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DMSP Cycling in Terrestrial Plant Environments

A thesis submitted for the degree of Master of Science by Research in Biomolecular Science

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ABSTRACT

Dimethylsulphoniopropionate (DMSP) is an abundant marine organosulphur compound produced by many marine microbes that acts also as a precursor to dimethyl sulfide (DMS). This project began when the longstanding belief that the majority of DMS synthesis occurs as a result of DMSP degradation via a cleavage pathway commonly utilised by marine bacterial and algal species, was challenged thanks to the identification of new terrestrial plant DMSP synthesis enzymes. Knowing that DMSP production was in fact widespread in plants and their soil environments, it was decided that this project would endeavour to clarify our understanding of DMSP cycling and challenge the dogma that it is a primarily marine process by characterising the microbial species existing in close proximity to DMSP-synthesising plants and potentially identifying DMSP-catabolism.

The aim of this project is to identify microbial DMSP degraders in terrestrial environments, specifically focusing on maize and rye plants as plants that produce low levels of DMSP, and Spanish Cane plants, that produce significantly higher levels of DMSP. This thesis details a year-long period of work, that showed 181 DMSP-degrading bacteria and 4 fungal strains to be present in environments such as the phyllosphere and rhizosphere environments of maize, rye and Spanish Cane plants, all of which produce significant (albeit varied) levels of DMSP. A variety of these strains were shown to either possess known *ddd* genes or belong to genera where *ddd* genes have been identified. This revelation provides strong evidence that terrestrial DMSP cycling is more significant than previously believed, highlighting a need for further investigation into the organisms within these environments that facilitate said cycling.

CHAPTER 1

INTRODUCTION

1.1 The Sulfur Cycle

All life on Earth requires sulfur as a nutrient to survive, evidenced by the fact that it accounts for ~1% of the dry mass of organisms on the planet (Sievert et al, 2007). For life, this essential element is vitally important for the role it plays in amino acids such as methionine and cysteine providing structural importance to a multitude of proteins. Globally, a large part of what makes the element so crucial is its ability to switch valence from -2 to +6, changing from sulfide to sulfate, and it is this alone that allows for the complex sulfur cycle we observe in nature to exist.

This biogeochemical cycle involves the conversion of sulfur to a number of derrivative products via different pathways comprised of various redox reactions. The cycle sees sulfate reduced to sulfide, which then acts as an electron donor in oxidation reactions that yield organic sulfur and sulfate once again. Specifically, the dissimilatory sulfate reduction pathway (figure 1) is commonly utilised by bacteria as a form of anaerobic respiration. This chemolithotrophic process sees sulfate reduced into hydrogen sulfide by bacterial and archaeal species, bridging a vital gap that would otherwise render the sulfur cycle incomplete and proving the imporatnce of microbes in global sulfur cycling (Santos *et al*, 2015). It is, in part, thanks to this microbial action that organosulfur compounds are so abundant in marine environments, where marine microbes are known to carry out synthesis and subsequent catabolism of organosulfur compounds. This has resulted in the Earth's oceans acting as large stores of sulfur in the form of derrivative compounds such as sulfate (Sievert *et al*, 2007). Mineralisation of sulfur means that over time, oxidative erosion leads to a release of sulfates (Farquhar *et al.,* 2000) which producers within food chains use in metabolic reactions. Eventually, as said producers are consumed and the sulfur products contained move between trophic levels, sulfate will be returned to the soil as saprotrophic decomposers break down dead organisms. Over time, the sulfur in the soil will be returned to marine environments, carried by water runoff. It is here that sulfate is assimilated into various products, one of which is dimethylsulfoniopropionate (DMSP).

1.2 Dimethylsulfoniopropionate (DMSP)

Dimethylsuplphionoproprionate (DMSP) is a prevalent organosulphur compound in marine environments with studies showing that $2x10^9$ tons of DMSP are produced per year (Ksionzek *et al*., 2016), making up 10% of the global marine primary production (Howard *et al*., 2006). Originally purified from a *Polysiphonia fastigiata* and *Prevotella nigrescens* in marine environments (Challenger and Simpson, 1948), it was believed that DMSP was only synthesised by marine eukaryotes including: algae and single celled marine phytoplankton, corals and many angiosperms. More recent work has

since revealed that alphaproteobacterium such as *Labrenzia aggregata* LZB033 also synthesise DMSP (Curson *et al.,* 2017).

Synthesised by a variety of microorganisms, DMSP itself has multiple suggested roles in the microbes making it. For example, it has been shown that the rate of DMSP synthesis increases at lower temperatures, which combined with the proof that the metabolite has a stabilising effect on enzymes implies that DMSP may act as a cryoprotectant (Bullock *et al*., 2017). High intracellular concentrations (2M in some dinoflagellates) suggests that DMSP may also be an osmoprotectant, supported by evidence that its production is heavily regulated by environmental salinity (Stefels, 2000).

There are multiple other suggested roles of DMSP, with its impact on protist grazing implying it is a grazing deterrent (Laroche *et al.,* 1999) and with a separate study showing that it may act as a signalling molecule for certain phytoplankton (Seymour *et al.,* 2010). Beyond the marine environment, DMSP has been shown to have potential anti-cancer properties through stimulating an immune response in mice against mammary carcinomas (Nakajima and Nakajima, 2011). As stated previously, it is an abundant marine metabolite which makes it not only a common source of sulfur, but also carbon among marine bacterial populations. It's been shown to provide up to 13% of the require carbon in surface-water marine populations (Kiene *et al.,* 2000), while also providing protein structural integrity through sulfur for amino acids.

When DMSP is released into the enviornment it a key source of reduced sulfur and carbon for marine microorganisms via two degradation pathways, one of these pathways further, the major source of the climate active gas dimethyl sulfide (DMS), as shown below. Note some of the organsims that produce DMSP also can catablise DMSP. REF

1.3 DMSP as a precursor to DMS and why this is important

Despite its inherent supposed properties, DMSP is so important as it is the major precursor to the climate cooling gas DMS (*Lovelock et al.,* 1972). DMS makes up roughly 60% of the natural reduced sulfur released into the atmosphere (Stefels *et al.,* 2007), with estimations showing anywhere between $15x10^{12}$ to $33x10^{12}$ g S y-1 (Kettle and Andreae, 2000). This atmospheric DMS is oxidised to form sulphur dioxide which in turn forms sulfate aerosols. These aerosols attract condensation to form clouds (hence they are deemed 'cloud condensation nuclei' or 'CCN') which reflect solar radiation (as can be seen in figure 1). Therefore, DMS is classified as a climate cooling gas (Arnold *et al.,* 2013).

In a study published April of 1987 (Charlson *et al*., 1987), it was hypothesised that the warming of the planet, driven by solar radiation, has generated more regions in which phytoplankton capable of DMSP synthesis are able to survive. The study proposed that the subsequent increase in DMSP-producers would see an increase in DMSP abundance and thus an increase in the concentration of DMS released into the atmosphere. Therefore, as the planet warms, more DMS is released which leads to more cloud cover, reflecting more solar radiation and eventually cooling the global temperature.

While the extent to which this CLAW hypothesis (named after the authors of the original study) remains highly debated (Quinn *et al.,* 2011), it remains a fact that DMS is a CNN and thus a climate cooling gas, further cementing DMS and its precursor, DMSP, as compounds that play a large role in the maintenance of global ecosystems

