THE BIOGEOCHEMISTRY OF DIMETHYLSULPHONIOPROPIONATE (DMSP) IN ESTUARINE SEDIMENTS

by

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ABSTRACT

FACULTY OF SCIENCE
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A number of techniques have been developed for the measurement of dimethylsulphoniopropionate (DMSP), dimethylsulphide (DMS) and other sulphur species commonly occurring in estuarine sediments. A reaction-based flow injection analysis (FIA) system that was developed enabled the detection and quantification of trace levels of dissolved sulphide, DMSP and DMS precursors, exhibiting detection limits of ca 10^{-6} M. An acid reaction step permits the measurement of sulphide species such as S^{2-}. A base hydrolysis was found to be selective toward DMSP, whilst a borohydride reaction widened the range of DMS precursors that could be measured. Gas chromatography was used to determine elemental sulphur and DMS.

A DMSP lyase that cleaves DMSP to DMS and acrylate was purified and characterised from the green macroalga, *Enteromorpha intestinalis* that is a dominant species on many estuarine intertidal mudflats. The DMSP-lyase is located both as a soluble and detergent-released protein. The molecular weight of the soluble protein was approximately 25.2 kDa. Three hydrophobic variant forms were isolated from the detergent-solubilized activity. The optimum pH value of this DMSP lyase is pH8.0 and the highest measured enzyme activity was observed at 37 °C. However, the influence of NaCl concentration is insignificant. Based on the results of inhibition studies, it is suggested that this DMSP lyase is a metal dependant enzyme but not a thiol-based enzyme. The enzyme activity increased with $MgCl_2$ concentration but decreased with $CaCl_2$ concentration. The K_m and V_{max} of the soluble DMSP lyase were 0.286 mM and 21.9 nmole DMS/min/mg protein at room temperature, respectively.

Green algae were a major contributor of DMSP to intertidal sediments of the Tamar estuary. High concentrations of DMSP were found in the surface sediments, accompanied by high DMSP lyase activities. The DMSP lyase activities decreased with decreasing DMSP content throughout the sediment depth indicating that there was a strong relationship between them. Due to its very low concentration DMSP is unlikely to be a major source of sulphur in the sediments. Elemental sulphur is the major sulphur compound in sediments accounting for about 30 to 40 % of the total sulphur which is ca 1.8 wt % of dry weight sediment. Metal sulphides may control the low levels of 'dissolved' sulphide in sediment interstitial water observed in this study.

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Declaration

All the studies presented in this thesis were carried out by the author.

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Howard, A.G. and C.Y, Yeh, *Anal. Chem.*, 1998, 70: 4868-4872.

Abbreviation

3-MPA	3-mercaptopropionic acid
3-MTPA	3-methylthiopropionate
CCN	cloud condensation nuclei
CH_3SH	methylmercaptan
$CH_3SO_2CH_3$	dimethylsulfone
CH_3SSCH_3	dimethyldisulfide
*p*CMB	*p*-chromercuribenzoate
p-CMBS	*p*-chloromercuribenzenesulfonic acid
COS	carbonylsulfide
CS_2	carbon disulphide
DI	Deionized
DFPD	dual flame modification of the FPD
DMDS	dimethyl disulphide
DMS	dimethylsulphide
DMS-2-MP	dimethylsulphonio-2-propionate
DMSAcet	dimethylsulphonioacetate
DMS-pr	DMS precursors
DMSO	dimethylsulphoxide
DMHSB	4-dimethylsulphonio-2-hydroxybutyrate
DMSP	dimethylsulphoniopropionate
DMSPent	dimethylsulphoniopentanoate
DTT	dithiothreitol
ECD	electron capture detector
EDTA	ethylenediaminetetraacetate
FABMS	fast atom bombardment mass spectrometry
FIA	flow injection analysis
FID	flame ionisation detector
FPD	flame photometric detection
FPLC	fast protein liquid chromatography
FW	fresh weight

