues to the root tissues, based on the localisation of DMSP to chloroplasts (Trossat *et al.,* 1998). This may be because the root tissues are the first exposed tissues to osmotic stresses in the soil.

Furthermore, the lack of supporting data from plants in their natural soil conditions makes these conclusions somewhat reductionist. It is unknown whether other soil components might affect the production of DMSP or what role rhizosphere microorganisms might have on the relationship between nitrogen availability and DMSP production. DMSP production has been reported to increase in response to drought in *M. biflora, Arundo donax* and *S. lycopersicum* (Storey *et al.,* 1995; Haworth *et al.,* 2017; Catola *et al.,* 2016), supporting the theory that it has an osmoprotectant function.

<u>DMSP for cryoprotection</u>

Broadly related to osmotic protection is DMSP's function as a potential cryoprotectant. DMSP concentrations have been consistently shown to be higher in polar species of macroalgae than tropical or temperate (Bischoff *et al.,* 1994; Karsten *et al.,* 1992; Kirst *et al.,* 1991), suggesting an evolutionary advantage in freezing conditions. In addition, Extracted DMSP from the polar macroalgae *A. arcta* was shown to stabilised bacterial extracts of the enzyme lactate dehydrogenase during freezing and thawing (Karsten *et al.,* 1996). This suggests that DMSP may have a cryoprotecant function in macroalage, however this method has limited usefulness for determining the scope DMSP as cryprotectant within a bacterial cell. Although in bacteria, other compatible solutes have been shown to protect against cold stress, such a glycine betaine in *Bacillus subtillis* (Hoffman & Bremer, 2011), there is no evidence to suggest DMSP has a similar function.

In diatoms, the same study that determined DMSP increases in the sea-ice diatom *F. cylindrus* in hypersaline conditions also noted that the DMSP concentrations significantly increased in the sea-ice species compared to temperate species (Lyon *et al.,* 2011). This may suggest that DMSP is upregulated by multiple conditions, including freezing. In phytoplankton, it has been suggested that DMSP can act as an antifreeze compound – lowering the temperature at which the cytosol freezes (Kirst *et al.,* 1991). Another study investigating the effects of melting sea ice on DMSP concentrations in

Phaeocystis antartica showed that there was significantly more intracellular DMSP in samples from sea ice than other marine environments (Kameyama *et al.,* 2020). However, they attributed this to the hyper-saline channels within the sea ice and that DMSP acted as an osmoprotectant, rather than a cryoprotectant.

There is no direct evidence to suggest that DMSP has a cryoprotectant function in higher plants, although in their review of DMSP in higher plants, Otte *et al.* suggest that based on the distribution of *S. alterniflora* in the northern hemisphere to regions as close to the arctic as Newfoundland, it may have some benefit in maintaining cellular integrity in response to freezing (Otte *et al.,* 2004). Therefore, DMSP as a plant cryoprotectant is considered speculative and there is no research to confirm this.

<u>DMSP as an antioxidant</u>

Another protective role of DMSP and its catabolites are against oxidative stress. When subjected to solar radiation, hydrogen peroxide and high concentrations of copper ions to induce hydroxyl radicals, it was found that *E. huxleyi* had between 9-15-fold more DMS per cell volume than controls (Sunda *et al.,* 2002). The authors suggested that DMS and acrylate can scavenge hydroxyl radicals. This is supported by the increase in intracellular DMSP when cultures of the same species were exposed to direct UV-A and UV-B radiation, compared to an unexposed control (Slezak & Herndl, 2003). The same study that investigated the effects of salinity on various macroalgae also showed that in all species, intracellular DMSP concentrations were higher in samples exposed to 55 photons.mol.m^{-2}.sec^{-2} compared to those kept in the dark (Karsten *et al.,* 1992). This suggests that DMSP has a protective role against free radicals generated by photooxidation. The mitigation against damage by reactive oxygen species (ROS) generated in response to UV was also demonstrated in the high producing phytoplankton *Phaeocystis globose* and *Heterocapsa triquetra*. Both phytoplankton produced significantly higher intra-chlorophyll DMSP and suffered less cellular damage by lipid oxidation products than the low DMSP-producer *Skeletonema costatum* (Gypens *et al.,* 2020).

The *Symbiodinium* dinoflagellates found in the mantles of Giant Clams (*Tridacnid spp.*) are also proposed to produce DMSP as an antioxidant. Methanol extraction showed intracellular DMSP concentrations to be significantly higher compared to molluscs without symbionts (Hill *et al.,* 2016). The authors also noted that within the same species, DMSP concentrations were higher in the mantle tissues (37.4 µmol.g^{-1})

compared to the gills or adductor muscles (33.3 μmol.g^{-1} and 4.4 μmol.g^{-1}, respectively). They suggest that this is because DMSP has antioxidant properties that protect against ultraviolet (UV) radiation stress (Hill *et al.,* 2016).

In their review of the role of DMSP in corals and the ecological effects thereof, Jackson et al suggest that corals are more likely to produce DMSP as an antioxidant (Jackson *et al.,* 2020), owing to the higher quantity of research showing DMSP is upregulated in response to bleaching, thermal and UV stress. For example, the coral *Acropora millipora* was also found to have inversely correlated intracellular DMSO and extracellular salt concentrations – with decreased salt concentration leading to the production of singlet oxide radicals that are scavenged by DMS, resulting in DMSO (Gardener *et al.,* 2016). Also, previously mentioned, in their study of dominant osmolytes in corals (Yancey *et al.,* 2009), the authors note the levels of DMSP compared to other intracellular osmolytes is significantly lower, and suggest it is produced for other cellular functions. Furthermore, headspace analysis of volatile organic compounds (VOCs) in mucus from *Acropora aspera* were inversely correlated with dissolved oxygen levels (Swan *et al.,* 2016). The same species was shown to have significantly increased concentrations of DMSP when nubbins of the coral were treated as follows: increased temperature, direct sunlight and exposure to air compared to the control nubbins (Deschaseaux *et al.,* 2014). Nubbins exposed to increased salinity showed no significant differences, leading the authors to conclude that intracellular DMSP in coral has an antioxidant, rather than an osmotic, effect.

High concentrations of DMSP and DMSP-synthesis enzymes are localised to chloroplasts and mitochondria in higher plants, as well as in macroalgae (Karsten *et al.,* 1992; Trossat *et al.,* 1998). This supports the theory that DMSP protects against oxidative stress, as these organelles generate reactive oxygen species (Ott *et al.,* 2007).

<u>DMSP as a chemoattractant</u>

Free DMSP in the marine environment has been shown to attract bacteria and bacterioplankton (Miller *et al.,* 2004; Seymour *et al.,* 2010). It is suggested this is due to the sulfur content, that gives it a strong odour. It has also been demonstrated that *Roseobacter* species associate with DMSP producing dinoflagellates (Miller & Belas, 2004). This is potentially due to the *Roseobacters* ability to detect DMSP or downstream products and for DMSP-catabolsing bacteria, this confers an evolutionary advantage. A laboratory study using isolates from the Northwest coast of the USA also

found that the addition of 20 µM DMSP to the water containing single predator-prey species caused between 28-75% reductions in ingestion of the prey species by the predator compared to a control (Frederickson *et al.,* 2009). Additionally, Motile phytoplankton *Dunaliella tertiolecta* and *Micromonas pusilla* have been shown to move towards high concentrations of DMSP in seawater (Seymour *et al.,* 2010). DMS is also reported to be a chemoattractant in the heteroptrophic bacteria *Alicaligenes* strain M3A and *Vibrio alginolyticus*, the latter using high DMS concentrations to locate marine algae of which they are pathogens (Amsler and Iken, 2001).

Furthermore, the unique odour of DMS acts as chemoattractant macrofauna. Firstly, to Procelliform seabirds that graze on the zooplankton that congregate around DMSP-releasing phytoplankton, such as Albatrosses, Petrels and Shearwaters (Savoca *et al.,* 2016). It is also a key component of the characteristic corpse-like smell of the Dead-Horse Arum (*Helicodiceros muscivorus*), which attracts carrion flies in the family *Calliphoridae* as pollinators (Stensmyr *et al.,* 2002). Finally, the Black Truffle (*Tuber melanosporum*) generates DMS as part of the complicated cascade of mating pheromone release and has been shown to be the primary compound by which pigs and dogs locate said delicacy (Talou *et al.,* 1990).

DMSP as a grazing deterrent

Almost in opposition to its role as a chemoattractant, both DMS and DMSP are reported to be grazing deterrents in marine organisms. It seems that the product of DMSP catabolism that confers a deterrent effect varies depending on the predator-prey interaction in question. On one hand, a laboratory study testing the effect of additional acrylate, DMS and DMSP on the grazing behaviours of three dinoflagellates and a ciliate on *E. huxleyi* showed that all four predator species had their feeding inhibited by the addition of DMSP, whereas DMS and acrylate had no significant effects (Strom *et al.,* 2003). The inhibitory effect of DMSP was determined to be because *E. huxleyi* releases DMSP on cell lysis and in response to mechanical stress from grazing (Wolfe and Steinke, 1996; Wolfe *et al.,* 1997). However, acrylate is a toxic compound and acrylate derived from DMSP catabolism has been shown to protect the marine bacterium *Puniceibacterium antarcticum* from the ciliate *Uronema marinum* (Teng *et al.,* 2021). Acrylate concentrations were positively correlated with intracellular DMSP, and membrane-bound DMSP-lyase enzymes, causing the ciliate to shift towards preying on bacteria that did not contain DMSP (Teng *et al.,* 2021).

Similarly, to phytoplankton, DMSP has also been proposed as grazing deterrent in *S. alterniflora*, although there is little experimental evidence to support this theory. A study looking at the effects of nitrogenous fertilisers in *S. alterniflora* incidentally noticed that plants treated with nitrogen produced less DMSP and were more likely to be consumed by rats than those that were untreated (Otte *et al.,* 2004), but these findings were never followed by any experiments on the effect of DMSP and rat grazing behaviours. The same school of thought that suggests DMSP degradation to acrylate would act as a grazing deterrent in macroalgae (Van Alstyne and Houser, 2003) has also been proposed for higher plants (Otte *et al.,* 2004). In their review, the authors suggest that degradation of DMSP to DMS and acrylate would also deter herbivores from grazing on the leaves *Spartina* (Otte *et al.,* 2004).

<u>DMSP as a nutrient source</u>

DMSP may also function as a nutrient source for marine microorganisms. It is estimated that between 30-90% of dissolved DMSP is immediately metabolised by marine bacteria, depending on season and other biogeochemical parameters (Seymour *et al.,* 2010). This is likely because DMSP is an easily accessible source of carbon and sulfur, and the sheer abundance of bacteria that contain DMSP-catabolism genes supports its importance as a nutrient (Curson *et al.,* 2018; Sun *et al.,* 2021). It is also an essential source of sulfur, and a secondary source of carbon, for SAR11 marine bacteria, that lack the necessary suite of genes to otherwise assimilate sulfur (Tripp *et al.,* 2008). It is estimated that DMSP constitutes 13% of the marine bacterial carbon load in surface waters (Kiene *et al.,* 2000).

A suggested function of DMSP in the Prymnesiophyte alga *Phaoecystis spp.* is that of a storage molecule to sequester carbon and sulfur (Keller *et al.,* 1989). High intracellular concentrations of DMSP – up to 260 mM - were found in *Phaoecystis globose*, suggesting that considerable amounts are produced and retained (Keller *et al.,* 1989). DMSP was also found in the secretory vesicles of *P. antartica* and it was suggested that they are released in regulated quantities to condense the excreted mucous that holds phytoplankton blooms together (Orellana *et al.,* 2010).

DMSP has also been proposed as a sink for excess sulfur in higher plants, which is toxic as it inhibits cytochrome c oxidase of the electron transport chain (Lamers *et al.,* 2013). This would enable the plant to maintain a balance of sulfur and nitrogen (Mullholland and Otte, 2000). A study showing that DMSP production increased with higher

concentrations of sulphide in *S. anglica* concluded that this was due to its function a sulfur sink (Diggelen *et al.,* 1986). However, a later study suggested that the increased DMSP concentration in plant tissues were relative to the mass of tissue. As the plant's growth had been stunted by the excess sulfur, this was insufficient evidence to demonstrate DMSP functions as a sink (Otte and Morris, 1994).

DMSP Biosynthesis

DMSP-producing phytoplankton

Phytoplankton are widely regarded as the most important and prolific DMSP producing organisms but the levels of DMSP they produce varies depending on the organism. Haptophytes are generally considered to be high producers of DMSP as are dinoflagellates (Miller & Belas, 2004). Of the haptophytes, the prymnesiophytes *Emiliania huxleyi* and *Phaecystis spp*, are major producers in estuarine and coastal waters (Thariath *et al.,* 2019; Yoch, 2002; McParland and Levine, 2017), producing between 100-300 mM DMSP cm^{-3} cell volume (Keller, 1989). Dinoflagellates such as *Crypthecodinium cohnii* are also high producers in surface waters, with intracellular DMSP concentrations measured at 376.9 mM DMSP cm^{-3} cell volume (Keller, 1989). Furthermore, a bloom of the dinoflagellate *Akashiwo sanguinea* in 2016 resulted in the highest DMSP concentrations recorded from a marine sample (Kiene *et al.,* 2019). In contrast, diatoms are generally thought to produce and accumulate low levels of DMSP e.g., *Cylindrotheca closterium* (up to 35 μmol l^{-1}) found in deep benthic regions of the ocean (Bergiejk *et al.,* 2003) and *Nitzschia frigida* (1.7 pg $cell^{-1}$) found in lower layers of Arctic ice (Levasseur *et al.,* 1994).

Thus, there is vast variability in the DMSP levels made withing these diverse algae. Most algae are thought to utilise the transamination pathway due to experimental work done in Rhodes *et al.,* (1997) where representatives of these algae were shown to accumulate the key DMSHB intermediate of the transamination pathway. Furthermore, more recent work has shown most of these algae with genomic or transcriptomics resources to contain, DSYB and or TpMMT, diagnostic enzymes of the transamination pathway (Curson *et al.,* 2018). Until any genes or enzyme are identified in the decarboxylation pathway, (see below), it is impossible to predict how widespread this pathway is in organisms. Given the high diversity of DMSP biosynthesising organisms in the marine environment alone, it is highly unlikely that this pathway is isolated to just to the dinoflagellate *C. cohnii*.

DMSP-producing Macroalgae and Corals

Other DMSP producers include corals and macroalage, which are again predicted to utilise transamination pathway on account of the transcriptomes containing the key transamination pathway reporter gene *dysB* (Curson *et al.,* 2018; Gage *et al.,* 1997). Reef building corals are a symbiotic partnership between Anthozoan polyps and intracellular *Symbiodinium spp.* dinoflagellates. DMSP assays of polyp fragments showed a range of DMSP concentrations, from 16 – 500 nmol DMSP per polyp (Yost and Mitchelmore, 2010). Both symbiotic partners appear to be able to synthesize DMSP, depending on the species involved. When inoculated with *Symbiodinium bermudense*, the anemone *Aiptasia pallida* produced an average of 4.2 µmol g-freshweight DMSP compared to uninoculated anemones that had no detectable DMSP concentrations (Van Alstyne *et al.,* 2008). However, a later study showed that juvenile polyps in species lacking *Symbiodinium* partners - *Acropora millipora* and *Acropora tenuis* – produced between 10-25 nmol m^{-2} DMSP (Raina *et al.,* 2010).

As previously mentioned, DMSP was first discovered in macroalgae, commonly known as seaweed, and indeed the transamination pathway was also discovered in these organisms (Rhodes *et al.,* 1997). The red algae – or rhodophyte – *Polysiphonia hendryi* has been shown to produce between 0.1-0.4% fresh weight, whereas multiple species of chlorophytes have been shown to produce significantly higher volumes of DMSP, ranging from 0.18-1.68% fresh weight (Van Alstyne *et al.,* 2001). Brown algae – or phaeophytes – have also been shown to produce DMSP, but at significantly lower levels than other species. For example, *Fucus vesiculosus* was shown to produce DMSP at an average of 0.033% fresh weight (Saha *et al.,* 2012). The DMSP synthesis enzymes within these organisms have not been elucidated yet.

DMSP producing Higher Plants

Compared to marine microorganisms, a relatively small number of higher plants have been shown to produce high concentrations of DMSP, and initially it was only shown in three taxonomically unrelated genera (Fig. 1.8): *Saccharum officianarum, Spartina* and *Melanthera biflora,* producing between 6-70 µmol g^{-1} DMSP (Hanson *et al.,* 1994; Otte and Morris, 1994; Paquet *et al.,* 1994). Prior to this thesis no gene/or enzymes for DMSP synthesis had been identified in plants. Later studies have discovered other high producing plants, such as aquatic Neptune grass (*Posidonia oceanica*), whose leaves were found to contain between 25-265 µg g^{-1} fresh weight (Richir, 2020). Subsequent

research has found a wide variety of flowering plants, that produce DMSP, albeit at two to four orders of magnitude lower than that of the four highest producers. These include crop plants, such as Maize (*Zea mays*) and Tomatoes (*Solanum lycopersicum*) (Ausma *et al.,* 2017; Catola *et al.,* 2016). Much work needs to be done to see if these plants contain the primary genes to allow them to synthesize DMSP. Due to the complexity of plant genomes compared to that of microorganisms, it is also entirely possible that DMSP might be a byproduct from the secondary activity of genes not primarily associated with DMSP biosynthesis.

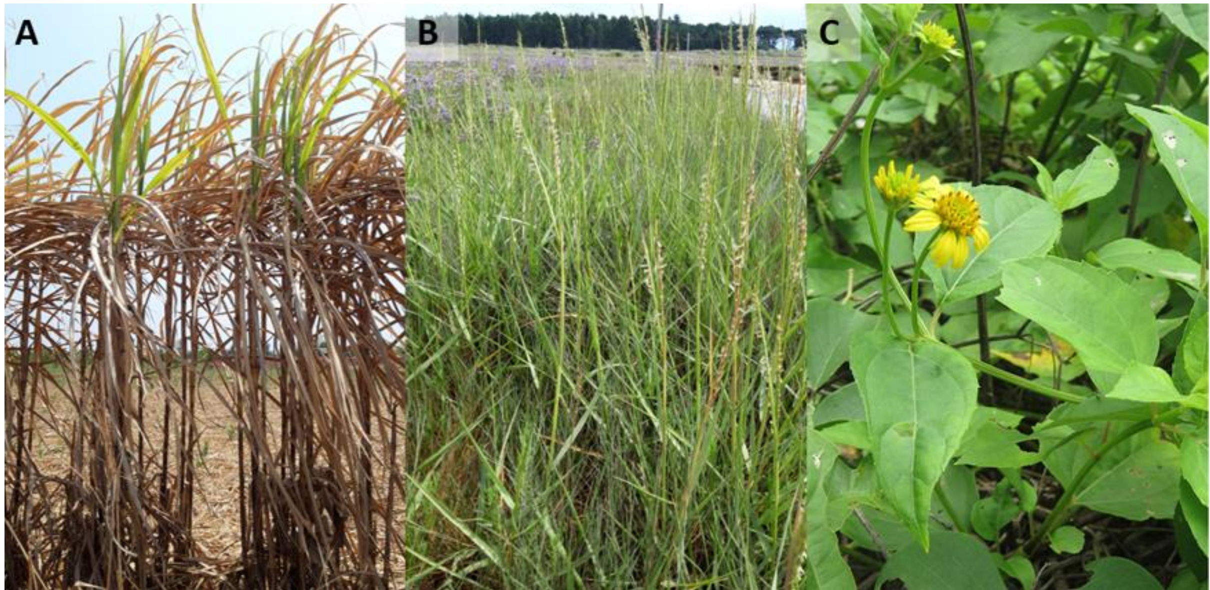


Figure 1.8: The most widely known higher plant producers of DMSP: A) Sugarcane – *Saccharum officianarum* B) Cordgrass – *Spartina anglica* C) Sea Daisy – *Melanthera biflora* Image of *Melanthera biflora* licensed © Wikimedia.commons/Vinayaraj

DMSP synthesis Pathways

In the photic zones of the ocean, the primary producers of DMSP are thought to be eukaryotic marine phytoplankton. These can be divided into four major classes: the green algae and the microalgae (diatoms, dinoflagellates, coccolithophores). The key difference between these microalgae is the composition of their cell walls: diatoms are silica based, dinoflagellates are cellulose based and coccolithophores have calciferous exoskeletons (Lalli and Parsons, 1993).

Three major pathways for DMSP synthesis have been proposed (Fig. 1.9). DMSP abundance was thought to globally increase 250 million years ago, with the emergence of DMSP producing dinoflagellates and the pathways diverged broadly in two families;

the green methylation lineage containing higher plants and the red transamination lineage containing algae, diatoms and bacteria (Bullock *et al.,* 2017). However, it is worth noting that this hypothesis does not consider the comparatively understudied decarboxylation pathway. The methylation pathway associated with higher plants splitting into two different proposed pathways and bacteria using it instead of the more typical transamination pathway (Williams *et al.,* 2019).

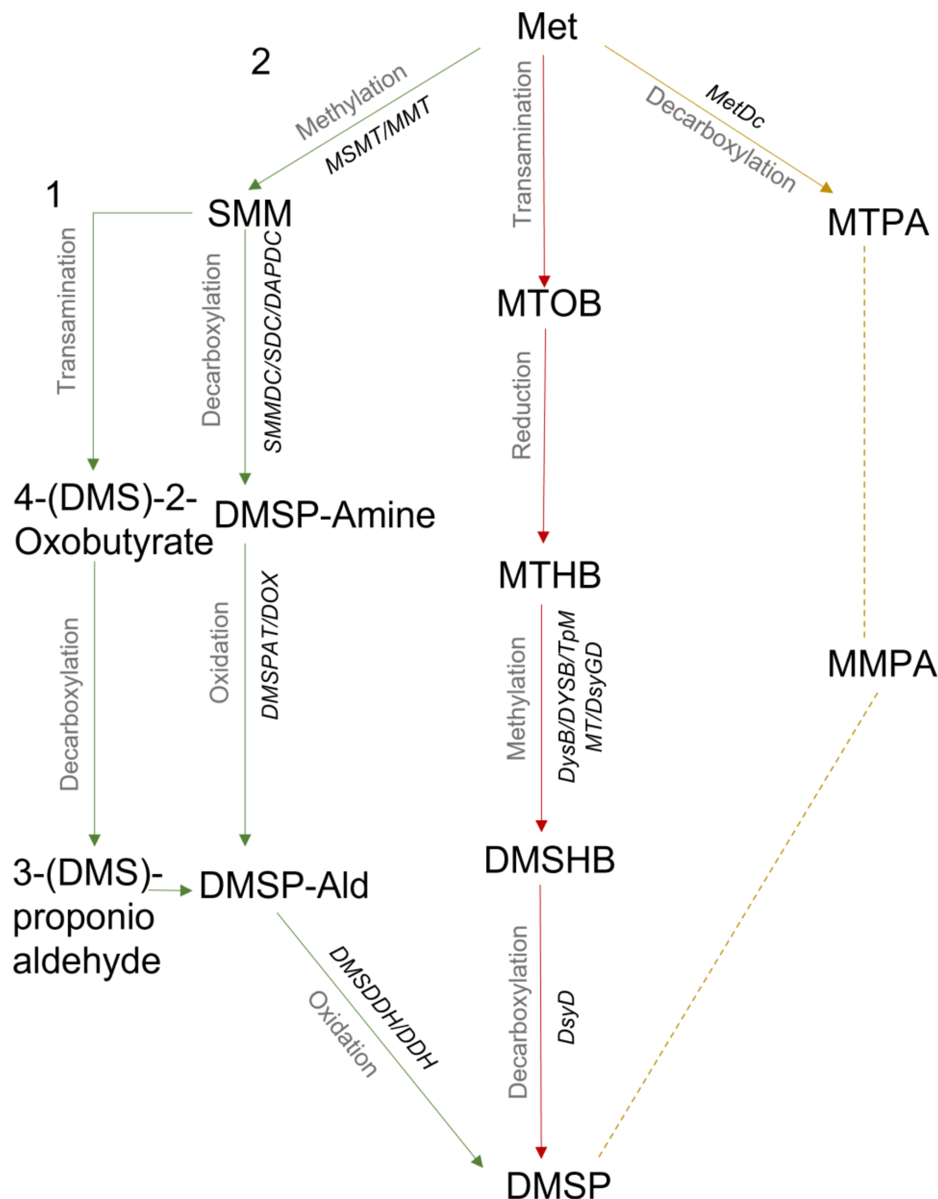


Figure 1.9: Summary of DMSP pathways: the products, genes (italicised) and reaction type. Green arrows represent the methylation pathways used by higher plants (pathway 1 used by *Melanthera biflora* and pathway 2 used by *Spartina spp.* and *Saccharum spp.*), red arrows represent the transamination pathway used by macroalgae, diatoms and bacteria and yellow lines the unknown pathway used by the dinoflagellate *Crypthecodinium.* Adapted from Williams *et al.*, 2019.

The Transamination pathway

The transamination pathway was initially identified using pulse chase experiments and radiolabelled ^{35}S methionine in the green macroalgae *Ulva intestinalis*, to determine the intermediate metabolites in this pathway (Gage *et al.,* 1997). The isolated intermediates were analysed by mass spectrometry. The authors determined that the amine group of methionine is transferred to a hydrogen atom donated by NADPH to generate ammonia and replaced by an oxygen atom in a transamination reaction, resulting in the unstable molecule 4-methylthio-2-oxobutyrate (MTOB). MTOB is rapidly reduced to 4-methylthio-2-hydroxybutyrate (MTHB), (Dickschatt *et al.,* 2015; Gage *et al.,* 1997).

MTHB is methylated to 4-(dimethylsulfonio)-2-hydroxy-butanoate (DMSHB) by an *S*-adenosine-methionine (AdoMet) dependent MTHB S-methyltransferase enzyme. This is the rate limiting and first committed step of the pathway (Curson *et al*., 2018). Furthermore, it is the only step for which genes/enzymes have been identified, see blow. The final step of the transamination pathway is the decarboxylation of DMSHB to DMSP, with the first enzyme ratified for this step found recently in the rhizobacterium *Gynuella sunshinyii*, termed DSYD (Wang *et al.,* 2023). Through radiolabelling experiments, it was determined that this final step of the pathway is also a committed step (Gage *et al.,* 1997).

There are multiple gene/enzymes identified for the MTHB S-methyltransferase enzyme but only one other gene/enzyme of the transamination pathway. Additionally, there are no genes linked to *DsyB* expression, either up or downstream, elucidated. The first of these was *dysB* identified in the marine alphaproteobacteria *Labrenzia aggregata*, and subsequently in ~ 100 other marine alphaproteobacteria (Curson et al., 2017). Incidentally, that seminal study was the first to show that bacteria could produce DMSP. BLAST analysis of the DSYB protein showed homologues in marine eukaryotes, including in most dinoflagellates, haptophytes and corals and some diatoms (Curson *et al.,* 2018). More recently an isoform MTHB *S*-methyltransferase was identified in the diatom *Thalassiosira pseudonana*, and termed TpMMT (Kageyama *et al.,* 2018). Consequently, the presence of *DsyB* alone is considered sufficient to positively identify DMSP producing species. Relevant to this project, none of these MTHB *S*-methyltransferase genes are found in any plant genomes or transcriptomes.

Recent studies have also demonstrated that DMSP is produced by a small proportion of marine and coastal prokaryotes (Fig. 1.9), such as *Oceanicola batensis* have been shown

to produce DMSP and cleave it to generate DMS in the East China Sea (Curson *et al.,* 2017). Of these, some species such as *E. huxleyi* and *Labrenzia aggregata* have been shown to contain both *DsyB* and known DMSP catabolic enzymes, such as *Alma1* and *dddL*, respectively (Johnston *et al.,* 2016; Zhong *et al.,* 2021). Since the recent identification of DMSP producing bacteria in notably *L. aggregata*, a recent study established procedures to enrich for – and isolate - such bacteria from a variety of environments sources, most relevant to this study, salt marsh sediments (Williams *et al.,* 2019). *Novosphingobium spp.* was shown to produce DMSP in salt-marsh environments (Williams *et al.*, 2019). Furthermore, Gammaproteobacteria and Actinobacteria, such as *Halomonas sp.* and *Aggrococcus sp.* respectively also produce DMSP in marine environments (Liao and Seebeck, 2019; Williams et al., 2019). Fascinatingly, *Novosphingobium spp*, lacked *DsyB* in their sequenced genome, which supports the hypothesis of their containing a distinct DMSP production gene/enzyme and/or pathway (Williams *et al.,* 2019).

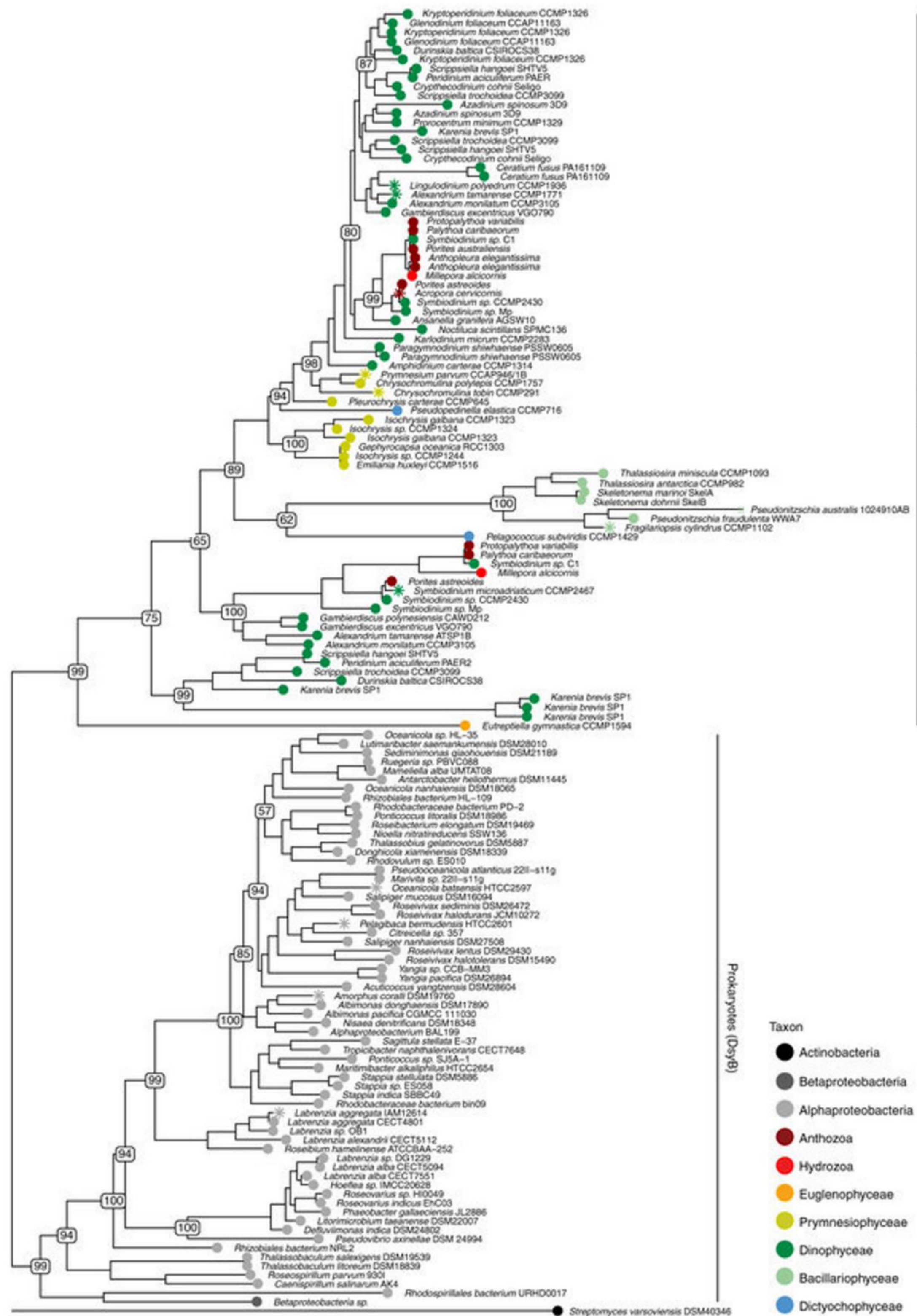


Figure 1.10: Maximum likelihood phylogenetic tree of DsyB/DSYB proteins involved in the Transamination pathway in both prokaryotes and eukaroytes. Species are colour-coded according to taxonomic class as shown in the key, with proteins shown to be functional marked with an asterisk. Figure adapted from Curson *et al.*, 2018.

The Methylation Pathway

The methylation pathway found in some bacteria is the only ratified DMSP production pathway found in plants. However, there are two variations on the pathway proposed (Fig. 1.9).

The first intermediate of the methylation pathway (Fig. 1.9) was determined to be S-methylmethionine (SMM) in both *M. biflora* and *S. anglica* by isotope-labelling studies. Using ^{14}C, pulse-chase experiments showed a kinetic pattern consistent with the methylation of methionine (Tab 1.1). The product was determined to be SMM through Fast Atom Bombardment Mass-Spectrometry (Hanson *et al.,* 1994; Kocsis *et al.,* 1998). Similar techniques were used to determine the next steps in the pathway in *S. anglica* but using ^{35}S as the radiolabelled element. Through this, DMSP-amine was determined to be the next intermediate (Kocsis *et al.,* 1998). The enzymes S-methylmethionine decarboxylase (SDC) and DMSP-amine oxidase (DOX) were proposed following a series of radiolabelled enzyme assays that showed these enzymes had significantly higher activity in *S. anglica* when supplied with methionine compared to *S. patens* which does not produce DMSP (Kocsis and Hanson, 2000).

SDC was identified following the measurable release of CO_2 following the conversion of SMM to DMSP-amine. The enzyme activity was assayed using Thin-Layer Chromatography (TLC) to detect conversion of SMM to DMSP-amine. DOX was proposed based on the high rate of conversion from DMSP-amine to DMSP-aldehyde and the increase in DMSP concentration when NADP was added. The enzyme activity was characterised using fractionation assays to determine the volume of DMSP-aldehyde extract produced. The final step in the pathway, BADH or DDH, was determined by ion-exchange chromatography and TLC to follow the conversion of the conversion of DMSP-aldehyde to DMSP (Trossat et al., 1996).

Table 1.1: Summary of parameters for each enzyme in the Methylation pathway based in *Melanthera biflora* for MMT and DDH, *Spartina alterniflora* for SDC and DOX (James *et al*., 1995; Kocsis and Hanson, 2000; Trossat *et al*., 1996). The cofactors and inhibitors listed are those that resulted in the greatest increase and decrease in activity, respectively.

Enzyme	**Specific Activity (pmol h^{-1} mg^{-1} protein)**	**Vmax (nmol min^{-1} mg^{-1} protein)**	**Optimal Conditions**	**Cofactor**	**Inhibitor**
MMT	3.9	0.1 (nkat mg-1 protein)	pH 7.2 25 °C	Cysteine	*N*-ethylmaleimide (NEM)
SDC	23.9	0.28	pH 7.2 23 °C	Pyridoxal-5’-Phosphate (PLP)	Ornithine
DOX/POP2	10800	0.37	pH 8.0 23 °C	α- ketoglutarate	Glycolate Oxidase
ALDH/BADH/DDH1/DDH2	99600	76200	pH 7.0 25 °C	NAD/NADP	γ- Aminobutyraldehyde

However, in *M. biflora* the same experiments that determined SMM to be the first step in the pathway also showed that there was a significant level of interconversion between methionine and SMM, and that overall, the DMSP production pathway did not produce high concentrations of DMSP (Hanson *et al.,* 1994). As the intermediates associated with decarboxylation of SMM were not identified in *M. biflora*, the researchers used ^{15}N labelled methionine to identify downstream products. They determined that the amino group of SMM derived from the radiolabelled methionine was predominantly incorporated into glutamate (Rhodes *et al.,* 1997). The presence of radiolabelled glutamate is consistent with a transamination reaction, leading them to propose the alternative pathway of transamination resulting in 4-DMS-2-oxobutyrate (DMSOB) as the intermediate (Fig. 1.9). The radiolabelling and pulse-chase experiments had been used to determine that DMSP-aldehyde was the final intermediate product in the methylation pathway (James *et al.,* 1995). The researchers concluded that the next step in the pathway to get from DMSOB to DMSP-aldehyde must be decarboxylation (Rhodes *et al.,* 1997).

The methylation pathway, identified in plants, was also recently found to operate in diverse bacteria (Liao and Seebeck, 2019; *Williams et al*., 2019). In these bacteria, SMM was found to enhance DMSP production and not transamination pathway intermediates. Some marine *alphaproteobacteria* - such as *Novosphingobium* - and actinobacteria - such as *Streptomyces mobaerensis* - were found to utilise this pathway and enhance DMSP production under raised salt conditions (Liao and Seebeck, 2019; Williams *et al.,* 2019). Homologues to the plant *mmt* were identified in the genomes of these diverse DMSP-producing bacteria at 25-60% sequence homology levels to *mmt* from *Melanthera biflora* (a plant known to produce DMSP) (Liao and Seebeck, 2019; Williams *et al.,* 2019). The corresponding gene in *Novosphingobium* and alphaproteobacterial *Thalassospira profundimaris* was designated *mmtN* (Williams *et al.,* 2019) and *mmtN*$^-$ were shown to no longer produce DMSP. The bacterial *mmtN* sequences are shorter than their plant homologues, suggesting that they have evolved independently (Williams *et al.,* 2019).

Importantly, *mmtN* in *S. mobaerensis* and *Novosphingobium* are linked to genes that encode the enzymes for the downstream steps in DMSP synthesis via the methylation pathway (Fig. 1.11). These clusters of genes, or operons, indicate that each step of the DMSP biosynthesis pathway are regulated co-ordinately. The other genes in these

operons are one of the criteria that determines how the *mmtN* genes are divided into three groups (Peng *et al.,* 2022). In reverse order, Group III are large *mmtN* sequences, more than double the length of most *mmtN* sequences, that are commonly found in plants. Group II are those found in the following organisms, that whilst functional are not predicted to produce DMSP: the archaea *Candidatus Woesearchaeota*, Candidate Phyla Radiation (CPR) bacteria, and the rotifer *Adineta steineri*. Finally, Group I comprises all remaining *mmtN* genes, split into subcategories based on the presence or absence of the non-ribosomal peptide synthesis gene within the operon (Peng *et al.,* 2022).

Indeed, these enzymes from *S. mobaerensis* were shown to have 80 µM min^{-1} catalytic activity when expressed in *E. coli* and a mutation in the *Thalassospira profundimaris mmtN* gene significantly knocked down DMSP production. These MmtN enzymes are classified depending on the methyl-accepting atom, within the substrate and comprise *O-, N-, C-,* and *S*-directed methyltransferases (Liscombe *et al.,* 2012). Of these, the *S*-methyltransferases will be of interest in future chapters.

Fig. 1.11: Gene maps showing genomic locations of *mmtN* in selected *mmtN* containing bacteria. The species names of the bacteria are indicated with their strain identifier. The *mmtN* gene is shown in orange within the operons. Grey arrows indicate genes that are not currently known or predicted to be involved in DMSP synthesis. Gene are to scale. (Williams *et al.*, 2019).

Most recently these genes encoding DMSP-amine aminotransferase and DMSP-aldehyde dehydrogenase from *Streptomyces mobaraensis* (Liao and Seebeck, 2019) were found to be part of a non-ribosomal peptide synthase producing DMSP as an intermediate in the production of a virulence factor in *pathogenic betaproteobacteria Burkholderia* (Trottman *et al*., 2020). This first demonstration of DMSP as an intermediate in toxin production, also identified novel SMM decarboxylase (BurI) and Met *S*-methyltransferase (BurB) enzymes, confined to *Burkholderia,* and proposes that DMS is produced during the production of the cyclopropanol warhead (Trottman *et al.,* 2020).

<u>The Decarboxylation Pathway</u>

There is a putative decarboxylation pathway potentially used by the dinoflagellate *Crypthecodinium cohnii*. The first step of the reaction was determined by radio-tracing experiments to be an oxidative decarboxylation via L-methionine decarboxylase converting methionine into 3- methylthiopropanamine (MTPA). This enzyme was purified from *C. cohnii* extracts but its identity is unknown (Kitaguchi *et al.,* 1999). The final intermediate was predicted to be 3- methylmercaptopropionate (MMPA) (Dickschatt *et al.,* 2015), but this has not been ratified and it is unknown why MMPA was predicted. It is noteworthy that the transcriptome of *C. cohnii* contains five DsyB homologues, therefore this mechanism of DMSP synthesis requires further study (Curson *et al.,* 2018).

DMSP Transport

DMSP transport is largely unknown in organisms other than bacteria. Within bacteria, the type of transporter used to move DMSP into and out of cells varies depending on the species. There are two main types of transporters for DMSP; the ATP-binding cassette proteins (ABC) and the betaine-carnintine-choline transporters (BCCT). ABC superfamily proteins are ubiquitous transmembrane proteins that require energy in the form of ATP to move molecules against their concentration gradient (Rees *et al.,* 2009). These proteins have a highly conserved obligate dimer structure of substrate binding domains for the molecule of interest, hydrophobic cores spanning the membrane and an intracellular nucleotide binding domain that dephosphorylates ATP (Dickschat *et al.,* 2015). The DMSP-specific transporter DmpXWV, first identified in *Ruegeria pomeroyi*, was found to be an ABC-transporter through structural analysis (Li *et al.,* 2023). The

substrate binding domain DMPX is found in bacteria across the globe, suggesting that DmpXWV type transporters may be the most widespread DMSP-transporter in pelagic bacteria (Li *et al.,* 2023). Furthermore, downstream of DMSP-lyase genes, *Bukholderia ambiferia* encodes ABC transporters predicted to transport DMSP (Sun *et al.,* 2012). Additionally, osmolyte-specific ABC transporters OpuB and OpuC in *Bacillus subtilis* were also shown to have a high affinity for DMSP, amongst other compatible solutes (Teichmann *et al.,* 2017).

BCCT superfamily proteins specifically transport osmolytes across cell membranes as symporters, moving the substrate against the concentration by coupling the movement to a molecule moving with the concentration gradient – or antiporters that move two molecules against opposing concentration gradients (Dickschat *et al.,* 2015; Ziegler *et al.,* 2010). These proteins usually consist of three separate monomers, each with 12 transmembrane sections that make up a hydrophobic core (Ziegler *et al.,* 2010). BCCT transporter genes are associated with *ddd* DMSP lyase genes in *α-proteobacteria* such as *Halomonas spp., Marinomonas spp., Sulfitobacter sp.* and *Roseovarius nubinhibens* (Sun *et al.,* 2012). This was also shown in the *α-proteobacteria Vibrio spp.* through BCCT knock-out mutants that had diminished growth in DMSP-containing bacteria compared to the WT control (Gregory *et al.,* 2021).

DMSP Catabolism

As previously discussed in the roles of DMSP, the breakdown of DMSP into DMS, is of critical importance to the global sulfur cycling, the formation of CNN and supporting the trophic structures of both marine and terrestrial systems. There are two DMSP catabolic pathways; demethylation and cleavage (Curson *et al.,* 2011a). These are thought to be mainly associated to bacterioplankton once DMSP has been released into the marine environment by phytoplankton through viral lysis (Hill *et al.,* 1998), natural cellular death (Stefels & van Boekel, 1993), grazing by zooplankton (Wolfe & Steinke, 1996) or excretion in faecal pellets (Yu *et al.,* 2023) (Fig.1.12). However, selected species of both macro- and micro-algae contain DMSP lyase enzymes, such as *Ulva mutabilis*, that contains an Alma1 homologue designated UM030_0039.1 (Pesante *et al.,* 2023).

There is a growing body of evidence to suggest that marine phytoplankton have a greater role in DMSP lysis to DMS than was hitherto thought. In vitro studies of the coccolithphore *Emiliana huxleyi* showed that they are capable of comparatively low

levels of DMS production (0.1-150 nM DMS.h^{-1}) (Steinke *et al.,* 2002). Despite these low levels per organisms, large numbers of phytoplankton are associated with DMS spikes and the functional DMSP cleavage enzyme Alma1 was identified in *E. huxleyi* (Alcolombri *et al.,* 2015). Other dinoflagellates closely related to *E. huxleyi* have also shown to be DMS producers, such as *Symbiodinium microadriaticum* (Yost & Mitchelmoore, 2009) and *Gephyrocapsa oceanica* (Franklin *et al.,* 2010).

The demethylation pathway is exclusively a bacterial process, that is thought to be the most prevalent DMSP catabolic pathway. It is predicted to account for up to 80% of global DMSP catabolism (Kiene *et al.,* 2000). In this pathway, DMSP undergoes a four-step series of reactions, starting with the removal of one of the methyl groups catalysed by the demethylase enzyme DmdA (Howard *et al*., 2006). This reaction requires tetrahydrofolate (FH_4) as a co-substrate/methyl acceptor molecule, and results in the production of methyl-mercaptoprionate (MMPA) and methyl-FH_4 (Reisch *et al.,* 2011b). DMDA, was first identified in *Ruegeria pomeroyii* the model *Roseobacter,* as a glycine T-cleavage enzyme, but was found to have high specificity towards DMSP (Howard *et al.,* 2006). Further study proved DmdA to be consistently present in *Roseobacters* and SAR11 bacteria (Hérnandez *et al.,* 2020; Zeng *et al.,* 2016). These are abundant in the marine environment, making up an estimated 20-30% of the marine bacterial community (Brinkhoff *et al.,* 2008) and supports the view that DMSP demethylation is the major catabolic pathway. After initial demthylation, MMPA next undergoes two stages of reduction: first demethiolation to 3-methylmercaptopropionyl-CoA (MMPA-CoA) by the DmdB enzyme, then MMPA-CoA to methylthioacryloyl-CoA (MTA-CoA) by the DmdC enzyme (Reisch *et al.,* 2011b). Finally, MTA-CoA undergoes hydrolysis to form the end products acetaldehyde, methanethiol (MeSH), CO_2 and Coenzyme A (Co-A or HS-CoA) by the enzyme DmdD (Bullock *et al.,* 2017; Reisch *et al.,* 2011b). MeSH is a particularly important end product, as it is a volatile, climate active gas in its own right DMS, contributing significantly to atmospheric SO_2 concentrations (Novak *et al.,* 2022). MeSH is also the major source of reduced sulfur scavenged by SAR11 bacteria, as they lack the necessary genes to assimilate sulfur to reduce sulfate to hydrogen sulfide (Moran & Durham, 2019).

Initially, all these enzymes were identified in *R. pomeroyi*, although an additional enzyme homologue to DmdD, known as AcuH, has also been identified in *Roseovarius nubinhibens* (Curson *et al.,* 2011a). Indeed, DmdABCD enzymes are found consistently across marine *Roseobacters* in a diverse range of environments – from the Blue Lagoon

in Iceland to the Caribbean Sea (Petursdottir and Kristjansson, 1997; González et al., 2003; Bullock et al., 2014), which is consistent with *Roseobacters* using the demethylation pathway to break DMSP down into DMS and MeSH to meet their sulfur and carbon needs through sulfate assimilation (Shao *et al.,* 2019).

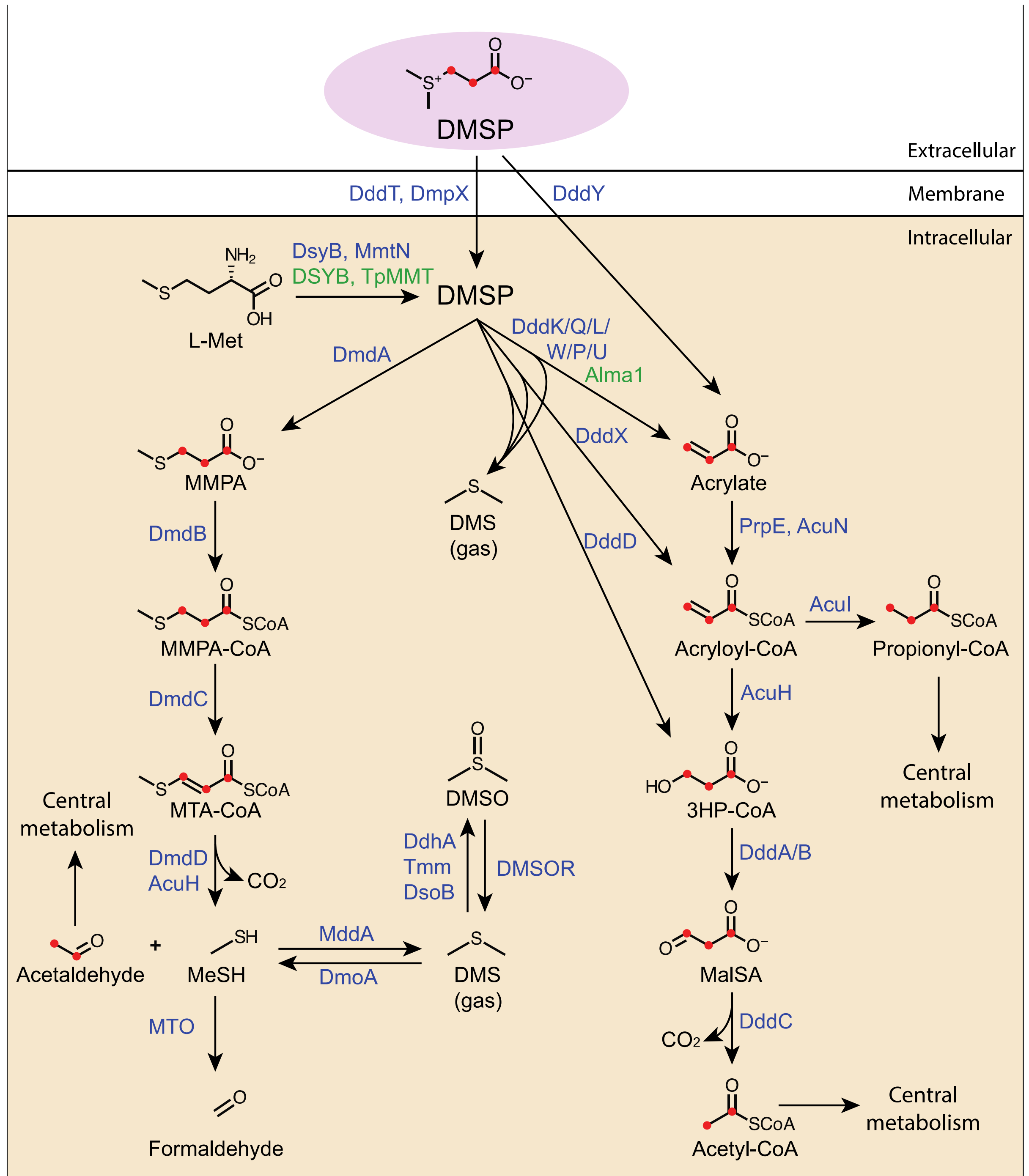


Fig. 1.12 Pathways of DMSP synthesis and degradation. DMSP can be synthesised by both phytoplankton and bacterioplankton from methionine (L-Met). Enzymes from phytoplankton and bacteria are shown in green and blue, respectively.

For most enzymes, the cleavage pathway results in the cleavage of the β-carbon:sulfur bond in DMSP to produce DMS and a 3C co-product; mainly acrylate, but in the cases of DddD and DddX, 3-hydroxypropionate-Coenzyme A (3-HpCoA) and acryloyl-CoA respectively (Bullock *et al.,* 2017; Curson *et al.,* 2011b; Li *et al.,* 2021). The exceptions to this are DmdA and an unknown enzyme that oxidises DMSP, which generate the 4C products MMPA and DMSOP respectively (Li *et al.,* 2021). In contrast to DMSP demethylation with only DmdA, there is huge biodiversity in DMSP cleavage, with 10 distinct DMSP lyase enzyme having been isolated to date. The DMSP lyase enzymes belong to one of 5 protein families (Tab 1.2).

