Developing novel tools to explore the marine volatilome

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Summary

Key volatile organic compounds (VOCs) such as dimethyl sulfide (DMS) and isoprene have been extensively studied for their importance as semiochemicals and in climate regulation. In contrast, disentangling the myriad of signature VOCs in gas chromatographic data has received much less attention. This thesis aims to develop novel analytical tools that utilise chromatographic information for the understanding of dynamic behaviour and interactions of organisms in the environment, assuming that every organism produces a characteristic volatile profile that reflects its physiological status. Five lab-based experiments combined with one final field study took place to begin building the foundations of such a tool. Studies were conducted with the aim to collect data on species specific volatile profiles, to comprehend what effects biophysiological factors may have on the organisms' volatilomes, and to learn if there is any seasonality in VOCs just as there are in plankton succession. During lab-based experiments, VOCs characteristic to species as well as biological and physical factors could be observed. Stress from heat resulted in higher production of VOCs that were likely to be induced by stress and mortality. Competition reduced VOC production amongst the two species grown together, and grazing behaviour reflected on VOC profiles obtained as numbers and concentrations would increase in samples that included individuals with high mortality. Field based studies showed a link between seasonal succession of plankton, with trends in VOCs prevalent over 8 months sampling time. Taking the results of the lab-based and field-based study into consideration, it can be said that there is potential in utilising VOC profiles to develop a novel, non-invasive tool to be used for environmental monitoring.

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Abbreviation list

ANOVA	Analysis of Variances
ASW	Artificial Seawater
BVOC	Biological volatile organic compound
CCMP	Center for the Cultivation of Marine Phytoplankton
CCN	Cloud condensation nuclei
CF	Cystic Fibrosis
DD	(2E,4E/Z)-didecadienal
DMS	Dimethyl sulfide
DMSP	Dimethyl sulphide propionate
ESAW	Enriched Seawater Artificial Water
GC-FID	Gas chromatography – Flame ionisation detection
GC-FPD	Gas chromatography – Flame photometric detection
GC/MS	Gas chromatography / Mass spectrometry
GNP	Gold nanoparticle
HS	Headspace
LOD	Limit of Detection
LOQ	Limit of Quantification
ROS	Reactive Oxygen Species
SASW	Sterile Artificial Seawater
SPME	Solid phase micro-extraction
NMHC	Non-methane hydrocarbons
PCA	Principal Components Analysis
UV	Ultraviolet
VHC	Volatile halogenated compound
VOC	Volatile organic compound

Contents

Chapter 1: Introduction

1.1 Overview

Climate change, habitat degradation and pollution are all examples of environmental problems that negatively influence ecosystem health. Growing concerns have brought attention to volatile metabolomics as an important approach in environmental science. Monitoring conditions within an ecosystem can be logistically challenging, whether it be the observation of species distribution, abundance or interactions. Biological volatile organic compounds (BVOCs) are produced in response to physical, chemical and biological disturbances experienced by an organism and therefore have potential in serving as an indicator for ecosystem health.

It is also important to understand what happens to these BVOCs after they are released as the likelihood is that they will influence the environment themselves (Carpenter *et al.*, 2012). They may also degrade over time or react with other chemicals upon reaching the atmosphere. Key studies of VOCs such as isoprene (Exton *et al.*, 2013a), dimethyl sulfide (DMS) and species of organohalogens (bromo/chloro/iodo compounds) (Hopkins *et al.*, 2010) are suggested to play important roles in atmospheric processes such as depletion of stratospheric ozone, production of tropospheric ozone and increased cloud formation (Bates, 1987; Quack *et al.*, 2004; Sanderson *et al.*, 2003). Some of these VOCs, such as DMS, are also thought to be involved in intra/intercellular signalling where VOCs act as infochemicals used for intra/interspecific communication. VOCs and their precursors have also been shown to change in production rates as a response to environmental

stimuli (Alcolombri *et al.*, 2015; Holzinger *et al.*, 2000). If quantitative and qualitative information in regards to sources of varying BVOC concentrations may be gathered, there is potential to non-invasively monitor the conditions or health status of an organism, population or even a community (Jud *et al.*, 2018; Kasal-slavik *et al.*, 2017). This would not be dissimilar to medical diagnosis of a patient.

1.2 Metabolomics

Compounds are classified as volatiles if they have high vapour pressure (0.01 kPa or higher) under room temperature (20°C). Such compounds consist of molecules that tend to change from their solid or liquid form into gaseous phase. Over the years it has been debated as to how to refer to the study of volatile compounds. Primarily the term volatilomics and volatolomics have been used; volatilomics being the study of all volatile compounds regardless of its origin, while volatolomics being the study of volatile organic compounds (VOCs) alone that are produced as a product of biological processes. For example, CO_2, which is produced as a result of respiration.

Following on from the definition of volatolomics, metabolomics is the study of metabolites in organisms, tissues, or fluids. It is a strategy to investigate cellular metabolites and uses technology to explore the purpose of these and how they are produced. Volatolomics is a sub-field of metabolomics whereby only compounds that are volatiles are studied.

To clarify, in this review the focus will be on volatolomics. Measurements of the volatolome serve as biomarkers in a series of fields such as medical diagnosis (Raquel, 2017), biodiversity assessment (Steinke *et al.*, 2018), and food quality control (Mayr *et al*., 2003) and recent volatolomics may exceed in potential compared to other biomarkers due to it offering less invasive and yet immediate results. As such, advancements in volatolomics has provided the scientific community with more novel approaches in medical and environmental research.

1.3 Medical Metabolomics

Human physiology is complex, and the many reactions and processes inside the body result in the formation and release of volatile metabolites. Not only do metabolic reactions in cells, tissues or fluids cause volatiles to be released, but the complex microbial communities that reside within the body also produce their own volatiles (Majchrzak *et al.*, 2018). The composition of such volatiles collected from healthy and unhealthy patients are often dissimilar and due to this, volatiles collected from exhaled breath, urine and faecal matter can deliver significant information about the metabolic processes that are occurring within the patient (Garner *et al.*, 2007; Gunther *et al.*, 1999; Miekisch *et al.*, 2004).

The measured BVOCs can be identified as well as quantified; they can serve as biomarkers and allow for the early detection of possible diseases such as bacterial infections, cancers and other genetic disorders like Alzheimer’s disease (Amann *et al.*, 2014; Mazzatenta *et al.*, 2015). To continue refining this non-invasive and immediate

diagnosis method, research in medical volatolomics has advanced greatly. In the following paragraphs, methods used as well as three different examples of the use of medical volatolomics will be introduced.

1.3.1 Medical volatilomics methods

Changes in VOC production may be because of a whole range of diseases. There are many different sample options that are tailored for the diagnosis of each condition. Examples of these include skin headspace, cell tissue, exhaled breath, urine and faeces (Amann *et al.*, 2014). Below is a compilation of sample collection and analysis methods that have currently been used in medical research of VOCs.

1.3.1.1 Sample collection

Skin Headspace When studying skin headspace, samples are often collected into thermal desorption tubes. Subjects are instructed to wash their hands thoroughly with distilled water for 2 minutes prior to sample collection, after which an air pump is attached to the hand. This directly pumps any VOCs emitted from the skin cells into the thermal desorption tube ready for sampling (Broza *et al.*, 2014).

Cell Tissue Headspace Although VOCs collected directly from cell tissue are not generally used for diagnosis in applied medicine, collection and analysis methods are still worth being briefly mentioned in this chapter. Cell lines of interest grow in vitro if

provided with the correct medium. Once cells have grown the headspace is collected into an absorption device attached to the culture plate for storage or immediate thermal desorption for analysis (Sponring *et al.*, 2010).

Exhaled Breath There is an abundance of research on respiratory diseases and signature VOCs that are frequently used as biomarkers for diagnosis. Breath samples collected need to be of alveolar air rather than respiratory dead space air. Inhaled air also needs to be clean of ambient contaminants and therefore subjects are asked to repeatedly inhale up to total lung capacity for approximately 3 minutes through a mouthpiece connected to a scrubber. Breath samples are then collected by filling 750 ml Mylar bags with breath from the end of the exhalation to avoid collecting any respiratory dead space air and instead the desired alveolar air. Immediately after the collection, VOCs in the breath samples are trapped and pre-concentrated into sorption tubes ready for analysis (Broza *et al.*, 2014).

Urine Urine samples are usually collected in the morning into 20 mL glass vials that must be closed immediately after. In addition to this, a smaller subset of urine is transferred into plastic urine monovette vessels if creatinine analysis is also to take place. Urine from the glass vials are then either stored or transferred into septa vials. Prior to analysis however, vials need to be cleaned with distilled water and pH balanced with phosphate buffered saline. The amount depends upon the size of the vials. Anti-foam agent is also desirable to prevent the urine from foaming. An appropriate amount of urine can then be transferred into the vial using a Hamilton syringe and analysed for VOCs (Mochalski & Unterkofler, 2016).

Faeces Faecal matter is sampled by directly transferring a small amount (the weight of which is dependent on the type of analysis) into crimp seal vials with a septum. These are then placed in a 60°C water bath for 1 hour to allow the VOCs to diffuse into the headspace. An appropriate SPME fibre can then be used to extract the VOCs by being exposed to the headspace for a given period (again, dependent upon the type of analysis). This is then ready for thermal desorption (Dixon *et al.*, 2011).

1.3.1.2 Volatile organic compound (VOC) analysis

In analytical chemistry, GC-MS is now commonly used within medical practice for its ability to identify and quantify a complex mixture of chemicals. This method can be used on its own or in combination with other techniques (Dean, 2015).

An example of such other techniques is a gold nanoparticle (GNP) sensor. Although GC-MS alone may be able to detect differences between cancer patients and healthy control groups, the additional tests using GNP sensors allow for an easy method to further distinguish between lung, breast, colorectal and prostate cancers (Peng *et al.*, 2010).

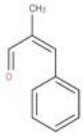
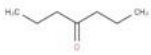
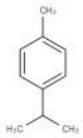
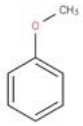
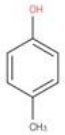
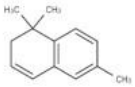
1.3.2 Current use of medical volatile metabolomics

Compilation of research on a variety of different bodily fluids has reported the presence of 1764 different VOCs in humans so far (Broza *et al.*, 2015). Medical fields such as

oncology, pathology, neurology, and gastrology are only a few of the many fields that have started to utilise the presence of such VOCs to investigate a patients symptoms.

Breast Cancer Among women, breast cancer is the most widespread medical condition and is a major cause of female fatality. However, mortality rates can be significantly reduced if detected at early stages. In a study by Silva *et al*. (2011), the headspace of cultured breast cancer cells (cell lines: T-47D, MDA-MB-231, MCF-7) and healthy mammary epithelial cells were sampled using solid phase microextraction (SPME) techniques followed by analysis using GC-MS. Volatiles of 60 types were identified belonging to chemical groups such as acids, alcohols, aldehydes, alkanes, ketones and benzene derivatives. Out of these volatiles, 13 were found to be in both normal as well as cancerous cell lines. Five were present in normal cells only, while six compounds (2-pentanone, 2-heptanone, 3-methyl-3-buten-1-ol, ethyl acetate, ethyl propanoate, and 2-methyl butanoate) were present only in cancerous cell lines (Silva *et al*. 2017). Cell line MCF-7 showed two unique compounds. This suggests that VOCs released from cells may serve as a biomarker for early diagnosis of breast cancer.

Table. 1.1. Compilation of compounds present in cancer patients and healthy control groups based on literature (Silva *et al.*, 2011).

Cancer Group	Control Group
2-methyl-3-phenyl-2-propenal 	4-heptanone 
p-cymene 	methanethiol
anisole 	
4-methyl-phenol 	
1,2-dihydro-1,1,6-trimethyl-naphthalene 	

Cystic fibrosis VOCs can also be indicative of pathogenic diseases. Cystic fibrosis (CF) causes the increased production of reactive oxygen species (ROS) during airway inflammation. This in turn causes the release of VOCs that can then be used to discriminate between CF patients and healthy control groups (Robroeks *et al.*, 2010). Patients with CF often suffer from mucus build up that increases the risks of secondary infections by *Pseudomonas* species that colonise the lungs. One of these species is *Pseudomonas aeruginosa* and it is possible to find signature VOCs that are specifically produced in its presence. Deciphering between patients that do or do not suffer from an infection of *P. aeruginosa* has become a much simpler process and VOCs have proven

to be useful as a monitoring tool for airway infection and inflammation/oxidative stress (Robroeks *et al.*, 2010).

Gastrointestinal Diseases Compared to breath and urine, there are fewer findings on VOCs emitted from faeces. One study found however, that abnormal VOCs are detected in faecal matter of patients with gastrointestinal disease. In fact, the study looked to differentiate between the VOCs emitted from faeces of three different patient types; those with ulcerative colitis, *Campylobacter jejuni*, and *Clostridium difficile*. Results showed that not only could they differentiate between patients and healthy control groups, they were able to differentiate between each separate disease. When a principal component analysis was used to visualise the contrast, it was evident that all three released VOCs of different identities at different quantities. These “signature” volatiles now have potential to be used as tools for diagnosis (Garner *et al.*, 2007).

Volatolomics has become more and more applied in the medical field, often used for early diagnoses of patients. Despite this, much less research has been undertaken on the approach of using volatolomics in an applied setting for environmental science. A lot of ecological monitoring is very time consuming and often even an invasive process. If volatolomics could be utilised in understanding nature, as is in medicine, more could be understood about the biotic and abiotic processes that are occurring in the world.

1.4 Environmental volatilomics

Each VOC has potential to change the environment (Exton *et al.*, 2015). They can either cause changes on their own, or have a combined effect when put together with another compound. It is important not only to understand processes that occur due to one compound, but just as much the combined results (Paasonen *et al.*, 2013; Williams, 2004).

There is a magnitude of volatiles present in the atmosphere and many are released by microalgae (Achyuthan *et al.*, 2017). Some examples of such volatiles include nitrous oxide (N_2O), which is a potent scavenger of stratospheric ozone; volatile hydrocarbons, most of which are trihalomethanes; methanol, which is an oxygenated VOC; geosmin and 2-methylisoborneol, both of which are considered to have a distinctive scent and produced mainly by cyanobacteria; isoprene, most of which are produced by plants and also by microalgae; and sulphurous compounds such as DMS, methanethiol, and carbonyl sulfide (Achyuthan *et al.*, 2017). This thesis will mostly discuss the production of sulfur compounds (DMS, methanethiol, carbon disulfide) and isoprene.

The VOCs present in the atmosphere are not only species specific, but depend on other spatiotemporal factors such as season, tidal cycle, and wind speed due to the variability they cause in water (Bravo-Linares & Mudge, 2009). Bravo-Linares *et al*. (2009) discovered that species were seasonally dependent in their population sizes and shifts in communities, which in turn further influenced the VOCs produced; tidal height affected the extent to which macroalgae and sediments are exposed to air and in turn affected

the type and amount of VOCs released; windspeed affected flux of VOCs to the atmosphere and distribution of VOCs on the surface layer (Bravo-Linares & Mudge, 2009). Of these factors, this thesis will mostly discuss seasonal fluctuations in VOCs.

There is still debate as to what role DMS plays in climate regulation. Research shows its existence can result in formation of cloud condensation nuclei (CCN) and help in increasing cloud albedo (Bates, 1987). There are two contradicting hypotheses that exist in relation to this. One is known as the CLAW hypothesis named after the four researchers; Charlson, Lovelock, Andreae and Warren. The other, known as the anti-CLAW hypothesis that was published by Lovelock in response to the CLAW hypothesis. The CLAW hypothesis predicts that temperature elevation will cause a rise in DMS emissions. This would then increase cloud albedo and then CCN, cooling the environment creating a negative feedback loop as can be seen in Figure. 1 (Charlson *et al.*, 1987). The anti-CLAW hypothesis on the other hand counters this statement with the argument that there are factors to consider other than temperature such as nutrient depletion which may perhaps result in a positive feedback loop instead. The DMS production would be reduced as ultimately all the phytoplankton would diminish due to lack of nutrients, increasing temperature of the environment (Liss, Peter S, 2007).

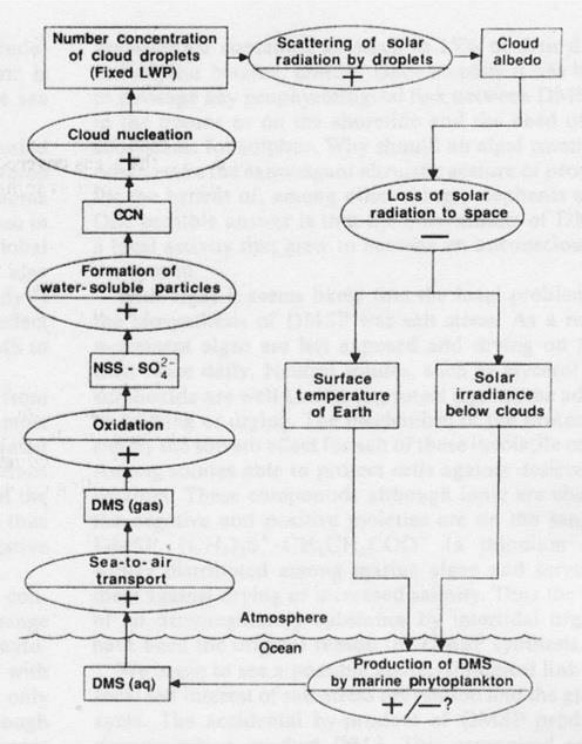


Figure. 1.1: Negative feedback loop portrayed by the CLAW hypothesis taken from literature (Charlson *et al.*, 1987)**.**

Ozone removal and production are also important atmospheric processes. Volatile halogenated compounds (VHCs) produce halogen atoms in a process of photolysis that then have the capability to catalyse the removal of stratospheric ozone (Molina & Rowland, 1974). Amongst the VHCs, brominated VHCs are more efficient catalysts (Prather *et al.*, 1984) which makes marine organisms that produce bromo-carbons particularly worthy of studying.

VHCs however; contribute to CCN. For example, biogenic iodine emissions form aerosols from alkyl halides (O'dowd *et al.*, 2002). Molecular iodine is released by many Laminariales, in this study *Laminaria digitata* that efflux iodide in response to oxidative

stress. These form iodide oxides that eventually become particles that are precursors of CCN (Küpper *et al.*, 2008).

Production of ozone in the troposphere can take place by, isoprene, in the presence of high concentrations of $NO_{(x)}$ (Kroll *et al.*, 2005). This can have detrimental effects on crop growth (such as closing of stomata) and human health (such as dysfunction of respiratory functions) (Ashworth *et al.*, 2013; Galizia & Kinney, 1999; Hill & Littlefield, 1969), which is why quantifying and monitoring such VOC production may be useful for future impact estimations.

There are other oxygenated volatile organic compounds including non-methane hydrocarbons that are prominent in marine environments, and a number of them are produced by seaweeds (Broadgate *et al.*, 2004). Emissions released were mostly dominated by alkenes and were found to be species, temperature, and light dependent (Broadgate *et al.*, 2004). Understanding the emission of these compounds will in turn help instruct how the presence of particular species may alter the number of atmospheric oxidants in their environment and how that in turn may affect atmospheric chemistry within the area.

Understanding seasonal influence on BVOC production and then further monitoring the consequences of anthropogenic activities on seasonality could help predict future changes in the atmosphere. DMS, isoprenoids and organohalogens have previously

been monitored to fathom how and why they fluctuate in different seasons (Bates, 1987; Orlikowska & Schulz-bull, 2009).

Production of isoprene and other reactive hydrocarbons (propane and 1-butene) has been shown to be seasonally dependent. Non-methane hydrocarbons (NMHCs) including isoprene, propane and 1-butene were seen to exhibit a maximum mean concentration from spring to summer in the North Sea and Southern Ocean but correlation coefficients between chlorophyll *a* concentration and other NMHCs were poor. This suggests that isoprene is produced by a different natural process than propane and 1-butene (Broadgate *et al.*, 1997). Many factors co-vary in different seasons, which make it difficult to identify the reason for differences between these gases (Broadgate *et al.*, 1997). There are also studies on concentrations of bromocarbons such as bromoform ($CHBr_3$) and dibromomethane (CH_2Br_2) (Hughes *et al.*, 2009). Measurements were taken at a time series station in a coastal bay on the western Antarctic Peninsula between February 2005 and March 2007. Again, strong seasonality is found in both bromocarbons. Concentrations increase during Austral summer during annual microalgal blooms. It is notable that timing of bromocarbon increase and decrease is dependent upon sea ice retreat and from the onset of a microalgal bloom (Hughes *et al.*, 2009). DMS also exhibits seasonal fluctuation. The North Sea has been investigated for seasonality in DMS fluctuation. Concentrations vary between winter and summer (1985) and range from 1 to 1100 ng S (DMS) $litre^{-1}$. DMS levels are very low during winter (mean = 4 ng S (DMS) $litre^{-1}$) and high during summer (mean = 220 ng S (DMS) $litre^{-1}$). Species composition are analysed during summer of which results indicated that main source of DMS are coccolithophores, various unidentified dinoflagellates and identified *Gyrodinium aureolum* (Turner *et al.*, 1988).

It would be enlightening to do similar studies on a spectrum of gases. This approach would be complex, but if signatures or individual BVOCs could be identified and monitored then understanding could be gained on interactions within communities of a habitat in question and how the ecosystem is influenced by seasonal change of the volatile metabolome.

1.4.1 Methods used to study environmental volatiles

Gas chromatography coupled with mass spectrometry (GC-MS) is commonly used alongside GC-FPD and GC-FID to analyse concentrations of environmental VOCs (Lara-Gonzalo *et al.*, 2008). There are also different pre-concentration techniques prior to analysis, sometimes a necessity to reduce the volume of sample VOCs. These can range from cryogenic enrichment of compounds using a purge and trap system (Franchini & Steinke, 2016) to thermal desorption techniques (Pankow *et al.*, 1998). A study conducted by Vogt *et al.* (2007) attempted to compare two of these pre-concentration techniques; in-vial purging using a purge and trap system, and solid phase micro-extraction (SPME). The study revealed that both techniques had their advantages and disadvantages (Vogt *et al.*, 2008).

The use of SPME was advantageous because of its versatility; samples could either be analysed immediately after collection or stored up to 20 days. VOC loss was less likely and sampling was less invasive. The disadvantages were that if only one compound was

of interest, the fibre could become over saturated with absorbance of other compounds, which could lead to the targeted compound being missed entirely. The conditioning before and after the fiber was used meant that it was time-consuming to utilise, and fibres have limited use of circa 100 times (Vogt *et al.*, 2008).

Purge and trap methods, in this case by in-vial purging, had scope for improving detection limit and precision of samples with lower concentrations. Calibrations were also not very time consuming, and the system itself was very long-lasting. Once it was built and tested, it could be used over and over without constant replacements. Compared to SPME fibres which were costly, a purge and trap system was cheaper in the long run as it was only expensive in the initial stage when being built. The disadvantages on the other hand were that there was mechanistical potential to produce different concentrations of gases. Concentrations were likely to be higher during sample collection. The number of samples collected were limited and it was not recommendable to store samples for more than a few hours (Vogt *et al.*, 2008). This comparison gave a good outlook on how the two methods varied in use. It is still early to conclude which method is better, but presently it is thought that SPME is more suitable for a wide array of situations (Vogt *et al.*, 2008).

Examples of non-volatile based ecological monitoring tools include species identification, whereby the presence (or absence) of pre-determined index species is observed to understand the state of environmental health (Carignan & Villard, 2002), and large-scale ecosystem monitoring methods such remote sensing (Pettorelli *et al.*, 2015).

1.4.2 Current Knowledge of Environmental Volatiles

VOCs released by an organism may serve many purposes, one of which can be as a semiochemical, or otherwise referred to as an infochemical. Infochemicals can be detrimental to the emitter (known as kairomones) or detrimental to the receptor (known as allomones) (Brown *et al.*, 1970) and are used as communication tools to spread information about itself, or another individual, intra or interspecifically (Schmidt *et al.*, 2015; Vet & Dicke, 1992). Sometimes these compounds (for example isoprene) are released unintentionally by the emitter as a product of biological processes, while other times can be released intentionally (for example 6,10,14-trimethylpentadecan-2-one (TMPD-one) and the corresponding alcohol, 6,10,14-trimethylpentadecan-2-ol (TMPD-ol)) as pheromones for mating behaviour (Wallin *et al.*, 2020).

1.4.3 Chemical Communication

Intraspecific

Intraspecific communication using infochemicals can commonly be seen among phytoplankton (Amin *et al.*, 2015). For example, during algal bloom succession, chemical signals are used by *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* among their populations as a defence mechanism against grazers. The allomone they emit is called (2E,4E/Z)-didecadienal (DD), an antiproliferative aldehyde that is harmful to recruitment of marine polychaetes (*Arenicola marina* and *Nereis virens*) and echinoderms (*Asterias rubens* and *Psammechinus miliaris*) (Caldwell *et al.*, 2003). It is primarily produced by these diatom species when exposed to stress, or when wounded.

Once released, this chemical can trigger the production of NO in surrounding diatoms and is thought to be part of a surveillance system amongst bloom populations that help alert surrounding cells for the presence of harmful grazers (Vardi *et al.*, 2006).

VOCs are sometimes produced also as a preliminary response to grazing. A study by Caldwell *et al.* (2003), investigated the oceanic bloom-dominating diatoms, *Skeletonema costatum* and *Nitzschia commutata*. These species released polyunsaturated short-chain aldehydes in late stages of their life cycle as a defence against common grazers. In this case female brine shrimps, *Artemia salina,* to which the chemical alone was seen to have teratogenic effect (Caldwell *et al.*, 2003). This suggested that grazers would be affected regardless of whether they did or did not graze upon their prey. Nutrient deficiency was also seen to contribute in enhancing production of this chemical which meant grazers were especially susceptible to damage towards the end of a phytoplankton bloom (Ribalet *et al.*, 2007).

Interspecific

The best example for interspecific reactions between grazers and their prey would be the use of chemodetection. The nutritionally dilute oceans are a harsh place to find food for tiny grazers like zooplankton. Therefore, chemodetection is a method used to detect their prey which is a necessity for survival. A study by Steinke *et al*. (2006) utilised female calanoid copepods, *Temora longicornis* to investigate prey capture by detection of DMS (Steinke *et al.*, 2006). Behavioural responses such as tail flapping was observed and quantified after DMS was injected into water. Controls consisted of the same filtered and aged natural seawater except with no DMS exposure. Results showed that number of

tail flaps were seen to increase with DMS injections during the first 2-seconds post exposure. Calculated average numbers of tail flaps were three times higher over the initial 1-second period after which these responses doubled for the 2-second period after. A threshold concentration is not yet known and is where further investigation is required. It is thought however that tail flaps are associated with up- or down- gradients more so than absolute concentration of this compound. Perhaps rapid changes in DMS concentration is what affects the behaviour of these species (Steinke *et al.*, 2006).

A study conducted by Maibam *et al.* (2015) further suggested that some VOCs were utilised as chemodetectors, where *Cetropagus typicus* (copepod) were shown to respond to such compounds produced by plant-like matter such as macro and microalgae (Maibam *et al.*, 2015). These chemical cues were used as signals to trace the location of their preferred food and to detect predators. In this study, three planktonic diatoms (*Skeletonema marinoi*, *Pseudonitzchia delicatissima* and *Chaetoceros affinis*) and a dinoflagellate (*Prorocentrum minimum*) were used to produce their corresponding VOCs as these chemical cues. pH was monitored and grazers were exposed to both treatments (normal = 8.10 and acidified = 7.76). Grazers used chemicals as cues as expected, but it was also noted that change in acidity changed their preference in prey. At normal pH, copepods were attracted to VOCs produced by *P. delicatissima* and *P. minimum*, but this attraction was lowered in the acidified water. Contrary to that, under normal pH, copepods were repelled by odours produced by *S. marinoi* but were attracted to them in acidified water (Maibam *et al.*, 2015).

Production of these VOCs, particularly DMS, is increased greatly by prey in response to grazing. Two strains of *Emiliania huxleyi* (CCMP 370 and CCMP 373) were grown under laboratory conditions. Although they produced DMSP at the same rates, there were some differences. Strain 373 had high activity while strain 370 had low activity. The grazer of choice was *Oxyrrhis marina*, a dinoflagellate species. DMS production was imminent when the two strains of *E. huxleyi* were grazed upon. This is likely due to the prey initiating the conversion of DMSP inside their cells to DMS and acrylate by DMSP lyase. To ensure that the production was only due to direct grazing, healthy algal cells were placed with grazer exudates and associated bacteriato observe if these promoted any DMS production. It was clear that only when cells were chemically or physically damaged that they produced DMS. Strain 373 produced more than strain 370 which lead to the belief that those with higher activities of DMSP lyase were likely to produce more DMS than those with lower enzyme activities (Wolfe & Steinke, 1996)

It seems counter intuitive that prey would release a VOC that attracts more grazers, but the actions of organisms higher up the food chain in response to detection of this VOC explains why algal cells increase their production when grazed upon. DMS is used by procellariform seabirds for navigation (Nevitt *et al.*, 1995; Nevitt & Bonadonna, 2005). Physiological and behavioural studies were conducted to investigate the extent to which DMS had an influence over these birds. Ten birds were exposed to DMS, after which their heart rates were monitored. DMS (3-4 nM) triggered a consistent increase in heart rate for all 10 out of 10 birds (Nevitt & Bonadonna, 2005). Twenty five birds were also tested for their orientation when exposed to DMS in a Y-maze. Out of these 25, 20 made the choice of orientating to DMS (Nevitt & Bonadonna, 2005). These experiments confirmed that procellariform seabirds do use DMS of biologically relevant concentrations

as an orientation cue in an experimental context (Nevitt & Bonadonna, 2005). Similar experiments on homing pigeons and other birds also suggested that birds placed in unfamiliar location can orientate because of atmospheric trace gases and so this information was not restricted to seabirds only (Wallraff & Andreae, 2000).

1.4.4 Impact of abiotic variables on volatile profiles

Investigation on species-specific VOC emissions have been studied previously (Colomb *et al.*, 2008); occurrence of BVOCs characteristic to species could be a good indicator of their presence, and such information could be used to interpret water conditions and trophic state. In a study, a eutrophic shallow lake was analysed for BVOCs to see if any could be linked to a species. Results showed that the presence of geosmin, argosmin isomers, heptadec-*cis*-5-ene and a sesquiterpene could be correlated with *Aphanizomenon gracile*, as could cyclocitral with *Microcystis wesenbergii* (Jüttner, 1984).

There are several environmental factors that can cause a change in production rates of BVOCs. Some examples are desiccation, temperature, salinity, light, acidity and oxidative stress (Bravo-Linares *et al.*, 2010). These can affect production rates alone but can also combine to enhance or inhibit the effects each would have alone.