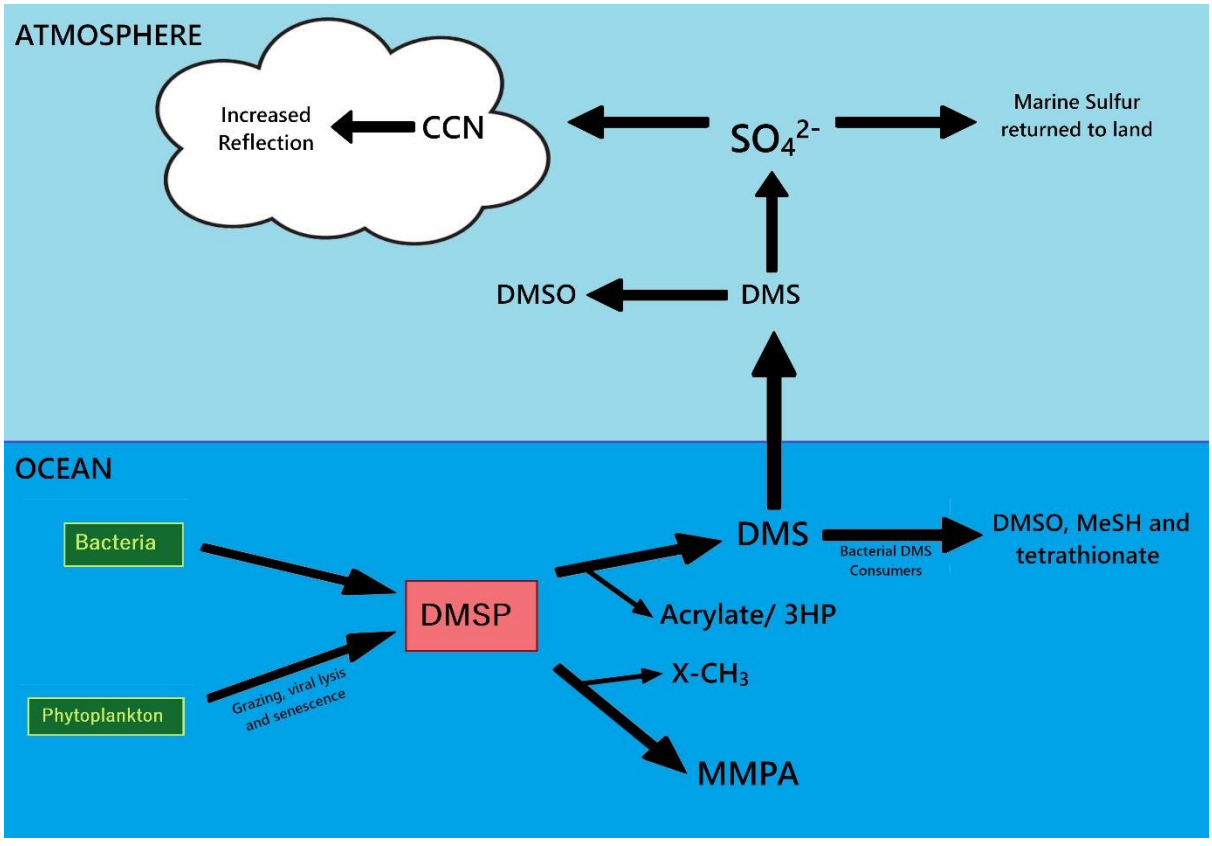

Figure 1: The vital roles DMSP and DMS play in the biogeochemical sulfur cycle. Phytoplankton and bacteria produce DMSP which is released into the marine environment in several different ways. The two major pathways through which DMSP is degraded are represented above, with the demethylation pathway forming MMPA and the cleavage pathway forming DMS. From here, DMS can be transformed by marine microbes or released into the atmosphere to form DMSO or airborne sulfate which can cause cloud formation by acting as nuclei. Adapted from (Curson *et al.,* 2011).

1.4 Synthesis of DMSP

DMSP was believed only be made by algae and unicellular phytoplankton (Stefels, 2000), corals (Raina *et al.,* 2013) and a few coastal angiosperms (Bullock *et al.,* 2017) at significant levels. It was later discovered that alphaproteobacterium is also capable of DMSP synthesis (with *Labrenzia aggregata* LZB033 being the first of this class to be identified) (Curson *et al.,* 2017). Despite a handful of angiosperms being reported to produce DMSP in terrestrial environments, the vast majority of said producers inhabit saline environemnts such as saltmarshes. This led to the belief that DMSP synthesis is an overwhelmingly marine process.

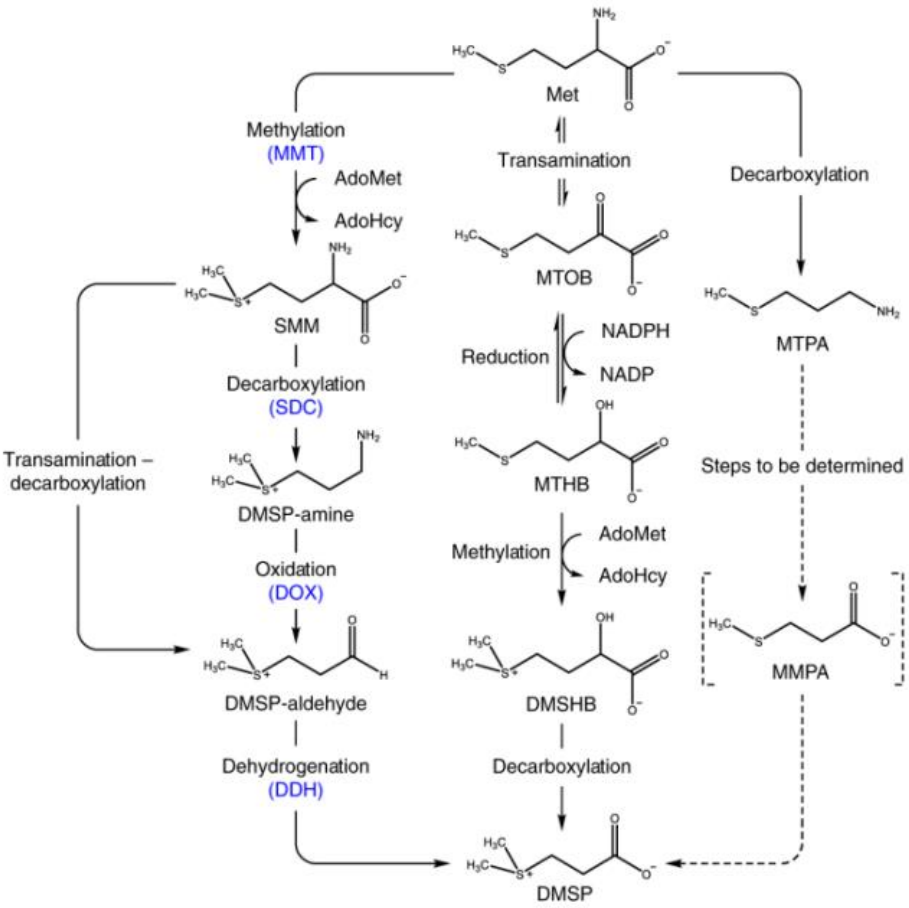

Figure 2: DMSP synthetic pathways; methylation (left), transamination (middle), decarboxylation (right). The methylation pathway is used by organisms that contain the *mmtN* synthesis gene, the transamination pathway is used by organism that contain the *dsyB* synthesis gene. The single dinoflagellate *Crypthecodinium* is known to use the decarboxylation pathway – the dotted line represents a suggested (but as yet unconfirmed) pathway. Figure adapted from Kotaras, 2020

Here, I will go into detail explaining the known synthesis pathways of DMSP (as depicted in figure 2). The first synthesis pathway is a methylation pathway used by some higher. There is an initial methylation of methionine to form S-methymethionine and a final pair of oxidation reactions

responsible for converting DMSP-amine into DMSP-aldehyde, which is then converted into the final DMSP product (Hanson *et al.,* 1994). The second of these synthesis pathways is a methionine transamination pathway (figure 2) utilised by various phytoplankton and macroalgae (Gage *et al.,* 1997). Finally, the third of these pathways is a used by dinoflagellates, involving methionine decarboxylation. Not all steps of the pathway are currently known, but it is understood that methionine is decarboxylated to form 3-methylthiopropionate (Kitaguchi *et al.,* 1999).

1.4.1 Methylation Pathway

The methylation synthesis pathway (figure 3) begins with the conversion of *L*-methionine to *S*-methylmethionine (SMM) in a methylation reaction. This step is catalysed by *S*-adenosylmethionine:methionine S-methyltransferase (MMT) (James *et al.,* 1995), an enzyme which triggers the donation of a methyl group by S-Adenosyl-L-methionine (AdoMet) (Hanson *et al.,* 1994). The SMM generated from this methylation is subsequently transferred from the cytosol (Trossat *et al.,* 1996)., into the site of DMSP synthesis (typically the chloroplast in organisms where this methylation pathway has been identified (Stefels, 2000). At this juncture, it is important to recognise that while the methylation pathway has been observed in the *Novosphingobium* BW1 bacterium (Williams *et al.,* 2019), as of writing the pathway has been observed primarily in higher plant species. Studies on this methylation pathway have been centred on a select group of these higher plants, including *Saccharum* (Gramineae), *Wollastonia biflora* (Compositae) and a species of *Spartina.* This is important as the following step in the pathway differs between species.

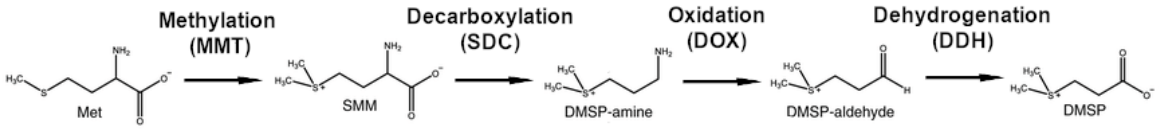

Figure 3: The stages of the DMSP synthetic methylation pathway.

The next step observed in the methylation pathway of *Saccharum* (Gramineae) sees SMM decarboxylated by the enzyme pyridoxal 5'-phosphate (PLP) to form DMSP-amine (Kocsis *et al.,* 2000) which then has its amino group removed. This removal occurs due to an oxidative deamination which is believed to be catalysed by an enzyme reliant on the presence of oxygen (Dickschat *et al.,* 2015), a reaction which transforms the DMSP-amine into DMSP-aldehyde.

Differing to the stages seen in *Saacharum* (Gramineae) after relocation of SMM to the chloroplast, the methylation pathway used by *Wallastonia biflora* (Compositae) sees the amino group of the SMM

molecule transferred to 2-oxoglutarate to form DMSP-aldehyde. This step notably lacks the intermediate DMSP-amine seen in *Saacharum* (Gramineae), instead utilising a pyridoxal 5'-phosphate dependent transamination-decarboxylation of the SMM. It has been shown that the CO_2 generated by said reaction is used in a decarboxylation reaction, but the intermediate formed as a result prior to the final synthesis of DMSP-aldehyde remains undiscovered (Dickschat *et al.,* 2015). The final stage of the pathway is identical in higher plants (Stefels, 2000), with the DMSP-aldehyde undergoing a dehydrogenation reaction to produce DMSP.