Firstly, the largest family is the cupin superfamily, with a characteristic barrel-like β-structure (Lei *et al.,* 2017). DddL, DddQ, DddW and DddK contain copper ions in their active site at the C-terminal domain, known as cupin pockets (Curson *et al.,* 2011a), however for the remaining DMSP-cleavage enzymes, their structures remain unknown. These cleave DMSP into acrylate and DMS. Acrylate and 3-HP can be converted to acryloyl-CoA by demethiolation or hydrolysis respectively, which is further reduced to propionyl-CoA as an essential component of the bacterial methylmalonyl-CoA pathway (Bullock *et al.,* 2017; Reisch *et al.,* 2011b).

Secondly, the M24 metallopeptidase family. The sole known member of this family that lyses DMSP is DddP, which cleaves the carbon-sulfur bond to generate acrylate and DMS (Curson *et al.,* 2011a). It is slightly structurally different to other members of its family, as the gene encodes for a simple lyase, rather than a peptide hydrolase (Wang *et al.,* 2015) and does not require metal cofactors (Curson *et al.,* 2011a). Despite being a family of one, it is in fact one of the most frequently encountered DMSP lyases in marine metagenome analysis (Curson *et al.,* 2011a; Todd *et al.,* 2011).

Thirdly, the aspartate racemase superfamily, that can also convert L-aspartate ↔ D-aspartate (Yamauchi *et al.,* 1992). Alma1 is also the sole member of this family and like the two families before, also produces DMS and acrylate as its co-product, by catalysing proton removal at the β-carbon (Alcolombri *et al.,* 2015). Fourthly, the Type III acyl-CoA transferase family, so named because it transfers an acyl group onto DMSP to cleave it (Curson *et al.,* 2011a). This is the only enzyme that releases 3-HP and DMS and is named DddD, (Curson *et al.,* 2011a). Finally, the acyl-CoA synthetase superfamily that catalyses an acetyl-CoA reaction and thus is the only indirect method of DMSP lysis (Alcolombri *et al.,* 2015). In all known cleavage systems, DMS is released into the environment.

Table 1.2 Accession numbers of previously ratified enzymes involved in the cleavage of DMSP to show the range of both enzymes and bacterial species identified. Adapted from Liu *et al.*, 2022.

Protein	Superfamily	Co-product	Ratified strains	Accession number	Reference
DddD	Type III acyl CoA transferase	3HP-CoA	*Marinomonas* sp. MWYL1	ABR72937	Todd *et al.*, 2007
			Oceanimonas doudoroffii	AEQ39135	Curson *et al.*, 2012
			Psychrobacter sp. J466	ACY02894	
			Halomonas sp. HTNK1	ACV84065	Todd *et al.*, 2010
			Sinorhizobium fredii NGR234	AAQ87407	Todd *et al.*, 2007
			Burkholderia ambifaria AMMD	WP_011659284	
			Pseudomonas sp. J465	ACY01992	Curson *et al.*, 2010
DddL	Cupin	Acrylate	*Sulfitobacter* sp. EE-36	ADK55772	Curson *et al.*, 2008
			Rhodobacter sphaeroides 2.4.1	YP_351475	
			Labrenzia aggregata LZB033	KP639184	Curson *et al.*, 2009
			Ahrensia marina LZD062	KP639183	Liu *et al.*, 2010
DddP	M24 Metallopeptidase	Acrylate	*Roseovarius nubinhibens* ISM	EAP77700	Todd *et al.*, 2009

			Ruegeria pomeroyi DSS-3	WP_044029245	Todd *et al.*, 2011
			Phaeobacter inhibens DSM 17395	AFO91571	Burkhadt *et al.*, 2017
			Oceanimonas doudoroffii DSM 7028	AEQ39091	Curson *et al.*, 2012
			Oceanimonas doudoroffii DSM 7028	AEQ39103	
			Aspergillus oryzae RIB40	BAE62778	Todd *et al.*, 2009
			Fusarium graminearum PH-1	XP_389272	
DddQ	Cupin	Acrylate	*Ruegeria pomeroyi* DSS-3	WP_011047333	Todd *et al.*, 2011
			Roseovarius nubinhibens ISM	EAP76002	
			Roseovarius nubinhibens ISM	EAP76001	
			Ruegeria lacuscaerulensis ITI1157	WP_005978225	Li *et al.*, 2014
DddW	Cupin	Acrylate	*Ruegeria pomeroyi* DSS-3	AAV93771	Todd *et al.*, 2012
DddY	Cupin	Acrylate	*Alcaligenes faecalis* M3A	ADT64689	Curson *et al.*, 2011b
			Desulfovibrio acrylicus	SHJ73420	van der Maarel *et al.*, 1996
			Acinetobacter bereziniae	ENV21217	Li *et al.*, 2017b

			Ferrimonas kyonanensis DSM 18153	WP_028114584	Lei *et al.,* 2017b
			Shewanella putrefaciens CN-32	ABP77243	Curson *et al.,* 2011a
DddK	Cupin	Acrylate	*Candidatus* Pelagibacter ubique HTCC1062	AAZ21215	Sun *et al.,* 2016
			Candidatus Pelagibacter ubique HTCC9022	WP_028037226	
			alphaproteobacterium_HIMB5	AFS47241.1	
DddX	Acyl-CoA Synthetase	Acryloyl-CoA	*Marinobacterium jannaschii*	WP_084332639.1	Alcolombri *et al.,* 2015
			Pelagicola sp. LXJ1103	WP_109384856.1	
			Psychrobacter sp. P11G5	WP_068035783.1	
			Sporosarcina sp. P33	WP_081242855.1	
Alma1	Aspartate Racemase	Acrylate	*Emiliana huxleyi* CPMP1516	XP_005784450	Alcolombri *et al.,* 2015

There is also evidence to suggest that other groups of organisms contribute significantly to DMSP lysis. Functional DMSP lyases have been identified in the macroalgae *Polysiphonia paniculata* (Nishigucchi & Goff, 1995) and *Ulva curvata* (De Souza & Yoch, 1995), but the output of DMS and genes encoding them have yet to be ratified (Reisch *et al.,* 2011b). Additionally, two species of terrestrial Ascomycota fungi have also been shown to catabolise DMSP. The filamentous fungi *Aspergillus oryzae*, used commercially as a fermenting agent in the production of miso and sake (Matsushima, 2020) and the highly toxic pathogen of wheat and barley, *Fusarium graminearium*

(Goswami & Kistler, 2004), have both been shown to catabolise DMSP through the decarboxylation action of *dddP* (Todd *et al.,* 2009). There is no evidence to suggest that higher plants catabolise DMSP, so far.

Knowledge Gaps and Research Aims

The importance of DMSP in both aquatic and terrestrial systems to global sulfur cycling is well understood, however the systems underpinning DMSP production in terrestrial systems is by no means as well characterised as the marine environment. As there is a growing body of evidence to prove that terrestrial systems such as salt marshes are very high producers of DMSP, and likely to contribute significantly to global sulfur cycling, this project aims to uncover the critical role of plants and their associated rhizospheres in the biosynthesis of this important climate active gas. This work aims to investigate the following research gaps:

1. The prevalence of DMSP production within higher plants and the relationships between high producers.
2. Ratification of the genes that encode for enzyme required for DMSP biosynthesis and the regulation of pathway.
3. The mechanisms of DMSP production within plants, with a focus on the first step of the methylation pathway.
4. The parameters that affect DMSP production in higher plants, with a focus on the model organism *Arabidopsis thaliana.*
5. The relationship between rhizosphere bacteria and the higher plant *Spartina anglica* in a uniquely high DMSP-producing terrestrial environment, the saltmarsh.

Chapter 2 – Materials and Methods

Media Recipes

Table 2.1 Media Recipes and Essential Additives used, with formulas calculated per litre. All company names and product codes are given in backets.

Media Name	Formula Per Litre
Basal Media	Dipotassium hydrogen 233.2 mg (Fisher P/5240/53) Tris 48.6 g (Fisher BP 152-1)
Luria Broth (LB)	NaCl 10 g (Sigma-Aldrich S9888) Tryptone 10 g (Formedium TRP03) Yeast 5 g (Formedium YEA02)
LB Agar	As above with Agar 15 g (Sigma 05040)
Marine Basal Media (MBM)	Basal Media 250 ml Sea Salts 20g (Formedium FSS10)
MBM Agar	As above agar 20 g (Sigma 05040)
¼ Murashige & Skoog (MS) Agar	MS with Vitamins 1.1 g (Duchefa Biochemie M0222) Sucrose 10 g (Thermo Scientific J65148.A1) Agar 8 g (Sigma 05040)
Super Optimal broth with Catabolite repression (S.O.C)	SOC Broth 31.5 g (Formedium SOC0201)
Vitamin Supplement for MBM	p-Aminobenzoic Acid 50 mg (Sigma A9878) Biotin 20 mg (Sigma B4639) Cyanocobalamin 1 mg (Merck V6629) Folic Acid 20 mg (Merck F8758) Nicotinic Acid 50 mg (Sigma N4126) Pantothenic Acid 50 mg (Sigma P9153) Pyridoxine-HCL 100 mg (Merck P5669) Riboflavin 50 mg (Sigma R4500) Thiamine 50 mg (Acros 148990100)
Yeast Tryptone Sea Salts (YTSS)	Tryptone 2.5 g (Formedium TRP03)

	Sea Salts 20 g (Formedium FSS10) Yeast Extract 4 g (Formedium YEA02)
YTSS Agar	As above with Agar 15 g (Sigma 05040)

Polymerase Chain Reaction Programmes

Table 2.2: Phusion PCR reaction conditions.

Step	Temperature (˚C)	Time (sec)	Repetitions
Initialisation	98	30	1
Denaturation	98	10	30
Annealing	72	45	30
Extension	52	20	30
Final Extension	72	300	1

Table 2.3: Bounce PCR Reaction Conditions

Step	Temperature (˚C)	Time (sec)	Repetitions
Initialisation	94	120	1
Denaturation	94	30	40
Annealing	60 decreasing by 1 for 15 cycles, then increasing by 0.6 for 25 cycles.	40	40
Extension	72	210	40
Final Extension	72	360	1

Table 2.4: Colony PCR reaction conditions.

Step	Temperature (˚C)	Time (sec)	Repetitions
Lysis	98	600	1
Denaturation	98	10	30
Annealing	53	20	30
Extension	72	120	30
Final Extension	72	300	1

Table 2.5: PCR Conditions for the amplification of extracted *A. thaliana* DNA to genotype MMT- and WT plants.

Step	Temperature (°C)	Time (sec)	Repetitions
Lysis	94	180	1
Denaturation	94	30	35
Annealing	54	30	35
Extension	72	30	35

Table 2.6: Primer Sequences (5' -3'). Genes are denoted in bold and both associated primers to amplify the gene given. Where a primer exists singly, the name is not bold.

Gene / Primer Name	Forward Primer	Reverse Primer
MMT	gtggaagacttaggtatgGCGGTGAATGGAC	GTGGAAGACTTAAGCTCATTTATCAACCAT
SDC/DAPDC	gtggaagacttaggtatgGCGGCTACAC	GTGGAAGACTTAAGCTTACAGACCTTCA
DOX	gtggaagacttaggtatgGCCAAGATTAC	GTGGAAGACTTAAGCTTACTTCTTCTGA
ALDH	gtggaagacttaggtatgGCGATTCC	GTGGAAGACTTAAGCTCACAGCTTTG
BADH	gtggaagacttaggtatgGCAAATCG	GTGGAAGACTTAAGCCTAATTCTTTG
DDH1	gtggaagacttaggtatgGCGTTTCG	GTGGAAGACTTAAGCTTAAAGCCAAGC
DDH2	gtggaagacttaggtatgGCAGCTC	GTGGAAGACTTAAGCCTATATCCAAG
MMT IF.01	GTAGGATGCATACCTCAGG	-
LBb1		GCGTGGACCGCTTGCTGCAACT
GoldengateL2	GCGGACGTTTTTAATG	-
BM0189		
27F	AGAGTTTGATCCTGGCTCAG	-
1429R	-	GGTTACCTTGTTACGACTT
515F	GTGCCAGCMGCCGCGGTA	-
806R	-	GGACTACNVGGGTWTCTAAT

Agarose Gel Electrophoresis

Unless otherwise specified, PCR products were loaded into the wells of 1% Agarose Gel (10 g of Agarose per 1L of 1x Tris-Acetate-EDTA (TAE) buffer) alongside 6 µl of 2-log ladder (1 kbp+ ladder with loading dye). The gel was run at 120V for 35 mins and post-stained in 0.5 µg/ml ethidium bromide for between 5-30 mins. DNA was visualised using Typhoon FLA 9500 laser scanner at a wavelength of 532 nm.

Making Chemically Competent Cells

E. coli DH5α (Invitrogen) were streaked from glycerol stocks maintained at -80 °C onto LB-Agar and incubated at 37 C, overnight. Single colonies were removed from the plate using aseptic techniques and inoculated into 5 ml LB. Cultures were incubated at 37 °C, 1 rcf until OD600 of 0.6 was reached. Cultures were incubated on ice for 15 mins, after which cells were spun down at 3580 rcf, 10 mins at 4 °C and the pellet resuspended in 20 ml 0.1 M $CaCl_2$. Cells were incubated on ice for 30 mins, then spun down as before. The pellet was resuspended in 250 µl 1M CaCl2, 1 ml 50% Glycerol and 1.25 ml H_20. Aliquots of 100 µl were made, flash frozen in N_2 (l), and stored at -80 °C.

Transformation of Competent Cells

To transform cells with the appropriate construct, 1 µl of cloned product was added to 20 µl of competent *E. coli* DH5α glycerol stocks of cells and incubated on ice for 30 mins. The cells were transformed by heat shocking at 42 ˚C for 30 seconds and recovered on ice for 1 min. To each transformation reaction, 500 µl of warm S.O.C media was added, and cells allowed to grow at 37 ˚C with shaking at 1 rcf for 60 mins.

Gas Chromatography

Head space analysis was conducted on all samples using 2 ml glass vials containing 300 µl liquid samples and sealed with PTFE/rubber crimp caps, unless otherwise specified. To measure DMS in liquid samples, vials were crimp sealed immediately and incubated at 22°C for 24 h in the dark. The head space was then directly measured.

To measure DMSP samples first underwent alkaline lysis. DMSP was first lysed to DMS with the addition of 100 µl 10 M NaOH to 200 µl culture. Vials were sealed and incubated as before, before the head space analysed.

Prepared samples in vials were assayed using by GC using a flame photometric detector (Agilent 7890A GC fitted with a 7693 autosampler), with head space samples carried through an HP-INNOWax 30 m x 0.320 mm capillary column (Agilent Technologies J&W Scientific) using hydrogen as a carrier gas. Peak areas at approximately 2.9 mins retention time were recorded as indicative of DMS production. DMSP concentrations (nmol) were determined using the formula:

$$DMSP\ (nmol\ g^{-1}FW) = \left(\frac{\sqrt{peak}\ area}{R}\right) \div g\ in\ Vial$$

Where R = the GC calibration curve as set by measuring standards of known DMSP concentration.

Eight-point calibration curves of DMS standards produced by the alkaline lysis of known DMSP standards in water. The curve was produced using known concentrations of DMSP ranging from 0.015 nmol to 30 nmol, added to 100 µl 10M NaOH and 200 µl sterile water, then incubated as before.

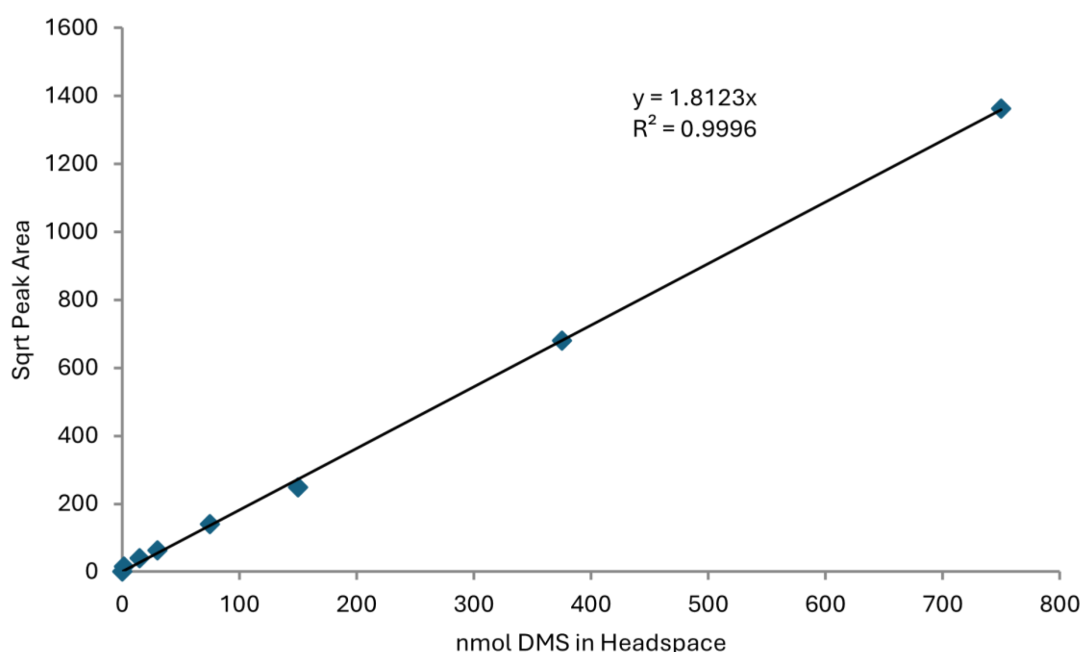


Figure 2.1: A typical eight-point calibration to calculate the concentration of DMS, which is also used as a proxy measurement for calculating the concentration of DMSP. The R measurement is the gradient of the line, determined by the formula y=Rx+c.

Calibration curves of MeSH were produced by preparing a stock solution of 50 mM by weighing 0.035g sodium methanethiolate in 10 ml methanol. A range of standards were then produced by serial dilution of the stock solution in 1 ml of methanol. The liberated MeSH was measured using the purge-and-trap method as described in Franklin *et al.,*

(2010). Sulfur gases were sparged from the sample with nitrogen – in which gaseous nitrogen is bubbled through the sample - and trapped in a loop of tubing immersed in liquid nitrogen. The trapped gases were desorbed with hot water (above 90 °C) and analysed by GC (Franklin *et al.,* 2010; Williams *et al.,* 2019)

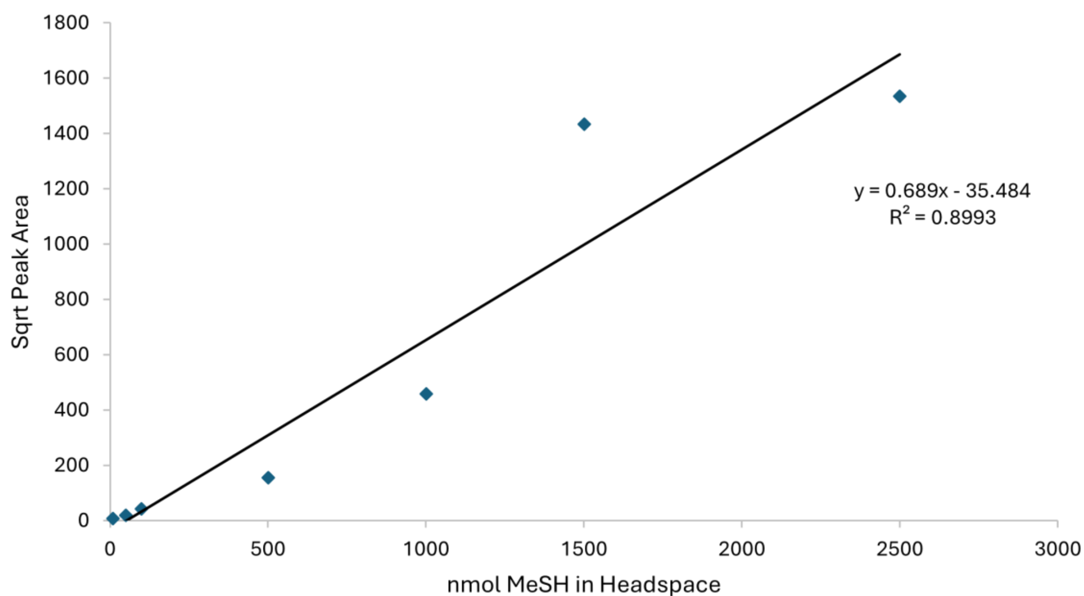


Figure 2.2: A typical eight-point calibration to calculate the concentration of MeSH. The R measurement is the gradient of the line, determined by the formula y=Rx+c.

The detection limits for headspace DMS and MeSH were 0.015 nmol and 0.10 nmol respectively.

Chapter 3 – The Range of Plants that Produce Dimethylsulfonio-propionate

Introduction

Sulfur Uptake in Higher Plants

A small proportion of a plants requirement for sulfur are met through absorption of atmospheric sulfur containing gases, such as H_2S and SO_2 (Aghajanzadeh *et al.,* 2016; Herschbach *et al.,* 1994; Sue *et al.,* 2002). The gases enter the leaves through the stomatal openings, the rate of which depends on the atmospheric concentrations of the gases and the metabolic needs of the plants (Noland & Kozlowski, 1979; By *et al.,* 1996). The SO_2 undergoes rapid hydration in the cytosol of the mesophyll cells, producing hydrogen sulfite (HSO_3^-) that is either reduced and enters the chloroplasts or is oxidised and enters the sulfur assimilation cycle (Noland & Kozlowski, 1979). H_2S, by contrast, is poorly soluble but rapidly dissociates in the atmosphere to H^+ and HS^- (By *et al.,* 1996). It is therefore uncertain whether the H_2S directly enters the cell, of the HS^- is oxidised to another gaseous compound before uptake.

Most of the sulfur plants need is taken up as inorganic SO_4^{2-} by the roots (Chorianopoulou & Bouranis, 2022; Li *et al.,* 2020; Ren *et al.,* 2022). This occurs through specific Sulfate Transport proteins (SULTRs), which are a large and diverse family of membrane spanning proteins (Ding *et al.,* 2016; Takahashi, 2019). All members of this family have a trans-membrane subunit with twelve domains, and a C-terminal sulfate-binding subunit, known as an anti-sigma factor (Ding *et al.,* 2016). SULTRs are split into four distinct groups, depending on their affinity for SO_4^{2-} and mechanism. Group 1 are high-affinity H^+/SO_4^{2-} symporters, which are expressed in sulfur deficient conditions (Takakashi *et al.,* 2012; Takahashi, 2019). Group 2 are the comparatively abundant, low-affinity Na^+/SO_4^{2-} symporters, that are expressed under optimal soil conditions (Ding *et al.,* 2016; Takahashi *et al.,* 2012). Group 3 are the most functionally diverse, with representatives found in chloroplast membranes as well as root cells, but are generally known to be anion anti-transporters (Ding *et al.,* 2016; Takahashi *et al.,* 2012, Takahashi, 2019). Finally, group 4 are the vacuolar ABC-type transporters, requiring energy in the form of ATP to translocate SO_4^{2-} from the cytosol in the vacuole for storage (Ding *et al.,* 2016; Takahashi *et al.,* 2012, Takahashi, 2019). The expression of the genes for SULTRs is regulated by the cellular concentration of sulfur-containing compounds, such as gluthathionine (GSH) and cysteine, as well as the Ethylene-Insensitive family transcription factor known as *SLIM1* (Li *et al.,* 2020; Takahashi 2019).

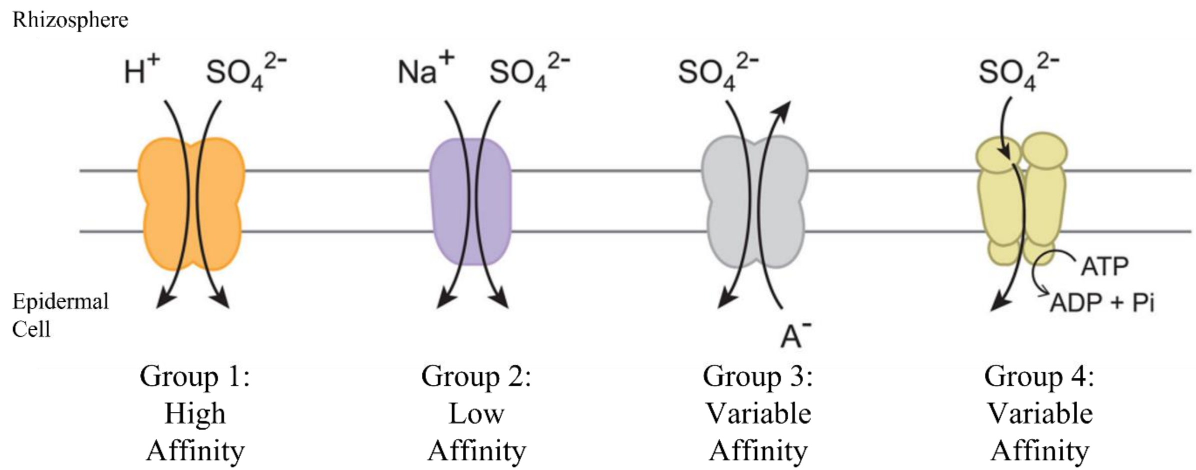


Figure 3.1: The four known categories of sulfur transporter found in plants cells and their substrates (adapted from Takahashi *et al.*, 2012).

SULTRs are not required to translocate SO_4^{2-} from root to shoot, after absorbing it from the soil (Takahashi, 2019). Instead, absorbed SO_4^{2-} diffuses directly from the epidermis to the xylem, which moves SO_4^{2-} along with water to the leaf xylem parenchyma cells, via capillary action (Takahashi, 2019). From there, SO_4^{2-} enters the sulfur-assimilation cycle in the chloroplasts and vacuole via Group 3 and 4 transporters (Takahashi, 2019; Yeo & Flowers, 2012).

<u>The Role of Sulfur in Higher Plants</u>

Outside of DMSP synthesis, the sulfur-containing compound gluthathionine (GSH) is a key regulator of plant hormones. Salicylic acid is a phenolic plant hormone that stimulates plant responses to abiotic stresses, such as sub-optimal temperatures and salt stress (Hassoon & Abdulsattar-Abduljabbar, 2020). GSH is found as a redox couple in plants cells, where the reduced form is in a higher proportion to the oxidised form (Noctor *et al.,* 2011; Rausch & Wachter, 2005). When abiotic stress results in the formation of reactive oxygen species, the ratio of reduced GSH: oxidised GSSG decreases, which increases the levels of salicylic acid produced (Hasanuzzaman *et al.,* 2018; Künstler *et al.,* 2020). Jasmonic acid is a plant-growth regulating hormone, that acts as an inhibitor of other plant hormones to limit growth under abiotic stress conditions (Wang *et al.,* 2020). Jasmonic acid also plays a key role in plant immunity, by stimulating production of defensive secondary metabolites in response to pathogens or wounding (Caarls *et al.,* 2017). Increased GSH levels in response to invasion or injury have been shown to increase production of jasmonic acid (Künstler *et al.,* 2020). Lastly, ethylene is a gaseous hormone that governs the lifespan of plants by inducing or

inhibiting senescence (Iqbal *et al.,* 2017). Sulfur is critical for the synthesis of ethylene. The ethylene biosynthesis pathway requires not only Methionine in its activated form, SAM, but SAM itself is upregulated by GSH (Künstler *et al.,* 2020).

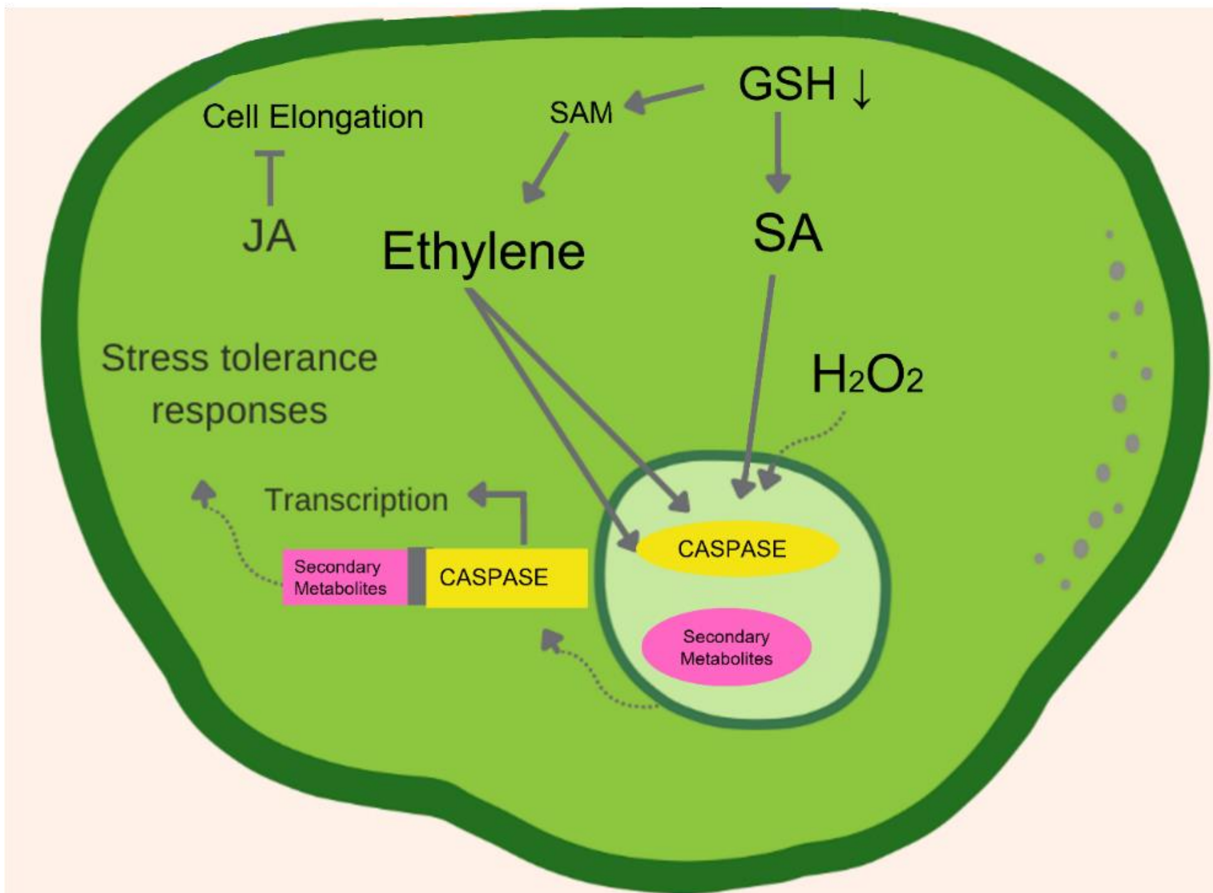


Figure 3.2: Diagram of phytohormone interaction and induction of cell death (senescence) genes and secondary metabolite production genes in response to salt stress. (Adapted from Gallego, 2023).

Additional to the role of jasmonic acid, sulfur is also critical for other plant immune responses. Sulfur-induced immunity against fungal diseases is a well-known phenomenon (Bloem *et al.,* 2015; Wang *et al.,* 2022). Crystal of sulfate have been commercially applied since the early 19th century (Forsyth, 1810), with the most famous product being copper (II) sulfate – $CuSO_4$ or Bordeaux Mixture – being applied to treat *Botrytis cinerea* infections in vineyards (Martin, 1933). Whilst the primary mode of action is the Cu^{2+} ions impeding fungal enzyme activity, the SO_4^{2-} component has been shown to improve disease resistance in plants such as *Vitis vinifera*, with a high sulfur requirement (Bloem *et al.,* 2015; Rausch & Wachter, 2005; Wang *et al.,* 2022). This may be due to increased cysteine production. In *Arabidopsis thaliana*, plants with the last enzyme of the cysteine biosynthesis pathway, *O*-acetylserine(thiol)lyase were shown to be more susceptible to *Botrytis cinerea* infections, as the hypersensitive

response to pathogens relies on cysteine to be triggered (Leustek *et al.,* 2000). Additionally, methionine is required for hydrogen peroxide generation, an important molecule for plant defence signalling cascades (Künstler *et al.,* 2020). Sulfur is also an essential structural component in anti-microbial compounds, such as phytoalexins, that directly attack pathogen cell membranes by binding to, and disrupting, cell walls via their sulfur-containing side chains (Glazebrook and Ausubel, 1994; Künstler *et al.,* 2020).

Sulfur is also essential for the regulation of other important micronutrients in plants (Chorianopoulou & Bouranis, 2022). Potassium ions (K^+) are required for SO_4^{2-} unloading from the xylem to the cytosol by Group 3 anion-exchange SULTRs (Chorianopoulou & Bouranis, 2022) and where sulfur is deficient, the intracellular concentrations of K^+ also decrease (Reich *et al.,* 2016). Sulfur and iron (Fe) also have well-established interactions in the form of Iron-Sulfur clusters, that are essential cofactors in the electron transport chains of chloroplasts and in nitrogen fixing root nodules (Fonseca *et al.,* 2020; Wang *et al.,* 2022). Sulfur is also critical in chelating heavy metal ions, that are toxic in large quantities (Chorianopoulou & Bouranis, 2022; Zakari *et al.,* 2021). SO_4^{2-} binds to heavy metal ions, such as manganese and molybdenum, to form immobilised sulfate compounds that can be excreted from the cells (Zakari *et al.,* 2021).

All these functions demonstrate the agricultural and economic importance of sulfur, and why the study of the metabolism of sulfur in all its forms, including DMSP, by plants can yield potential benefits.

<u>Plants that have been shown to produce DMSP</u>

Traditionally it was believed that DMSP synthesis was exclusively a property of marine microorganisms. However, three species of land plants were found to be high producers of DMSP- producing between 6-70 µmol g^{-1} DMSP (Hanson *et al.,* 1994; Otte and Morris, 1994; Paquet *et al.,* 1994), comparable to levels found in marine sediment (Williams *et al.,* 2019). The three species selected for testing for DMSP production were chosen for their halotolerance only, as no genes for DMSP synthesis in plants have been identified.

Saccharum officianarum, or sugarcane, are economically important tropical grasses used primarily to produce sugar and biofuel in equatorial regions (Zhang and Li, 2015). Of the first three plants demonstrated to produce DMSP, sugarcane is the only one not

to grow in saline soils. Samples of leaf tissue from wildtype Sugarcane plants were shown to produce up to 6 µmol g^{-1} DMSP using gas chromatography-mass spectrometry (GC-MS), twenty times the concentration in other grasses studied (Paquet *et al.,* 1994). This relies on treatment with 6-10 M NaOH to cleave the DMSP into DMS and acrylate. The volatile DMS gas is then detected by gas chromatography.

These concentrations were confirmed in a later study using capillary electrophoresis (Zhang *et al.,* 2004). Capillary electrophoresis separates DMSP and other compounds based on their charge, size and solubility in a buffer solution (Zhang *et al.,* 2004). The researchers suggested that as the concentrations of DMSP were ten-fold higher than glycine betaine, DMSP is likely to have replaced glycine betaine as a major compatible osmolyte in this cropped plant (Paquet *et al.,* 1994). There have been no further studies on DMSP production and cycling associated to sugarcane.

Moving to *Spartina* (also known as Sporobolus), which is the most studied of the DMSP-producing plants. *Spartina* are a family of halotolerant grasses, known colloquially as Cordgrasses, found in coastal regions and saltmarshes across the East coast of the Americas and the coasts of Western Europe (Doody, 2012), including the saltmarshes of the Norfolk Coast. They are ecologically important as both an invasive species and a means of mitigating coastal erosion (Doody, 2012), and as such their mechanism of halotolerance came under early discussion (Larher *et al.,* 1977). Not all species of *Spartina* produce DMSP. Whilst concentration of 4-70 µmol g^{-1} fresh weight of DMSP have been reported in the leaf tissues of *S. alterniflora, S. anglica, S. foliosa* and *S. maritima* (Otte *et al.,* 2004: Otte and Morris, 1994), no DMSP production has been found in *S. cynosuroides, S. patens* or *S. versicolor* (Otte and Morris, 1994: Rousseau *et al.,* 2017). This may be the consequence of DMSP producing species possessing specific genes, or suites thereof, that non-producing species do not. Although the genetic pathway for DMSP production has not yet been elucidated, genomic comparison between *Spartina spp.* to identify genes only present in DMSP producing species may help identify candidate genes.

Melanthera biflora (also known as *Wollastonia biflora*) is a member of the aster family. Commonly known as Sea Daisies, they are a halotolerant species found predominately on Indo-Pacific salt strands (Storey *et al.,* 1993). Samples of *M. biflora* from Japan, Samoa and the UK were shown to produce between 10-15 µmol g^{-1} fresh weight in the leaf tissues when grown in a controlled environment using Fast-Atom Bombardment and Mass Spectroscopy (FABMS) (Hanson *et al.,* 1994). This detected DMSP by

exposing samples to high energy atoms, resulting in ions that are identified by their mass to charge ratio. The concentration of DMSP doubled when treated with 80% w/v artificial sea water (Hanson *et al.,* 1994). *M. biflora* is not to be confused with Sea Aster (*Aster tripolium*), a Eurasian aster found in salt estuaries. Wildtype samples from Norfolk, England have also been shown to produce lower levels of DMSP: 0.02-0.8 µmol g^{-1} fresh weight in leaf and stem tissues (Williams *et al.,* 2019).

Seagrasses have also been shown to produce DMSP. Neptune Grass (*Posidonia oceanica*) is a higher plant forming large underwater fields in the Mediterranean (Borges & Champenois, 2015; Richir *et al.,* 2020). Neptune Grass leaves were found to contain between 25-265 µg g^{-1} fresh weight by gas chromatography analysis (Richir, 2020), and showed an intracellular increase during the summer months (Borges & Champenois, 2015; Richir *et al.,* 2020). The researchers did not draw any specific conclusions on the role of DMSP in Neptune Grass but commented that the intracellular concentrations were higher than that of any other higher plant (Richir *et al.,* 2020).

More recent studies have shown that the range of higher plants producing DMSP is broader than originally thought. A study using Headspace Solid Micro-Extraction – a variant of GC that using silicon-tipped fibre needles to extract liquid from GC vials and converts it to gaseous analytes – found that leaf tissues of *Solanum lycopersicum* (Tomato) produced 1 µg m^2 DMSP when subjected to drought (Catola *et al.,* 2016). The same researchers also found that the mildly halotolerant subtropical grass *Arundo donax* (Elephant Grass) produces more DMSP when drought stressed (1.2-1.4 µg g^{-1} dry weight in comparison to 0.8-1.0 µg g^{-1} dry weight in control plants), although compared to other higher plants, this is at the lower end of the DSMP concentration spectrum (Haworth *et al.,* 2017). Another important crop plant shown to produce DMSP is *Zea mays* (Maize). Both root and shoot tissues of seven-day old seedlings produced between 0.5 – 1.0 nmol g^{-1} freshweight of DMSP in control conditions (Ausma *et al.,* 2017). DMSP concentrations increased to between 1.5-2.0 nmol g^{-1} freshweight when seedlings were treated with 100 mM NaCl and grown in anoxic conditions (Ausma *et al.,* 2017).

There are many other plants reported to have detectable DMSP concentrations. A review of wild type plants collected in the Netherlands by Ausma *et al.,* showed a wide range of monocots and dicots had trace DMSP concentrations in their leaf tissues using gas chromatography (Tab. 3.1).

Table 3.1: Review of all species assayed for DMSP production and the maximum concentration of DMSP detected. DMSP concentrations were detected by NaOH/GC with the following exceptions: [a]FABMS, [b]Capillary Electrophoresis and [c]HS-SPME.

Species	**Common Name**	**Maximum DMSP concentration (µmol g^{-1} freshweight)**	**DMSP Production Level**	**Publication**
Alopecurus pratensis	Meadow Foxtail	2.3 x10^{-3}	Medium	Ausma *et al.*, 2017
Anthriscus sylvestris	Cow Parsley	2.1 x10^{-3}	Medium	Ausma *et al.*, 2017
Artemisia maritima	Sea Wormwood	9.0 x10^{-4}	Low	Ausma *et al.*, 2017
Arundo donax	Elephant Grass	1.0	High	Haworth *et al.*, 2017
Aster trifolium	Sea Aster	0.8	High	Williams *et al.*, 2019
Brassica napus	Rapeseed	1.0 x10^{-3}	Medium	Ausma *et al.*, 2017
Bromus hordaceus	Soft Brome	2.8 x10^{-3}	Medium	Ausma *et al.*, 2017
Cannabis sativa	Hemp	1.0 x10^{-3}	Medium	Ausma *et al.*, 2017
Carex appropinquata	Fibrous Tusock-Sedge	1.8 x10^{-3}	Medium	Ausma *et al.*, 2017
Carex echinata	Star Sedge	7.0 x10^{-4}	Low	Ausma *et al.*, 2017
Carex nigra	Common Sedge	5.0 x10^{-4}	Low	Ausma *et al.*, 2017
Convallia majalis	Lily of the Valley	1.4 x10^{-3}	Medium	Ausma *et al.*, 2017
Dactylis glomerata	Cat Grass	9.7 x10^{-3}	Medium	Ausma *et al.*, 2017

Dryopteris dilatata	Broad-Buckler Fern	1.5×10^{-3}	Medium	Ausma *et al.*, 2017
Elytrigia atherica	Sea Couch	4.0×10^{-4}	Low	Ausma *et al.*, 2017
Equisetum arvense	Common Horsetail	1.7×10^{-3}	Medium	Ausma *et al.*, 2017
Festuca rubra	Red Fescue	1.2×10^{-3}	Medium	Ausma *et al.*, 2017
Holcus lanatus	Yorkshire Fog Grass	8.0×10^{-4}	Low	Ausma *et al.*, 2017
Iris pseudacorus	Yellow Iris	4.8×10^{-3}	Medium	Ausma *et al.*, 2017
Juncus effusus	Soft Rush	4.2×10^{-3}	Medium	Ausma *et al.*, 2017
Juncus gerardii	Saltmarsh Rush	7.0×10^{-4}	Low	Ausma *et al.*, 2017
Leucanthemum vulgare	Ox-Eye Daisy	5.0×10^{-4}	Low	Ausma *et al.*, 2017
Limonium vulgare	Common Sea Lavender	1.8×10^{-3}	Medium	Ausma *et al.*, 2017
Lolium perenne	Perennial Rye-Grass	1.7×10^{-3}	Medium	Ausma *et al.*, 2017
Melanthera biflora [a]	Sea Daisy	15	High	Hanson *et al.*, 1994
Narcissus pseudonarcissus	Wild Daffodil	1.0×10^{-3}	Medium	Ausma *et al.*, 2017
Poa annua	Annual Meadowgrass	2.4×10^{-3}	Medium	Ausma *et al.*, 2017
Posidonia oceanica	Sea Grass	130	Very High	Richir *et al.*, 2020
Puccinellia maritima	Common Saltmarsh Grass	1.4×10^{-3}	Medium	Ausma *et al.*, 2017
Quercus robur	English Oak	3.9×10^{-3}	Medium	Ausma *et al.*, 2017

Ranunculus repens	Creeping Buttercup	9.0×10^{-4}	Low	Ausma *et al.*, 2017
Sacharum officianarum [b]	Sugarcane	6.0	High	Paquet *et al.*, 1994
Solanum lycopersicum [c]	Tomato	6.1×10^{-6}	Very Low	Catola *et al.*, 2016
Spartina alterniflora	Smooth Cordgrass	48	Very High	Otte and Morris, 1994
Spartina anglica	Common Cordgrass	21.7	Very High	Otte and Morris, 1994
Spartina foliosa	California Cordgrass	8.2	High	Otte and Morris, 1994
Spartina maritima	Small Cordgrass	Presence confirmed but not quantified	-	Otte *et al.,*2004
Taraxacum officinale	Common Dandelion	6.0×10^{-4}	Low	Ausma *et al.*, 2017
Zea mays	Maize	1.0	High	Ausma *et al.*, 2017

Whilst an increasingly wide variety of species of higher plants have been identified as producing DMSP (Tab. 2.1), there is a lack of experimental evidence in model plant systems - such as *Arabidopsis thaliana* and *Nicotiana benthamiana*. Model organisms are beneficial for research due to their short lifespans, ease of transformation and small genomes (Cesarino *et al.,* 2020). Simple genomes and low ploidy levels, combined with fully sequenced genomes make gene identification more efficient compared to plants such as octoploid *Spartina spp,* with no sequenced genome. It is unknown whether these plants have the capability to produce DMSP at all, and if so whether DMSP is produced under normal growth conditions or is inducible by different environment cues. In addition, for all the plants species in which DMSP has been detected, the majority use NaOH cleavage of DMSP to DMS, which is then detected by gas chromatography. This method does not directly detect DMSP, which leaves open the possibility that DMS is produced from a different precursor molecule.

The broad range of plants tested by Ausma *et al.,* suggests that many more species than are currently recognised may produce DMSP. However, when taxonomically comparing high producers amongst themselves, two families are more commonly represented: *Poaceae* and *Asteraceae*. However, membership of these families is not in itself indicative of high DMSP production. For example, whilst Maize and Cordgrass are high *Poaceae* producers, Yorkshire Fograss and both Common and Star Sedges are low producers (Tab. 3.1). Furthermore, whilst Sea Daisy and Sea Aster are high *Asteraceae* producers, Ox-Eye Daisy is a low producer (Tab. 3.1). Additionally, Neptune Grass is a very high producer, but is a member of *Posidoniaceae*, rather than *Poaceae* as the common name implies. Therefore, there is no taxonomic link for DMSP production, and it is a poor predictor.

Without a strong taxonomic link, the next most obvious link between high DMSP production is environmental factors. As previously discussed, DMSP is suggested to be an osmolyte, which would indicate that plants in saline environments must be high producers. Whilst this is true for *Spartina spp.,* Neptune Grass and high producing Asters, this is not true for Sugarcane (Tab. 3.1). Sugarcane does not grow in coastal regions and is a glycophyte (any plant not considered a halophile), showing low tolerance for salt (Wahid *et al.,* 1997). Furthermore, known halophiles such as Sea Lavender and Common Saltmarsh Grass are medium producers, four orders of magnitude lower than *Spartina anglica* (Tab. 3.1). This suggests that environmental factors are also poor predictors of DMSP production.

This perhaps leads to the conclusion that high producers may have increased quantities of DMSP production genes. As such a wide range of plants have been shown to produce DMSP even to low levels, it is probable that there is a ubiquitous suite of genes that are upregulated in high producers. Gene regulation is a complex mechanism, occurring at multiple stages of gene expression: epigenetic, transcriptional, post-transcriptional, translational and/or post-translational (Hoopes, 2008). It may also be that in addition to such a suite of genes, high producers may have extra genes that are not found in medium or low producing plants. This is why the establishment of a DMSP-producing model organism with a sequenced genome and is amenable to genetic manipulation is essential to determine the genetic pathways of DMSP production.

Aims and Objectives

The aims of this chapter were to explore a range of commercially and scientifically important plants for their ability to produce DMSP, including model organisms. Having ascertained suitable model organisms that produce DMSP, this chapter further aimed to determine the optimal experimental procedures to assay DMSP production in plants; growth requirements for DMSP production, sample preparation techniques and how DMSP production and or transportation might be impacted by plant growth stage.

Methods

Sampling

Plants were sampled from the following locations in Norfolk, UK; Stiffkey Saltmarsh (52.957907, 0.923546), The University of East Anglia (UEA) lake (52.618567, 1.235058) and The Worstead Estate (52.763619, 1.453913) (Tab 3.2) between May and July 2020. The mean daily temperature for this period was 18 °C, with a mean daily rainfall of 9.6 mm. Where plants species could not be obtained from wild or agricultural environments, samples were purchased as commercial products or grown in a controlled environment for 4 weeks at 23°C, 16 h photoperiod. Whole plant samples were removed, before being transferred to the lab for immediate analysis. In all cases, the sampling sites were within a one-hour drive from the laboratory, ensuring samples were fresh when processed. Subterranean tissues (roots and tubers) were rinsed with deionised water to remove rhizosphere soil before further processing. Tissue was homogenised by grinding fresh, unfrozen aerial tissue with a pestle and mortar and transferring immediately to a to 1.5 ml glass crimp-top gas chromatography vials to an approximate depth of 200 µl. Vials were weighed using a Fisherbrand PS-60 balance to 4 decimal places before and after addition of homogenised plant tissue to determine fresh weight of tissue in the vial.

Table 3.2: The species assayed for DMSP production and the method by which they were obtained. Species were selected based on one or more of the following properties: is a commercially important crop plant [a], is a model organism of scientific interest [b], is a basal land plant [c] and therefore more likely to be reliant on osmolytes, is a species likely to be halotolerant [d] so may produce DMSP as an osmolyte and/or is known for a high sulfur content [e].

Species Name	Common Name	Method of Obtaining Sample
Allium ampeloprasum **[a,e]**	Leek	Commercial product
Allium cepa **[a,e]**	Red Onion	Commercial Product
Allium sativum **[a,e]**	Garlic	Commercial Product
Aloe barbadensis **var. Miller [a]**	Aloe vera	Grown at UEA
Anthriscus sylvestris **[d]**	Cow Parsley	UEA Lake
Apium graveolens **[a]**	Celery	Commercial Product
Asparagus officinalis **[a,e]**	Asparagus	Commercial Product
Avena sativa **[a]**	Oat	Commercial Product
Bellis perennis **[d]**	Common Daisy	UEA Lake
Beta vulgaris **[a,d]**	Beetroot	Commercial Product
Brassica napus **[a,e]**	Oilseed Rape	UEA Lake
Brassica oleracea **var. Botrytis [a,e]**	Cauliflower	Commercial Product
Brassica oleracea **var. Capitata [a,e]**	Cabbage	Commercial Product
Brassica oleracea **var. Italica [a,e]**	Broccoli	Commercial Product
Brassica oleracea **var. Italica "Purple Sprouting" [a,e]**	Purple Sprouting Broccoli	Commercial Product
Calystegia sepium **[d]**	Hedge Bindweed	UEA Lake
Coffea arabica **[a]**	Coffee	Grown at UEA
Dactylis glomerata **[d]**	Cocksfoot	UEA Lake
Daucus carota **[e]**	Carrot	Worstead Estate
Epilobium hirsutum **[d]**	Great Willowherb	UEA Lake
Eupatorium cannabinum **[d]**	Hemp Agrimony	UEA Lake
Foeniculum vulgare **[a]**	Fennel	Commercial Product
Heracleum sphondylium **[d]**	Hogweed	UEA Lake

Hordeum vulgare [a,d]	Barley	Worstead Estate
Jacobaea vulgaris [d]	Ragwort	UEA Lake
Lepidium sativum [a]	Cress	Commercial Product
Leucanthemum vulgare [d]	Ox-Eye Daisy	UEA Lake
Limoneum paradoxicum [d]	Sea Lavender	Stiffkey Saltmarsh
Luzula sylvatica [d]	Great Wood Rush	UEA Lake
Malus pumila [a]	Apple	Commercial Product
Malva sylvestris [d]	Common Mallow	UEA Lake
Marchantia polymorpha [c]	Marchantia	Grown at UEA
Matricaria discoidea [d]	Wild Chamomile	UEA Lake
Medicago truncatula [b]	Barrel Clover	Grown at UEA
Musa acuminata [a]	Banana	Commercial Product
Nicotiana benthamiana [b]	Benth	Grown at UEA
Phragmites australis [d]	Common Reed	UEA Lake
Pinus sp. [c]	Pine	UEA Lake
Pisum sativum [a]	Pea	Commercial Product
Poa annua [d]	Annual Meadowgrass	Worstead Estate
Polypodiophyta sp. [c]	Fern	UEA Lake
Quercus ruber [a]	English Oak	UEA Lake
Salicornia europaea [d]	Samphire	Stiffkey Saltmarsh
Secale cereale [a]	Rye	Worstead Estate
Solanum tuberosum [a]	Potato	Commercial Product
Sorghum bicolor [a,d]	Sorghum	Grown at UEA
Spartina anglica [d]	Common Cordgrass	Stiffkey Saltmarsh
Trifolium repens [d]	Common Clover	UEA Lake
Urticaria dioica [d]	Nettle	UEA Lake
Vitis vinifera [a]	Grape	Commercial Product
Zea mays [a,e]	Maize	Worstead Estate

Arabidopsis

Generation of *A. thaliana* seedlings

Arabidopsis thaliana wildtype Colombia (Col-0) seeds were treated using a solution of sodium hypochlorite, Triton and deionised water applied for 12 mins to sterilise. This was removed and the seeds rinsed with sterile water five times before plating onto solid ¼ MS Agar (Methods).