In environments, such as intertidal zones where exposure to air is frequent, BVOC fluctuations are strongly prominent (Jiao & Li, 2004). An increased concentration of bromocarbons is a common occurrence because of desiccation/submersion of

macroalgae and to further investigate this, a study by Leedham *et al*. (2014) took place to understand emissions of dibromomethane (CH_2Br_2) and bromoform ($CHBr_3$) from two temperate macroalgae commonly found in intertidal zones; *Fucus vesiculosus* and *Ulva intestinalis* (Leedham Elvidge *et al.*, 2014).Emissions from both species increased during desiccation with a rapid pulse of emissions, 10 minutes post-exposure after which it plateaued or decreased within 1-3 h after they were exposed. Impacts of rain were also investigated by rewetting both with freshwater as a simulation of rainwater. It is estimated that rain may increase emissions of bromo-carbons; however further studies must be conducted to fully understand how rain may affect emissions in field rather than as a laboratory study (Leedham Elvidge *et al.*, 2014). Salinity is also noted to affect DMS production. Coccolithophore, *Hymenomonas carterae* was exposed to different osmolarities using sucrose or salt. Measurements were made approximately 3 hours after they were transferred into the new medium. Increase in DMS production was observed with increasing salinity, and when transferred from high salinity to low salinity. The quantity of DMS that was released depended upon the difference in salinity between the media that the sample was kept in and the media that the sample was transferred into. This was considered to be an adaptation of the species against osmotic stress; increased DMSP content resolved the difference in water potential, preventing crenation (Vairavamurthy *et al.*, 1985). As such, many BVOCs show change during exposure to salinity fluctuation.

A study by Yassaa *et al.* (2008) suggests that monoterpene production is seen in marine phytoplankton (Colomb *et al.*, 2008). These are a class of terpenes that consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. Laboratory incubation experiments of nine phytoplankton monocultures (Coccolithophorid: *Emiliania huxleyi*;

Diatoms: *Chaetoceros neogracilis, Ch. debilis*, *Phaeodactylum tricornutum*, *Skeletonema costatum* and *Fragilariopsis kerguelensis*; Chlorophyte: *Dunaliella tertiolecta*; cyanobacteria: *Synechococcus* and *Trichodesmium*) and shipboard measurements were taken in the Southern Atlantic Ocean. To avoid terrestrial influence, detection of monoterpenes were also made in areas sufficiently far from land. Out of the nine cultures some produced exceedingly high amounts of monoterpenes, while others differed very little from cultureless controls. Monoterpenes detected included; *α*-pinene, *β*-pinene, myrcene, camphene, sabinene, limonene and ocimene (Yassaa *et al.*, 2008).

Table. 1.2: Emission rates of monoterpenes from the nine algae species as shown in Yassaa *et al*. (2008).

Phytoplankton species	Emission rates (nmol g [chlorophyll *a*]$^{-1}$ day^{-1})
Diatom	
Chaetoceros neogracilis	3.7
Chaetoceros debilis	0.8
Phaeodactylum tricornutum	68.1
Skeletonema costatum	0.3
Fragilariopsis kerguelensis	43.1
Coccolithophorids	
Emiliania huxleyi	0.3
Chlorophycae	
Dunaliella tertiolecta	225.9
Cyanobacteria	
Trichodesmium	0.8
Synechococcus	1.1

To follow on, further studies by Meskhidze *et al.* (2015) took place to then look at effects of irradiance and temperature on production of these monoterpenes as well as on isoprene (Meskhidze *et al.*, 2015). Six phytoplankton monocultures were prepared each for irradiance experiments and temperature experiments. Those cultured for irradiance experiments included; diatom strains (*Thalassiosira weissflogii* and *Thalassiosira*

pseudonana), prymnesiophyte strains (*Pleurochrysis carterae*), dinoflagellate strains (*Karenia brevis* and *Prorocentrum minimum*) and cryptophyte strains (*Rhodomonas salina*) (Meskhidze *et al.*, 2015). Only the diatom strains out of these was used for temperature experiments. When subjected to low irradiance, production rates rapidly increased, while only gradually increased to high irradiance. Temperature variability did not affect production at low irradiance, but emissions did increase with temperature. Suggestions could be made from these results that monoterpene and isoprene production is enhanced because of fluctuations in environmental conditions. Possibly as an attempt to physiologically acclimate to its environment (Meskhidze *et al.*, 2015).

Light intensity influences photosynthesis and as light intensity decreases so does the rate of photosynthesis. This leads to decrease in O_2 concentration that in turn forms an anaerobic environment. Such processes are rather commonplace amongst algal mats. Decomposition rates increase under anaerobic conditions which result in an increase in release of volatile sulfur compounds (Sela-Adler *et al.*, 2016; Zinder *et al.*, 1977).

DMS production is also thought to increase in the presence of light, as studies suggest that cleavage of DMSP has a role as an anti-oxidant that reacts to hydroxyl radicals which are produced in the process of photosynthesis (Sunda *et al.*, 2002). pH can influence volatile production, and some gas types such as DMS and halocarbons have been studied in relation to changes in acidity. Elevated CO_2 reduces oxidative stress and causes DMS concentrations to decrease; however when combined with increased temperature, remains the same (Arnold *et al.*, 2013). Halocarbons on the other hand are not affected by elevated acidity directly (Hopkins *et al.*, 2013).

Production of BVOCs are sometimes produced in response to an organisms' attempt at resisting oxidative stress. Laminariales are exceptional at iodide accumulation and play a role for kelp species as an inorganic antioxidant. ROS are scavenged by iodides upon oxidative stress, which result in an efflux of iodide as molecular iodine (Küpper *et al.*, 2008). DMSP as well as its breakdown products are also considered to be possible antioxidants. DMS, acrylate, dimethylsulphoxide, and methane sulphinic acid are all scavengers of hydroxyl radicals and ROS. Production and enzymatic cleavage of DMSP may be part of an in-built anti-oxidant system of marine algae and explain the increased algal DMSP concentrations in presence of oxidative stressors and light (especially considering hydroxyl radicals are produced as a by-product of photosynthesis) (Sunda *et al.*, 2002). Areas with organisms experiencing high amounts of oxidative stress could perhaps be detected or monitored by measuring iodide and DMS concentrations.VOC concentrations can sometimes change simply due to aging of the organisms. Mesocosm systems were studied under various conditions to monitor community growth and DMS concentrations. The study showed that the relationship between phytoplankton growth and DMS production has long been considered but DMS release rates correlate more with senescence than growth alone (Kwint & Kramer, 1995).

1.5 Conclusion

To conclude, there are a vast number of resources that have helped investigate the production of BVOCs. Medical science has advanced their knowledge and use of BVOCs as a diagnosis method, and if information on BVOC production can be accumulated over

the years, then perhaps so can environmental volatile metabolomics use this information as a monitoring tool. The studies documented in the next few chapters will go through a step by step process of how to build the foundations of such a tool (Figure. 1.2). To start with, VOCs will be investigated as unialgal cultures, then mixed cultures, to then be brought to *In situ* field sampling to bring together the information gained from lab studies to field studies to see if there are any similarities and links between the two (Figure. 1.2).

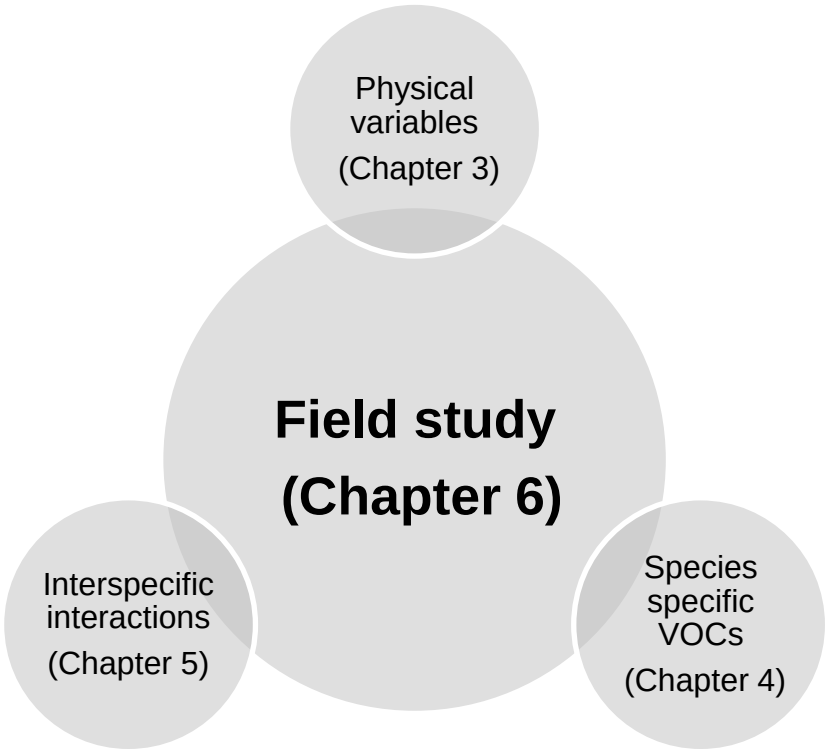


Figure. 1.2: Infographic representation of aims in each chapter and how they relate to the final study in which in-situ samples will be taken. Experiments increased in complexity with each chapter.

Chapter 2: Methods of Marine Trace Gas Analysis

2.1 Introduction

A series of steps are involved in the analysis of VOC concentrations. The VOCs of interest typically require being extracted and concentrated prior to analysis in order to reduce the volume necessary. After VOCs are extracted and concentrated, they are passed through a gas chromatograph (GC). Compounds are then separated, detected and quantified. In the following chapter, information on the range of extraction methods, concentration methods, as well as detection methods will be explored.

2.2 Analytical Procedures, Materials and Methods

2.2.1 Culture preparation

Thalassiosira pseudonana, *Emiliania huxleyi*, *Dunaliella tertiolecta, Rhodomonas lacustris* and *Oxyrrhis marina* were grown in batch cultures. Samples were then transferred to be grown in 1 L and 750 mL conical flasks for chapter 3 and 5 respectively, while in chapter 4 were transferred to be grown in purpose built 750 mL silica screw cap bottles.

In chapter 3, two prepared batches of *E. huxleyi* (CCMP 373) obtained from the Provasoli-Guillard Center for the Cultivation of Marine Phytoplankton (CCMP, West

Boothbay Harbour, Maine, USA) originating in the Sargasso Sea and isolated in 1960, were grown and kept under 16 and 26 °C. These were then transferred into six different 1 L conical flasks (3 per each temperature) with sterile artificial seawater (SASW) supplemented with f/2 nutrients and vitamins (Guillard & Ryther, 1962) in volumes of 400 mL. The controls consisted of SASW only but was treated and kept under the exact same conditions as the live sample bottles. These were then placed as biological triplicates in two growth rooms, one set at 16°C and the other set at 26°C. Cultures within each bottle were swirled slowly for 10 seconds daily to be mixed evenly, and a systematic routine of swapping physical locations of bottles in a clock-wise manner allowed for maintenance in homogeneity.

Cultures for chapter 4 were prepared and kept differently. *D. tertiolecta, E. huxleyi, R. lacustris,* and *T. pseudonana* were grown in 16°C growth rooms in batches prior to experiments. These were then transferred to three purpose built 750 mL silica screw cap bottles (n=3) to reach starting volume of 400 mL. These bottle lids were modified with gas tight gas supply and waste tubes (1/8 inch, approximately 3.2 mm OD Teflon tubes) that allowed cultures to be supplied with compressed air that bubbled at a flow of 60 mL min and allowed for sample collection using tedlar bags. The media used for *D. tertiolecta, E, huxleyi* and *R. lacustris* were SASW with f/2 nutrients and vitamins, while media used for *T. pseudonana* was the same used as the aforementioned but supplemented with silica. Controls consisted of media only.

For chapter 5, *D. tertiolecta, E. huxleyi* and *T. pseudonana* were grown the same as in the previous chapters. For competition experiments, *E. huxleyi* and *T. pseudonana* were

transferred and set up so that there were two unialgal cultures of *E. huxleyi* and *T. pseudonana*, and a mixed culture within which the two were mixed. *O. marina* was grown in 1 L Duran bottles alongside their prey (*D. tertiolecta*) to feed on and kept in a dark room under black cloth to prevent any autotrophic activity and ensure all of prey were removed prior to experiments. These were then transferred into 750 mL conical flasks to a starting volume of 400 mL.

Light intensity was measured in both growth rooms (16 and 26°C) at 300±15 µmol photons m^{-2} s^{-1} supplied by cool-white fluorescent tubes (TL-D 840, Philips) on a 14:10 light: dark cycle.

2.2.2 Gas extraction and pre-concentration techniques

During this study, a variety of VOC extraction and pre-concentration methods were explored. Due to VOC concentrations being very dependent upon where they are extracted from, a variety of extraction techniques exist to fit the range of concentration levels. Pre-concentration is necessary if concentrations are below the limit of detection. To successfully extract and pre-concentrate your VOC of interest, a search through literature for estimated concentrations may need to take place prior to sampling. Techniques that were used during the experiments carried out in this thesis include Headspace Direct Injection (HS-DC), Tedlar-bag Purging and In-tube purging (Franchini & Steinke, 2016).

All purging techniques are pre-concentration methods that utilise a “purge and trap” system. Purge and trap is a technique used to extract VOCs from seawater samples for subsequent analysis by gas chromatography. This can be directly from within the water, or from the headspace above. If the concentrations of VOCs are high enough, the VOCs from the headspace can be injected directly into the GC but if not, purge and trap techniques are required. These techniques involve passing through an inert gas such as nitrogen through the sample, whether it be in the water or the headspace above, to carry the VOCs into the gas stream. These are then cryogenically “trapped” for further analysis. The lower the VOC concentrations, the more purging is required prior to analysis. Headspace purging is the least effective while in-tube purging is the most effective and best used when sampling seawater that contains miniscule amounts of VOCs. Of course, this can be very time consuming and considerations must be made as to how long the sample needs to be purged. It is also important to note that the efficiency of extraction is dependent on the Henry’s Law coefficient (H) of the VOCs in mind, which is defined as the concentration in air divided by the concentration in water when at equilibrium. Lower H values mean that extraction is more efficient when compared to compounds with a higher H value.

Once VOCs are extracted from the sample or its headspace, compounds are pre-concentrated by either cryogenic enrichment, or by accumulation onto absorptive material such as solid phase microextraction (SPME) fibres and thermal sorption tubes. However, in this thesis the focus is more on cryogenic enrichment using purge and trap. VOCs can be cryogenically enriched by purging the VOCs into the gas stream onto a cryogenic loop that is maintained at a temperature of -150°C. Once the appropriate, pre-determined period of time for purging has passed, the cryogenic loop is immediately

heated up to 90°C using freshly boiled water to flush the concentrated sample into the GC oven for separation and detection of the VOCs. It is vital that the process of heating the loop is in sync with sending the sample into the oven, as otherwise results may be skewed.

To make VOCs from chromatograms comparable, an R package called GCalignR was used. As a measure for quality control, following the statistical alignment, each peak was compared qualitatively with raw chromatograms to ensure that the values were aligned correctly. Data was then organised by removing control values from actual values and then normalised to cell density, cell volume, or chl *a* as appropriate.

2.3 Sulfur Compounds Analysis

2.3.1 Sample collection

Samples were collected from both in-lab cultures as well as directly from the field (Brightlingsea Harbour) and so separate techniques were used. Production was measured using tedlar bags, high concentration samples were measured using head space injections or flushing, concentration was measured using in purge flushing as was the case with field studies also.

2.3.1.1 Cultured Plankton

VOCs from lab-grown cultures were collected in two forms. Samples of experiments in chapter 3 and 5 were collected as volumes of liquid from which the gases were purged out, while those from chapter 4 were collected directly using tedlar bags that would then be pushed straight through the purge and trap system as described in 2.2.2.

Either glass syringes of suitable size, dependent on the experiment, were used to extract samples from conical flasks (chapters 3 and 5) or tedlar bags were attached directly onto purpose-built bottles for a pre-determined period of time (chapter 4). The purpose-built bottles were made by altering the bottle caps, Two holes were drilled through, one to connect Teflon tubing to the compressed air supply, the other to collect the sample in tedlar bags. All connections needed to be gas tight so that the VOCs could only exit from the second tubing to which tedlar bags were attached for sample collection. Prior to sample collection, tedlar bags were flushed with nitrogen and vacuum sealed to ensure that any possible contaminants were removed. Cultures were left to grow until they reached exponential phase.Once exponential phase was reached, tedlar bags (that has a maximum capacity of 3000 ml) were attached to the culture bottles and left for 20 minutes. This allowed for 1200 ml of the headspace to be collected.

After the samples were collected into the tedlar bags, these were attached one at a time to a purpose-built Teflon purge and trap system (Franchini & Steinke, 2016). Gases were then concentrated in a cryotrap held at -150°C using a purpose-built liquid nitrogen heater for 20 minutes at a flow rate of 60 ml/min. VOCs were then desorbed as described in 2.2.2.

The samples that were directly extracted from live cultures required in-tube purging to cryogenically enrich any dissolved VOCs. In chapters 3 and 5, from each sample, a pre-determined amount of the culture was extracted and filtered through a glass fibre filter (Whatmann, GF/F, 25 mm) into the purge tube. Nitrogen flow was adjusted, and the samples were left to purge for a set time. Both parameters varied depending on the type of experiment undertaken in these chapters. Once samples had finished purging, the condensate was thermally desorbed by immersing the loop in freshly boiled water heated to 90°C. The sample was flushed into the GC equipped with an FPD for sulfur compounds, and an FID for other compounds. Temperatures for the GC oven and how long it ran also varied between experiments.

2.3.1.2 Field Samples

Three 10 litre polycarbonate aspirators were used to collect water samples from Brightlingsea harbour. There were a few additions including teflon tubing attached to the tap, and 200 µm mesh attached to the opening. Each aspirator was inverted with the taps open and gently pressed against the sea surface. This allowed for water to gently fill the aspirator with very few gas bubbles. Large detritus and zooplankton were excluded from entering due to the 200 µm mesh. Once the aspirators were full, the tubing attached was placed at the bottom of each of the three 500 ml Duran bottles, after which the taps were turned on to allow the water from the aspirator to flow into the Duran bottles. Large aliquots were taken and shaken around the bottle for rinsing. After this, water was then filled from the bottom of the bottle to then overflow for approximately three times the total

volume of the bottle. Once overfilled enough, the bottle was carefully removed and capped, avoiding headspace and bubbles as much as possible. These bottles were then placed in cooler boxes in the dark and transported to the laboratory. Bottles were kept in the dark at a constant room temperature of 10-12°C.

When ready for sampling, 50 ml seawater samples were drawn into a glass syringe (60 ml) and then slow filtered through a 25 mm GF/F filter directly attached to the glass purge vessel. The seawater sample was then preconcentrated by bubbling it with nitrogen for 20 minutes at a rate of 80 ml/min to carry the VOCs out of the solution into the stream of gas. VOCs were then desorbed as described in 2.2.2.

2.3.2 Dimethyl sulfide (DMS) Analysis

The identity of each sulfur compound may not be clear when using a GC-FPD, but known standards can be run through to identify a compound. In this case, the retention time in which a dimethyl sulfide peak would appear was estimated by injecting 250 µl of 30 µM DMS directly into the GC. This however can only reveal the identity of that peak and regular calibrations must be run if the compound is to be quantified. The oven temperature was set to start at 40°C and ramp up to 120°C over 20 minutes and the detector was set to 250°C. However, this could vary and needs to be changed depending on the GC used and would need to be tested prior to the experiment if it is to be repeated.

2.3.3 Dimethylsulphoniopropionate (DMSP) Analysis

As sulfur compounds were quantified, including dimethyl sulfide (DMS), when possible, DMSP was also quantified to coincide with results for DMS analysis. When sampling for DMSP, the oven temperature was set to 120°C, the injector temperature was 200°C, and the detector temperature was 250°C. However, this could vary and needs to be changed depending on the GC used and would need to be tested prior to the experiment if it is to be repeated.

2.3.3.1 Cultured Phytoplankton

A 20 ml glass syringe was used to extract 10 ml from each bottle and was slow-filtered through 25 mm GF/F filter. With flat forceps, the filter was carefully folded and put into 4.92 ml vials that were prepared and filled with 3 ml of 0.5 M sodium hydroxide (NaOH). Vials were then immediately closed with a gas-tight silicone/Teflon septa and incubated at 30°C for a minimum of 24 hours to complete equimolar alkaline hydrolysis of DMSP to DMS. Pre-concentration for DMSP samples was not necessary, and therefore headspace injection methods were used and calibrated for as described in 2.3.6..

2.3.4 Purge and Trap System

The purge and trap system for sulfur analysis was constructed of:

- 1/8 in (about 3.2 mm) outer diameter PTFE tubing.

- Stainless steel fittings, metering valves (part number SS-SS2-A), and PTFE fittings (Diba Omnifit; Kinesis Ltd, St Neots, UK).
- Glass tube filled with saline treated glass wool to remove aerosols, although this is typically replaced with a glass condenser tube immersed in ice-water mixture if sample volumes are larger than 100 ml.
- Nafion counter flow drier (1.8 m length, Perma Pure part number MD-050-72F-2; Fluid Controls Ltd, Aldermaston, UK).
- PTFE 6 port, 2-way distribution valve (Diba Omnifit part number 001127-6LI; Kinesis Ltd, St Neots, UK).
- Purge tubes consisting of glass tubes measured at 18-30 mm diameter and variable volume, with a porous glass fit (porosity size 3; 16-40 µm poresize) at the bottom and Quickfit glass stopper at the top, both equipped with ¼ inch (about 6.4 mm) glass connectors to connect in-line with the purge gas.
- Purpose-built temperature controller to adjust cryotrap to -150°C

All the above information regarding the parts of the trap system was based on previous work (Franchini & Steinke, 2016).

On a side note, the purge system was built in duplicate and consists of two concurrent purge systems. This allows for two samples to be purged simultaneously.

2.3.5 Gas Chromatography - Flame Photometric Detector (GC-FPD) System and Methods

Flame photometric detectors work based on the concept of a photochemical process known as chemiluminescence. Reactions of sulfur atoms in the hydrogen/air flame is used as a source for analytical detection. As the molecules are excited, light is produced and serves as the FPD's signal. The end of a GC column leads into the reaction chamber that is in front of the FPD. The FPD is contained within a combustion chamber fuelled by hydrogen and air that flow through at a rate of 50 ml/min and 60 ml/min respectively. Within the FPD is also a thermal filter that separates the UV and visible radiation emitted by the flame. The FPD also contains an optical filter that works to isolate the sulfur wavelength emission. GC-FPDs are only able to analyse sulfur VOCs and therefore should not be used if analysing non-sulfur compounds.

2.3.6 GC-FPD System Calibrations

To quantify the VOCs of interest, the GC-FPD system needed to be calibrated. However; calibrations were required for each different method used, which were necessary for both samples that were pre-concentrated and those that were not. Therefore, three types of GC-FPD calibrations were run. To quantify the sulfur compounds, DMSP standard solutions hydrolysed in 0.5 M NaOH were used.

For headspace injection (for samples that were not pre-concentrated), DMSP standards of a range of concentrations were prepared by carefully mixing a set amount of DMSP-

HCl solution (ranging from available concentrations of 7.5 µM to 7.5 mM) with 3 ml of 0.5 M NaOH solution. These were left to incubate for 12 hours prior to being injected into the GC-FPD. This method was used for DMSP sampling in chapter 5 whereby DMSP-HCl solutions of concentrations 7.5 to 75 µmol were used for the calibrations performed.

For in-tube purging, DMSP primary stocks with a concentration of 5.39 µM were used to make a secondary stock with a concentration of 5 nM. These were made by gas tightly injecting the DMSP primary stock into a crimp-sealed 21 ml vial filled with milliQ water and a small vial to aid with mixing. These were left overnight to allow the primary stock to mix with the milliQ completely. The purge tube was then filled with 2.5 ml of NaOH along with an appropriate amount of milliQ water and secondary stock so that there was a final volume of 50 ml in the purge tube with a final target concentration dependent on the study (literature was used to estimate what sample concentrations were expected), after which the purge top was immediately closed to prevent any DMS from escaping. After purging, the DMS was desorbed and flushed through into the GC for measurement as explained in 2.2.2.This method was used for chapters 3, 5 and 6. In chapter 3 and 5, calibrations were achieved by creating a primary stock of 40 µM by gas tightly injecting 112 µL of 7500 µM DMSP-HCl solution into milliQ and left till mixed. The purge tube was then filled with 19 ml of ASW and 1 ml of NaOH. The purge tube was injected with a range from 25 to 400 µL of this primary stock to achieve concentrations of 40 to 640 nM. In chapter 6, the same was conducted except the secondary stock was gas tightly injected into the purge tube to achieve concentrations ranging from 1 and 30 nM.

2.4 Broader VOC Analysis

In the sea, there is a much broader range of VOCs not only limited to sulfur compounds. This meant that a different GC was necessary to gain a better insight into what VOCs were present in the environment at that moment in time. Although GC-FPDs can only detect sulfur compounds, GCs with a flame ionisation detector (FID) can detect a much wider range of compounds (although not all). Thus, to collect better field data, analysis was undertaken using a GC-FID in conjunction to a GC-FPD.

2.4.1 Preconcentration

Preconcentration techniques used were the same as those used in 2.3.6 but using a stainless steel purge and trap system instead of a PTFE one. In-tube purging was used, and the period of time required, varied from experiment to experiment.VOCs were pre-concentrated by purging the sample with nitrogen at the rate of 80 ml/min.

2.4.2 Purge And Trap System

PTFE could not be used when building a trap system for FID analysis due to it having the capacity to absorb some of the VOCs of interest. This would make the sample far from being representative of the environment which is not ideal. To fix this problem, the trap was made the same way, except using 1/16 inch stainless steel tubing and fittings to replace those made out of PTFE.

2.4.3 Isoprene Analysis

Although GC-FIDs can detect a larger range of VOCs, they cannot identify the compound as GCs coupled with mass spectrometers (MS) may. However, standards of known concentrations can be run through the GC-FID to identify and quantify that compound. One of these compounds in addition to DMS is isoprene. The GC oven was set to start at 80°C and ramp up to 180°C over 36 minutes, and the detector was set at 250°C. However, this could vary and needs to be changed depending on the GC used and would need to be tested prior to the experiment if it is to be repeated.

2.4.3.1 Isoprene Calibration

In-tube purging alone was used as a pre-concentration method when using the GC-FID and so it was appropriate to only conduct calibrations for this method. A primary stock of 4.78 mM was made by injecting 10 µl of isoprene (solubility in water of 642 mg/l at 25 ºC) using a 10 µl gas tight syringe into a crimp sealed vial (20 ml nominal volume, Chromacol; Fisher Scientific, Loughborough, UK) filled with milli Q and the smaller vial. This was then shaken to mix completely, after which a second stock of 455 µM was made by injecting 2 µl of the primary stock into a second crimp seal vial using the same methods.

To generate a calibration curve, duplicates of 10 different concentrations were run through the GC. For each individual run, the purge tube was filled with 50 ml of milliQ

water followed by a known volume (10-160 µl) of the secondary stock and then purged for 20 minutes with a nitrogen flow of 80 ml/min. The calibration needed to yield a linear regression (r^2) of > 0.99.

2.5 Preparing Equipment for Use

The GC-FPD and GC-FID as well as the purge and trap systems attached respectively needed to be tested for any problems prior to analysis. Experiments would be affected if there were leaks, blockages or contaminants as it would distort the data making it less accurate. In the next few paragraphs, I will explain how these potential problems were tested for resolved.

2.5.1 Leaks

Occasionally there may be a leak in the purge and trap system. This may be because fittings are not connected properly within the system. If the flow is much lower than it should be and remains low even when the gas flow or pressure is increased, there is a slight chance that there could be a leak in the system. Whether there is a leak or not can be tested by using an 80:20 mixture of water and detergent (Fairy liquid for example). A small amount of this can be pipetted on all connections to see if it foams. If it does, there is a good indication that, there is a leak. If there is a leak in between connecting fittings, tighten the fitting and do this again. If it no longer foams then the leak may be fixed, so

try measuring the flow again. If the flow is the rate at which is should be at the correct pressure, then the leak problem has been resolved.

2.5.2 System Blockage

If the above does not work or if there was no leak at all, there is a chance that it could be a block. There are a couple of signs that could indicate that there is a block in the system. One is if the flow is still low after a leak test, the second is if a standard of a known VOC is injected, that the peak area is much smaller than expected or will not appear at all. This problem is resolved by cleaning the system. The nafion dryer must first be removed beforehand. Dilute (1%) nitric acid is then used for cleaning PTFE purge systems, while methanol is used for the stainless steel systems. Cleaning reagents are run through the system by syringing it through all the tubing. Once the system has been rinsed with the cleaning reagents, the same thing is repeated except with milliQ water. It is important to flush the system with lots of milliQ water to ensure none of the cleaning reagents remain. After this is completed, the system is dried completely by flushing it through with nitrogen gas.

2.5.3 Contamination

The purge and trap system may be contaminated if foreign peaks are seen after a blank analysis. A blank analysis is where nitrogen gas is preconcentrated using the system and then run through the GC. Normally there should not be any obvious peaks but sometimes if there is a contaminant within the system, peaks will appear on chromatograms generated. If this is the case, the first step is to flush the system with copious amount of nitrogen overnight. The system should then be tested for the second time. If there are still foreign peaks, then the system needs to be cleaned as described in 2.5.2. If the system is still contaminated, then parts of the system may need replacing, or it could be contamination in the dryer.

The nafion dryer should be visually inspected first for any solids or visible deposits, a clean soft cloth may be used to wipe off any dust or liquid residues. If the dryer is likely to be contaminated with oil or organic compounds, then it must be rinsed using a hydrophobic organic solvent such as hexane. After the system has been rinsed, it must be blow dried with nitrogen gas. Afterwards, deionised water must be used to rinse through the system to ensure it has been fully cleaned of any remaining compounds. The dryer can then be reattached for testing. If this does not work, a new dryer may be required.

2.6 Other Measurements

To accompany volatile analysis, chlorophyll *a* concentrations were determined, phytoplankton identification took place for field samples, and cells were counted daily for cultured samples.

2.6.1 Chlorophyll *a*

For cultured samples, 15–50 ml of each bottle was slow filtered through a GF/F filter using a 20–100 ml syringe. The filter was then folded using flat tipped forceps and put into 15 ml centrifuge tubes wrapped in aluminium foil. These were then stored at -20°C. The same procedure took place with the field samples, except 50 ml was filtered through instead of 15 ml.

For field studies, 50 ml of each sample was slow-filtered through a glass fibre filter (Whatman GF/F, 25 mm) using a 100 ml glass syringe. With forceps, the filter was placed into 15 ml centrifuge tubes and stored in -20°C.

Stored samples were extracted by soaking in 100% methanol for 24 hours and stored in the dark at -20°C. After 24 hours the samples were centrifuged at 3000 *g* for 10 minutes to separate out particles from the methanol/chlorophyll *a* solution. The centrifuge was pre-cooled prior to centrifugation. Afterwards the samples were immediately analysed by spectrophotometry (cultured samples) or spectrofluorometry (field samples) using a band

width of 632 - 750 nm (Riemann & Ernst, 1982). Readings were then converted to chlorophyll *a* measurements using established equations from Ritchie (2008) (Ritchie, 2008).