1.4.2 Methionine Transamination Pathway

The first step in the methionine transamination pathway (figure 4) sees methionine transformed to 4-methylthio-2-oxobutyrate (MTOB) in a reversible transamination (Gage *et al.,* 1997). After MTOB has been formed, the molecule is then reduced to form 4-methylthio-2-hydroxybutyrate (MTHB) by NADPH-linked reduction (Summers *et al.,* 1998). S-Adenosyl-L-methionine (AdoMet) is then involved as it transfers a methyl group to the sulfur molecule on MTHB in an irreversible methylation reaction (catalysed by an S-methyltransferase) that converts it to 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB). It should be noted that while all previous stages of this synthesis pathway are present in microoorganisms that do not produce DMSP, the aformentioned stage is exclusively found in DMSP-producers (Ito *et al.,* 2011). Once the DMSHB intermediate is formed, a final oxidative decarboxylation occurs to form DMSP (Kettles *et al.,* 2014).

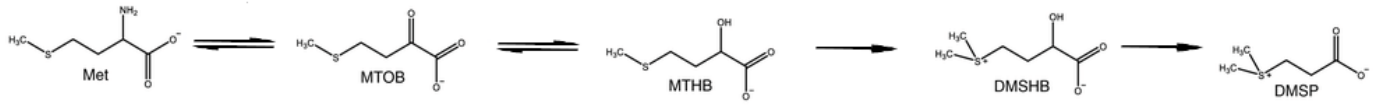

Figure 4: The stages of the DMSP synthetic methionine transamination pathway.

Studies have shown that this pathway is the most commonly utilised of the three, its use found to be primarily by various species of red and green algae. It has been shown that bacteria (Curson *et al.,* 2017) and coral (Raina *et al.,* 2013) also utilise said pathway. The first major gene of the DMSP synthesis pathway to be identified was *dsyB* in *Labrenzia aggregata*. This gene encodes a methyltransferase-like protein which catalyses the SAM-dependent methylation of MTHB to DMSHB in *L. aggregata*. It has been shown that *dsyB* is found in 0.5% of bacteria in marine metagenomes, proving that it is a key step in said pathway. Furthermore, the presence of the methyltransferase enzyme 'DSYB' (a homologue of *dsyB*) has been confirmed in marine organisms including a selection of corals and certain phytoplankton (Curson *et al.,* 2018).

1.4.3 Decarboxylation Pathway

As stated previously, the steps involved in this synthesis process (figure 5) remains relatively unknown. A study on *Crypthecodinium cohnii*, a marine dinoflagellate species, first revealed that DMSP could be synthesised in this way. This was a significant revelation as dinoflagellates are responsible for a large proportion of the DMSP found in marine environments due to their high intracellular conenctrations of the compound which are leeched into ocean waters during algal blooms. Currently, it is understood that an enzyme known as L-methionine decarboxylase sees methionine decarboxylated into 3-methylthiopropylamine (MTPA) (Uchida *et al.,* 1996). Beyond this, the reactions involved in the decarboxylation pathway remain undiscovered. A common speculation is that an intermediate product is currently undetermined and that its discovery would allow for the full pathway to be confirmed (Dickschat *et al.,* 2015).

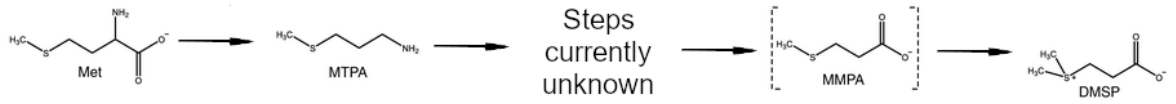

Figure 5: The stages of the DMSP synthetic decarboxylation pathway.

1.5 Degradation of DMSP

The many proposed properties of DMSP combined with the fact that it is an extremely abundant food source for microorganisms would explain why it's so commonly utilised throughout the marine environment (Howard *et al*., 2006). Other than its obvious role as a sulfur source, the molecule also acts as a source of carbon for many marine microbes while playing a role in chemosynthesis for others (Yoch, 2002). In fact, roughly 10% of microbial carbon in surface water environments is derrived from DMSP (Kiene *et al.,* 2000). Its diversity in this regard cement DMSP as a molecule vital to the survival of innumerate marine bacterial species by facilitating a range of metabolic pathways. To do so, the compound must be catabolised so that its base components can be accessed, something that is achieved via one of two major pathways in bacterial species.

1.5.1 DMSP Lysis

Also known as the cleavage pathway, DMSP lysis (figure 6) is achieved through the use of Ddd enzymes in bacteria and fungi and Alma1 in some algae. This group of proteins comprises of DMSP-lyases found in bacterial DMSP degraders, except for the lyase enzyme 'Alma1' found in algae such as *Emiliania huxleyi* (Alcolombri *et al.,* 2015). It is worth noting that the bacterial strains that possess *ddd* genes are not exclusively found in marine environments (Curson *et al.,* 2011).

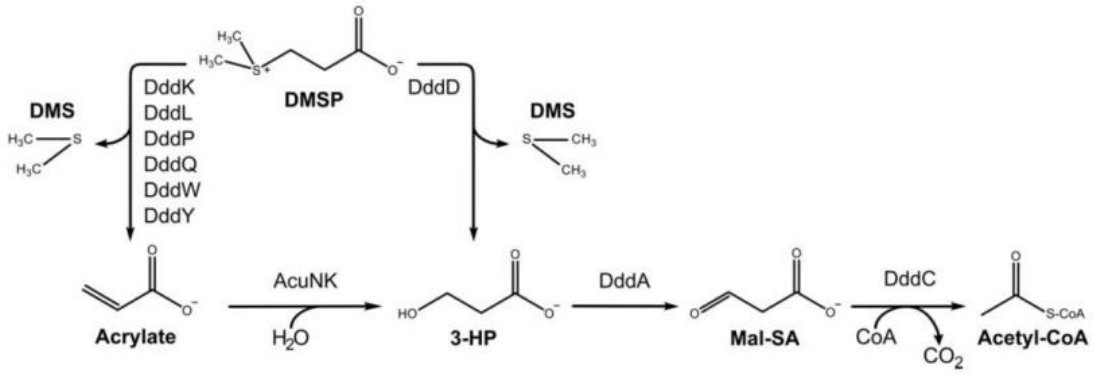

Figure 6: The lysis pathway for DMSP degradation. Lysis of DMSP yields DMS via one of two ways controlled by various ddd genes. Enzymes controlling the lysis of DMSP to acrylate are DddK/L/P/Q/W/Y. Acrylate is then converted to 3-HP via AcuNK. Alternatively, DddD, catalyses the lysis of DMSP to 3-HP directly. Once the 3-HP molecule has been formed, DddA catalyses its oxidation to Mal-SA where DddC catalyses the addition of a coenzyme A moiety to form actetyl-CoA. Figure adapted from Kotaras, 2020.

The first of these enzymes to be identified was DddD, a group of DMSP-lyase enzymes originally discovered in *Marinomonas sp.* MWYL1 (a marine bacterium) (Todd *et al.,* 2007). While identified DMSP lysis pathways tend to lead to the formation of DMS (with acrylate), the way in which they reach this point differs. For pathways utilising DddD, a CoA transferase cleaves a DMSP molecule, leading to the creation of Mal-SA after the resultant 3-HP is oxidised. Finally, the Mal-SA molecule is converted to Acetyl-CoA after a DddC enzyme adds a coenzyme A (Curson *et al.,* 2011).

Other enzymes, including Dddl, DddQ, DddY, DddK, DddP and DddW, instead catalyse the cleavage of DMSP by breaking the carbon-sulfur bond so that a molecule of DMS is produced (Curson *et al.,* 2008). DddX is another type of lyase which catalyses the ligation of DMSP and CoA, an intermediate which is then cleaved in a different way so that acrylol-CoA and DMS are formed (Li *et al*., 2021).

1.5.2 DMSP Demethylation

More common than the aforementioned cleavage pathway, DMSP demethylation pathway (figure 7) sees DMSP demethylated to form a molecule of 5-methyl-THF as well as a molecule of methylmercaptopropionate (MMPA) by the enzyme DmdA (Curson *et al.,* 2011). MMPA is then demethiolated before being converted to MTA-Coa via a dehydrogenation reaction (catalysed by methylmercaptopropionyl-CoA ligase) (Reisch *et al.,* 2011). This MTA-Coa molecule undergoes hydration and then hydrolysis in quick succession, creating a molecule of acetylaldehyde which is finally dehydrogenated to form acetate.

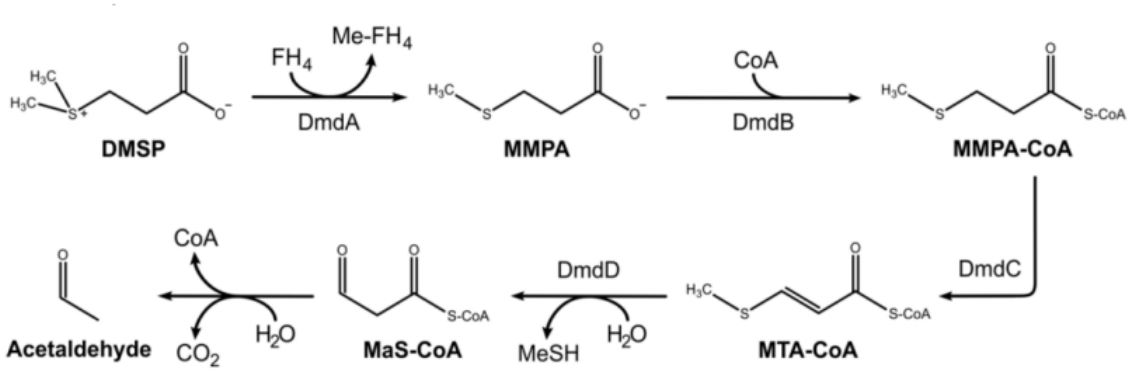

Figure 7: The demethylation catabolic pathway for DMSP degradation. Conversion of DMSP to MMPA is catabolised by the tetrahydrofolate (THF) dependent enzyme DmdA. Subsequent addition of coenzyme to MMPA to form MMPA-CoA is catalysed by DmdB. The MMPA-CoA undergoes oxidation to MTA-CoA via DmdC, followed by transformation by DmdD through addition of water which forms a brief intermediate. Release of MeSH transforms the intermediate into MaS-CoA which is finally converted to acetaldehyde via hydrolysis reaction releasing CoA and carbon dioxide. Figure adapted from Kotaras, 2020.