Seeds were stratified by wrapping the plate in aluminium foil and storing at 4 ˚C for a minimum of 48 hours. The foil was removed, and plates transferred to a Sanyo Versatile Environment Test Chamber at 23 ˚C, 16 h photoperiod with 800 W fluorescent lights for 7 days.

Growth of *A. thaliana* seedlings on DMSP-inducing media

Seedlings were removed from the plates using flame-sterilised forceps and 15 seedlings were placed 30 cm from the top of a 100 mm square plate containing 50 ml of ¼ MS Agar, approximately 5 mm apart. The root tip was marked on the plate with a black dot. This was repeated for all 6 treatment conditions. Plates were placed upright in a 23 ˚C growth cabinet for 7 days before root growth was measured from black dot to root tip. Root and shoot tissue were harvested separately using a sterile scalpel and pooled for all 15 plants per plate in previously weighed 1.5 ml Eppendorf tube. Tubes were weighed again to determine the mass of fresh weight (g) for the total tissue from each plate. Tissues were homogenised in the Eppendorf tubes using a plastic tissue homogeniser tool, before transferring to a 1.5 ml glass crimp-top gas chromatography vial to an approximate depth of 200 µl. Vials were weighed using a Fisherbrand PS-60 balance to 4 decimal places before and after addition of homogenised plant tissue to determine fresh weight of tissue in the vial.

Final concentration per plate of 100 mM NaCl and 0.25 mM methionine were determined from pilot studies that tested the effects of different concentrations on growth. The NaCl was added to test if saline conditions would induce DMSP production. Methionine was added as DMSP precursor molecule. A final concentration of 1.5 mM $MgSO_4$ was added to the media as indicated as this concentration was shown to be optimal for growth (Guo *et al.,* 2015).

Time Course Experiment

To determine the optimum developmental stage at which *A. thaliana* should be harvested for maximum DMSP concentration per g fresh weight, a time course experiment was set up. Seedlings were grown and plated on ¼ MS-Agar as before, with the following life stages selected for their ease of identification (Lièvre *et al.,* 2016).

Table 3.3: Life stages of *A. thaliana* and the identifying physiological features to determine when each stage is reached. Days at which each stage is reached after plating the stratified seedlings onto ¼ MS-Agar are averages determined across the time course experiment.

Life Stage	Identifying Feature	Average Time (days)
Early Growth	First fully formed true leaf	10
Vegetative Growth	First full formed rosette (5 adult leaves and 3 transitional leaves)	14
Budding	First unopened flower bud	20
Flowering	First fully opened flower bud	24
Fruiting	First intact silique of >0.5 mm	28

Each life stage had 15 seedlings per separate plate, to avoid contamination by taking plants of different life stages from the same plant. Each plate per life stage only contained seedlings of that had been sterilised and stratified together, to ensure a comparable starting growth stage. Plants were grown in a Sanyo Versatile Environment Test Chamber at 23 ˚C, 16 h photoperiod with 800 W fluorescent lights for up to 28 days.

Preparation techniques for Gas Chromatography of Plant and Soil Samples

To determine the most efficient method of sample preparation that does not compromise tissue concentrations of DMSP, fresh homogenised shoot and root tissues of *A. thaliana* were compared to flash frozen tissues. Fresh tissues were separated into shoots and roots using a sterile scalpel and prepared as before. Frozen tissues were first separated in shoots and roots in 1.5 mL Eppendorf tubes, then flash frozen in liquid nitrogen. The tissue was ground to powder using a sterile plastic tissue homogeniser in the Eppendorf

tubes. The harvested plant tissue powder was mixed with 600 µL of sterile water and 200 µL aliquots transferred to 1.5 mL glass crimp-top gas chromatography vials.

To establish whether the plants also produce DMSOP, assays were prepared on *A. thaliana* shoot and root tissue (Pascual *et al.,* 2020). Homogenised fresh tissues were transferred to 1.5 mL glass crimp-top gas chromatography vials (without lids) and 100 µL of dH_20 added. Vials were heated to 80 ˚C for 10 mins to remove residual DMS. Vials were allowed to cool to room temperature before 200 µL of 0.33 mM tin (II) chloride ($SnCl_2$) was added. The vials were immediately crimped to form a gas tight seal and heated to 55 °C for 90 mins to reduce DMSO to DMS. The sample was then assayed by gas chromatography automatic injection method 530_HP-PLOT_SPLITDMS2_MESH_0.1M.

To establish whether DMS detected is the breakdown product of plant intracellular DMSP, or DMSP from residual soil, bulk and rhizosphere soil samples were collected from *A. thaliana* tissues and pooled. Dry soil was weighed into 1.5 ml glass crimp-top gas chromatography vials using a sterile spatula and a Fisherbrand PS-60 balance to 0.3 g. Fresh homogenised shoot and root *A. thaliana* tissues were harvested as before, and 0.3 g weighed into each vial. To each vial, 300 µL of 10 M NaOH were added and immediately sealed by crimping. Vials were left in the dark for overnight to allow DMS gas to generate and the sample assayed by gas chromatography as before.

Statistical Analysis

Concentrations of DMS production for all experiments were tested for normality using Shapiro-Wilke tests. Data sets not normally distributed were transformed using Log_{10} and reanalysed for equal variances (Levene’s Test) and equal distributions (Kolmogorov-Smirnov Test). Normally distributed data sets underwent Analysis of Variance (ANOVA) for DMSP production, with post-hoc Tukey analysis for pair-wise comparisons between the same tissues with different treatment conditions. Pairwise comparisons between roots and shoot for the same treatment condition were analysed using Student’s T-Test. Data sets that were not normally distributed and could not be transformed were analysed using Kruskal-Wallis with post-hoc Dunn-Bonferroni corrections for pairwise comparisons.

Statistics were carried out in R (R Core Team, 2014), SPSS Version 28.0 (IBM, 2021) and Microsoft Excel (Microsoft Corporation, 2018).

Results

To understand how plants produce DMSP, it was necessary to further study the wide range of plants that might produce DMSP and compare their concentrations thereof. This study determined only the concentrations of DMS found in higher plants derived from DMSP, in a range of 10 – 5250 nmol DMSP g^{-1} fresh weight, and not DMSOP.

This was to try and uncover if there are any overlooked taxonomic or environmental links to DMSP production. Plants sampled were divided into three categories: crops and plants of scientific interest, basal land plants and wild plants. For the purposes of this chapter, basal land plants are defined as primitive plant species that have a requirement to grown near water and gymnosperms. Plants of scientific interest are synonymous with model organisms in plant research.

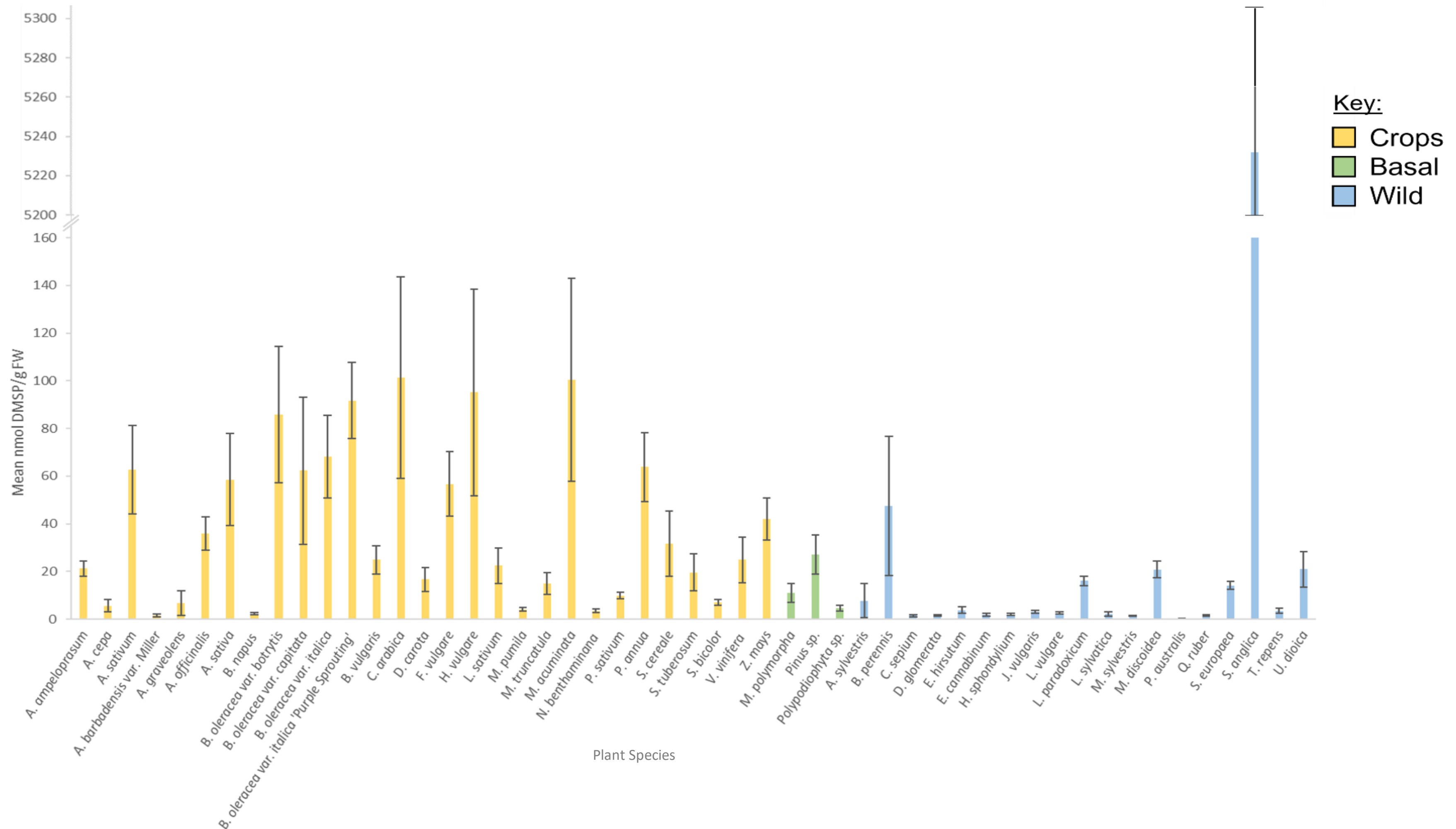


Figure 3.3: Mean concentration of DMSP from combined tissues of a wide variety of plants (n = 395), categorised as crops and scientifically important plants (yellow), basal land plants (green) and wild plants of suspected or known halotolerance (blue). Error bars are ± 1 SEM.

The crop and scientifically important plants produced significantly higher concentrations of DMSP than the wild, halotolerant plants (Dunn Test, $p < 0.001$) but there were no significant differences between crop plants and basal land plants, or basal land plants and wild plants (Dunn Tests, $p = 0.559$ and $p = 0.465$, respectively). All the plants sampled across all categories were significantly lower than the three known high producing species; *S. anglica*, Sugarcane and Sea Daisy.

There was a lot of variability across replicates of the same plants and between species. To account for the large variation, plants were pooled into two categories; halophytes and non-halophytes (Tab.3.2) and compared using a Student's T-Test. There were no significant differences between DMSP concentrations (T-Test, $p = 0.580$). This confirms that there are no clear taxonomic or environmental differences between high DMSP-producing plants and other species.

Understanding DMSP in the model organism *Arabidopsis thaliana*

Out of the plants surveyed, model organisms including *Arabidopsis thaliana* were shown to produce DMSP, albeit to significantly lower levels than *Spartina anglica.* Having established that *A. thaliana* samples liberated DMS when incubated with NaOH, it was necessary to determine if the concentrations of DMSP were derived from the plant or if they were from the soil, as there is the possibility that rhizosphere bacteria might degrade DMSP to DMS, or that there are sulfur containing compounds making up the elemental profile of the soil.

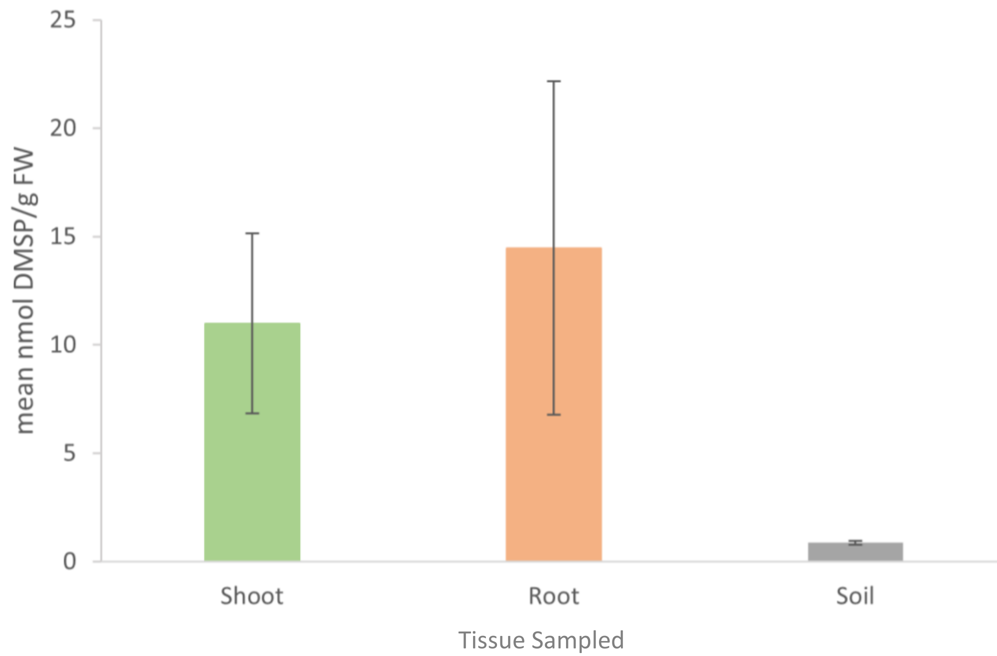


Figure 3.4: The mean concentrations of DMSP in fresh *A. thaliana* root and shoot tissues, alongside the soil they were grown in (n = 54). Error bars are ± 1 SEM.

Fresh shoot tissues produced significantly higher concentrations of DMSP than the soil (Student's T-Test, p = 0.003), but there was no significant difference between fresh root tissues and the soil (Student's T-test, p = 0.111). This indicates that the DMS detected is from the plant tissues and not the soil they were grown in. Although there were no significant differences between root and shoot tissues, DMSP concentrations were higher in the roots. This is the first of repeated results that demonstrate DMSP concentrations are higher in the root tissues than shoot tissues in *Arabidopsis thaliana*.

Knowing that the DMSP was from the plants themselves, it was then necessary to determine if the differences in DMSP concentration were dependent on the method of tissue homogenisation and GC preparation.

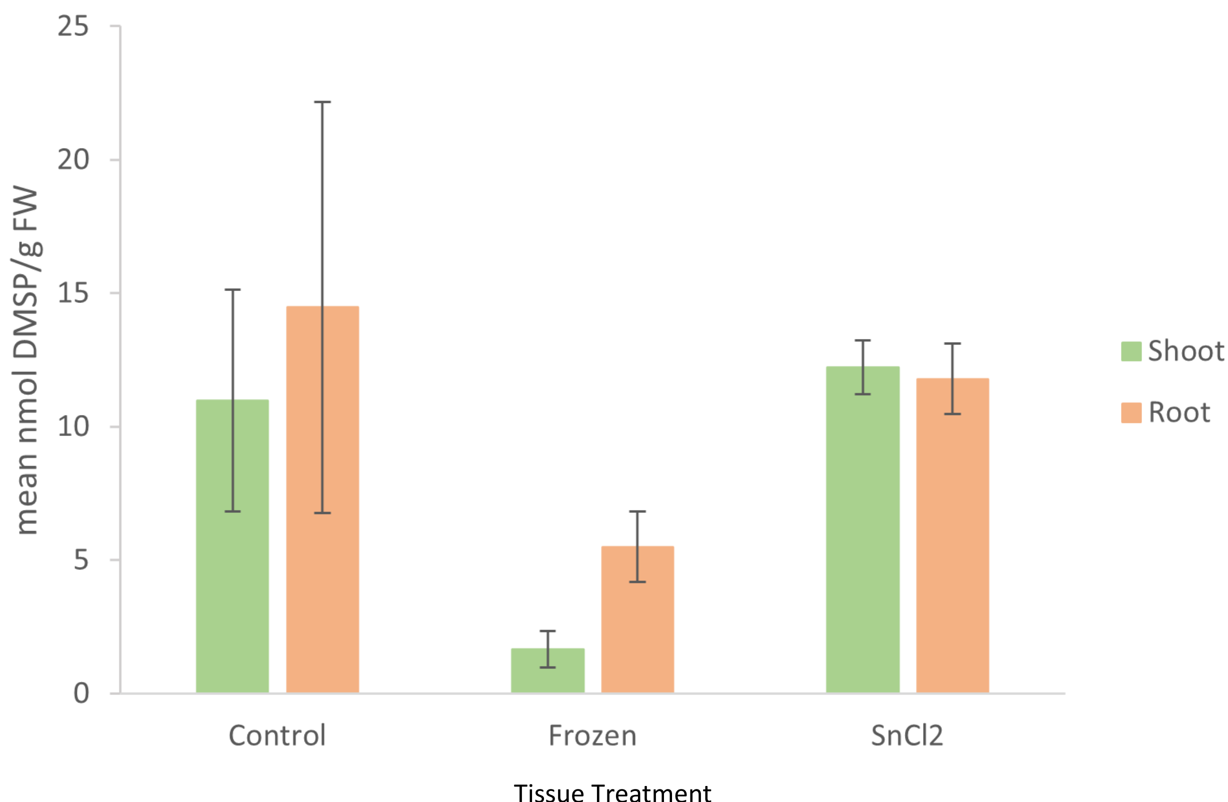


Figure 3.5: The mean concentrations of DMSP in *A. thaliana* shoot and root tissues for freshly homogenised tissues, flash frozen and fresh tissues treated with $SnCl_2$ (n = 54).

There were no significant differences between shoot and root tissues for the different treatment conditions (Student's T-Tests; Control p = 0.381, Frozen p = 0.510, $SnCl_2$ p = 0.420), but again there was a higher concentration of DMSP detected in fresh and frozen tissues, consistent with the previous result.

Fresh shoot tissues and those treated with $SnCl_2$ produced significantly higher concentrations of DMSP than frozen shoot tissues (Tukey Test, p = 0.025 and p = 0.012 respectively). There was no significant difference between fresh shoot tissues and those treated with $SnCl_2$ (Tukey Test, p = 0.788). This suggests that the DMS detected by GC may not be the result of alkaline lysis of DMSP, but rather DMSOP. There were no significant differences between root tissues for any treatment condition (ANOVA, p = 0.543). This demonstrates that the freezing process reduces the DMSP detectable, and the fresh tissues are optimal for analysis.

Consequently, *A. thaliana* aerial tissues were analysed by Liquid Chromatograpy-Mass Spectrometry, to verify that the DMS was derived from DMSP, with preliminary results recorded.

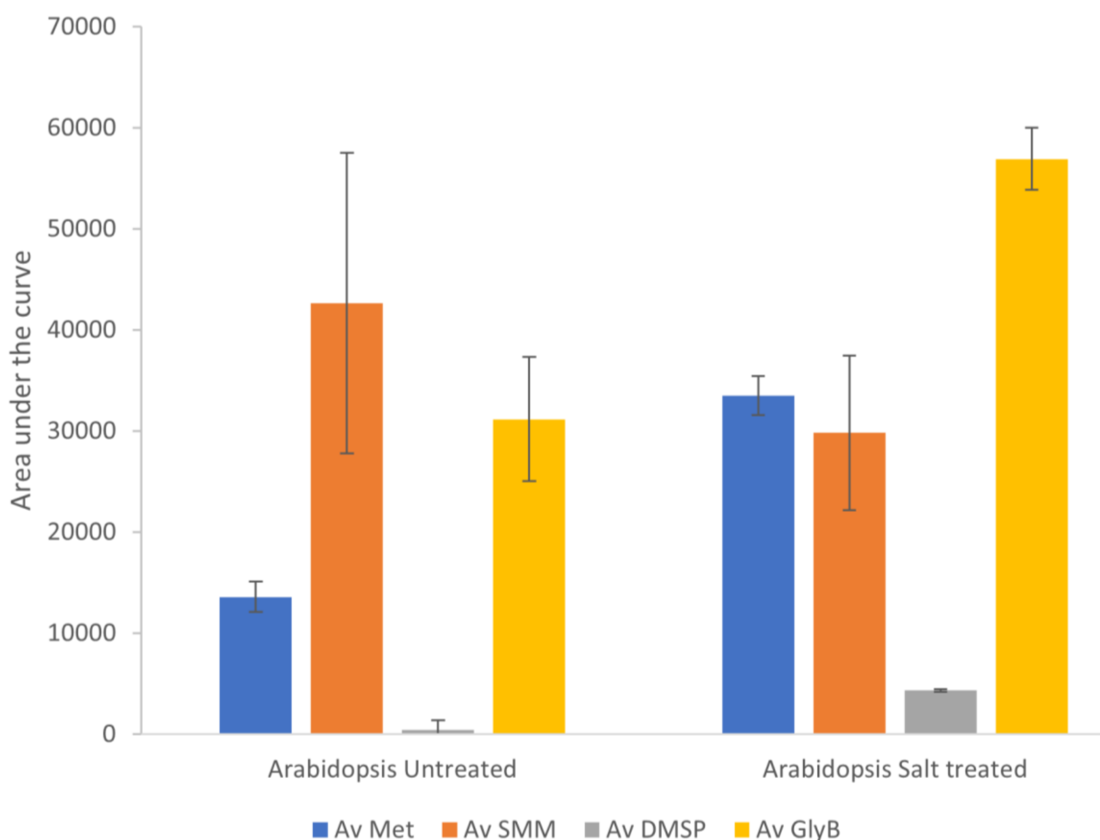


Figure 3.6: Mean integration of the relative concentrations of Methionine (blue), SMM (orange), DMSP (grey) and glycine betaine (yellow) present in *A. thaliana* aerial tissues detected by LC-MS. Error bars ± 1 STE. (Personal communication).

Although significantly lower than glycine betaine, DMSP was detectable in *A. thaliana*, indicating that the DMS detected by GC is the consequence of alkaline lysis of DMSP. Additionally, the presence of the precursor molecule Methionine and the intermediate SMM suggests that DMSP is biosynthesised through the methylation pathway. These preliminary results were from personal communications, and the mass of samples used were not supplied to determine the concentration of DMSP. It should be noted that although this strongly indicates that the detected DMS is derived from DMSP, the use of a purge-trap GC system would be required to determined if any DMS present was derived from DMSP.

This was considered sufficient evidence to establish that fresh tissues are optimal for DMS production and that the DMS produced comes from DMSP. Thus, we examined

how DMSP might be differentially accumulated in the roots and shoots over different periods of its growth cycle.

To determine the effects of growth on DMSP concentration, pairwise comparisons of between the life stages across the tissue types were performed.

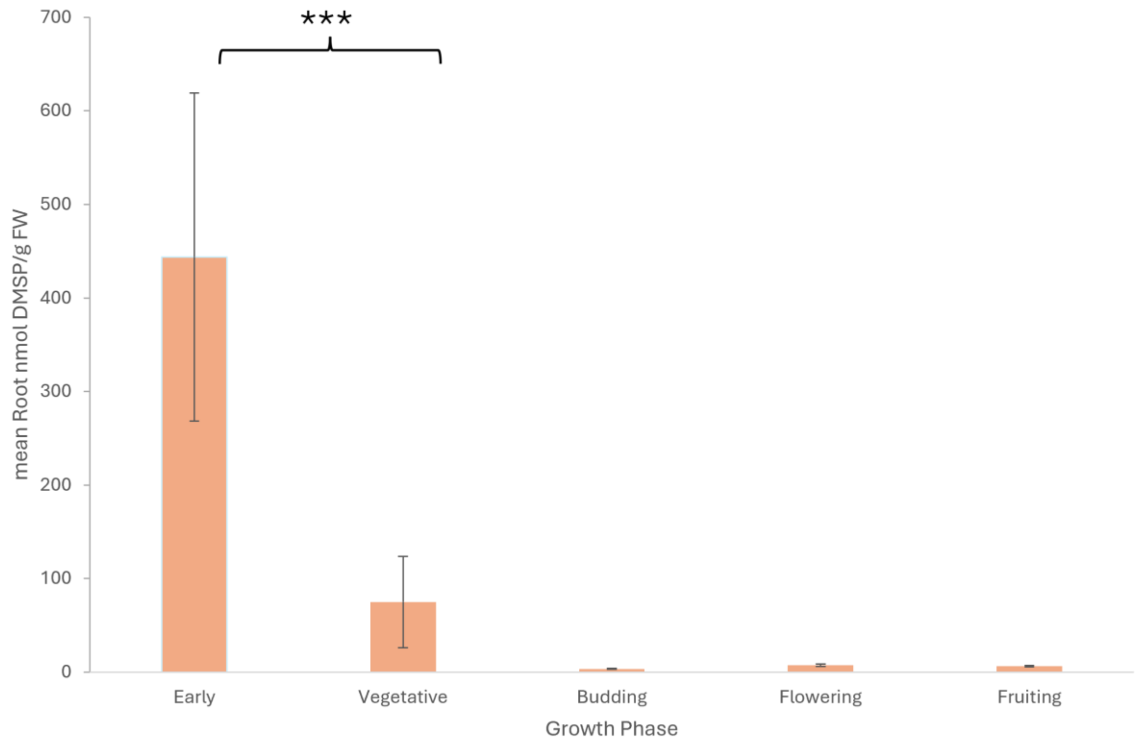


Figure 3.7: Concentration of DMSP produced by *A. thaliana* root tissue (nmol per gram fresh weight) for five life stages, grown on MS-Agar without additional NaCl or DMSP precursors (n = 115). Error bars are ± 1 SEM.

Plants in the early and vegetative stages of growth accumulated significantly higher levels of DMSP per g fresh weight than those in the later growth stages. Roots in the early stage produced significantly higher DMSP concentrations than those in the vegetative stages (Tukey Test, $p < 0.01$) and budding, flowering, and fruiting stages (Tukey Test, $p < 0.01$).

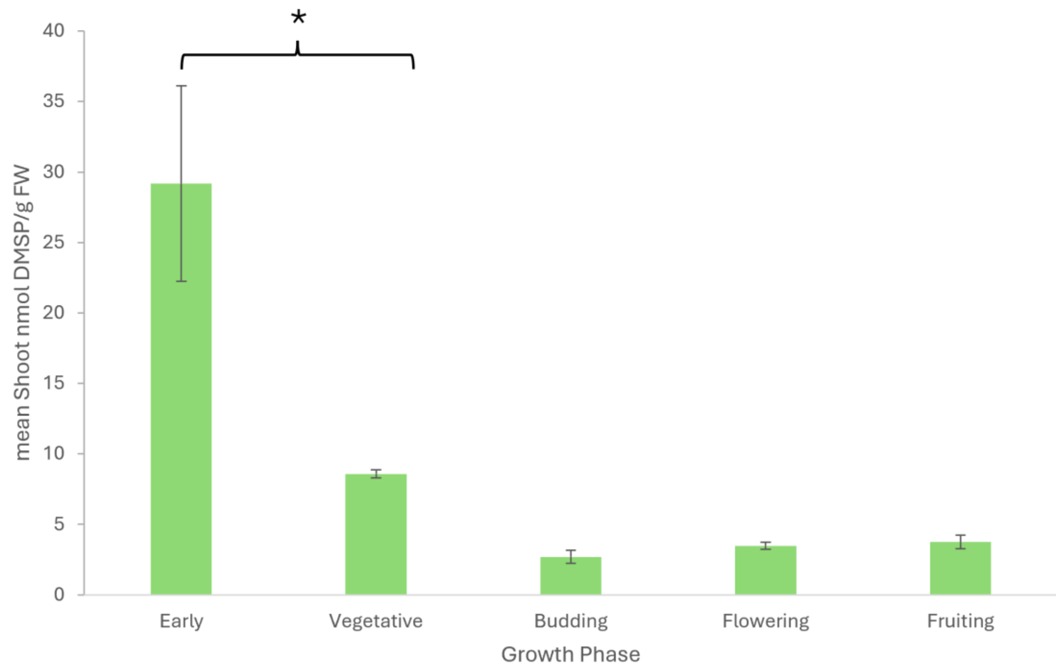


Figure 3.8: Concentration of DMSP produced by *A. thaliana* shoot tissue (nmol per gram fresh weight) for five life stages, grown on MS-Agar without additional NaCl or DMSP precursors (n = 115). Error bars are ± 1 SEM.

Shoots in the early stage produced significantly higher DMSP concentrations than those in the vegetative stages (Tukey Test, $p = 0.035$) and budding, flowering, and fruiting stages (Tukey Test, $p < 0.01$). There were no significant differences between budding, flowering, or fruiting stages, for either roots or shoots. Therefore, *Arabidopsis thaliana* plants should be harvested between 10-14 days post-germination to maximise detectable DMSP concentrations.

In the early, vegetative and flowering stages of growth, root tissues produced significantly higher concentrations of DMSP compared to shoot tissues at the same stage of growth (Student's T Test, $p = 0.04$, $p = 0.031$ and $p = 0.022$, respectively). There were no significant differences between root and shoot DMSP concentrations in budding and fruiting stages. This is consistent with the previous results that also found DMSP concentrations were higher in the roots.

DMSP Production in response to Salinity and DMSP Precursors

Having established that young plants produced significantly higher concentrations of DMSP, we moved on to examine whether environmental conditions such as salinity and increased DMSP precursor (Methionine) concentration can influence the accumulation of DMSP in plants.

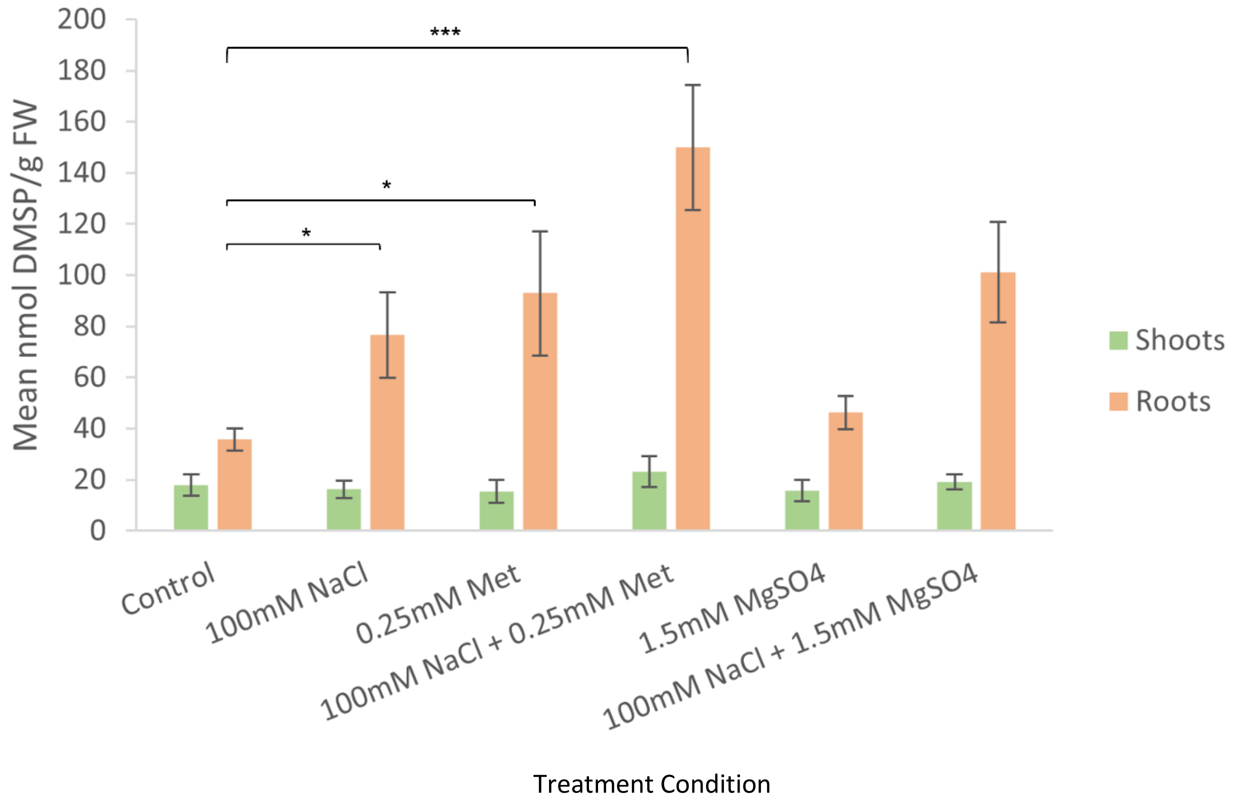


Figure 3.9: Mean concentration of DMSP produced by *A. thaliana* shoot (green) and root (orange) tissue (nmol per gram fresh weight) for each DMSP precursors and the addition of NaCl (n = 120). Error bars are ± 1 SEM.

In all conditions, *A.* thaliana plants produced significantly more DMSP in their roots compared to their shoots (Student's T-Test, p<0.001 for all conditions). This suggests that DMSP is produced in the roots and translocated to the shoots, or that DMSP synthesis is upregulated in the roots. This is also consistent with previous results that show root production exceeds shoot production.

There were no significant differences in shoot DMSP accumulation across any of the treatment conditions compared to each other or the control (ANOVA, p = 0.83).

A. thaliana plants treated with NaCl produced significantly more DMSP than control plants (unpaired t-test, p = 0.04), suggesting that DMSP is upregulated by salinity. *A.*

thaliana plants treated with Methionine also produced significantly more DMSP compared to the control (unpaired t-test, $p = 0.04$) indicating that DMSP is synthesized from Methionine, in common with marine microorganisms. Significantly more DMSP was produced in root tissue in plants treated with salt and methionine compared to salt only (unpaired t-test, $p < 0.01$), but there was no significant difference in DMSP production between salt only and methionine only (unpaired t-test, $p = 0.59$), showing that the regulatory effects are cumulative.

The addition of sulfate had no effect on DMSP production, compared to the addition of Methionine. There was no significant difference in DMSP production between plants treated with magnesium sulfate only and the control (unpaired t-test, $p = 0.21$). Looking at each pair of treatment conditions, there were no significant differences between salt only and magnesium sulfate only (unpaired t-test, $p = 0.13$) or methionine only and magnesium sulfate only (unpaired t-test, $p = 0.10$). This suggests that plants lack the ability to utilise sulfur outside of being contained in Methionine for DMSP production. It is, however, consistent with previous studies that determined Methionine was the first step of the DMSP production pathway in *S. anglica*, Sugarcane and Sea Daisy.

Discussion

Proposed Explanations for Selected High DMSP Producers

The first finding of this study is that a wide variety of previously untested plant species can produce DMSP, proving that DMSP production in higher plants is a more common phenomenon than had previously been thought and is not limited to a few coastal species (Fig 3.3).

However, there was extremely high variability within the replicates of the species sampled, making direct comparisons between mean DMS detected less robust. This is likely due to three factors; small sample sizes, inability to control for environmental factors and variability in the time between harvesting and assaying. Although a minimum of three biological replicates with two technical replicates were taken for each species and tissues, plants were not controlled for environmental factors such as age, hydration or fertilisation. To grow all the species sampled within a controlled environment was beyond the scope of this study, but for further investigation, taking a greater number of replicates would minimise in species variability. Furthermore, although care was taken to minimise differences in time between harvesting and assaying, it was not always feasible to use the machine exactly 24 hours after alkaline

hydrolysis of samples. Samples therefore had to be flash frozen and stored, which significantly reduces the DMS detected in aerial tissues (Fig 3.4). It is therefore recommended that for further study, only fresh tissues are used.

This high variability between species may be why on average there was no significant difference in mean DMSP concentrations between halophytic wild plants and glycophytic crop plants (Tab. 3.2[d]). This is a surprising finding, as the prevailing wisdom is that DMSP functions primarily as an osmolyte in higher plants. However, the generally high variability combined with some hitherto unknown high producing species in the glycophyte group are may have caused the lack of differences, which may not be reflective of overall trends in plant populations. Perhaps a more likely explanation is that these plants use other compatible solutes, such as glycine betaine, as their primary osmolyte (Ghosh *et al.,* 2021; Singh *et al.,* 2022). Where DMSP concentration increase with salinity (Fig. 3.8), it may be that DMSP has another role in coping with general stress, such as a signalling or antioxidant role.

High variability notwithstanding, all the plants analysed had orders of magnitude lower levels of DMSP than *Spartina anglica* (52281.64 nmol DMSP g^{-1} FW). As previously commented, this might be the consequence of increased upregulation of ubiquitous DMSP production genes, possibly the consequence of functional redundancy, or have extra genes that are not found in medium or low producing plants. Following the train of thought that low DMSP producing plants do not use DMSP as a primary osmolyte, possibly *Spartina anglica* does.

Although the findings are reassuring that any DMSP detected comes from the plants themselves, and not the surrounding soil (Fig. 3.7), it was unlikely that the DMSP would be made in the soil. The significantly higher DMSP in the plant tissues compared to the soil are indicative of intracellular synthesis. If DMSP was transported from the soil, the difference in concentrations would be much lower, as molecular transport across a membrane will always tend towards equilibrium. This hypothesis could be tested by studying DMSP synthesis rates in plants, to track the progression of DMSP concentrations.

The lack of significant difference in detected DMS in plants treated with $SnCl_2$ compared to those that were only subjected to alkaline hydrolysis is less promising (Fig. 3.4). This means that the DMS detected may not be from DMSP, but rather DMSO. This seems very unlikely, given that DMSP and its precursors in the methylation

pathway were detectable by LCMS in *A. thaliana* (Fig 3.6). This demonstrates that even if the plants are capable of synthesising DMSO, they certainly biosynthesise DMSP and thus alkaline lysis and headspace analysis is an appropriate technique. Further investigation using techniques that can discriminate between precursors, such as Nuclear Magnetic Resonance (NMR) or HPLC would be beneficial to confirm this finding.

It is probably that plants that can produce DMSP may also produce DMSO. A study analysing the ratio of DMSP to DMSO in Neptune Grass showed that DMSP:DMSO was a 3:1 ratio in the leaf tissues (Champenois & Borges, 2019). Additionally, both DMSP and DMSO were found in all tissues of *Spartina anglica*, although the authors attributed this to DMSP degradation to DMS and its subsequent oxidation (Husband & Kiene, 2007). The presence of DMSO and the ratios of DMSP:DMSO are likely to vary between species, and as such the proportion of DMS detected that comes from DMSP or DMSO is also likely to vary between species. This highlights the necessity to understand the genetic pathways that lead to DMSP synthesis in higher plants, to determine which precursor produces DMS detected, and if both, in what proportions.

Unexpected Medium Producers of DMSP

Of the species sampled, the following were still lower in their DMSP production to *Spartina anglica* (102.79 nmol DMSP g^{-1} FW) but were significantly higher than average of our samples: *Brassica oleracea* var. italica 'Purple Sprouting' (91.67 nmol DMSP g^{-1} FW), *Coffea arabica* (101.33 nmol DMSP g^{-1} FW), *Hordeum vulgare* (95.12 nmol DMSP g-1 FW) and *Musa acuminata* (100.31 nmol DMSP g^{-1} FW). These would be considered medium producers (Tab. 3.1) but are hitherto untested species and therefore useful in furthering the understanding between DMSP production and taxonomy or the environment.

Brassica oleracea var. italica 'Purple Sprouting', commonly known as Sprouting Broccoli, is an annual brassica, selected for its anthocyanin-based pigment (Monero *et al.,* 2010). Broccoli has been extensively studied for its high content of non-enzymatic antioxidants, particularly polyphenols in the flavonoid family (Faller & Fiahlo, 2009; Lin & Chang, 2005). Brassicas are known for their dependence on sulfur metabolism (Friedrich *et al.,* 2022) and the production of VOCs, including DMS (Akpolat and Barringer, 2015; Danner *et al.,* 2015). The high quantities of DMS may be due to the overall high production of antioxidants to scavenge ROS in Sprouting Broccoli. DMSP

has been shown to localise to chloroplasts in higher plants, which are organelles under high oxidative stress, supporting the theory that DMSP may function as an antioxidant in higher plants (Ott *et al.,* 2007; Trossat *et al.,* 1998).

Another explanation for the high concentrations of DMS measured as a proxy for DMSP in Sprouting Broccoli is that it is a breakdown product of the high concentrations of sulfur-containing compounds, for which Brassicas are well known. Brassicas produce the compound S-methyl cysteine sulfoxide (SMCSO) (Coode-Bate *et al.,* 2019; Frank *et al.,* 2018). SMCSO is broken down intracellularly by cysteine lyase to methanesulfenic acid (Frank *et al.,* 2018). Methanesulfenic acid is further broken down to methanethiol, which dimerises to form DMS (Coode-Bate *et al.,* 2019; Frank *et al.,* 2018). A limitation of this study is that DMS released from SMCSO would not be driven off by tissue treatment with NaOH, designed to cleave DMSP to DMS. Therefore, the high DMS in Sprouting Broccoli, and indeed the other brassica species sampled, may not be the result of high intracellular DMSP concentrations.

Coffea arabica, commonly known as Coffee, is a flowering plants native to the basalt-rich alkaline soils of the East-African highlands (Arndt & Menzies, 2005; Moat *et al.,* 2020). Coffee might produce DMSP in response to the alkaline environment of its native soils, as alkaline soils have excessive Na^+ accumulation around plant roots, known as pooling (Hayward and Wadleigh, 1949). Production of DMSP as an osmolyte to cope with Na^+ pooling would provide an evolutionary advantage for Coffee plants, and indeed they are one of the few plants that thrive on alkaline-basalt soils (Singtuen *et al.,* 2021). Furthermore, Coffee beans have been shown to produce DMS in the green (unprocessed) stage (Leitner & Ringer, 2020). In a study of the sulfur-containing aromatic compounds that flavour coffee and the effects of the roasting process, flash frozen coffee beans were subjected to GC-MS and sulfur chemiluminescence detection. The only detectable VOC in the raw, green beans was DMS (Leitner & Ringer, 2020). However, another study using X-ray absorption near edge structure (XANES) spectroscopy determined that the predominant sulfur compound in coffee beans was DMSO (Lichtenburg *et al.,* 2007), suggesting that the measured DMS may not have been the result of DMSP production. This confirms the growing evidence from this study that GC, using DMS as a proxy, is not sufficiently robust to draw conclusions about the intracellular DMSP content.

Hordeum vulgare, commonly known as Barley, is a temperate grass and the fourth most cultivated cereal, globally (FAO, 2018). Barley had the highest measured DMS of all

the crop plants sampled. Again, whether this is the consequence of DMSP production within the plants is debatable. A study of SMM and MMT in Barley suggested that Barley does not accumulate DMSP (Pimento *et al.,* 1998), however there is no experimental evidence to corroborate this statement. Furthermore, the study did determine the presence of the enzyme MMT, which is the first step of DMSP production. SMM is produced in the embryo of Barley during germination, which is synthesised to DMSP during the development of malt (Annes & Bamforth, 1989), indicating that in the embryo at least, Barley has the capability to produce DMSP.

The final high producer was *Musa acuminata,* commonly known as Banana. Banana plants are an important crop and the largest herbaceous flowering plant, native to South-East Asia (Williams, 2017). Although the benefits of DMSP production in bananas are not immediately apparent, there are some possible explanations why this might prove to be an evolutionary advantage. Firstly, tropical plants are more at risk of osmotic stress, as they are closer to their upper thermal tolerance limits, which leads to increased evapotranspiration and thus cellular plasmolysis (Cramer *et al.,* 2011; Sentinella *et al.,* 2020). Having additional osmolyte production in the form of DMSP may be beneficial in ameliorating these effects. Additionally, banana is known to be a ruderal (also known as a pioneer) species; hardy and fast-growing species that are the first to colonise disrupted areas, where all other biomass has been destroyed (Agbeshie *et al.,* 2010). Banana can rapidly colonise barren soils following severe abiotic stress, such as wildfires and flood damage (Marod *et al.,* 2010). Wildfires increase the pH of soils, leading to alkaline sodium pooling (Agbeshie *et al.,* 2010) and flooding will dramatically increase the water content of soils, both of which make having high osmolyte production an advantage. Indeed, several ruderal species (Fig. 3.1, blue columns) were found to produce DMSP, albeit at lower levels.

However, there is always the possibility that the DMS measured might come from other sources. A study of banana plant holobionts showed that in all compartments (bulk soil, rhizosphere, pseudostem and leaf) the dominant bacterial classes were Alpha- and Gamma-proteobacteria (14-85% and 6-43% respectively) (Birt *et al.,* 2022). Although the study did not detail the species within these classes, representatives of *Alpha-* and *Gamma-proteobacteria* are well characterised as DMSP producers and degraders (Carrión *et al.,* 2023; Liu *et al.,* 2021). Therefore, the possibility that the DMS detected was a consequence of bacterial sulfur metabolism cannot be discounted.

The range of families in these medium producers is consistent with our previous conclusion that there is no strong taxonomic link for DMSP production and is therefore not a good means of predicting DMSP production. Additionally, none of these plants are known halophytes and only Coffee and Banana have natural tolerance to saline-adjacent environments, suggesting that a single environmental factor, such as salinity, is also an insufficient predictor of DMSP production. As climate change increases soil salinity due to salt-water intrusion, it may be that future studies will find increased DMSP production in a variety of plants as a coping mechanism and the link to saline environments will be a stronger predictor, but currently these results show that we are no closer to being able to predict high DMSP production. This again demonstrates the need for robust genetic analysis of model organisms, to determine if specific genes are present in high producers, or if a ubiquitous suite of genes are upregulated.

Developing a model for DMSP assaying by GC in higher plants

Another critical finding of this study is that wildtype *Arabidopsis thaliana* produces DMSP (Figs. 3.4- 3.8), although the concentrations are considerably lower than in the other species reported in Tab. 3.1 (nano-molar compared to micro-molar concentrations). *A. thaliana*, or Mouse-Ear Cress, is a wide-spread Brassica, prominent as a ruderal species and model organism (Durvasula *et al.,* 2017). As previously stated, Brassica species are known for DMS production (Akpolat & Barringer, 2015; Danner *et al.,* 2015) and if further experimentation with different conditions demonstrates that *A. thaliana* can be triggered to produce higher concentrations of DMSP, it could prove to be a useful model organism to study how plants synthesise and use DMSP. Compared to the most commonly studies plants in relations to DMSP – *Spartina spp*. – *A. thaliana* has considerable benefits: it has a fully annotated genome, it is diploid as opposed to hexaploid and even dodecaploid *Spartina* (Rousseau *et al.,* 2017) and comparatively easy to grow and maintain.

The ideal stage to harvest *A. thaliana* for the highest levels of DMSP was found to be at the earliest stage of growth before the formation of a full rosette (Fig. 3.7). This is in keeping with other studies that suggest that DSMP concentrations are inversely correlated with plant age. This was investigated over a 15-month period in *Posidonia oceanica* and the researchers found significantly higher DMSP concentrations in shorter (younger) leaves (Richir *et al.,* 2020). The authors suggest that this may be to deter grazers during the growth stages. During the early photosynthetic stages, plants upregulate amino acid synthesis to meet the protein demands of growing cells

(Hildebrandt *et al.,* 2015). An increase of methionine in these stages may therefore contribute to increased DMSP synthesis. Increased production of DMS may also be a consequence of plant growth hormones upregulating DMSP production. In a study of *Spartina alterniflora*, DMSP production was found to increase in response to additional Salicylic Acid (Kiehn & Morris, 2010). Salicylic Acid is a hormone commonly associated with plant immunity, but also stimulates vegetative growth and flowering (Demspey & Klessig, 2017). However, other plant hormones are not shown to have any effect on intracellular DMSP (Kiehn & Morris, 2010).

In determining the best tissue to sample for DMSP production, root tissues consistently had higher detectable DMS than shoots (Figs. 3.4 – 3.8). The overall higher concentrations of DMS in the root tissue compared to shoot tissues (Figs 3.4 – 3.8) is contrary to other research that demonstrates DMSP production in other species is increased in leaf tissues only and not in the roots. It has been proposed that DMSP production in leaf tissue is higher because DMSP localises to the chloroplasts (Trossat *et al.,* 1998). However, roots contain leucoplasts, that may be able to synthesise or localise DMSP, as they have the same structure as chloroplasts except for a lack of pigment (Barton *et al.,* 2018). Although research in *M. biflora* has also demonstrated that isolated root tissues can still produce 15-35 µmol g^{-1} freshweight concentrations of DMSP (Otte *et al.,* 2004), it doesn't explain why the concentration in the *A. thaliana* tissues is significantly higher than in shoot tissues and of those reported in isolated root tissues. It may be that in *A. thaliana* the roots are induced to increase DMSP production as they are the most sensitive tissues to ionic stress and sulfur deficiencies (Narayan *et al.,* 2022).

Another possible explanation for this may be the result of DMSP translocation from the photosynthetic tissue to the roots. This has been reported in *Spartina alterniflora* in response to 2 mM in the environment (Mulholland and Otte, 2000), and as the base nitrogen content of MS Agar is 60 mM (Zhang *et al.,* 2019) this could have contributed to the higher concentrations in the root tissue. Given that plants assimilate SO_4^{2-}, the principle DMSP precursor, via their roots (Chorianopoulou & Bouranis, 2022; Li *et al.,* 2020; Ren *et al.,* 2022), it is plausible that DMSP production is localised to the roots.

Understanding the effect of DMSP Precursors on Detectable DMS

Although sulfate is the principle DMSP precursor, our findings suggest that increased SO_4^{2-} does not increase intracellular DMSP, whereas increased methionine does (Fig. 3.8). This may be because plants were harvested at the early growth stage of approximately 7 days post germination. The enzyme that catalyses the first step of the pathway, adenylation of SO_4^{2-} to APS, is ATP sulfurylase (ATPS) (Davidian & Kopriva, 2010). However, it has been shown that ATPS activity does not start until three weeks post germination in *Glycine max* (Adams & Rinne, 1969). Thus, *A. thaliana* plants might have been harvested too young for the assimilated sulfur to be activated. The addition of methionine bypasses this step, which might allow for plants to synthesise DMSP faster.