2.6.2 Phytoplankton Identification

Phytoplankton were settled in settling chambers overnight (approximately 8 hours per 10 ml) and placed under a dissecting microscope. Taxa were then identified using a taxonomy guide.

2.6.3 Phytoplankton Counts

To assess whether cultures had reached exponential phase and then stationary phase, cells were counted daily then plotted onto a graph. From each culture, 2.7 ml was extracted and 30 µl of lugol's solution was added if cells were motile. These were counted under a light microscope using a Neubauer haemocytometer at x200 magnification. Cell volumes were also calculated in shapes of a sphere, cylinder or ellipsoid using the diameter and heights measured using a calibrated eye piece graticule.

Using a coulter counter was considered, but Arnold *et al.* (2013) revealed the underestimation of cell counts at cell densities above 500,000 cells ml^{-1}. Cell densities were calculated by multiplying an average of 3 haemocytometer counts, where each haemocytometer count was an average of cell counts across all 5 haemocytometer grids.

These haemocytometer counts were then multiplied by total media volume to give total cell density. Specific growth rates were estimated using calculated cell densities following the equation:

$$\mu = \ln (x_1 - x_0) / t_1 - t_0$$

Where x_1 and x_0 refer to cell densities at t_1 and t_0 respectively.

2.7 Summary

Trace gases were extracted using multiple purging techniques suited for each experiment, cryogenically trapping compounds of interest. For cultured samples, the VOCs produced were compared among the species. For field samples, temporal comparisons were made in search of seasonal change. DMS and isoprene were quantified during this study. Using these methods in the following chapters, different types of factors and how they influence the VOC spectrum will be explored in detail. For all VOC measurements and quantification, peaks were determined for analysis based on the Signal-to-Noise approach in which the signal is divided by the noise. If the value obtained is above three, it is considered acceptable and above the limits of detection (LOD), while if it is above 10, it is considered acceptable and above the limits of quantification (LOQ) (Currie, 1968).

Chapter 3 – Comparison of VOCs released by *E. huxleyi* grown under two temperatures of 16 and 26 °C

3.1 Introduction

3.1.1 Ecological monitoring in the past

There are several methods developed for ecological monitoring, each adapted for different purposes. A common method is to look for index species to comprehend the state of health in community structures. Traditional methods such as taxonomy are still used to accomplish this, but new methods such as metagenomics and next generation sequencing may also be used if such species are difficult to identify. Another method is to measure factors such as various nutrient levels and pH to compare with values collected previously when the ecosystem in mind was healthy.

Although useful, these techniques can be logistically and experimentally challenging. This leads to the potential use of volatile metabolomics. If measurements for volatile organic compounds (VOCs) could be used to disentangle the species present, then traditional taxonomy that has a chance of error, and metagenomics that can be expensive, could be replaced by a more accurate and cost-effective method of volatolomics. Changes in abiotic factors often affect the inhabitants of an ecosystem and therefore in turn the volatile composition. The detection of such changes, or lack of changes, may be used as a bio-indicator for fluctuations that occur in the environment.

With these points in mind, the development of volatolomic tools for “environmental diagnosis” would be a great addition to current monitoring techniques.

3.1.2 Influence of temperature on organisms

Abiotic factors such as temperature affect the biological processes of all living organisms. The existence of varied temperatures has made room for niches to be filled. Such temperature windows in which organisms can survive are thought to be determined by activity of key cellular components. Upper and lower limits are determined by balance between protein denaturisation and production (Rothschild & Mancinello, 2001; Nedwell, 1999). Temperature levels rising often lead to an increase in protein denaturisation, which without adaptations, can result in disruption of cellular function. Lower limits are thought to be determined by decreased or loss of membrane function (Nedwell, 1999). These temperature windows may be broad or narrow depending on the taxa. Some species are specialists that are able to survive in a narrow range, whilst some are generalists that have a much wider temperature range for growth (Rothschild & Mancinello, 2001). Extremophiles must have a set of adaptations that allows them to survive in harsh environments. For example, *Chlamydomonas raudensis* is a psychrophilic phytoplankton species adapted to survive in low temperatures. They have highly unsaturated fatty acids within their photosynthetic membranes, increased plasticity and structural flexibility in their enzymes, and produce ice-binding proteins to serve as antifreezes, ice recrystallisation inhibitors and ice nucleators (Dolhi *et al.*, 2013). *Pyrolobus fumarii* on the other hand, is a hyperthermophilic, nitrate-reducing chemolithotroph archaea species capable of growing up to temperatures of 113°C

(Rothschild & Mancinelli, 2001). Although there are a number of archaea and bacteria species able to grow in high temperatures, the upper limits of eukaryotes are known to be ~60°C (Rothschild & Mancinelli, 2001). Unlike obligate thermophiles and obligate psychrophiles, mesophiles have a wider geographic distribution due to their survivability in habitats between 20–45°C; a temperature range found within a much larger number of environments (Willey *et al.*, 2009). In particular, *E. huxleyi* is considered an ubiquitous species with a wide temperature range of 2–27°C (Fielding, 2013). This is thought to be because of the formation of many ecotypes as an adaptation to each respective environment which has allowed the species to survive in a wide array of temperatures (Paasche, 2002).

3.1.2.1 Influence of temperature on specific VOCs

There are already many experiments that display the impact that temperature has on VOCs; however, these focus on specific, known volatiles. For example, there is a lot of information on the production of isoprene and dimethyl sulfide. This could be expanded to gaining an understanding of volatile profiles and how those grown in two temperatures may differ from one another.

Much research has been carried out on isoprene and DMS. A study by Shaw *et al*. (2003) explored the production of isoprene by a marine phytoplankton (*Prochlorococcus spp*) in response to increased temperature whereby an exponential increase in isoprene production from 12°C up to 23°C was observed, followed then by a decline (Shaw *et al.*, 2003). Isoprene production was also studied in 21 strains of different phytoplankton

(Exton *et al.*, 2013a). Each species had its own optimum temperature range but despite each being in their optimal conditions, species that favoured higher temperatures (26°C) released more isoprene than those that favoured lower temperatures (-1°C). This was likely due to isoprene production being an adaptation of those that can survive in higher temperatures (Exton *et al.*, 2013a; Singsaas *et al.*, 1997).

DMS is considered to increase in production when phytoplankton are exposed to sub-optimal temperatures (Loreto & Schnitzler, 2010). An experiment was conducted by Arnold *et al.* (2013) that investigated the effects of global warming on DMSP concentration and DMS production in strains of *Emiliania huxleyi*. The results showed that DMS production was higher by 36.2 ± 2.58 μmol L^{-1} cell volume h^{-1} in cultures grown at 21ºC compared to those grown at 17ºC (sub-optimal vs optimal) (Arnold *et al.*, 2013). Similarly, cultures exposed to temperatures lower than their optimum values also caused a rise in DMS production. Four species of phytoplankton were grown under two temperatures of 1 and -1.6°C (optimal and sub-optimal respectively). Results showed that DMS production was higher in those grown in -1.6°C compared to 1°C and indicated that cultures exposed to temperatures lower than what they were acclimatised to would result in a rise in production (Baumann *et al.*, 1994). DMSP concentration followed both patterns in that cultures increased DMSP production when exposed to temperatures that are sub-optimal, whether it be higher than, or lower than temperatures in which they are found naturally. (Arnold *et al.*, 2013; Baumann *et al.*, 1994; Van Rijssel & Gieskes, 2002). Similar results have been seen in studies of coral holobionts (Deschaseaux *et al.*, 2014). When *Acropora aspera* was exposed to thermal stress DMS production was triggered (Deschaseaux *et al.*, 2014). Studies such as these, further supported the idea that DMS and DMSP increases due to stress.

3.1.3 Hypotheses and aims

The next step in developing further understanding of VOCs is to consider the volatile profile of organisms. Their VOC production rates are likely to be dependent on parameters within their environment. Information gained by comparing volatile profiles of the same species grown under two separate temperatures may be used to progress research on using VOCs as biomarkers for heat stress.

The aim of this study was to investigate how the environment in which organisms grow may shape the VOCs they release. Temperature was chosen to be the differing abiotic factor and *E. huxleyi* (strain 373) was chosen as the test species. *E. huxleyi* (strain 373) was chosen as it is a known DMS producer and is easy to culture. Overall there is evidence for the species to grow over a range of temperatures between 0–30°C (Figure. 3.1) (Conte *et al.*, 1998; Rosas-Navarro *et al.*, 2016; Van Rijssel & Gieskes, 2002). Most cultures began increasing in growth rates between 10–20°C, while decreasing from 20°C upwards to the limit of 30°C (Figure. 3.1). This suggested that molecular instability increased when cultures were grown under temperatures higher than 20°C. With this in mind, we considered growing strain 373 at 16°C, a temperature in which lipids and proteins of the species are thought to function well, and 26°C, a temperature in which molecular instabilities begin to occur and growth rates begin to slow as a response, to compare these differences in the context of VOC concentrations (Nedwell, 1999).

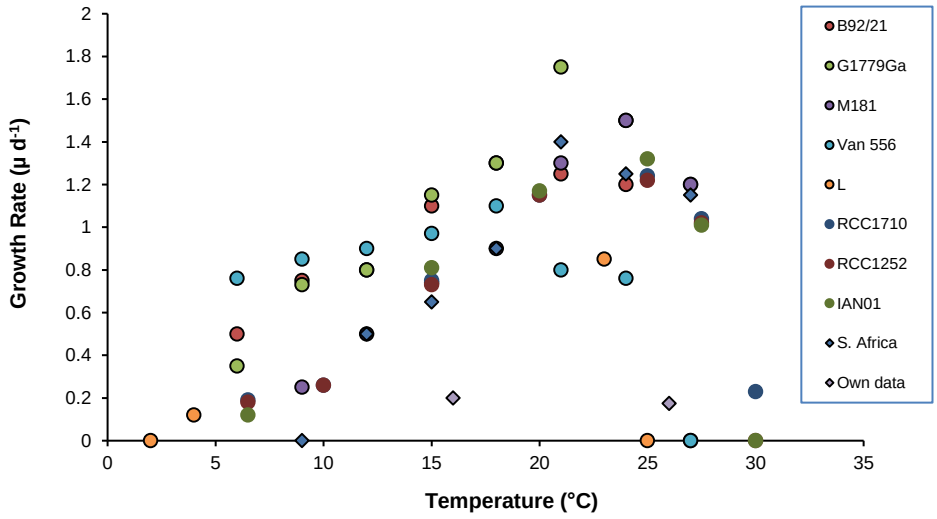


Figure. 3.1: Growth rates of a range of *E. huxleyi* strains grown at different temperatures. Own data refers to strain 373 grown for this study, and all others were collected from the literature (Conte *et al.*, 1998; Rosas-Navarro *et al.*, 2016; Van Rijssel & Gieskes, 2002).

With this in mind, the following hypotheses were addressed:

1. Growth rate of cultures will be lower in those grown at 26°C than those grown at 16°C as previous literature shows that many strains exhibit a decline in growth rates of those grown in 26°C when compared with their optimal temperatures
2. The number of different VOCs released will be higher at 26ºC compared to 16°C due to 26°C being a more stressful temperature for the organisms
3. Isoprene and DMS concentrations will both be higher in cultures grown under 26°C compared to those in 16°C because isoprene is seen to increase in

production with temperature, and DMS is seen to be released when individuals are subjected to stress

3.2 Material and Methodology

3.2.1 General experimental setup

Cultures were grown in growth rooms maintained at temperatures set to 16°C and 26°C prior to experiments. The species chosen was *Emiliania huxleyi* (CCMP 373) obtained from the Provasoli-Guillard Center for the Cultivation of Marine Phytoplankton (CCMP, West Boothbay Harbour, Maine, USA) originating in Sargasso Sea and isolated in 1960. Media used consisted of sterile artificial seawater (SASW) supplemented with f/2 nutrients and vitamins (Guillard & Ryther, 1962). A volume of 400 ml of cells/media mix with a starting cell density of 100,000 cells ml^{-1} was prepared in triplicates into these flasks. The control consisted only of the media. Light intensity was measured at 300±15 µmol photons m^{-2} s^{-1} supplied by cool-white fluorescent tubes (TL-D 840, Philips) on a 14:10 light:dark cycle. Cultures were maintained as described in 2.2.1, and samples were collected as described in 2.3.1.1.

3.2.2 Cell Growth

Cell growth was monitored daily throughout the experiment as described in 2.6.3

3.2.3 Trace Gas Analyses

A purge and trap method combined with gas chromatography was used to analyse dissolved VOCs in each samples (Franchini & Steinke, 2016; Lara-Gonzalo *et al.*, 2008). From each sample, 20 ml was injected using a 20 ml glass syringe through a glass fibre filter (Whatmann GF/F, 25 mm) into the purge tube. Nitrogen flow was adjusted to 60 ml min^{-1} and samples were left to purge for 25 minutes. Once the samples finished purging, the sample was flushed into the GC-FID. The GC oven was run to increase in temperature from 80 to 180°C over 36 minutes. Control values were subtracted from data values and then normalised by cell density to calculate the values per cell. Chromatograms were analysed upon collection by examining each detectable peak.

3.2.4 Isoprene and DMS calibrations

Due to technical issues, the GC was not calibrated during the period that the VOCs were measured; however, a calibration was run on the same machine using the same parameters later in the year to attempt to quantify the amount of isoprene and DMS found in samples.

DMS and isoprene calibrations were achieved by following steps described in 2.3.6 and 2.4.3.1.

3.2.5 Statistical Analysis

To make VOCs from chromatograms comparable, an R package called GCalignR was used. As a measure for quality control, following the statistical alignment, each peak was compared qualitatively with raw chromatograms to ensure that the values were aligned correctly. Data was then organised by removing control values from actual values and then normalised to cell density.

For all analyses, a series of statistical tests were used to check the data for normal distribution prior to comparison. A Levene's test for equality of variances was carried out to check for homogeneity in data followed by a Shapiro-Wilk test to confirm whether data was normally distributed.

A one-tailed t-test was used to check for statistical differences between the two temperature treatments on cell density, growth rates, and cell doubling time.

Comparison of DMS concentrations between phases and temperatures was undertaken using a Kruskal-Wallis test followed by a Dunnett's test, while the comparison of isoprene concentrations between phases and temperatures were undertaken using an analysis of variances (ANOVA) followed by a Tukey test.

Other VOCs were compared in normalised area sizes of numbered peaks using the same methods as those used to compare DMS concentration. VOCs were also visually explored by generating a heat map for a generalised overview.

As a significance threshold for all analytical tests, a p value of p = 0.05 was used as a probability value, and all were performed using R studio (Version: 1.1.463).

3.3 Results

3.3.1 Culture Growth

Cultures grown at 16°C showed a rapid increase in number and a steeper exponential slope while cultures in 26°C showed a much slower increase in number with a shallower exponential slope that decreased soon after in samples taken after the 2nd and 3rd dilutions (Figure. 3.2A). Both treatments were seen to decrease in overall growth of cell numbers over time, but the cultures grown in 16°C had on average a higher number of cells ($2.5 \times 10^5 \pm 0.10$ cells/ml) compared to cultures grown in 26°C ($1.7 \times 10^5 \pm 0.17$ cells/ml), $t(180) = 3.69$, $p = 0.000149$ (Figure. 3.2A). Cultures grown in 16°C overall, statistically grew twice as fast (0.194±0.13) as cultures grown in 26°C (0.095±0.17), $t(166) = 2.99$, $p = 0.0016$. Both temperature treatments began with a higher growth rate where cultures grown in 16°C begun at a rate of 0.53 d^{-1} and those grown in 26°C begun at a rate of 0.36 d^{-1} but both gradually decreased over time (Figure. 3.2).

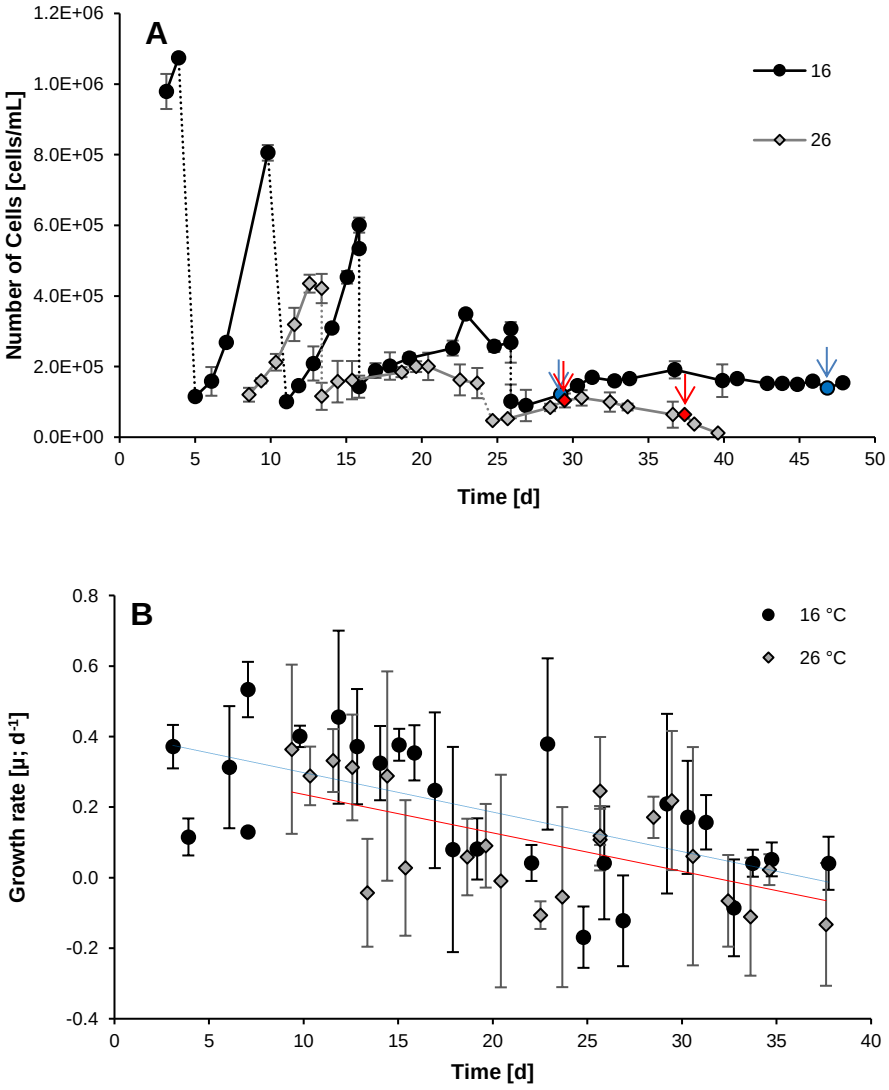
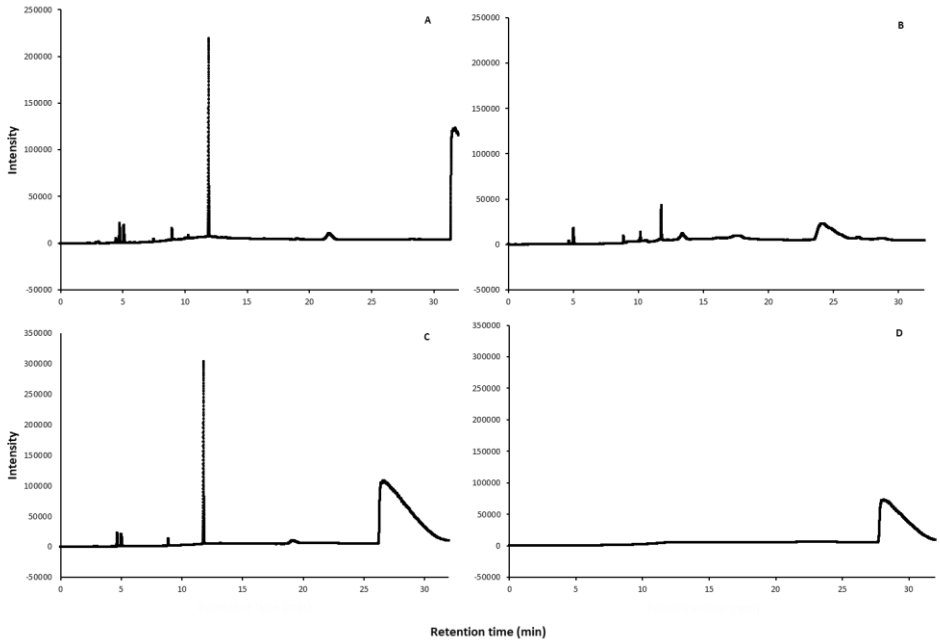


Figure. 3.2: A) Cell densities (±SE) of *Emiliania huxleyi* cultures grown under temperatures 16 and 26°C (n = 4) over time. Cultures were diluted during stationary phase (shown by dotted lines). Arrows show when gas samples were taken. Blue = 16°C, while red = 26°C. B) Calculated specific growth rates (±SE) over time. Lines show linear regressions that demonstrate a decrease in growth rate over 40 days. Blue = 16°C and red = 26°C.

3.3.2 VOC Analysis

As many of the compounds were unidentified, areas were used to compare the “amount” of these between the two temperature treatments. Peaks were normalised by subtracting control values from sample values. Example chromatograms can be seen in Figure. 3.2.



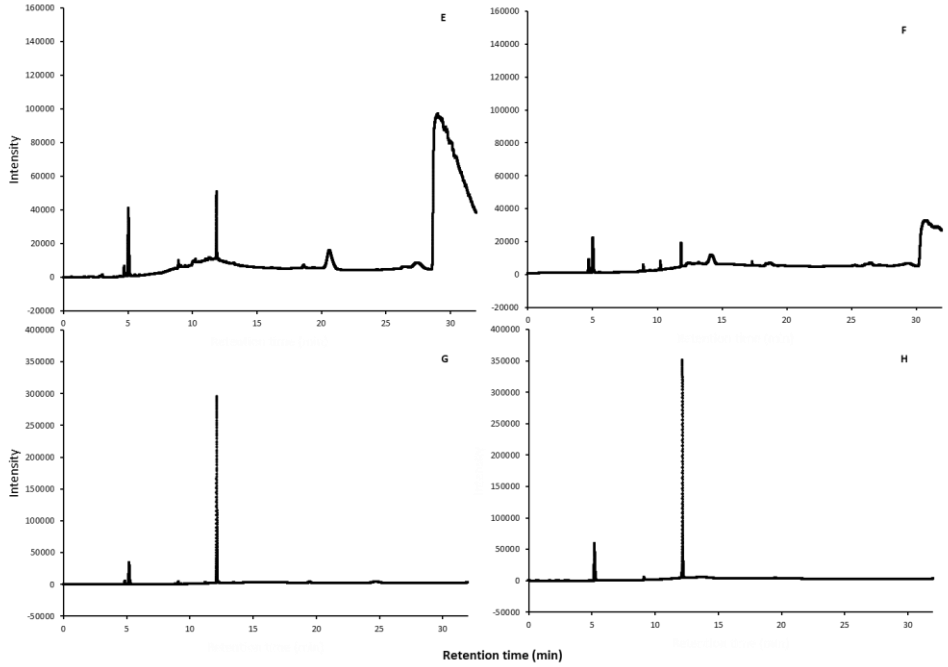


Figure. 3.3: Example traces of raw data obtained from A) cultures grown in 16°C during exponential phase; B) controls for A; C) cultures grown in 16°C during lag phase; D) controls for D; E) cultures grown in 26°C during exponential phase; F) controls for E; G) cultures grown in 26°C during stationary phase; H) controls for H.

To explore the effect of temperature on VOC composition, a PCA was performed. Clear separation between clusters were seen in the PCA, with VOC profiles of cultures grown in 26°C during stationary phase (26S) displaying the largest variation of any group (Figure. 3.3). Whilst cultures kept at 16°C during stationary phase (16S) had the most distinct VOC profiles, forming a cluster far from the other treatments, cultures kept at 16°C during exponential phase (16E) and cultures kept in 26°C during exponential phase (26E) both demonstrated clear groups. Similarly, the VOC profiles of 16S and 26S also

formed distinct groups although with less separation (although with great variability between replicates); 26S was indistinguishable from 16E due to high amounts of variation in the data (Figure. 3.3). Both 26°C and 16°C showed a shift along the horizontal access when comparing exponential phase with stationary phase VOCs, where a plethora of VOCs were different (Figure. 3.3). However, the 16°C exponential and stationary clusters showed a much clearer shift than the 26°C exponential and stationary groups (Figure. 3.3). Overall, it could be deduced that growth stages and temperature, both had an influence on the VOC profiles.

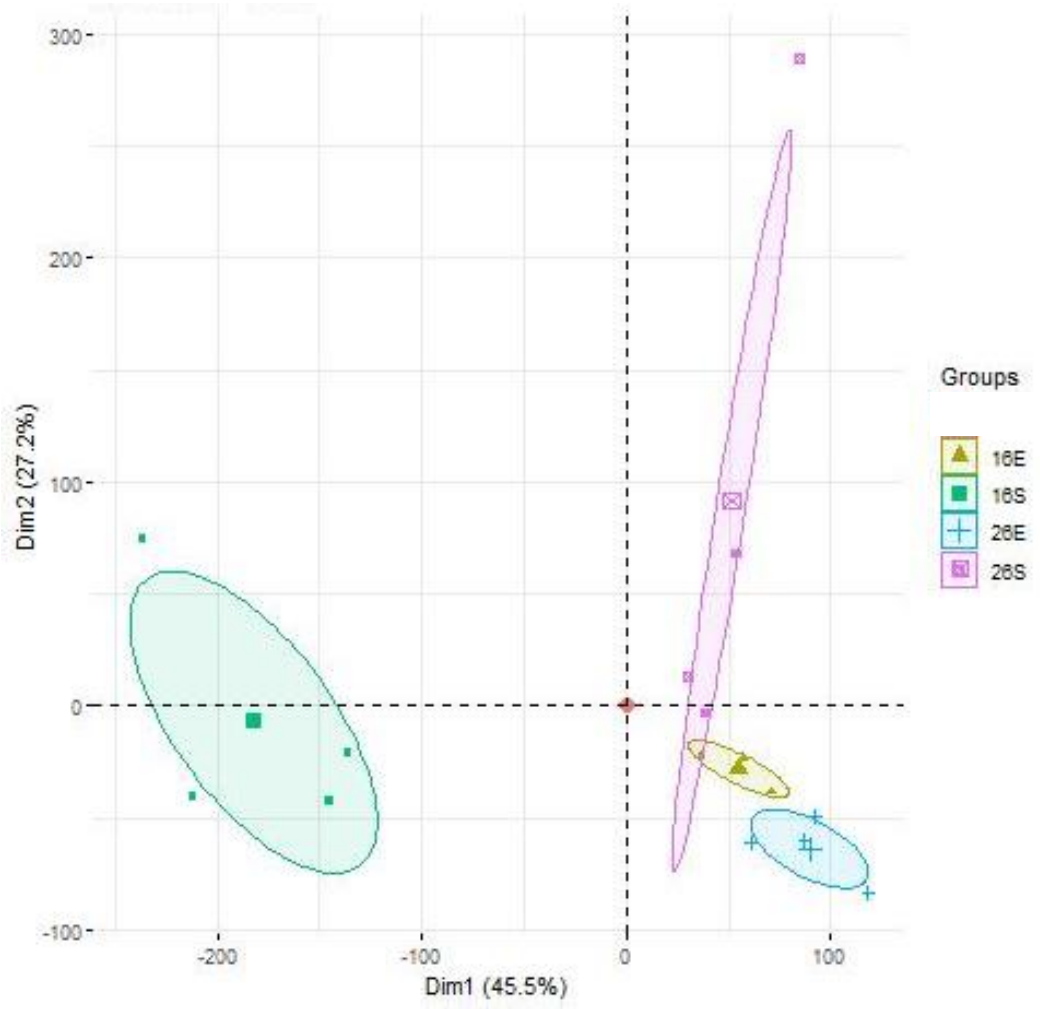


Figure. 3.4: Principal components analysis of VOCs for both exponential (E) and stationary (S) growth stages grown at 16°C and 26°C, respectively. Ellipses represent 95% CI, n = 4, group means are displayed

Production and consumption were both observed amongst VOCs present in the cultures. The number of VOCs released were highest during exponential phase in cultures grown under 26°C (54 compounds), while the other three treatments remained similar in numbers with there being 36 and 37 compounds in cultures grown at 16°C during exponential and stationary phases, and 36 during stationary phases for those grown in 26°C. Consumption, or alternatively degradation of VOCs was seen most within the

cultures grown in 16°C during stationary phase (39). While lowest in those grown under 26°C also during stationary phase (5) (Table. 3.1).

Statistically comparable peaks (compounds) were peaks 9, 13, 21, and 26 ($P = 2.2x10^{-16}$) (Table. 3.1). All were differences between phases and not between temperature treatments. Peaks 9, 21, and 26 were significantly different between the two phases of cultures in 16°C, and showed a higher peak area of compounds; 9, 21, and 26 during exponential phases with clear consumption present during stationary phases ($p = 0.0004$; $p = 0.0372$; $p = 0.0073$) (Table. 3.1). Compound 13 was the only one to show statistical differences between the phases of 26°C cultures but similar to the trend in 16°C cultures, concentrations were higher during the exponential phase, while consumption occurred during stationary phase (Table. 3.1).

Table. 3.1: Control normalised areas (n = 4) of 4 noteworthy (statistically different) compounds detected by the GC-FID. Numbers represent temperature (16 = 16°C, 26 = 26°C), and letters represent phases of growth (E = exponential, and S = stationary). Positive values represent production, while negative numbers represent consumption/degradation.

	Average area			
Compounds	16 E	16 S	26 E	26 S
9	7478.43	-14705.73	7405.50	2926.50
13	-13517.70	3089.00	78409.67	-191006.20
21	295.43	-465.08	103.23	0.00
26	5895.47	-10792.00	2067.48	0.00

Production and consumption were both observed amongst VOCs present in the cultures. The number of VOCs released were highest during exponential phase in cultures grown under 26°C (54 compounds), while the other three treatments remained similar in numbers with there being 37 and 40 compounds in cultures grown at 16 ºC during exponential and stationary phases, and 47 during stationary phases for those grown in 26°C (Table. 2.2). Consumption, or alternatively degradation of VOCs was seen most within the cultures grown in 16°C during stationary phase (40 compounds). While lowest in those grown under 26°C also during stationary phase (5 compounds) (Table. 2.2).

Table. 3.2: Number of compounds found in each culture type detected by the GC-FID. Numbers represent temperature (16 = 16°C, 26 = 26°C), and letters represent phases of growth (E = exponential, and S = stationary).