GC-MS	gas chromatography-mass spectrometry
GSC	gas solid chromatography
H_2S	hydrogen sulphide
HIC	hydrophobic interaction chromatography
IAA	iodoacetamide
KDMSB	2-keto-4-dimethylthiobutyrate
K_m	Michaelis-Menten constants
KMTB	2-keto-4-methylthiobutyrate
LOI	loss on ignition
M-3-MTPA	methyl-3(methylthio)-propionate
MeSH	methanethiol
Met	methionine
MMPA	3-methylmercaptopropionate
MSA	methanesulphonic acid (CH_3SO_3H)
MTHB	4-methylthio-2-hydroxybutyrate
MTOB	4-methylthio-2-oxobutyrate
NSS–SO_4^{2-}	non-sea-salt sulphate
$(NH_4)_2SO_4$	ammonium sulphate
PFPD	pulsed flame photometric detector
pI	isoelectric points
PMSF	phenylmethylsulphonylfluoride
PMT	photomultiplier tube
R-GHS	reduced glutathione
S^o	elemental sulphur
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SMM	S-methylmethionine
SO_2	sulphur dioxide
SO_4^{2-}	sulphuric acid
TLC	thin layer chromatography
TLE	thin layer electrophoresis
V_{max}	maximum velocity

CHAPTER ONE

The biogeochemical properties of dimethylsulphoniopropionate (DMSP)

1.1 The natural sulphur cycle

Sulphate is the most stable form of sulphur in the presence of oxygen and is the predominant sulphur species in soils, sediments, seawater and fresh waters. Sulphate reduction is therefore required for the production of volatile sulphur compounds to be released to the atmosphere. In the global biogeochemical sulphur cycle, reduction occurs by two biological pathways: assimilatory and dissimilatory routes. The two main purposes of biological sulphate reduction are to biosynthesise organosulphur compounds which can be used by algae and plants and to use sulphate as a terminal electron acceptor to support respiratory metabolism under anaerobic conditions. The former process is called assimilatory sulphate reduction and the latter is dissimilatory sulphate reduction.

H_2S is the major product of dissimilatory sulphate reduction which is carried out by anaerobic bacteria in the absence of O_2. The large amount of H_2S which is produced in the marine or coastal environments cannot usually be transported to the atmosphere, and is either reoxidised abiotically with O_2, Fe oxides and Mn oxides producing a combination of oxidation products including $S_2O_3^{2-}$, elemental sulphur, SO_3^{2-} and SO_4^{2-} at the oxic-anoxic interface, or precipitated in the form of metal-sulphides and locked up in sediments and sedimentary rocks. Once produced, these oxidised intermediates $S_2O_3^{2-}$, elemental sulphur and SO_3^{2-} are actively metabolised.

They can undergo further intermediate oxidation, intermediate reduction and disproportionation to sulphide and sulphate (Jorgensen, 1991; Jorgensen, 1990; Canfield, 1996).

In oxic environments, a variety of organosulphur compounds and amino acids are produced by assimilatory sulphate reduction by bacteria, algae and plants (Anderson, 1980). The mechanisms of this reduction have been intensively studied using the green alga *Chlorella* (Andreae, 1990). The first organosulphur metabolite cysteine was assimilated from sulphate by a complex process (Fig. 1.1). The sulphur-containing amino acids, namely, cysteine, homocysteine and methionine, are incorporated into proteins. Cysteine and methionine are the major sulphur amino acids in plants and represent a very large fraction of the sulphur content of biological materials (Giovanelli et al., 1980). In marine algae, dimethylsulphoniopropionate (DMSP) is produced by a multi-step process from methionine, whereas S-methyl-methionine,which is also a precursor of dimethylsulfide (DMS), is produced from methionine by terrestrial plants.

In 1935 Haas (Haas, 1935) identified an odoriferous gas emitted from the marine red alga *Polysiphonia lanosa* as DMS. The source of the DMS was subsequently shown by Challenger and Simpson (Challenger & Simpson, 1948) to be evolved from DMSP. This was the first direct linkage between DMS and DMSP in the natural sulphur cycle.