This does demonstrate that DMSP is upregulated by the presence of the methionine precursor, consistent with previous pathway analyses that concluded plants use the methylation pathway (Hanson *et al.,* 1994; Otte and Morris, 1994; Paquet *et al.,* 1994) and not another pathway, such as the 4-methylthio-2-oxobutyrate (MTOB) pathway used by coral (Raina *et al.,* 2013). In the context of previous experimentation into the DMSP production pathway, it is perhaps unlikely that plants use the Demethylation or Decarboxylation pathways, but without proper identification of the enzymes involved, this cannot be ruled out. It is possible that like bacteria that use multiple DMSP production pathways (Williams *et al.,* 2019), plants may also show family or species level variation in the DMSP production pathways. Therefore, elucidating the genetic pathway of DMSP synthesis in the model organism *Arabidopsis thaliana* is the most logical next step in research.

Global Implications of Higher Plant DMSP Production

Although these findings have not discovered a new DMSP producing plant that is comparable to *Spartina anglica*, or Sugarcane, it has nevertheless opened a new line of thought into the importance of terrestrial systems in global sulfur cycling. Current estimates suggest that plants make up 80% of the Earth's biomass (Thompson, 2018), the majority of which are terrestrial. Even if most plants only produce a small concentration of DMSP, the sheer volume of terrestrial plants globally will have an enormous impact on the global flux of DMSP cumulatively. Thus, this study is at the beginning of an exciting new paradigm, that shows terrestrial systems are of far more importance to global sulfur cycling, and thus global cooling, than previously thought

Chapter 4 – Plant Methionine Methyltransferases and their Role in DMSP Biosynthesis

Introduction

Economic and Biological Impacts of Salinization

Salinization is the increase in dissolved solutes in environments, predominantly of Na^+ ions e.g., in underground, water-bearing rock layers or soil (Brindha & Schneider, 2019). Naturally occurring saline layers, or aquifers, are found beneath freshwater aquifers (Martens & Wichmann, 2007). When the fluxes between the different areas of the hydrosphere and lithosphere are altered, such as increased evapotranspiration (Fig. 1.1), freshwater aquifers can become contaminated with saline water (Brindha & Schneider, 2019). Anthropogenic climate change is also increasing the rate of salinisation, as rising sea levels lead to saltwater intrusion further inland (Moore & Joye, 2021) and fertiliser leaching increases the ionic load (Fohrer & Chícharo, 2011).

Salinization of land is one of the leading threats to arable agriculture, with an estimated 1/3 of currently irrigated land affected by salinity levels too high for most plants to tolerate (Jamil *et al.,* 2011). This is defined by the electrical conductivity of soil (EC_e in dS m^{-1}), with a conductivity of greater than 4.0 and a pH of less than 8.5 resulting in conditions that restrict the yields of most crops (Abrol *et al.,* 1988). This causes a significant reduction in yields, with glycophytic crops such as beans, rice and maize experiencing yield reductions of between 50-80% (Panta *et al.,* 2014). Indeed, 60% of the calories consumed by humans come from three species; rice, wheat and maize, none of which are halotolerant (FAO, 2022; Khan *et al.,* 2006; Zörb *et al.,* 2018). Furthermore, extreme salinization causes soil degradation to such an extent that even halophiles struggle to grow, leading to the abandonment of previously cultivated land (Panta *et al.,* 2014). This is most prominent in regions that are already economically and agriculturally fragile; such as the Bay of Bengal, the Aral Sea Basin, and Sub-Saharan Africa (Qadir *et al.,* 2009; Vashev *et al.,* 2010; Zingore *et al.,* 2015). The economic importance of yield loss and land abandonment are critical, with an estimated £139-282 million cost in three European countries due to yield reduction (Montanarella, 2007), and a global cost of £21 billion due to land losses (FAO, 2011; Ruto *et al.,* 2021). Therefore, a greater understanding of the mechanisms of halotolerance in higher plants is critical to mitigating against the effects of salinization.

Plants and halotolerance

The earliest response to increased uptake of environmental Na^+ is the Calcium signalling cascade (Fig. 4.1). In response to increased sodium uptake in root cells, sphingolipid molecules bind to Na^+ triggering increased uptake of Ca^{2+} via transporters such as MOCA1 and ANN4 (Park *et al.,* 2016; Zhao *et al.,* 2021). The increased intracellular Ca^{2+} concentration is detected by a complex network of calcium-dependent protein kinases (Fig. 4.1), that phosphorylate sensor relay proteins, such as calmodulin, which in turn interact with signal transducing kinases (CIPKs) (Tansley *et al.,* 2023). This swiftly triggers the Salt-Overly Sensitive (SOS) pathway, detected by SOS3/SCaBP8 complexes that signal to SOS2 sodium transporters to extrude Na^+ from the cytosol to restore osmotic balance (Tansley *et al.,* 2023; Zhao *et al.,* 2021). Another early point of salt stress detection is loss of turgor pressure within the cells, detected by cell wall proteins such as Ferronia Receptor kinases that also trigger rapid Ca^{2+} influx to mobilise the SOS pathway (Liao *et al.,* 2017; Zhao *et al.,* 2021).

Plants have a complicated and varied response to salt-stress induced calcium signals. The most immediately deleterious effect on plant cells is the loss of turgor pressure, or plasmolysis, caused by loss of water from the vacuole to counter the increased Na^{2+} ions (Lang *et al.,* 2014; Park *et al.,* 2016; Zhao *et al.,* 2021). To maintain turgor pressure, actin cytoskeletal filaments rearrange in the cell wall, to form a stabilising network around the protoplast (Lang *et al.,* 2014). Additionally, to offset the increased Na^+ concentrations, plants reduced water loss by closing their stomata in response to reactive oxygen species (ROS) (Park *et al.,* 2016; Zhao *et al.,* 2021), such as hydroxyl radical (HO•) and hydrogen peroxide (H_2O_2) (Kesawat *et al.,* 2023). Salt stress induces organelles – primarily the mitochondria and chloroplasts (Kesawat *et al.,* 2023) to produce ROS, which although can be damaging, have a secondary function as signalling molecule to initiate downstream stress response cascades (Zhao *et al.,* 2021). Under moderate salt stress conditions, plants will also produce antioxidants to ameliorate the damaging effects of ROS, such a glutathionine and peroxidases (Kesawat, 2023).

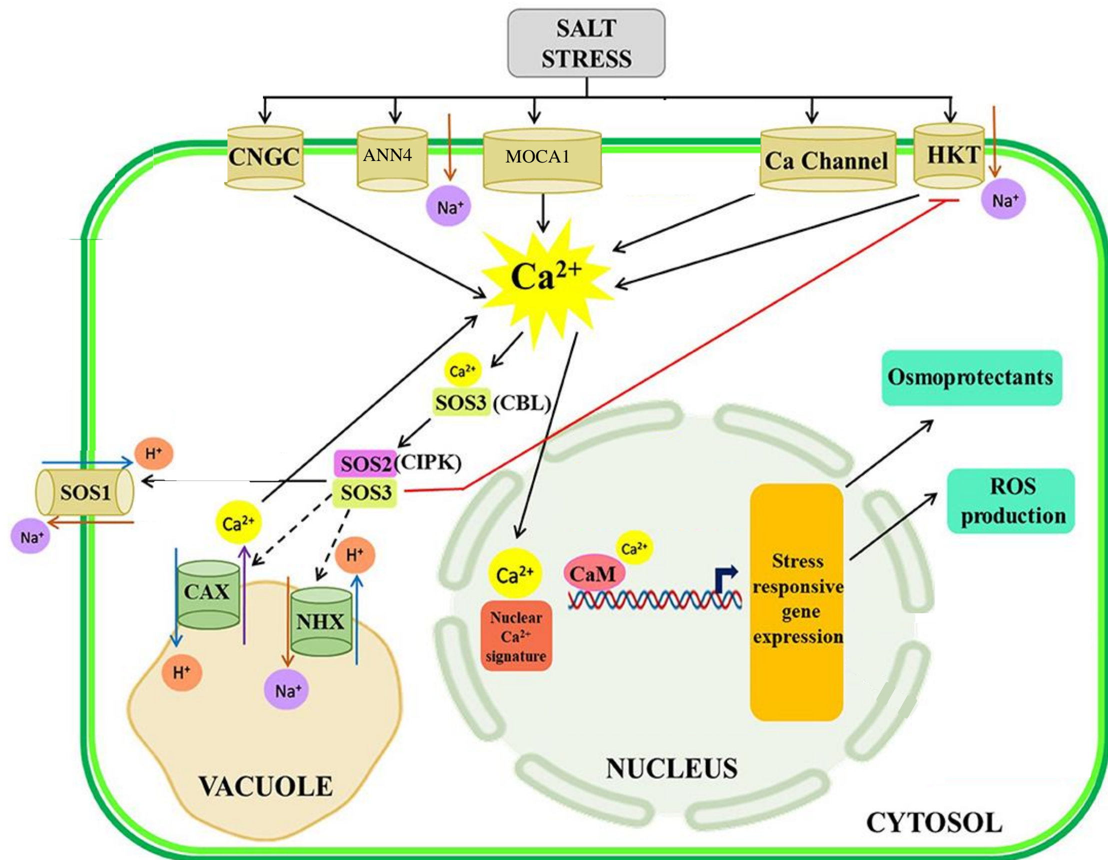


Figure 4.1: Summary diagram of cellular calcium signalling in response to salt stress, and the movement between membrane-bound organelles within the plant cell (adapted from Patra *et al.*, 2021).

Phytohormones are also a critical secondary response to salt stress. Abscisic acid is an isoprene derived molecule that rapidly accumulates in response to increased intracellular Na^+ concentrations (Chen *et al.,* 2019). Abscisic acid mobilises cytosolic Ca^{2+} channels to maintain SOS signalling, as well as inducing H_2O_2 synthesis to trigger stomatal closure (Chen *et al.,* 2019; Ullah *et al.,* 2017; Yu *et al.,* 2020). Auxin, also known as indole-3-acetic acid, slows root meristem elongation to limit the surface area for Na^+ uptake (Chen *et al.,* 2019), as well as upregulating antioxidant production to counter ROS damage (Iglesias *et al.,* 2010). Salicylic acid has also been shown to induce antioxidants in response to salt-dependent ROS production, notably the synthesis of salicylate hydroxylase (Borsani *et al.,* 2001). Salicylic acid also has some more idiosyncratic responses to salt stress. Firstly, salicylic acid induces germination in salt stressed *A. thaliana* (Lee *et al.,* 2010), perhaps to ensure reproduction before plant death due to salt stress. Additionally, salicylic acid protects nitrogen fixation from being inhibited in *Medicago truncatula* under high salinity (Palma *et al.,* 2013), by producing antioxidant enzymes in root nodules.

Plant osmolytes are a key response to salt stress and are not limited to DMSP (Fig. 4.2). These low molecular weight compounds have multiple functions; to act as signalling molecules for phytohormones (Singh *et al.,* 2022), scavenge ROS (Ghosh *et al.,* 2021; Singh *et al.,* 2022) and increase intracellular osmotic pressure (Auton *et al.,* 2011). The major osmolytes in plants are sugar derivatives, such as trehalose and mannitol, which are easily water soluble to rapidly establish isotonic cellular conditions (Ghosh *et al.,* 2021; Singh *et al.,* 2022). Plants may also produce amino acids as osmolytes, notably proline (Ghosh *et al.,* 2021; Singh *et al.,* 2022). In response to salt stress, proline rapidly accumulates in the chloroplasts, to act as a proton-shuttle for downstream high-energy processes involved in protection (Kavi Kishor *et al.,* 2022) and maintaining $NADP^+$/NADPH ratios, critical for photosynthetic electron transfer (Hare & Cress, 1997). Arguably though, the quintessential osmolyte is the DMSP homologue, glycine betaine.

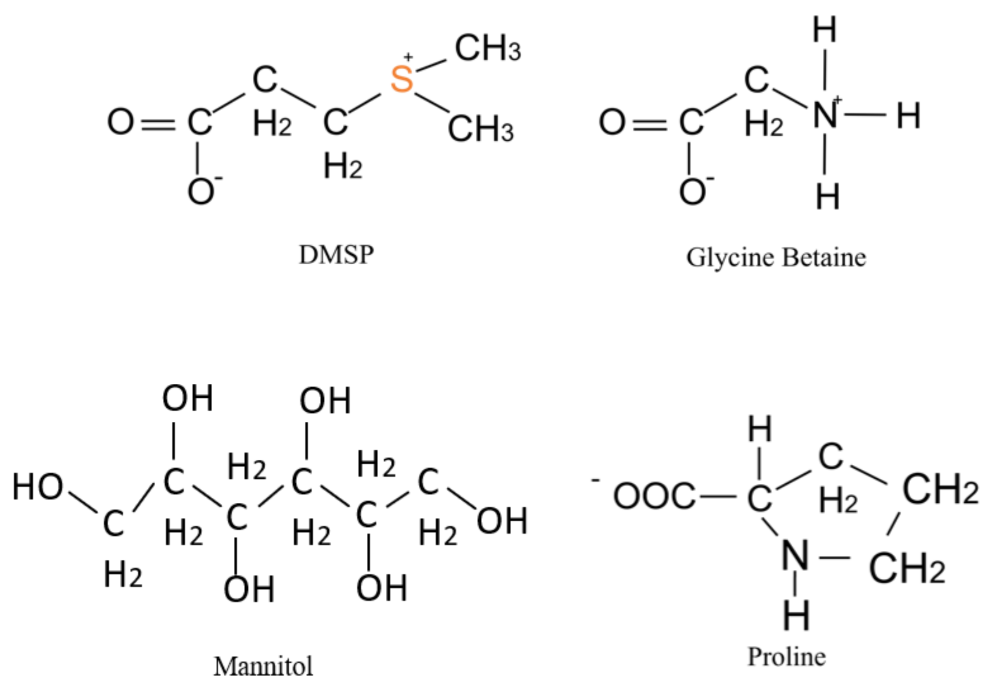


Figure. 4.2: The Lewis Structures of common plant osmolytes, all of which exhibit the characteristic short carbon chains and overall small molecular weights.

Glycine betaine is a structural homologue to DMSP. It is produced by the oxidation of choline (Nahar *et al.,* 2016; Valenzuela-Soto & Figueroa-Soto, 2019) in the cytoplasm and transported to the chloroplast thylakoid membranes (Valenzuela-Soto & Figueroa-Soto, 2019). Glycine betaine protects plants cells against osmotic stress not only by maintaining chloroplast isotonicity, but also by upregulating expression of D-family

repair proteins that stabilise the tertiary structures of photosynthetic electron transport proteins in response to oxidative stress (Fan *et al.,* 2012; Murata *et al.*, 2007).

The *S*-methionine Methyltransferase pathway for DMSP synthesis and its Role in Plant Halotolerance

The *S*-methionine Methyltransferase pathway of DMSP biosynthesis is thought to dominate in higher plants (see Chapter 1). The first enzyme of this pathway, Methionine Methyltransferase (MMT) is a ubiquitous enzyme in higher plants (Ranocha *et al.,* 2001), and has multiple functions above DMSP synthesis. MMT methylates methionine to yield SMM (Fig. 4.3), in a reaction that requires SAM as the methyl donor (Amir, 2010). SMM can then be used as a methyl donor itself, to generate methionine from homocysteine, requiring the enzyme homocysteine *S*-methyltransferase (HMT) (Amir, 2010; Ranocha *et al.,* 2001). This SMM cycle is necessary to prevent depletion of the free methionine pool, by excessive S-adenosine-methionine (AdoMet) synthesis, as a compensatory mechanism for the lack of an AdoMet specific negative feedback loop (Ranocha *et al.,* 2010). SMM itself is considered both a sink and a transporter for excess sulfur molecules (Hesse *et al.,* 2004). Additionally, MMT is required for the conversion of inorganic selenium to bioactive gaseous forms, such as dimethylselenide (Tagmount *et al.,* 2002), an essential element for amino acid formation, such as selenocysteine (Hall *et al.,* 2022).

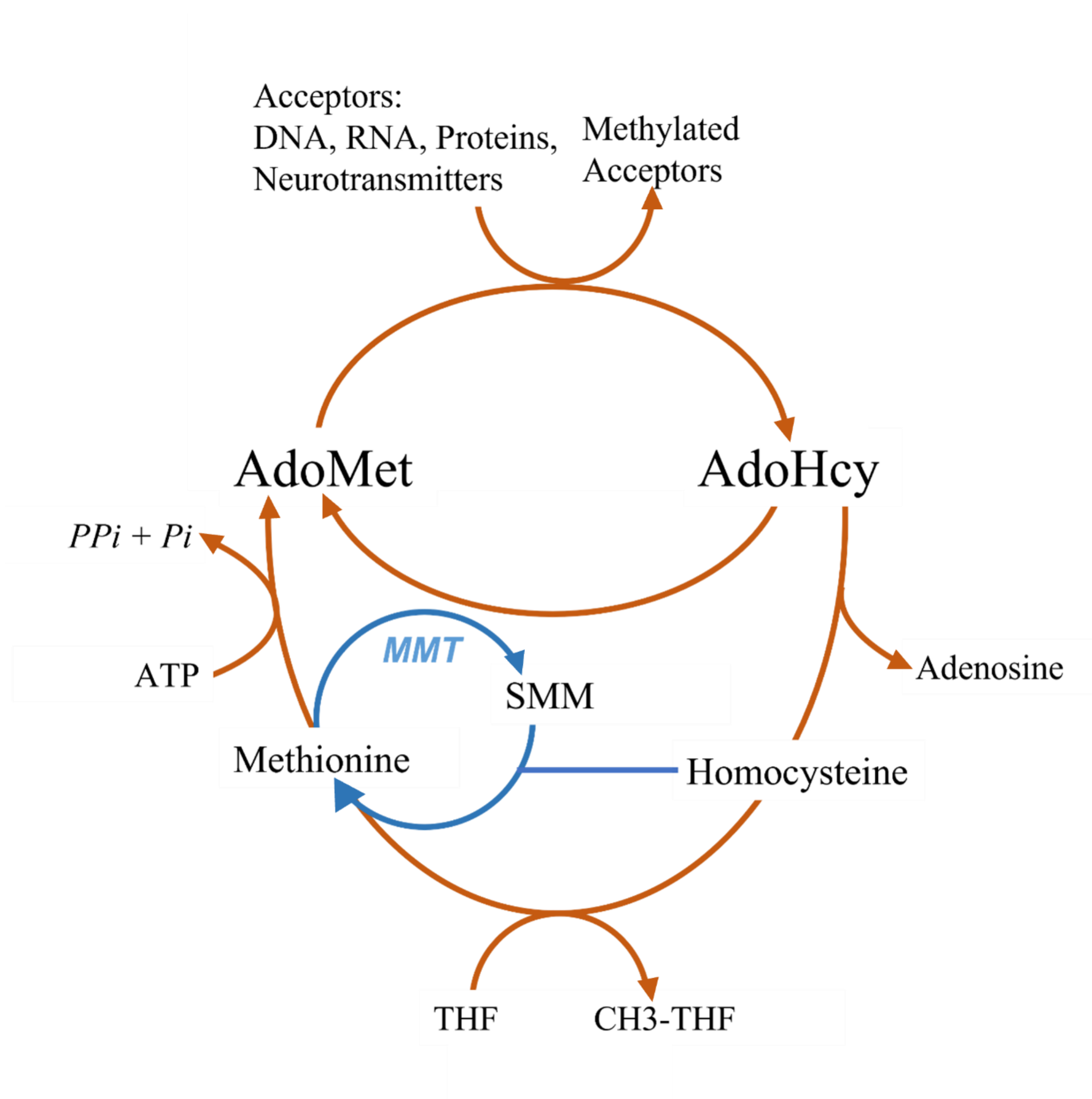


Figure 4.3: The SMM Cycle, embedded within the Methyl cycle. Reactions requiring the enzyme MMT are in blue. Adapted from Kocsis *et al.*, 2003.

The MMT structure is typical of the methyltransferase family enzymes, with a highly conserved 130 amino acid Class I Rossmann-fold region which binds SAM (Gana *et al.,* 2013; Schluckebier *et al.,* 1995). This is characterised by alternating α-helices and β-pleated sheets, forming a seven strand β-sheet flanked by three α-helices on either side, known as the core motif (Martin & McMillan, 2002). These may be found as dimers and trimers (Peng *et al.,* 2022).

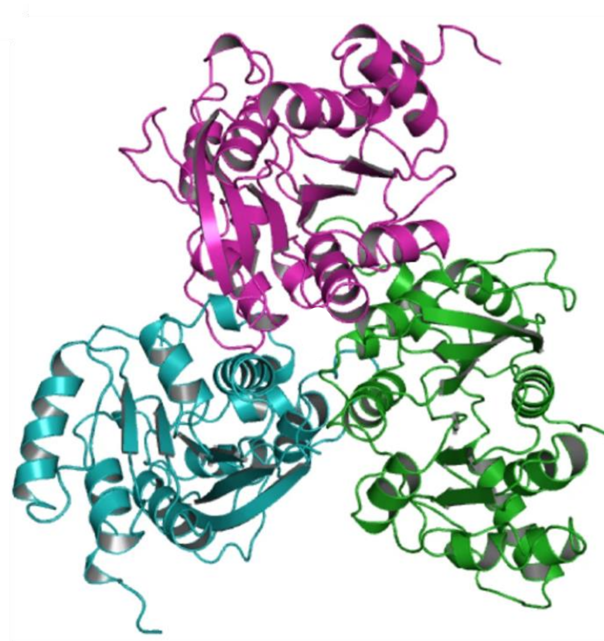


Figure 4.4: The 3-Dimensional crystal structure of the bacterial *mmTn* homotrimer from *Roseovarius indicus* (PDB 7vv), with the characteristic Rossmann-fold shown as alternating α-helices (spirals) and β-pleated sheets (arrows) on each domain (coloured pink, green and blue). Each monomer is composed of six β-strands and eleven α-helices.

Within the core motif, methyltransferases show considerable variability, excepting Motif II, which is a conserved Ω-loop (Gana *et al.,* 2013; Martin & McMillan, 2002), which is assumed to stabilise MMT-SAM interactions (Pal & Dasgupta, 2003). Additionally, there is a consensus sequence at the N-terminal end (Fig. 4.4) known as Motif I (Gana *et al.,* 2013), which is a characterised by a glycine-dense region (D/ExGxGxG) and is highly conserved across plant, bacterial and archaeal MMT and *mmtN* sequences (Long, 2021; Kozbial *et al.,* 2005).

The presence of MMT has been associated with halotolerance in plants and bacteria. The presence of the MMT homologue, *mmtN*, is a reliable indicator of DMSP biosynthesis in marine and salt marsh bacteria (Liao & Seeback, 2019; Williams *et al.,* 2019). In higher plants, SAM-dependent MMT's have been found to contribute directly to halotolerance in Australian Saltbush (*Atriplex nummularia*), as the glycine betaine pathway is reliant on SAM as a methyl donor, necessitating MMT as part of its regulatory mechanism (Tabuchi *et al.,* 2005). In Cotton plants (*Gossypium spp.*), increased numbers of identified methyltransferase genes are associated with salt tolerance, because of increased secondary metabolite production and increased cellulose

fibre production (Hafeez *et al.,* 2021). The *S*-methionine methyltransferase has also been shown to confer increased salt tolerance in the model organism *Arabidopsis thaliana*. In Col-0 ecotype *A. thaliana*, plants with the *Mmt* gene knocked out had significantly lower germination rates and shoot fresh weights compared to wildtype plants (Ogawa & Mitsuya, 2012). Additionally, transgenic *A. thaliana* with overexpressed *Mmt* showed increased salt tolerance (Ezaki *et al.,* 2016; Liu *et al.,* 2014; Ma *et al.,* 2017). Col-0 plants transformed with *Mmt* from Broomsedge (*Andropogon virginicus*) showed increased tolerance to reactive oxygen species (characteristic of salt stress) and heavy metal concentrations, compared to the wildtype (Ezaki *et al.,* 2016). Furthermore, *A. thaliana* was transformed with *Mmt* genes from Sweet Potato (*Ipomoea batatas*) and Sugar Beet (*Beta vulgaris*) in separate studies. Both studies found that transgenic *A. thaliana* plants had significantly increased root and shoot fresh weights, and significantly decreased chlorosis and tissue death compared to wildtype plants (Liu *et al.,* 2014; Ma *et al.,* 2017). These results suggest that *Mmt* is a critical gene to study for the production and importance of DMSP and other osmolytes in plants, but also that *A. thaliana* is a good model organism for such study.

Aims and Objectives

To continue the work in establishing *A. thaliana* as a suitable model organism for DMSP biosynthesis in higher plants and ratify the genes in DMSP synthesis in higher plants, the following aims were established:

1. To compare plant *Mmt* genes to known bacterial *mmtN* sequences, to establish sequence similarity and find suitable candidate genes for knock out work.
2. To generate transgenic *Escherichia coli* expressing plant *Mmt* genes and to characterise the MMT activity of the resulting strains.
3. To generate *A. thaliana* mutant plants with *Mmt* knocked out and to measure the difference in DMSP concentrations and compare phenotypes, when grown in saline conditions.

Methods and Materials

Cloning of *A. thaliana* DMSP synthesis genes into *Escherichia coli*

Generation of Synthesised Genes

Candidate *Arabidopsis thaliana Mmt* gene sequences were identified from The Arabidopsis Information Resource (TAIR) and then compared to the following bacterial *mmtN* sequence using BLAST: *Roseovarius indicus* DSM26383 and *Thalassospira profundimaris* DSM1734 (Liao & Seebeck, 2019), to compare sequence homology. These specific *mmtN* sequences were selected due to their similarity to plant *Mmt* sequences, with a minimum of 30% sequence homology to the N-terminal domain and amino acid count greater than 1000, to give greater than 20% query coverage of the much larger plant *Mmt* proteins. This was in accordance with findings of previous studies (Williams *et al.,* 2019), to minimise effort in searching for the best candidate bacterial species. The best candidate *A. thaliana Mmt* sequence was selected by highest query cover, followed by highest percentage identity. The gene locus number for the selected *A. thaliana Mmt* is AT5G49810.1.

To test the function of *A. thaliana Mmt,* it was introduced into an organism that does not have any DMSP Synthesis capabilities. Gene sequences were domesticated by silently mutating bases to remove internal restriction sites for Goldengate Cloning and a 5' ribosome binding site added for pET vector cloning. Gene sequences were synthesised by Invitrogen GeneArt and diluted to 100 ng/µl.

Goldengate Cloning and Transformation

Escherichia coli DH5α was used as the host organism for both level 0 and level 2 Goldengate Cloning. Synthesised DNA products were used as templates in a Phusion PCR (see Chapter 2). A master mix of 11.9 µl water, 4 µl Phusion 5x HF Buffer, 1.6 µl 2.5 mM dNTPs, 0.5 µl 100% dimethylsulfoxide (DMSO), 0.5 µl 50 mM $MgCl_2$ and 0.2 µl Phusion DNA Polymerase was made. To each 18.7 µl aliquot, 0.5 µl of synthesised DNA and 0.4 µl each of 20 µM gene specific forward and reverse primers (Tab. 2.4) were added. Phusion PCR reaction mixes were incubated in the thermocycler, using the Bounce PCR method (Mugford & Hogenhout, 2018) according to the method detailed in Chapter 2 (page 42).

Successful amplification was checked by running all 20 µl of the Bounce PCR products on a 1% Agarose gel (see Chapter 2, page 43).

Mmt DNA was recovered from the gel by visualising with a 254 nm Clearview UV transilluminator (Cleaver Scientific) and cutting out the band of appropriate size from the gel with a scalpel. The DNA was extracted by adding 300 µl of QC buffer to the removed band and incubated at 55 ˚C with 32 rcf for 2 mins in a Multi-Therm^{TM} Shaking Incubator. Once the gel was dissolved, 100 µl of isopropanol was added, followed by 10 µl of silica dioxide. The mix was inverted, and DNA allowed to bind for 2 mins. The silica pellet was resuspended by centrifuging at 6450 rcf for 13 sec and the supernatant removed. The pellet was resuspended in 500 µl of Column Wash Solution, vortexed and centrifuged at 6540 rcf for another 13 sec. The supernatant was removed, and the DNA eluted in 20 µl of water, the pellet thoroughly broken up and incubated at 70 ˚C for 2 mins. The elution product was centrifuged at 18400 rcf for 2 mins to pellet the silica, and the DNA extracted from the remaining supernatant.

The extracted DNA product was measured using a Nanodrop and DNA with concentrations greater than 100 ng/µl were diluted to 100 ng/µl. Goldengate assemblies were constructed as follows.

Table 4.1: Goldengate Cloning reaction components, the reaction level at which each component required and the individual volumes for a 17 µl total reaction volume.

Component	Goldengate Level	Volume (µl)
Plasmid 41264 Vector Backbone	0	1
Plasmid pMal 1039 Vector Backbone	2	1
Extracted DNA Assembly Piece	0 and 2	3
10x NEB T4 Buffer	0 and 2	1.5
10 x Bovine Serum Albumen (BSA)	0 and 2	1.5
NEB T4 Ligase (400000 cohesive end unit/ml)	0 and 2	1
BpiI restriction enzyme	0	1
BsaI restriction enzyme	2	1
Distilled water	0 and 2	6

The assembly reaction mix was placed in the thermocycler and incubated at 37 ˚C for 3 mins followed by 16 ˚C for 4 mins for 25 repetitions. This was followed by incubating at 50 ˚C followed by 80 ˚C for 5 mins each.

Following the level 0 Goldengate reaction, 1 µl of *A. thaliana Mmt* was added to 20 µl of chemically competent *E. coli* DH5α to transform them.

Selection of Positive Colonies

Transformed cells were plated on LB Agar containing the appropriate antibiotic (spectinomycin for level 0 constructs and ampicillin for level 2 constructs) and final concentrations of 100 µg/m isopropyl β- d-1-thiogalactopyranoside (IPTG) inducer and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) pigment to perform blue-white screening. Plates were incubated overnight at 37 ˚C.

White colonies were tested for successful cloning into the plasmid using colony PCR. A reaction mix of 5 µl of Promega GoTaq® G2 Green Master Mix, 3 µl of water, 1 µl of each appropriate forward and reverse primers from 20 µM stock and 1 white colony were generated. Each selected colony was streaked onto LB Agar with the appropriate antibiotic before addition to the reaction and the streaked plate was incubated at 37 ˚C overnight. Colony PCR reaction mixes were incubated in the thermocycler (see Chapter 2, page 42).

Plasmid DNA Purification and Sequencing

Successful colonies containing the level 0 construct were grown in 5 ml of LB broth with 5 µl of spectinomycin at stock concentration overnight at 37 ˚C with 16 rcf. DNA was purified using Promega Wizard ® Plus SV MiniPrep DNA Purification System and the concentration measured using NanoDrop. DNA with concentrations greater than 100 ng/µl were diluted to 100 ng/µl. This extracted DNA was then used as the assembly piece for level 2 Goldengate, which was repeated as above (see Table 4.1 for components).

To ensure that the cloned gene was of the correct sequence and quality, 8 µl of DNA were combined with 1 µl of the appropriate primer (goldengate 2 for level 0 and BM0189 for level 2) and 8 µl of sterile water. This was sent to Eurofins for Sanger Sequencing. Sequencing results were viewed using BioEdit software and compared to the in-silico plasmid map for each construct using Ape Plasmid Editor. Sequences were

compared to each other and reference databases using Basic Local Alignment Search Tool (BLAST).

Protein Extraction and Purification

Tab 4.2: Protein Purification and Extraction Buffer Formulas (exclusive of dH_2O to make up the total volume).

Buffer Name	Volume (ml)	Formula
Laemelli Buffer	20	Bromophenol Blue 0.004g Glycerol 4 ml Dithiothreitol 1M 4 ml SDS 0.8 ml Tris (pH 6.8) 0.5M 5ml
Lysis Buffer	1000	DTT 1M 1 ml EDTA 0.5M (ph 8.0) 2 ml NaCl 11.7g Tris-HCl 1M (pH7.4) 20ml
SDS-PAGE Running Buffer	1000	Glycine 14.42 g SDS 10 ml Tris 3.28 g
SDS-PAGE Resolving Gel	10	APS 10% 200 µl Bis-Acrylamide 6.7 ml Tetratmethylethylenediamine 10 µl Tris 1.5 M (pH 8.8) 5ml SDS 0.2 ml
SDS-Stacking Gel	2.97	APS 10% 60 µl Bis-Acrylamide 1.005 ml Tetratmethylethylenediamine 6 µl Tris 1.5 M (pH 6.8) 1.5 ml SDS 60 µl
Washing Buffer 1	1000	EDTA 0.5M (pH 8.0) 2 ml NaCl 11.7g Tris-HCl 1M (pH 7.4) 20ml
Washing Buffer 2	1000	NaCl 11.7g Tris-HCl 1M (pH 7.4) 20ml

E. coli BL21 was transformed with the pmal1039 Goldengate level 2 plasmid containing *Ammt*, or the empty plasmid as before and grown on LB Agar with 100 mg/ml carbenicillin overnight at 37 ºC. Single colonies were inoculated into 10 ml LB with 100 mg/ml carbenicillin and 0.1% glucose at 37 ºC, 16 rcf for a minimum of 2.5 h, until OD_{600} of 0.6 was reached. Half the replicates of each treatment condition were then inoculated with 5 µl IPTG to induce protein expression, the others with 5 µl of dh_2O as a control. All cultures were then incubated at 16 ºC, 2 rcf overnight.

Protein was extracted from cultures by addition of 20 µl Laemelli buffer to 100 µl of culture and boiling at 95 ºC for 10 mins to lyse bacterial cells.

SDS-PAGE gels were made as described to a 1.5 mm thickness by pouring between glass plates using the mPAGE® Gel Caster (Millipore) to generate 83 mm × 101 mm gels (see Tab. 4.2 for components). The resolving gel of 10% acrylamide was poured first and allowed to solidify for 45 minutes with an isopropanol cover. Once set, the isopropanol cover was removed by micropipette and the stacking gel of 5% acrylamide was poured on top, a 15 well comb inserted, and allowed to solidify for 1 hour. Plates were inserted into a vertical gel tank and covered with running buffer. The first well contained 5 µl of protein ladder, with 20 µl of sample added to subsequent wells. The gels were run at 130V in an mPAGE® Gel tank (Millipore) for 2 hours to allow for sufficient resolution of proteins. Gels were then removed and post-stained with Ultrabrilliant Blue dye at 1 rcf, until the dye turned brown. Gels were visualised Biorad GelDoc XR+ image system with Image Lab™ software version 6.1 for Windows.

To purify proteins using a Maltose Binding Protein column, 200 ml of cultures were pelleted by centrifugation at 4 ºC, 8400 rcf for 20 mins. Pellets were resuspended in lysis buffer (Tab 4.2) and lysed by G-M® High Pressure Cell Disruption French press (Glen Mills). Samples were separated into soluble and insoluble fractions by centrifugation at 18000 rcf for 30 minutes, and the soluble fractions were then incubated for one hour with amylose resin (New England Biolabs E8021S) washed with washing buffer (Tab 4.2) four times. After incubation with amylose resin samples were centrifuged at 220 rcf for 1 min and the supernatant removed. The resin was washed four times with washing buffer, and then poured into an empty PD10 with filter (Cytiva). Wash buffer was allowed to run through, followed by a wash with washing buffer 2 (Tab 4.2). Columns were then incubated for 5 minutes with 10mM maltose (Sigma-Aldrich 6363-53-7) dissolved in washing buffer 2 and eluate collected.

Phenotyping of MMT Homozygous Knock-Out *A. thaliana*

Generation of mutant plants and Genotyping

Mmt Homozygous knockout *A. thaliana* Col-0 seeds of the germplasm line SALK_023362C were ordered from the Arabidopsis Biological Research Centre. The *Mmt* gene at locus AT5G49810 was generated via *Agrobacterium tumefaciens* transformation, allowing for a tDNA insert in the fifth exon to disrupt gene function. Successfully transformed plants were selected using kanamycin (Alonso *et al.,* 2003).

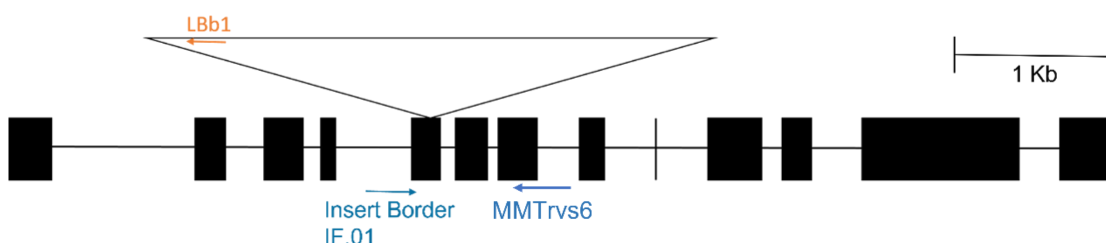


Figure 4.5: To scale diagram of the MMT gene where black boxes denote exons and white spaces denote introns. The tDNA insert to knock out the function of the MMT gene is shown above in exon five.

Plants were grown from sterilised seeds on ¼ MS-Agar (See Chapter 2 – Methods and Materials). To verify the homozygous *Mmt* knockout phenotype, DNA was extracted from adult plant leaf tissue using the protocol outlined in Edwards *et al.,* 1991. A single leaf of fresh weight between 3-5 mg was placed in 200 µL of extraction buffer (10% Edwards Solution in TE Buffer) and crushed. The solution was centrifuged at 18400 rcf for 5 mins to separate the plant material from the supernatant and the recovered.

Table 4.3: One-Step DNA Extraction Buffer (Edwards *et al.*, 1991) components, made up using dH_2O.

Solution	**Component**	**Concentration (mM)**
Edwards Solution	Tris-HCL (pH 7.5)	200
	NaCl	250
	EDTA	25
	SDS	0.5% of total reaction volume
TE Buffer	Tris-HCL (pH 8.0)	10
	EDTA	1

Extracted DNA was amplified using a reaction mix of 5 µl of Promega GoTaq® G2 Green Master Mix, 7.5 µl of water, 1 µl of each forward primer *Mmt* IF.01 and reverse primer LBb1 from 20 µM stock and 0.5 µl of supernatant from the DNA extraction. Reaction mixtures were amplified in a thermocycler (See Chapter 2).

All 15 µl of the amplified DNA were loaded into the wells of a 2% Agarose Gel (20 g of Agarose per 1L of 1x Tris-Acetate-EDTA (TAE) buffer) and run as before.

Growth of MMT- and WT *A. thaliana* and their responses to salt stress

Arabidopsis thaliana wildtype Colombia (Col-0) seeds were treated using a solution of sodium hypochlorite, triton and deionised water applied for 12 mins to sterilise. This was removed and the seeds rinsed with sterile water five times before plating onto solid ¼ MS Agar in round plates. Seeds were stratified by wrapping the plate in aluminium foil and storing at 4 ˚C for a minimum of 48 hours. The foil was removed, and plates transferred to a Sanyo Versatile Environment Test Chamber at 23 ˚C, 16 hour photoperiod with 800 W fluorescent lights for 7 days.

Seedlings were removed from the plates using flame-sterilised forceps and 15 seedlings were placed 30 mm from the top of a 100 mm square plate containing 50 ml of ¼ MS Agar at salinities (Tab. 4.4), approximately 5 mm apart. This was repeated for all five treatment conditions, for both *Mmt*- and WT plants. Plates were placed upright in a 23 ˚C growth cabinet for 10 days.

Table 4.4: Supplementation of 5M NaCl to ¼ MS-Agar to attain phenotyping concentrations for a total volume of 50 ml.

Final NaCl concentration (mM)	**H_2O Added (µl)**	**NaCl added (µl)**
0	2000	0
50	1500	500
100	1000	1000
150	500	1500
200	0	2000

Root and shoot tissue were harvested separately and pooled for all 10 plants per plate in previously weighed 1.5 mL glass crimp-top gas chromatography vials. Tubes were weighed again to determine the mass of fresh weight (g) for the total tissue from each

plate. Once weighed, 300 µL of 10M NaOH was added, to convert DMSP to DMS by alkaline lysis. The vial tops were immediately crimped to form a gas tight seal. Vials were left in the dark for overnight to allow DMS gas to generate and the sample assayed by Gas Chromatography

Results

The protein sequences of ratified bacterial DMSP synthesis enzymes from the methylation pathway (Liao & Seebeck, 2019; Williams *et al.,* 2019) were used as probes in BLASTP searches against the *A. thaliana* genome to identify candidate plant DMSP synthesis enzymes. This was because although *Mmt* activity had been identified in plants as the first step of the methylation pathway (Hanson *et al.,* 1994; Kocsis *et al.,* 1998), no gene ratified gene sequences existed for any of the known high producing plants and no reference genomes existed.

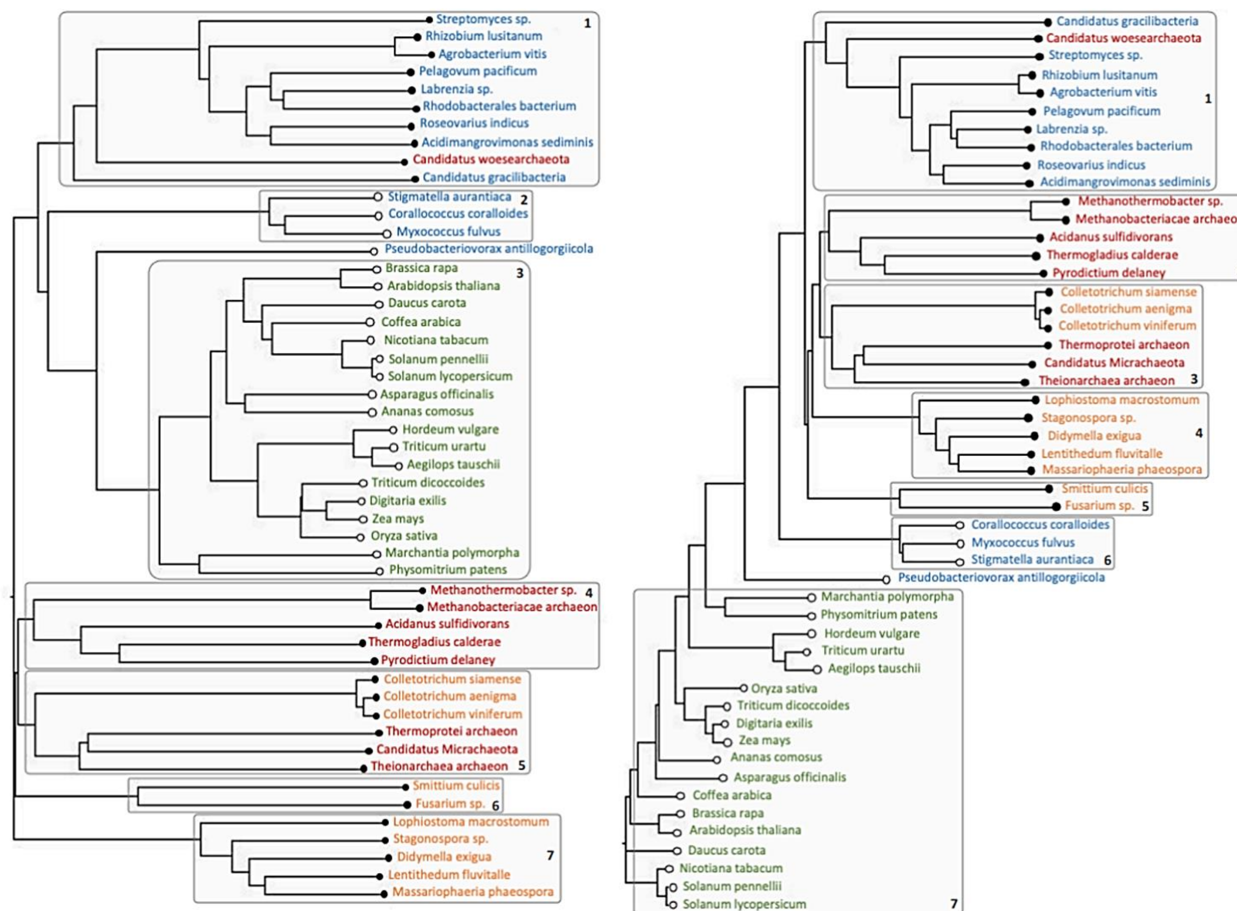


Figure 4.6: Phylogenetic analysis of the relatedness between Methionine Methyltransferase (*Mmt*) and Methione *S*-methyltransferase enzyme (*mmtN*) sequences. Organisms are grouped as follows: Green = Plant, Blue = Bacteria, Red = Archaea, Orange = Fungi. Identified clades (deriving from a single common ancestor) are outlined and numbered. The left tree was generated from analysis of methyltranferase sequences in full. The right tree was generated using the N-terminal domain. White circles at the end of a branch refers to an *Mmt* sequence. Black circle refers to an *mmtN* sequence (Long, 2021, reproduced with permission).

Roseovarius indicus mmtN was selected from bacterial Clade 1 (Fig. 4.4) for use as probe because it is a well characterised DMSP producer using the methylation pathway. *mmtN* genes from bacteria *Streptomyces mobaraensis* and *Thalassiospira profundimaris* (not to be confused with the diatom, *Thalassiosira*) were also selected for comparison, due to their known *mmtN* activity in DMSP biosynthesis (Kageyama *et al.,* 2018; Liao & Seebeck, 2019). These were then compared against the *Ammt* gene, identified from the TAIR database.

Table 4.5: Similarity Matrix of percentage identity between the candidate *A. thaliana MMT* gene and selected bacterial *mmtN* sequences. Query represents the percentage of the *A. thaliana* gene overlapping the bacterial reference genes. % represents the percentage of matching base pairs over the aligned genes.

	R. indicus		***S. mobaraensis***		***T. profundimaris***	
	Query	**%**	**Query**	**%**	**Query**	**%**
MMT AtMMT_AT5G49810.1	25.00	26.12	23.00	24.37	23.00	28.52

The query cover for *A. thaliana Mmt* sequences compared to all the reference sequences were low (<30%), suggesting that they are not closely related. This is to be expected as plant MMTs are double-domain proteins, and sequence homology is therefore only expected at the conserved N-terminal methyltransferase domain, that makes up the entirety of the single domain MMTN proteins. Additionally, the percentage identities between *A. thaliana Mmt* and the reference sequences were low (<30%), suggesting that the primary sequences are not well conserved. This is likely due to evolutionary divergence of domains, although the active site conserved.

Having established that the *Ammt* candidate gene has some, albeit low, levels of sequence homology, the *Ammt* gene was synthesized and introduced into the model bacteria *E. coi* through goldengate cloning. *E. coli* has no DMSP synthesis activity, so any activity post transformation must be due to the presence of *Ammt*.

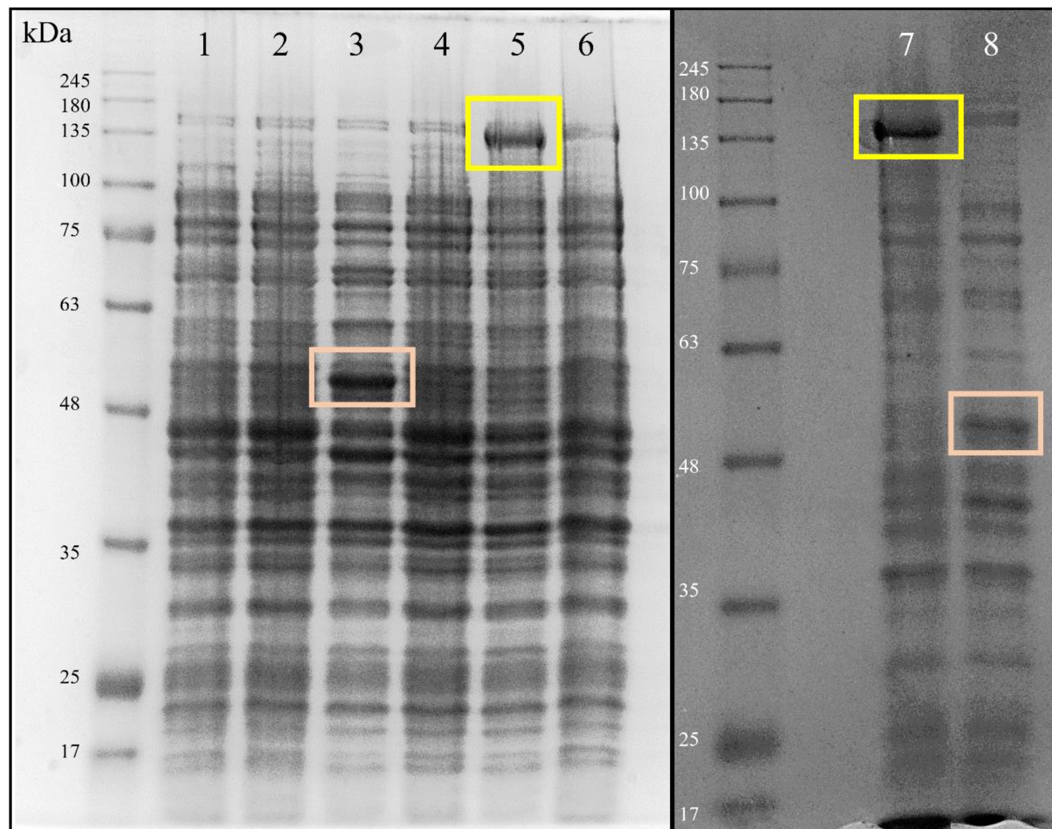


Figure 4.7: SDS-PAGE showing overexpression of AMMT in *E. coli*. Lane 1) Unpurified *E. coli* WT induced with IPTG 2) Unpurified *E. coli* WT without induction 3) *E. coli* transformed with empty pmal1039 + IPTG 4) *E. coli* transformed with the empty pmal1039 plasmid - IPTG 5) *E. coli* transformed with pmal1039/AMMT + IPTG 6) *E. coli* transformed with pmal1039/AMMT - IPTG 7) Purified protein extract from *E. coli* transformed with pmal1039/AMMT 8) Purified protein extract from *E. coli* transformed with empty pmal1039.

E. coli BL21 was successfully transformed with pmal1039 and AMMT expression induced by the addition of IPTG. Controls were included to which no IPTG was added. As can be seen in Fig. 4.5, bands at approximately 135 kDa (in lanes 5, 6 and 7) represent the overexpressed MMT protein. This estimated size is considerably smaller than the predicted 164 kDa of the plant MMT and may be due to protein folding in its native state, the protocol not fully denaturing the protein and/or the MMT enzyme being processed/degraded in *E. coli*. The *E. coli* containing only the empty vector expressed maltose binding protein, visible at 40 kDa (Lanes 3 and 8), but no bands corresponding to any *Mmt* genes. The WT *E. coli* negative control also showed no MMT-sized bands. This demonstrates that *E. coli* can express plant methyltransferases as a proof of concept.

The next step was to see if *E. coli* expressing AMMT could produce SMM from Methionine as the first step of the methylation pathway.