	Number of compounds	
Type	**Produced**	**Consumed/degraded**
16E	37	8
16S	40	40
26E	54	12
26S	47	5

3.3.3 DMS and isoprene concentrations

Observations on the quantities of DMS showed that there were contrasting amounts of DMS released in the two phases of cultures grown in 26°C where they were highest during stationary phase, and lowest during exponential phase ($p = 0.016$) (Figure. 3.5). Not as much difference could be seen in cultures grown under 16°C, but slightly more

DMS was released during exponential phase than during stationary phase ($p>0.05$) (Figure. 3.5). Samples in 26°C released less DMS during exponential phase ($p = 0.038$) but more during stationary phases ($p > 0.05$) (Figure. 3.5).

Results on isoprene quantities showed that both cultures grown in 16 and 26°C did not show a clear difference in concentrations between the two phases of growth ($p>0.05$) (Figure. 3.5). However, cultures grown in 16°C had a slightly higher concentration of isoprene when compared to 26°C during both growth phases ($p>0.05$) (Figure. 3.5).

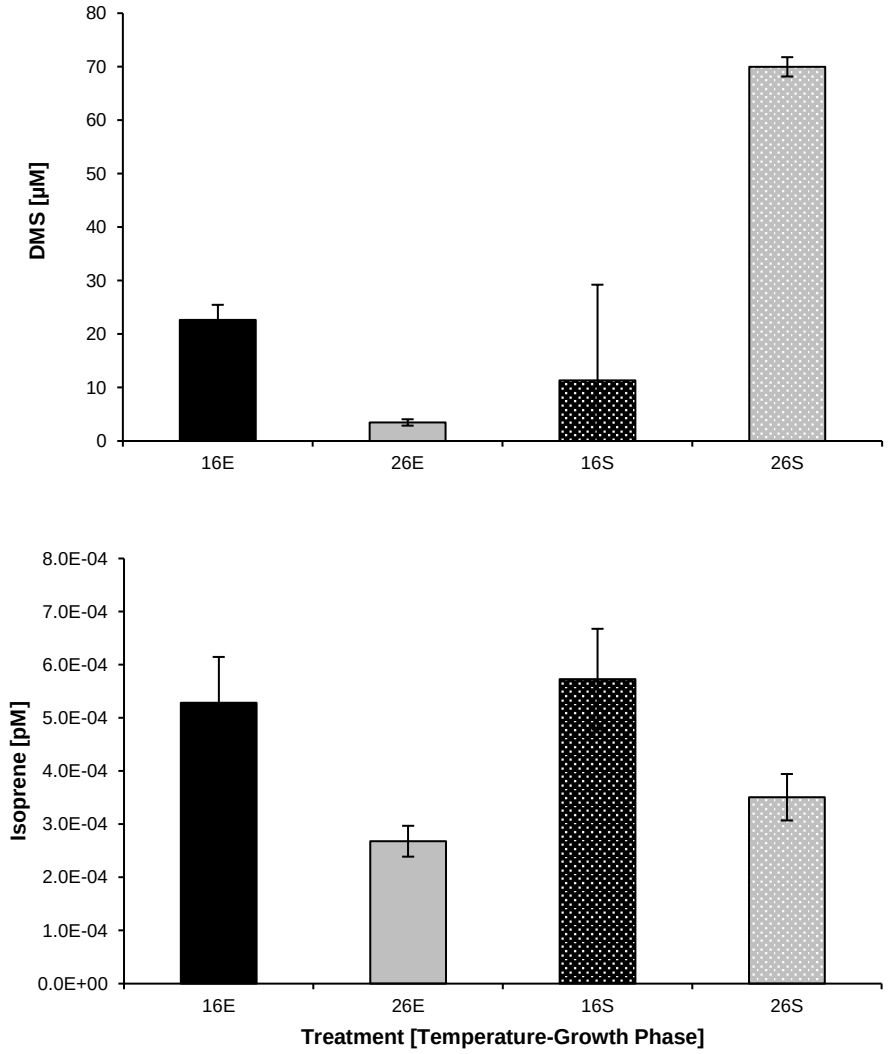


Figure. 3.5: Concentration of DMS and isoprene (±SE) during exponential and stationary phases of cultures grown in 16 and 26°C (n = 4). Black represents those grown in 16 °C, while grey represents those grown in 26 °C. Solid colours = measurements taken during exponential phase, patterned fill = taken during stationary phase.

3.4 Discussion and Conclusion

3.4.1 Culture Growth

E. huxleyi is known as a cosmopolitan species and is distributed widely across the world. Investigations have taken place on specific growth rates (μ) of each strain which overall have been seen to grow successfully between 0 and 30°C; strain 373 was noted to grow best between 10 and 20°C where in the 16°C range the growth rates would be increasing, while in the 26°C range the growth rates would typically be on the decline (Conte *et al.*, 1998; Rosas-Navarro *et al.*, 2016; Van Rijssel & Gieskes, 2002) (Figure. 3.1). This agrees with the higher growth rates of individuals grown in 16°C when compared with 26°C (Figure. 3.2) and could suggest that the 20°C region would be considered the optimum temperature for *E. huxleyi* 373 to grow in.

Specific growth rates began to plateau quickly from the 3rd generation of cultures and onwards (Figure. 3.2). Initially the conclusions made were that it was due to 26°C being a temperature range that strain 373 may not grow very well in, but this pattern could then also be seen in cultures grown in 16°C (Figure. 3.2). Another potential explanation was that overall coccolith coverage of cells may have changed during the experiment, as this is thought to influence growth rates of cultures (Buitenhuis *et al*, 2008). However, it is difficult for this to support any explanation on growth rates as two studies showed contrasting results whereby naked cells were 15% slower in growth rates in one study (Paasche, 1999), while 15% faster in growth rates in another (Lecourt *et al.*, 1992). It was later explained that genetic variation is present among *E. huxleyi* isolates which may

have resulted in such contradictory results. This lead to the final possibility of nutrient depletion which was the most likely. When following the instructions to create ASW with supplementary f/2 nutrients (Guillard & Ryther, 1962), selenium was not added due to it being a requirement specific to only 16 species known thus far, and was left out unnoticed (Harrison *et al.*, 1988). If this experiment were to be repeated, media used must include selenium as a supplement nutrient to allow the individuals to grow better.

We hypothesised that growth rates will be higher in cultures grown in 16°C due to previous studies showing an increase in growth of a variety of *E. huxleyi* strains between temperatures of (5 to 30°C). As expected, growth rates in *E. huxleyi* 373 were higher in cultures grown under 16°C than those grown under 26°C (Figure. 3.2). The possible reasons for this have been explained above. Many organisms may grow faster under higher temperatures, but to a certain extent after which they begin to decline in growth rates often from denaturing proteins. For *E. huxleyi* 373, 26°C was a little higher than that threshold. To understand how much temperature influences growth rates, it may be interesting to further study cultures of different species and inspect how they may differ. It is likely that VOCs will also be influenced by temperature alongside growth rates.

3.4.2 VOCs released

Alongside phytoplankton, it is known for bacteria associated with such phytoplankton to also produce and consume VOCs, much like *Azospirillum brasilense* and *Bacillus pumilus* that enhance the performance of *Chlorella sorokiniana* (Amavizca *et al.*, 2017).

It was hypothesised that the number of VOCs would be higher in cultures grown under a higher temperature treatment of 26°C. Results shown agreed with this statement as more VOCs could be seen in cultures grown under 26°C regardless of growth phase (Table. 3.2). This was likely due to the fact that those grown in 26°C were more exposed to heat stress than those grown in 16ºC and therefore possibly promoted the release of VOCs whether it be as a stress mechanism or due to increased likelihood of cell death. This could also be seen in the growth rates whereby throughout the experiment those grown optimally in 16°C had a higher cell density as well as specific growth rate than those grown in 26°C (Figure. 3.2). This finding relates to the CLAW hypothesis whereby DMS concentrations were predicted to rise with temperature (albeit rebutted by the anti-CLAW hypothesis), as the same can be said on the diversity of VOCs that may be produced, which in this study has been shown to also rise with temperature. If compounds highlighted could be identified, this information could be then utilised to see if they may influence detrimental or beneficial atmospheric processes. From such information, better prediction on changes in climate could be made.

VOC concentrations were normalised by removing control area values from sample values and then dividing by cell volume. When control values were higher than sample values, the presumption was made that there was biological consumption of compounds occurring, whereby VOCs originally present in the media were consumed by some other organism, possibly bacteria associated with the phytoplankton species being studied. In the results, it was highlighted that consumption was higher in cultures grown under 16°C than in those grown under 26°C (Figure. 3.5). It was concluded that this was likely due

to there being more cells, and therefore more bacteria which could be consuming the VOCs in question. Although there have been a number of studies that explore consumption of VOCs in soil microbial systems (Cleveland & Yavitt, 1998; Ramirez *et al.*, 2010), less is understood on consumption by marine microbial communities. It is essential to consider not only gas exchange between the ocean and atmosphere, but also the utilisation of certain VOCs. Whilst atmospheric VOCs and their influence on atmospheric processes are important concepts to understand, it is essential to comprehend the fluctuation of VOCs involved as it will affect such processes just as much by increasing or decreasing the frequency and rates at which they may occur.

3.4.3 DMS and Isoprene Concentrations

As expected, DMS concentrations were seen to be highest in cultures grown under 26°C during stationary phase. This is likely due to the conditions being the most stressful for this strain as seen in Figure. 3.1. It was expected that DMS concentrations will also be higher from those grown in 26°C during exponential phase; however, this was not the case (Figure. 3.5). Possible explanations are that cells did not invest energy in DMSP production as they focused on acclimation and growth as was seen in studies by (Paasche, 1999).

In contrast to predictions, isoprene concentrations were higher in cultures grown at 16°C (Figure. 3.5). This could be due to 26 °C being higher than the maximum isoprene production threshold. Isoprene does increase in production with temperature (Shaw *et al*, 2003; Singsaas *et al*, 1997), but only up to a certain point after which it declines

(Kuzma *et al.*, 1995). Another possibility is that there were isoprene degrading communities of bacteria present on the algal cells (Alvarez *et al.*, 2009). Cultures were not axenic and therefore it is possible for cultures to have been contaminated. If this is the case, those grown in 26°C provide more of an optimal environment for such communities and therefore have a likelihood of having higher rates of isoprene degradation occurring than those grown in 16°C. To eliminate this possibility, the experiment would need to be repeated with axenically grown algal cultures.

3.4.4 Possible Improvements

There are a few points that could be improved to ensure scientific accuracy and precision within this study. Additional information on cell size and genetic alterations that occur may explain why growth rates decreased over time as well as explain the molecular background that are likely to influence VOC concentrations.

It has been noted that in the future, f/2 media made for *E. huxleyi* must always include the addition of selenium to ensure maintenance of health.

Further investigations to the bacterial level using sequencing techniques would bring light to how bacteria that grow in association with microalgae may have an impact on VOC profiles, particularly those known to consume identified VOCs.

Finally, a GC coupled with a mass spectrometer would allow for potential identification of compounds which would help generate identified volatile profiles. Even if standards could be used to narrow down some of the compounds it would be a huge addition to this study.

3.4.5 Conclusion

In conclusion, the results agreed with predictions 1 and 2 stating that; 1) Growth rates would be lower in 26°C, and 2) The number of compounds would be higher in cultures grown in 26 than 16°C; partial agreement was seen in 3. That isoprene and DMS production be higher in cultures of 26°C than 16°C, as DMS was seen to be higher, but isoprene on the other hand was lower and therefore the null hypothesis was agreed upon instead. VOCs were also much higher in cultures of 26°C than 16°C, which leads to the belief that it is indeed stress that results in increased VOC concentrations possibly due to higher metabolic activities.

This study was a good start in the journey to developing a method using volatolomics for environmental diagnosis. It was an addition to the data pool for lab-based use of VOCs as indicators for temperature dependent culture growth. The high presence of the certain unidentified compounds potentially suggests lack of health in maintained cultures, and lack of signals a healthier batch, or bacterial infection. The next chapter will move on from the influence of abiotic factors to characterising any species-specific trends in sulphur VOCs.

Chapter 4: Characterising sulfur compounds produced in four species of phytoplankton

4.1 Introduction

4.1.1 Sulfur compounds in phytoplankton

Sulfur is an important element to all phytoplankton due to its role as a vital building block for proteins, coenzymes, lipids, polysaccharides, iron-sulfur clusters, secondary metabolites, and other biological molecules. A wide diversity of sulfur compounds are found in aquatic systems and can be in both particulate as well as dissolved forms. Some of these compounds are produced by phytoplankton such as dimethyl sulfoniopropionate, methane thiol, hydrogen sulfide, dimethyl sulfide and polysulfides. In this study, the production of these compounds will be quantified and analysed for better understanding of the sulfur volatolome (Watson & Jüttner, 2017).

4.1.2 Methane thiol

Methane thiol is often found in anoxic environments and is thought to be produced by bacteria. The marine bacterium *Pelagibacter* has been found to catabolise DMSP into both DMS and methanethiol by either cleavage or demethylation, although demethylation was suggested to be the main, preferred pathway (Sun *et al.*, 2016). The pathway DMSP takes is determined in response to sulfur demand in bacteria. According to a study by Varaljay *et al.* (2015), methanethiol production was seen to increase during dinoflagellate

blooms while DMS production was higher in presence of mixed diatom and dinoflagellate communities (Varaljay *et al.*, 2015). This agreed with estimations that bacteria determined which pathway depending on how abundant DMSP is. In dinoflagellate blooms where DMSP concentrations are high, it is more energy cost effective for the bacteria to use the demethylation pathway to utilise the DMSP, contrasting to the duration of diatom and dinoflagellate blooms whereby DMSP concentrations are lower, and therefore investing in the use of DMSP as a sulfur source is worthwhile (Varaljay *et al.*, 2015).

4.1.3 Dimethyl sulfide and Dimethylpolysulfides

Dimethyl sulfide is commonly released by algae during catalytic breakdown of DMSP (Stefels & Dijkhuizen, 1996). There are numerous explanations as to why DMSP is cleaved to DMS. Some sources say that DMSP plays a role as a long term osmolyte (Kirst, 1996). Only some clades of phytoplankton produce DMSP such as Prymnesiophytes and Dinophytes. Bacillariophytes and Chlorophytes are also known producers but not to the extent of the aforementioned two (Keller, 1989a). Another argument is that DMS is not only produced as a result of DMSP cleavage but also as a consequence of cell lysis that may occur due to grazing or viral outbreaks (Wolfe *et al.*, 1994). These variables also need to be taken into consideration if aims for the use volatilomics as a method for ecological analysis is to be achieved.

Dimethyldisulfides (DMDS) have been found in dense cyanobacterial cultures such as *Anabaena* spp. in eutrophic ponds after remnants of *Spirogyra* spp. and *Oedogonium*

spp. mats degrade (Jüttner, 1984). DMDS was also found in some dinophytes such as *Peridinium gatunense*, a freshwater species (Ginzburg *et al.*, 1998). Although less studied, some bacillariophytes and cryptophytes have also been documented to produce DMDS albeit in trace amounts (Ginzburg *et al*., 1998).

4.1.4 Hydrogen sulfide

Larger sulfur compounds can break up into smaller ones, one of these is hydrogen sulfide (H_2S), which is produced by some phytoplankton such as *Synechoccocus* spp. (Walsh *et al.*, 1994). Studies have showed that H_2S production in *Synechoccocus* spp. increased alongside concentrations of uncomplexed trace metals in the media; from this it was deduced that the H_2S was primarily produced as a result of metal detoxification mechanisms (Walsh *et al.*, 1994).

4.1.5 Miscellaneous compounds

Many other sulfur compounds are likely to be released by species of plankton and their associated bacteria. Their identities may not be clear yet, but with time, more research will be conducted on the search and identification of compounds produced by marine organisms that will be added to the overall literature.

4.1.6 Characterising other compounds

Investigations have begun on other sulfur compounds. These include examples mentioned above as well as more obscure compounds like 3-methylthio-propanal, benzothiazole, 3-methyl-6-methylsulfanyl-hexa-1 and 5-dien-3-ol (Watson & Jüttner, 2017). What their roles are or why they are produced have yet to be explored, but even characterising VOC 'fingerprints' will allow us to move one step closer to developing a non-invasive method to monitor ecological health.

4.1.7 DMS production

DMS is an organosulfur compound commonly released by algae (Bentley & Chasteen, 2004). In particular, phytoplankton are major contributors of DMS emissions and contribute 19.6 Tg S 21^{-1} to the atmosphere which accounts for 90% of biogenic sulfur emissions from the oceans (Land *et al.*, 2014). DMS production is an important process to consider due to the impact it has on the atmosphere. Once reaching the atmosphere, it has the capability to promote the formation of cloud condensation nuclei (CCN) and increase albedo (Vallina & Simó, 2007). This knowledge once lead to beliefs that DMS would positively influence its environment by having a cooling effect on the earth (Charlson *et al.*, 1987), although later hypothetically disproved by Lovelock. DMS is also responsible in determining the lifetimes and oxidation pathways of many other atmospheric VOCs such as ammonia, isoprene, methane and ozone (Chen & Jang, 2012). It is also involved in the formation of sulfur and methanesulfonic acids (Barnes *et al.*, 2006) which in turn, further promotes CCN formation.

4.1.8 Previous studies on DMS production

In the past, DMSP and DMS concentrations from a variety of classes have been monitored and used to estimate DMS production (Keller, 1989a) but direct production of the secondary compound is yet to be measured. Such information gaps must be filled as it is understood that DMS is not only produced due to DMSP cleavage, but also as a result of cell lysis from grazing behaviour or viral outbreaks. Methods previously used, only takes DMSP cleavage into consideration which could mean that values may not be entirely accurate. This study also aims to further investigate this to confirm the validity of calculated values, and to understand the degree of impact cell lysis may have on production rates.

4.1.9 Contributing information on fundamental sulfur profiles

Aquatic ecosystems as a whole are extremely complex and are influenced by many variables. In order to understand the VOC profiles of such systems, it is important to start with an understanding of fundamental trophic levels such as individual species of primary producers. Findings from this will then allow for further development of knowledge on the effects that ecological interactions and physical variables may have on the biology and therefore VOC profile of each individual within a population, a community, and eventually even of an ecosystem.

4.1.10 Hypotheses and aims

The aim of this study was to start characterising species specific VOCs using *Thalassiosira pseudonana*, *Rhodomonas lacustris*, and *Emiliania huxleyi* as model organisms. These species were chosen due to their preferred environmental conditions being similar as well as their variety in estimated DMS production. Primary aims were to advance current knowledge on species specific sulfur production, but DMS production rates were also measured and quantified for the aforementioned reasons. VOCs were sampled in exponential as well as stationary phases of growth in all species that were estimated from growth curves plotted prior to experiments. Information gained from this chapter will add to the current knowledge on sulfur production; it will lead to the development of a broader and better understanding of VOC profiles produced by phytoplankton. Furthermore, there are very few studies that investigate the changes that occur in VOC profiles among unialgal populations when exposed to other planktonic species. This will be investigated in chapter 5 with the aid from data collected in this study.

By following these aims and conducting this experiment, the following hypotheses were addressed:

1. Growth rates will vary between species
2. Sulfur VOCs will be unique in type and quantity for each species
3. DMS production rates and DMSP concentration will differ for each species with an expected order from highest production downwards to be *E. huxleyi*, *T.pseudonana, D. tertiolecta,* then *R. lacustris.*

Hypotheses were based upon known DMSP and DMS released by these species in a study conducted by Keller *et al.* (1989) as well as on sulfur compounds released that were characterised by Watson *et al*. (2017).

4.2 Experimental Setup, Material and Methodology

4.2.1 General Experimental Setup

Cultures of *E. huxleyi, R. lacustris,* and *T. pseudonana* were obtained from the Provasoli-Guillard Center for the cultivation of Marine Phytoplankton (NCMA, West Boothbay Harbour, Maine, USA) and grown in growth rooms set and maintained at 16°C prior to experiments.

Cultures were mixed with autoclaved artificial seawater (ASW) supplemented with f/2 nutrients and vitamins (Guillard & Ryther, 1962) to ensure that the resulting volume would be 400 ml with a starting density of 2×10^5 cells mL^{-1}. These were grown in unialgal batch cultures in biological triplicates. The control consisted of media only. The bottle lids were modified with gas tight gas supply and waste tubes (1/8 inch, approximately 3.2 mm OD Teflon tubes). Light was delivered by white fluorescent light tubes (TL-D 36W 840, Phillips) which yielded a light intensity of 100±15.2 µmol photons m^{-2} s^{-1} on a 14:10 light:dark cycle. Cultures within each bottle was supplied with compressed air bubbling at a flow of 60 ml min. Cell growth was monitored daily by haemocytometry to estimate when exponential and stationary phases were reached. 50 ml was extracted from all

bottles to be used for DMSP and chl *a* extractions. VOCs were also collected using tedlar bags that could be attached to each bottle.

4.2.2 Cell Growth

Cell growth was monitored during the experiment. Cells were counted daily in replications of 10 using a Neubauer haemocytometer under a light microscope, the data of which was used to calculate specific growth rates using the equation:

$$u = \ln (x_1 – x_0) / t_1 – t_0$$

Where x_1 and x_0 refer to cell densities at t_1 and t_0 respectively.

4.2.3 Chl *a* Analysis

15 ml of each culture was filtered through a glass fibre filter (Whatman GF/F, 25 mm) using a 20 ml syringe. With forceps, the filter was carefully folded and put into 15 ml centrifuge tubes that were then stored at -20°C. These were quantified spectrophotometrically after extraction in 100% neutralised methanol following methods described in Riemann *et al.* (1982) and established equations from Ritchie (2008) (Riemann & Ernst, 1982; Ritchie, 2008).

4.2.4 DMSP analysis

Ten ml of the cultures from each bottle was filtered through a glass fibre filter (Whatman GF/F, 25 mm) using a 20 ml syringe. With flat forceps, the filter was carefully folded and put into 4.92 ml vials filled with 3 ml of 0.5 M NaOH. Vials were immediately closed with gas-tight silicone/Teflon septa and incubated at 30°C for a minimum of 24 hours for complete equimolar alkaline hydrolysis of DMSP to DMS. After incubation, the same methods as the DMS analysis was used to analyse the DMSP samples, however headspace purging was used instead of tedlar bag trapping and samples were purged for 3 minutes at 60 ml/min instead of 20 minutes. Calibrations were performed using known DMSP standards as described in Franchini and Steinke, 2016.

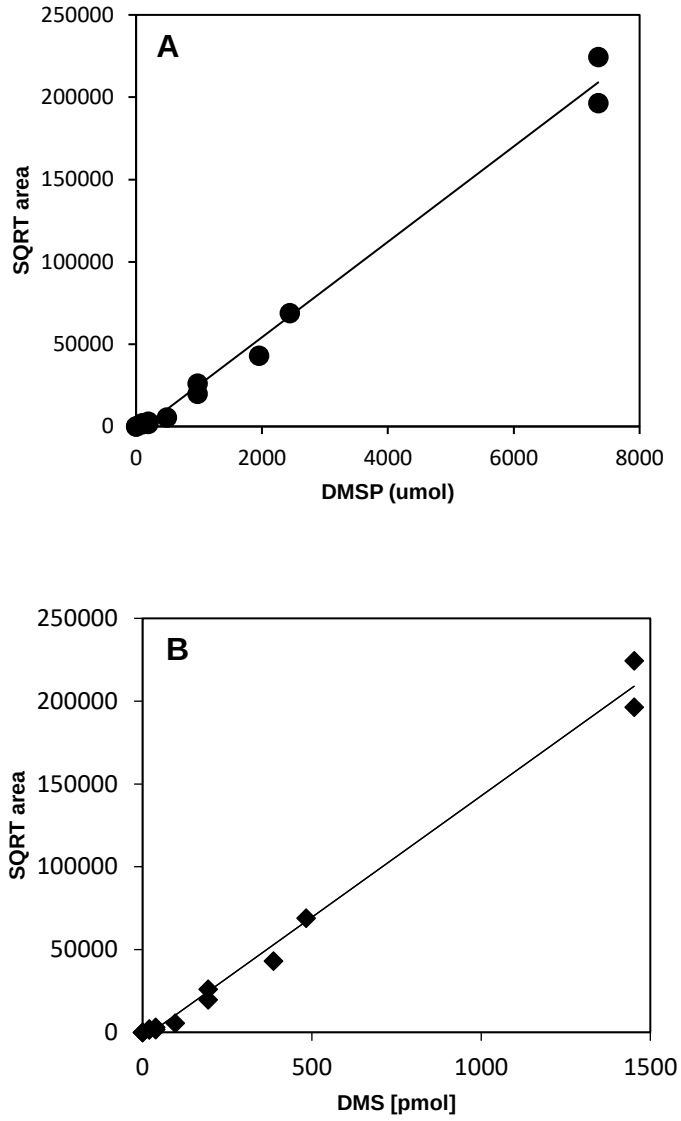


Figure. 4.1: A) Calibration by headspace flushing methods. Yielded a calibration equation of y = 147x - 3783 with an r-squared value of 0.99. The limit of detection was calculated to be 0.059 pmol. B) Calibration by headspace flushing methods. Yielded a calibration equation of y = 29x - 3783 with an r-squared value of 0.99. The limit of detection was calculated to be 0.059.

4.2.5 Trace Gas Analysis

A continuous flow of compressed air (British Oxygen Company) was bubbled through each culture bottle at a fixed rate of 60 ml/min for at least 2 days before start of gas sampling to ensure equilibrium was reached between aqueous medium and gaseous headspace. 3000 ml tedlar bags were flushed with nitrogen and vacuum sealed to ensure possible contaminants were removed. These were then attached to the outlet of the culture bottles and left for 20 minutes to collect 1200 ml of gas sample. Once samples were collected, Tedlar bags were attached one at a time to a purpose-built Teflon purge and trap system (Franchini and Steinke., 2016). Gases were concentrated in a cryotrap held at -160°C using a purpose-built liquid nitrogen heater for 20 minutes at a flow rate of 60 ml/min. The cryotrap was then heated to 90°C using freshly-boiled water. This process thermally desorbed collected gases before being flushed with a carrier of helium gas into a gas chromatograph with flame photometric detection (GC-FPD) for quantification of sulfur (GC-2014; Shimadzu). The injector was set at a temperature of 200°C, the detector at 250°C, and the oven temperature program (30 min total) was set to an initial temperature of 40°C for 7 minutes, and increased to 120°C at 5°C/min. Calibrations were performed using headspace purging of DMSP standards as described in Franchini and Steinke, 2016 (Franchini & Steinke, 2016).

4.2.6 Statistical Analysis

A Levene test for equality of variances was carried out to check homogeneity of data. A Shapiro-Wilk test then used to confirm whether data was normally distributed. Once it

was confirmed that data was parametric, an analysis of variance (ANOVA) was used to check for statistical differences in growth rates and DMS production between the three species followed by a Tukey post hoc test. t-tests were also used to check for statistical differences in DMSP concentrations between species. As a significance threshold, a p value of p = 0.05 was used as a probability value.

To analyse all sulfur compounds, firstly the chromatograms were aligned using an R package called GCalignR (Ottensmann *et al.*, 2018). Then using DMS as a standard, the areas were converted into estimated concentrations for compounds containing only one sulfur atom. All statistics were performed using R studio.

4.3 Results

4.3.1 Growth Rates

T. pseudonana had the highest growth rate during exponential phase (0.36 μ; d^{-1}), while *E. huxleyi* had the highest growth rate during late exponential phase nearing stationary phase (0.02 μ; d^{-1}) (Figure. 4.2).

Maximum growth rates (μ) showed little difference between the four species during exponential phase with an average of 0.38 d^{-1} ($p > 0.05$) (Figure. 4.2). However, the period required to reach maximum μ during stationary phase was significantly different between the species (*D.teriolecta*: 5 days, *E. huxleyi*: 7 days, *R. lacustris*: 5 days, *T.*

pseudonana: 3 days) (Figure. 4.2). Growth rates slowed down over time for all cultures, however, the third replicate of *E. huxleyi* showed no sign of reaching stationary phase during the experiment. Differences could also be seen in maximum cell densities [$F(2, 6) = 19.616$, $p = 0.002$] particularly between *R. lacustris* and *T. pseudonana* ($p = 0.002$), between *D. tertiolecta* and *E. huxleyi* ($p = 0.02$) as well as between; *R. lacustris* and *E. huxleyi* ($p = 0.03$). Maximum density was highest for *E. huxleyi* (6.1×10^6 cells^{-1} mL^{-1}) followed by *T. pseudonana* (2.2×10^6 cells^{-1} mL^{-1}), *R. lacustris* (8.0×10^5 cells^{-1} mL^{-1}), and then *D. tertiolecta* (6.2×10^5 cells^{-1} mL^{-1}) (Figure. 4.2). Alongside growth rates, no difference was seen in doubling time between the four species ($p>0.05$). All species had doubling times within the range of 1-4 ± 0.2 (cells day^{-1}) without any being notably higher or lower (Figure. 4.2).

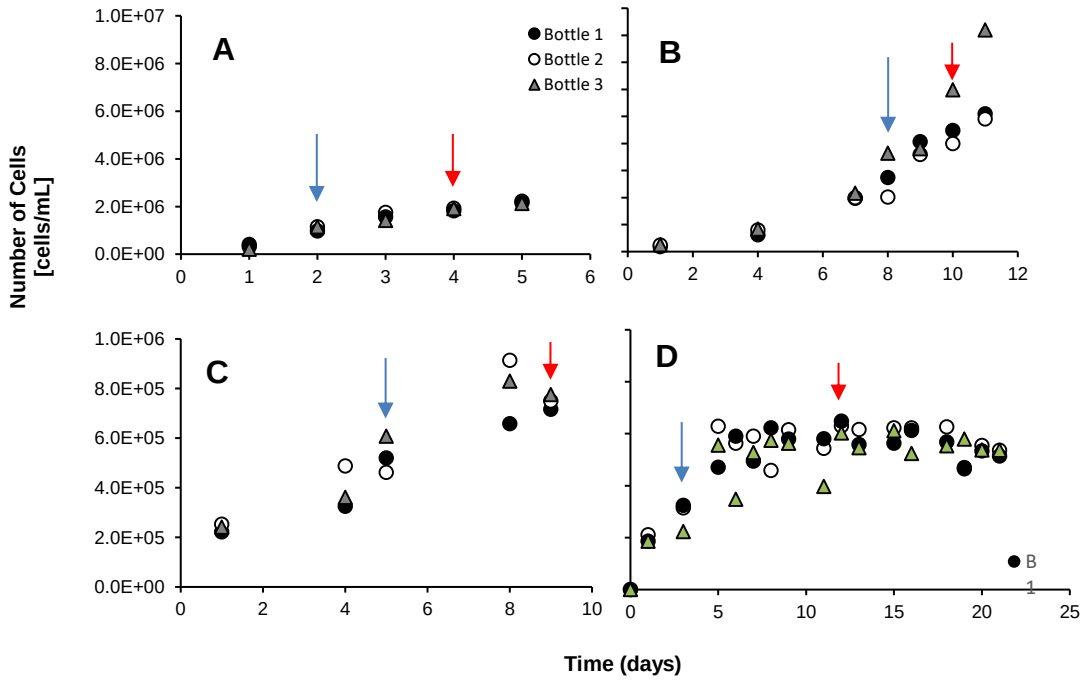


Figure. 4.2: Growth curve of cells plotted over time. Data points of n = 3 were plotted to visualise variance. Letters represent: A) *T. pseudonana*, B) *E. huxleyi*, C) *R. lacustris*, and D) *D. tertiolecta*. Blue arrows represent the time when the first

VOC sample was taken, and the red arrows represent the time when the second VOC sample was taken.