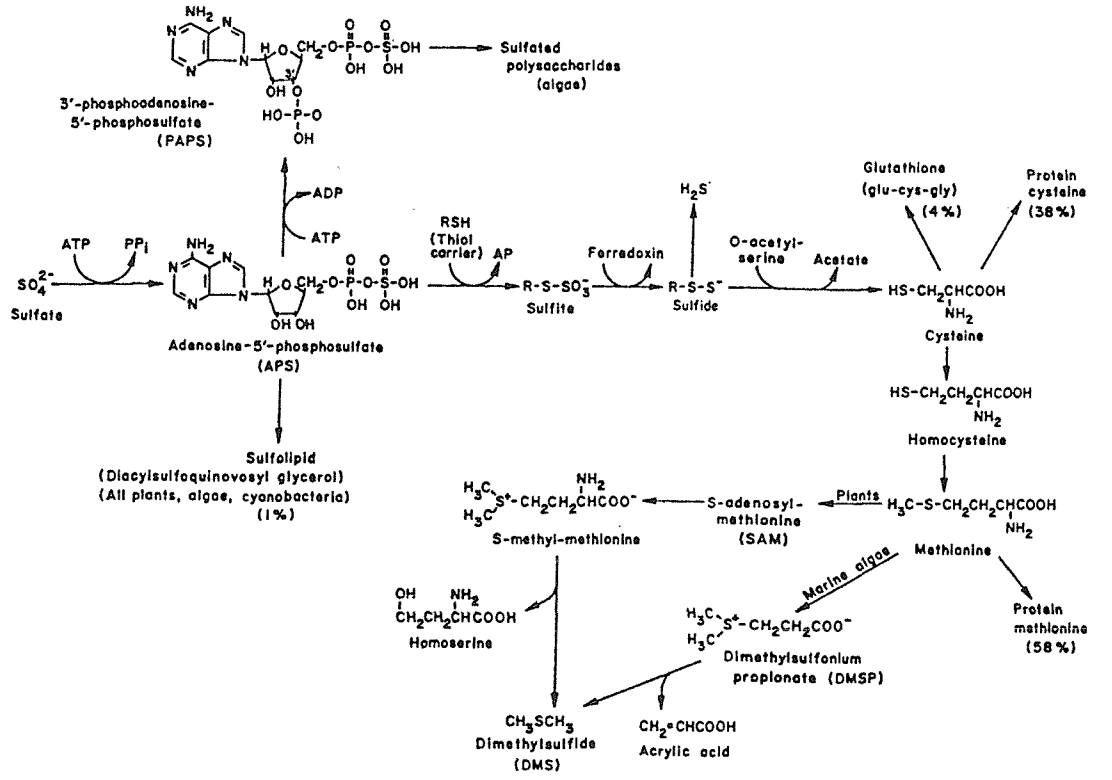


Figure 1.1 Major metabolic pathways of sulphur in algae and plants. The percentages represent the approximate distribution of the major organosulphur compounds in *Chlorella* (Andreae, 1990).

In order to complete the global sulphur cycle a volatile or gaseous sulphur species is required to transport the element from the sea through the atmosphere to land. This role was originally assigned to the hydrogen sulphide (H_2S) released from coastal areas, but the 0.2 ppt that was required to balance the budget was never found. In 1972, Lovelock *et al.* (Lovelock et al., 1972) measured the concentration of H_2S in seawater and found that the ocean water is too oxidising for H_2S to exist in sufficiently large concentrations. This is believed to be due to sulphide being oxidised with a half-life of 20 minutes (Ostlund & Alexander, 1963). Lovelock carried out the first measurement of DMS in seawater and suggested that DMS filled the important missing link in the natural sulphur cycle between the ocean and the atmosphere (Lovelock et al., 1972).

In addition to DMS, other volatile sulphur compounds have also been found in ocean waters including carbonylsulfide (COS), carbon disulphide (CS_2), methylmercaptan (CH_3SH) and dimethyldisulfide (CH_3SSCH_3). DMS, however, is usually dominant (Andreae et al., 1983). The flux of DMS from the oceans (0.6-1.6 Tmol S $year^{-1}$) is believed to be responsible for about 90% of the total emissions of gaseous sulphur from oceans to the atmosphere (Andreae, 1990). The rate of transfer is calculated from the concentration difference across the ocean-air interface and the transfer velocity. The transfer velocity is dependant on wind speed, sea state, water temperature, sea waves and biomass in the surface water (Andreae et al., 1985, Liss & Slater, 1974).