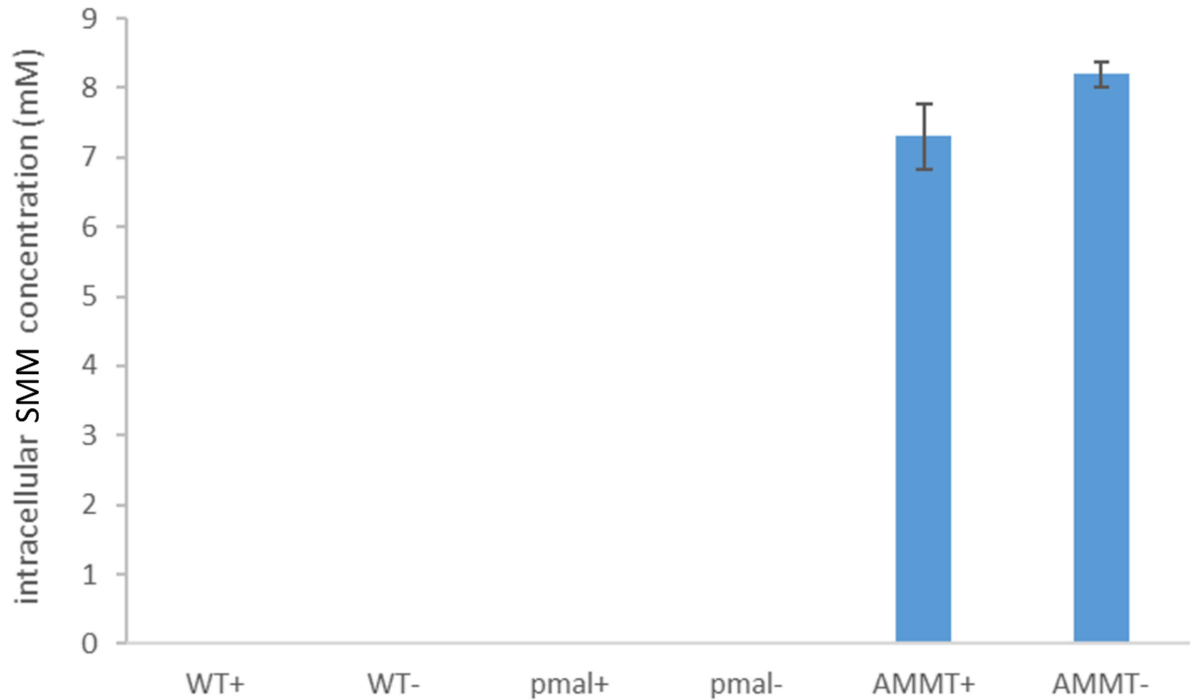


Figure 4.8: Intracellular SMM concentrations of WT *E. coli*, *E. coli* transformed with the empty pmal1039 vector and *E. coli* transformed with pmal1039 containing the AMMT grown in liquid culture with additional Methionine. Cultures induced with IPTG (+) were done so before inoculation into the methionine-containing growth media. Error bars are ± 1 SEM.

E. coli transformed with pmal1039 containing *Ammt* produced significantly more SMM compared to untransformed *E. coli* and *E. coli* transformed with only the pmal1039 vector (Student's T-tests, $p < 0.001$), which did not produce any detectable SMM. This suggests that methionine is successfully converted to SMM, which is converted to detectable DMS by alkaline lysis at 80C.

<u>The effect of salinity on *A. thaliana MMT-* mutant phenotype and DMSP production</u>

To further demonstrate *Mmt* function as the first step of DMSP biosynthesis in *A. thaliana*, *Mmt* knockout plants (henceforth referred to as *Mmt*-) were tested for their DMSP synthesis capabilities and salt tolerance compared to WT plants. Firstly, the plants grown from mutant seeds need to be proven homozygous knockouts, to ensure there is no *Mmt* activity.

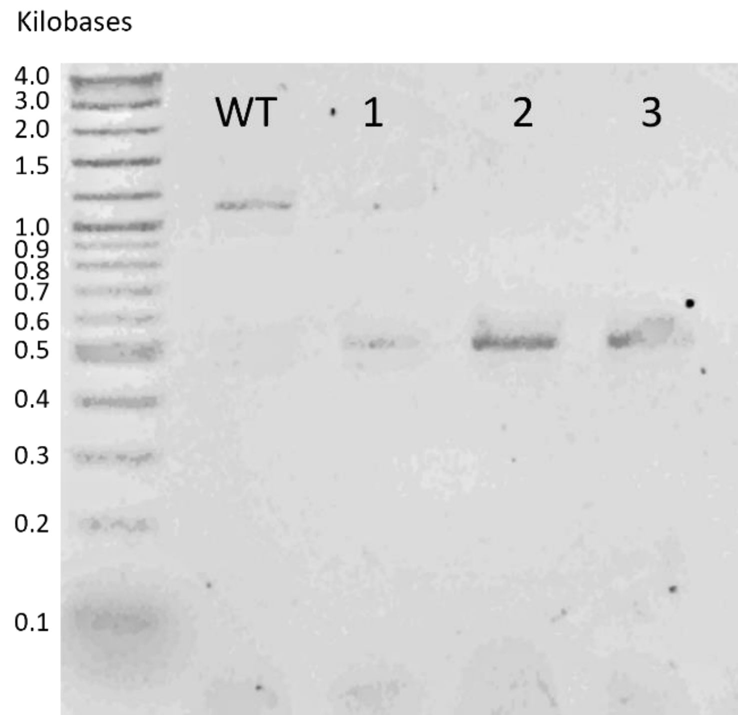


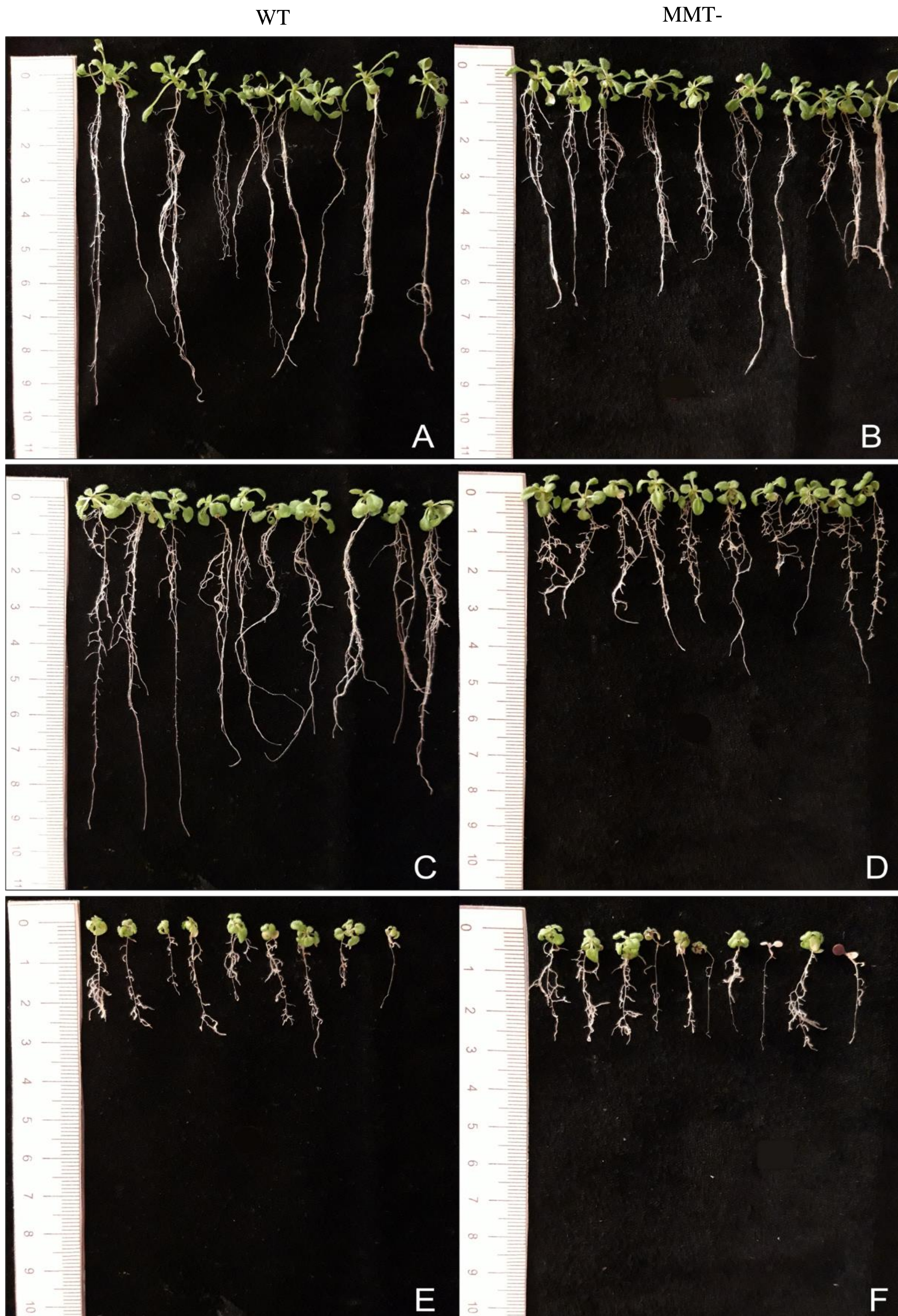
Figure 4.9: Agarose gel to confirm that the *A. thaliana Mmt-* strain is a homozygous knockout mutant. The Gel Electrophoresis of extracted and amplified *A. thaliana* DNA confirms the WT in the WT lane, and the *Mmt-* plants (Lanes 1, 2 and 3).

The presence of the 1.2 kb amplified region in the WT DNA and its absence in the *Mmt-* DNA indicates that the plants grown from the mutant seeds are indeed homozygous knock out (Alonso *et al.,* 2003). This is known as a "Wild-Type PCR" and is a standard method of confirming the presence of a tDNA insert (O'Malley *et al.,* 2015). This uses a combination of a gene specific primer pair (IF.01 and MMTrvs6). Thus, the "Wild-Type PCR" tests for the ability to amplify a genome region that is present in wild type and heterozygous mutants, but not in homozygous lines (O'Malley *et al.,* 2015). This is because the bacterial tDNA insert in the amplified region is 24,000 bp (Barker *et al.,*1983; Gielen *et al.,* 1999), making the DNA too large to effectively amplify, resulting in the 1.2 kbp region in the WT.

This is used in combination with the "T-DNA PCR". This tests for the presence of a specific smaller region between the gene specific forward primer (IF.01) and a standard left border primer that anneals to a 25 bp flanking region of the insert (LBb1) (O'Malley *et al.,* 2015) The smaller bands in mutants of 500 bp are indicative of this region.

The WT and homozygous knockout *Mmt- A. thaliana* plants were then grown on a series of increasing salinities, to determine if there were phenotypic differences that

might indicate increased susceptibility to salt stress. Additionally, the intracellular DMSP concentrations were tested, to see if there were a) differences between DMSP production between WT and *Mmt*- plants and b) if DMSP production scaled with increasing salinity.



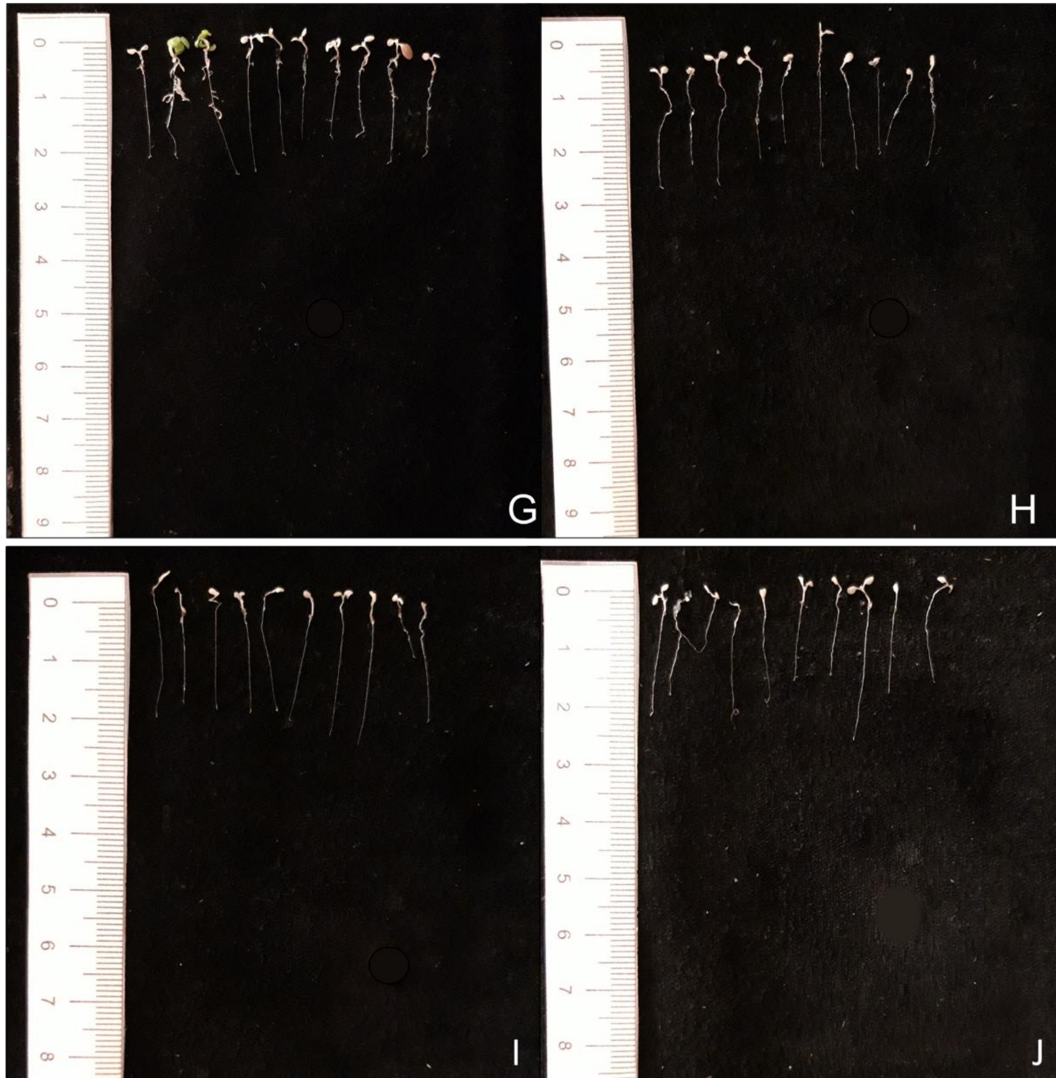


Figure 4.10: Phenotypes of *A. thaliana* plants harvested after 10 days of treatment on MS-Agar with additional NaCl. The left-hand column shows WT plants grown on 0 mM (A), 50 mM (C), 100 mM (E), 150 mM (G) and 200 mM (I). The right-hand column shows *Mmt-* plants grown on 0 mM (B), 50 mM (D), 100 mM (F), 150 mM (H) and 200 mM (J).

Phenotypic differences are most distinct at 50 mM NaCl, where *Mmt-* plants (D) have shorter roots and moderately more wilted leaves than the WT (C). This indicates that *Mmt-* plants are more susceptible to salt stress. This is also evidenced in 100 mM and 150 mM NaCl conditions, where more *Mmt-* plants were bleached and did not progress past the cotyledon (F and H) stage compared to WT (E and G).

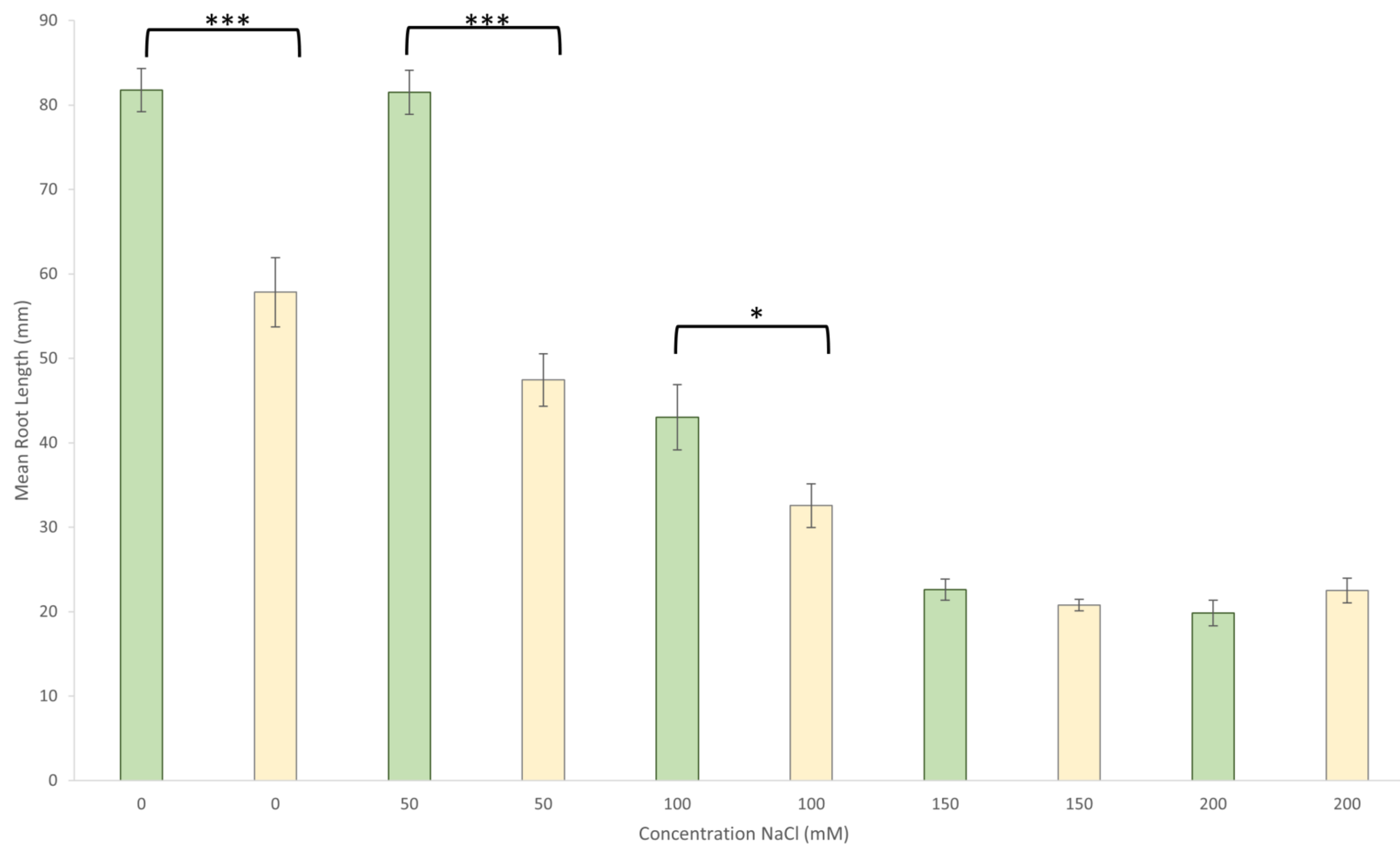


Figure 4.11: Mean root length of WT (green) and *Mmt-* (yellow) *A. thaliana* plants grown on increasing concentrations of NaCl (n = 60 for each treatment condition). Error bars are ± 1 SEM. Significance is denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Mmt- plants did indeed have significantly shorter roots compared to WT in 0 mM, 50 mM and 100 mM NaCl conditions ($p < 0.001$, < 0.001 and $= 0.030$ respectively). Although not statistically significantly different in 150 mM and 200 mM ($p = 0.216$ for both), the general trend of *Mmt*- having shorter roots was consistent. Furthermore, root length was inversely proportional to NaCl concentrations, suggesting that stunted root growth is indeed a consequence of salinity.

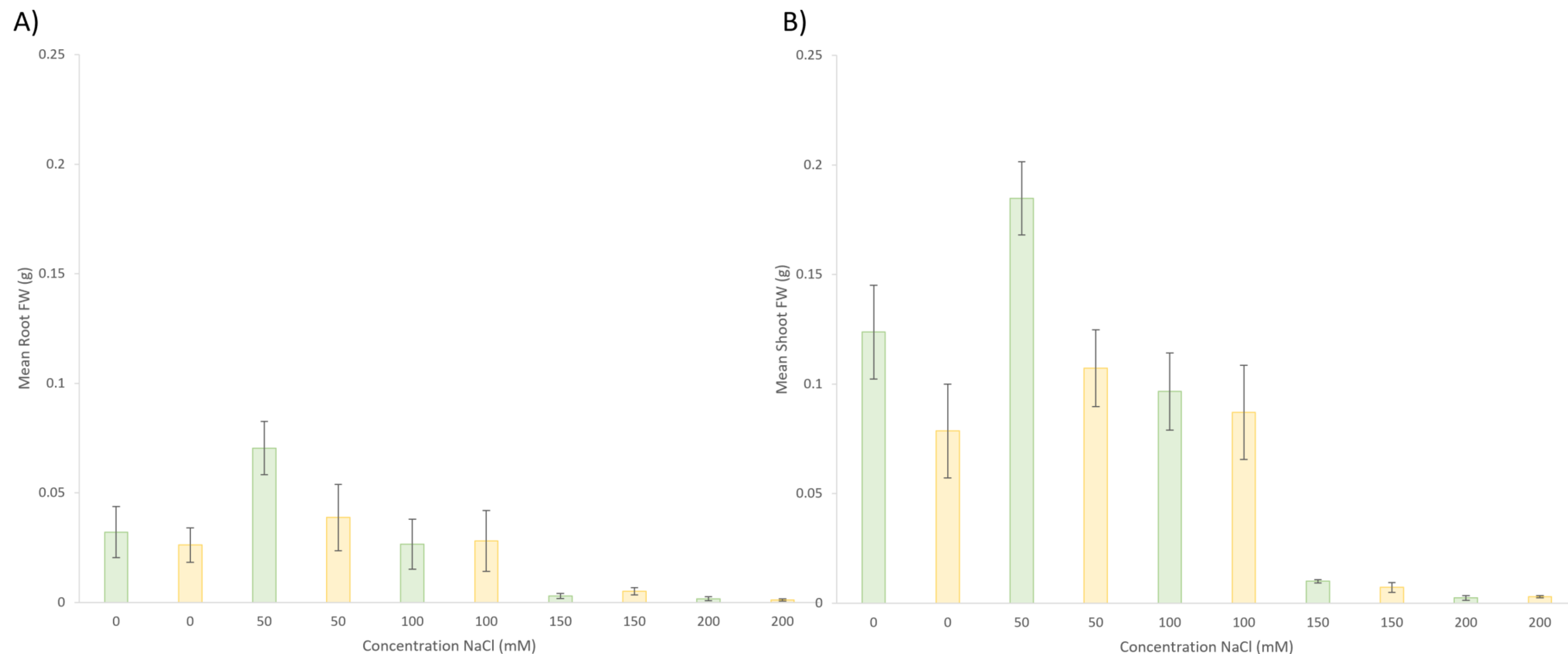


Figure 4.12: A) Mean root fresh weight and B) Mean shoot fresh weight of WT (green) and *Mmt-* (yellow) *A. thaliana* plants grown on increasing concentrations of NaCl (n = 60 for each treatment condition). Error bars are ± 1 SEM.

There were no significant differences between root or shoot fresh weights, when pairwise comparisons were made (Mann-Whitney U, $p = 0.820$ for both data sets), but interestingly both WT and *Mmt*- plants had greater tissue masses in 50 mM NaCl compared to 0 mM NaCl. This might be due to low level NaCl stimulating nutrient and water uptake to ameliorate the effects of NaCl, whereas at higher concentrations (100 mM – 200 mM), the NaCl is sufficiently high to cause tissue damage, resulting in decreased biomass.

To determine if DMSP concentrations were affected by salinity and if knocking out the *Mmt* gene did indeed prevent DMSP production, plant material was subjected to alkaline lysis and assayed for DMS derived by alkaline lysis of DMSP by gas chromatography.

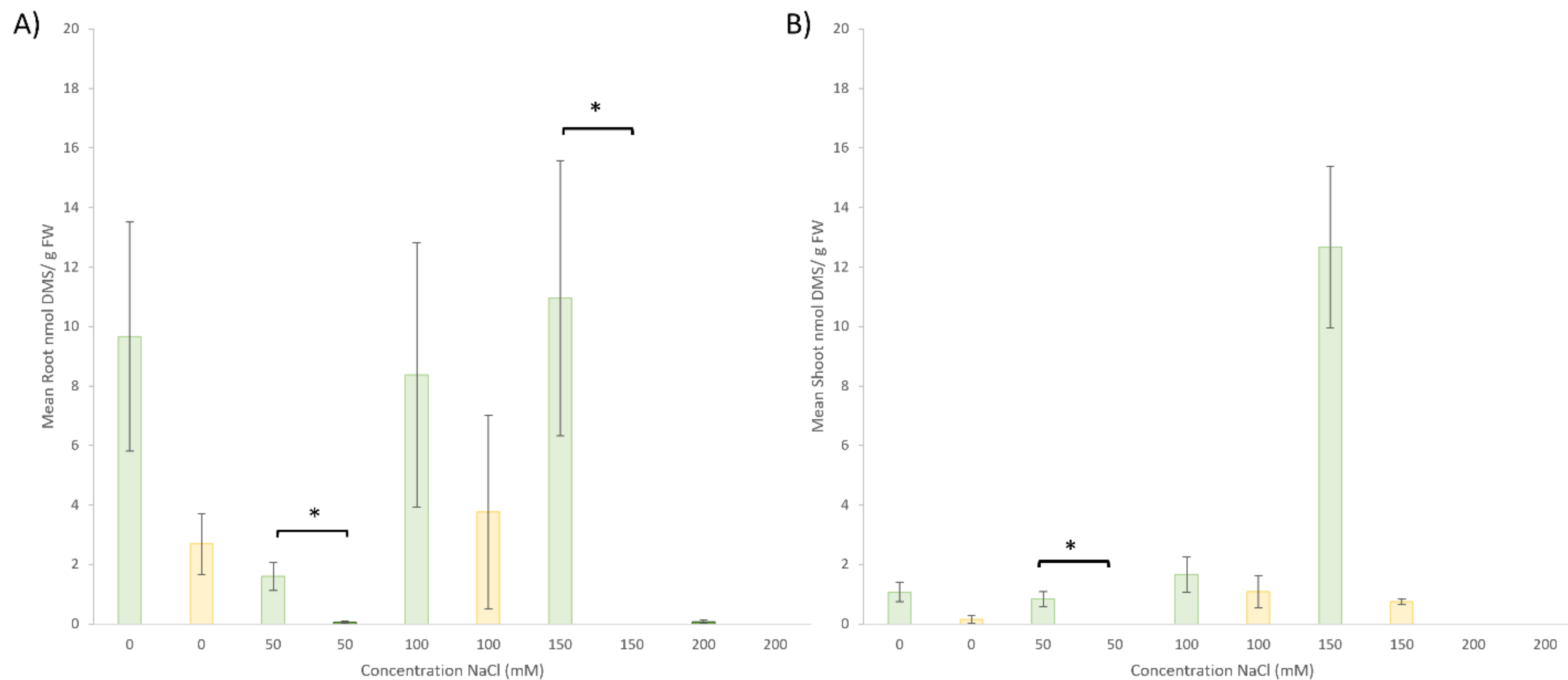


Figure 4.13: A) Mean root nmol DMSP g^{-1} FW and B) Mean shoot root nmol DMSP g^{-1} FW of WT (green) and *Mmt-* (yellow) *A. thaliana* plants grown on increasing concentrations of NaCl (n = 60 for each treatment condition). Error bars are ± 1 SEM.

In all conditions WT plants produced more DMSP than *Mmt*- plants. In the cases of root tissues of plants grown at 50 mM and 150 mM and shoot tissues grown at 50 mM, the WT plants produced significantly higher concentrations of DMSP (Mann-Whitney U tests, $p = 0.037$ in all cases). This suggests that whilst homozygous *Mmt*- plants do make less DMSP than their WT counterparts, knocking out the *Mmt* gene does not knock out DMSP production entirely. This may be because there is functional redundancy of the *Mmt* gene, or because SMM is generated by another pathway, which can then be used in the production of DMSP. Additionally, plants may produce other compounds, such as DMSO, as sources of DMSP. Clearly, more sensitive assays need to be developed to discriminate between potential sources of SMM and DMS, to ensure that future work is more conclusive.

DMSP production increases in WT plants between 50 mM – 150 mM, which might indicate that more is produced in response to increasing salt stress and would support the theory that DMSP functions as an osmolyte in higher plants. However, between 0 mM – 50 mM the DMSP concentrations decrease slightly, which contradicts this hypothesis. The lack of DMSP production in 200 mM is likely due to most of the plants in both conditions dying at such high NaCl concentrations. Therefore, there is no correlation between DMSP concentrations and salinity.

Discussion

Transformation of *E. coli* with *A. thaliana Mmt*

The first finding of this study is that when transformed with the *A. thaliana Mmt* gene, *E. coli* can synthesize SMM. There is no evidence to suggest that wild type *E. coli* can synthesise SMM (Thanblicher *et al.,* 1999), which confirms our results that the SMM detected was the consequence of transformation with plant MMT genes. This result is confirmed by previous studies that show MMT is the first step of the methylation pathway in DMSP synthesis (Kocsis & Hanson, 1998; Liao & Seebeck, 2019; Williams *et al.,* 2019). Regarding *A. thaliana* specifically as a model organism to study DMSP production, this result shows that the first step of the methylation pathway is present, although the remaining steps in the pathway would need to be confirmed by similar approaches.

The ubiquitous nature of *Mmt* and its role in synthesising *S*-methylmethionine are well characterised in relation to the activated methyl cycle and the generation of

homocysteine (Ranocha et al., 2001). Consequently, the production of SMM cannot be interpreted as indicative of DMSP biosynthesis in isolation. However, this represents the exciting first stage of an avenue of improving salt tolerance in crops using transgenic approaches. The ability for crop plants to produce additional and/or increased osmolytes in response to salinization is critical for ensuring food security (Jamil *et al.,* 2011). Transgenic approaches to achieving this have been steadily increasing in popularity and sophistication (Kotula *et al.,* 2000) and the ability to transform plants with the genes in the DMSP biosynthesis pathway may be a mechanism to further this. Successful transformation of *E. coli* with the first step of this pathway from high DMSP producing plants, such as Sugarcane, is the first proof of concept. To further this approach, this would need to be repeated with the remaining putative genes in the methylation pathway. Once proving that each step converts the precursor into the next product in *E. coli*, the next step in completing this first stage is to prove that low DMSP producing plants, such as Wheat, would be able to produce each intermediate of the pathway, and DMSP, in increased levels compared to untransformed plants.

Subsequent, gene function can would need to be further ratified within a plant model. This can be achieved by increasing the gene copy number through floral dip. This method transforms plant ova by immersing inflorescences in sucrose solution containing *Agrobacterium tumefaciencens* with additional copies of the gene in question in their plasmid, resulting in transformed plants within one or two generational cycles (Zhang *et al.,* 2006). In this scenario, to further ratify *Mmt* function, the expected result in response to increased copy number would be a higher intracellular concentration of DMSP. Another approach is to increase transcription of potential genes using a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 to introduce a tag to the activation domain to recruit multiple copies of the transcriptional activators (Casas-Mollano *et al.,* 2023). Increased transcription of genes in the pathway will lead to increased intracellular concentrations of DMSP.

Saline tolerance in MMT- *A. thaliana* compared to Wildtype

Mmt- A. thaliana plants were shown to be significantly less salt tolerant than wildtype plants. *Mmt*- plants had more bleached aerial tissues than their WT counterparts (Fig. 4.10); chlorosis and necrosis being key indicators of stress and nutrient deprivation in many plant species (Colin *et al.,* 2023). This may be the consequence of a lack of DMSP to ameliorate the increased reactive oxygen species in response to salt stress (Kesawat *et al.,* 2023), which causes chlorophyll degradation (Taïbi *et al.,* 2016). This

is supported by the inverse experiments that found that *Mmt* overexpression in higher plants results in decreased chlorosis (Liu *et al.,* 2014; Ma *et al.,* 2017) and increased tolerance to the effects of ROS (Ezaki *et al.,* 2016).

Potentially there may be a direct link between DMSP and chlorophyll concentrations. In photosynthetic microalgae *Gyrodinium impudicum* (Belviso *et al.,* 2000) and *Phaeocystis globosa* (Royer *et al.,* 2021), the intracellular concentrations of DMSP:Chlorophyll-*a* are directly proportional. This is believed to be a mechanism to maximise energy capture from sunlight, whilst protecting against UV radiation (Bell *et al.,* 2010). This mechanism has yet to be proven in higher plants but is a possible explanation for the increased lack of pigment in *Mmt- A. thaliana.*

Mmt- A. thaliana plants also had significantly shorter roots than their wildtype counterparts (Fig. 4.11), a known characteristic of salt-stressed plants (Chen *et al.,* 2019). Additionally, *Mmt- A. thaliana* plants had significantly decreased shoot and root fresh weights at 50 mM NaCl compared to those grown at 0 mM NaCl (Fig. 4.12). This is supported by previous studies that showed the inverse; plants that over-express *Mmt* had increased tissue fresh weights (Liu *et al.,* 2014; Ma *et al.,* 2017; Ogawa & Mitsuya, 2012). This may be the consequence of increased salt sensitivity due to the lack of DMSP, supporting the theory that DMSP functions as an osmolyte in higher plants. Furthermore, DMSP is inversely correlated with relative foliar water content in *Arundo donax* (Haworth *et al.,* 2017) under drought conditions. Drought and salt stress produce similar phenotypic responses in higher plants (Cao *et al.,* 2023; Mahajan & Tuteja, 2005). It is possible that a decrease of DMSP in *Mmt- A. thaliana* results in increased water loss from leaf tissues, which is reflected as a decrease in the fresh weights of *Mmt-* plants compared to the WT.

However, there is also the possibility that increased salt sensitivity may be because by knocking out *Mmt*, the whole SMM cycle is disrupted. Although previous studies showed the *Mmt- A. thaliana* grew and reproduced normally, compared to the WT counterparts (Koscis *et al.,* 2003), this study did not assess the effect of disrupting the SMM cycle on saline tolerance. SMM has been implicated in salt tolerance (Hafeez *et al.,* 2021). For example, glycine betaine, a well characterised osmolyte, requires SMM as part of its synthesis (Tabuchi *et al.,* 2005). Disruption to the SMM cycle could lead to decreased glycine betaine as an off-target effect, reducing salt tolerance. Furthermore, glycine betaine acts as a growth-regulating phytohormone, and promotes cell elongation (Hernandez-Leon & Valenzuela-Soto, 2023) which may result in a shorter root

phenotype. Additionally, the loss of homocysteine due to AdoMet not being converted may also produce a stressed phenotype. Homocysteine has been shown to confer drought tolerance in Barley by increasing SAM production, which can scavenge reactive oxygen species (Qiu *et al.,* 2023). It is not unreasonable to think that it may contribute to tolerating other abiotic stresses, and thus the increased salt sensitivity in *Mmt- A. thaliana* cannot be conclusively attributed to a reduction in intracellular DMSP.

DMSP Production in MMT- *A. thaliana* compared to Wildtype

The most critical finding of this investigation is that in all salinities, *Mmt- A. thaliana* produced less detectable DMSP compared to their WT counterparts (Fig. 4.13). This, combined with the salt-stressed phenotype, supports the growing body of evidence that DMSP functions as an osmolyte in higher plants. The significant increase in DMSP production in plant tissues when exposed to salt is in keeping with a study that found that salt stressed *A. thaliana* Col-0 seedlings showed increased expression of *Mmt*- and consequently significantly increased SMM concentrations – compared to control seedlings (Ogawa & Mitsuya, 2011). The authors of this study suggest that the increased salt tolerance is the result of SMM directly stabilising cell membranes or its conversion to S-adenosylmethionine. However, they did not determine the fate of the SMM. It is possible that the increases in salinity in their study are the result of upregulation of the Methylation pathway, of which SMM is the first intermediate (Kocsis & Hanson, 1998; Liao & Seebeck, 2019; Williams *et al.,* 2019), supporting the argument that DMSP is an osmolyte in *A. thaliana*. In general, the root tissues produced more DMSP than the shoot tissues across all treatment conditions and genotypes. This is consistent with the findings of the previous chapter and is likely due to the root tissues being in direct contact with the NaCl-containing media (Narayan *et al.,* 2022).

However, although *Mmt*- plants showed decreased DMSP production compared to the WT, it was not a full knock out of DMSP production. There is no evidence to suggest that DMSP is produced through the decarboxylation or transamination pathways used by dinoflagellates and marine bacteria, respectively (Kocsis & Hanson, 2000; Kocsis et al., 1998). Furthermore, SMM is exclusively produced through the methylation of methionine (Amir, 2010; Ranocha *et al.,* 2001), supporting that *A. thaliana* produces DMSP through the methylation pathway (James et al., 1995; Kocsis & Hanson, 2000; Kocsis et al., 1998). There is also no evidence to suggest that any of the downstream intermediates (DMSP-amine and DMSP-aldehyde) are produced by any other pathways, that might intercede even without SMM (Carríon *et al.,* 2023; Kocsis & Hanson, 2000;

Stefels, 2000). Without compensatory pathways to supply intermediates when the pathway is disrupted by *Mmt*- loss of function, this suggests that there are multiple *Mmt* genes that can methylate methionine to SMM.

Given the ubiquitous nature of *Mmt* in higher plants and the multiple functions it possesses (Ranocha *et al.,* 2001), it is highly likely that plants would have more than one methyltransferase that could compensate for the loss of the *Mmt* gene knocked out. Gene redundancy is common in plants, including *A. thaliana* (Gottlieb, 2003). This prevents mutations in important genes, such as those that encode signalling compounds, from causing a lethal phenotype (Briggs *et al.,* 2006). It is therefore possible that another SAM-dependent methyltransferase of the large family that has been identified in plants (Lashley *et al.,* 2023) may be able to compensate for the loss of the specific *Mmt* mutated, to confer partial redundancy (Briggs *et al.,* 2006). For example, homocysteine-S methyltransferase has three isoenzymes that provide a compensatory phenotype when one is mutated to lose function (Cohen *et al.,* 2017). To fully test this hypothesis, a comprehensive reverse genetic approach is required in which all possible genes with sequence homology are identified and knocked out, to test the downstream SMM production in *A. thaliana*.

Another approach would be to utilise Stable Isotope Probing (SIP). This method will be discussed at length in the next chapter, but briefly, tracks of compounds through a metabolic pathway using isotope enrichment. This requires the generation of a heavier isotope – in this case either ^{13}C or ^{35}S, incorporated into the substrate likely to be used by the gene of interest, here Methionine. If *Mmt* is indeed functional, plants grown on substrate containing the labelled methionine would be used to synthesize DMSP, which would also incorporate the heavy isotopes. The successful incorporation of the heavy isotope can be analysed by LC-MS of extracted DMSP and would indicate functional plant *Mmt.* This approach has been successfully undertaken for other osmolytes, namely glycine betaine (Wilhelm *et al.,* 2022).

Furthermore, ratification of the next steps of the pathway is also essential to conclusively piece together the entire pathway. Potential genes for the next stages of the methylation pathway have already been identified (Fig. 1.9) through interrogation of the TAIR database for homologues to known bacterial genes in the methylation pathway. The previously mentioned approaches to ratifying *Mmt* would also apply to these downstream candidate genes, but there is a large suite of metabolomic approaches to which *A. thaliana* has shown itself to be amenable. For example, the use of high-

definition mass spectrometry in conjunction with transcriptomic analysis has been used to assess biochemical changes in response to a multitude of abiotic stresses (Garcia-Molina & Pastor, 2024). Another approach is Direct Analysis in Real Time – Time Of Flight Mass Spectrometry (DART-TOMFS). This elegant system is widely used in dendroforensics (analysis of wood specimens), that utilises electronic excitement of ions in a specimen, which causes ion-molecule reactions with the sample molecules to produce analyte ions that then be analysed directly (Time of Flight) Mass Spectrometry. This technique has the advantage that it can be done in ambient temperatures and pressures, without the need for extensive sample preparation and is ideal for analysing gases present in plant samples (Parades-Villaneuva *et al.,* 2018; Roepenak-Lahaye *et al.,* 2004). This technique could well be applied to the analysis of gaseous intermediates in the DMSP methylation pathway.

Suitability of *A. thaliana* as a model organism for studying DMSP biosynthesis in plants

To summarise, these initial studies into the DMSP synthesis pathway in *A. thaliana* suggest that it can be used a model to sequentially knock out all the genes in the pathway, to test their DMSP production and phenotypic differences. However, given the comparatively low intracellular DMSP concentrations compared to other species, such as *S. anglica*, and the partial DMSP production in MMT- homozygous knockouts, it may not be the most robust model for future work. The benefits of its other characteristics that make it a model organism may not outweigh the drawbacks DMSP-biosynthesis specific research.

Despite these drawbacks, these findings contribute to the hypothesis that DMSP biosynthesis is ubiquitous in higher plants, and that even relatively small concentrations still contribute to global sulfur flux, as a cumulative effect. Further research into DMSP production from higher plants is therefore essential to understanding how terrestrial systems contribute to climate cooling effects and possible amelioration of the effects of salinization in crop plants.

Chapter 5 - Analysis of microbes using DMSP as a carbon source in the *Spartina anglica* rhizosphere by DNA-Stable Isotope Probing

Introduction

Salt Marsh Ecology – An Overview

Salt marshes are globally distributed coastal ecosystems, found in the intertidal zone between the sea and upland (Adam, 1990). Subject to regular flooding by incoming tides, salt marshes experience daily periods of submergence, causing hypoxia, and salt intrusion (Adam, 1990; Woodroffe, 2002). It is estimated that there are approximately 5.5 million hectares of salt marsh distributed globally (Mcowen *et al.,* 2017), with the majority found in temperate and sub-arctic zones of the Northern hemisphere (Mcowen *et al.,* 2017; Adam, 1990). Of specific interest to this chapter, salt marshes are known to be high producers of DMSP, both from the predominance of high producing plants such as *Spartina sp.* (Rousseau *et al.,* 2017) and sediment bacteria, such as *Novosphingobium*, *Oceanicola* and *Alteromonas* (Williams *et al.,* 2019).

Salt marshes are characterised by a unique dominance of halophytic species. Macrofauna, such as wading birds and crustaceans, are found in salt marshes across the globe (Rinke *et al.,* 2022), although regional naturally variation exists. An interesting variety of halophytic plants are found in salt marshes, in some cases uniquely. Southern hemisphere salt marshes are characterised by a predominance Mangrove species (*Rhizophora*) (Veldkornet, 2023). Northern hemisphere salt marshes have less tree-sized plants, but a large variety of *Poaceae*, such as cordgrasses (*Spartina spp*.), rushes (*Juncaceae*) and reeds (such as *Arundo donax* and *Phragmites australis*) (Adams, 1990; Davy, 2008; Frid & James, 1989). Additionally, there are abundant flowering plant species, such as Samphires (*Salicornia spp.*), Sea Daisies (*Aster tripolium*) and Sea Lavender (*Limonium vulgare*) (Adams, 1990; Davy, 2008; Frid & James, 1989; Williams *et al.,* 2019).

Plant Adaptations to the Salt Marsh Environment

With shallow surface sediments deposited by the tides (French, 2019), much of the flora of saltmarshes have the following physiological adaptions: salt glands, the ability to form rhizome networks as anchor points, highly aerated roots or aerial roots (Adam, 1990; Davy,

2008). These are additional to mechanisms of halotolerance previously discussed on page 76. Plant salt glands are aerial, epidermal structures (Flowers & Colmer, 2015), that in their cellular composition are similar to trichomes (Esau, 1965). These can be split into two sub-groups; vacuolar bladders that collect salt and store it away from the other leaf cells (Ding *et al.,* 2010) or secretory vessels that directly channel salt onto the leaf surfaces (Fig. 3.1C) (Breckle, 1990). The former are typical of salt-marsh plants such as Ice-Plants (*Aizoaceae*) found in the southern hemisphere salt marshes (Dassanayake & Larkin, 2017). The latter are more widely distributed amongst higher plants and found in a range of salt-marsh plants such as Sea Daisies (*Asterids*) and Mangroves (*Rosids*) (Veldkornet, 2023).

Adaptations to root structures are another common feature in salt-marsh plants. Many salt-marsh plants form dense networks of horizontal rhizomes (Fig. 3.1B) that form interlocking meshes with neighbouring clumps (Granse *et al.,* 2022; Roberts, 2008). This is particularly prominent in cordgrasses (*Spartina spp.*) and allows them to withstand the movement of sediment by the tide, as well as limiting erosion of sediment (French, 2019). A unique feature of wetland plants is that they have large air-filled intercellular spaces, known as aerenchyma, within their roots (Fig. 3.1A), which allows for oxygen to continue to be transported in the stem even in hypoxic, waterlogged conditions (Granse *et al.,* 2022). In some species, these air-filled, root networks may also be found above the surface of the sediment to further increase oxygen availability and reduce osmotic disruption, a characteristic feature of Mangrove species (Nguyen *et al.,* 2023). These aerial roots, known as pneumatophores, have stomata-like structures, or lenticels, that allow for oxygen diffusion into spongy tissues of the roots, for diffusion into the cells (Kazemi *et al.,* 2021).

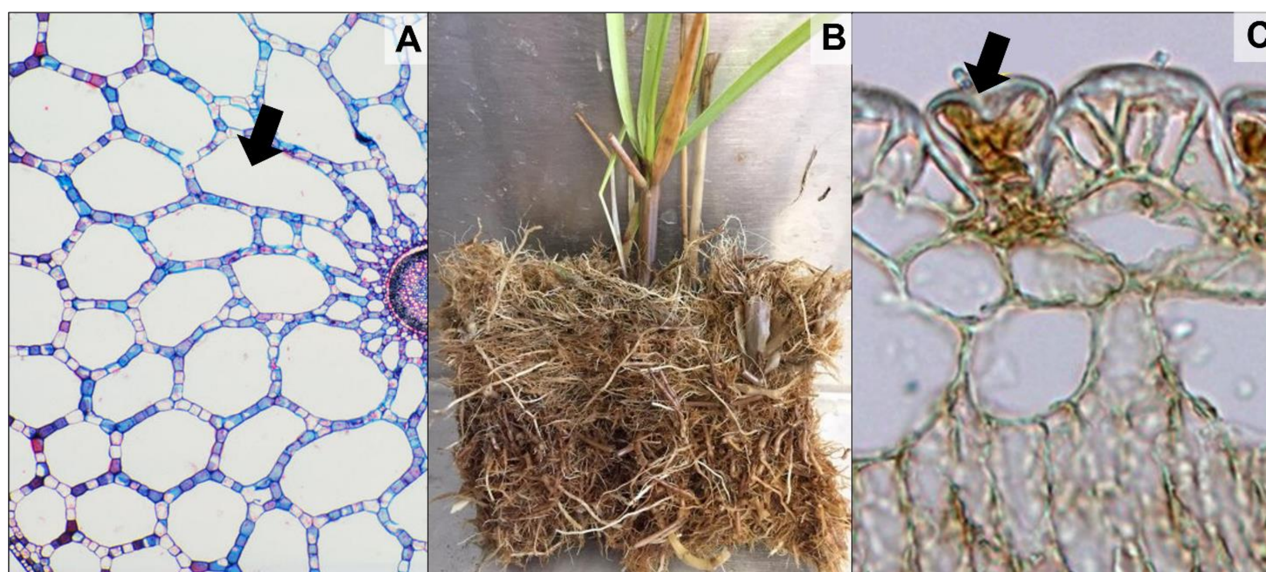


Figure 5.1: Physiological adaptations of halophytes and hydrophytes associated with salt marshes. A) Light micrograph transverse section of Mare's Tail (*Hippuris vulgaris*) stem, showing the large, air-filled aerenchyma (indicated by the black arrow). Image credit: Walker, 2013. B) Photograph of cross section of Common Cordgrass (*Spartina alterniflora*) rhizome, showing the dense network of adventitious roots and rhizomes. Image credit: Davis, 2017. C) Confocal micrograph of Indian Mangrove (*Avicennia officinalis*) upper epidermal surface with a salt gland (indicated by the black arrow). Image credit: Tan *et al.*, 2015.

The Rhizosphere: Plant-Microbe Interactions

In addition to the biochemical and physiological adaptations to the salt marsh environment, wetland plants also rely on symbiosis with microorganisms in the soil surrounding the root, known as the rhizosphere. The rhizosphere is defined as the soil immediately influenced by root exudates and is typically not more than 10 mm from any root surface (Walker *et al.,* 2003). It is more diverse in terms of species, compared to the microbiome of the aerial tissue, known as the phyllosphere, (Kroll *et al.*, 2017) as the soil is a more chemically stable and nutrient rich environment (Bodenhausen *et al.,* 2013). Salt marsh soils are characterised by poor drainage and clay-rich soils (Woodroffe, 2002). Levels of bioavailable organic carbon are often plentiful due to deposition of carbon-fixing benthic microalgae (Middleburg *et al.,* 1997) but are subject to seasonal fluxes (Tobias and Neubauer, 2009). Additionally, iron levels are typically high due to the presence of iron rich pools (Williams *et al.,* 2019), making

SO_4^{2-} ions a valuable chemical commodity for the reduction of iron oxides (Tobias & Neubauer, 2009).

There are multiple factors that affect the species present in the rhizosphere. The plant genotype influences the members of the microbiome through the secretion of different carbon sources that microorganisms can utilise (Emmet *et al.,* 2017), as well as a variety of anti-microbial compounds that select for different species (Breen *et al.,* 2015). The microbiome also varies with the age of the host plant (Wagner *et al.,* 2016), with older plants typically having greater species diversity (Zhang *et al.,* 2023).

Also of relevance to this chapter, abiotic factors also critically shape the microbiome, including Ph, temperature, oxygen concentration, metal ion concentration and water availability (Santoyo *et al.,* 2017). A fundamental factor that influences the soil microbiome, as well as plant health, is the elemental composition of the bulk soil. Plants require seventeen key elements for growth and development, of including sodium and sulfur (Singh and Schulze, 2015). Saline soils found in coastal regions are characterised by elevated metal chloride and sulphate concentrations, notably sodium chloride, which lead to more alkaline conditions and poor water availability (Gupta and Abrol, 1990), as discussed at length in Chapter 2. This is a challenging environment for plants to be able to assimilate enough oxygen, nitrogen and water (Hingole and Pathak, 2016), as well as cope with increased concentrations of metal ions, leading to cellular toxicity (Serrano and Rodriguez-Navarro, 2001).

<u>The Rhizosphere and its Role in Plant Stress Adaptation</u>

The plant rhizosphere is known to play important roles in other types of plant stress. This can be through modulation of plant signalling. For example, the bacteria *Pseudomonas putida* degrades the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) by deamination, preventing ethylene derived leaf abscission (Glick, 2005). Perhaps one of the best characterised examples of the rhizosphere in stress tolerance is that of pathogen suppression through a wide variety of mechanisms. The bacteria *Pseudomonas fluorescens* suppresses the pathogenic fugus *Fusarium oxysporum* by the degradation of fungal cell walls, the secretion

of iron-chelating siderophores (León *et al.,* 2009) and release of the diacetylphloroglucinol, an anti-fungal compound (Meyer *et al.,* 2009).

The rhizosphere is critical for plant survival in saline conditions. Halotolerant strains of different phyla of bacteria, such as *Proteobacteria* and *Firmicutes* are unsurprisingly dominant in saline bulk soil (Naylor and Coleman-Derr, 2018), but the species present in halophile rhizospheres are less well characterised. Whilst there are many well explained examples of single species, the rhizosphere holobiont is less understood. There are multiple bacterial species that confer resistance to drought conditions, a common feature of saline soils. For example, *Achromobacter piechaudi* modulates ethylene production using the same ACC deaminase as *P. putida*, which increases the fresh weight of *Solanum lycopersicum* grown in arid environments (Mayak *et al.,* 2004). In salt marsh environments, where drought is often of far a less of concern to plants compared to the overwhelming volume of sea water they are regularly flooded with, there are distinct patterns of bacterial distributions that correspond to the proximity to the ocean (Blum *et al.,* 2004; Bowen *et al.,* 2009; Wang *et al.,* 2017).