4.3.2 DMS production

Differences in DMS production were found between the four species [$F(2,6) = 18.48$, $p = 0.003$], specifically between *E. huxleyi* and *R. lacustris* ($p = 0.049$). During exponential phase, mean (±SE) production was highest in *E. huxleyi* ($1.20 \pm 0.227 \times 10^{-1}$ µmol [g Chl *a*]$^{-1}$ h^{-1}) followed by *R. lacustris* ($1.85 \pm 0.29 \times 10^{-3}$ µmol [g Chl *a*]$^{-1}$ h^{-1}), *D. tertiolecta* ($1.50 \pm 0.17 \times 10^{-3}$ µmol [g Chl *a*]$^{-1}$ h^{-1}), and then *T. pseudonana* ($1.22 \pm 0.63 \times 10^{-3}$ µmol [g Chl *a*]$^{-1}$ h^{-1}).

Differences between *D. tertiolecta*, *R. lacustris*, and *T. pseudonana* were negligible, but *E. huxleyi* showed to differ when compared to all three species ($F(2, 12) = 17.74$, $P< 0.001$) (Figure. 4.3). *E. huxleyi* produced more DMS than *D. tertiolecta*, ($P<0.001$) *R. lacustris* ($P<0.001$), and *T. pseudonana* ($P<0.001$). Similar results could be seen during stationary whereby *E. huxleyi* produced higher than all other species ($P<0.001$); however *D. tertiolecta* and *R. lacustris* produced no DMS, while *T. pseudonana* produced DMS albeit a small amount ($9.89 \pm 4.15 \times 10^{-4}$ µmol [g Chl *a*]$^{-1}$ h^{-1}) (Figure. 4.3).

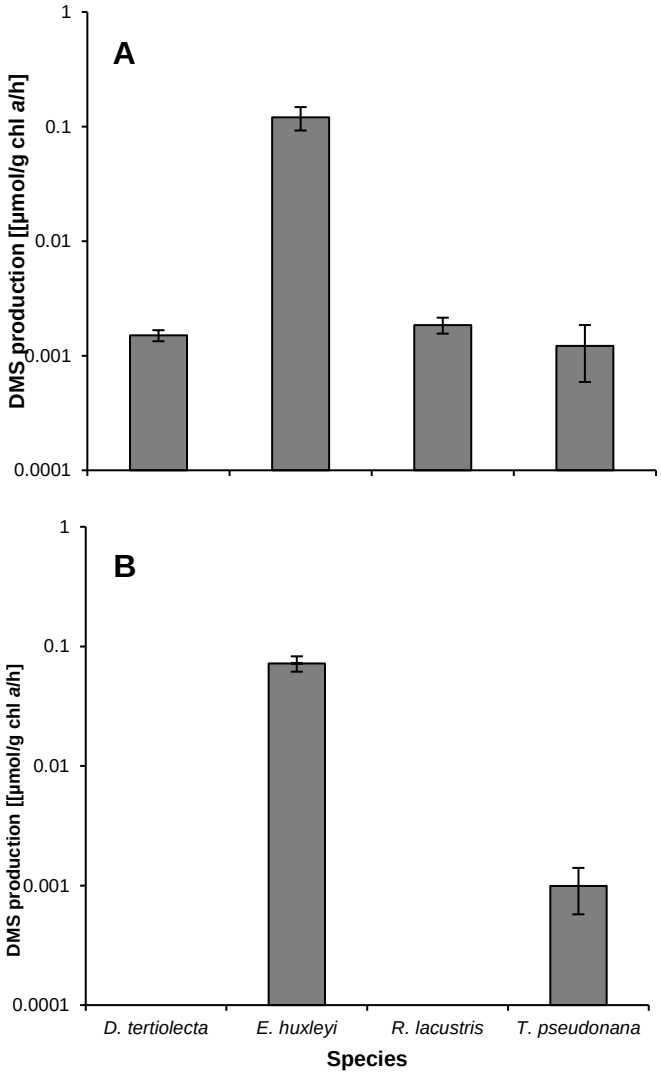


Figure. 4.3: DMS production rates of laboratory grown *D. tertiolecta*, *E. huxleyi*, *R. lacustris*, and *T. pseudonana*. Log scale data of mean DMS production (±SE) normalised to chl *a* is shown for each species taken during A) exponential and B) stationary phases (n = 3).

4.3.4 Trace Gas Analysis

A diversity in both compound type and production/consumption rates were observed in the sulfur VOC samples. There was a production of hydrogen sulfide (H_2S), methane thiol (MT), carbon disulphide (CS_2), and dimethyl disulphide (DMDS) alongside DMS as well as consumption of H_2S, MT, and CS_2 (Figure. 4.4). During exponential phase, *E. huxleyi* was shown to have the highest production rate of MT and DMDS, while CS_2 was produced most by *D. tertiolecta* (Figure. 4.4). H_2S was produced most by *R. lacustris*. There was consumption (or degradation) of H_2S and CS_2 by *E. huxleyi*, and MT by *T. pseudonana*. *D. tertiolecta* and *R. lacustris* did not show any sign of consumption/degradation (Figure. 4.4). Both production and consumption was species specific; *D. tertiolecta* did not produce any MT or DMDS, *R. lacustris* did not produce any MT or CS_2, *T. pseudonana* did not produce any DMDS and consumed MT, while *E. huxleyi* did not produce any H_2S or CS_2 but consumed it instead (Figure. 4.4).

A series of changes occurred when cultures reached stationary phase. *E. huxleyi* stopped producing MT, DMDS decreased in production, and consumption/degradation rates decreased for both H_2S and CS_2 (Figure. 4.4). *D. tertiolecta* continued to produce H_2S and CS_2 albeit at a slower rate and still showed no sign of consumption/degradation. *T. pseudonana* stopped producing H_2S and instead began to consume/degrade the compound. MT continued to be consumed/degraded, but in smaller amounts. *R. lacustris* stopped production completely (Figure. 4.4).

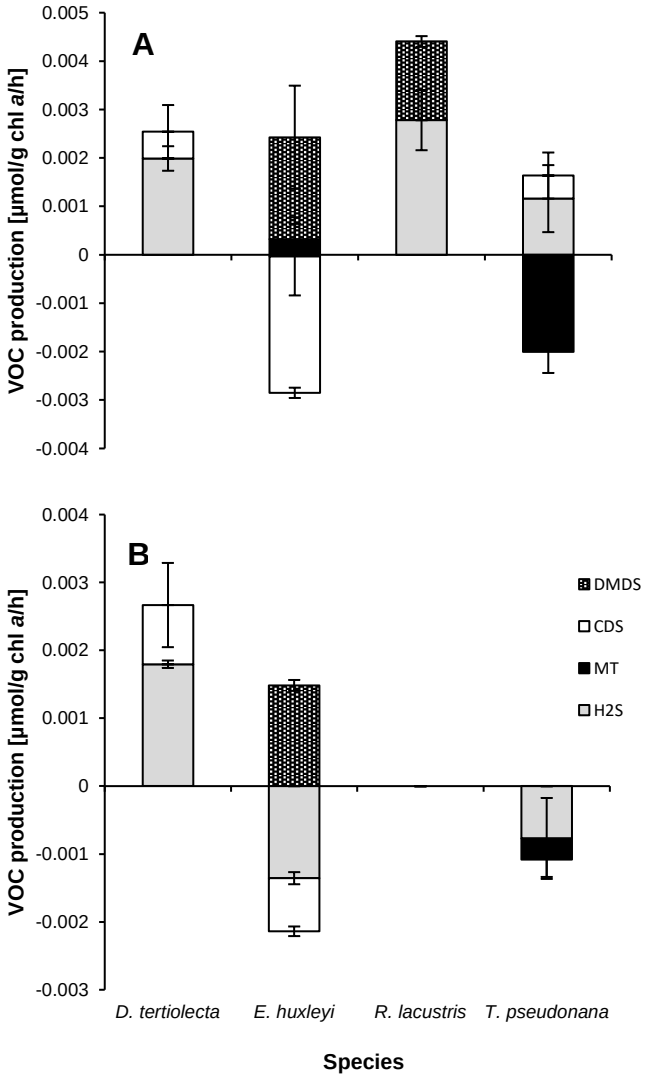


Figure. 4.4: VOC production (positive values) and consumption/degradation (negative values) normalised to chl *a* (±SE; n = 3) in unialgal cultures of four species during A) exponential, and B) stationary phases. CDS refers to CS_2 and H_2S refers to H_2S. Negative values were obtained by subtracting area values obtained from controls of media only, from area values obtained from samples of growing cultures.

4.4 Discussion

4.4.1 Growth rate

The maximum cell density varied between the three species of phytoplankton (Figure. 4.2). This is likely due to each species having their own species specific growth rates. Also, they all will not have the same biological processes, and their life strategies vary to ensure that they are competitively viable (Crossetti & Bicudo, 2008). Ecologically speaking, phytoplankton composition can be complex due to the unique advantages each group exhibits (Sommer, 1986). There are small, fast growing “undergrowth” species that can quickly increase in population but are edible to a wider range of herbivorous zooplankton, and there are larger, slow growing “canopy” species that, although may not increase rapidly in population, are not as easily ingested and therefore can avoid being culled by non-specialist herbivores (Sommer, 1986). Fast growing groups include cryptophytes and small centric diatoms, while slow growing groups consist of large dinoflagellates and large diatoms (Sommer, 1986).

Growth rates slowed over time and this is probably due to them reaching stationary phase from nutrient limitation in combination with increased waste (Figure. 4.2) (Schltiter *et al.*, 2018).

4.4.2 DMS production

Results agreed with hypothesis 3, and DMS production was highest in *E. huxleyi*, followed by *T. pseudonana*, *D. tertiolecta*, and *R. lacustris* (Figure. 4.3) This agrees to studies by Keller which suggests that DMS production from cell lysis and other means do not contribute to a significant extent when compared to production from DMSP cleavage alone (Keller, 1989b).

Contrary to the hypothesis, there was no difference for the production rates of DMS between *T. pseudonana* and *E. huxleyi* (Figure. 4.3). Although Figure. 4.3 visually shows a large difference in production between these two species, statistically this was not the case. This could be due to several reasons but one that may dominate the others is that there was a large variation in data for *E. huxleyi*. Another explanation was that because stationary phase was not quite reached, it could be argued that VOC samples taken for comparison were for early exponential and late exponential phases. Statistical differences were however seen between *E. huxleyi* and *R. lacustris*.

The positive relationship seen between DMS production and chl *a* concentration in *E. huxleyi* and *T. pseudonana* is likely because measurements for these two were taken just before stationary phase was reached, while for *R. lacustris* the measurements were taken just after. It may suggest that DMS production increases with population density up to a certain point. This is something that needs to be further investigated.

It is further confirmed that *E. huxleyi* contribute a lot of DMS to the atmosphere, while *D. tertiolecta* less so, with *T. pseudonana* and *R. lacustris* producing close to none (especially during stationary phase), the result of which also coincides with previous studies (Figure. 4.3) (Keller, 1989b). This does not however mean that the latter two are not worth studying from the standpoint of sulfur production, as data has shown that they do still release notable amounts of DMDS and H_2S, as well as CS_2 (Figure. 4.4). Considering the rate at which these species are able to grow, particularly *D. tertiolecta* and *R. lacistris* that produced a lot of other sulfur. Overall, they contribute greatly to global sulfur levels.

4.4.4 Other sulfur compounds

Data collected on other sulfur compounds supported the hypothesis that VOCs would differ in type and quantity. It is clear that *T. pseudonana* produced a narrower range of VOCs compared to *E. huxleyi,* for example (Figure. 4.4). There were also differences in quantity produced; however, this is only representative of a small group of VOCs (Figure. 4.4). If these were explored further using a flame ionisation detector, it would be possible that in fact *T. pseudonana* produced more volatiles overall. More sulfur compounds were produced during exponential phase than they were in stationary phase (Figure. 4.4), this could be due to there being more metabolic activity during exponential phase as they are healthy and doubling rapidly. This is something that may have an influence on VOC production.

4.4.5 Future work

There are several aspects of this experiment that would be interesting to further work on. In this study the species were all temperate, if the same experiment was conducted on polar and tropical species the DMS production may be very different as DMSP generation and cleavage may not be the same (Keller & Korjeff-Bellows, 1996). Polar species such as *Nitzschia lecointeii* have distinct adaptations to help them survive in their natural environment, one of them being a high DMSP content (Kirst, 1996). Due to this, compared to temperate species they may have a higher overall DMS production. However, DMSP is not known only as a cryoprotectant, but also as an antioxidant that eliminates reactive oxidation species (ROS), so species that endure a lot of ROS related stress may also have a high DMS production rate. It is important to note however, that they do not necessarily play a role in reducing temperature induced ROS (Exton *et al.*, 2013b) and as such species that endure higher temperatures will not necessarily be higher producers of DMS.

Lastly, it would be interesting to compare methodology to that which has been used previously to measure VOC production. Isoprene production rates have been measured in the past by taking VOC samples at two different time points, which afterwards were used to calculate the production rate (Exton *et al.*, 2013a).

4.4.6 Improvements to be made

There were several aspects that could be improved. It would be desirable to have more replicates. As the cultures are not axenic, there was a possibility for DMS degrading bacteria that could affect the gas samples that are tested last. There is also the consideration of true volumes to be made. Although the volume of *T. pseudonana* was accounted for, the fact that they have a much larger vacuole than other species was not (Raven, 1987). It is understood that DMSP is not stored in the vacuole which means much of the volume may not be contributing to DMS production (Wolfe *et al.*, 1994).

Developing a more robust and accurate DMS data set could help us understand more about physiological processes of DMS production by phytoplankton. Collecting and identifying other compounds would also help us learn more about why these compounds are produced and in turn use this knowledge to understand our environment a little more.

Chapter 5: Characterising volatile organic compounds produced in mixed plankton populations

5.1 Introduction

5.1.1 Interspecific interactions

The ecology of aquatic systems are incredibly complex, with strong interactions taking place between often biodiverse trophic levels, also influenced by seasonal flux of nutrients (Platt *et al.*, 2003). The population dynamics are ever-changing and are likely to influence volatile organic compounds (VOCs) that are produced, and VOCs produced are likely to influence the population dynamics in return. A variety of phytoplankton co-exist because of their differences in growth requirements/rates and survivability, which points to potential mechanisms used in order for some species to remain competitively viable (Hobson, 1988). Contrasting species may come together to enhance one another's survival or live off of the other (Schmidt *et al.*, 2015). Examples of this are seen as commensalism among species of phytoplankton, whereby one species may secrete nutrients (e.g. B_{12}, thiamine, and biotin) that can then be utilised by another species (Hobson, 1988). Interestingly, competitive behaviour can take place in a similar form, which instead of beneficial nutrients, inhibitory molecules are produced to outcompete others of another species (Hobson, 1988). In upwelling conditions, such competitive behaviour is more common, and fewer instances of coexistence are seen, with those that do, requiring a period of dormancy as a form of temporal heterogeneity (Hobson, 1988). With this in mind, it is possible that in a similar manner, VOCs are produced as kairomones or allomones.

Grazing activity is also prominent in which smaller phytoplankton are predated upon by larger phytoplankton, or zooplankton. Such predator-prey interactions will inevitably influence VOCs and need to be considered just as much as interactions within phytoplankton communities. Such ecological interactions are made viable through chemical communication.

5.1.2 Phytoplankton interactions

Even amongst phytoplankton the competition for resources is harsh. This means that for survival, each species must find a way to outcompete the others. Good examples of this are dinoflagellates. Previously it was questioned as to how they managed to maintain their biomass when in comparison to diatoms their growth rates are slow and require more energy. Experiments showed however that there is a possibility that some species of dinoflagellates release compounds that are allelopathic (Castrec *et al.*, 2018). Red tides are particularly harmful to many species including humans, which aids the emitter in survival as it allows their populations to out-compete other species that cannot tolerate the compound (Kubanek *et al.*, 2005). Another example is interactions between cyanobacteria. An allelopathic effect was seen in a compound released by *Tychonema bourrellyi* against *Microcystis aeruginosa.* β-Ionone was considered likely to be the inhibitory compound that decreased the maximum electron transport rate (ETR_{max}) to elevate reactive oxygen species (ROS) in the cells (Shao *et al.*, 2013).

5.1.3 Grazing behaviour

VOCs can also have an effect on grazing behaviour. Studies have noted swimming behaviour to alter in heterotrophic plankton grazers such as *Oxhyrris marina* and *Temora longicornis*. When exposed to dimethyl sulfide (DMS), clear signs of chemodetection are seen such as changes in swimming patterns (Steinke *et al.*, 2006). Multicellular organisms can also be influenced by VOCs produced by plankton. Cyanobacterial biofilms have been shown to act as both attractants and repellents for free-living nematodes. Chemical cues as attractants are usually formed when the biofilms will be beneficial by offering structure, shelter, and food which the emitter utilises (Höckelmann *et al.*, 2004).

5.1.4 Hypothesis and aims

Chemicals and their roles in communication are evident as can be seen in previous research. Again, most of this research singles out one or two compounds, where accumulating knowledge on a bouquet of them at once may help us understand how these microenvironments operate.

With this in mind, studies were conducted with two aims. The first study was a competition experiment whereby two species of phytoplankton were grown together to observe whether VOC profiles vary compared to when each species was grown independently. This was accomplished by comparing two single cultures of *E. huxleyi*

and *T. pseudonana,* and co-cultures of the two. The second study consisted of a grazing experiment in which *D. tertiolecta* was grazed upon by *O. marina*, and VOC samples were collected to observe the effects of grazing on VOC profiles.

E. huxleyi and *T. pseudonana* were chosen due to their rapid growth rates and ease in maintenance as well as knowledge on their difference in VOC profiles when grown independently. Both *E. huxleyi* and *T. pseudonana* are ecologically widespread and are found to coexist in late spring (Benedetti *et al.*, 2019; Perrot *et al.*, 2016). *D. tertiolecta* and *O. marina* were chosen for similar reasons but also because *D. tertiolecta* is a species commonly used as food for *O. marina* prior to investigations. For example, the fact that they do not produce any DMS makes them ideal prey to feed prior to an experiment monitoring DMS concentrations/production. However, they may produce other compounds which could affect studies in which these other VOCs are of the target interest instead. Understanding how they may influence signature VOCs may be valuable information to have when planning experiments that use *O. marina* as the investigative species. In both experiments VOC samples were taken during lag and exponential phases of growth.

By following these aims and conducting this experiment, the following hypotheses were addressed:

1. *T. pseudonana* would out-compete *E. huxleyi* when mixed due to their higher growth rates when independently grown
2. DMS concentrations will be higher in mixed cultures compared to unialgal cultures because of the competitive stress experienced as well as higher mortality rates

resulting in DMS production via cell lysis in conjunction to the breakdown of its precursor DMSP

3. As *O. marina* increases in growth, *D. tertiolecta* will decline
4. DMS production will increase a few days after *O. marina* are introduced into *D. tertiolecta* cultures due to catalytic break down of DMSP from being grazed
5. VOC profiles will alter as soon as prey are introduced due to grazing activity resulting in alterations in biological processes
6. Volatile profiles of mixed cultures will consist of both addition and subtraction of peak areas depending on the compound

Hypotheses 1, 2, and 6 were based on known information of growth rates and defence mechanisms in both *T. pseudonana* and *E. huxleyi* under optimal conditions. DMS production is commonly caused by stress on the individual and other VOCs may be released for similar reasons, or even be utilised under other unknown circumstances. Bacteria that grow in association may also produce VOCs, and are known to use VOCs produced by some phytoplankton (Todd *et al.*, 2007). Hypotheses 3 and 4 were based on the fact that grazing promotes the release of DMS. There is a possibility that other VOCs may be released in a similar manner that is not yet known.

5.2 Experimental Setup, Material, and Methodology

5.2.1 General Experimental Setup

Cultures of *T. pseudonana*, *E. huxleyi, D. tertiolecta, and O. marina* were obtained from the Provasoli-Guillard Center for the cultivation of Marine Phytoplankton (NCMA, West Boothbay Harbour, Maine, USA) and grown in growth rooms set and maintained at 16°C at a light intensity of 100±15 µmol photons m^{-2} s^{-1} supplied by cool-white fluorescent tubes (TL-D 840, Philips) on a 14:10 light:dark cycle, prior.to experiments.

All cultures were grown in autoclaved artificial seawater (ASW) supplemented with f/2 nutrients and vitamins (Guillard & Ryther, 1962) in biological triplicates alongside a control of media only. For mixed autotroph experiments, three types of cultures were set up consisting of two unialgal cultures of *E. huxleyi* and *T. pseudonana*, and a mixed culture where the two species were grown together with cell densities of 15 x 10^5 cells ml^{-1} for *E. huxleyi* and 5 x 10^5 cells ml^{-1} for *T. pseudonana*. Independent cultures of *E. huxleyi* and *T. pseudonana* were grown with a starting cell density of 10 x 10^5 cells ml^{-1} and 30 x 10^5 cells ml^{-1} respectively. These numbers were chosen so that both species would have the same starting biomass in both unialgal cultures and co-cultures. Grazing experiments were set up similarly except with *D. tertiolecta* and *O. marina* that were set up to start at densities of 5 x 10^5 cells ml^{-1} and 10 x 10^5 cells ml^{-1}, and a mixed culture with starting densities of 15 x 10^5 cells ml^{-1} for *D. tertiolecta* and 5 x 10^5 cells ml^{-1} for *O. marina*. These densities were chosen using a study by Steinke *et al* (1996) as reference material. *O. marina* cultures were grown on *D. tertiolecta* when cultivated alone but were

starved prior to the experiment. This was confirmed by monitoring the presence of *D. tertiolecta* using microscopy. All starting volumes were 400 ml set up in 750 ml conical flasks positioned so that the light intensity was 100±15 µmol photons m^{-2} s^{-1} supplied by cool-white fluorescent tubes (TL-D 840, Philips) on a 14:10 light:dark cycle. Cell growth was monitored daily by haemocytometry. Extractions of 120 ml during lag and exponential phases was used for VOC sampling as well as DMSP and chl *a* extractions.

5.2.2 Cell Growth

Cell growth was monitored throughout the experiment using haemocytometry. Cells from both unialgal and mixed cultures were counted daily up to 300 cells using a Neubauer haemocytometer under a light microscope. For cells that move such as *D. tertiolecta* and *O. marina*, 2% lugol's solution was added to halt movement. Collected data was used to calculate the specific growth rates (µ) of each culture using equations:

$$\mu = \ln (x_1 - x_0) / t_1 - t_0$$

Where x_1 and x_0 refer to cell densities at t_1 and t_0 respectively.

5.2.3 Chl *a* Analysis

From each growing photosynthetic culture, 50 ml was filtered through a glass fibre filter (Whatman GF/F, 25 mm) including *D. tertiolecta*, *E. huxleyi*, and *T. pseudonana* using a 100 ml glass syringe. The filter was folded with blunt end forceps and put into 15 ml centrifuge tubes that were stored at -20°C. These were spectrophotometrically quantified

after extraction in 100% methanol (neutralised with $MgCO_3$) following methods described in Riemann (1982) and established equations from Ritchie (2008).

5.2.3 DMSP analysis

From each growing culture, 50 ml was filtered through a glass fibre filter (Whatman GF/F, 25 mm) using a 100 ml glass syringe. The filter was folded using blunt end forceps and put into 4.92 ml vials prepared with 3 ml of 0.5 M NaOH. Vials were immediately closed with gas-tight silicone/teflon septa and incubated at 30°C for a minimum of 24 hours for complete equimolar alkaline hydrolysis of DMSP to DMS. After incubation, DMSP samples were analysed using a headspace injection method. Calibrations were performed using DMSP standards prior to analysis as described in Franchini & Steinke. (2016). DMSP concentrations were normalised to chl *a* as this was more representative than cell density considering the difference in cell sizes of the two autotrophic species that were observed under the microscope.

5.2.4 Trace Gas Analysis

A purge and trap method combined with gas chromatography was used to analyse dissolved VOCs in each samples (Franchini & Steinke, 2016; Lara-Gonzalo *et al.*, 2008). From each sample, 50 ml was injected using a 100 ml glass syringe through a glass fibre filter (Whatman GF/F, 25 mm) into the purge tube. Nitrogen flow was adjusted to 80 ml min^{-1} and samples were left to purge for 20 minutes during which any dissolved VOCs

were collected in the cryogenic loop maintained at a temperature of circa -150°C. Once purged, the condensate was thermally desorbed by immersing the loop in freshly boiled water bringing the temperature up to 90°C. This allowed the VOCs to be flushed into the GC. VOCs were measured using both a GC equipped with a flame photometric detector, as well as a GC equipped with a flame ionization detector. The GC ovens were run to increase in temperature from 40 to 120°C over 36 minutes. Chromatograms were analysed upon collection by examining each detectable peak.

5.2.5 Statistical Analysis

Each sulfur compound was compared between unialgal cultures as well as mixed cultures. For analysis, a series of statistical tests were used. A levene's test for equality of variances was carried out to check for homogeneity in data. This was followed by a shapiro-wilk test to confirm whether data was normally distributed. The data was confirmed to be non-parametric, so a Kruskal-wallis was used to check for statistical differences between the species for all data collected followed by a Dunnet's test. As a significance threshold, a p value of 0.05 was used as a probability value.

5.3 Results

5.3.1 Growth rates

In the samples of the mixed autotrophs experiment, difference in cell density between sample types were statistically insignificant ($P>0.05$)(Figure. 5.1). They all increased in cell numbers and then plateaued after c. day 6. Maximum cell density was highest in unialgal *E. huxleyi* ($1.6x10^6 \pm 1.8x10^4$ cells/ml) followed by unialgal *T. pseudonana* ($1.0x10^6 \pm 2.2x10^4$ cells/ml), mixed *E. huxleyi* ($6.7x10^5 \pm 1.8x10^4$ cells/ml), and lastly mixed *T. pseudonana* ($5.8x10^5 \pm 2.2x10^4$ cells/ml)(Figure. 5.1). Unialgal cultures tended to have a steeper exponential phase than the mixed cultures and higher density throughout both growth stages (Figure. 5.1).

In samples of the grazer experiment, separately grown cultures followed a classic sigmoid curve (Figure. 5.1). *D. tertiolecta* cells showed signs to continue growing for longer ($2.2x10^5 \pm 1.8x10^4$ cells/ml), while *O. marina* cells passed exponential phase and entered early stages of death phase by day 5 ($1.5x10^4 \pm 2.4x10^3$). In mixed cultures, *O. marina* followed a similar pattern to their unialgal counterparts where they increased up to day 3 ($1.1x10^4 \pm 2.6x10^3$ cells/ml) to slow down by day 4 ($1.0x10^4 \pm 1.7x10^3$ cells/ml) and drop on day 5 ($4.6x10^3 \pm 2.2x10^3$ cells/ml) *D. tertiolecta* on the other hand behaved the complete opposite to those grown unialgal as they consistently decreased in both life stages (Figure. 5.1).

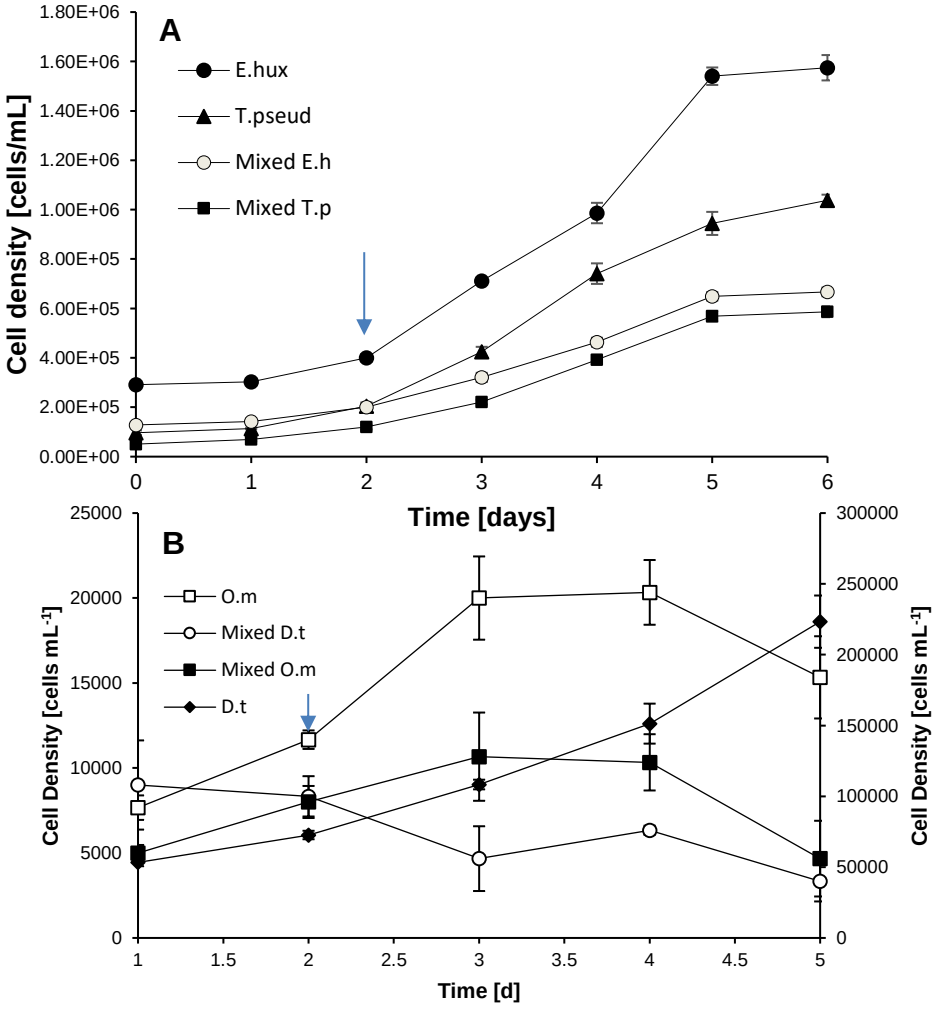


Figure. 5.1: Cell density (±SE) in cultures prepared for two separate experiments; A) three cultures of unialgal *E. huxleyi*, and *T. pseudonana*, and one culture with both of these grown together (n = 3). B) three cultures of *D. tertiolecta* alone, *O. marina* alone, and one culture with both of these grown together (n = 3). The primary y axis represents *O. marina* numbers, while the secondary axis represents *D. tertiolecta* numbers. Lag phase refers to the first day that the cultures were introduced to fresh media, and for the mixed cultures, also the first day that the separate unialgal cultures prepared were mixed together. Exponential phase refers to points at which samples were taken, represented by blue arrows.