1.6 Terrestrial DMSP Cycling

For many years, DMSP cycling has been viewed as a primarily marine process, mainly due to the eight billion tonnes of DMSP produced annually from marine environments (Ksionzek *et al.,* 2016) and the fact that the majority of identified metabolic enzymes involved in DMSP-degradation have been found in marine organisms. Thanks to investigations such as the one conducted by the lab of Professor Jonathan Todd at the University of East Anglia, it has been shown that DMSP production is far more widespread in terrestrial plants than initially understood, with a selection of soil samples exhibiting significantly higher levels of DMSP than surface seawater. While it has been shown that saltmarshes are greater DMSP and DMS producers per M^2 than ocean surface waters (Dacey *et al.,* 1987) with around 10% of global DMS being produced in said environments, this is likely due to the fact that these are environments with high salinity (combined with the prevalance of DMSP-producing

Spartina plants that grow there) (Steudler and Peterson, 1984). However, the work of Professor Todd's lab and the details laid out in Ausma *et al.,* 2017 provide strong evidence that diverse plants that do not grow in equally salty environments also produce DMSP. Species of *Saccharum* (sugarcane), another terrestrial plant, has been shown to contain concentrations of 6 μmol g^{-1} DMSP in its leaves, indicating that is a high producer of DMSP (Otte *et al.,* 2004). Although an outlier, a range of other terrestrial plants have been shown to produce low levels of DMSP, indicating that there could indeed be far more cycling of DMSP occurring than once believed.

1.6.1 DMSP production in *Zea mays* (Maize) and *Secale cereale* (Rye)

Zea mays, more commonly referred to as simply 'corn', is a cereal grain belonging to the *Gramineae* family (as does sugar cane). A staple of the global food supply, 1.6 billion tonnes of *Zea mays* is cultivated for food alone (FAO, 2022). *Z. mays* and the many food products derrived from it see increasing levels of production every year, with an increase of around 11% seen in the weight of *Z. mays* produced between 2014 and 2016 (US Department of Agriculture, 2016).

Secale cereale, known more commonly as 'rye', is similar in that it is a grain used in many food products. Unlike *Zea mays, S. cereale* belongs to the *Poaceae* family and saw roughly 15 million metric tonnes produced worldwide in 2020 (FAO, 2022). This follows a trend of declining production rates since the early 1990s when global demands shifted to other crops such as *Z. mays*.

1.6.2 DMSP production in *Arundo donax* (Spanish Cane)

The higher plant *Arundo donax*, more commonly referred to as Spanish Cane, is a type of reed that originates from Middle Eastern countries (CABI 2020) and grows in soil exposed to either fresh or moderately saline water (Global Invasive Species Database, 2020).The plant is extremely resilient as it has no major pests, does not suffer from competition when grown in close proximity to other weed species and is capable of surviving in a variety of soil types (Cosentino *et al.,* 2006). The plant is so resilient in fact, that the Centre for Agriculture and Bioscience International's (CABI) Invasive Species Compendium lists the species as being amongst the top 100 most invasive alien species in the world.

Arundo donax is also capable of rapid growth thanks to a high rate of photosynthesis facilitated by comparatively efficient levels of CO_2 assimilation when compared to similar higher plants (Haworth *et al.,* 2017). This, coupled with *A. donax's* biomass calorific mean value (i.e. the calorific value gained from combustion of a dry plant sample) of 17 MJ kg^{-1} makes the reed excellent candidate as a renewable biofuel in the future (Badr and El-Sharabasy, 2014). These factors indicate that the

cultivation of *A. donax* will likely increase over the coming years as the world moves toward generating more renewable energy sources in a world with rapidly changing climate conditions.

Similar to other higher plant species, *A. donax* has been shown to upregulate DMSP-synthesis to produce comparatively moderate concentrations of the compound as part of a response to environmental stresses such as drought (Haworth *et al.,* 2017). When experiencing the conditions of a drought, *A. donax* plants grown in Italy were shown to see concentrations of DMSP in leaf samples increase from around 850 ng g^{-1} to 1380 ng g^{-1} (dry weight) (Haworth *et al.,* 2017). This proves that even under normal conditions, *A. donax* plants contain comparatively moderate concentrations of DMSP and as such, there is a likelyhood that some level of DMSP catabolism is being performed by organisms existing on the surface of the plant and within the surrounding bulk soil.

1.7 Plant-linked DMSP Catabolism by *Glutamicibacter sp.*

Despite common beliefs, the fact that DMSP synthesis is increasingly observed in a number of terrestiral higher plants likely indicates that terrestrial environments play a far more significant role in global DMSP cycling and as a result, considerably influence levels of atmospheric DMS. Recent work conducted by the lab of Professor Jonathan Todd at the University of East Anglia has shown that a strain of the terrestrial bacterium *Glutamicibacter sp.* found on sugarcane leaves and in surrounding soil containing µM DMSP levels is capable of growing on DMSP as a sole carbon source, catabolises it to produce DMS, but when sequenced shows no match to any known *ddd* genes. This presents a novel research opputurtunity to investigate the DMS-synthesising organisms that exist in proximity to terrestrial higher plants that synthesise DMSP in hopes of discovering the extent to which terrestrial DMS production contributes to atmospheric DMS levels, while simultaneously providing oppurtunity to discover novel *ddd* genes that are perhaps exclusive to terrestrial organisms.

1.8 Objectives

The main objectives of my master's thesis are to study the extent to which terrestrial DMSP-cycling is conducted by microorganisms associated with maize, rye and Spanish cane. This will involve the investigation of a large number of terrestrial microorganisms found in the bulk soil and rhizospheres of these three plants, characterising a large selection of isolates native to the soil surrounding low and moderate producers of DMSP in an attempt to identify novel DMSP-degradation enzymes unique to terrestrial environments.

The main focus of the project was to isolate and characterise microbial isolates from the bulk soil and rhizospheres of rye and maize (lower producers of DMSP), as well as Spanish cane (a moderate producer of DMSP) with the ability to use DMSP as a source of carbon. It was hypothesised that a diverse range of DMSP-degrading bacteria associated to maize, rye and Spanish cane exist within the soil environments where these plants grow. To test this, bacteria was grown on media with DMSP as a sole carbon source to prove that they primarily utilise the DMSP lysis pathway. It was also hypothesised that any bacteria that consistently grows with DMSP as a sole carbon source would produce significant levels of DMS. To test this, gas chromatography assays involving the measurement of headspace DMS produced as a result of DMSP degradation was carried out using 0.3ml liquid samples of microbial cultures isolated from maize, rye and Spanish Cane soil samples. Growth curves were then performed for the highest producers of DMS seen in the gas chromatography assays. These growth curves will elucidate the extent to which isolated microbes use DMSP as a carbon source in an environment where other sources of carbon (for example, succinate) are available.

CHAPTER 2

METHODS & MATERIALS

2.1 Media Preparation

Unless specified otherwise, the growth media used in this project was Rhizobial Minimal Media (RM) (Vogel and Bonner, 1956) with the addition of 10mM NH_4Cl to provide a source of nitrogen for microbes, and 5mM DMSP to act as a sole carbon source. This ensures that bacterial isolates have access to nutrients necessary for growth, but that only DMSP-metabolising organisms are cultivated. By referencing a study conduceted by Gonzalez *et al.,* 1999, yeast extract concentrations that had proven suitable for similar bacterial growth were determined to be 0.1µg per millilitre media.

2.2 Isolation of Bacteria from Bulk Soil and Rhizosphere Soil Samples

Soil samples were collected from the rhizosphere and bulk soil surrounding a variety of *Zea mays* and *Secale cereale* plants growing at the Worstead Estate in Norfolk, England. This sampling took place on the morning of June 26th, 2020. 1g of soil from each sample was added to 100ml liquid RM media containing 5mM DMSP as the sole carbon source. These samples were then incubated at 30 °C 140 rpm for one week before being diluted 1/1000 into another 100ml of RM containing 5mM DMSP. This process was repeated a further 2 times with the incubaction period being reduced to 2 days and it constited an enrichment experiment for bacteria with the ability to grow on DMSP as sole carbon source. Finally, a dilution series was conducted and 100µl (10^0-10^{-4}) of cells were spread onto fresh RM agar media containing DMSP at 5mM as the sole carbon source.

The RM Agar plates grown from these soil enrichments were then incubated at 28 °C for one week before colonies of varying colour, size and morphology were selected. Selected colonies were restreaked onto fresh RM agar media. After incubation for a week at 28 °C, this re-streaking process was repeated and following a further week of incubation at 28 °C, repeated once more with a final week of incubation at 28 °C to check for contamination of isolates.