Additionally, the species of plant and distribution of heavy metals are also critical factors in the species of bacteria present. A study of spatial differences in *S. alterniflora* and *S. patens* rhizobacterial abundance and community composition suggests that the increasing alkalinity of the soil correlating with proximity to the sea was the primary driver of changes in community composition (Bowen *et al.,* 2009). The study did not, however, document that species present. This limits the conclusions drawn as there may be species specific adaptations at play that were unrelated to the soil Ph. Another study contradicts this hypothesis and following comparisons of bacterial communities from a variety of salt marsh plants, including three *Spartina* species and *Phragmites australis* (as surveyed by us in Chapter 1) that the species of plant has a greater influence on the rhizobacteria present (Blum *et al.,* 2004). It is to be noted that proximity to the sea will affect the plant species present (Adam, 1990), although it is probable that multiple factors will affect the rhizobacterial composition.

Furthermore, there is an emerging body of evidence that suggests the microbiome may confer specific salt stress resistance (Fig. 3.2). *Spingomonas sp.* LK11 has been shown to upregulate glutathione production in *Solanum pimpinellifolium,* which binds to Reactive Oxygen Species and protects the plants against salt stress induced damage (Khan *et al.,* 2017). Also, Plant Growth Promoting Rhizobacteria (PGPR) have been found to module host phytohormones that results in upregulated salt compartmentalisation and proton pump production (Ilangumaran and Smith, 2017). However, it is clear from reviewing the literature that comparatively little is known about the specific bacterial species present in the salt marsh rhizospheres, and that mechanisms by which a symbiotic relationship exists between plants and bacteria is poorly understood. DMSP has been proposed as an infochemical for signalling between plants and bacteria (Schmidt & Saha, 2020). It has also been demonstrated that salt marsh sediments are high producers of DMSP (Williams *et al.,* 2019) and that it can be used as an osmolyte by sediment bacteria, but its role as specifically as a rhizobacterial compound in plant symbiosis has not been specifically explored. Thus, this chapter aims to identify specific microbial groups utilising DMSP from *Spartina anglica* as a carbon source, what genes and pathways they use to do so and if such bacteria possess the potential to interact with the host plant.

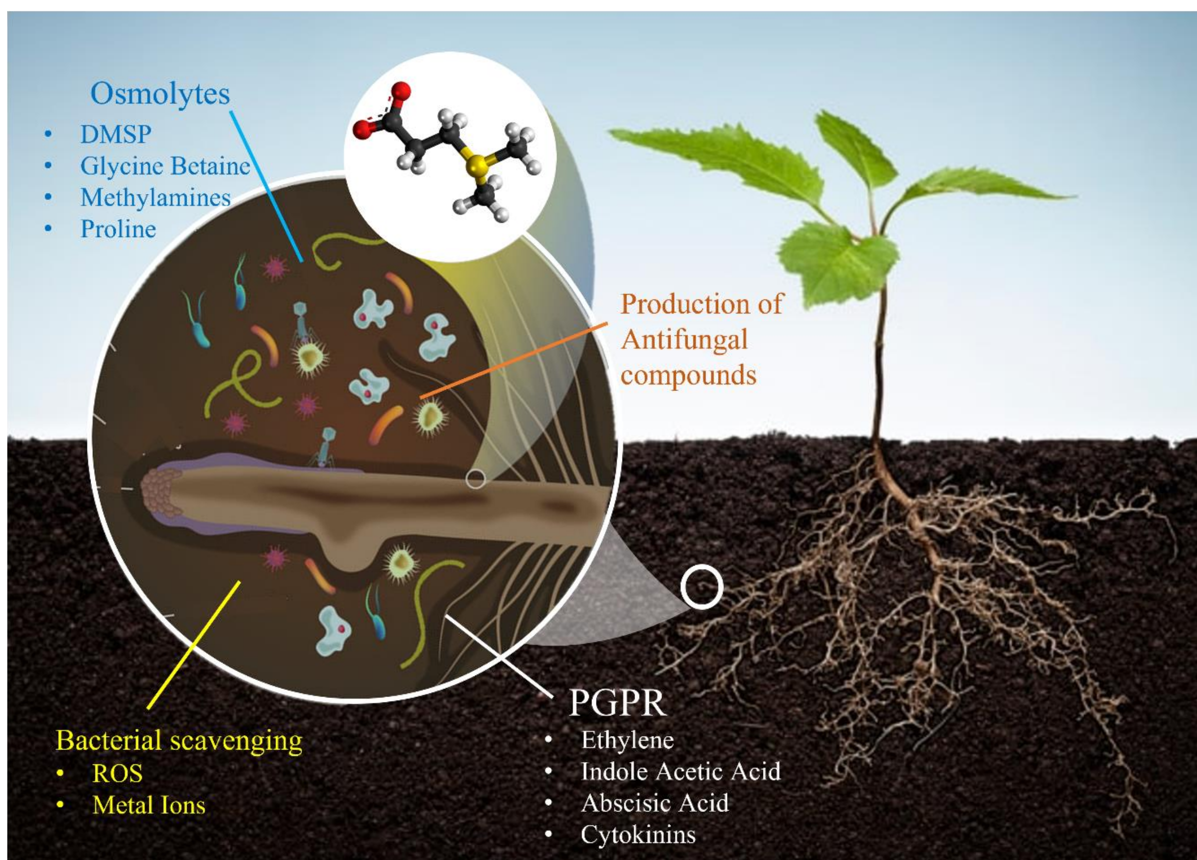


Figure 5.2: Summary diagram of the beneficial activities of rhizosphere associated bacteria in response to saline soil environments of salt marshes. Image adapted from White III *et al.*, 2017.

Molecular Ecology Techniques to Survey DMSP-producing Microorganisms

To this end, it is essential to employ techniques that can generate links between bacterial phylogeny and DMSP metabolism, which traditional methods of culture and 16S rRNA gene amplicon DNA sequencing are unable. Shotgun metagenomic sequencing will allow predictions but it is possible that only a very small proportion of the DNA sequenced will be from microbes that are actually degrading the DMSP. Therefore, a technique such DNA-Stable Isotope Probing (SIP) that captures the breadth of groups of bacteria present in the *Spartina anglica* rhizosphere that assimilated the ^{13}C in the labelled substrate of choice (DMSP in this case) (Radajewski *et al.,* 2000). In addition, we also plan to use culture-dependent techniques to uncover model bacteria that we can study their response to DMSP etc to support our hypotheses we generate (Fig. 3.3).

DNA-SIP is an elegant technique that enables the tracking of compounds through a metabolic pathway using isotope enrichment (Dumont & Murrell, 2005). The compound of interest, in this case DMSP, is generated with a stable, heavy isotope of carbon, ^{13}C, on each of the 3 carbons in acrylic acid. This can be introduced into culture of bacteria obtained from a natural environment at regular intervals. Bacteria that catabolise the compound of interest will subsequently incorporate the ^{13}C into their DNA, which can be separated by buoyant density (or isopycnic) centrifugation (Dumont & Murrell, 2005; Neufeld *et al.,* 2007). The heavy fraction of DNA can then be sequenced with the light fraction, to find out which bacteria are able to catabolise the compound of interest.

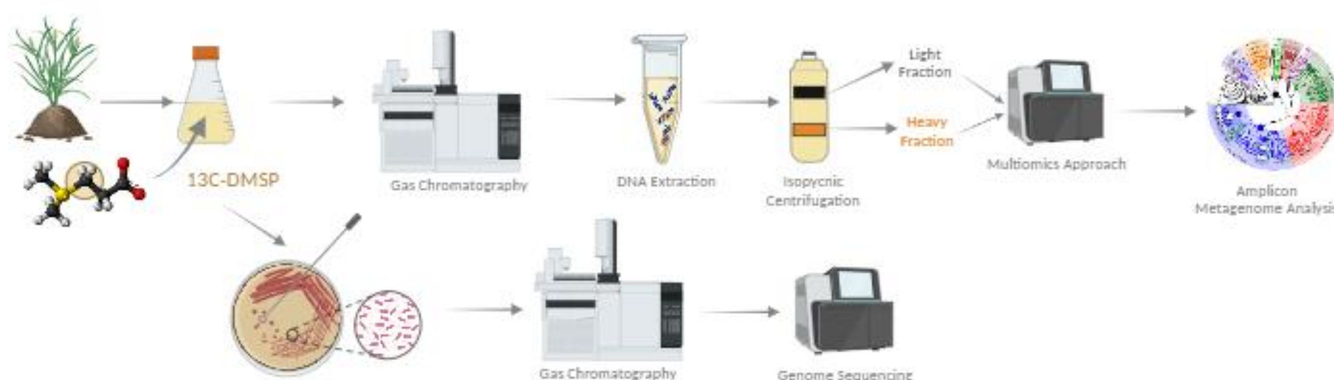


Figure 5.3: Work-Flow of DNA-SIP in which rhizosphere cultures are grown with ^{13}C-DMSP as a sole carbon source, allowing for assimilation into the DNA of DMSP-catabolising bacteria. The extracted DNA from these samples undergoes isopycnic centrifugation through a Caesium Chloride (CsCl) matrix, that separates DNA fractions based on density. The fractions are eluted separately, sent for Next Generation Sequencing to enable metagenome analysis of the relatedness of DMSP-metabolising bacteria to be determined. Figure adapted from Liu *et al.*, (2022).

The use of ^{13}C was first successfully employed in elucidation of soil methylotrophs using Methanol ($^{13}CH_3OH$) and Methane ($^{13}CH_4$) (Radajewski *et al.,* 2005), and has subsequently been used to assess both rhizobacteria (Carrión *et al.,* 2020; Larke-Meija *et al.,* 2019) and bacteria that can metabolise DMSP in coastal seawater (Liu *et al.,* 2022). However, this chapter represents the first time that DNA-SIP has been used to combine these two areas and look specifically at DMSP-metabolising rhizobacteria associated with *S. anglica*.

This approach is enhanced by metagenome analysis. Metagenomics is the study of the collective genetic material of all microbes in a specific environment (Clark & Pazdernik, 2013). This does not rely on primers to be designed for known DNA sequences, unlike traditional amplicon sequencing, but rather fragments and sequences all the DNA isolated from the community, in an approach known as "shotgun" sequencing (Clark & Pazdernik, 2013). This is extremely useful in identifying unculturable bacterial species, as sequences obtained – or reads – can be readily compared to existing genome sequences. Additionally, this approach also allows for large scale analysis of groups of bacteria to see if they contain homologues to genes associated with specific metabolic processes. Traditional culture-dependent methods to study the same would take a great deal of time and resources, compared to a metagenomics approach. Naturally, this process is also time consuming, and requires specialist bioinformatics skills, but when combined with DNA-SIP and culture dependent work, this presents a comprehensive study on the microbial diversity and metabolic capabilities in a given environment.

Aims and Objectives

The aims of this chapter were firstly to uncover the diversity of bacteria within the natural *Spartina anglica* rhizosphere and what genes they express via metatracriptomics. Secondly, to quantify DMSP catabolism and production through measurement of produced DMS and MeSH through a DMSP DNA SIP experiment. Thirdly, to investigate the abundance, expression and taxonomy of DMSP catabolic genes with the bacterial community through metagenomic analysis in the natural and DMSP DNA SIP samples.

Materials and Methods

Sampling

The enrichment and isolation research described in this study was performed on rhizosphere samples from *Spartina anglica* obtained from Stiffkey saltmarsh, UK (latitude 52.949156° N, longitude 0.926132° E). Sampling was carried out on the 16th May 2022, at low tide. The average temperature for that day was 14 °C, with no rainfall. Triplicate samples of rhizoplane

and ectorhizosphere soil were collected from the rhizome/root cluster by shaking the cluster to remove loose soil and discarded. The soil adhering to the cluster up to a 4 mm distance was removed using ethanol sterilized scalpels (Bouray *et al.,* 2021). DMSP content was also measured in samples (200 µl) taken from the bulk soil 1 m away from the root/rhizome cluster for comparison.

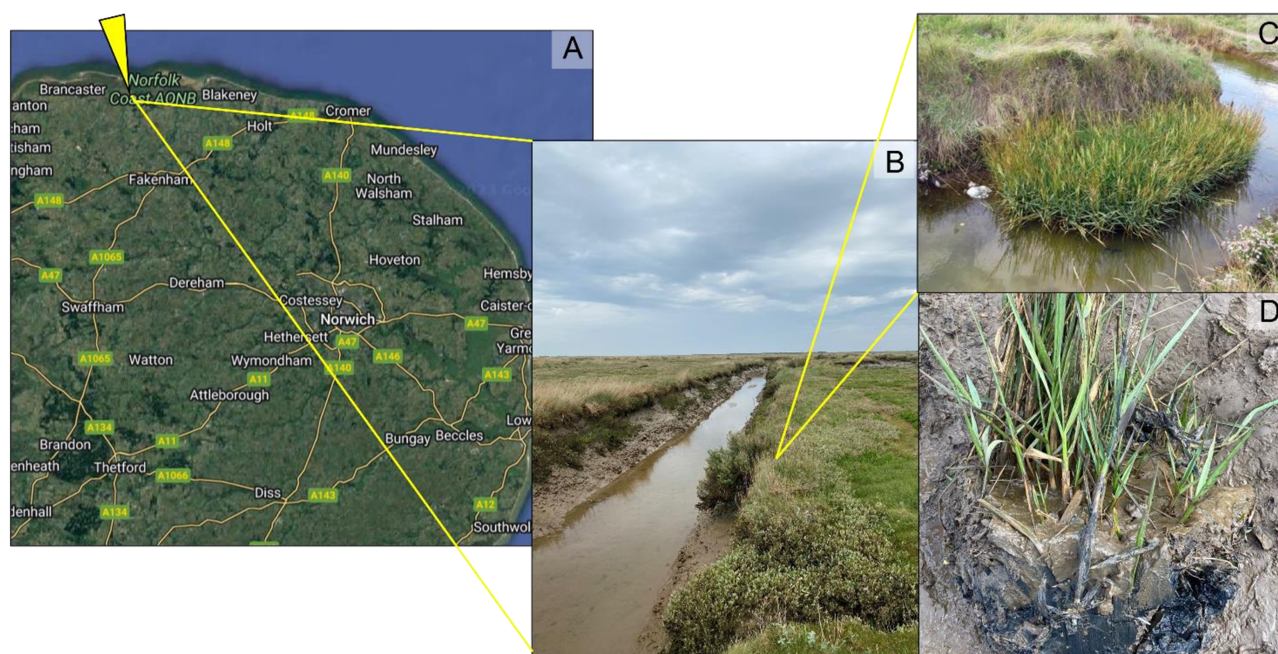


Fig. 5.4: The sampling site. A) An aerial satellite image of Norfolk, showing the location of Stiffkey Salt Marsh marked with a yellow arrow. B) The landscape of Stiffkey Salt Marsh, showing a characteristic tidal inlet flanked by mudflats and vegetation, including *Spartina anglica.* C) A closer image of a clonal clump of *S. anglica*, situated within a tidal inlet, clearly indicating the density of plants. D) The sample of *S. anglica* from which samples were taken, immediately after excavation, showing the rhizosphere intact and compacted within a protective layer of black bulk soil.

Stable Isotope Probing

Synthesis of ^{13}C-DMSP

^{13}C-DMSP was synthesised in house from $^{13}C_3$-acrylic acid and DMS (Merck) as in Todd *et al.* (2010).

DNA-stable isotope probing

Bulk soil associated to *Spartina anglica* plants was discarded by shaking and rhizosphere soil (<4 mm from roots) was sampled using sterile scalpels. For DNA-SIP experiments, 4 g of rhizosphere soil were placed in 125 ml vials containing 40 ml of sterile seawater collected from Stiffkey saltmarsh ponds. Vials were set up in triplicate and immediately sealed after the addition of 100 µM of either ^{12}C- or ^{13}C-DMSP. Vials with autoclaved rhizosphere soil were set up in triplicate as controls to account for possible abiotic degradation of DMSP. All samples were incubated at 25 °C with shaking (1 rcf). DMSP consumption and DMS production was monitored by gas chromatography (GC) as described below. DMSP in the microcosms was replenished when ≥ 95% had been consumed (approximately every 4-8 hours).

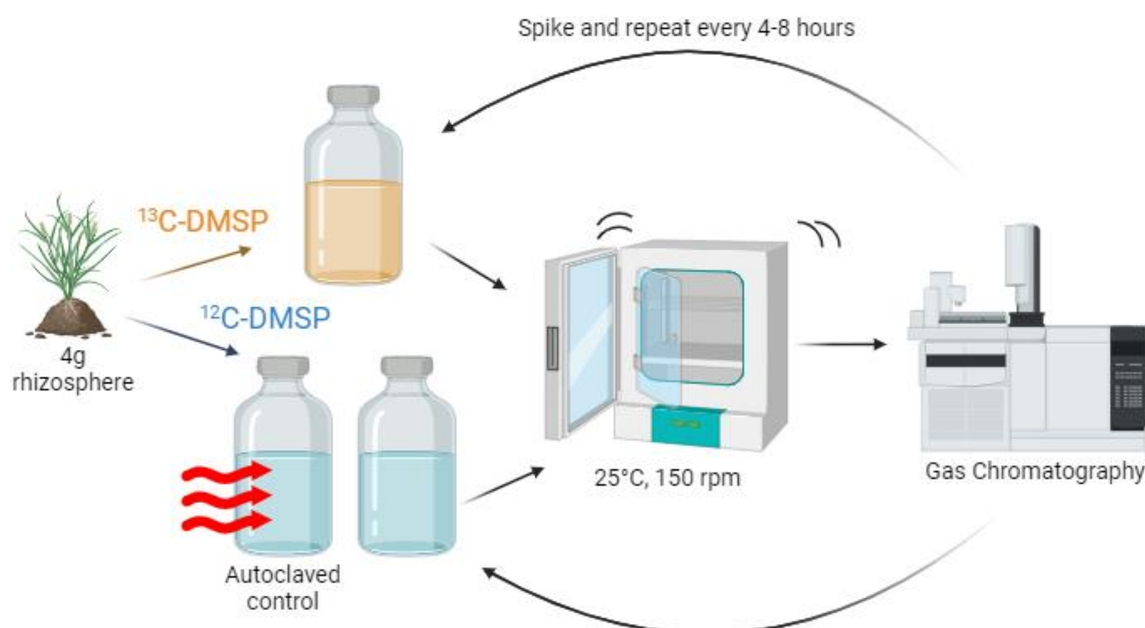


Fig 5.5: Illustration of the first stage of DNA-SIP incubation. Note that all samples were in triplicate, not shown on the figure for simplicity.

At T0 and after 5 days of incubation (T1; 75 µmol C assimilated·g^{-1}), 10 ml of DNA-SIP microcosms were spun down and supernatants discarded. Then, soil pellets were used for DNA and RNA extraction using the DNeasy PowerSoil Pro Kit (Qiagen) and the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Subsequently, 4 µg DNA of T1 samples were separated into heavy (^{13}C-labelled) and light (^{12}C-unlabelled) DNA by isopycnic

centrifugation as in El Khawand *et al*. (2016). DNA retrieved in each fraction was quantified using a Qubit dsDNA HS assay kit (ThermoFisher Scientific). Density of DNA fractions was estimated by refractometry using a Reichert AR200 refractometer (Reichert Analytical Instruments). Heavy and light DNA fractions from each sample were identified by plotting the percentage of DNA retrieved vs the refractive index and subsequently used for 16S rRNA gene amplicon and metagenomic analysis.

Quantification of DMSP, MeSH and DMS by Gas Chromatography (GC)

DMSP, DMS and MeSH in DNA-SIP incubations were quantified by gas chromatography using a flame photometric detector (Agilent 7890A GC fitted with a 7693 autosampler) and a HP-INNOWax 30 m x 0.320 mm capillary column (Agilent Technologies J&W Scientific). Eight-point calibration curves of DMS and MeSH standards were used and the detection limit for headspace DMS and MeSH were 0.015 and 0.10 nmol respectively (see Chapter 2 - Material and Methods: Gas Chromatography for a more detailed methodology).

DMSP concentration in DNA-SIP incubations were measured by extracting 0.2 ml of sample with a sterile syringe and a 0.5 mm diameter needle. Aliquots were then placed in 2 ml vials and heated at 80 °C for 10 min to remove possible DMS present in the samples. Vials were left to cool down before the addition of 0.1 ml of NaOH 10 M to measure DMSP content via alkaline lysis as in Liu *et al.,* after which they were immediately sealed. No residual DMS was detected in heated control vials without NaOH added.

To measure DMS and MeSH concentrations in the DNA-SIP microcosms experiments, 50 µl of headspace were manually injected in the gas chromatograph with an SGE® gas-tight syringe (Trajan).

Isolation of Bacteria from ^{13}C samples

After 13 days of enrichment with ^{13}C-DMSP, bacteria were isolated by removing a 100 µl aliquot from the serum vial, for culture dependent characterisation. A serial dilution was made and 200 µl administered to plates containing MBM Agar + 0.5 mM DMSP as the sole carbon source. Plates were incubated for 48 hours at 28 °C.

Nine single colonies with distinct morphologies were streaked onto MBM Agar + 0.5 mM DMSP + 5 mM Succinate as the carbon sources and incubated for 48 hours at 28 °C. Isolates were tested for purity using Phase Contrast Microscopy. Cell morphology was visualised using a Zeiss AxioScope A1 microscope at 1000x magnification (lamp: HXP 120C, filter set: Zeiss, FS #49) (Images in Appendix).

Characterisation of Bacterial DMSP Catabolising Activity

Growth on DMSP as Sole Carbon source

Growth on DMSP as a sole carbon source was assessed by aseptic inoculation of each isolated species into 5ml YTSS and incubated at 28 ˚C for 24 h. The OD_{600} of the rich media culture was measured and adjusted to OD_{600} of 0.8 in 1 ml of MBM. The cells were washed by centrifuging the culture at 4000 rcf, for 4 min and the supernatant discarded. The pellet was resuspended in MBM minimal media with no carbon source (Baumann & Baumann, 1981) and the washing process repeated three times.

The pellet was resuspended in MBM and a final culture of 4% inoculum in MBM with 0.5 mM DMSP as the sole carbon source was incubated at 30 °C for 5 days. Controls of MBM with 5 mm Succinate as the sole carbon source and MBM with no carbon source were prepared as before. Cell growth was measured by comparing OD_{600} at T0 and T96.

DMS and MeSH Production by Bacterial Strains from DMSP

Growth on DMSP as a sole carbon source was assessed by aseptic inoculation of each isolated species into 5ml YTSS and incubated at 28 ˚C for 24 h. The OD_{600} of the rich media culture was measured and adjusted to OD_{600} of 0.8 in 1 ml of MBM. The cells were washed by centrifuging the culture at 4000 rcf, for 4 min and the supernatant discarded. The pellet was resuspended in MBM minimal media with no carbon source (Baumann & Baumann, 1981) and the washing process repeated three times.

The pellet was resuspended in MBM and a final culture of 4% inoculum in MBM containing 10 mM NH_4Cl, 2.5 mg/ml FeEDTA, 0.01 mg/ml vitamin mix (Baumann & Baumann, 1981) and a combined carbon source of 0.5 mM DMSP and 10 mM Succinate. A 300 l aliquot of

liquid bacterial culture was added to 2 ml glass GC vials and immediately sealed with 11 mm crimp caps with rubber/PTFE septa. Cultures were incubated at 30 °C, 2 rcf for 24 hrs.

DMS and MeSH production from DMSP catabolism were measured by Gas Chromatography (GC) headspace analysis on an Agilent Technologies 7890B Gas Chromatography System using a flame photometric detector fitted with a 7693 autosampler) and a HP-INNOWAX 30 m × 0.320 mm capillary column (Agilent Technologies J&W Scientific) using automatic injection method 530_HP-PLOT_SPLITDMS50_MESH_0.1M at 60 ˚C. Peak areas at approximately 2.1 and 3.3 mins retention time were recorded as indicative of MeSH and DMS production, respectively.

To calculate the concentration of DMSP produced per mg protein per minute, cellular protein content was estimated by sonication of liquid cultures for 20 seconds at 20 kHz and spectrophotometric analysis with Bradford's reagent, according to the manufacturer's instructions (BioRad) at 595 nm.

DMSP Production by Bacterial Isolates

DMSP biosynthesis by bacterial isolates was assessed by aseptic inoculation of each isolated species into 5ml YTSS and incubated at 28 ˚C for 24 h. The OD_{600} of the rich media culture was measured and adjusted to OD_{600} of 0.8 in 1 ml of MBM. The cells were washed as before.

The pellet was resuspended in MBM and a final culture of 4% inoculum in MBM, adjusted to 35 PSU, containing 0.5 mM NH_4Cl, 2.5 mg/ml FeEDTA, 0.01 mg/ml vitamin mix (Baumann & Baumann, 1981), 5 mM methionine and 5 mM succinate. Additionally, 0.1 µl/ml yeast extract (González *et al.,* 1999) was added to enhance growth. The cultures were incubated at 30 °C for 5 days and growth analysed by comparing OD_{600} at T0 and T96.

A 200 l aliquot of liquid bacterial culture was removed from each T96 sample for alkaline lysis. The aliquot and 100 µl 10M NaOH were added to 2 ml glass GC vials and immediately sealed with 11 mm crimp caps with rubber/PTFE septa. Cultures were incubated at 28 °C, 1

rcf for 30 mins in the dark, and measured by Gas Chromatography (GC) headspace analysis as before.

DMS consumption by Bacterial Isolates

Consumption of DMS as a sole carbon source was assessed by aseptic inoculation of each isolated species into 5 ml YTSS and incubated at 28 ˚C for 24 h. The OD_{600} of the rich media culture was measured and adjusted to OD_{600} of 0.8 in 5 ml of MBM. The cells were washed as before.

The pellet was resuspended in MBM and a final culture of 4% inoculum in 20 ml MBM, adjusted to 35 PSU, containing 10 mM NH_4Cl, 2.5 mg/ml FeEDTA, 0.01 mg/ml vitamin mix (Baumann & Baumann, 1981) was prepared in 125 ml serum vials. The vials were sealed and injected with 1% DMS per volume and incubated at 30 °C for 48 h. DMS consumption was measured at T0 and T48 by Gas Chromatography (GC) headspace analysis as before.

Inducibility of DMSP Catabolising Genes

Inducibility of DMSP cleavage was assessed. Each isolated species was inoculated into 5ml YTSS and incubated at 28 ˚C for 24 h. The OD_{600} of the rich media culture was measured and adjusted to OD_{600} of 0.8 in 1 ml of MBM. The cells were washed as before.

The pellet was resuspended in MBM and a final culture of 4% inoculum in MBM, adjusted to 35 PSU, containing 10 mM NH_4Cl, 2.5 mg/ml FeEDTA, 0.01 mg/ml vitamin mix (Baumann & Baumann, 1981), 0.1 µl/ml yeast extract (González *et al.,* 1999) and either 5 mM Succinate as a sole carbon source or 5 mM Succinate and 0.5 mM DMSP. The cultures were incubated at 30 °C for 5 days and growth analysed by comparing OD_{600} at T0 and T96.

A 300 l aliquot of liquid bacterial culture was removed from each T96 sample for alkaline lysis. Aliquots were added to 2 ml glass GC vials and immediately sealed with 11 mm crimp caps with rubber/PTFE septa. Cultures were incubated at 28 °C, 1 rcf for 30 mins in the dark, heat-killed at 80 °C for 10 mins and measured by Gas Chromatography (GC) headspace analysis as before. Cellular protein content was estimated by sonication of liquid cultures and

spectrophotometric analysis with Bradford's reagent, according to the manufacturer's instructions (BioRad).

Sequence Analysis of Bacterial Isolates

Purified isolates were identified by amplification of their 16S rRNA gene (using the primers 27F and 1492R (Baumann & Baumann, 1981; DeLong, 1992) and the resultant PCR products were PCR purified using High Pure PCR Product Purification Kit (Roche) and sequenced by Eurofins Genomics (Munich, Germany). Isolates were taxonomically identified using Basic Local Alignment Search Tool (BLASTn) (http://blast.ncbi.nlm.nih.gov). Genomic DNA from bacterial isolates, *Vibrio spartinae Haloarcobacter arenosus, Thioclava nitratireducens* and *Alteromonas oceani* was sequenced by Microbes NG (Birmingham, United Kingdom) using Illumina HiSeq technology. Publicly available genomes of the most closely related reference strains (Table 1) were screened for the presence of homologous DMSP lyase genes. Homologous sequences to ratified proteins (Table S2) were identified using local BLASTp, with thresholds set as $E \leq 5e-30$, $\geq 50\%$ amino acid identity and $\geq 75\%$ coverage.

Metagenome Analysis

Amplicon, Metagenomic and Metatranscriptomic Sequencing

DNA and RNA were extracted using the PowerSoil DNA Isolation Kit (Qiagen) and the Zymo Direct-zol RNA Kit, respectively. For 16S rRNA amplicon sequencing, the V4 region of the bacterial 16S rRNA genes were amplified with primers 515F and 806R (Apprill *et al.,* 2015; Walters *et al.,* 2016). All amplicons (amplified 16S rRNA genes), metagenomes (total DNA) and metatranscriptomes (total RNA) samples were subsequently sent to Novogene (Beijing, China) for quality control, libraries construction and Illumina high-throughput sequencing.

Amplicon Analyses

Qiime2 platform (Bolyen *et al.,* 2019) was used to analyze the 16S rRNA gene amplicon data, with "dada2" module to remove low quality sequences and cluster sequences at 100% identity to get the representative amplicon sequence variants (ASVs), and "classify-sklearn"

module to assign taxonomy for each ASV. Finally, a ASV table was generated by Qiime2 and used for reveal community composition in different samples.

Metagenome and Metatranscriptome Analyses

For both metagenomes and metatranscriptomes, raw data were primarily quality controlled by fastp (Chen *et al.,* 2018) and were then assembled with MEGAHIT v1.0.2 (Li *et al.,* 2016) with the default parameters. Assembled contigs were used to call genes using Prodigal (Hyatt *et al.,* 2010) with '-meta' option for metagenomes and using FragGeneScan (Rho *et al.*, 2010) for metatranscriptomes. Then, these predicted genes from metagenomes and metatranscriptomes were independently clustered at 95% identity using CD-HI (Fu *et al.,* 2012) to remove the redundant genes. Phyloflash (Gruber-Vodicka *et al.,* 2020) was applied to determine the prokaryotic community from metagenomes and metatranscriptomes based on 16S rRNA gene reads with the Silva v138.1(Quast *et al.,*2013) as the reference database.

MAG (metagenome assembled genome) Recovery

To improve the quality of recovered MAGs, three metagenomic replicates from each sample were coassembled by MEGAHIT v1.0.2 (Li *et al.,* 2016) with the default parameters. The co-assembly results of each sample were imported to MetaWRAP (Uritsky *et al.,* 2018) to recover bacterial and archaeal MAGs. Both the bin_refinement and reassembly_bins modules from MetaWRAP (Uritsky *et al.,* 2018) were performed to refine the recovered MAGs and improve completion and N50 of the newly recovered MAGs, respectively. All recovered MAGs were dereplicated using dRep v2.3.2 (Olm *et al.,* 2017) with the default settings. Genome completeness and contamination were estimated by CheckM v1.0.12 (Parks *et al.,* 2015). Only MAGs with completeness ≥ 50% and contamination ≤ 10% were retained for downstream analysis. Taxonomic assignment of each genome was determined by 'classify' module of GTDB-Tk v1.7.0 (Chaumeil *et al.,* 2019). Gene calling and annotation of each MAG was performed by Prokka v1.12 (Seeman, 2014). The relative abundances of MAGs in metagenomes and metatranscriptomes were estimated using CoverM (v0.6.1, https://github.com/wwood/CoverM), which mapped metagenomic and metatranscriptomic sequences to the MAGs with default parameters.

Identification of DMSP/DMS Cycling Related Genes

The functionally ratified protein sequences (not shown) of enzymes involved in DMSP/DMS cycling (Appendix) were used as reference for searching homologues in metagenomes/metatranscriptomes/MAGs using hmmsearch (http://hmmer.org/) with an evaule of 1e-10. Due to complex structure of eukaryotic genes, it is quite hard to predict them in metagenomes. Thus, for eukaryotic DMSP/DMS-cycling related genes, we only searched their homologues in metatranscriptomic gene set. To further validate the environmental sequences retrieved from these marine metagenomes/metatranscriptomes/MAGs, potential sequences of interest were crosschecked by BLASTp and only those sequences had a minmum 40% amino acid identity and 70% query coverage to the corresponding ratified proteins were retained. Gene relative abundances (Copies/transcripts per million reads) of those retained genes were determined by CoverM v0.6.1 (https://github.com/wwood/CoverM). Briefly, the copy/transcript number of each gene was calculated by the "jgi_summarize_bam_contig_depths" module integrated in CoverM and was further normalized by total mapped reads of each metagenome. Taxonomic assignment of those retained genes based on the contigs they located in were conducted by CAT (von Meijenfeldt *et al.,* 2019) (parameters: --top 30 --range 5 --fraction 0.3).

Statistical Analysis

Statistical analysis was performed using SPSS Software Package (IBM, 2021) unless otherwise stated. Data sets were assessed for normality using the Kolmogorov-Smirnov test and equal variances with the Levene's test. Data could not be transformed to be normally distributed, so non-parametric equivalent tests were used for all data sets. Pairwise comparisons for each species between the test condition and its control condition were carried out using Mann-Whitney U tests with a 95% confidence interval.

Results

Characterisation of *Spartina* rhizosphere

Firstly, it was necessary to quantify the base line concentrations of DMSP present in the *Spartina* rhizosphere through alkaline lysis, to ensure that the amounts of DMSP that we add to the SIP-incubations were excessively high compared to that which exists in the natural environment.

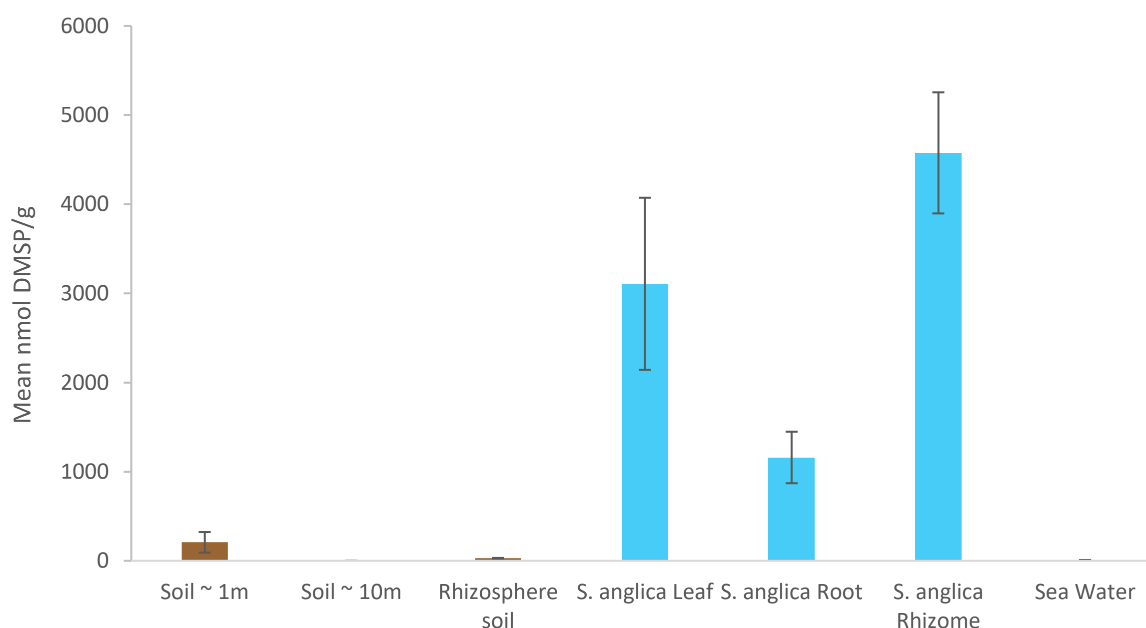


Figure 5.6: Mean nmol DMSP per gram of sample, from standing stocks of soil and other surrounding environment to the *Spartina anglica* rhizosphere. Bulk soil was taken from both ~ 1m and ~ 10m distances from the *S. anglica* clump sampled. This was to provide a baseline for comparison of microbial metabolism of DMSP Error bars indicate ±1 S.D. No DMS was detected in any of the standing stocks.

The tissues of *S. anglica* showed significantly higher concentrations of intracellular DMSP compared to the rhizosphere and bulk soil, as expected ($n = 6$, $p < 0.001$ in all cases). Soil of approximately 1m distance from the sampled clump had higher standing stocks of DMSP compared to the rhizosphere, but not significantly so ($n = 6$, $p = 0.057$). The reason for this is unknown.

Having established that the rhizosphere contained micro molar levels of DMSP, it was decided that the addition of 100 µM of either ^{12}C- or ^{13}C-DMSP would not be such a great enhancement on the standing stocks as to be unrepresentative of natural environments and thus was appropriate for the SIP incubations. Samples were divided into those enriched with ^{12}C-DMSP and ^{13}C-DMSP, with autoclaved controls to ensure that decreases in DMSP were due to bacterial catabolism and not gas leakage. Samples were taken for quantification of DMSP by alkaline lysis, and the headspace analysed for DMS at regular intervals.

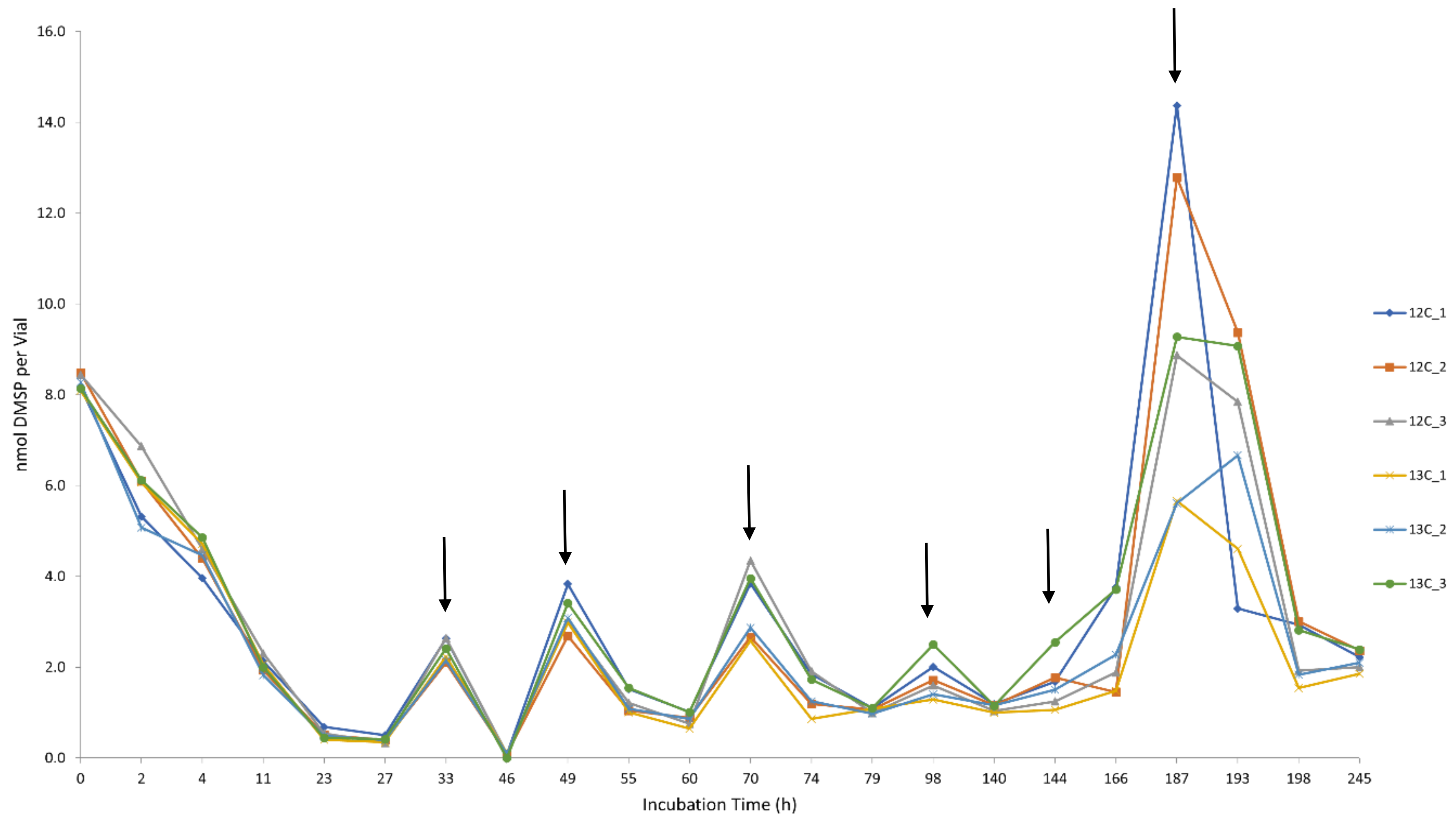


Figure 5.7: DMSP degradation during the incubation in the SIP experiments. Autoclaved controls were not shown as the DMSP was not degraded and thus concentrations consistently increased over the course of the experiment to orders of magnitude higher than the live cultures. Increases in DMSP correspond to the addition of fresh DMSP (spikes) as the sole carbon source and are denoted by black arrows.

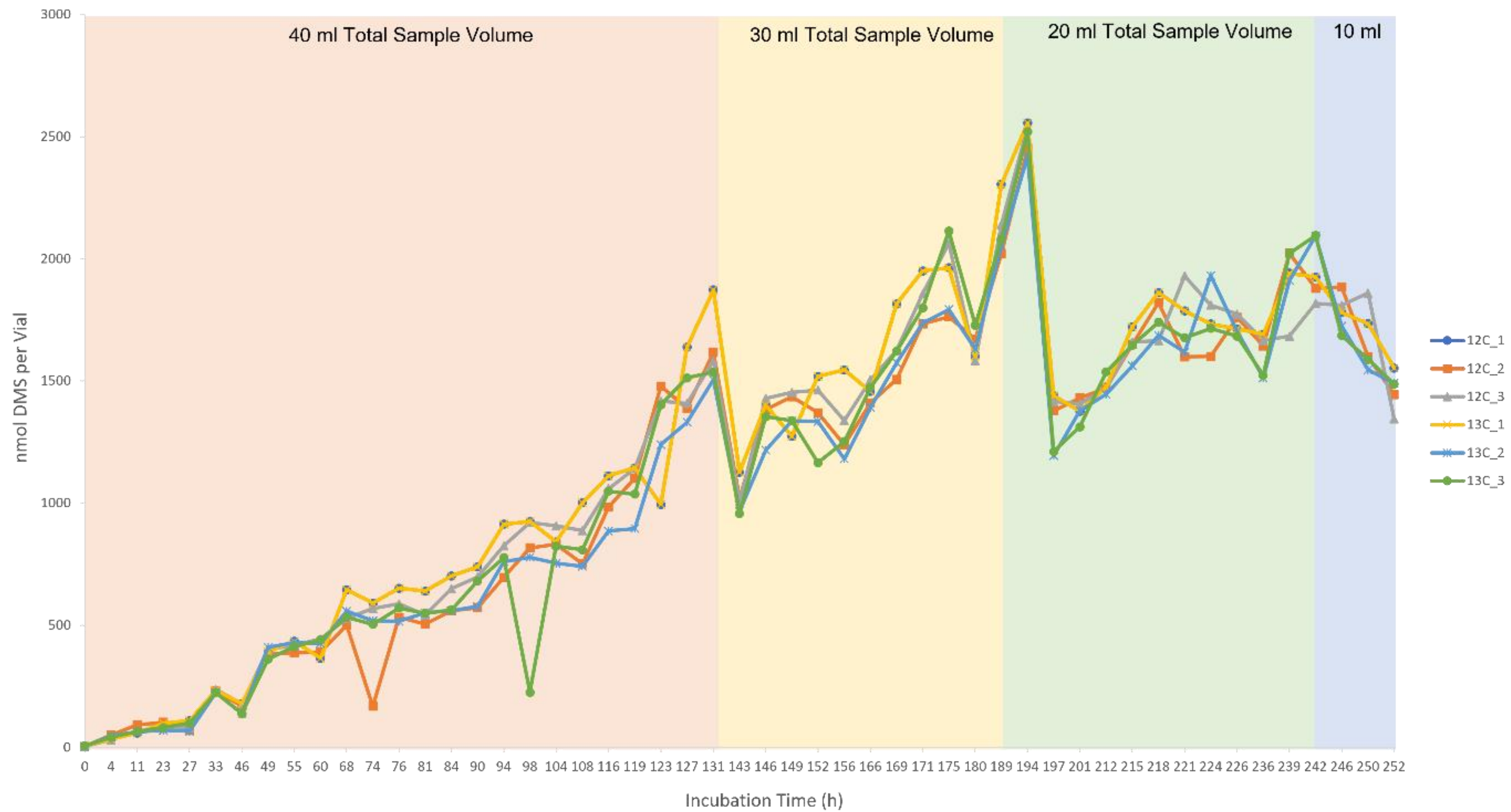


Figure 5.8: DMS production during the incubation in the enriched cultures. Autoclaved controls were not shown as the DMS concentrations stayed consistent. The times at which 10 ml samples were removed for DNA extraction are shown as coloured blocks, as sudden decreases in DMS production correspond to the loss of volume.

DMSP was consistently degraded after spiking in both the ^{12}C – and ^{13}C – DMSP cultures, indicating bacterial catabolism of DMSP. This corresponded with increases in headspace DMS as a breakdown product of DMSP catabolism and shows that there were rhizobacteria capable of utilising DMSP as a sole carbon source. Note, unlike in the previously published DMSP SIP experiments there was no observed decrease in the levels of DMS over time, likely indicating a lack of enhanced DMS catabolism. This is a good predictor of DMSP cleavage. Working on the hypothesis that DMSP was being degraded - most likely by bacteria from the *S. anglica* rhizosphere that metabolised DMSP - the next step was characterise the species present and the abundance of genes associated DMSP metabolism in the samples. DNA was extracted following isopycnic ultracentrifugation for metagenome analysis, when 77 µmol DMSP L^{-1} (231 µmol C · L^{-1}) was assimilated and DMS levels were no longer increasing, (by 0.7 ± 0.006 µmol · h^{-1} during the last 24 h of incubation).

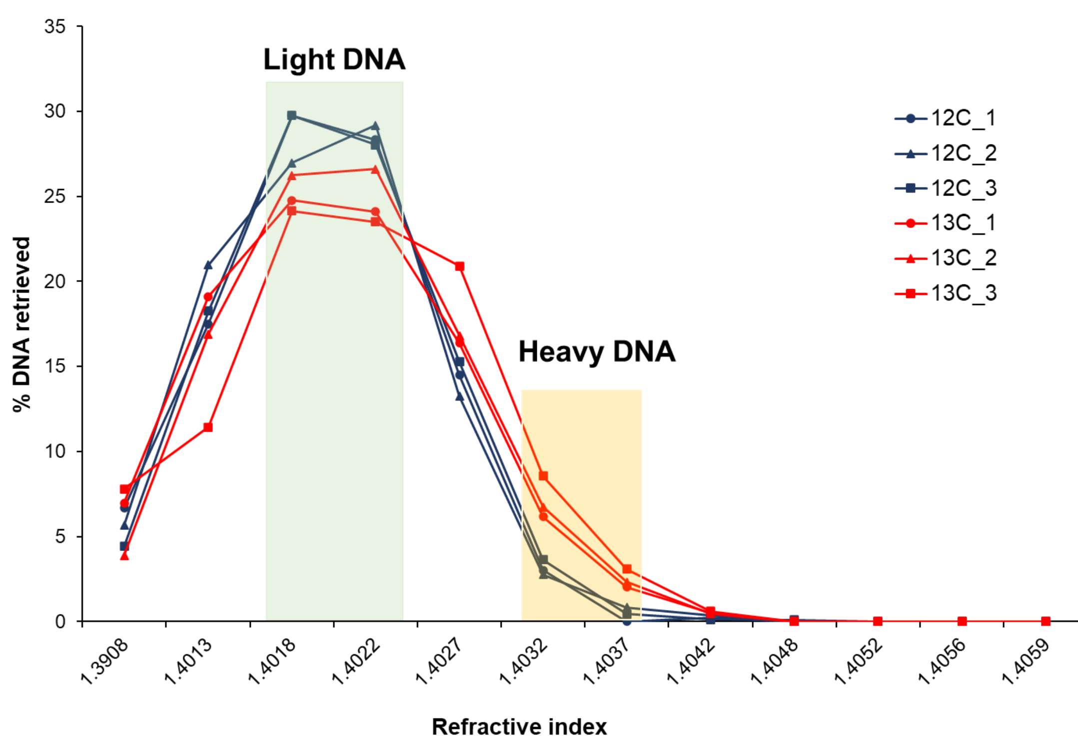


Fig. 5.9: DNA retrieved as function of refractive index of each fraction following isopycnic ultracentrifugation. Samples incubated with ^{12}C-DMSP are represented in blue. Samples amended with ^{13}C-DMSP are shown in red. Light and heavy DNA fractions used for subsequent downstream analysis are shadowed in green and yellow, respectively. Figure produced by Ornella Carrión.

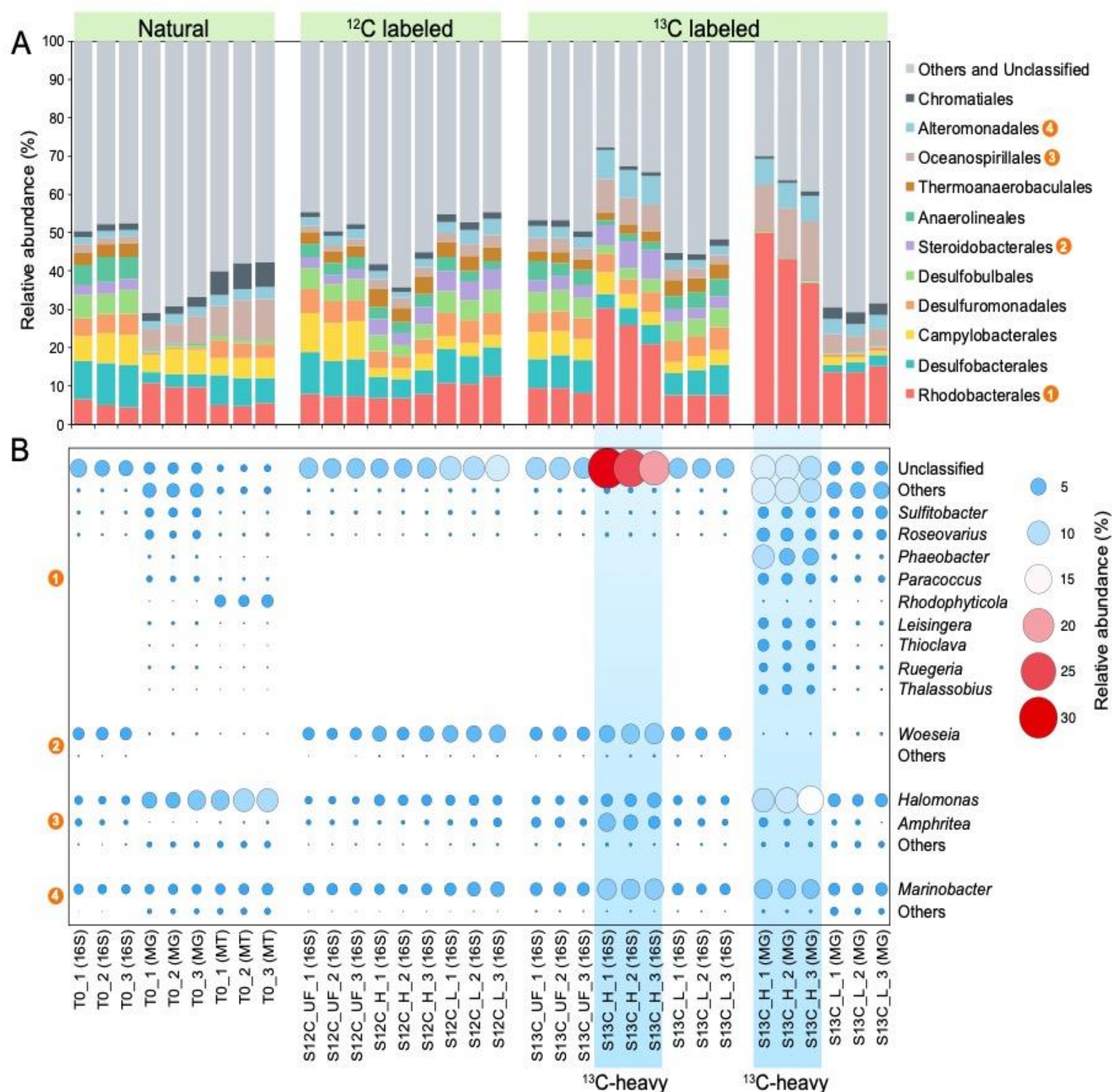


Fig.5.10: Microbial community profile of spartina rhizosphere samples at the order: (**A**) level revealed by 16S rRNA gene amplicon (16S rRNA) and metagenomic (MG) sequencings. T0 samples and unfractionated (UF), heavy (H) and light (L) part of incubation samples were analysed. "_1", "_2" and "_3" after the sample name represent biological replicates. Four orders that significantly enriched in the ^{13}C-heavy samples compared to the ^{13}C-light samples were numbered with 1-4. **(B)** The classification of selected orders at the genus level. Figure produced by Xiao-Yu Zhu.