Specific growth rates of all cultures showed a similar trend of increasing up to a maximum point (c. day 4) to then decrease after, however *E. huxleyi* reached the maximum point after *T. pseudonana* by c. 0.5 day (*E. huxleyi*, 0.58±0.02 μ; d^{-1}; *T. pseudonana*, 0.74±0.05 μ; d^{-1})(Figure. 5.2). *T. pseudonana* in unialgal cultures had the highest maximum growth rate (0.74±0.05 μ; d^{-1}) followed by *T. pseudonana* in mixed cultures (0.61±0.05 μ; d^{-1}), *E. huxleyi* in unialgal cultures (0.58±0.02 μ; d^{-1}), and then by *E. huxleyi* in mixed cultures (0.47±0.10 μ; d^{-1}) (Figure. 5.2). Overall, *T. pseudonana* had a higher growth rate than *E. huxleyi* in both unialgal and mixed cultures; growth rates compared between species were lower in those found in mixed cultures than unialgal ones (Figure. 5.2).

Specific growth rates of *O. marina* and mixed cultures decreased with time, but cultures of *D. tertiolecta* grown alone increased (Figure. 5.2). *O. marina* grown with *D. tertiolecta* dropped the most by day 5 in contrast to those grown alone which also decreased but to a much smaller extent (Figure. 5.2). *D. tertiolecta* in mixed cultures had the highest growth rate (0.64±0.52 μ; d^{-1}) followed by unialgal *O. marina* (0.52±0.07 μ; d^{-1}), *O. marina* in mixed cultures (0.46±0.10 μ; d^{-1}), and then unialgal *D. tertiolecta* (0.40±0.07 μ; d^{-1}) (Figure. 5.2).

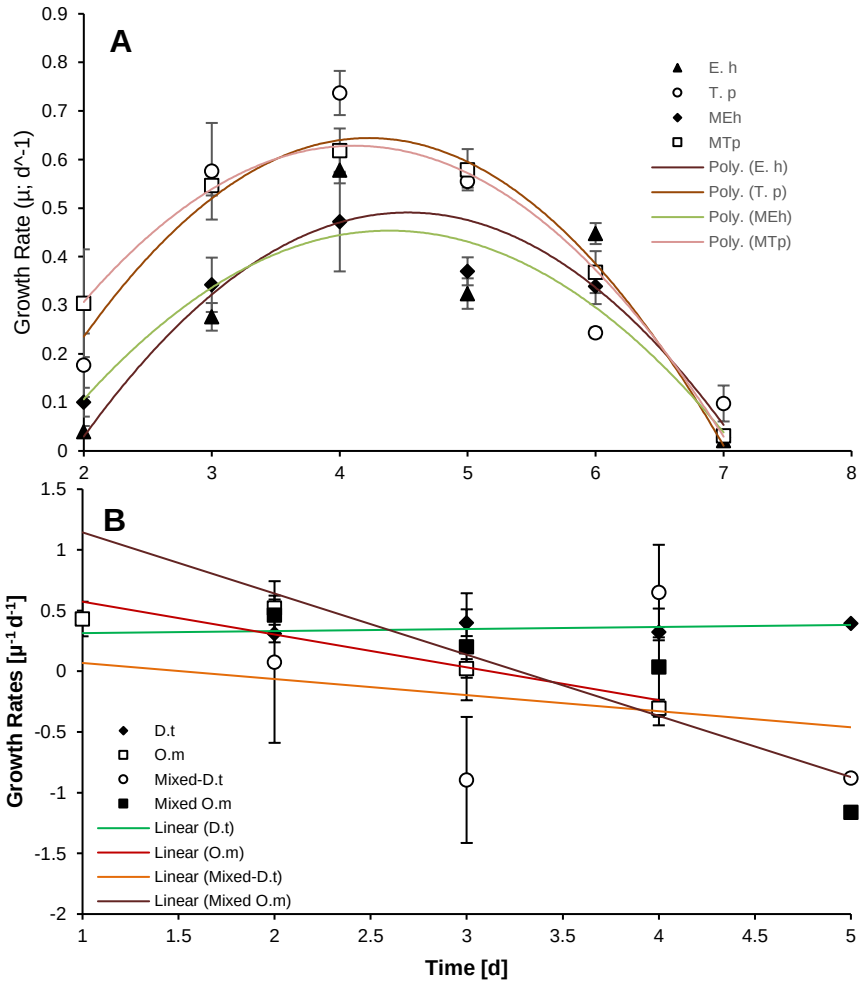


Figure. 5.2: Specific growth rates (±SE) in cultures prepared for two separate experiments; A) three cultures of unialgal *E. huxleyi* and *T. pseudonana*, and one culture with both mixed together (n = 3). B) three cultures of *D. tertiolecta* alone, *O. marina* alone, and one in which both are mixed together (n = 3). Growth rates were calculated using cell density values. Lines in A) show linear trends, while B) show polynomial trends.

5.3.2 Chl *a*

Chl *a* concentration was slightly lower in *E. huxleyi* when compared to *T. pseudonana* and mixed cultures (Figure. 5.3). The highest was seen in mixed cultures during exponential phase (1.24±0.11 Chl *a* mg L^{-1}), while the lowest was seen in unialgal *T. pseudonana* cultures during lag phase (0.67±0.06 Chl *a* mg L^{-1}) as can be seen in Figure. 5.3. The biggest difference observed between lag and exponential phase was in mixed cultures (+0.47 Chl *a* mg L^{-1} d^{-1}) followed by unialgal *T. pseudonana* (+0.44 Chl *a* mg L^{-1} d^{-1}) and then *E. huxleyi* in which very little change could be seen (+0.02 Chl *a* mg L^{-1} d^{-1}) (Figure. 5.3). Overall, chl *a* concentration was higher in exponential phases than lag phases; however such results obtained were not statistically significant (P>0.05) (Figure. 5.3).

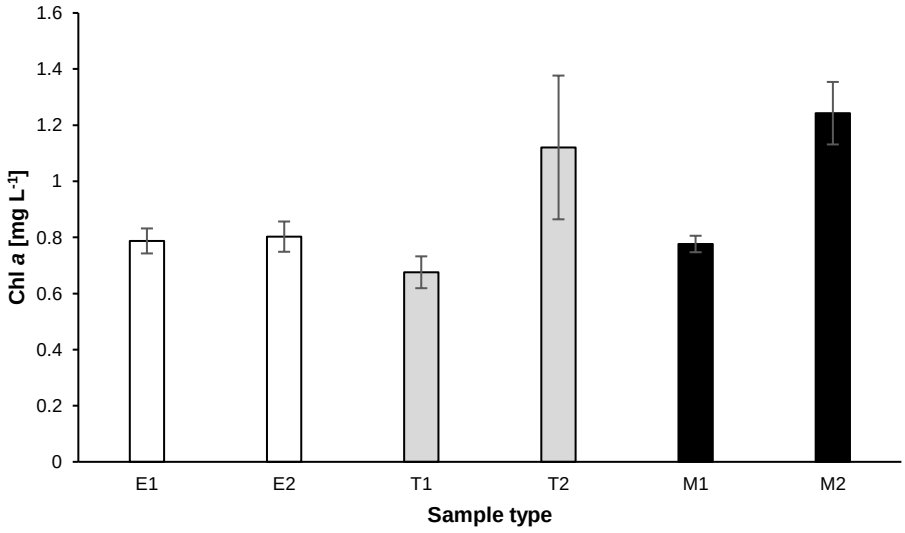


Figure. 5.3: Chl *a* samples from cultures of *E. huxleyi* (E), *T. pseudonana* (T), and two mixed together (M) (±SE). Numbers refer to lag (1) and exponential (2) phases. All samples were in triplicates (n = 3). Lag phase refers to the first day that the cultures were introduced to fresh media, and for the mixed cultures, also the first

day that the separate unialgal cultures prepared were mixed together. Exponential phase refers to points at which samples were taken, represented by blue arrows shown on Figure. 5.1.

5.3.3 DMSP

DMSP concentration (µmol) during both lag (first day that cultures were introduced to fresh media, and for mixed cultures, the first day that unialgal cultures prepared were mixed together) and exponential phases (points represented by blue arrows on Figure. 5.1) were measured at the time when VOCs were sampled per mg of chl *a* per one litre. DMSP concentration was highest in unialgal *E. huxleyi* cultures during exponential phase (40.88±4.30 µmol mg^{-1} Chl *a* L^{-1}), while lowest in unialgal *T. pseudonana* cultures during lag phase (0.53±0.23 µmol mg^{-1} Chl *a* L^{-1}), with mixed cultures being in between (16.79±1.34; 21.19±2.46 µmol mg^{-1} Chl *a* L^{-1}) (Figure. 5.4). Samples taken during exponential phase always had a higher DMSP concentration than those taken during lag phase, although insignificant ($P>0.05$) (Figure. 5.4). The biggest changes in DMSP concentration between lag and exponential phase was observed in unialgal *E. huxleyi* (+9.82 µmol mg^{-1} Chl *a* L^{-1} d^{-1}) cultures, followed by mixed cultures (+4.40 µmol mg^{-1} Chl *a* L^{-1} d^{-1}), and then *T. pseudonana* cultures (+0.76 µmol mg^{-1} Chl *a* L^{-1} d^{-1}) (Figure. 5.4). Statistical differences were seen between *E. huxleyi* and *T. pseudonana* cultures during both phases ($p=0.03$), but not when compared to mixed cultures ($p>0.05$) (Figure. 5.4).

Chl *a* could not be measured for *O. marina* cultures, so in the grazer experiment, DMSP concentration was calculated using biovolume instead. DMSP concentration during both lag and exponential phases were sampled at the same time as when VOCs were being sampled.

The three culture types contained different amounts of DMSP ($p=0.01$, $df=2$). Highest DMSP concentration was seen in mixed cultures during lag phase ($1.52x10^{-6}±3.22x10^{-6}$ µmol/um^3), while lowest was seen in *O. marina* cultures which produced none during both lag and exponential phase (lag, $p<0.0005$; exponential, $p=0.03$) (Figure. 5.4). The biggest change, although insignificant, that occurred in DMSP concentration was in mixed cultures whereby concentrations of DMSP dropped during exponential phase of growth ($-7.88x10^{-6}$ µmol/µm^3; $p>0.05$) (Figure. 5.4). No change occurred in *O. marina* cultures as none were produced, and only a small change was seen in *D. tertiolecta* cultures ($-2.06x10^{-7}$ µmol/µm^3; $p>0.05$) (Figure. 5.4). DMSP concentration was always higher during lag phase than exponential phase (Figure. 5.4).

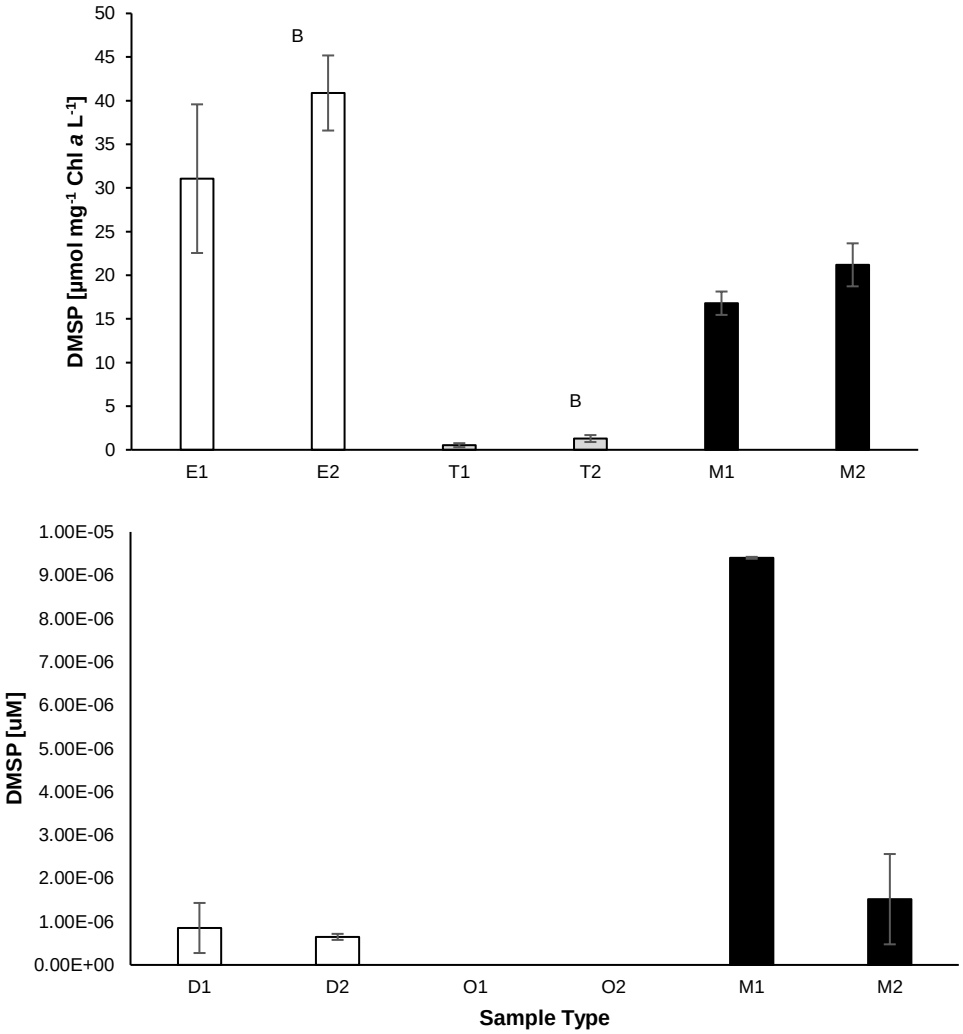


Figure. 5.4: DMSP concentration (±SE) in cultures prepared for two separate experiments; A) three cultures of unialgal *E. huxleyi* (E), *T. pseudonana* (T), and one with both mixed together (M) in triplicates (n = 3). B) three cultures of *D. tertiolecta* (D) alone, *O.marina* (O) alone, and one in which both are mixed together (M) in triplicates (n = 3). Numbers refer to lag (1) and exponential (2) phases. Lag phase refers to the first day that the cultures were introduced to fresh media, and for the mixed cultures, also the first day that the separate unialgal cultures prepared were mixed together. Exponential phase refers to points at which samples were taken, represented by blue arrows on Figure. 5.1.

5.3.4 DMS

In the experiment with mixed autotrophs, all three culture types showed statistically different DMS concentrations (p=0.01, df=2) (Figure. 5.5). Concentration was highest in *E. huxleyi* (lag, 0.01±0.002; exponential, 0.009±0.001 mmol Chl a^{-1}), followed by mixed cultures (lag, 0.004±0.0002; exponential, 0.006±0.001 mmol Chl a^{-1}), and then *T. pseudonana* (lag, -0.001±0.003; exponential, 0.0003±0.005 mmol Chl a^{-1}) (Figure. 5.5). During lag phase, *E. huxleyi* and *T. pseudonana* were statistically different (p=0.003), while during exponential phase, Mixed cultures and *T. pseudonana* were statistically different (p=0.046) (Figure. 5.5).

In the experiment with grazer-prey interactions, there were statistical differences amongst the three cultures types (p=0.00, df=2) (Figure. 5.5). *D. tertiolecta* produced the most DMS, more during exponential phase (0.0021±3.26x10^{-11} µM/$µg^3$ p>0.05) than during lag phase (0.0016±3.07x10^{-9} µM/$µg^3$; p>0.05). A much smaller concentration was produced in mixed cultures during lag phase (9.04x10^{-12}±6.28x10^{-12} µM/$µg^3$; p>0.05) and none in exponential phase (Figure. 5.5). *O. marina* did not produce any DMS in either phase. Statistically, unialgal *D. tertiolecta* was higher than solely *O. marina* in lag phase (p=0.02), while higher than both *O. marina* alone (p=0.03) and mixed cultures (p=0.03) during exponential phase (Figure. 5.5). However, differences observed between *O. marina* and mixed cultures were not statistically significant during both lag and exponential phases (p>0.05) (Figure. 5.5).

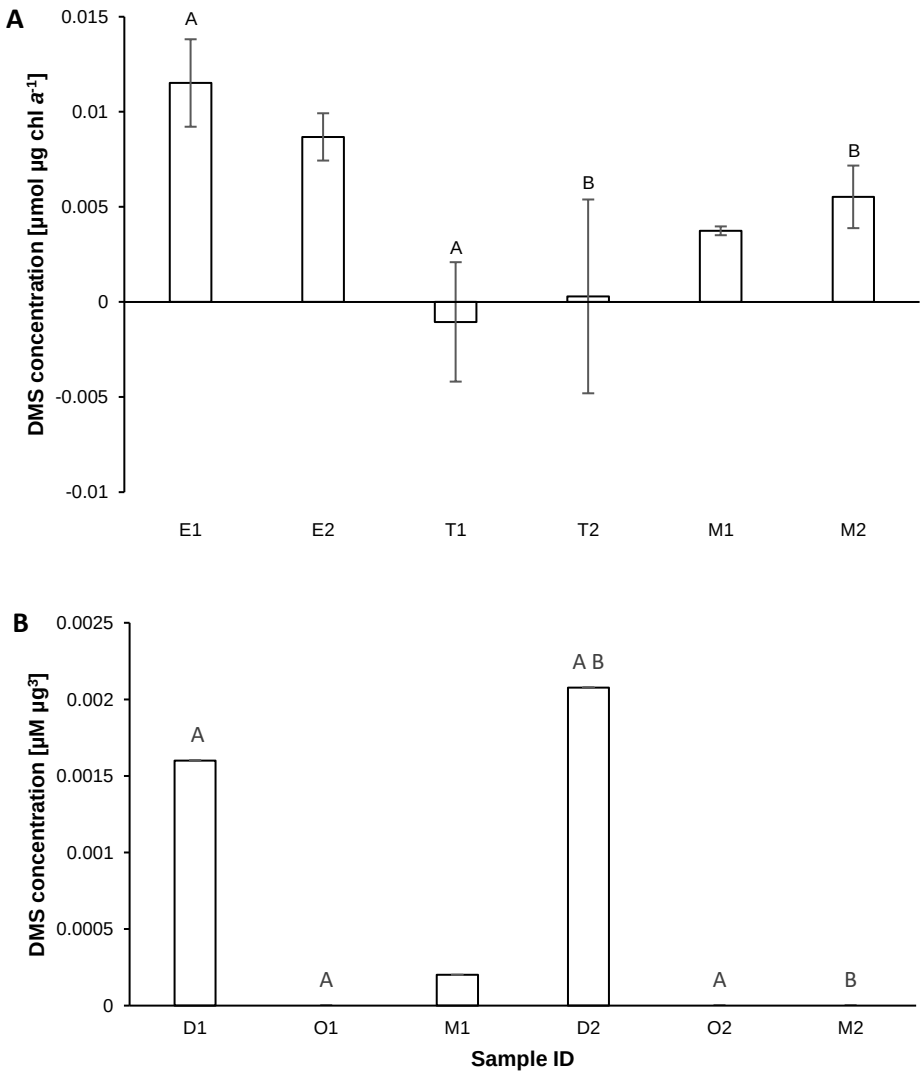


Figure. 5.5: DMS concentrations (±SE) in cultures prepared for two separate experiments; A) three cultures of unialgal *E. huxleyi* (E), *T. pseudonana* (T), and one with both mixed together (M) in triplicates (n = 3). B) three cultures of *D. tertiolecta* (D) alone, *O. marina* (O) alone, and one in which both are mixed together (M) in triplicates (n = 3). Numbers refer to lag (1) and exponential (2) phases. Matching letters represent statistically significant differences between those data sets presented here that have matching letters. Lag phase refers to the first day that the cultures were introduced to fresh media, and for the mixed cultures, also the first day that the separate unialgal cultures prepared were mixed together.

Exponential phase refers to points at which samples were taken, represented by blue arrows on Figure. 5.1.

5.3.5 Other sulfur compounds

Hydrogen sulfide was consumed in all culture types, highest being in unialgal *E. huxleyi* cultures during lag phase ($-4.53 \times 10^{-4} \pm 2.28 \times 10^{-4}$ mmol Chl a^{-1}), and lowest being in the same culture type but instead during exponential phase ($-3.06 \times 10^{-5} \pm 2.22 \times 10^{-4}$ mmol Chl a^{-1}) (Figure. 5.6). With time, a decrease in consumption was seen in the *E. huxleyi* cultures, while the other two culture types showed an increase in consumption instead (Figure. 5.6). There was little difference in concentration between *T. pseudonana* and mixed cultures ($p>0.05$) (Figure. 5.6). For DMDS, a trend was seen amongst all culture types in which it was present in lag phase but consumed/degraded when exponential phase was entered ($p>0.05$) (Figure. 5.6).

A minute amount of H_2S was present in *D. tertiolecta* during lag phase ($2.76 \times 10^{-5} \pm 3.53 \times 10^{-10}$ µM/µg^3), but not in exponential phase where it was consumed/degraded instead ($-1.2 \times 10^{-4} \pm 8.59 \times 10^{-12}$ µM/µg^3; $p=0.01$) (Figure. 5.6). Similar amounts were produced in both *O. marina* and mixed cultures during both phases (O1, $4.5 \times 10^{-4} \pm 7.36 \times 10^{-12}$; O2, $4.7 \times 10^{-4} \pm 2.40 \times 10^{-12}$; M1, $5.2 \times 10^{-4} \pm 1.43 \times 10^{-11}$; M2, $4.0 \times 10^{-4} \pm 6.41 \times 10^{-12}$ µM/µg^3) (Figure. 5.6). CS_2 was produced moderately in *D. tertiolecta* cultures during both phases (D1, $8.1 \times 10^{-4} \pm 9.52 \times 10^{-10}$; D2, $4.3 \times 10^{-4} \pm 9.98 \times 10^{-12}$ µM/µg^3), although more during lag phase ($p>0.05$) (Figure. 5.6). Meanwhile, CDS was consumed/degraded immensely in *O. marina* cultures during lag phase ($-8.7 \times 10^{-4} \pm 1.44 \times 10^{-11}$ µM/µg^3),

produced in small amounts in exponential phase ($5.63x10^{-5}\pm6.95x10^{-12}$ µM/µg^3), and only consumed/degraded in mixed cultures (M1, $-4.0x10^{-4}\pm3.29x10^{-11}$; M2, $-4.0x10^{-4}\pm1.24x10^{-11}$ µM/µg^3) (Figure. 5.6). DMDS was consumed/degraded in all (D1, $-4.23^{-9}\pm7.55x10^{-12}$; O1, $-4.0x10^{-4}\pm1.75x10^{-12}$; O2, $-1.7x10^{-4}\pm9.56x10^{-13}$; M1, $-4.0x10^{-4}\pm2.40x10^{-12}$; M2, $-1.7x10^{-4}\pm1.54x10^{-12}$ µM/µg^3) except *D. tertiolecta* cultures during exponential phase ($8.66^{-5}\pm2.40x10^{-12}$ µM/µg^3) (Figure. 5.6). It was lower/more negative during lag phase than it was exponential phase in all cultures that consumed/degraded it (Figure. 5.6).

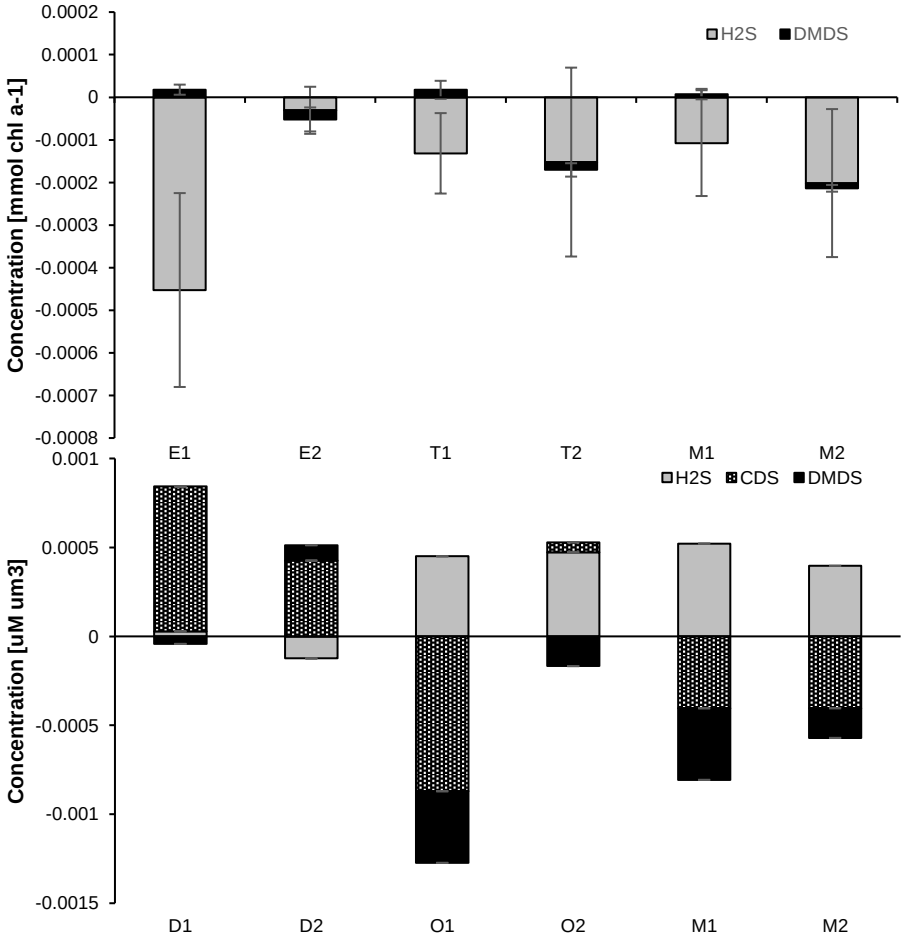


Figure. 5.6: Proportions of each known sulfur compound (±SE) in cultures prepared for two separate experiments; A) three cultures of unialgal *E. huxleyi* (E), *T. pseudonana* (T), and one with both mixed together (M) in triplicates (n = 3). B) three cultures of *D. tertiolecta* (D) alone, *O. marina* (O) alone, and one in which

both are mixed together (M) in triplicates (n = 3). Numbers refer to lag (1) and exponential (2) phases. H2S, H_2S; DMDS, Dimethyl disulfide; CDS, CS_2. Lag phase refers to the first day that the cultures were introduced to fresh media, and for the mixed cultures, also the first day that the separate unialgal cultures prepared were mixed together. Exponential phase refers to points at which samples were taken, represented by blue arrows on Figure. 5.1. Negative values represent "consumption/degradation" whereby VOCs already present in the media, as seen in control values, were consumed/degraded when mixed with live cultures.

5.3.6 VOC concentrations

5.3.6.1 Mixed autotroph

There was no clear separation between the samples; all VOC samples overlapped with VOCs produced by *E. huxleyi* cultures during lag phase (ehlag) (Figure. 5.7). It was ehlag that also showed the most variation both horizontally and vertically (Figure. 5.7). The most distinct cluster, although still overlapping others, was VOCs produced by *E. huxleyi* cultures during exponential phase (ehexp) (Figure. 5.7). Overall, groups were indistinguishable from one another with very little separation (Figure. 5.7).

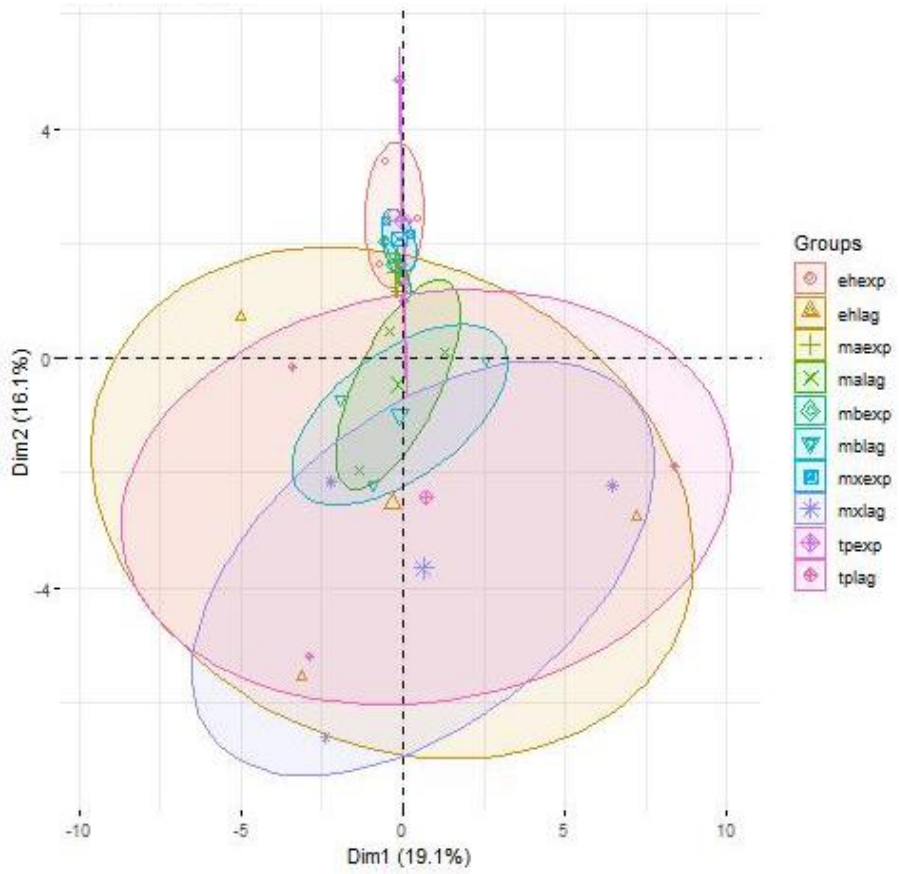


Figure. 5.7: Principal component analysis of VOCs for both lag (lag) and exponential (exp) growth stages of three sample types; unialgal *E. huxleyi* (eh); unialgal *T. pseudonana* (tp); mixed cultures of both. Lag phase refers to the first day that the cultures were introduced to fresh media, and for the mixed cultures, also the first day that the separate unialgal cultures prepared were mixed together. Exponential phase refers to samples taken 2 days after those taken during lag phase. Samples for mixed cultures were normalised to total chl *a* (mx). Ellipses represent 95% CI, n = 3, group means are displayed.

There were some compounds that were present in small amounts in all cultures (10, 17, and 35), while others that were only present in one or two (23; Table. 5.1). A common

pattern seen was that in *E. huxleyi* production would be higher in lag phase and lower in exponential phase, while the other two cultures would show the reverse in which production would be higher in the later phase (14, 32, 34, and 37); however significance for these differences were only seen in compound 34 (p=0.0056; p=0.0049; Table. 5.1). Compound 21 was produced hugely in mixed cultures but not others, while compound 31 was hugely consumed/degraded by *E. huxleyi* (-45026) and mixed cultures (-75599) but produced greatly in *T. pseudonana* (331604) all in lag phase (Table. 5.1). Compound 33 was found to be consumed in minute amounts during lag phase (E, -2271; T, -3631; M, -2890; Table. 5.1). Compounds that dominated most in production were 34 and 32, while those that dominated most in consumption/degradation were 31 and 14 (Table. 5.1).