2.3 Isolation of Fungi from Bulk Soil and Rhizosphere Soil Samples

Spanish cane leaf and root cuttings were imported and arrived at UEA 48 hours after collection in Barcelona. Samples were added to 100ml of liquid RM media with DMSP as sole carbon source at 5mM. Samples were then agitated via vortex for 2 minutes to ensure full coverage of the sample by the media. These samples were then incubated at 30 °C 140 rpm for one week before being diluted 1/1000 into another 100ml of RM containing 5mM DMSP. This process was repeated a further 2 times with the incubaction period being reduced to 2 days and it constited an enrichment experiment for bacteria with the ability to grow on DMSP as sole carbon source. Finally, a dilution series was

conducted and 100µl (10^0-10^{-4}) of cells were spread onto fresh RM agar media containing DMSP at 5mM as the sole carbon source.

While the initial intention was to isolate bacterial strains from the Spanish Cane samples, every agar plate innoculated with RM washate from the cuttings was quickly dominated by organisms with morphologies clearly belonging to fungal species. To counter this, 1ml 100% ethanol with 355.4mM cycloheximide was created due to its antifungal properties (Kominek, 1975), 200µl of which was added to 100ml RM media. 2ml washate from each Spanish cane cutting was used to inoculate this media in triplicate, providing a total of 12 liquid cultures that were incubated at 30 °C 140 rpm for one week. Fresh media was generated in this way as cycloheximide has potent antifungal properties. The new liquid cultures were diluted in series to 10^{-4} and 100µl of each dilute was spread onto fresh solid RM agar media. Despite this, the agar plates continued to yield fungal species. Since these fungi were growing on DMSP as a sole carbon source and were present on the leaves and roots of Spanish Cane (a known DMSP producer), it was decided that the fungi would be screened for DMS production and characterised.

2.4 Sequencing of bacterial 16S rRNA gene

First developed in 1977 by Frederick Sanger, Sanger sequencing is a process that allows for the formation of extension DNA products of various lengths terminated with dideoxynucleotides at the 3' end (Sanger *et al.,* 1997). This technique was chosen to amplify the 16S gene of bacterial isolates so that individual strains might be characterised as belonging to either a bacterial species or genus for further investigation.

2.4.1 Colony PCR of Bacterial Isolates for 16S Sequencing

Colonies were picked to 30µl of nuclease-free water and cell lysis was performed by microwaving the suspension for 30 seconds. Following this, 1µl of the suspension was added to 25µl of PCR master mix comprised of 12.5µl MyFi Master mix, 0.5 µl 27F primer, 0.5µl 1492R primer and 10.5µl sterile water. The 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (1492R 5'-GGTTACCTTGTTACGACTT-3') primers (Lane, 1991) were included in this PCR master mix as they are commonly used for the amplificiation of the desired 16S rRNA gene which would later be used for species-level (Frank *et al.,* 2008). This mix was then run on a thermal PCR cycler using a suitable 16S PCR program.

Eschericia coli colonies were used as a positive control. Following the PCR program, PCR products were verified to be unique isolates via gel electrophoresis using an agarose gel. Once isolates were confirmed to be unique, they were sent for forward Sanger sequencing externally.

2.4.2 Colony PCR of Fungal Isolates for Sequencing using ITS Primers

Colonies were picked to 30µl of nuclease-free water and cell lysis was performed by microwaving the suspension for 30 seconds. Following this, 25µl of PCR master mix was created comprised of 4% cell lysis solution, 50% MyFi Master mix, 2% ITS1F 5'-CTTGGTCATTTAGAGGAAGTAA-3' primer, 2% ITS4 5'-TCCTCCGCTTATTGATATGC-3' primer and 42% sterile water.

This mix was then run on a thermal PCR cycler using a suitable ITS PCR program. Following the PCR program, PCR products were verified to be unique isolates via gel electrophoresis using an agaroseº gel. Once isolates were confirmed to be unique, they were sent for forward Sanger sequencing externally.

2.5 Gas Chromatography

All of the gas chromatography (GC) assays involved measurement of headspace DMS using a flame photometric detector (Agilent 7890A GC fitted with a 7693 autosampler) and a HP-INNOWax 30m×0.320mm capillary column (Agilent Technologies, J&W Scientific). Unless otherwise stated, all GC measurements were performed using 2ml glass vials containing 0.3ml liquid samples and sealed with PTFE/rubber crimp caps. To quantify DMSP, 0.2ml of overnight culture was added to a 2ml vial, 0.1ml 10M NaOH was added, vials were crimped immediately, incubated at 22 °C overnight in the dark and monitored by GC. An eight-point calibration curve was produced by alkaline lysis of DMSP and SMM standards in water. The detection limit for DMSP (per 300 µl sample in 2ml GC vial) was 0.015nmol.

2.5.1 Liquid Growth Experiments

Before gas chromatography screening of the bacterial isolates could occur, optimal growth conditions first had to be determined. It was quickly apparent that isolates that grew on RM plates with DMSP as a sole carbon source could not grow in liquid RM (i.e. without agar). This lead to growth experiments being devised to investigate conditions where the isolates gained a growth advantage by having DMSP present in the presence of background levels of yeast extract.

2.5.2 Gas Chromatography Screening for Rate of DMSP-lyase activity in *Arundo donax*

To ascertain if microbes and or *Arundo donax* catabolise DMSP and to estimate the rate of DMSP catabolism the following work was done. A gram of replicate leaf and root sample were vortexed for two minutes in 10ml of liquid RM media with only 10mM NH_4Cl added to provide a nitrogen source. The resultant washate was then split evenly into two gas chromatography vials which were immediately crimped. 5mM DMSP was then added to one of the two vials in triplicate. The vial with no added DMSP was to study DMSP catabolism from the background DMSP likely originating prom the plant. A media-only negative control with DMSP added was also created. Manual GC injections were taken from the headspace at 4 time points across a period of 28 hours (0 hours, 5 hours, 24 hours and 29 hours) so that the rate at which DMS and/or MeSH was produced in the headspace over time could be recorded.

2.5.3 Gas Chromatography Screening for Rate of DMSP-lyase activity from pure cultures

This screening was conducted using the processes detailed in section 2.5. All bacterial isolates from *Zea mays, Secale cereale* and *Arundo donax* had single colonies picked from RM growth plates prior to assay. These picked colonies were incubated for 72 hours at 30 °C 150rpm in liquid RM media containing 5mM succinate, 1mM DMSP, 5mM NH_4Cl and 0.1 µg/ml yeast extract to ensure sufficient growth for assay. All bacterial liquid cultures were then adjusted to equal optical densities (OD600) through dilutions with further liquid RM media and triplicate samples of 500µl were created for each isolate. These samples were crimped in 2ml GC vials and incubated for 24 hours at room temperature before being run on a split 100 gas chromatography screen. All fungal liquid cultures were screened in a similar way, with the subsequent fungus cultivated after the 72 hour incubation period in RM media being pelleted and adjusted to equal dry weights before being resuspended (in the same liquid RM media used for bacterial isolates) with triplicate samples created for each isolate.

CHAPTER 3

RESULTS

3.1 Sample Collection

Under the supervision of Professor Jonathan Todd and Dr Ben Miller, soil sampling was conducted at the Worstead Estate in Norfolk, England. This was to be carried out so that bacterial strains present in the soil, growing in close proximity to DMSP-producers, could be characterised and screened to check for DMSP-degradation. Soil was taken from the rhizosphere and bulk soil surrounding a variety of *Zea mays* and *Secale cereale* plants. These maize and rye plants were shown to be low producers of DMSP, with maize roots containing around 28nmol DMSP/g freshweight while rye roots contained around 9 nmol DMSP/g freshweight. Similarly, both rye and maize rhizospheres were shown to contain around 3nmol DMSP/g freshweight with bulk soil containing around 2nmol DMSP/g freshweight. While low, these concentrations of DMSP are significant and were still hypothesised to support the growth of local DMSP-degrading bacteria.

3.2 Isolation of Bacterial Strains and Generation of PCR Products

By plating diluted soil suspensions on RM agar plates with DMSP as a sole carbon source, we ensured that only DMSP-degraders would grow. Individual colonies of unique morphology were then picked and replated, this process repeated three further times to ensure proper isolation of the strains. Before 16S sequencing could be performed to characterise these bacterial isolates, colony PCR had to first be performed. Colonies were picked to nuclease-free water and cell lysis was performed by microwaving the suspension for 30 seconds. Following this, 1µl of the suspension was added to 25µl of PCR master mix (full details of which are provided in section 2.4.1. These PCR master mixes were run on a thermal PCR cycler using a suitable 16S PCR program. By including 27F and 1492R primers in the master mixes, the 16S rRNA gene was amplified to allow for species-level characterisation.

Escherichia coli colonies were used as a positive control. Following the PCR program, PCR products were verified to be unique isolates via gel electrophoresis using an agarose gel. Since the primers used were modelled according to the 16S rRNA gene of *Escherichia coli,* a sample of *Escherichia coli* was used as a positive control for each series of PCR, with master mix containing no cellular contents acting as the negative control. As shown below in figure 8, an image was taken of each agarose gel after gel electrophoresis of PCR products had been performed. Visible bands aligning with the band representing the PCR product derived from the *Escherichia coli* sample confirmed that the 16S rRNA gene of any given isolate had been successfully amplified.