Metagenome analysis showed that of previously identified orders DMSP metabolising bacteria, such as *Oceanospiralles* and *Alteromondales* were abundant in all samples, but the most prevalent – especially in the ^{13}C-DMSP enriched samples – were *Rhodobacterales*. However, the most abundant bacteria were either unknown or single representatives of a small family, classified as others. This demonstrates how diverse the *S. anglica* bacterial community is.

In the natural sample, the 16S rRNA sequencing revealed a more even distribution of bacterial orders compared to the metagenome analysis (Fig 3.9A), with relative abundances of between 2-5% of *Anearolineales, Steroidobacterales* and *Desulfobulbales* in 16S rRNA samples, compared to less than1% in metagenome analysis and metatranscriptome. Additionally, the *Oceanospiralles* were less abundant in the 16S rRNA samples at approximately 2%, compared to the metagenome with relative abundances of between 5-10%. Furthermore, in the 16S rRNA, *Thermoanaerobacules* were present with a relative abundance of between 3-5%, whereas they were completely absent in the metagenome analysis.

The 16S rRNA samples for both unfractionated ^{12}C- and ^{13}C-DMSP were very similar to the natural 16S rRNA, in terms of both the orders represented and their percentage relative abundances. Additionally, both the heavy and light fractions of ^{12}C-DMSP showed no striking differences to the unfractionated ^{12}C-DMSP or natural sample, except that the heavy fraction had more unknown bacterial orders (between 5-10%) compared to the light and unfractionated 16S rRNA samples. The most notable difference was in the ^{13}C-DMSP heavy fraction, in which *Roseobacters* were considerably more abundant compared to the ^{13}C-DMSP light fraction and unfractionated samples, as well as the ^{12}C-DMSP samples, 30%, compared to 10%. This suggests that *Roseobacters* are significant catabolisers of DMSP in the *S. anglica* rhizosphere.

In the ^{13}C-DMSP heavy fractions, there was considerable differences between the relative abundances in the 16S rRNA and metagenome samples. In both cases, the *Roseobacters* were the most abundant order, but they were considerably more abundant in the metagenome samples (up to 50% compared to 30%). Additionally, there was considerably less diversity of

orders in the heavy fraction metagenome, with *Thermoanaerobacules, Anearolineales, Steroidobacterales*, *Desulfobulbares, Desulforomonas, Campylobacters* and *Desulfobacterales* all at less than 1% abundance, if present at all, compared to the 16S rRNA samples. This indicates that *Roseobacters, Oceanospiralles* and *Alteromondales* are very likely to be the most active DMSP catabolising bacteria in the *S. anglica* rhizosphere.

Having established the most diversity and abundance of bacterial families present, the diversity and abundance of DMSP-cycling related genes needed to be determined, as well as their expression in the samples.

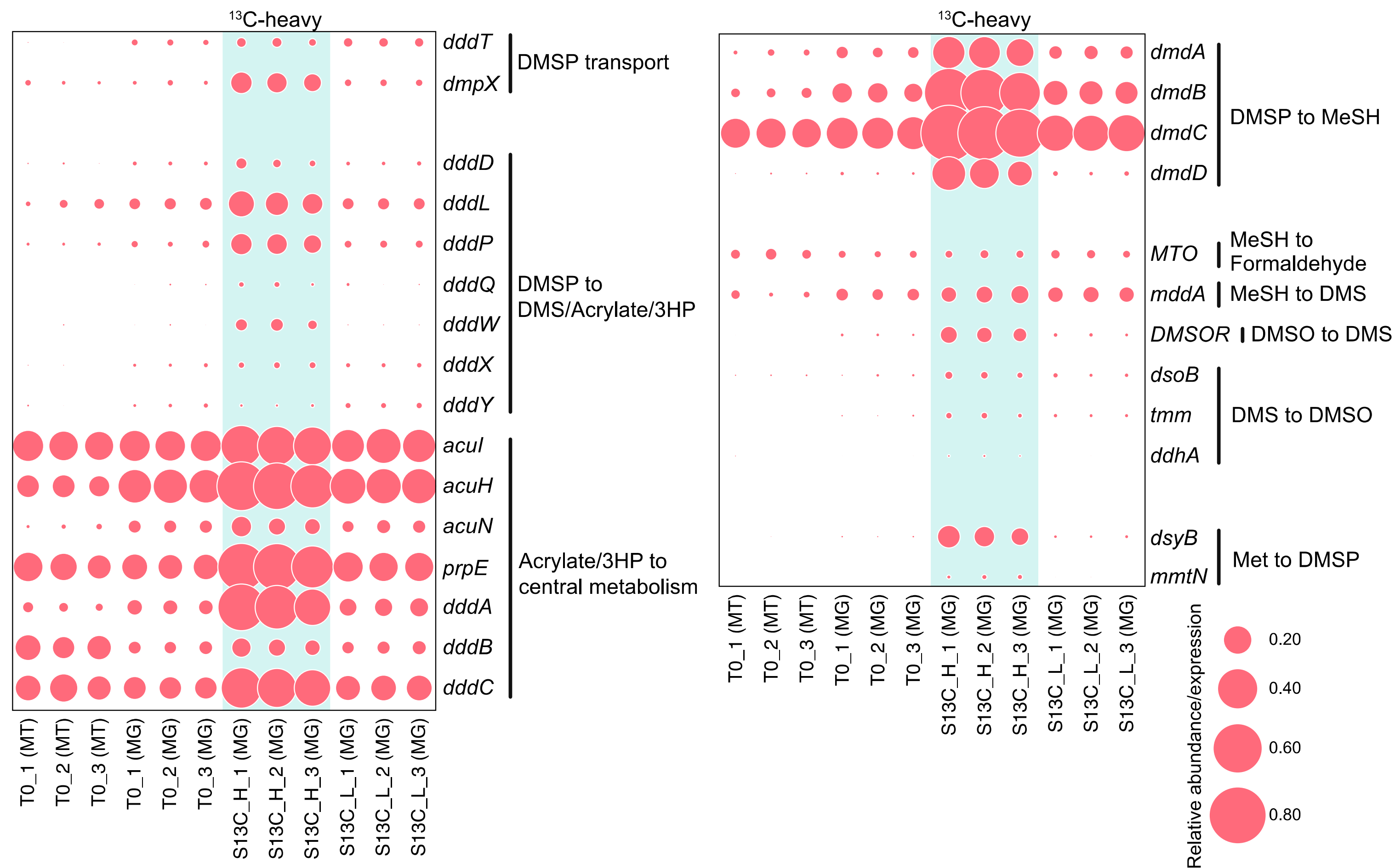


Fig.5.11: Relative abundance and relative expression of DMSP/DMS cycling related genes retrieved from rhizosphere metagenomes revealed by metagenomes and metatranscriptomes, respectively. Relative abundance and relative expression of these genes were normalized to those of 10 single-copy genes. "_1", "_2" and "_3" after the sample name represent biological replicates. The taxonomic assignments of these genes can be seen in the supplementary table. Figure produced by Xiao-Yu Zhu.

In the natural sample metagenome analysis, only *dddL* and *dddP* DMSP lysis genes were present, and both with relative abundance/expression of less than 0.20 (Fig. 3.10). Genes associated with DMSP demethylation had greater relative abundance/expression in the natural sample compared to DMSP lysis genes, of between 0.20 to 0.40. Additionally, genes related to the breakdown of Acrylate for use in the central metabolism were also abundant, especially *acuH*, *prpE* and *dddA*. This indicates that multiple metabolic pathways are being used to catabolise DMSP.

In the ^{13}C-DMSP enriched samples, all genes had a higher relative abundance/expression of each gene, compared to the natural sample. Additionally, in all cases the heavy fraction had had a higher relative abundance/expression of each gene, compared to the light fraction. The primary genes enriched in the heavy fraction were again the DMSP lysis genes *dddL* and *dddP*, and DMSP demethylation genes, including *DmdA*.

To uncover the genera within the orders of abundant bacteria in the *S. anglica* rhizosphere that are the most prominent degraders of DMSP, analysis of metagenome assembled genomes (MAG's) was performed.

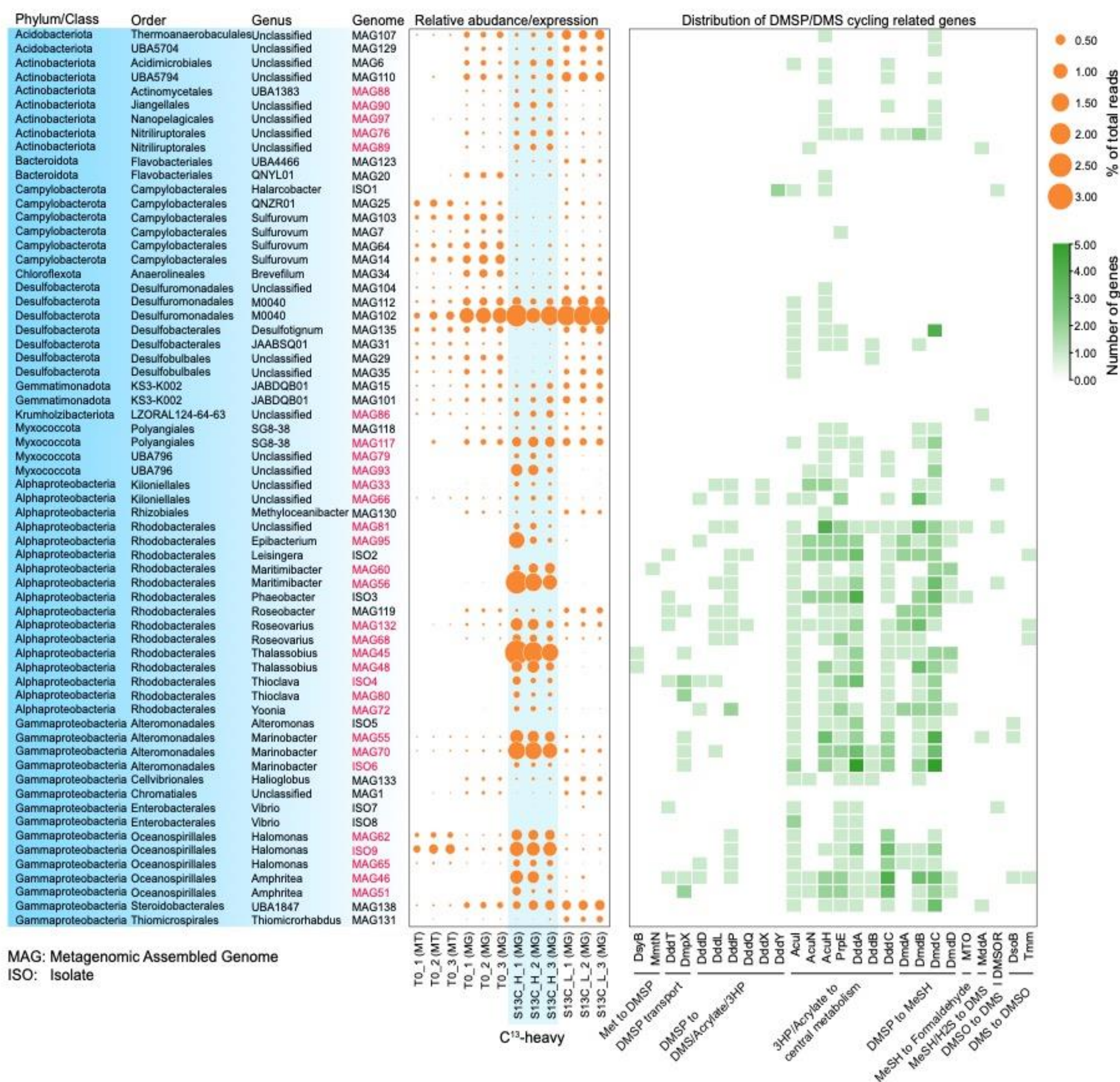


Fig. 5.12: Genomic analyses of MAGs (metagenome assembled genomes) and isolated strains from rhizosphere samples. The left panel shows their taxonomic affiliations. The middle panel shows their relative abundance/expression (Copies/Transcripts per million reads) in different samples. Genomes with higher relative abundance/expression in the ^{13}C-heavy samples were marked with red. The right panel shows the distribution of DMSP/DMS cycling related genes in these genomes. Figure produced by Xiao-Yu Zhu.

Of the *Rhodobacterales*, the genomes with the highest relative abundance/expression in the heavy fraction were *Maritimibacter*, *Roseovarius*, *Thalassobius* and *Thioclava*. In all cases, these contributed between 1.5 – 2% of total reads, with DMSP demethylation genes being most highly expressed. *DmdA*, the most important DMSP demethylation gene, had a lower distribution compared to other associated genes, such as *dmdC*, with both a reduced number of genera expressing it, and a smaller number of genes where it is expressed. *DmdA* was also expressed in *Halomonas*, with a single *DmdA* found in the isolate.

Furthermore, the *Rhodobacterales* also had greatest distribution of DMSP lysis genes, predominantly *dddP* and *dddL*. *DddP* was also represented in the genera *Halomonas* within the heavy fraction, which contributed between 0.5 – 1.5% of total reads. DMSP lysis genes were exclusive to *Alphaproteo* – and *Gammaproteobacteria*, except for a single *Haloarcobacter* isolate, that possessed the only *dddX* gene found in this study.

Overall, there was a clear correlation between bacteria abundant in the ^{13}C-DMSP enriched samples and high expression of DMSP to MeSH catabolic genes, with a consistent but lower level of expression of DMSP lysis genes, clustering in the *Rhodobacterales* and *Oceanospiralles*.

Culture-Dependent Characterisation of Selected Species from the Heavy Fraction

Nine bacterial strains with distinct morphologies were isolated after incubations of ^{13}C-DMSP enriched rhizosphere samples with DMSP as the sole carbon source, all of which were able to catabolise DMSP yielding DMS. None of the isolates produced MeSH. This suggests that they are using DMSP lysis pathway, and not the DMSP demethylation pathway, which is inconsistent with the metagenome analysis that showed higher enrichment of the *dmd* pathway genes compared to DMSP lysis genes. The sequenced strains were screened for DMSP lyase and demethylation genes.

Table 5.1: Characteristics of bacterial strains with DMSP-degrading activity isolated from *Spartina anglica* rhizosphere enriched with ^{13}C when grown with the DMSP. No species produced Hydrogen Sulfide (H_2S) nor did any species catabolise Dimethyl Sulfide (DMS). Isolates whose genomes were sequenced in this study are indicated in bold. Homology to ratified DMSP lyase proteins was determined for each species using BLASTP, with a minimum of 75% query coverage and a maximum E value of 5E-50.

Top-hit taxon (taxid)	**Assembly Accession Number**	**16S rRNA DNA gene identity (%)**	**Top-hit taxonomy**	**DMS production rate (pmol DMSP.mg protein^{-1}.min^{-1}) (±1 STD)**	**MeSH production rate (pmol MeSH.mg protein^{-1}.min^{-1}) (±1 STD)**	**Growth on DMSP as a sole carbon source in liquid culture?**	**Top-hit Homologous DMSP-Lyase Gene**	**Amino Acid Sequence Homology (% Identity)**
***Vibrio spartinae* (1918945)**	GCA_900149295.1	99.3	*Gammaproteobacteria;Vibrionales;Vibrionaceae*	0.018 (0.001)	0	No	DddX - *Pelagicola sp.* (WP_109384856.1)	27.73
***Haloarcobacter arenosus* (2576037)**	GCA_005771535.1	97.2	*Epsilonproteobacteria;Campylobacterales;Arcobacteraceae*	0.288 (0.002)	0	No	DddY *Arcobacter nitrofigilis* (WP_013133925.1)	77.6
***Thioclava nitratireducens* (1915078)**	GCA_001940525.2	98.7	*Alphaproteobacteria;Rhodobacterales;Paracoccaceae*	0.168 (0.001)	3.829 (0.061)	Yes	DddL *Thioclava pacifica* (WP_051692700.1)	84.2

***Halomonas aesturii* (1897729)**	GCA_001886615.1	99.80	*Gammaproteobacteria; Oceanospirillales; Halomonadaceae*	0.007 (0.0001)	1.828 (0.036)	No	DddD *Oceanimonas doudorofii* (AEQ39135)	34.2
***Phaeobacter piscinae* (1580596)**	GCA_000826835.2	99.6	*Alphaproteobacteria;Rhodobacterales;Roseobacteraceae*	0.110 (0.006)	11.431 (0.367)	Yes	DddP *Rosebacter nubinhibens* (EAP77700.1)	80.3
***Leisingera caerulea* (506591)**	GCA_000473325.1	98.2	*Alphaproteobacteria;Rhodobacterales;Roseobacteraceae*	0.402 (0.005)	0	Yes	DddP *Phaeobacter inhibens* (AFO991571)	83.4
***Vibrio kanaloae* (170673)**	GCA_002876865.1	98.9	*Gammaproteobacteria;Vibrionales;Vibrionaceae*	0.026 (0.002)	3.970 (0.760)	No	DddP *Oceanimonas doudorofii* (AEQ39091)	79.27
***Alteromonas oceani* (2071609)**	GCA_003731635.1	98.8	*Gammaproteobacteria;Alteromonadales;Alteromonadaceae*	0.013 (0.001)	2.028 (0.253)	No	DmdA marine gammaproteobacterium HTCC2080	27.91

							(WP_007233625)	
***Marinobacter flavimaris* (262076)**	GCA_003363485	99.2	*Gammaproteobacteria;Alteromonadales;Marinobacter*	0.023 (0.002)	0	Yes	DmdA Ruegeria pomeroyi (AAV95190)	33.70

Of these DMSP-degrading isolates, the majority were *Gammaproteobacteria* (56%) with the remaining identified as *Alpha-* (33%) or *Epsilonproteobacteria* (11%). This is consistent with previous studies that showed the rhizosphere and bulk soil of *S. anglica* are dominated by *Gammaproteobacteria* (50.8% and 37.5%, respectively), although this study was concerned with the identity of DMS-degrading bacteria (Kröber *et al.,* 2022).

Having identified the species present by Sanger Sequencing, their DMSP catabolic abilities were tested following growth with DMSP in liquid culture, and the DMSP assessed by alkaline lysis and headspace analysis.

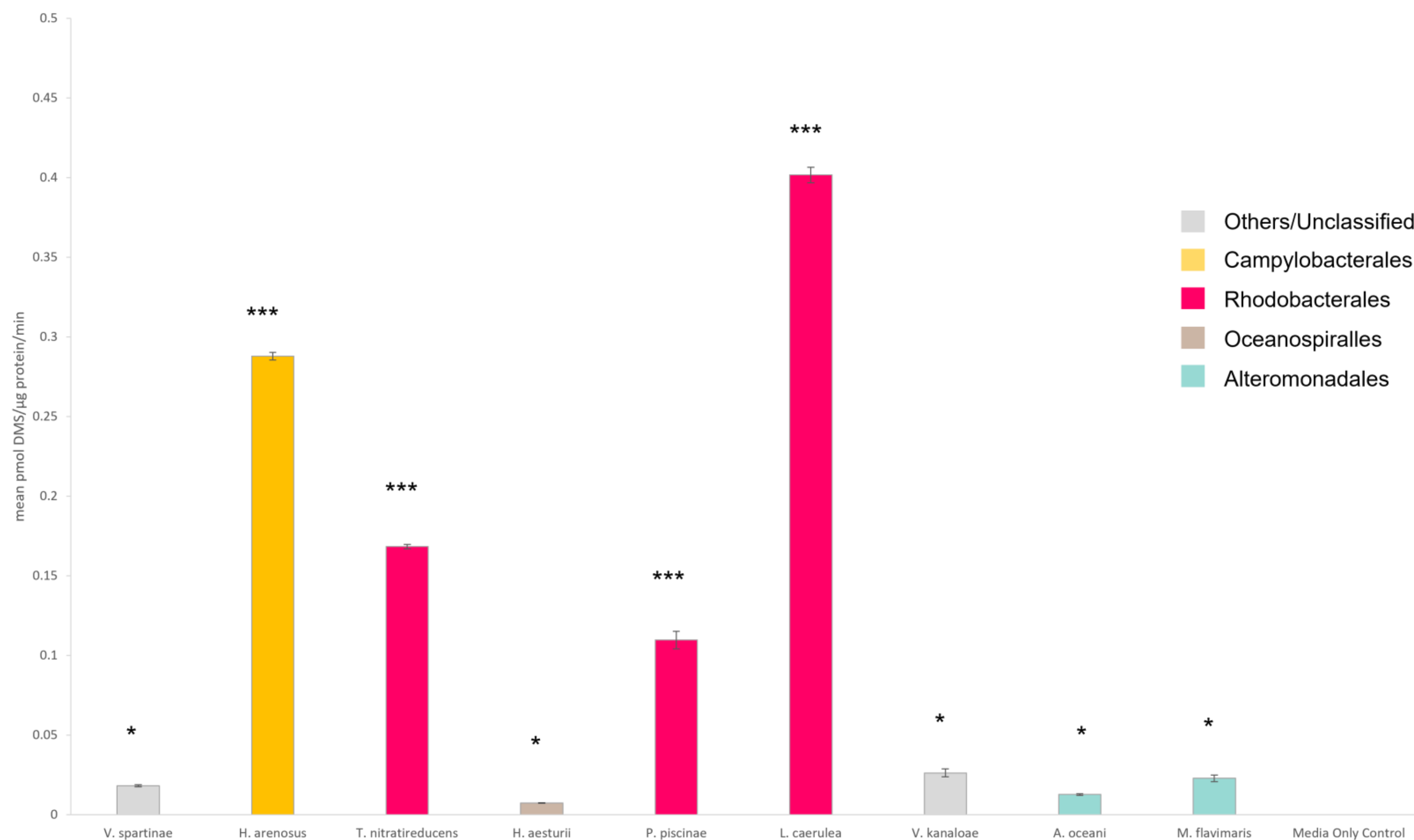


Figure 5.13: Mean rate of DMS production (pmol DMS, µg protein^{-1}, min^{-1}) in the nine species isolated from enriched samples after growth with 0.5 mM DMSP and 10 mM (n = 30 biologically independent samples). Error bars are ± 1 STD. Analysis was carried out using Mann-Whitney U tests for pairwise comparisons against the Media Only Control. Significance is denoted as follows * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In all cases, the bacterial isolates produced significantly higher volumes of detectable DMS than the media only control, consistent with the catabolism of DMSP, by DMSP lyases. Additionally, MeSH production was examined as many of the highly abundant bacteria in the heavy fraction had highly expressed genes associated with the catabolism of DMSP to MeSH, such *DmdA*.

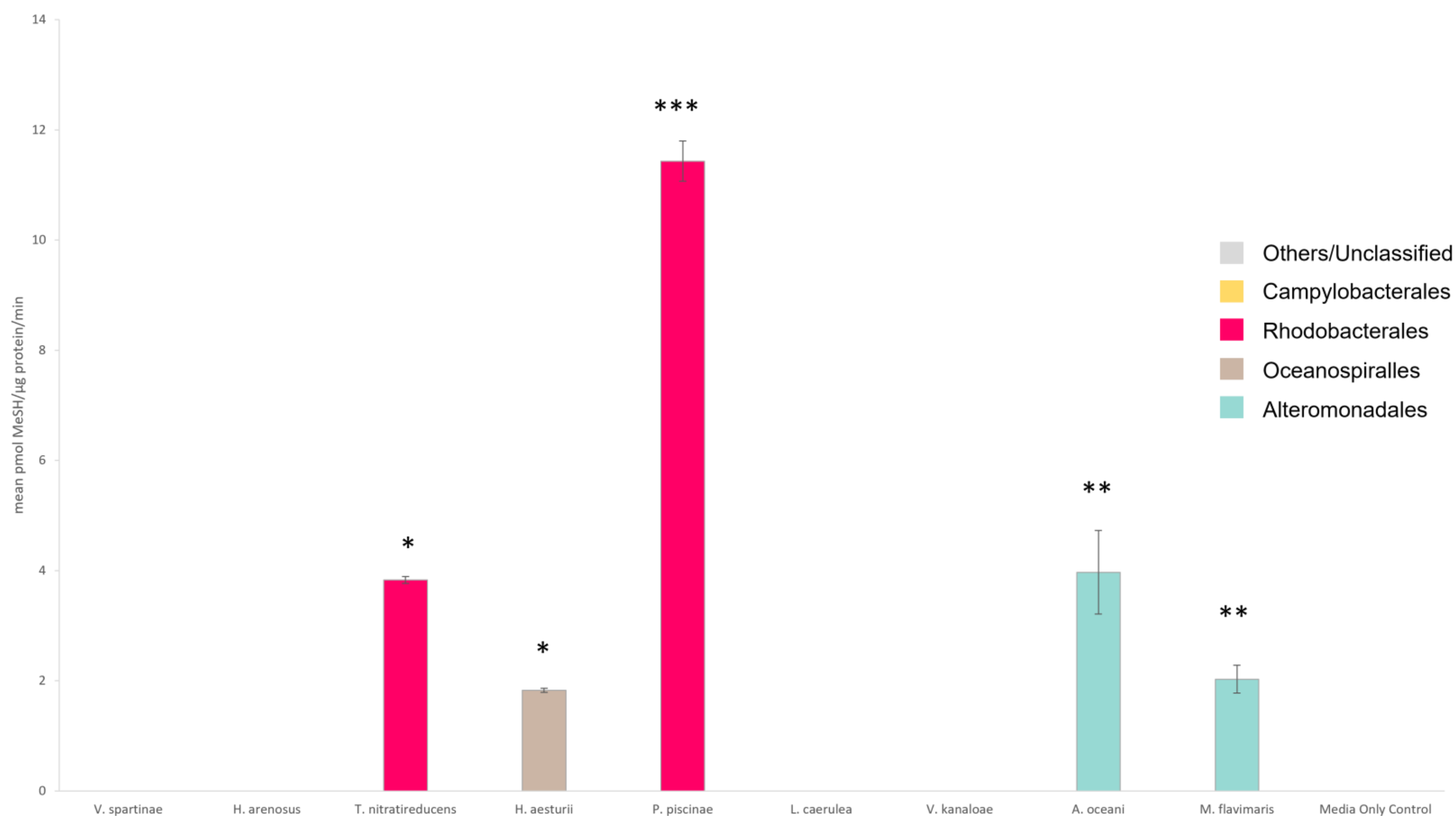


Figure 5.14. Mean rate of MeSH production (pmol MeSH, mg protein^{-1}, min^{-1}) in the nine species isolated from enriched samples (n = 39 biologically independent samples). Error bars are ± 1 STE. Analysis was carried out using Mann-Whitney U ($p = 0.05$) tests for pairwise comparisons against the Media Only Control. Significance is denoted as follows * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

MeSH production was inconsistent, with some species not producing any, suggesting DMSP catabolism via *ddd* gene activity, generating Acrylate as a by-product. Of those that did produce MeSH, *P. piscinae* was the highest producer, consistent with high expression of *DmdA* associated with it in the metagenome analysis.

Substrate inducibility of DMSP-lyase activity was assessed by transferring cultures grown with a mixed carbon source into fresh media with either DMSP + Succinate or Succinate as a sole carbon source.

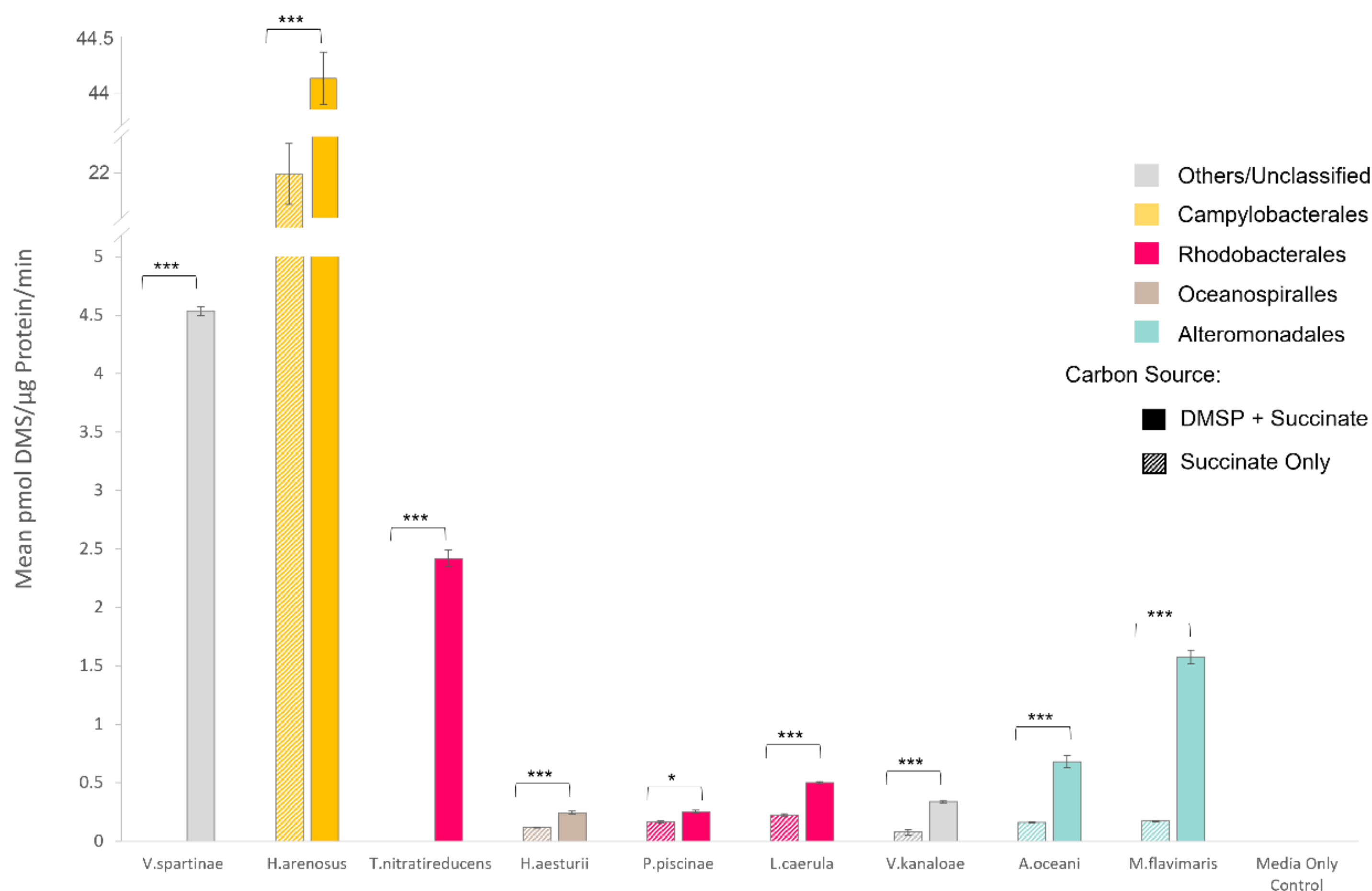


Figure 5.15: Mean rate of DMS production (pmol DMS, mg protein^{-1}, min^{-1}) in the nine species isolated from enriched samples after growth in DMSP + Succinate compared to Succinate as a sole carbon source (n = 54 biologically independent samples). Error bars are ± 1 STD. Analysis was carried out using Mann-Whitney U tests for pairwise comparisons (p = 0.005). Statistically significance differences are denoted as follows: * $p \leq 0.05$ *** $p < 0.001$.

In all cases, DMS levels were higher in the cultures grown with DMSP. However interestingly, in some species DMS was detected in the succinate only condition, which suggests that a low level of induction takes place during the incubation time.

To further characterise the catabolic abilities of the bacteria, isolates were grown on DMSP as a sole carbon source and the cell density compared to those grown in Succinate and with no carbon.

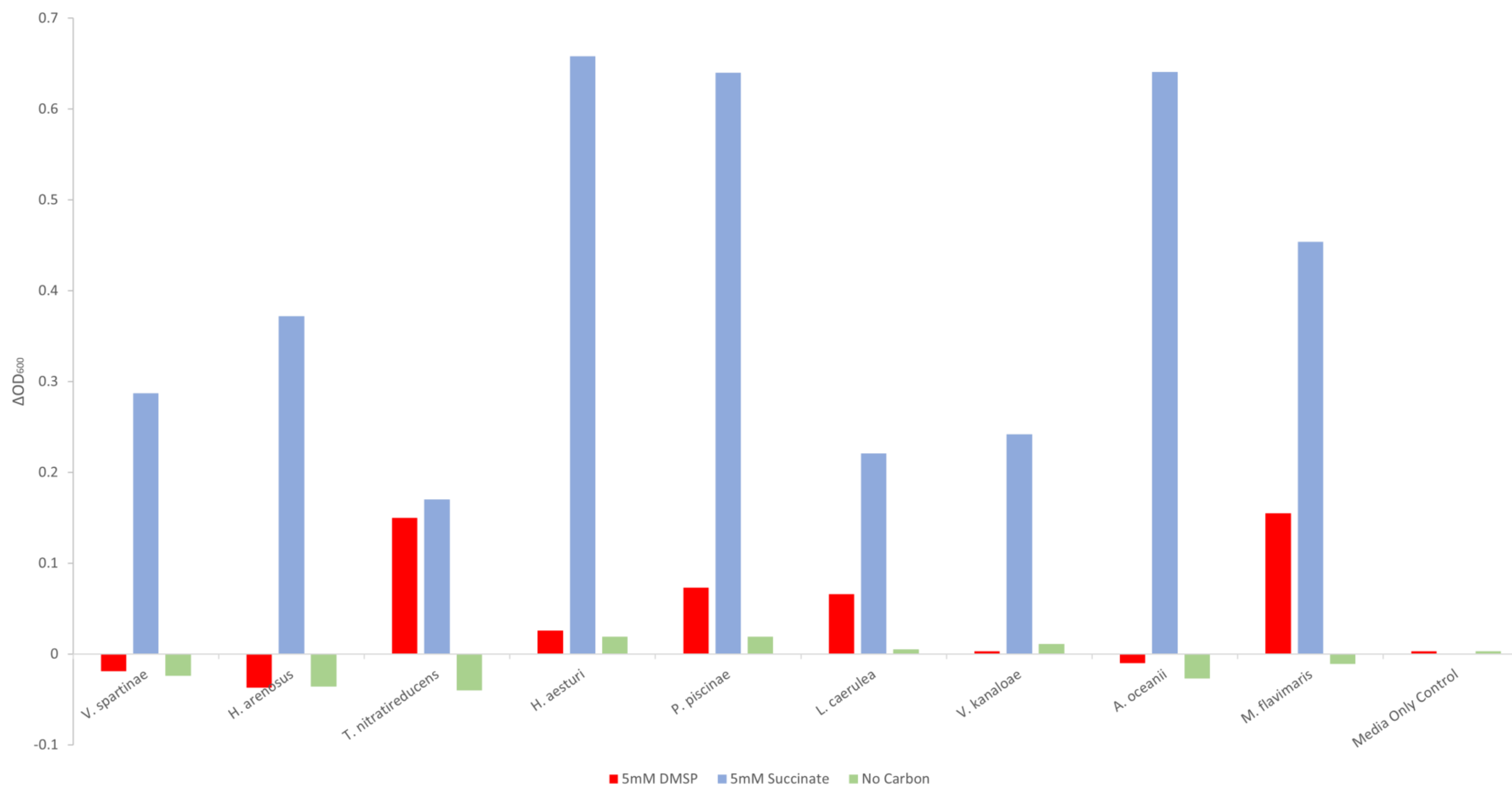


Figure 5.16: The change in OD_{600} (Δ) between T0 and T96 for each of the bacterial isolates (n= 30 biologically independent samples) grown in MBM + 5mM DMSP as a sole carbon source (red) compared to MBM + 5mM Succinate (blue) and MBM with no carbon source (green).

Isolates deemed capable of being able to grow on DMSP as a sole carbon source were determined by those with a positive ΔOD_{600} when in grown in 0.5 mM DMSP, that was also greater than ΔOD_{600} when grown with no carbon source. Of the nine isolates, four were identified: *T. nitratireducens, P. piscinae, L. caerulea* and *M. flavimaris*.

Discussion

Standing stocks

Unsurprisingly, the intracellular concentrations of DMSP in all *S. anglica* tissues were significantly higher than the rhizosphere and the bulk soil, due to the high levels of DMSP biosynthesis known to be produced by *S. anglica* (Mullholland & Otte, 2002; Rousseau *et al.,* 2017). What was more surprising was that bulk soil collected from approximately 1m away had higher DMSP concentrations than the rhizosphere samples (Fig. 3.5). This may be due to presence of macroalgae species, such as Sugar Kelp (*Laminaria saccharina*) and Double Ribboned-Weed (*Enteromorpha linza*) that have been shown to produce up 161 nmol DMSP g^{-1} FW and are more prevalent in the mudflats away from *S. anglica* clumps (Russell & Howard, 1996). This environment is consistent with where the bulk soil samples were extracted, as care was taken to avoid vegetation. Another explanation is that the rhizosphere is more likely to be anoxic compared to the bulk soil (Mavrodi *et al.,* 2018; Zedler *et al.,* 2008), due to the water retention by the rhizome clumps causing hypoxia (Mendelssohn *et al.,* 1981), and DMSP concentrations have been shown to correlate with oxygen levels at Stiffkey salt marsh (Williams *et al.,* 2019).

Analysis of DMSP Catabolism Genes in Bacterial Isolates

All DMSP-degrading alphaproteobacterial isolates were *Rhodobacteraceae* (Fig. 3.9), whose ability to catabolise DMSP is already well established (Curson *et al.,* 2008; Wagner-Döbler & Biebl, 2006). This is also consistent with previous analyses of sediment at the sample site, which found that *Rhodobacterales* such as *Ruegeria sp.* and *Oceanicola sp.* were abundant (Kröber *et al.,* 2022; Tebbe *et al.,* 2023; Williams *et al.,* 2019). The *Roseobacter* and *Paracoccus* isolates were characterised as containing homologous DmdA, DddL and/or DddP genes, compared to the reference strains, also produced DMS from DMSP at rates of 0.11 – 0.40 pmol DMS, µg protein^{-1}, min^{-1} (Fig. 3.12). This finding is partially consistent with the adaptation of *Paracoccus spp.* to non-

marine environments, despite *Rhodobacters* being primarily marine bacteria (Simon *et al.,* 2017).

However, *Roseobacter spp.* are typically characterised as marine bacteria exclusively and the identification of *Phaeobacter piscinae* and *Leisingera caerulea* within the rhizosphere is therefore a novel finding. The metagenome analysis (Figs. 3.9-3.11) were consistent with the culture dependent work, identifying *Roseobacters* as the most abundant order in both the natural sample and the ^{13}C-DMSP enriched samples. *Roseobacters* are known for forming anaerobic biofilms on shallow marine waters (Ding *et al.,* 2023). It is possible they infiltrate the soils through the extensive network tidal channels that are characteristic of salt marshes (Schwarz *et al.,* 2022). As anaerobic bacteria, they are well adapted to the oxygen-deprived rhizome mesh characteristic of regularly flooded *Spartina spp.* (Mendelssohn *et al.,* 1981). Additionally, *Alteromonadaceae* were abundant in both the natural rhizosphere and enriched samples (Fig. 3.9) which is consistent with previous studies that showed *Alteromonads* such as *Marinobacter sp.* and *Alteromonas sp.* were abundant in the sediment of Stiffkey salt marsh (Kröber *et al.,* 2022; Williams *et al.,* 2019; Tebbe *et al.,* 2023).

Of the genes with the highest relative abundance/expression, genes associated with lysis of DMSP to MeSH were prevalent, such as *DmdA*, *dmdB* and *dmdC* (Fig. 3.10). Dmd – or DMSP-Dependent Demethylase – proteins are tri-domain monomers of approximately 40 kDa (Reisch *et al.,* 2008; Schuller *et al.,* 2012) that cleaves the methyl group from DMSP, resulting in MMPA and Methyl-Tetrahydrofuran (CH_3-THF) (Howard *et al.,* 2006). This family of proteins are commonly associated with DMSP catabolism by ocean-dwelling *Roseobacters* (Hernández *et al.,* 2020; Howard *et al.,* 2006), consistent with the metagenome analysis that showed *Roseobacters* are abundant in the rhizosphere samples (Fig. 3.9). This suggests that *Roseobacters* are a novel rhizosphere species for DMSP cycling, and their presence warrants further investigation into the important role of salt-marshes, and more specifically salt-marsh rhizospheres, into global DMSP cycling.

DMSP lysis directly to DMS, generating Acrylate as a by-product were less abundant, but were moderately abundant the ^{13}C enriched fraction (relative abundance of 0.20, compared to 0.40 of *DmdA* (Fig. 3.10). Ddd – or DMSP-dependent DMS – proteins are a superfamily of lyases (Todd *et al.,* 2007). Of these, *dddL* and *dddP* were abundant. These two lyases are entirely structurally different, with *dddL* encoding for enzymes

with a characteristic β-barrel fold, or cupin domain (Wang *et al.,* 2023). In contrast, *dddP* encodes for a metallopeptidase that requires Zn, Mn or Co cofactors (Todd *et al.,* 2009). However, both genes are associated with *Rhodobacterales*, such as *Roseobacter spp.*, (Fig. 3.11) consistent with the species abundant in the rhizosphere (Liu *et al.,* 2023; Wang *et al.,* 2023).

Curiously, *DddD* genes were not abundant in any fraction, contrasting with previous studies that have found them to prevalent in *Marinomonas* bacteria associated with the *S. anglica* rhizosphere (Todd *et al.,* 2007). *Marinomonas spp.* were not abundant in this study. A possible explanation is seasonal variation in rhizobacter species, as there is evidence to suggest that considerable abiotic fluxes occur in response to the seasons, such as changing sulfate and salinity (Gamble *et al.,* 2010), which shifts the microbial profile between *Gammaproteobacteria*, such as *Marinomonas sp.* in the growing season, to *Alphaproteobacteria*, such as *Roseobacters*, in the flowering season (Gamble *et al.,* 2010). The complexity of the *S. anglica* rhizosphere and the fascinating seasonal fluxes of gene abundance/expression alone makes this a good candidate for future research.

<u>Culture-Dependent Characterisation of Rhizosphere DMSP Metabolism</u>

The DMSP-degrading gammaproteobacterial isolates were identified as *Vibrionales* (40%), *Alteromonadales* (40%) and *Oceanospiralles* (20%). The *Vibrio* isolates were characterised as producing DMS from DMSP at rates of 0.018-0.026 pmol DMS, µg protein^{-1}, min^{-1}, however no sequence homology was shown to any DMSP cleavage or DMSP oxidation genes. This is inconsistent with previous studies that showed an unresolved *Vibrio sp.* G41H has been shown to degrade DMSP and was predicted to contain Ddd and Dmd family genes, based on sequence homology (Zhang *et al.,* 2022). Low level homology was shown to downstream genes of the Acrylate catabolism pathway, notably AcuI, PrpE and DddA. This suggests that DMSP is cleaved to DMS, allowing for the acrylate by products to be utilised as part of the central metabolism, however the lack of homologous genes for direct DMSP catabolism means that this cannot yet be set down as certain. Potentially, it has the exciting potential to elucidate a hitherto unknown DMSP lyase, and further analysis is warranted of these species. All species showed significantly increased DMS production when incubated afresh with DMSP + Succinate compared to when incubated with Succinate only (Fig.7). This is

consistent with the *in silico* analysis of the species genetic potential for DMSP catabolism, and was particularly marked in *V. spartinae* (0 - 4.54 pmol DMS, mg protein^{-1}, min^{-1}) and *H. arenosus* (21.96 – 44.31 pmol DMS, mg protein^{-1}, min^{-1}).

The genera *Vibrio* has been associated with *S. anglica* previously; it was found to be moderately abundant in the phyllosphere of *S. anglica* at the sampling site (Kröber *et al.,* 2022) through SIP analysis. Additionally, *Vibrionaceae* were found in the sediment of the same site and were considered likely to have genetic potential for DMSO respiration (Tebbe *et al.,* 2023). It is therefore reassuring that the findings of this study are consistent with previous findings. However, neither study resolved the *Vibrio* to species and therein lies the novelty of this finding. *Vibrio spartinae* is a relatively newly identified species, isolated from *Spartina maritima* rhizospheres in Spanish salt-marshes (Lucena *et al.,* 2017). Not only is this the first identification of *V. spartinae* in the UK and in association with *S. anglica*, but no characterisation of its DMSP degrading or synthesizing capabilities have been undertaken prior to this study. *V. spartinae* is a halophile exclusively associated with halophyte rhizospheres (Lucena *et al.,* 2017; Mateos-Naranjo *et al.,* 2020; Vitale *et al.,* 2020). It has been characterised as having plant-growth promoting properties, such as nitrogen fixation and solubilisation of phosphates (Mateos-Naranjo *et al.,* 2020), as well as production of prodigiosin, an anti-microbial compound that gives it its characteristic red colour (Vitale *et al.,* 2020). It is possible that *V. spartinae* catabolises DMSP to DMS as part of this suite of plant-growth promoting mechanisms, and this exciting discovery warrants further investigation.

Vibrio kanaloae is marine pathogen of molluscs, causing large scale outbreaks of lethal hepatopancreatitis in bivalves (Huang *et al.,* 2021; Romalde *et al.,* 2014; Xin *et al.,* 2022). This is the first instance of *V. kanaloae* recorded in terrestrial systems, and its presence may be the consequence of infection of mollusc species found in salt marsh sediments. Indeed, *S. alterniflora* has been found to have a mutualistic relationship with Atlantic Ribbed Mussels (*Geukensia demissa*) in salt marshes (Bilkovic *et al.,* 2017), congregating around the base of the stems and the upper rhizome layers (Nielsen & Franz, 1995), which would bring pathogenic species such as *V. kanaloae* into proximity with the *Spartina spp.* rhizospheres. The purpose of metabolising DMSP in *V. kanaloae* is speculative, but as bivalves are known to experience high levels of cellular hypoxia

(Donaghy *et al.,* 2013), it may produce an advantage in surviving long enough to colonise molluscan cells over other pathogens.

Curiously, although both *Vibrio spartinae* and *Vibrio kanaloae* had strong sequence homology to known DMSP lyase genes (DddX and DddP – see Tab. 5.1), neither were able to grow on DMSP as a sole carbon source. A similar phenomenon was found in *Desulvovibrio acrylicus*, which contained the DMSP lyase similar in function to DddY (van der Maarel *et al.,* 1996[a]). This *Vibrio* species cleaved DMSP to DMS and acrylate, but instead of fermenting acrylate to propionate and acetate, in common with other anerobic bacteria, instead uses it as an electron acceptor for anaerobic respiration (van der Maarel *et al.,* 1996[b]). Given this precedent in *Vibrio* species, it may be that *V. spartinae* and *V. kanaloae* would grow on DMSP as a sole carbon source in anaerobic conditions, using this mechanism.

Of the *Alteromonadales* isolates (*Alteromonas* and *Marinobacter*), both produced DMS from DMSP at a rate of 0.013-0.023 pmol DMS, µg protein^{-1}, min^{-1} respectively, and both had low homology to DmdB and DmdC genes, although none to DmdA. This suggests that DMS is generated from DMSP catabolism to MeSH. *Marinobacter spp.* and *Alteromonas spp.* have been previously identified associated with *S. anglica* rhizospheres at this site and this study confirms previous findings of their DMSP catabolic capabilities (Williams *et al.,* 2019). *Alteromonas alba* is an aerobic bacterium, commonly associated with cyanobacteria in open waters (Feng *et al.,* 2021; Sun *et al.,* 2019), so its presence in the rhizosphere is curious. Again, it is possible that is has been carried into the sediment through sea water ingress via tidal channels (Schwarz *et al.,* 2022) but it might have migrated from the surface cyanobacteria that is commonly found across the sample site.

The *Oceanospiralles* isolate, *Halomonas aesturii*, produced 0.007 pmol DMS, µg protein^{-1}, min^{-1} and was found to have homology the strongest homology to DmdA, but also DddD and DddP. This is consistent with previous findings that *Oceanospiralles* species are important DMSP catabolisers in coastal environments (Kröber *et al.,* 2022; Liu *et al.,* 2022; Tebbe *et al.,* 2023). The *epsilonproteobacteria* identified, *Haloarcobacter arenosus* (formerly known as *Arcobacter arenosus* (Pérez-Cataluña *et al.,* 2018)) produced DMS from DMSP at the second highest rate of 0.288 pmol DMS, µg protein^{-1}, min^{-1} and had homology to DddY, compared to the reference strains. This

is consistent with a small number of *Arcobacter* species have been previously identified as DMSP-catabolisers, such as *Arcobacter nitrofigillis*, that were shown to harbour DddY genes (Curson *et al.,* 2011).

Additionally, five out of the nine isolates produced significantly higher volumes of MeSH compared to the media only control (Fig. 3). With no additional DMSO, this is consistent with the demethylation of DMSP by DmdA homologues to MeSH and DMS, as opposed to oxidation of DMS to MeSH by DmoA homologues.

P. piscinae was the highest producer of MeSH with 11.43 pmol MeSH, mg protein^{-1}, min^{-1} despite no sequence homology to DmdA, although downstream DmdB and DmdC were represented, consistent with the catabolism of DMSP via the Dmd pathway identified in marine *Roseobacters* (Howard *et al.,* 2006). *L. caerulea* did not produce MeSH, despite sequence homology to DmdA and being part of the *Roseobacteraceae*, which are well characterised for DMSP catabolism to MeSH (Howard *et al.,* 2006; Howard *et al.,* 2008). This may be because it is more energetically efficient to produce DMS through a single-step lysis pathway, and instead relies more on the activity of Ddd genes. Alternatively, DMSP is catabolised by a DmdA homologue, but there is total conversion of MeSH to DMS, consistent with the high concentration of DMS produced by both species (Fig. 2). *H. aesturii* produced the lowest concentration of MeSH, consistent with also producing the lowest concentration of DMS. This suggests that whilst it has DMSP catabolic activity, it does so at low rates and is therefore less reliant on DMSP metabolism than the other isolates.