On release of DMS into the atmosphere it is rapidly photo-oxidised, principally by hydroxyl and nitrate radicals (Fig. 1.2). The major oxidation products which have been identified are sulphur dioxide (SO_2), sulphuric acid (SO_4^{2-}), methanesulphonic acid (CH_3SO_3H; MSA) and dimethylsulfone ($CH_3SO_2CH_3$). The effects of these compounds are two-fold. From the climatic viewpoint, sulphate and MSA resulting from DMS form a non-sea-salt sulphate ($NSS-SO_4^{2-}$) aerosol which produces potential cloud condensation nuclei (CCN), causing the nucleation of cloud droplets. The increased concentration of CCN leads to an increase in cloud albedo either by light scattering or reflectance from nucleated clouds. The result is that less sunlight reaches the Earth's surface, in turn decreasing the surface temperature. Therefore, the rate of photosynthesis and production of DMS by marine phytoplankon is reduced. This effect has been put forward as a negative feedback mechanism (Fig. 1.3). According to the geophysiology or Gaia theory this could regulate the Earth's climate to yield the best conditions for the living biomass (Charlson et al., 1987). Other sources of sulphate aerosols such as anthropogenic and volcanic SO_2 emission are also capable of increasing cloud albedo, but this is only regionally important (Charlson & Rodhe, 1982, Falkowski et al., 1992).

The secondary effect of DMS release is the increased acidity of atmospheric aerosols and precipitation (Andreae, 1990; Charlson, 1982; Grosjean & Lewis, 1982). The natural pH of rainfall is about pH 5.6. It was originally thought that the main

contributor to this natural acidity was the dissolution of CO_2 and that lower pH values were due solely to the anthropogenic input of acidic gases such as SO_2. Hence, once it was found that DMS could be photooxidised to SO_2 and MSA in the marine atmosphere, then it was logical to expect that the localised regions with increased DMS production might experience a decrease in the pH of precipitation. Based on the above findings, Robinson proposed an explanation of the extinction of dinosaurs due to the dramatic increase of biogenically produced DMS resulting in increased precipitation acidity in the late Cretaceous (Robinson, 1995).

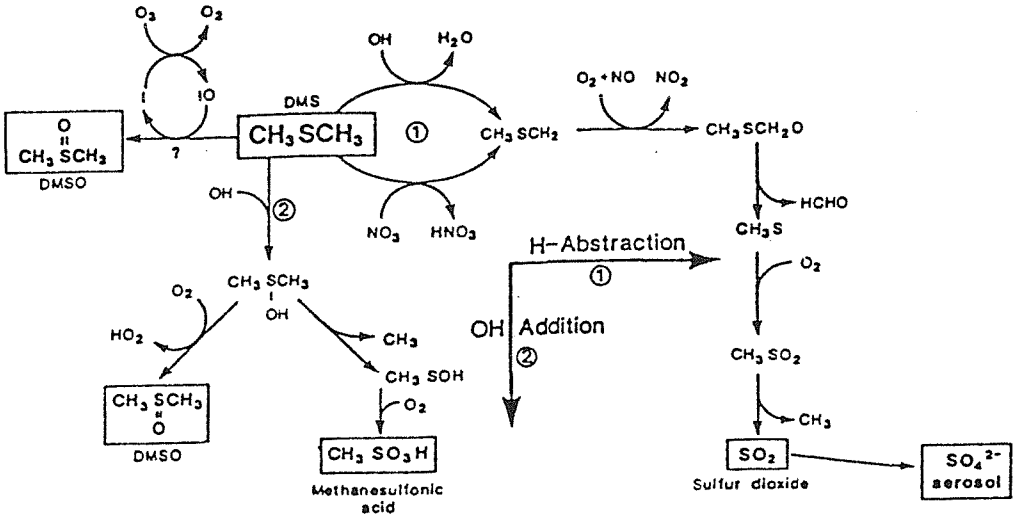


Figure 1.2 Reaction pathways for the oxidation of DMS in the atmosphere (Malin et al., 1992).