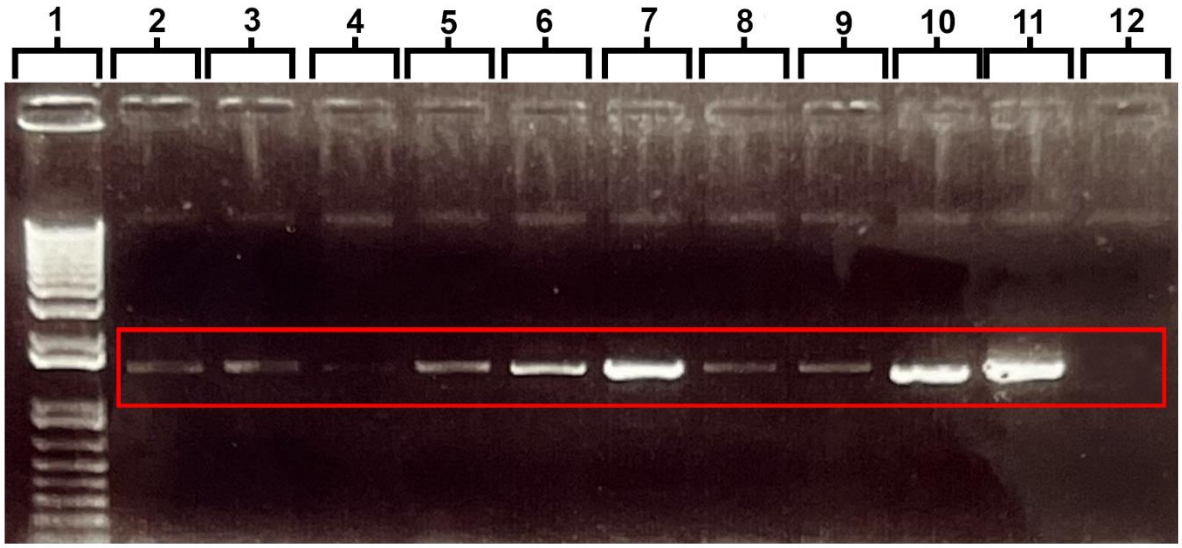

Figure 8: An image taken of an agarose gel used to perform gel electrophoresis at 120V on PCR products. Lane 1 contains the 1Kb DNA ladder, while lanes 2-10 contain the PCR products produced after the 16S rRNA PCR cycle used to amplify the DNA of bacterial isolates. Lane 11 contains the PCR product derrived from *Escherichia coli* to act as a positive control, while lane 12 contains the master mix with no cellular content added to act as a negative control. Wells 2-12 all contained 5 µl of PCR product and 3 µl of a loading dye. The red box highlights the DNA bands for the desired 16S rRNA gene, with brighter bands signifying a higher level of amplification and dark / non-existant bands signifying poor or unsuccessful amplification.

3.3 Characterisation of Bacterial Isolates

In total, 181 bacterial strains were isolated from *Zea mays* and *Arundo donax* soil samples. Following PCR (as detailed in section 3.2), 16S sequencing in exclusively the forward direction, it was discovered the 181 isolates all belonged to one of 22 different bacterial species from across five separate classes:

- Actinobacteria
- Betaproteobacteria
- Flavobacterium
- Gammaproteobacteria
- Sphingobacteria

Table 1 shows the identified species, the class that they belong to and what type of soil the species. This table was generated using BLAST analysis of the 16S rRNA sequences produced by colony PCR of the bacterial isolates. By using BLAST, the closest % ident match 16S rRNA gene from a species to the 16S rRNA gene amplified from any given isolate was found, confirming that the isolate was of that same species. Only 16S sequences of isolates that showed a 70% or higher homology to a species are listed, with the highest % ident match of sequences being considered to identify the species. As each isolate was clearly categorised following the soil sampling stage of this project, it was possible to then list the environment in which the species was found at the Worstead Estate.

Table 1: Species isolated from rhizosphere and bulk soil samples with characterised species and relevant class.

Soil from which bacteria was isolated	Species of bacterial isolate	Class of bacterial isolate
Secale cereale Bulk Soil	*Achromobacter spanius*	Betaproteobacteria
Secale cereale Bulk Soil	*Actinobacterium sp.*	Betaproteobacteria
Secale cereale Bulk Soil	*Flavobacterium sp.*	Flavobacterium
Secale cereale Bulk Soil	*Pedobacter sp.*	Sphingobacteria
Secale cereale Bulk Soil	*Pseudomonas fluorescens*	Gammaproteobacteria
Secale cereale Bulk Soil	*Rhodococcus erythropolis*	Actinobacteria
Secale cereale Bulk Soil	*Rhodococcus sp.*	Actinobacteria
Secale cereale Bulk Soil	*Streptomyces sp.*	Actinobacteria
Secale cereale Bulk Soil	*Streptomyces sp.*	Actinobacteria
Secale cereale Bulk Soil	*Streptomyces tendae*	Actinobacteria
Secale cereale Bulk Soil	*Streptomyces tendae*	Actinobacteria
Secale cereale Rhizosphere	*Achromobacter aegrifaciens*	Betaproteobacteria
Secale cereale Rhizosphere	*Achromobacter sp.*	Betaproteobacteria
Secale cereale Rhizosphere	*Flavobacterium sp.*	Flavobacterium
Secale cereale Rhizosphere	*Halomonas sp.*	Gammaproteobacteria
Secale cereale Rhizosphere	*Pseudomonas graminis*	Gammaproteobacteria
Secale cereale Rhizosphere	*Variovorax paradoxus*	Betaproteobacteria
Secale cereale Rhizosphere	*Variovorax sp.*	Betaproteobacteria
Zea mays Bulk Soil	*Achromobacter marplatensis*	Betaproteobacteria
Zea mays Bulk Soil	*Achromobacter marplatensis*	Betaproteobacteria
Zea mays Bulk Soil	*Achromobacter spanius*	Betaproteobacteria
Zea mays Bulk Soil	*Massilia sp.*	Betaproteobacteria
Zea mays Bulk Soil	*Micromonospora sp.*	Actinobacteria
Zea mays Bulk Soil	*Mucilaginibacter sp.*	Sphingobacteria
Zea mays Bulk Soil	*Pedobacter panaciterrae*	Sphingobacteria
Zea mays Rhizosphere	*Achromobacter xylosoxidans*	Betaproteobacteria
Zea mays Rhizosphere	*Pedobacter sp.*	Sphingobacteria
Zea mays Rhizosphere	*Pseudomonas frederiksbergensis*	Gammaproteobacteria

By using BLAST analysis, the genomes of these characterised species were then analysed to ascertain a percentage match to the known DMSP-lyase genes. Table 2 displays the percentage match of DNA sequences of characterised species from *Zea mays* and *Secale cereale* soil samples compared to the known ddd proteins. Ddd proteins from the following species were used for comparison agains the isolated 16S mMRNA genes amplified from the soil isolates:

- DddK – *Pelagibacter bermundensis*
- DddD – *Halomonas elongata*
- DddP – *Fusarium graminearum*
- DddQ – *Ruegeria pomeroyi*
- DddW – *Ruegeria pomeroyi*
- DddL – *Sulfitobacter pontiacus*
- DddY – *Alcaligenes faecalis*
- DmdA – *Ruegeria pomeroyi*

Table 2: Characterised species from *Zea mays* and *Secale cereale* soil samples compared to the percentage match to the known ddd genes. Percentages represent the 'percentage identical' figure yielded from BLAST analysis of the known *ddd* genes against genomic sequences of the characterised isolates.

Species	DddK	DddD	DddP	DddQ	DddW	DddL	DddY	DmdA
Achromobacter aegrifaciens	0%	37.68%	0%	37.10%	0%	0%	0%	0%
Achromobacter marplatensis	0%	37%	0%	0%	0%	0%	0%	0%
Achromobacter sp.	36.23%	40.20%	0%	38.71%	0%	0%	0%	0%
Achromobacter spanius	34.78%	37.62%	0%	36.67%	0%	0%	0%	0%
Achromobacter xylosoxidans	0%	36.06%	0%	37.10%	0%	0%	0%	0%
Actinobacterium sp.	42.59%	42.54%	51.84%%	45.90%	47.41%	45%	0%	51.35%
Flavobacterium sp.	28.12%	38.71%	0%	42.86%	38.89%%	0%	0%	0%
Halomonas sp.	43.30%	100%	0%	30.77%	33.60%	48.33%	0%	40.41%
Massilia sp.	35.71%	39.62%	0%	45.65%	37.74%	41.18%	0%	0%
Micromonospora sp.	35.06%	36.87%	0%	44.90%	45.10%	0%	0%	0%
Mucilaginibacter sp.	42.22%	37.21%	0%	42.11%	0%	0%	0%	0%
Pedobacter panaciterrae	30.11%	0%	0%	0%	0%	0%	0%	0%
Pedobacter sp.	31.52%	37.98%	0%	42.31%	37.29%	0%	0%	0%
Pseudomonas fluorescens	32.93%	57.04%	81.26%%	38.46%	36.47%	28.95%	0%	0%
Pseudomonas frederiksbergensis	0%	34.43%	42.44%	34.62%	0%	0%	0%	0%
Pseudomonas graminis	31.88%	32.83%	0%%	31.58%	0%	0%	0%	0%
Rhodococcus erythropolis	0%	36.54%	0%	0%	0%	0%	0%	0%
Rhodococcus sp.	30%	36.95%	0%	30.67%	38.98%	39.06%	0%	0%
Streptomyces sp.	36.99%	38.39%	0%	47.62%	41.27%	0%	0%	0%
Streptomyces tendae	0%	33.83%	0%	0%	0%	0%	0%	0%
Variovorax paradoxus	0%	37.96%	0%	40.38%	0%	0%	0%	0%
Variovorax sp.	0%	41.67%	0%	44.23%	39.06%	0%	75.56%	0%

The ddd proteins listed in table 2 play vital roles in different steps of DMSP degradation and synthesis pathways. Enabling the DMSP lysis pathway, DddY, DddK, DddL, DddP, DddQ, DddW and DddY all facilitate the lysis of DMSP to form acrylate (figure 6). DddD has also been observed to lyse DMSP, but instead this sees the production of 3-hydroxypropionate instead of acrylate (Todd *et al.,* 2007). DmdA instead facilitates demethylation of DMSP to MMPA in the demethylation pathway that allows for DMSP degradation (figure 5).