The *Alteromonadales* isolates (*Alteromonas* and *Marinobacter*) both produced MeSH, consistent with the presence of DmdB and DmdC homologous genes, despite no homology to DmdA. This is contrary to previous studies, that have found DddD genes to be more representative of DMSP lysis strategies by *Oceanospiralles* (Liu *et al,* 2022). Possibly terrestrial species utilise more than one lysis mechanism, which highlights the potential of terrestrial systems for DMSP cycling.

All the *Rosebacterales* had an increased OD_{600} after four days of growth with DMSP as a sole carbon source, although *Roseobacters* are well known for their ability to catabolise DMSP (Curson *et al.,* 2011), this result in inconsistent with previous studies that have not found *Roseobacters* isolated by SIP as capable of using DMSP as a sole carbon source (Liu *et al.,* 2022). Traditionally, it was thought that *Roseobacters*

catabolise DMSP purely for their sulfur content (Liu *et al.,* 2022; Todd *et al.,* 2012; Wagner-Döbler & Biebl, 2006). Additionally, the *Oceanospiralles*, *M. flavimaris* also showed an increase in OD_{600}. This is consistent with previous studies, that found the *Oceanospiralles* species *Marinobacter sediminum* and *Marinobacter rhizophilum* were able to grow on DMSP as a sole carbon source (Liu *et al.,* 2022).

All the *Roseobacters* grown with 5 mM DMSP as a sole carbon source showed an increase in growth compared to their species counterparts grown with no carbon source. In the case of *M. flavimaris*, there was no increase in growth in either condition, both remaining at the starting OD_{600}. This suggests that the *Roseobacters* do catabolise DMSP for the carbon content, and not just the sulfur as previously thought. *T. nitratireducens* showed the most prominent growth, reaching exponential phase at 25 h (Fig. 5). *T. nitratireducens* is a relatively newly identified species, isolated from the Bering Sea (Liu *et al.,* 2017) and as such its DMSP catabolising activity has not previously been studied. Other *Thioclava* species, notably *Thioclava pacifica*, have been identified as thiosulfate-catabolising chemoautotrophs that grow without a carbon source (Sorokin *et al.,* 2005), so the ability to utilise sulfur sources is not unexpected in *T. nitratireducens.*

Overall DMSP production was significantly lower (0.043 – 0.199 pmol DMS, µg $protein^{-1}$, min^{-1}) than when grown with DMSP as the sole carbon source (Fig. 6), however there was no significant difference in DMS production with Met compared to the media only control ($p < 0.05$ for all pair-wise comparisons). This suggests that these species do not synthesize DMSP. This is consistent with previous findings, that have not identified these species as having the genetic potential for DMSP-synthesis within the *S. anglica* rhizosphere (Williams *et al.,* 2019).

Additionally, the media only control had detectable DMS (0.056 pmol DMS, mg $protein^{-1}$, min^{-1}), not found in any other assay. Methionine requires enzyme activity to form DMS, whether that is through DMSP biosynthesis, or through lysis and oxidation to MeSH and subsequently DMS (Higgins *et al.,* 2008). Consequently, the presence of DMS in the media only control suggests that the Methionine stocks may have been contaminated with either DMS or DMSP.

Chapter 6 – Final Discussion and Recommendations for Future Work

DMSP Production by Higher Plants

DMSP Biosynthesis is More Prevalent in Higher Plants than Previously Thought

In line with the growing body of literature on the subject, our findings demonstrate that even more species of higher plants produce DMSP (Chapter 1). This is highly significant for the field in showing that terrestrial DMSP production is critical for global sulfur fluxes and thus contributes more heavily to the climate cooling effects of DMSP than hitherto thought. Previously, the most widely held opinion was that DMSP cycling was predominantly by marine eukaryotes, specifically phytoplankton, (Kiene et al. 2000), with species such as *E. huxleyi* producing it at consistently high levels without much regulation (Sunda et al. 2007). However, this thesis has contributed to the changing paradigm of terrestrial environments being as significant, and the wide variety of higher plants that produce DMSP suggests that quite possibly all plants can synthesise DMSP to some level.

As previously discussed, if all higher plants, or even a large majority, produce only a small concentration of DMSP, the overwhelming global biomass of terrestrial plants will contribute tremendously to the global flux of DMSP. For example, this study demonstrated that Barley is a moderate producer of DMSP (91.67 nmol DMSP/g FW). For an individual plant, this may not seem particularly important, but in the UK alone, Barley fields cover 1,104,000 hectares (DEFRA, 2022). This is a not an insignificant volume of DMSP, and this accounts for one species, in one country only. The global production of DMSP from higher plants cumulatively is likely to account for a significant part of the global sulfur budget.

Additionally, this thesis has shown that the range of higher plants producing DMSP to moderate levels is greater than previously thought. This study corroborates the data put forward by Ausma *et al.,* (2017) that a wide range of wild angiosperms, native to the UK, produce low to moderate concentrations of DMSP. As well as expanding the range of wild plants tested, we have also shown that a wide variety of crop plants from a range of families also produce DMSP. This includes members of the *Brassicaceae* and *Allioideae* families, noted for their high sulfur content (Akpolat and Barringer, 2015; Danner *et al.,* 2015), non-native crops such as Banana and scientifically important model species, such as *Arabidopsis thaliana, Medicago truncatula* and *Nicotiana*

benthamiana. This further supports our conclusion that terrestrial environments, and higher plants therein, are significantly producers of DMSP.

It worth continuing in this vein of study, to fully assess the breadth of DMSP production. Going forwards, the findings of the work, in regard to specific families of crop plants that reasonably high and detectable DMSP will allow for more specific studies to be generated. Indeed, at the time of writing, a study characterising DMSP production in Barley in salt-afflicted fields, and a comparison between stress phenotypes when treated with DMSP-containing fertiliser is already underway (Li *et al.,* personal communication). Additionally, expanding these studies to in detail the role of DMSP in ruderal species such as Banana in coping with abiotic stress is critical to work centred on the repair of damaged ecosystems, especially in neo-tropical regions that are already suffering from the effects of rising sea levels and salinisation. Full metabolomics profiles of osmolytes would be the ideal for such species, using the methods proposed in the discussion of Chapter 4, and would enable a greater understanding of the role DMSP plays in such plants, relative to other compounds.

There are No Clear Taxonomic Links between High DMSP Producers

Having shown that a wide variety of plant species produce DMSP, what is more curious is that there are no strong taxonomic links between higher producing species. Although some basal land plants were assayed, most plants surveyed were flowering plants. Of these, moderate to high producers were represented in the Grass (*Poales*) and Ginger (*Zingiberales*) orders within the monocots, and the Brassica (*Brassicales*), Gentian (*Gentianales*) and Daisy (*Asterales*) orders within the eudicots. The apparent unrelatedness of these orders, suggests that high levels of DMSP biosynthesis evolved convergently multiple times, and that environmental factors are a larger driver of this behaviour in plants, rather than phylogeny.

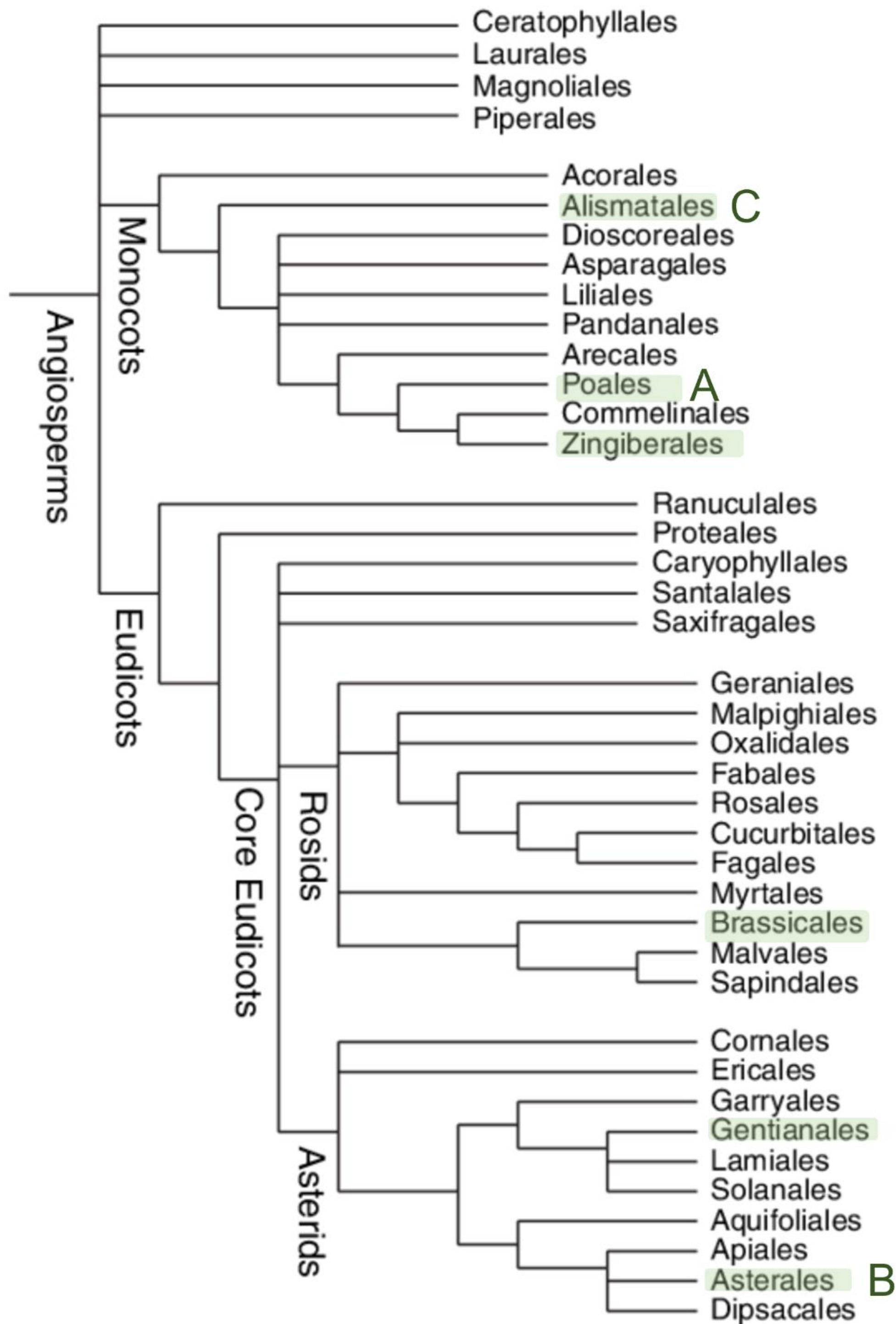


Figure 6.1: Phylogenetic tree of plant families, with moderate and high DMSP producing species families highlighted in green. Families marked with a letter indicate they contain a representative the four highest producing species: A) *S. anglica* and *S. officianarum* B) *M. biflora* and C) *P. oceanica*. Figure adapted from Guiltinan *et al*., 2008.

The range of species shown to be moderately high producers from our study supports the theory that DMSP is used in higher plants as an osmolyte and/or as an antioxidant. In common with salt marsh plants, such as *S. anglica*, Banana and Coffee plants are

from natural environments commonly associated with elevated sodium, albeit due to alkalinity rather than proximity to the sea (Agbeshie *et al.,* 2010). Potentially, the slightly higher levels of DMSP found in the wild plants sampled by Ausma *et al.,* (2017) are a function of plant saline resistance, as these hedgerow species are adapted to increased saline and sulfur, from road surfaces and agricultural run-off (Green *et al.,* 2008). Additionally, the Brassicas are known for high intracellular antioxidant concentrations (Faller & Fiahlo, 2009; Lin & Chang, 2005), supporting the theory that DMSP functions as an antioxidant. The natural conclusion, therefore, is that DMSP has different primary roles in different species.

Since the conclusion of this study, preliminary work has indeed been done to expand the understanding of the quantities of DMSP produced in basal land plants. Payet *et al.,* (2024) conducted a comprehensive sampling of bryophytes (mosses) and gymnosperms (e.g. pine trees) to assess intracellular DMSP concentrations. These results are in review, but once again found a large degree of variation between genera and species, although species known for production of aromatic compounds, such as Monkey Puzzle Trees (*Araucaria araucana*) producing amounts comparable to that of Barley (Payet *et al.,* 2024). This highlights the lack of understanding of the multitude of roles played by DMSP in higher plants, and trialling DMSP production in response to other abiotic stresses would help to characterise this more thoroughly.

Arabidopsis thaliana is Novel Model Organism for DMSP Biosynthesis

An exciting outcome of assaying a variety of species for DMSP production is the discovery that the favoured model organism for plant biology, *Arabidopsis thaliana*, biosynthesises DMSP (up to 450 nmol DMSP g^{-1} FW), albeit not to the same extent as *S. anglica*. Having a well-established model organism suitable for study is of great benefit to this area of research. Discovering that wild type *A. thaliana* is capable of synthesising DMSP opens a world of experiments to compare DMSP concentrations in a variety of environmental conditions that were beyond the scope of this thesis, such DMSP in response to heavy metal ions, oxidative stress and drought. Additionally, the fully sequenced genome and tractability for genetic manipulation makes it simpler and quicker to study the methylation pathway and ratify the remaining genes. This can be done through knock-out of candidate genes, or transformation with genes from a high producer, such as *S. anglica* to increase expression.

The optimisation processes to determine spatial-temporal changes in DMSP concentration carried out in this project will also significantly enhance future experiments in this field. Having confirmed that DMSP is present in both aerial and root tissues – with higher concentrations in roots – confirms previous work in higher plants (Ausma *et al.,* 2017; Catola *et al.,* 2016; Kocsis & Hanson, 2000), but with the additional understanding that optimal stage for harvesting is 10 days post germination, and that additional Methionine increases DMSP concentration, future experiments will be considerably more efficient. This is the first study to examine the effect of plant age on intracellular DMSP concentration, contributing significantly to our understanding of the complexity of DMSP production and regulation in higher plants. This work could be significantly enhanced in future studies by the use of metabolomics techniques such as DART-TOMFS, as detailed in the discussion of Chapter 4, to thoroughly understand real-time DMSP synthesis in live plants, without the need for destructive sample preparation methods.

Ratifying DMSP Production Pathways in High Plants

MMT is the Gene Encoding the First Step of the Methylation Pathway

Through knocking out *MMT* in *A. thaliana*, we were able to prove that DMSP production is decreased in *MMT*- plants compared to the wildtype (Chapter 2). Whilst this is not the first time *MMT* has been knocked out in *A. thaliana* (Lee *et al.,* 2008; Tagmount *et al.,* 2002), it is the first time this has been done in the context of DMSP biosynthesis and is consequently an important new experimental tool in this field. Firstly, this confirms previous studies that have determined the methylation pathway to be present and functional in higher plants, for DMSP biosynthesis (James et al., 1995; Kocsis & Hanson, 2000; Kocsis et al., 1998). Secondly, it demonstrates that *A. thaliana* is indeed a suitable organism for the study of DMSP biosynthesis.

Thirdly, and perhaps more excitingly, is that *MMT*- plants did not have a complete absence of DMSP, but a decreased concentration. This suggests that higher plants can accumulate and store DMSP or that there is another supplementary pathway at work. It is entirely possible that both mechanisms are at play, but this certainly brings new avenues of study to the field of DMSP biosynthesis and function in higher plants. Clearly, genetic and cellular mechanisms of DMSP biosynthesis in plants is an

understudied area and given the importance of terrestrial systems in DMSP cycling, the importance of continuing research in this area cannot be understated.

DMSP Production in the Model Organism *Arabidopsis thaliana* is Upregulated by Salt Stress

This study also demonstrated that DMSP does indeed confer salt resistance to higher plants, as *MMT- A. thaliana* plants that produced less DMSP than their wildtype counterparts were significantly more stressed. The reduction in biomass, root length and increase in total number of dead plants are all clear indications of salt stress and were correlated with decreased DMSP production. This supports the theory that DMSP does indeed function as an osmolyte in higher plants (Storey *et al.,* 1993; Trossat *et al.,* 1998). Whilst it has been previously established that MMT has a role in salt tolerance in *A. thaliana* (Ogawa & Mitsuya, 2011), this is again the first time that its role in DMSP biosynthesis has been studied in *A. thaliana*. It is also the first time DMSP and its effects salinity have been studied in this model organism. This study represents a lot of firsts in the fields; and is the start of a novel and fascinating avenue of studying DMSP in a new model organism.

This has the potential to be very impactful for engineering salt tolerance in glycophytic crops. For example, future work could study the effects of the application of exogenous DMSP on saline tolerance. This has been successfully proven with Glycine Betaine, a structural homologue (Hamani *et al.,* 2021) and it would be interesting to see if DMSP can confer increased salt tolerance when applied externally. Additionally, there is increased interest in the use of transgenic to engineer salt tolerance in plants (Kotula *et al.,* 2020), as current models of ocean level rises predict that salt incursion will increase (Chen & Mueller, 2018). As previously discussed, this will severely limit the land available to grow glycophytic species (Shrivastava & Kumar, 2015). Consequently, transgenic approached to enhance DMSP production in higher plants represents a possible mechanism of improving abiotic stress resistance and thus, agricultural productivity.

DMSP and the Salt Marsh Rhizosphere

Spartina anglica Rhizobacteria Synthesise and Catabolise DMSP

Having focused on the production of DMSP in higher, the move in the third chapter to the metabolism of DMSP in the Rhizosphere may seem to be a step away from the focus

of this project. However, plants do not exist in isolation and have complicated symbiotic relationships with the microbes surrounding them. Thus, the decision to study the role of the rhizosphere in DMSP production in a known high producer is critical to the holistic understanding of DMSP production in higher plants. Understanding this interplay between the plants and their microbiome to adapt to salt stress is becoming increasingly important conjunction with increased food insecurity and a rising global population (Ladeiro, 2012). The use of microbes to inoculate the soil and boost availability of nutrients has been long deployed through nitrogen fixing bacteria such as *Rhizobium*, and more recently phosphate solubilising bacteria such as *Bacillus* and *Pseudomonas* (Parnell *et al.,* 2016). Utilising plant microbiomes to enhance abiotic stress therefore has a precedent and there is a developing interest within the research community. Consequently, a thorough understanding of the mechanisms by which plants and microbes produce osmolytes such as DMSP can assist in engineering greater halotolerance in crop plants.

Thus, revealing that the *S. anglica* rhizobacteria are capable of metabolising DMSP supports previous studies on the importance of bacteria in terrestrial environments (Williams *et al.,* 2019) and also that there is a great deal more to DMSP biosynthesis in higher plants than had previously been thought. It is very likely that plants take up DMSP from their rhizobacteria in symbiotic relationship, and provide some benefit to the bacteria, such as vitamin production (Tariq & Ahmed, 2023). This again supports the idea that exogenous application of DMSP may be beneficial for conferring salt resistance in crop plants.

Of these DMSP Producing Rhizobacteria, finding *Roseobacters* present in a terrestrial system is a novelty. Although well characterised as marine DMSP-producers, their discovery as a dominant rhizosphere-associated bacteria demonstrates that there is a great deal of work to be done to fully characterise the terrestrial environment and accurately assess its role in global sulfur cycling.

Recommendations for Future Work

There is a lot of work that could be done to enhance our knowledge, in addition to the suggestions detailed previously in this chapter. Firstly, having established suitable a model organism, continuing the ratify the remaining genes in the methylation pathway is critical to understanding the intracellular and genetic mechanisms of DMSP biosynthesis. This can be achieved by generating knock out mutants for each candidate

gene and measuring the DMSP concentrations compared to the wildtype. Additionally, testing the concentration of the intermediate product from each step will prove that the methylation pathway is consistent in higher plants. This could be done by utilising the SIP methods that were so effective in Chapter 5, but instead applying it to the precursor molecule methionine. The converse could also be done, using agrobacterium transformation to overexpress the genes of the pathway and measure increases in DMSP production would ratify the genes.

Secondly, improving our understanding of the abiotic influences that regulate DMSP in higher plants will elucidate its roles. Experiments in which plants are subjected to drought, elevated or decreased temperatures, heavy metal stress or simulated grazing and the correlation to DMSP concentrations would be very interesting to identify other uses for DMSP. As a step towards improving crop resistance, the exogenous application of DMSP to the model organism *A. thaliana* would also be beneficial.

Finally, further studies on the rhizosphere of plants would be an interesting avenue to explore. Comparisons of the species and genes present in the rhizospheres of different plants, such as high producers compared to low producers would help to establish how influential the species present are. Additionally, how far the rhizosphere effects DMSP production could be analysed by comparing changes in the rhizosphere of the same plant species under different abiotic conditions. Additionally, much of the work has centred on Stiffkey Saltmarsh ((Kröber *et al.,* 2022; Tebbe *et al.,* 2023; Williams *et al.,* 2019) and the rhizosphere and sediments thereof are now extremely well characterized. It would therefore be interesting to replicate these studies in other geographic areas and to compare the microbial profiles of *S. anglica*. A comparison of these geographic differences would help to pinpoint factors that enhance or detract from DMSP cycling. Greater depth could be added to these studies with the addition of ionomic profiling of the rhizospheres and sediments, to establish in detail the effects of salinity, as well as other compounds that may induce a stress response, such as heavy metals (Bollman-Giolai *et al.,* 2024).

There is a wealth of methods that could be employed to produce novel and useful results. This exciting new field of DMSP cycling in higher plants, therefore, has a lot of potential in better understanding mechanisms of climate cooling and salt tolerance in higher plants.

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Appendix

1. Candidate Arabidopsis MMT Gene Sequence SALK_ AT5G49810.1

CTATGCCACAAATGTCTTATATAATTTGATTCCTTGCTGGTGAGAGTCACAAAACCCAAC

AGACTTCACACTTTCACTTTCATTCGCTTTTTCTTCTCTATTACTGCAATCACTTGTTCACG

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GCAGCTTATGGCGCCTTGCGATCGGTCTTGGAGCGGCTCGAGGATCCGAATACTCGATCT

AAGGCTCGGATCTTCTTGTCTGACATCTACAAACGCGTCGGATCTTCTGAGACGTCTCTC

CAAACCTACCATTTTCATATCCAGGACATCTATCTCGATCAATACGAAGGTAACATCATC

GATCCGATTCTTTGTTGTTCCTCTTTCACGATCCTTGAGAATTGTTCTGTGATTATTTGTCA

AATGGATTTGATTCAGCTGATTATACTGATTTGGTGATTCCCTGTGTGTAATCTCGCCGAT

CTTACGTTACTTTGTTTAGTTGACTTCATGATCGATTAAAATGATCTGATTTCTGGCTTCTT

TTGCTTGATTTCCCGGAGAATTGGGATGATTCCGGTATCAATCTGCGCTTTTGTTTACTGG

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GATCATGGTTTAAGGTTTGAATTCCAGACTTTTGGGAAACCATATGAGCTTTAGGTTTTA

CAAATTCAATACTGACACTGATATTTTTTTCTTATATGATTTGTGATCGTATTTTTAGAAC

AGTAAATTTATCCATAGCAGAAATTTTTACAAAACGTTCTGTTGCTATGAGCCGTATTGA

CAGAGAGGCATGATCATCAGGAGTTTAAGTTAAAATTGTTTTGGATGGTCCTTTTTTCTTT

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TTCTTGGATAAATTTGTACCTAAACGCTCTTGATGATAATGGCGAACCAGTCTATGACGA

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TTTATGCATGCTTCCTTGTATCTTATGCTCTTGTGGCAGATTCTTAATCCAAACCCAGAAG

CTATGTCTAAGCTGATCACAGAAAATGCAAGTGAGGAATTTCTCCATTCGCTGAGTAACT

ATTGTGCCCTTCAGGTAAGGGAATTTTTTCCCAGCAAAAATACTCTGAAGGTATCATTA

GTATGTTAGAAAGCTAGTTCCTAGAACTCTTCTGGTGCGTCCCAATTTCATGTTATATCAT

TCAATTGTCTAATCTATGATACCTCTTTTCTTCCTTCTCTATCAAACATCATGTGAAATCTC

ACATTGAGCTTTTGCGAGTATGATTGAGCCAGTCATATGCATGCCTGACATATCGCATT

TTCAACCTAAATGTTGCATGTTTGTGATGAGTATTTACATAAG**AAAACTTTTTCTCCTC**

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TTGCAGGCTTTATGAGGACATACCACCGTATACCAATTAATCAGGATGTAAGACTATTCT

CTATCTATGTTTTTGACAGTTTCTTAGAGTTTCCAGGCAATTACTTATTCAGACTCATAA

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CCTCGACTTGCAATTGTTGATGAGCATTTAACTCGACAACTTCCGAGGAGCTGGCTAACC
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CATTGTGCATTGCTGGTTCTGAGACTTAACACGTCGCTCTTCTTCTGCGTTCCTTTGTTCTG

TTGTTTCTAAATAACTCGCTTCCCGTTTCTTGGTCAGCCCCATAACGGGTTTACTTTTTGG

AAGTATGATATTTTCATGCTAATAAAGGAGTCATAAAACTTTGAATAAGTCTTATATTTT

AATGTGAATAAGTCTCCTTGTTTCTATGATGTTTTTTTCTTTTATGTTCGATCTTGGGCTCT

CTATGATGTTCCAGAAAAAAAGAATGTGTTCCAGAAAAGATATCAAATAAGTATCTCAA

AAT

Exon Intron Start Codon Stop Codon *UTR*

2. Bacterial mmtN Amino Acid Sequences

Roseovarius indicus DSM26383:

MTDFKTPETVGDSEEPVVTPHAPEFAFDPTDPWTETFQRGLEIAGLGGKRVYEVGIGTGINVA

FMLQICEAAVVSGSDLDPRLAGLAERNVRDLAPRRADRFHPVEGAVSLIDTPEARAQVGRSD

VIVGCLPQVGEPDDVRLRAFRTAQKAKLAKGADTRDEDHIAHYYPWAEFDSYPFNSVGLGL

NEALLRRTRATAPAADVVLNFGARVGSAVLFELFEANGYVPEKLHSQIVLQHAGTDISFFVA

LENALAQTGLEREFTCEFYGDPEGATRLSATEAQALVDTDSAAEIYHEVCVIRGRPALSETDP

SNR

Thalassiospira profundimaris DSM17340:

MLEESSETSSPYASDPETPDFAFDPDDPWTQTFQEGLARADLKDKTVYEVGVGTGINVAFILQ

SCGAKRVYGSDLDPRLVVLAERNIKILSPEHAKHFKPVHGSVSLVDTDEAREKIAKTDVVIAC

IPQVGEPSDARLTAFREAQSIELAEGAGDEAEDHIAHYYPWSLFDQYPYNSVGLGLNEALMR

RIREHAPKAELVMNFGCRIGTEIICECFEANGYKPEKIASKIVLQHSGTDISFFVSLEKALNGTE

YEKQLVCKFYGDPEGKQPLSATKAQEMINDDPNVPLYHEVAVIRGTPV

Streptomyces mobaerensis:

MGSSHHHHHHSSGLVPRGSHMPSEHTMLAPAPAPASVPDPAPASVPSFTFDPSDPWTVTFQA

GLERAGLRGRRVYEVGVGSGANVLHLLRRCGAAHVTASDLDPRLPPLARRFVMDAAPGLA

GRCRFIEGSVSLVDGPAATEAVVAADTVVACLPQVPDPGDAMYTRFRAAHLRTGPETGGPL

RITDHAAHYYPWSAFDDHPFNAVGLGLIEALLRRVRARAPRAEVVLNLGCRIGKDVLTRLFR

AHGYRPEELASRVVPQDGRTDITFFAALEAALRGTGHEKDFTCSFSADPEGRRPLSATEAADR

LAADPGTPVFHEICVLRGRPTAFDDVPDEEDRR

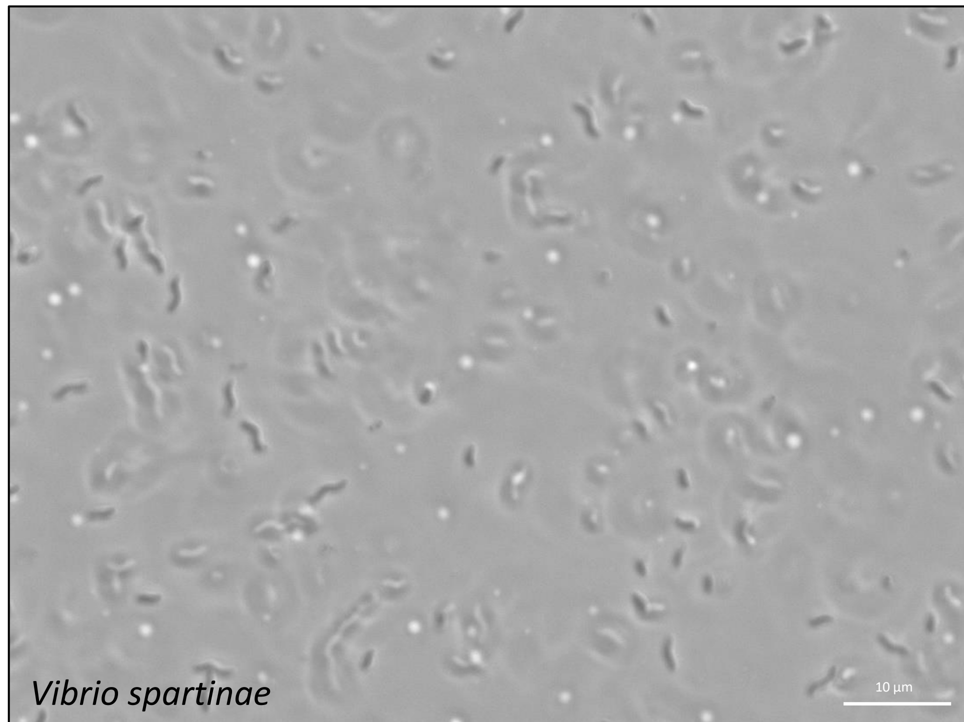
3. Accession numbers of previously ratified enzymes involved in the degradation of DMSP.

Protein	Ratified strains	Accession number	Reference
DmdA	*Ruegeria pomeroyi* DSS-3	AAV95190	Howard *et al.,* 2006
	Candidatus Pelagibacter ubique HTCC1062	WP_011281570	
	Dinoroseobacter shibae DFL 12	WP_012178987	Howard *et al.,* 2008
	marine gammaproteobacterium HTCC2080	WP_007233625	
	Candidatus Pelagibacter sp. HTCC7211	WP_008546106	Howard *et al.,* 2011
	Candidatus Puniceispirillum marinum IMCC1322	WP_013044947	
DddD	*Marinomonas* sp. MWYL1	ABR72937	Todd *et al.,* 2007
	Oceanimonas doudoroffii	AEQ39135	Curson *et al.,* 2012
	Psychrobacter sp. J466	ACY02894	
	Halomonas sp. HTNK1	ACV84065	Todd *et al.,* 2010
	Sinorhizobium fredii NGR234	AAQ87407	Todd *et al.,* 2007
	Burkholderia ambifaria AMMD	WP_011659284	
	Pseudomonas sp. J465	ACY01992	Curson *et al.,* 2010
DddL	*Sulfitobacter* sp. EE-36	ADK55772	Curson *et al.,* 2008
	Rhodobacter sphaeroides 2.4.1	YP_351475	
	Labrenzia aggregata LZB033	KP639184	Curson *et al.,* 2017
	Ahrensia marina LZD062	KP639183	Liu *et al.,* 2018
DddP	*Roseovarius nubinhibens* ISM	EAP77700	Todd *et al.,* 2009
	Ruegeria pomeroyi DSS-3	WP_044029245	Todd *et al.,* 2011
	Phaeobacter inhibens DSM 17395	AFO91571	Burkhardt *et al.,* 2017
	Oceanimonas doudoroffii DSM 7028	AEQ39091	Curson *et al.,* 2012
	Oceanimonas doudoroffii DSM 7028	AEQ39103	
	Aspergillus oryzae RIB40	BAE62778	Todd *et al.,* 2009
	Fusarium graminearum PH-1	XP_389272	
DddQ	*Ruegeria pomeroyi* DSS-3	WP_011047333	

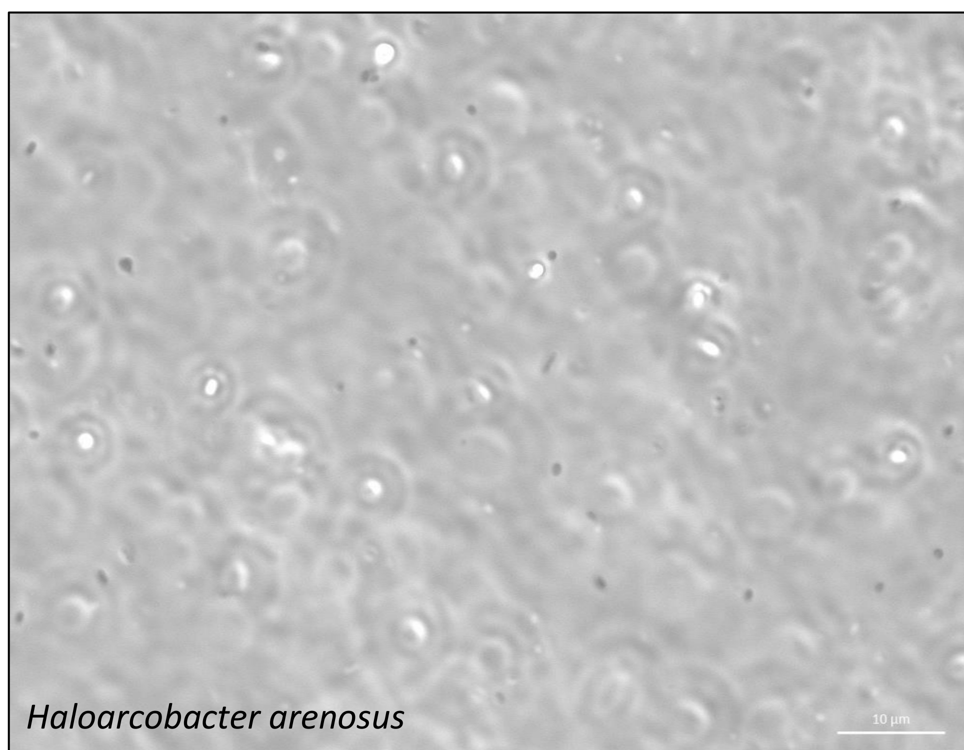
	Roseovarius nubinhibens ISM	EAP76002	Todd *et al.,* 2011
	Roseovarius nubinhibens ISM	EAP76001	
	Ruegeria lacuscaerulensis ITI1157	WP_005978225	Li *et al.,* 2014
	GOS_2632696	ECW91654	Todd *et al.,* 2011
	GOS_7860946	EBP74803	
	GOS_2469775	ECX82089	
DddW	*Ruegeria pomeroyi* DSS-3	AAV93771	Todd *et al.,* 2012
DddY	*Alcaligenes faecalis* M3A	ADT64689	Curson *et al.,* 2011a
	Desulfovibrio acrylicus	SHJ73420	van der Maarel *et al.,* 1996
	Acinetobacter bereziniae	ENV21217	Li *et al.,* 2017
	Ferrimonas kyonanensis DSM 18153	WP_028114584	Lei *et al.,* 2017
	Shewanella putrefaciens CN-32	ABP77243	Curson *et al.,* 2011b
DddK	*Candidatus* Pelagibacter ubique HTCC1062	AAZ21215	Sun *et al.,* 2016
	Candidatus Pelagibacter ubique HTCC9022	WP_028037226	
	alphaproteobacterium_HIMB5	AFS47241.1	
DddX	*Marinobacterium jannaschii*	WP_084332639.1	Li *et al.,* 2021
	Pelagicola sp. LXJ1103	WP_109384856.1	
	Psychrobacter sp. P11G5	WP_068035783.1	
	Sporosarcina sp. P33	WP_081242855.1	
Alma1	*Emiliania huxleyi* CCMP1516	XP_005784450	Alcolombri *et al.,* 2015
	Emiliania huxleyi CCMP1516	XP_005763983	

Ratified proteins were used to confirm sequences obtained from metagenomes, metagenome-assembled genomes (MAGs) and genomes of bacterial strains as functional genes of interest.

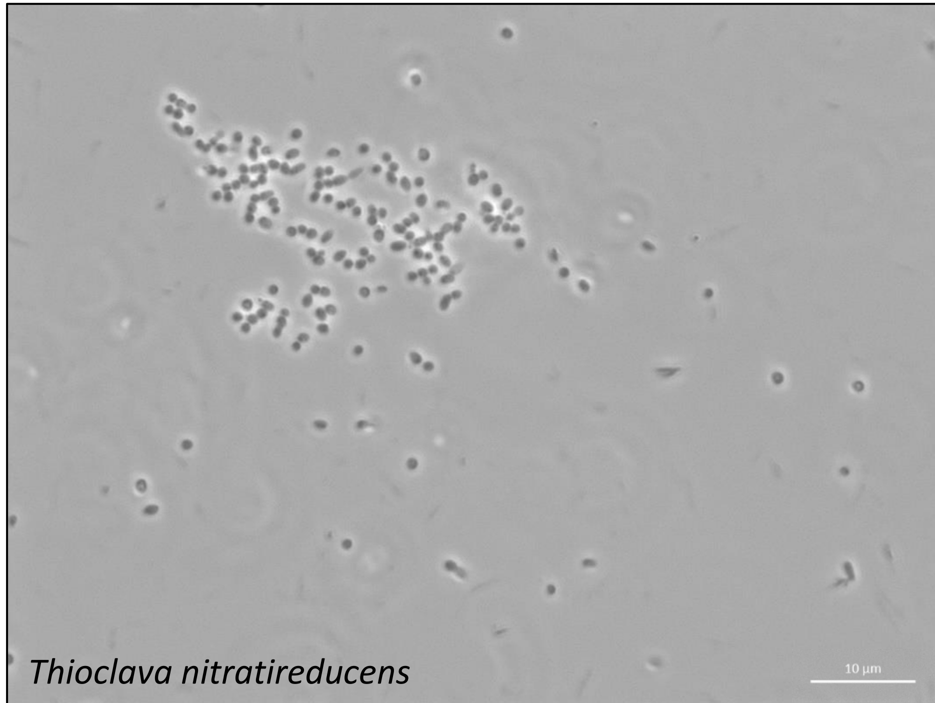
4. Phase Contrast Microscopy Images of Bacterial Isolates

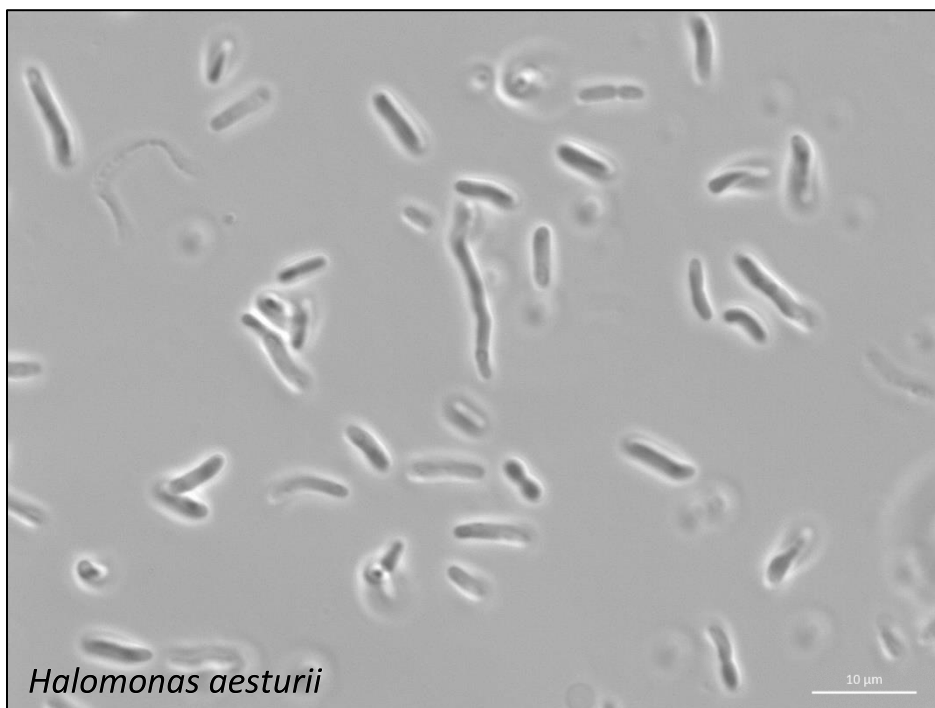


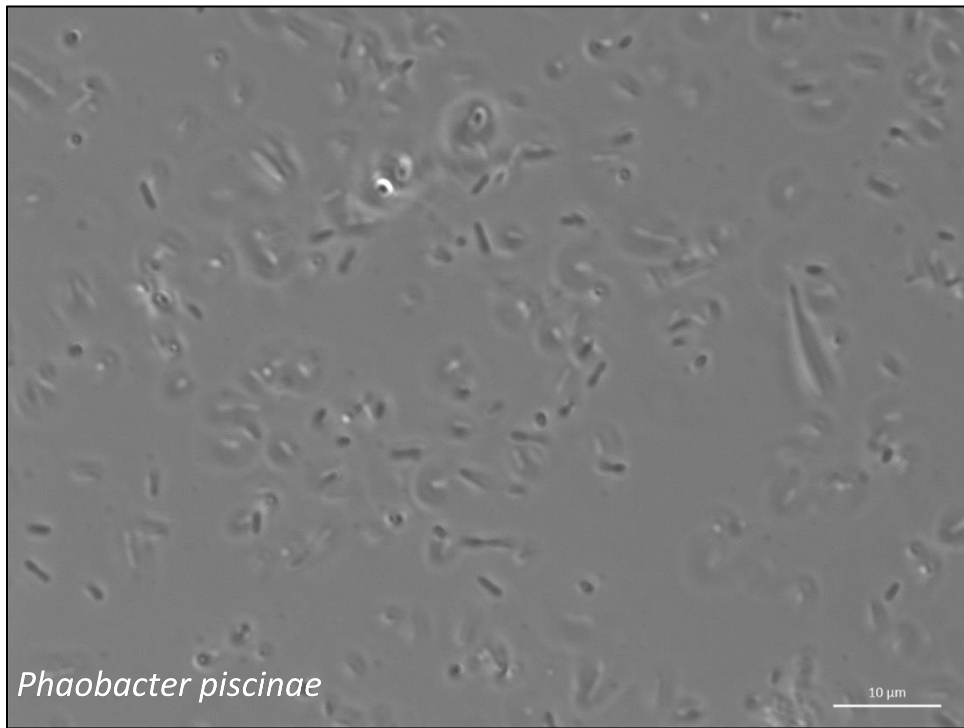
Vibrio spartinae

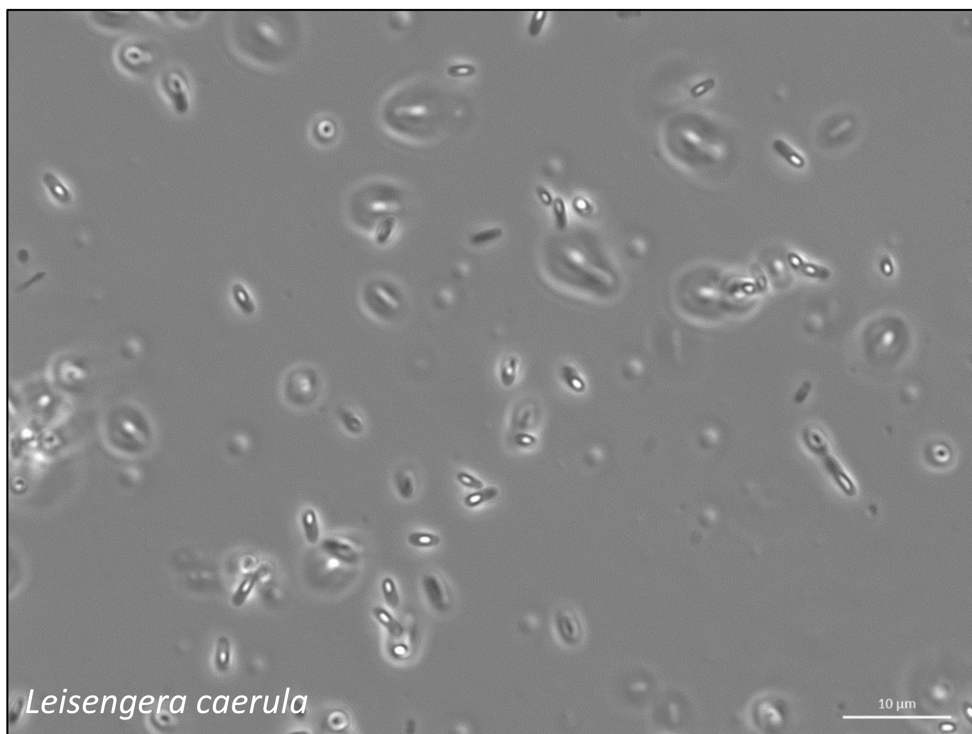


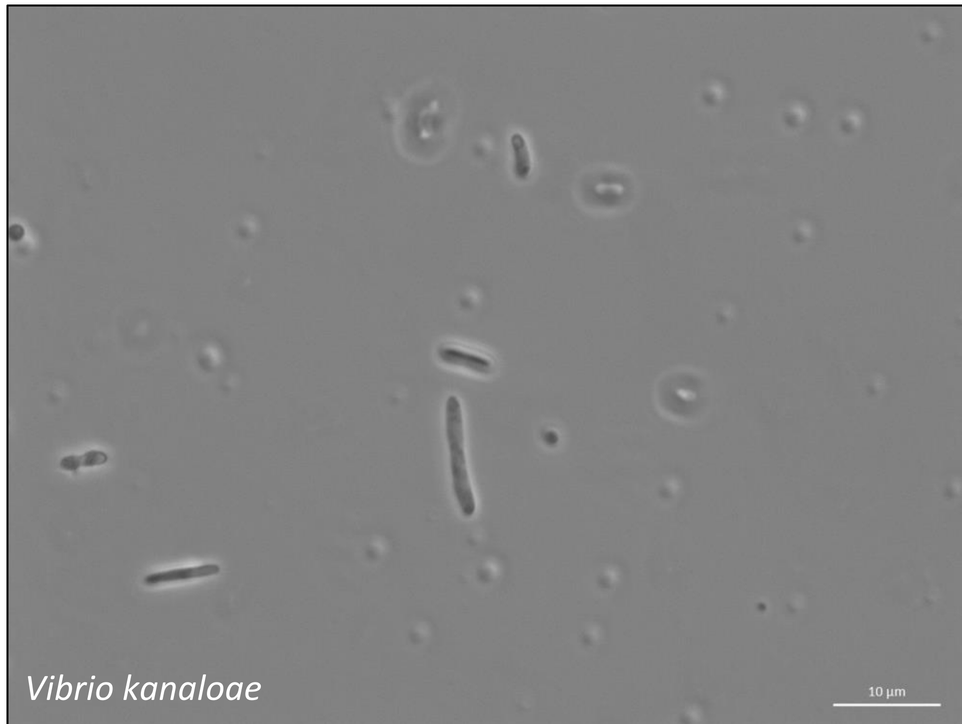
Haloarcobacter arenosus

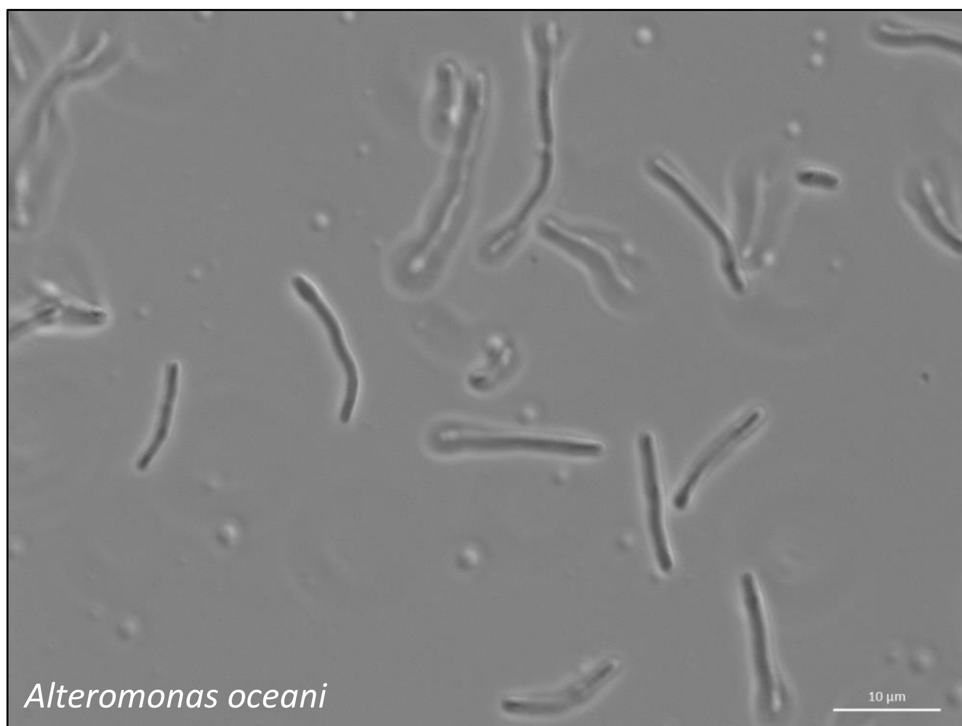

Thioclava nitratireducens
10 µm

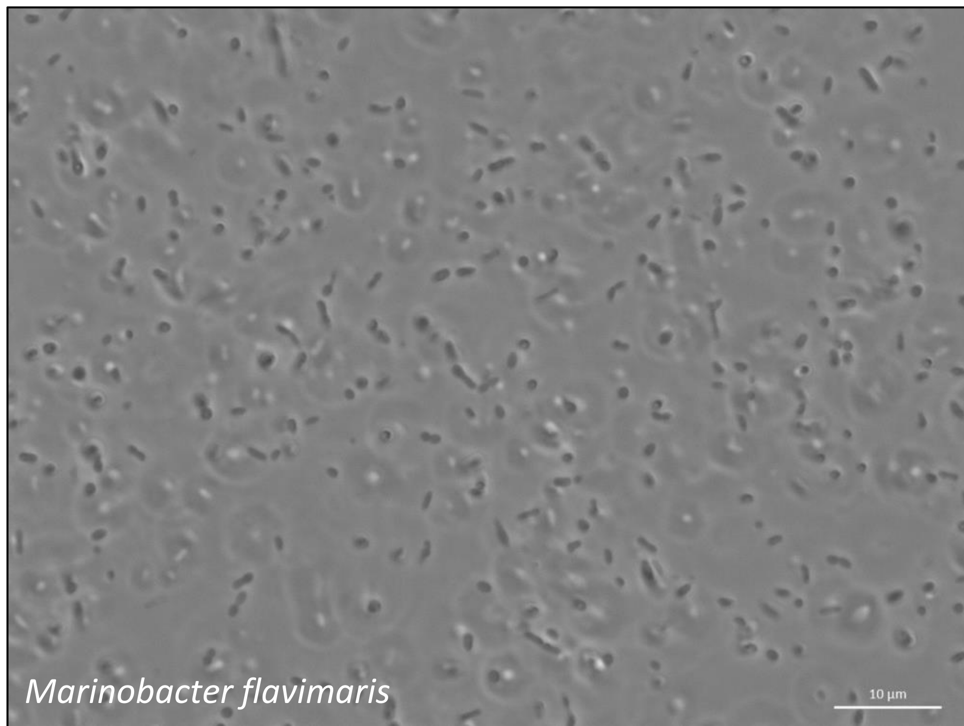

Halomonas aesturii
10 µm


Phaobacter piscinae
10 µm


Leisengera caerula
10 µm


Vibrio kanaloae
10 µm


Alteromonas oceani
10 µm


Marinobacter flavimaris
10 µm