Table. 5.1: Values of noteworthy unidentified VOCs out of total trace gas data measured by GC-FID. To normalise area values, control values of seawater media only were subtracted from live culture values and then divided by chl *a*. Three cultures were prepared; *E. huxleyi* (E), *T. pseudonana* (T), and one where the two were mixed together (M). All samples were collected in triplicates (n=3). Numbers refer to lag (1) and exponential (2) phases. Lag phase refers to the first day the cultures were mixed, and exponential phase refers to points at which samples were taken, represented by blue arrows on Figure. 5.1.

	Average area					
Compounds	E1	E2	T1	T2	M1	M2
10	3040.22	72.89	-138.45	316.51	-479.12	281.88
14	-2145.83	-7567.45	-1068.71	-10806.77	-1165.97	-6651.16
15	6378.78	-2403.45	0	-5953.82	0	-4059.99
17	-3761.91	10544.04	-16513.32	3488.27	-1017.29	7578.76
21	110.27	144.21	54.86	-38.74	-69.24	19266.03
23	0	811748.09	0	43.81	0	50.72
31	-45025.61	454.70	331603.87	0	-75599.13	0
32	30727.80	3417.01	2974.30	2694.40	2034.17	2011.64
33	-2271.64	0	-3631.80	0	-2889.60	0
34	21443.4596	14724.71	28962.53	20861.02	27405.62	21078.16
35	800.72	983.52	688.82	2358.17	-161.91	1665.634
37	0	-31025.57	0	10870.21	0	850.01

5.3.6.2 Predator-prey interactions

From the VOC analysis there was no clear separation between clusters of treatment types and species, and all overlapped. The cluster that showed the most variation was mixed cultures during lag phase (lagmi) horizontally, and mixed cultures during exponential phase (expmi) vertically. All clusters were indistinguishable; there were also no horizontal nor vertical shifts (Figure. 5.8).

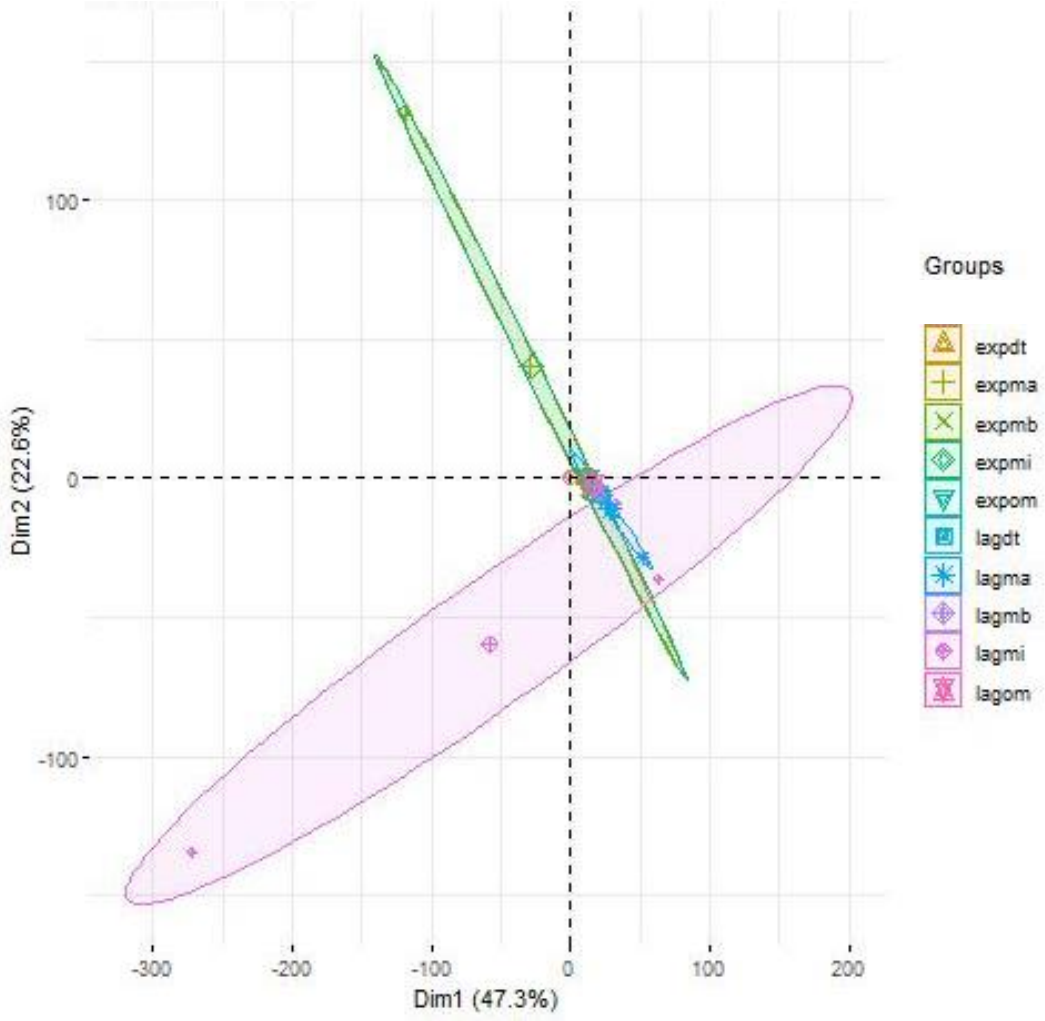


Figure. 5.8: Principal component analysis of VOCs for both lag (lag) and exponential (exp) growth stages of three sample types; unialgal *D. tertiolecta* (dt); unialgal *O. marina* (om); mixed cultures of both. Samples for mixed cultures were normalised to per µm^3 (mi), per µm^3 of only the estimated *D. tertiolecta* portion of the mixed culture (ma) and per µm^3 of only the estimated *O. marina* portion of the mixed culture (mb). Portions were estimates based off of mixed and unialgal cultures. Ellipses represent 95% CI, n = 3, group means are displayed.

In contrast to cultures in the mixed autotroph experiments, there were many compounds that existed only in one culture. Some of these largely dominated the overall volatile

profile of that culture (compound 50, $3.7x10^{-4}$; 55, $5.2x10^{-4}$; 58, $1.1x10^{-3}$; and 60, 0.02), while others not so much (Table. 5.2). There were also compounds that were broadly produced >5 cultures, just to a less extent compared to those found only in single culture types. Of all the aforementioned compounds, there were several noteworthy compounds. There was a statistical difference between *D. tertiolecta* and *O. marina* during lag phase in the amount of compound 52 (p=0.047) (Table. 5.2). A common trend was also observed in compound 4, which decreased in production (or increased in consumption/degradation) in mixed cultures during exponential phase (p=0.044); overall, VOCs dominated profiles of all three cultures much less than samples taken in the beginning during lag phase (Table. 5.2).

Table. 5.2: Noteworthy unidentified VOCs out of total trace gas data measured by GC-FID. Area values were normalised by subtracting control (seawater media only) from live culture values and calculated to biovolume (μm^3). Three cultures were prepared; *D. tertiolecta* (D), *O. marina* (O), and one where the two were mixed together (M). All samples were collected in triplicates (n=3). Numbers refer to lag (1) and exponential (2) phases. Lag phase refers to the first day the cultures were mixed, and exponential phase refers to points at which samples were taken, represented by blue arrows on Figure. 5.1.

	Average area					
Compound ID	D1	D2	O1	O2	M1	M2
4	1.85E-03	-1.83E-04	3.97E-06	-4.09E-05	0	-9.95E-05
26	0	-8.45E-04	0	-1.89E-04	0	-4.60E-04
34	1.95E-04	-2.97E-01	3.22E-05	8.20E-05	4.60E-06	4.24E-06
42	0	2.47E+02	0	7.75E-06	0	2.16E-05
48	8.19E-04	1.67E-01	2.42E-04	1.68E-04	1.78E-04	2.09E-04
50	0	0	0	0	3.75E-04	0
52	3.55E-04	-1.09E-01	1.51E-04	4.88E-04	3.69E-05	1.89E-03
55	0	0	0	5.19E-04	0	0
58	0	0	0	0	0	1.07E-03
60	2.29E-02	0	0	0	0	0

5.4 Discussion

5.4.1 Influence of competition on growth rates

E. huxleyi grew the most, closely followed by *T. pseudonana* (Figure. 5.1, 5.2). This is likely due to their ability to grow quickly because of their tendency to investment in reproduction, over growth in cell size (Marañón *et al.*, 2013). Each species in mixed cultures grew approximately half of that observed in the unialgal cultures, but this is likely due to starting densities being half of what they were in independent cultures and both having to share resources which would limit their growth (Figure. 5.1). They did not however lead the other to mortality which suggests that they can grow alongside one another.

In the grazing experiment, unialgal cultures of *O. marina* and *D. tertiolecta* grew as expected with plots following similar patterns to the classic sigmoid curve (Figure. 5.1). The difference however was that *O. marina* had already hit the death phase by day 5, whereas *D. tertiolecta* looked as if to continue growing for another few days (Figure. 5.1). This could be due to different dietary preferences. *O. marina* has evolved to be heterotrophic and is thought to be derived from a photosynthetic ancestor (Slamovits & Keeling, 2008). It can however survive without prey such as *D. tertiolecta* due to its tendency to turn to cannibalism (Montagnes *et al.*, 2011), and this would explain their ability to still grow. *D. tertiolecta* on the other hand is photosynthetic, meaning if there is plenty of light, CO_2, and nutrients, it may continue growing (Chen *et al.*, 2011), unlike *O.*

marina which is limited to population sizes for nutrition. *D. tertiolecta* had plenty of nutrients in the media as well as light, which is probably what allowed continued growth.

For both experiments, growth rates typically decreased with time (Figure. 5.2). This is characteristic of populations reaching stationary phases, which could be seen in the cell density growth curves (Figure. 5.1). The reasons stated above also explain the quicker diminishing in growth rates of *O. marina* compared to *D. tertiolecta*.

5.4.2 The effects of growth and competition on Chl *a* concentration

Chl *a* concentrations increased in *T. pseudonana* and mixed cultures as expected; however surprisingly, this was not seen in the *E. huxleyi* cultures (Figure. 5.3). Potential explanations lie in variability of chl *a* content. Chl *a* content is known to change with the type and concentration of nutrients (Riemann *et al.*, 1989). It is possible that nutrient content did not influence the chl *a* concentration of *E. huxleyi* cells as much as it did those of *T. pseudonana*. Mixed cultures may be particularly influenced as there was an element of competition for nutrients between the two species (Figure. 5.3). Also, if the cells decreased in size over time, this could also cause there to be a lower total chl *a* concentration. The same reason could explain the similar pattern seen in chl *a* of mixed cultures to *T. pseudonana* cultures. *T. pseudonana* may have been the only species to contribute to the increase while *E. huxleyi* remaining the same.

5.4.3 Influence of interspecific interactions on DMSP concentrations

Prymnesiophytes such as *E. huxleyi* are renowned for their high DMSP content, while Bacillariophytes such as *T. pseudonana* are known to produce very little (Keller & Korjeff-Bellows, 1996). It was important to confirm these results as changes in DMSP often have an influence on DMS concentration, in this case production, and it may also influence other sulfur compounds which are newly explored in this thesis. This content difference is confirmed yet again. Mixed cultures resulted in having DMSP concentrations of halfway between the DMSP concentrations of unialgal *E. huxleyi* cultures and unialgal *T. pseudonana* cultures (Figure. 5.4). This is likely, simply a result of there being half the number of both species in the mixed culture that meant that DMSP levels were expected to be the combined total of DMSP levels when half of the unialgal cultures were added together.

D. tertiolecta is known to produce a small amount of DMSP which can be seen from the data collected, but the noteworthy point is that the DMSP content in mixed cultures increases rapidly when the two species are combined (Figure. 5.4). This is likely because of grazing activity, as it is well documented that grazing initiates DMS release (Wolfe *et al.*, 2000; Wolfe & Steinke, 1996), which requires DMSP as a precursor. The concentration decreases in exponential phase as *D. tertiolecta* populations drop from being grazed upon, but even then the concentration is higher than *D. tertiolecta* unialgal cultures which shows how much of an impact grazers can have on DMS production (Figure. 5.4).

5.4.4 Influence of interspecific interactions on DMS concentrations

DMS concentrations closely resembled the patterns seen in DMSP concentrations (Figure. 5.5). This was expected as DMSP cleavage is what often leads to the release of DMS (Alstyne *et al.*, 2001). Again, *E. huxleyi* is a species renowned to produce DMS, while *T. pseudonana* is known to produce much less (Keller, 1989b). The same explanations could be made for DMS concentrations as was made for DMSP concentrations.

D. tertiolecta is known not to be a huge producer of DMS, with a calculated average DMS concentration of only 0.14 µm DMSP/cm^3 of cell volume, which compared to *E. huxleyi* (166.42 µm/cm^3) and even *T. pseudonana* (16.64 µm/cm^3) is a very small amount (Keller *et al.*, 1989), and the results support this statement in that there was very little DMS in the samples collected (Figure. 5.5). However, this also meant that they are still DMS producers, and could therefore potentially affect experiments investigating DMS production if used as a food source for *O. marina*. It was expected that mixed cultures would produce more DMS, but only showed to do so a little during lag phase (Figure. 5.5). This could be because the small amount that was detected was due to the *D. tertiolecta* proportion of the culture, and by the time samples were taken again at exponential phase, not as many were left because of grazing activity, resulting in there being no DMS at all. It was not expected for there to be DMS in the *O. marina* cultures as they are known for not producing any DMSP (Keller, 1989a), and therefore no DMS would be released from the cell.

5.4.5 Exploring other sulfur compounds

When samples were taken, there were sulfur VOCs which had increased in concentration, but also sulfur VOCs which had decreased in concentration (Figure. 5.6). This was calculated by subtracting the sulfur VOCs present in controls consisting of media only, from the sulfur VOC samples obtained from the live cultures. H_2S was potentially consumed/degraded in all culture types during both phases (Figure. 5.6). A possibility for this is that both cultures may contain bacteria that utilise H_2S as an energy source. DMDS was found to be present during lag phase but then had decreased in concentration (assumed to be consumed/degraded) by exponential phase (Figure. 5.6). This was also seen in grazer experiments and showed similar behaviour as was seen in *D. tertiolecta* cultures. There is little information on DMDS consumption, but a possibility is that there are organisms present in the culture (whether that be bacteria or alga is unknown), that are DMDS degraders or consumers. Alas, cultures were not checked for bacteria, which highlights a new area to further explore. Overall, when compared to samples obtained from grazer cultures, the autotrophic samples there had a lot more degradation/consumption occurring and less production (Figure. 5.6), which could be the difference present between autotrophic and heterotrophic plankton.

H_2S is known to be produced by certain species of anaerobic bacteria and archaea. It is also known to be a product of decomposing material (Watson & Jüttner, 2017). A possible explanation as to why there is so little in the *D. tertiolecta* cultures and more in the *O. marina* and mixed cultures is that *O. marina* can turn to cannibalism when no food is present which leads to possibility in higher cell mortality and decomposition than when

D. tertiolecta is grown alone. For mixed cultures it could be that the predation of *O. marina* on *D. tertiolecta* means that there is inevitably more debris from broken *D. tertiolecta* cells which again would be decomposed by sulfate reducing bacteria, releasing H_2S, something that could be tested for using next generation sequencing. Alternatively, it could be larger sulfur molecules such as CS_2, simply being broken down into smaller ones, although this is less likely. CS_2 is produced by some plants as a biocide (Feng Zhe & Hartel, 1996). Although research has not yet been undertaken specifically with algae, it is possible that some species release it, even if reasons are unknown. As for the decrease, CS_2 can be biodegraded into COS and H_2S. Considering H_2S increases when CS_2 decreases, it is possible that in the *O. marina* and mixed cultures CS_2 is being biodegraded, whether it be bacteria associated with *O. marina*, or *O. marina* itself. Little is known about degradation of DMDS, but It is possible that if bacteria that degrade DMDS are present, the amount of DMDS in the culture would decrease, much like what could be seen in the *O. marina* and mixed cultures. Again, if it is due to bacterial activity, *O. marina* has a larger surface area for such bacterial colonies to form, making them a better candidate for microbial communities that rely on biofilms, to grow on (Seymour *et al.*, 2017).

5.4.6 Other VOC compounds

There is likely to be most biological and metabolic activity in lag phases as the plankton have just been transferred into fresh media and need to adjust to their new environment (Kirst, 1996). As the phytoplankton adjust to their new environments, it is likely that they produce and consume VOCs in the process hence why so much change is seen

compared to during exponential phase (Kirst, 1996). The least activity is seen in mixed cultures possibly due to the competition between the two species. Each will inevitably limit the other from growing, which means there are fewer individuals to produce the same amount as those in monospecific cultures. The high consumption seen in lag phases are potentially because of there being a lot of VOCs in the fresh media ready to be utilised/consumed by bacteria present with the algal cultures, as they were not axenic (Cleveland & Yavitt, 1998). Additionally, the cell numbers were much higher in cultures at exponential phase which may have also influenced the overall VOC profiles.

The common trend observed was that *E. huxleyi* cultures produced more VOCs in lag phase, which dropped in exponential phase, while *T. pseudonana* and mixed cultures showed the reverse response (Figure. 5.6); this suggests a number of things. It could be that *E. huxleyi* increased VOC production in response to changes in their surroundings from being transferred to new media, while *T. pseudonana* was not as affected. It could be that *E. huxleyi* was able to normalise to their environment with more ease than *T. pseudonana*, which lead to the mixed cultures showing a milder version of the pattern in VOC change shown by *T. pseudonana* cultures. Compound 21 could be a product of competition as it was only released by mixed cultures during exponential phase and no other species or phases (Table. 5.1). Compound 31 was largely produced by *T. pseudonana* while being consumed by other species, all during lag phase (Table. 5.1). This could either be because this compound is characteristic to *T. pseudonana*, or alternatively to a microbe that happened to be associated with that *T. pseudonana* culture. It could also be the absence of a microbe that consumes the compound. It is hard to conclude the source of each VOC unless further molecular work to investigate bacterial species, such as polymerase chain reaction using bacterial primers, is carried

out. Compounds that are present in all cultures in small amounts could be background “noise” compounds that are not related to the plankton in question.

Part of the grazing experiment showed similar trends to mixed autotroph experiments. Activity in VOC production/consumption was higher in lag phase than exponential phase (Figure. 5.6), which again is likely because of their transition and adjusting to their new environments. Overall, the most activity was apparent in *O. marina* cultures during lag phase, while least was in *D. tertiolecta* cultures during exponential (Figure. 5.6). Possible explanations to this is, that *O. marina* cells are more sensitive to change than are *D. tertiolecta*. Not to mention, *O. marina* cells are prone for cannibalism and therefore with higher rates of cell lysis, it is not strange for there to be more VOCs present. Generally speaking, there were also more production than consumption/degradation. This could mean than VOCs were not used as resources by any organism as much as was in mixed autotroph experiments leading the production to be more prevalent. Mixed cultures showed interesting VOC behaviour. There was a large contrast between the cultures in lag phases and exponential phases. In lag phase there was a large amount of consumption/degradation, while the opposite was seen during exponential phase (Figure. 5.6). This could be the product of grazing behaviour. VOCs are largely produced once exponential phase is reached as more *D. tertiolecta* cells are grazed upon. As more cells reach their mortality, the cells result in releasing any VOCs trapped within.

There were VOCs that dominated in only one culture type and not present in any of the others. It’s debatable why this is the case; it may be that the compound is characteristic to that species and phase alone. There were also specific compounds that were

noteworthy and showed trends that could suggest a variety of possibilities as they presented themselves in all or most of culture types but in different amounts/proportions. Compound 26 was only seen to be consumed/degraded and not produced (Table. 5.2); this could be a compound that was already present in the media, formed chemically of the nutrients, and favoured by plankton present, or by the microbes on the plankton as an energy source. This would explain why there was a bigger portion in *O. marina* and mixed cultures as the surface area of *O. marina* for such microbes to form colonies is much greater than small *D. tertiolecta* cells. Compound 42 was massively produced by *D. tertiolecta* in exponential phase, but in small amounts by others (Table. 5.2). This could be because compound 42 may be a product of metabolic activity that occurs in *D. tertiolecta* but not so much in *O. marina*, much like the presence of DMSP lyase in *E. huxleyi* that is not present amongst *D. tertiolecta* cells (Niki *et al.*, 2000). The similarity seen in mixed is probably because by exponential phase the *D. tertiolecta* cells are mostly consumed by the *O. marina* cells. Another suggestion is that it is only produced in healthily growing *D. tertiolecta*, but this is another experiment that would be necessary to confirm. Compound 52 was consumed/degraded by *D. tertiolecta* while it was prevalent in *O. marina* and mixed cultures (Table. 5.2). This could be either because *D. tertiolecta* cells do not produce this compound while it is consumed by bacteria or chemically degrading. *O. marina* on the other hand may produce this compound at a higher rate than is consumed by such bacteria/chemically degraded. This may be seen in exponential phase more, due to the higher *O. marina* population. An overall common trend for this experiment, particularly seen in 4, 34, and 48, was the high concentration initially in lag phase that then decreases to be a smaller portion in exponential phases (Table. 5.2). The best explanation to this is the utilisation of “background” VOCs already present in the media for reasons unknown, measured in the control consisting of media

only. In order to confirm such use of VOCs, further investigations must be undertaken using axenic cultures and bacterial cultures isolated from cultures such as those used in this grazer response study. VOCs also need to be identified to some extent using MS techniques so that conclusions on why such VOCs are utilised can be made.

To conclude, the overall consensus was that although competition in mixed autotroph experiments may have had some role in VOC fluctuations, they did not necessarily interact with each other, but more stunted each other in growth and therefore VOC production/consumption. Grazing experiments showed a clearer possibility of interactions made that may have reflected in volatile profiles. Ultimately, either axenic cultures need to be used, or bacteria found on the surface of cells grown in non-axenic cultures need to be isolated for any conclusive statements to be made in regard to the behaviour of plankton species alone.

Chapter 6: Fluctuation in VOCs in response to seasonal succession of plankton

6.1 Introduction

Short term laboratory experiments are valuable, though have several limitations. They are unable to show results entirely relevant to the real world and therefore are more useful in filling unknown pockets of information than being representative of the overall environment. Due to this, data was collected from Brightlingsea twice a week over an 8-month period to understand the existence (if any) of seasonal shifts in VOC profiles.

Planktonic shifts alone have been heavily monitored and predicted in forms such as the plankton ecology group (PEG) model (Sommer, 1986). Seasonal succession of plankton is therefore relatively well documented which meant there is potential for VOC profile shifts to be present alongside such succession. Estuarine environments are of particular interest, as organisms that inhabit these areas are frequently exposed to alterations in their microenvironments as well as macroenvironments, making it a very complicated system with lots to learn from.

So far, the presence of seasonal variation has been noted in individual VOCs such as dimethyl sulfide where it has suggested that sulfur fluctuates between 1 to 1100 ng S/liter over the course of a year, with the fluctuation being likely due to shifts in taxa as there was only a weak correlation between chl *a* and DMS (Turner *et al*, 1988). Isoprene is also found to be seasonally dependent with there being a fluctuation between a minimum

of 0.7 pmol l^{-1} and a maximum of 54.3 pmol l^{-1} from the North Sea (Broadgate *et al.*, 1997). These are only two of many VOCs that are known to exist at ocean surfaces; investigating the seasonal fluctuation of multiple VOCs would prove to be a thought-provoking study.

6.1.2 Study Site Location

Brightlingsea harbour is a conservation area situated in Essex, England. It is close to the mouth of the Colne Estuary where it also meets two other estuaries: Blackwater estuary, and Thames estuary (Figure. 6.1). The pontoon from which samples were collected is used as a mixed leisure and commercial port. The waters are well mixed, as can be seen from comparison data collected at the surface, 3 m depth and 10 m depth with those taken right at the pontoon. This site was chosen as the sampling destination because of its ease of access.

Figure. 6.1: Location of the sample site in Brightlingsea. Samples were collected from surface waters at the pontoon (seen in the close-up view) in triplicates using aspirators.

6.1.3 Hypotheses and aims

The aim of this field research was to begin investigating trends in volatile profiles of Brightlingsea harbour and the explanations behind any identifiable patterns found. This location was chosen due to its turbulent waters that allowed stratification as a factor to be discounted. By following these aims and conducting the field study, the following hypotheses were addressed:

- There will be more types of VOCs during periods when plankton populations are high.
- There will be higher concentrations of compounds during periods when phytoplankton populations are stressed.
- Volatile profiles will not be the same throughout the year.

Hypotheses were based upon trends seen in compounds such as DMS and isoprene that have already been investigated for their seasonal fluctuations and with assumption that many other VOCs will behave a similar manner.

6.2 Materials and Methods

6.2.1 General information, locations, etc.

Seawater samples were collected from the pontoon in Brightlingsea Harbour every month during high tide for the duration of 8 months between mid-March to late October. An aspirator was altered to fit the purpose of the study by covering the top with 200 µm mesh to avoid any large debris from entering and attaching silica tubing to the tap. These were then acid washed prior to use. The prepared aspirators were then lowered into the water top first, after which the tap was opened for the air to gradually escape. This allowed for the water to gradually enter without disturbance. Once full, the aspirator was slowly reverted, and the end of the tubing was placed at the bottom of a 500 ml borosilicate duran bottle. The tap was then opened to let the water flow in and then left to overflow for 10-20 seconds before the tap was closed and the bottles closed with as little gas bubbles as possible. This process took place in order to limit the potential gas

escape. Chl *a* and UPLC samples were collected in the same way. Taxonomy samples were collected in a single medical flask with neutral lugol's solution for a resulting 2% concentration.

In order to test for similarity between open water (water accessed using a boat) and water collected from the pontoon, the exact same method was used to collect water from three different depths (surface, 3 m, and 10 m) and measured in the same fashion.

6.2.2 Chl *a* analysis

Fifty mL of each sample was slow filtered through a glass fibre filter (Whatman GF/F, 25 mm) using a 100 ml glass syringe. With forceps, the filter was carefully folded and put into 15 ml centrifuge tubes that were stored at -20°C. These were spectrofluorometrically quantified after extraction in 100% methanol following methods described in Riemann (1982) and calculated using values obtained from a calibration run prior to sampling (Riemann & Ernst, 1982).

6.2.3 Plankton Taxonomy

Samples were collected from Brightlingsea and preserved in 2% neutral lugol's solution prepared and stored refrigerated. The bottles were carefully swirled for 60 seconds before 10 ml was pipetted into a 10 ml settling chamber. Organisms were identified to the taxa level using a light microscope at 100x magnification.

6.2.4 Trace Gas Analysis

Immediately after transport to the lab, water samples were analysed for VOCs using a GC-FPD and GC-FID. 50 ml of seawater samples were filtered through a GF/F filter into the purge tube using a 100 m glass syringe. VOCs from samples were cryogenically enriched at -160°C using the purpose built purge and trap system. Samples were purged for 20 minutes at a flow of 80 ml/min on the FPD and 50 mL/min on the FID. The cryotrap was heated to 90°C using freshly boiled water which allowed for thermal desorption of collected gases before flushing into GC with a carrier gas of helium.

Once a minimum of 24 hours had passed, samples were brought out but maintained at 30°C using a water bath and sampled for DMS. In vial purging method was used for cryogenic enrichment. The DMS detected and quantified was then used to calculate the amount of DMSP within the sample. Calibrations were performed using known DMSP standards (Franchini & Steinke, 2016).

6.2.5 Statistical Analysis

FID data was organised using the same methods as mentioned in chapter 5, whereby peaks were automatically aligned using an R package called GCalignR (Ottensmann *et al.*, 2018), after which quality checked manually.

A Levene test for equality of variances was carried out to check homogeneity of data. A Shapiro-wilk test then used to confirm whether data was normally distributed. When data was parametric, an ANOVA alongside a post hoc was used to compare data sets. For non-parametric data, a Kruskal-Wallis test alongside a Dunnets test was used to compare results. All of this was conducted using R studio.

6.3 Results

6.3.1 Chl *a* and temperature

Temperature constantly increased from lowest in early March (5°C) up to late July (21°C) after which it decreased until the final measurement taken in October (14°C) (Figure. 6.2). Chl *a* on the other hand also increased from March (0.78±0.01 µg/L) but then peaked in late April (4.62±0.26 µg/L) and dropped in late May (1.95±0.003 µg/L) to then peak again for the second time in early July (4.98±0.52 µg/L) and finally gradually decreased (Figure. 6.2).

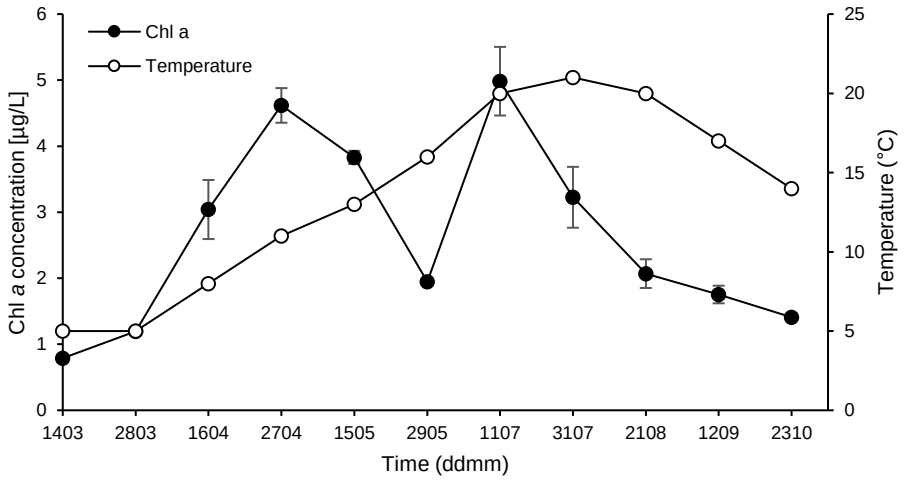


Figure. 6.2: Temperature and chl *a* concentration (±SE) over 8 months. Chl *a* samples were collected in triplicates (n = 3) and measured using a fluorometer and to then calculated using a calibration constructed in advance. Temperature data was collected from an online database that monitors and collects sea water temperature including that of Brightlingsea. Numbering system is as follows: first two digits = day, last two digits = month.