3.4 Characterisation of Fungal Isolates

Following the unexpected growth of fungus that was observed to be dominating RM growth plates innoculated with *Arundo donax* washate, it was decided that characterisation of said fungal

species would prove beneficial as it would reveal species capable of DMSP degradation. In total, four fungal cultures displaying unique morphologies when grown on RM media were sent for sequencing using the primers specified in section 2.4.2 of this thesis. Table 3 shows the identified fungal species, the class that they belong to and whether it was washate of the leaf or root of the Spanish cane that the species was isolated from. Interestingly, the Sordariomycetes class proved the most abundant, with three of the four identified species belonging to it. Fungi from this class appear in a variety of ecosystems as endophytes and animal and plant pathogens. More importantly, they're often characterised as saprotrophs involved in decomposition and nutrient cycling, which makes sense given their ability to degrade an organosulfur compound such as DMSP in this instance.

Table 3: Characterised fungal species isolated from *Arundo donax* leaf and root washate with relevant class.

Sample from which Isolated	Species	Class
Arundo donax Leaf Washate	*Rhinocladiella simlis*	*Eurotiomycetes*
Arundo donax Leaf Washate	*Sporothrix stylites*	*Sordariomycetes*
Arundo donax Root Washate	*Fusarium fujikuroi*	*Sordariomycetes*
Arundo donax Root Washate	*Purpureocillium lilacinum*	*Sordariomycetes*

3.5 Screening for DMSP-lyase Activity

A series of gas chromatography assays for DMS were conducted in order to ascertain the rate at which microorganisms were degrading DMSP into DMS. The first of these assays involved a screen of *Zea mays* and *Secale cereale* for rate of DMSP degradation/DMS synthesis. Table 4 shows the result of these assays combined with the results of Bradford assays to determine the rate of DMS production in nmol DMS per hour per milligram of protein.

A gas chromatography assay was also carried out via a series of manual injections taken from the headspace of *Arundo donax* washates in the presence of DMSP. Table 5 shows the nmol DMS recorded in the headspace of each washate vial, where the positive sample indicates that DMSP had been added and the negative sample indicates that no DMSP was added. This data has been plotted graphically in figure 9.

Table 4: Characterised species from *Zea mays* and *Secale cereale* soil samples with their calculated rate of DMS production listed in nmol DMS per hour per milligram protein.

Soil	Species	nmol DMS/hr/mg protein
Secale cereale Bulk Soil	*Rhodococcus erythropolis*	23.07307826
Secale cereale Bulk Soil	*Streptomyces sp.*	5.501228337
Secale cereale Rhizosphere	*Halmonas sp.*	4.718712009
Secale cereale Bulk Soil	*Rhodococcus sp.*	1.367081545
Secale cereale Bulk Soil	*Pedobacter sp.*	1.321999142
Zea mays Bulk Soil	*Massilia sp.*	1.126573921
Zea mays Bulk Soil	*Achromobacter marplatensis*	1.008292783
Secale cereale Rhizosphere	*Achromobacer aegrificiens*	0.794264363
Secale cereale Bulk Soil	*Streptomyces tendae*	0.661685092
Secale cereale Rhizosphere	*Flavobacterium sp.*	0.659616675
Zea mays Bulk Soil	*Achromobacter spanius*	0.537217287
Zea mays Bulk Soil	*Micromonospora sp.*	0.459343074
Zea mays Rhizosphere	*Pseudomonas frederiksbergensis*	0.407335725
Zea mays Rhizosphere	*Achromobacter xylosoxidans*	0.317957108
Zea mays Bulk Soil	*Pedobacter panaciterrae*	0.149096339
Secale cereale Rhizosphere	*Variovorax sp.*	0.027917544

Table 5: A table displaying the nmol DMS recorded in the headspace of vials containing washate of *Arundo donax* cuttings at four separate time points during a gas chromatography assay for DMS, as well as the average rate of DMS production per hour calculated using the four time points. Samples listed with a + contained DMSP, while samples listed with a – did not. The DMSP only control contained no *A. donax* washate.

	nmol DMS Recorded in Vial Headspace at Time Point				
Sample	**0hrs**	**5hrs**	**24hrs**	**29hrs**	**Rate (nmol DMS/hr)**
DMSP Only	3.035550698	4.901553771	3.620994874	4.016895838	0.033839488
Root A+	1.583287981	1.854330597	6.265418476	12.4145439	0.373491583
Root A-	0.950427065	1.015955515	1.063004426	1.178528491	0.007865566
Root B+	1.903499185	5.084348623	33.12176957	55.3143623	1.8417539
Root B-	0.859598256	0.903525867	1.112097257	0.906840508	0.001629043
Leaf A+	1.339180294	1.399515774	2.38179712	3.198238006	0.064105438
Leaf A-	0.91308541	1.226893452	2.389543677	2.843520728	0.066566735
Leaf B+	1.188772505	2.096320568	3.416513446	2.643679822	0.050169218
Leaf B-	0.931960862	1.668327928	2.745412538	5.256925053	0.149136696

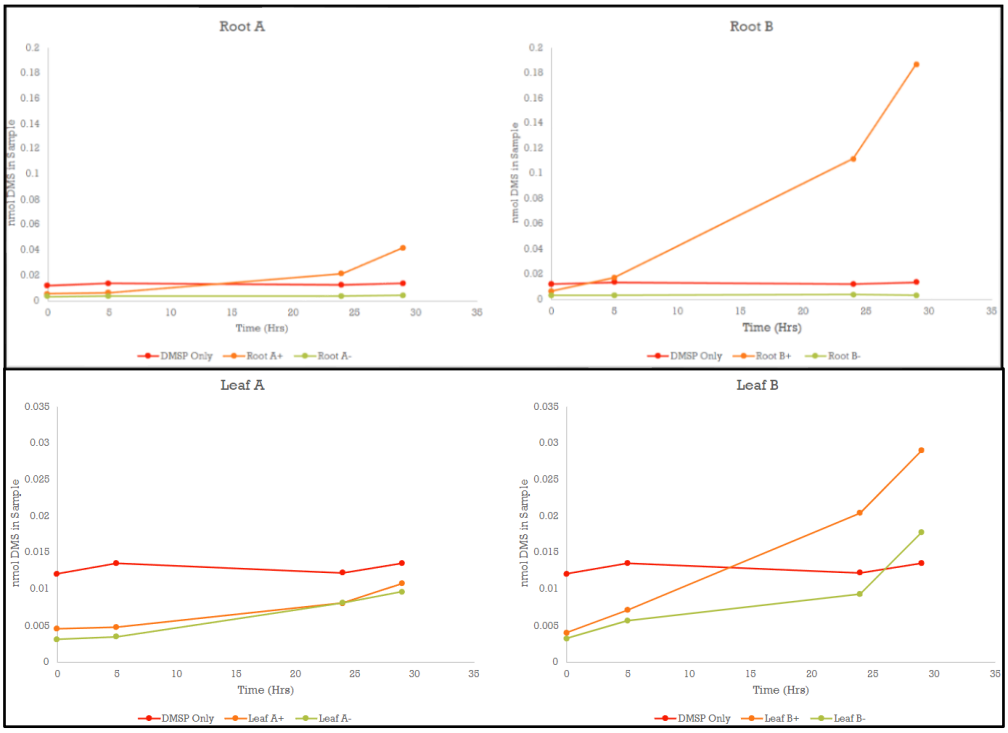

Figure 9: Scatter graphs representing the nmol DMS recorded in the headspace of vials containing washate of *Arundo donax* cuttings at four separate time points during a gas chromatography assay for DMS. Each graph displays results gained from screening washate generated from unique *A. donax* samples (leaf A, leaf B, root A and root B).

Once it had been confirmed that DMS was being produced by *Arundo donax* washate containing DMSP, a 24 hour gas chromatography assay was carried out on the fungal strains isolated from the leaf and root samples. Table 6 shows the results of said assay in conjunction with the weighing of the samples after they had been dried to provide a rate of DMS production in nmol DMS per hour per milligram of protein. Since the rate of DMS production in these fungal isolates was so low, it was decided that BLAST analysis of their respective genomes for potential sequence matches with known DMSP-lyase genes would be irrelevant and therefore no further analysis of the samples was performed.

Table 6: Characterised species isolated from *Arundo donax* leaf and root washate with the concentration of DMS recorded in the headspace of a vial used in a gas chromatography assay. The table also displays the nmol DMS produced per hour, as well as the nmol DMS produced per hour per milligram of protein. These rates were formulated in conjunction with Bradford assays to determine the weight of protein present in each sample assayed via gas chromatography.

Fungal Sample	nmol DMS in headspace after 24hrs	nmol DMS/hr	nmol DMS/hr/mg protein
Sporothrix stylites	0.099059355	0.004127473	0.041274731
Purpureocillium lilacinum	0.005115937	0.000213164	0.000112192
Rhinocladiella simlis	0.05538495	0.002307706	0.002884633
Fusarium fujikuroi	0.075455124	0.003143963	0.002858149

CHAPTER 4

DISCUSSION & CONCLUSION

This thesis and the project upon which it is written was carried out in hopes that the longstanding opinion that microbial DMS production is primarily a marine process would be challenged. By focusing on *Zea mays, Secale cereale* and *Arundo donax* (all terrestrial higher plants), the intention was that a representative sample of bacterial DMSP degraders could be identified in terrestrial environments where low and moderate levels of DMSP were present.