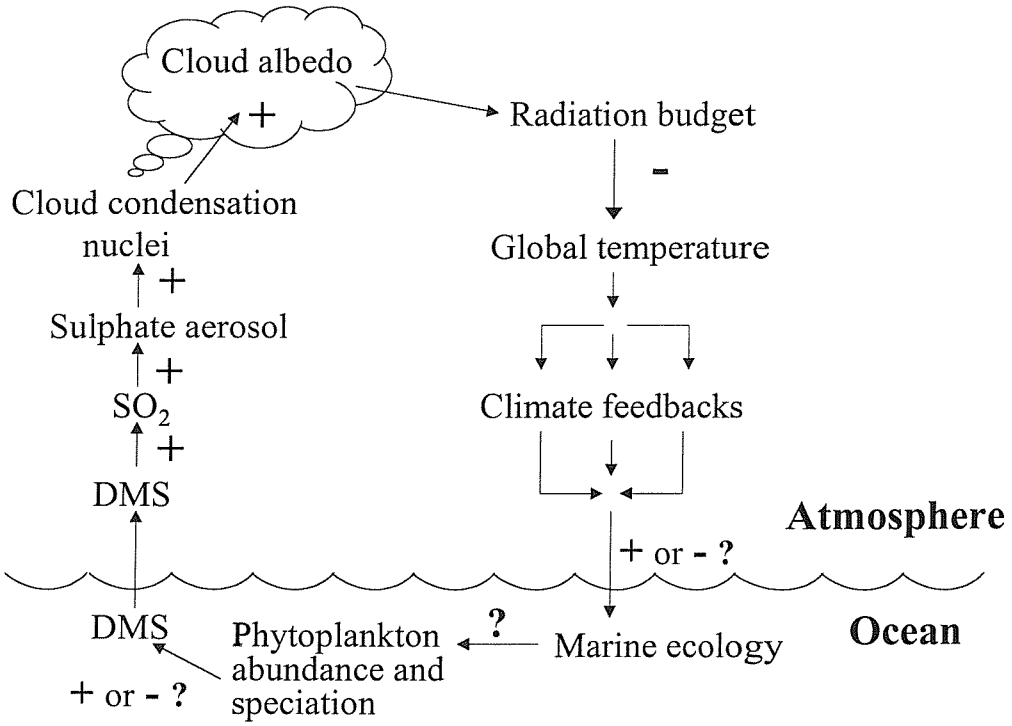


Figure 1.3 Proposed feedback cycle between climate and marine DMS production. The pluses and minuses indicate if an increase in the value of the preceding parameter in the cycle is expected to lead to an increase (+) or decrease (–) in the value of the subsequent parameter (Andreae, 1990).

Volatile sulphur compounds are also emitted by terrestrial biota, volcanoes and by anthropogenic activity. The manmade flux of SO_2 is mostly due to the combustion of fossil fuel. Andreae has estimated that the anthropogenic sulphur emissions (2.5 Tmol S $year^{-1}$) represent about 50% of the total flux of gaseous sulphur to the atmosphere (Andreae, 1990).

Significant non-biological natural sulphur fluxes of SO_2 and H_2S are emitted by volcanoes. Volcanic sulphur is estimated to produce 0.3 T mol S $year^{-1}$, about 10-20% of the biogenic sulphur fluxes from terrestrial biota. These are uncertain however due to the difficulty in obtaining representative data from land biomass, the low levels of sulphur species and the technical problems involved in accurately determining emission fluxes. The total flux of sulphur from soil and plants to the atmosphere is

believed to be ca. 0.15 to 0.5 T mol S $year^{-1}$; much lower than the flux from the oceans.

1.2 Dimethylsulphoniopropionate (DMSP)

1.2.1 Introduction

DMSP is a tertiary sulphonium betaine (Fig 1.4) which was first extracted and identified from the red alga *Polysiphonia fastigiata* in 1948 by Challenger and Simpson (Challenger, 1948). DMSP is a natural sulphonium compound which is produced mainly by marine algae and some other higher plants (Karsten et al., 1994, Karsten et al., 1990a, Pakulski & Kiene, 1992, Reed, 1983, Weber et al., 1991, White, 1982). DMSP has been found in a wide range of marine sources such as macro- and micro-algae, salt marsh plants (Dacey et al., 1994, Otte & Morris, 1994, Weber et al., 1991), fish (Dacey, 1994 ; Ackam, 1966; Ackam, 1972; Ackam, 1967), shellfish (Ackam, 1968 ; Iida, 1986), cyanobacteria (Vogt et al., 1998, White, 1982), plankton (Ackman et al., 1966, Dacey & Wakeham, 1986, Malin et al., 1993) etc. Frequently the presence of DMSP is recorded using an indirect method. Hence the DMSP occurrence in such cases is merely inferred and is not a true measurement of DMSP.

It has been suggested that DMSP is associated with osmoregulation and cryoprotection (Dickson et al., 1980, Dickson et al., 1982, Vairavamurthy et al., 1985). It is also believed to be the major natural precursor of atmospheric DMS, the oxidised products of which subsequently affect climate and weather.