Phytoplankton taxa fluctuated in population density throughout the 8 months during which sampling took place. Typically, one taxon would dominate over others for each season. Diatoms dominated during the colder months such as in early spring (March, 8.2 cells ml^{-1}) and mid-autumn (October, 11.7 cells ml^{-1}), while remaining low during the warmer seasons such as July and August (5.6 and 4.7 cells ml^{-1}) (Figure. 6.3). Dinoflagellates on the other hand showed contrasting results to diatoms whereby they would be very low during early spring but increase greatly in summer (Figure. 6.3). Ciliates remained relatively constant in numbers, but followed the trend of being low in winter (1.9 cells ml^{-1}) and increased in late spring (7.8 cells ml^{-1}); it did not increase or

decrease rapidly like others during any particular season except post winter (Figure. 6.3). Coccolithophores remained low throughout the year, and the similar pattern was seen in zooplankton populations. Another point to mention was that what seemed like zooplankton eggs seemed to be high during early spring and late autumn (Figure. 6.3).

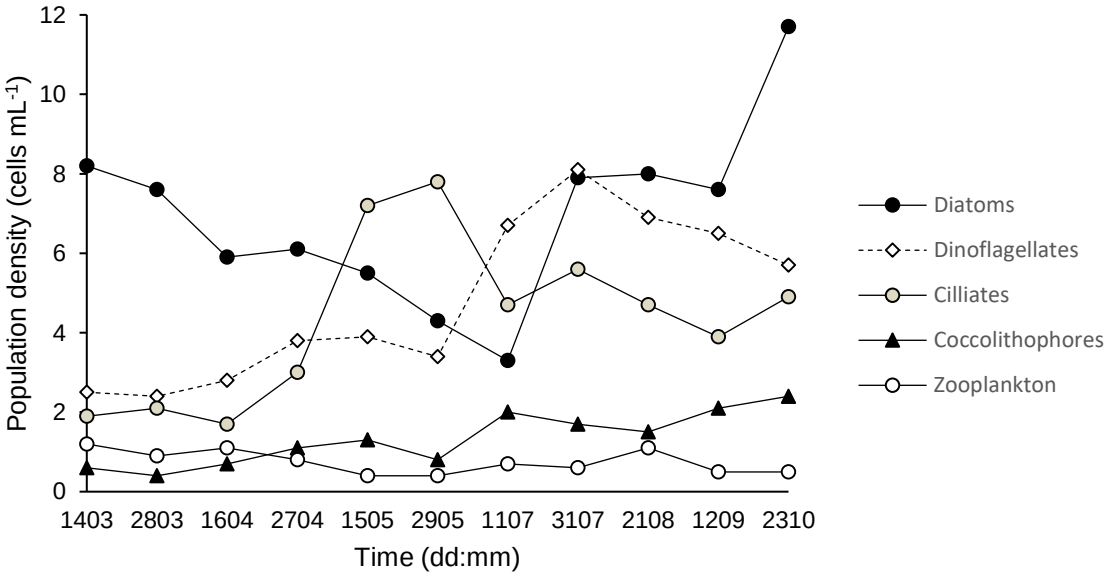


Figure. 6.3: Mean densities of four groups of plankton types identified in samples collected in Brightlingsea harbour over 8 months (n = 3). All taxa were preserved in lugol's solution and identified under a dissecting microscope after being settled in a settling chamber. The numbering system of the x axis is as follows: first two digits = date, last two digits = month.

6.3.2 Sulfur compounds:

DMS concentration was highest in early spring (2.59±0.02 nM) and early summer (3.43±0.51 nM), dropped towards the end of summer (0.80±0.89 nM) and then remained similar towards winter (1.04±0.12 nM; $p \leq 0.005$) (Figure. 6.4). H_2S however was the reverse, whereby it was high during autumn (0.87±0.05 nM) and winter (1.40±0.05), but decreased in later spring (0.00 nM) and summer (0.19±0.35 nM; $p \leq 0.03$). MT followed a similar pattern to DMS except delayed in the sense that it peaked at early and late summer (0.55±0.05; 0.38±0.11 nM; $p \leq 0.01$) (Figure. 6.4). Carbon disulfide (CS_2) was non-existent during the earlier months, but gradually increased in summer and remained a similar concentration into autumn ($p \leq 0.03$) (Figure. 6.4). Dimethyl disulfide (DMDS) followed a similar pattern to MT, except in lower concentrations ($p > 0.05$). Dimethyl trisulfide (DMTS) was only present during summer ($p > 0.05$) (Figure. 6.4).

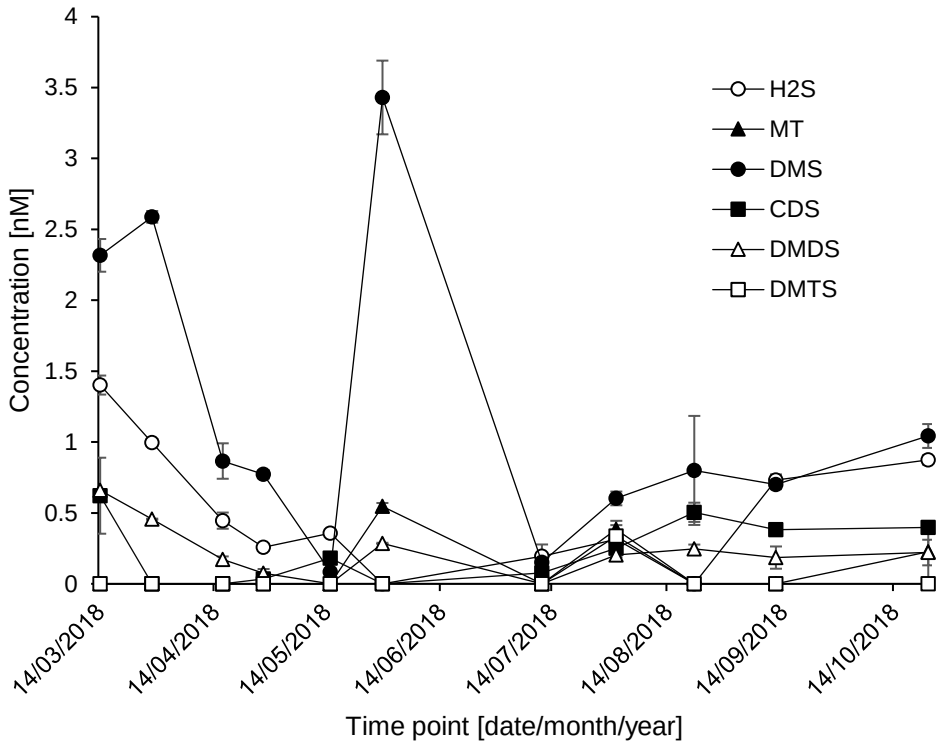


Figure. 6.4: Concentration of six different sulfur VOCs present in samples collected from Brightlingsea harbour over the course of 8 months (n = 3). Abbreviations are as follows: H2S, H_2S; MT, methane thiol; DMS, dimethyl sulfide; CDS, CS_2; DMDS, dimethyl disulfide; DMTS, dimethyl trisulfide.

There were the most diverse of compounds during mid-summer where there were all six types (including H_2S, MT, DMS, CDS, DMDS, and DMTS) in late July, whilst least were three types varying in each sample, tied between several time points throughout the year (Figure. 6.5). Total concentrations of the sum of all volatiles was highest during early spring in mid-March, while there was least during mid-summer in late-July (Figure. 6.5). The median, close to average amounts were seen between these seasons during mid spring in April, and early-autumn in September. Concentrations began to increase again in October towards the winter months (Figure. 6.5).

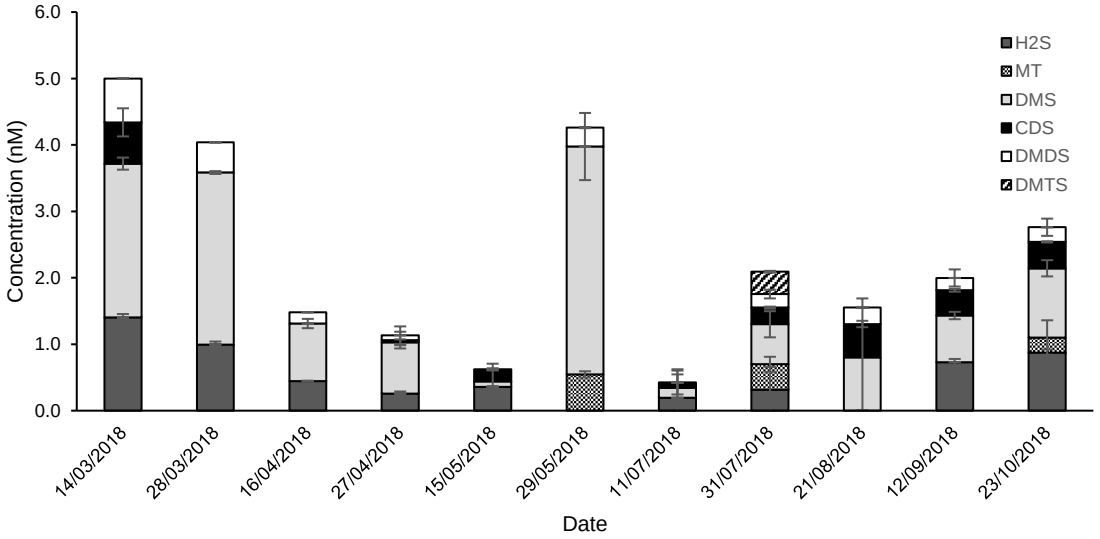


Figure. 6.5: Proportions and concentration of six different sulfur VOCs present in samples collected from Brightlingsea harbour over the course of 8 months (±SE; n = 3). Abbreviations are as follows: H2S, H_2S; MT, methane thiol; DMS, dimethyl sulfide; CDS, CS_2; DMDS, dimethyl disulfide; DMTS, dimethyl trisulfide.

6.3.3 Other organic compounds:

A high amount of variation was present in the VOCs collected, where most seasons overlapped greatly in their VOC profiles (Figure. 6.6). However, the samples taken on the 21st of August were distinct from any other groups, demonstrating a clear separation along the y axis (Figure. 6.6). Conversely, due to an overall lack of VOCs being detected, the samples of 23rd of Oct and 11th of July were largely consistent with one another and displayed very little variation (Figure. 6.6).

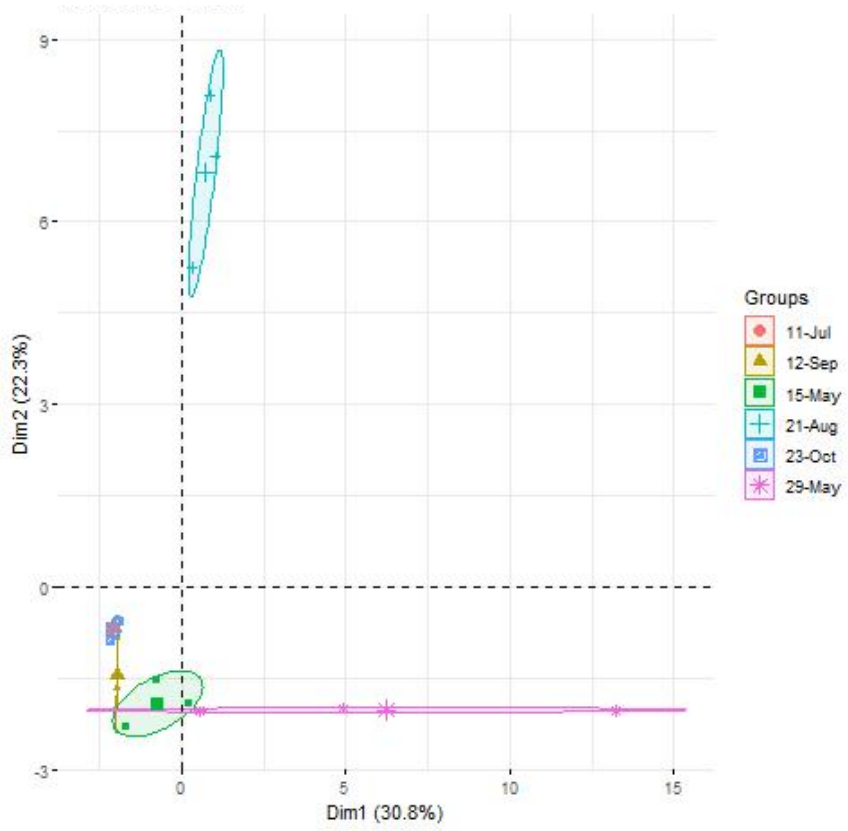


Figure. 6.6: Principal component analysis of VOCs gathered at Brightlingsea harbour from May to October. Ellipses represent 95% CI, n = 3, group means are displayed.

Compounds that heavily dominated the volatile profiles in many of the samples were compounds 19 and 35 (Table. 6.1). The only compound that was present in all time points samples was compound 8 (Table. 6.1). Compounds that hugely dominated in proportion within multiple time points were 26 during late spring, while compounds 19, 28, and 39 dominated during the summer and autumn months (Table. 6.1). There were also a handful of compounds that only dominated hugely during one time point (Table. 6.1).

Such compounds include compound 36 and 37 that only dominated during early May, and compound 46 that only dominated during early July (Table. 6.1).

Table. 6.1: Volatile profiles (area) of samples collected from Brightlingsea harbour, and sampled using GC-FID. Data was collected over 8 months. Notable peaks, numbered as compounds, were compiled. The numbering system is as follows: first two digits = date; last two digits = month; 3 m = 3 meters from surface; 7 m = 7 meters from surface.

	Average area								
Compounds	**1505**	**2905**	**1107**	**2108**	**1209**	**2310**	**surface**	**3 m**	**7 m**
8	231.67	2285.23	962.05	2572.50	68.20	573.90	292.60	272.90	170.10
19		4078.40	7915.40	13264.70	1488.40	1797.13	1809.70	1454.10	
26		13145.10	2724.40	1434.50					
28	252.07	1478.40		5097.47	481.33	39.97			21.90
35		59421.53	9445.20	8149.97		1135.13	747.60	796.30	657.00
36	2397.87		535.65	1093.43		252.13	124.70	204.70	
37	948.20		119.00	321.20					
39			1533.55		912.47	91.27	674.80		
46			8676.15	2826.43					

6.4 Discussion

6.4.1 Carbon disulfide

CS_2 is likely to be produced by a handful of plants and phytoplankton (Aneja *et al.*, 1979). It is also produced under anaerobic conditions by bacteria (Lovelock & Margulis, 1974) although this is unlikely the source in the samples taken for this study. Seasonally, it was higher in concentration during summer (Figure. 6.4), which is likely due to its sources being biogenic and there being higher biogenic activity in summer from the increased species richness, and phytoplankton populations.

6.4.2 Dimethyl sulfide

DMS was observed to be produced during early summer (May-June), and decrease in late summer to increase again a little in autumn (Figure. 6.4). There are a number of reasons as to why this seasonal trend was seen. One is that DMS is a biproduct of a sulfur based anti-oxidant system (Vallina & Simó, 2007). The high population of phytoplankton in late spring combined with the increasing solar radiation results in increased DMS production in early summer (Stefels & Dijkhuizen, 1995). Another is taxonomic composition. Some taxa, such as dinophytes and haptophytes, produce more than others. From the samples collected, in late spring, dinophytes tended to increase in population, while in colder seasons there were more bacillariophytes (Figure. 6.3) which do not produce as much DMS (Keller, 1989a). Vertical and horizontal mixing of waters can also affect DMS concentrations (Stefels *et al.*, 2007), although in this study it is

unlikely that the variability is due to this considering the estuary is relatively mixed at all times.

6.4.3 Hydrogen sulfide

H_2S concentration could possibly be present due to breakdown of larger compounds, but it is also known to be produced anaerobically by bacterial species such as *Desulfovibrio* spp. (Sisler & Zobell, 1951; Zinder *et al.*, 1977). Ingvorsen & Jorgensen. (1982) investigated the seasonal variation in H_2S emission from sediment and water surfaces, and discovered that it was higher during summer, and non-existent during winter (Ingvorson & Jorgensen, 1982). This contradicts the results found here in which concentrations were higher in winter and non-existent in summer (Figure. 6.4). This could however be due to methodological differences between the study by Ingvorson & Jorgensen. (1982) and the study conducted in this chapter. The methods documented in the study by Ingvorson & Jorgensen. (1982) utilised a Plexiglas box which collected samples in gaseous form from sediments. On the other hand, sampling methods used in the study conducted for this chapter consisted of seawater collected in the field, which was later bubbled through with nitrogen for gas collection. This creates the possibility for error in that not only are the gas samples carried from the study location to the lab, but also the organisms that produce it. It could be argued that this method was not as representative of the environment as methods used in the study conducted by Ingvorson & Jorgensen. (1982). It is also important to note that there would be a difference between microbial communities in sediments and those in the water column, and although it is

possible that microbial communities in sediments influence those in the water to some degree, it is difficult to make a direct comparison.

6.4.4 Methane thiol

As mentioned in chapter 4, MT is produced in algal blooms by dinophytes, while less so during more mixed populations (of dinophytes and bacillariophytes combined) (Varaljay *et al.*, 2015). The dinophyte population tends to increase during summer, while becoming more mixed in later seasons (Figure. 6.3). This may explain why the concentration was higher during summer than in the colder months (Figure. 6.4).

6.4.5 Phytoplankton succession

Seasonal succession is commonly seen in plankton populations. Each taxon favours certain biophysicochemical factors such as temperature, nutrient levels, predator population densities, light intensity, pH, and competition, and thrives when a particular level/balance is reached (Archer *et al.*, 2000; Sommer, 1986). Marine plankton are also influenced by vertical mixing that happens during spring and autumn, as well as the hibernation of zooplankton that predate on certain species over others (larger plankton with harder exteriors may be harder to predate on compared to smaller ones) (Sommer, 1986). In the data collected, trends dependent upon seasonality could be seen (Figure. 6.3). Diatoms were high in numbers during the winter (March considered winter) and autumn months, likely due to their preference of colder temperatures in combination with

their morphology - silica shells for defence against herbivores (Sommer, 1986) (Figure. 6.3). Ciliates flourished during warmer spring months (Figure. 6.3) likely due to their competitive advantage as they are able to grow much quicker than diatoms that invest a lot of energy in their frustules and size (Lewin, 1966). Dinoflagellates dominated during summer (Figure. 6.3), which was expected because of their mobility and size and they were able to escape herbivores while having a larger surface area to volume ratio for maximum uptake of nutrients (Sommer, 1986). There was also strong stratification during this season, which tends to promote niche partitioning; the nutrient limited surface water column are often dominated by nitrogen fixing picocyanobaceria, while the deep-water layers are dominated more by picoeukaryotes (Winder, 2009). This observation may not be seen as strongly in the samples collected for this study however, as the water studied was well-mixed. In Autumn, dinoflagellates were out-competed by diatoms that again dominated in colder seasons (Figure. 6.3), although other phytoplankton do manage to survive and exist in unison, probably due to the increased abundance of nutrients and optimum light levels. The sample site is an estuary that is constantly mixed in comparison to that which would be seen in a normal marine environment. This could explain why the succession is not so drastic. Also, coccolithophores and zooplankton were expected to be seen in much higher densities that also fluctuate throughout the season, but this was not the case. This could be due to the fact that samples were stored in 2% Lugol’s, and although neutralised, may have disintegrated the exteriors of fragile coccolithophores. Larger zooplankton were hard to count as often they were found in segments.

6.4.6 VOC concentrations

Certain taxa are known to produce more of a particular compound than others. For example, dinophytes tend to produce more DMS, while bacillariophytes are known to produce more isoprene (Dani *et al.*, 2016). In this way, the volatile profiles are likely to be dominated by different compounds throughout the year depending on the taxon that dominate at that point in time. This would explain the dominance of compound 35 during the summer months which could be produced mostly by taxa that favour summer, while 19 being produced by a taxon that favour cooler conditions found in autumn (Table. 6.1). There were also compounds that were only found to dominate in one time point such as compounds 36, 37, and 46 (Table. 6.1). This could be due to them being produced only under incredibly specific conditions that are not necessarily met at all times.

The diversity in the type of compounds seen during summer is probably due to there being a higher diversity in taxa during this period, contrasting to the taxa in autumn samples that were much less diverse (Figure. 6.3). Factors other than those that are biogenic could also have an influence on the volatile composition. Some compounds may not be directly produced by organisms but could be a product of physiochemical reactions in the water, or even in the air. Compound 8 is consistently present throughout and in similar concentrations (Table. 6.1). This could be a “background” VOC that’s present as a result of human actions, or a biproduct of nutrients/minerals interacting in the water.

6.4.7 Improvements

There were several improvements that could have been made. More time points would have helped understand seasonality better. No samples were taken during June or mid-winter, so critical information during those periods is missing. Formaldehyde may have been more suitable for the preservation of samples if treated with care. Although neutral lugol's was used, the counts of plankton with calcium carbonate skeletons or shells would have not been as accurate as if formaldehyde was used. For future studies, the use of other sampling methods such as solid phase micro-extraction, or thermal desorption tubes would be interesting. This could help understand what volatiles are in the atmosphere better, as the methods used in this study were only able to understand compounds within the water. The use of a mass spectrometric detector would also allow for further investigations as it could help in the attempts of identifying what the compounds are and possibly to calculate the concentrations.

6.4.8 Conclusion

In conclusion, VOCs were not necessarily higher in number during periods of high phytoplankton density, however they were high during periods in which phytoplankton would typically be stressed (such as summer in which nutrient levels can be limited). The volatile profiles did change throughout the year, where some compounds only seemed to be present for one month, although more data points would be necessary to confirm this information.

Chapter 7 General discussion

7.1 Research context

Ecosystems are complex and ever-changing (Bolliger *et al.*, 2005). Due to this, many factors must be considered when studying biophysiochemical phenomena in the environment. As fluctuations and changes occur on earth, research questions are constantly brought up that need answering, and alongside this the progress in ecological monitoring is prevalent. In response to this, it became apparent that the concept of utilising volatilomics as an environmental diagnosis method would contribute in advancing knowledge in environmental science. Through both experimental and field studies, in this thesis I have attempted to explore the volatile profiles from a specific level, gradually up to an ecological level with the aim of exploiting any information gained for future development of an explorative tool. This chapter will include summaries of major findings in this thesis, comparisons with similar studies undertaken in the past, and attempts to process information from results to direct future studies towards this ultimate aim of developing an environmental monitoring tool.

7.2 The impact of temperature on *Emiliania huxleyi*

The study described in chapter 3 represents the very first step in this journey; understanding how temperature may influence the overall volatilome of *E. huxleyi*. Previous studies have noted a change in growth rate and morphology of *E. huxleyi* which suggested that similar effects may also be seen in the VOCs produced

(Buitenhuis *et al.*, 2008; De Bodt *et al.*, 2010). The design of such experiments was similar to the ones conducted for chapter 3, whereby cultures were grown as batches in specific temperatures for comparison. In chapter 3, volatile profiles revealed that more types of VOCs were produced in higher temperatures (26°C), while more were consumed/degraded in optimal temperatures (16°C). Research on precursors of DMS by Spielmeyer and Pohnert (2012) revealed that DMSP production in *E. huxleyi* increase with temperature (Spielmeyer & Pohnert, 2012) from which it can be presumed that DMS production could also increase. Abe *et al* (2017) investigated the effects of temperature on methyl chloride production and noticed a higher production rate in those grown under 25°C compared to those in 10°C (Abe *et al.*, 2017). It is also well documented that isoprene increases the thermotolerance of its emitters and therefore is produced more in higher temperatures (Singsaas *et al.*, 1997). These are a handful of compounds that have been noted to increase with temperature and shows similarity to the results that were obtained in the chapter 3 study.

With this in mind, if similar experiments were repeated and the results obtained aligned with the ones here, it could be inferred that if environmental VOC samples contained more VOC types than usual, that the inhabitants of that location may be suffering from heat stress to a greater extent than expected. Although such a statement may seem obvious, it can lead to questions of what metabolomic activities may result in such changes of volatile profiles. Further investigations could be made on other species to see if this pattern is followed by a variety as opposed to just one, as *E. huxleyi* could be an exception. However, this is doubtful due to there being studies that display results in which production of particular VOCs increase also with temperature in species other than *E. huxleyi* (Baumann *et al.*, 1994). Another point to note is that despite the study

showing interesting results, cultures were deprived of selenium which could have significantly impacted the information collected and therefore the same study must be repeated before the results can be stated as conclusive.

7.3 Sulfur production rates in three species of phytoplankton

The next step in the development of this tool was to find out if there were any species-specific VOCs. To maintain simplicity, in this study only sulfur compounds were explored. The most well-investigated compound amongst the handful found was DMS. Some species are already known to produce more than others. For example, *E. huxleyi* is known to produce high amounts of DMS (Keller, 1989a), while *R. lacustris* is reported to produce none at all (Keller, 1989a). Again, the assumption was that if one compounds shows species specific behaviour, perhaps others may do so too.

The study set-up was a little different to the usual as production rates were investigated as opposed to just concentration alone. *E. huxleyi* produced the most VOCs in both type and concentration, while *T. pseudonana* produced the least. It was concluded however that although *T. pseudonana* may not be a huge producer of sulfur compounds, it could be a producer of non-sulfur VOCs and that a follow-up experiment should take place in which other non-sulfur VOCs are explored. This presumption was based on studies showing that, although DMS production is low in *T. pseudonana*, isoprene production is higher in *T. pseudonana* than in *E. huxleyi* (Exton *et al.*, 2013a; Keller, 1989a). If a broad range of species could undergo investigations such as this, then a data library could be

made within which characteristic volatile profiles are registered and used as suggested identifiers.

7.4 The effects of competition and grazing on VOC profiles

It is a known fact that phytoplankton living in the environment are never limited to a single species, but a collection of species within a community subjected to competition and predation that needs overcoming for survival. To aid individuals to thrive in such environments, there are metabolic activities that occur within the cell that results in the production of VOCs. DMS is a well-documented example, which is a product in response to DMSP cleavage that is initiated by grazing (Wolfe & Steinke, 1996). Mutualistic relationships between algae and bacteria can form as compounds associated with the algae may be used as energy sources, while bacteria may protect the individual from viral infection (Ramanan *et al.*, 2015). The presence of such bacteria can also have an influence on the VOC profiles obtained. This experiment was set up in a similar manner to that of chapter 3, but this time at same temperatures and with the addition of a sample in which two species were combined. Both production and consumption/degradation were seen in all samples. There is a possibility that the VOCs present already in the media are used as sources of energy (much like sulfur reducing bacteria) when exposed to the selected species and their associated bacteria. Production was assumed to be a result of both bacteria and plankton, while consumption was assumed to mostly be a result of bacterial activity as there is little research to support the idea of eukaryotic plankton utilising VOCs as an energy source. Amongst the mixed autotroph experiment samples, unialgal *E. huxleyi* samples produced the most compounds, while the range of

VOCs produced by unialgal *T. pseudonana* samples were much narrower. It is interesting to see a pattern that coincides with those seen in experiments that investigate DMS production in these select species (Keller, 1989a). In the grazer experiments, most activity was present in unialgal *O. marina* cultures, while least was seen in unialgal *D. tertiolecta* cultures. A probable explanation for this is the heterotrophic nature of *O. marina*. Even in cultures without prey, such as *D. tertiolecta*, they are known to turn to cannibalism (Montagnes *et al.*, 2011). This means that there is much more cell death/lysis that would inevitably release a variety of VOCs in the process. Mixed cultures showed contrasting behaviour in VOC activity. During lag phase, there was a huge amount of consumption/degradation, while during exponential phase a lot of VOCs were instead produced. This could be because of the VOCs produced from grazing activity. The initial consumption/degradation could be a result of bacterial consumption of VOCs already present in the media, while the production in exponential phase is likely to be the result of cell death and stress causing VOCs to be released from *D. tertiolecta* cells that were being grazed upon and *O. marina* cells that may have been subjected to cannibalistic behaviour. These fluctuations in VOCs indicate that grazing may have an influence over VOCs produced and consumed/degraded much like how DMS production increases in response to grazing (Wolfe *et al.*, 1994). Isolating bacteria present on the cells would help understand to the extent to which these populations, or even communities, affect VOC profiles. The reverse could also be investigated, whereby the same experiment would be conducted with axenic cultures. Doing this would help distinguish what VOCs are purely affected by the algal species from those affected by bacteria, and whether there are any influence in response to the combination of both.

7.5 Seasonal succession of VOCs in Brightlingsea harbour

The final study was investigating *in situ* VOCs collected and sampled from Brightlingsea. Information gained was then used to tie previous experiments together to be compared to samples found in naturally occurring environments. Similar studies have been conducted with the aim of utilising singular VOCs as a detective tool, this field study built onto this and required the investigation of VOC profiles as potential biomarkers. Seasonal succession of phytoplankton has been intensely researched (Sommer, 1986), which lead to the possibility of there also being trends in VOC composition that relates to annual shifts observed in taxa. There were compounds that dominated during one season, much like how dinophytes bloom during summer, and bacillariophytes bloom during colder months such as winter and autumn (Sommer, 1986). There were a broad range of compounds during summer which adhered to the diversity in taxa also seen during this season. On the other hand, late autumn samples did not contain as much VOCs, and the taxa were not as diverse. In this way, VOC samples responded in ways that sometimes aligned to the organisms present in the water at that point in time. To create a database of environmental VOC profiles, samples could be taken over more time points to visualise the activities that occur in the water throughout the year.

7.6 Conclusion

Although there is still much to be explored in the field, this thesis demonstrated that there is scope for new discoveries to be made in diagnostic environmental volatilomics as an up and coming discipline.

Studies conducted within this thesis have shown the influence physiological activity can have on volatile profiles of planktonic species and how such profiles could possibly in turn be used to understand or 'diagnose' specific habitats. This process could also be utilised in other environments. For example, there is growing evidence for plant-microbe interactions based on bacterial VOCs in the rhizosphere despite how little that is understood about the function of these metabolites (Kai *et al.*, 2016). Characterising the VOCs produced by bacteria and how they are used by the associated plant, could lead to the utilisation of these VOCs to help improve crop yield or their defence against environmental stress (Kai *et al.*, 2016). Another example is the use of VOC detection in ecotoxicology (Hidalgo *et al.*, 2019). In a study by Hidalgo *et al.* (2019), VOC profiles of two common strains of bacteria, *Pseudomonas fluorescens* strain SG-1 and *Bacillus megaterium* strain Mes11, were used to investigate the utilisation of their VOC profiles for the detection of pesticides in agricultural soils (Hidalgo *et al.*, 2019). The results showed that even short-term exposure of pesticides to the two species altered the VOCs produced leading to the belief that further exploration of this topic would be beneficial for agricultural purposes (Hidalgo *et al.*, 2019). Similarly, phytoplankton species distribution have been considered to be of potential use in assessing risk profiles of drinking water supplies (Rose *et al.*, 2019). This information could be studied further by investigating whether VOC profiles of particular phytoplankton populations may change when exposed to toxins in water, much like they did in soil-based bacteria observed in the study by Hidalgo *et al*. 2019 (Hidalgo *et al.*, 2019).

With more future efforts in building a database of volatile profiles, and identifying unknown VOCs produced, it is possible to develop a non-invasive, cost-effective method in which to monitor nature.

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