The first several months of the project were spent characterising the 181 bacterial strains isolated from *Zea mays* (maize) and *Secale cereale* (rye) rhizospheres, as well as the bulk soil surrounding them. The reason behind this was that the density of microorganisms tends to be lower in this bulk soil, but also because the compounds produced by a plant (such as DMSP) are less concentrated here. Therefore, by taking soil samples from here as well as the rhizosphere it would be possible to determine the concentration of DMSP required to facilitate DMSP-degrading bacteria.

As shown in table 2, the majority of successfully characterised bacterial isolates came from bulk soil samples as opposed to rhizosphere soil samples. General nutrient availability in rhizospheres tends to be higher than in bulk soil due to presence of plant roots, resulting in higher densities of bacteria growing there. This caused difficulties when isolating unique strains from growth medium inoculated with rhizosphere soil washate as many varied strains of bacteria sharing somewhat similar morphologies were growing on the agar gel and thus contamination of plates limited the number of successful rhizosphere isolates. In future experiments, a further dilution of the washate of rhizosphere soil samples may reduce the number of colonies grown and therefore make successful, uncontaminated isolation of unique strains more feasible.

Once cultures of the 181 bacterial isolates had been successfully grown without visible contamination, amplification of the 16S DNA of said cultures was performed using 27 forward and 149 reverse primers. The resultant PCR products were sent off for forward sequencing for third party genomic sequencing. At this point in the project, forward sequencing was deemed sufficient as the purpose of the sequencing was to get a general idea of the variety of microbes growing in the specified soil environments. Furthermore, the added cost of reverse sequencing wasn't warranted. Before doing so,

I was checking that the PCR had worked by running the products on ethidium bromide gels. If I could see the appropriate bands, I'd then purify the product and send it off.

Table 2 shows that bacterial species from across 5 different classes were identified, all gram negative except for *Actinobacter*. Once these species had been identified, unique instances of each had their genomes subjected to BLAST analysis so that 'Percentage Identical' (per. Ident.) figures could be determined for known DMSP-lyase (*ddd*) genes. Essentially, this process calculates how many nucleotides in aligned DNA sequences match. As shown by table 3, all characterised bacterial isolates show matches of varying degrees to known *ddd* genes in other bacterial species. Despite this, many of the species analysed in this way showed only low per. ident. matches. This could indicate that a similar, but currently undiscovered novel DMSP-lyase gene is primarily responsible for the cleavage of DMSP to DMS in these species.

The gas chromatography assays used to screen *Arundo donax* samples yielded results supporting the hypothesis that the plant is a comparatively moderate producer of DMSP and thus would support the cultivation of DMSP-degrading microbes. Figure 5 shows that washate generated using root sample B demonstrated a stronger rate of DMSP degradation, whether that be due to a higher number of microbes present in the solution or the types of microbes present. Nonetheless, both root samples showed significant DMSP degradation.

The data gained from washate generated using leaf samples was not as clear cut, with both leaf washates showing levels of DMS below that of the media only control. For the washate generated from leaf sample A, while not significant there was an increase in the DMS detected implying that some level of degradation is occurring. Washate generated from leaf sample B showed similar results. The assay showed that both leaf washates had more DMS present in the headspace of their respective vials by the end of the 28 hour period. This implies that the microbes within the washate are degrading the DMSP present, whether that be from the DMSP we added or residual DMSP from the washate. In the case of further investigation, extra caution when ensuring all samples, including media, remain uncontamined is crucial. Alternatively, it could be possible that DMSP was being catabolised by microbes in a way that does not generate DMS. For example, it could be the case that microbial demethylation of DMSP was occurring, resulting in the generation of acetate as opposed to DMS (Curson *et al.,* 2011).

Originally, the intention was to then characterise the isolates responsible for the DMS production in these samples in a similar way to the characterisation of the *Zea mays* and *Secale cereale* soil isolates. The use of the antibiotic cycloheximide was originally believed to be a sufficient precaution to avoid the fungal contamination expected from plant samples. This antibiotic exhibits potent antifungal properties (Kominek, 1975), but despite its repeated addition to growth media used when attempting microbial isolation from the *Arundo donax* washates, all cultures were quickly overrun with fungal contamination. Since the fungal contaminants were growing successfully and rapidly on DMSP as a sole carbon source, it was decided that they should be investigated to ascertain whether they were significant producers of DMS.

Before screening for DMS production, it was important that the fungi were characterised. The DNA of four unique morphologies was successfully amplified using ITSF and ITS4 primers, revealing 1 fungus belonging to the *Eurotiomycetes* class and 3 belonging to the *Sordariomycetes* class. As seen in table 7, all four characterised fungal species exhibited extremely low rates of DMS production when assayed via gas chromatography. It could be the case that this DMS synthesis has occurred due to natural cleaving of DMSP as opposed to any fungal-catalysed process. If this were the case, there is a possibility that the fungal species do metabolise DMSP, but via the DMSP demethylation pathway as opposed to the DMSP lysis pathway. This would explain why they are capable of growing on DMSP as a sole carbon source while producing insignificant amounts of DMS gas. If further investigation into these species was deemed relevant, it would be useful to assay samples for acetate as this would indicate that the use of the DMSP demethylation pathway.

Following the characterisation of the bacterial isolates from *Zea mays* and *Secale cereale*, the focus of the project shifted to determining which of these strains exhibited the highest rates of DMS production. After tiral attempts at gas chromatography screens at splits of 2:1 and 25:1 that were unable to record accurate DMS due to the concentration exceeding the detection limit, it was decided that a 100:1 split would be required to yield accurate results. A separate issue arose in the matter of finding a growth medium that all strains would grow on sufficiently while also ensuring that DMSP was being used as a sole carbon source. It was at this point that the addition of small concentartions of yeast extract to RM growth medium could provide the solution. Using previous studies on the benefits of yeast extract in bacterial cultivation (Gonzalez *et al.,* 1999) growth curves were planned using a plate reader to ascertain the ideal concentration to be added. By creating media in duplicate, with one sample containing DMSP and one lacking it, it was possible to see which concentration of yeast extract facilitated growth without the bacteria defaulting to it as a carbon source. Due to time constraints and

an unexplained lack of growth seen across two attempts, the route of using growth curves to determine this concentration was abandoned and instead, determining whether or not growth had occurred by observing plates with the naked eye was used. Although less accurate, it was decided that this provided a sufficient degree of accuracy for the purpose of developing an effective growth medium.

Once the composition of the RM media used when growing bacterial cultures for assay had been determined, the split 100:1 gas chromatography assay was performed. As shown in table 5, *Rhodococcus erythropolis* and *Streptomyces sp.* exhibited the highest rate of DMS production, both species belonging to the *Actinobacteria* class. Something worth noting is that 4 of the top 5 strains for DMS production were isolated from the bulk soil of *Secale cereale.* Considering that one would assume DMS concentrations to be lower in bulk soil than in rhizospheres, it could be possible that exposure to lower levels of environmental DMSP has forced these strains to adapt to metabolise the compound more efficiently. Given that none of these significant DMS producers showed significant per. ident. matches to known *ddd* genes, it could be possible that an undiscovered novel *ddd* gene is responsible for the high rates of DMSP lysis recorded in the assay. If further work were to be carried out on these isolates, it would seem appropriate to perform thorough genomic screening and analysis in hopes of finding novel *ddd* genes that facilitate the DMSP-lysis pathway in terrestrial bacteria.

The two main objectives of this thesis were to characterise a large number of terrestrial DMSP-degraders and to then screen them to show that they are in fact significant producers of the climat cooling gas DMS. Both of these goals were achieved, except for in *Arundo donax* where severe fungal contamination prevented the successful characterisation and subsequent screening of bacterial isolates. This is an area for future study that would certainly provide a more complete picture of DMS production and wider DMSP cycling in terrestrial environments. Although time constraints disallowed it, the identification of novel *ddd* genes seems like a logical next step, with the bacterial isolate showing the highest rate of DMS production from each class acting as model organisms for said study. This would include:

- *Rhodococcus erythropolis (Actinobacteria)*
- *Halomonas sp. (Gammaproteobacteria)*
- *Massilia sp. (Betaproteobacteria)*
- *Flavobacterium sp. (Flavobacterium)*
- *Pedobacter sp. (Sphingobacteria)*

Furthermore, before said genetic screening were to occur, full genome sequencing of these isolated strains would need to be performed. By discovering a novel *ddd* gene in this way, a case for significant DMSP cycling in terrestrial environments would surely be strengthened as a unique pathway for DMSP-lysis would have been discovered in bacterial species shown to be significant producers of DMS by this study.

As it stands, bacteria are not considered in dynamics models to predict the production of DMS and the effects of a warming climate. If the significant land-based microbial DMS producers identified in this project are confirmed to be widespread across terrestrial, DMSP-rich environments through further investigation, it could be that not only the dogma that DMS production is a marine process is challenged, but that the considerations of climate control shift to better incorporate said metabolic processes. The scope of investigation into terrestrial DMS producers is far wider than it may initially seem, and the results from further studies could have wide-reaching implications that impact not only the study of DMSP and DMS cycling, but also how we as a society combat climate change and help to maintain a functioning ecosystem through manipulation of the geochemical sulfur cycle.

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