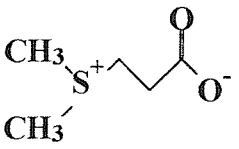


Figure 1.4 Dimethylsulphoniopropionate

1.2.2 Dimethylsulphide precursors

In 1935, DMS was found to be produced by the marine alga *Polysiphonia fastigata* (Haas, 1935), Challenger and Simpson first isolated and characterised DMSP from *Polysiphonia fastigata* in 1948 (Challenger & Simpson, 1948). Subsequently, they developed a presumptive test for DMSP based on the evolution of DMS by treatment with cold, concentrated alkali (Challenger & Simpson, 1948). As a result of these studies, they suggested that algal DMSP was a potential biological precursor of natural DMS. DMSP has since attracted particular interest as a biogenic precursor of DMS in the marine and estuarine ecosystem.

It is currently believed that DMSP is the most common DMS precursor. Besides DMSP, there are some other DMS precursors found in living organisms including S-methyl methionine (McRorie et al., 1954, White, 1982), 4-dimethyl sulphonio-2-methoxybutyrate (Patti et al., 1992, Sciuto et al., 1982), gonyol (Nakamura et al., 1993), gonyauline (Nakamura, 1990, Nakamura et al., 1992) and less certainly dimethyl sulphonio-5-pentanoate (Larher & Hamelin, 1979).

1.2.3 The biosynthesis of DMSP

In 1962, Greene exposed the macroalga *Ulva lactuca* to various radiolabelled methionines and showed that the methyl carbon, sulphur, hydrogen and α-carbon of methionine were incorporated in DMSP. This led him to suggest that DMSP may be synthesised from methionine by deamination, decarboxylation, oxidation and methylation (Fig. 1.5); the order of these steps, however is still unknown (Greene, 1962, Hanson et al., 1994). Since then, a wealth of research papers have reported that the mechanism of DMSP biosynthesis is performed differently in various marine algae and higher plants (Hanson et al., 1994, Uchida et al., 1996); the complete pathway has not, as yet been fully understood.

The biosynthesis of DMSP from methionine by the higher plant *Wollastonia biflora* proceeds via S-methylmethionine and dimethylsulphoniopropionaldehyde; whereas, the route utilised by the green alga *Enteromorpha intestinalis* and three phytoplankton species is completely different (Gage et al., 1997)(Fig. 1.6). The first reaction in this route is the loss of the amino group to produce the 2-oxo acid MTOB. Based on ^{15}N-labeling evidence it was suggested that the amino group is removed via transamination rather than deamination. The following reactions are reduction to MTHB, S-methylation to yield DMSHB and oxidative decarboxylation to yield DMSP. Substrate specific enzymes catalysing the first three reactions were detected and characterised in cell free extracts of the green alga *Enteromorpha intestinalis* by Summers et al. (Summers et al., 1998). Radiometric assays of these enzymes showed that their activities in other green algae were as high as those in *E. intestinalis,* but were more than 20 times lower in algae without DMSP. Moreover, the intermediate DMSHB of DMSP synthesis played an osmoprotectant role which shows that the DMSP biosynthesis pathway might be able to tolerate a wide range of fluctuating osmotic potentials.

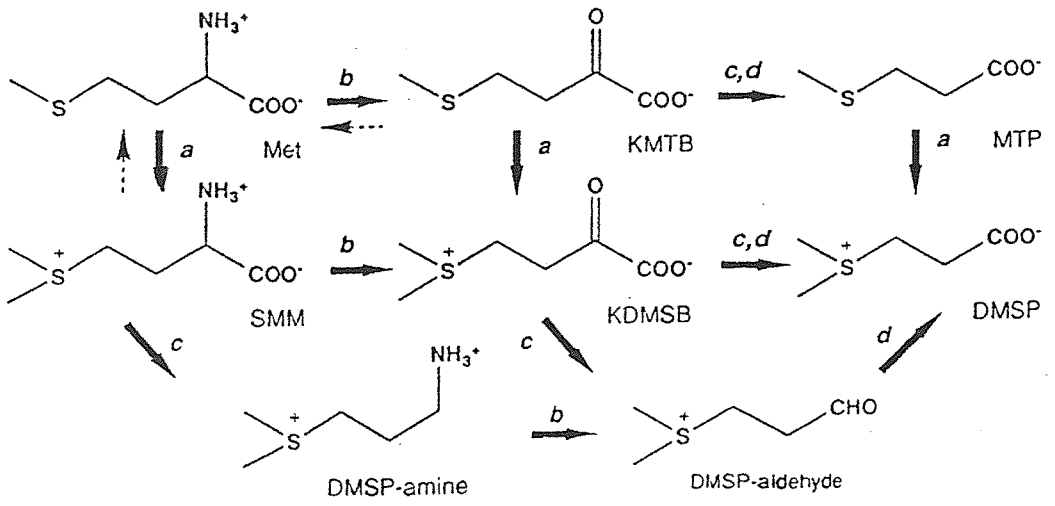


Figure 1.5 A metabolic grid showing possible pathways of DMSP biosynthesis from methionine (Met). The steps involved are methylation (a), oxidative deamination or transamination (b), decarboxylation (c), and oxidation (d), in various orders. Note the broken arrows denoting possible interconversion of Met and SMM via the SMM cycle and of Met and 2-keto-4-methylthiobutyrate (KMTB) via transamination. Except for 2-keto-4-dimethylthiobutyrate (KDMSB), all the hypothetical intermediates DMSP-aldehyde, 3-Dimethylsulfoniopropionaldehyde; DMSP-amine, 3-dimethylsulphoniopropylamine shown are known from plant, animal, or microbial metabolism; KDMSB would be expected to be unstable. (Hanson et al., 1994).

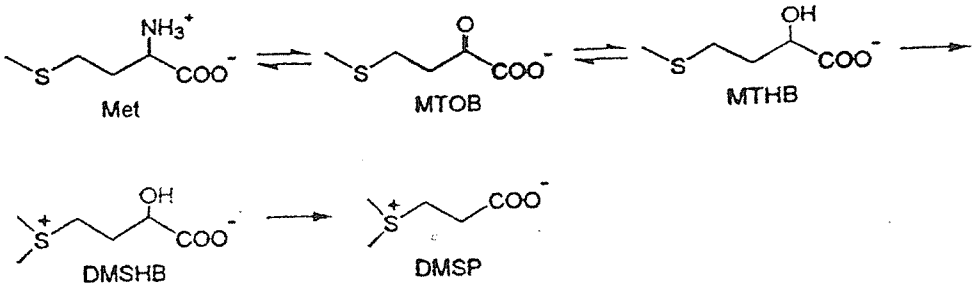


Figure 1.6 The biosynthesis of DMSP from methionine in marine algae (Gage et al., 1997).

1.2.4 The biological importance of DMSP

Since DMSP was first suggested as a DMS precursor by Challenger and Simpson (Challenger & Simpson, 1948) and subsequently found to be omnipresent in the marine ecosystem, it has been accepted as an important component of the global sulphur cycle. There is compelling evidence that DMSP has osmoregulatory and cryoprotectant functions in marine algae (Dickson et al., 1980, Dickson et al., 1982, Karsten et al., 1991a, Karsten et al., 1992). In addition, Cantoni and Anderson (Cantoni & Anderson, 1956) found a DMSP cleaving enzyme in algae. These findings all point to the biological roles of DMSP. The concentration of DMSP in algae is influenced by a wide range of variables such as temperature, light length, season, climate, salinity, water stress, species of algae, nutrients and populations and locations of phytoplankton (Malin et al., 1993, Reed, 1983, Storey et al., 1993, Turner et al., 1988).

1.2.4.1 Cryoprotection

Freezing can restrict many enzymatic reactions involved in photosynthesis. Some of these reactions can however still take place below 0°C as long as the cytoplasm is kept liquid. The increased cytoplasmic osmolyte concentration in some organisms resulting from the presence of DMSP results in a decreased freezing point, which in turn extends the tolerance of the organism to low temperatures. The variation of DMSP content with temperature in Antarctic phytoplankton has been interpreted as leading to DMSP having a cryoprotectant role (Karsten et al., 1992, Kirst et al., 1991). Five Antarctic Chlorophyceae were grown for a year at 10 °C and at 0 °C under various light source strengths in the laboratory. The DMSP levels were found to be five times higher under the lower temperature conditions (Karsten et al., 1992).

1.2.4.2 Osmoregulation

Organisms such as algae can experience environmental stress as a result of high or fluctuating salinity or desiccation in estuarine habitats. In order to continue their normal functions these organisms must adjust the composition of their intracellular fluids to maintain the balance of osmotic potential. It has been found that the DMSP content varies with salinity in some phytoplankton species, suggesting that DMSP may play an osmoregulatory role, protecting enzymes against inhibition and denaturation at high ionic strengths (Vairavamurthy et al., 1985).

In addition, from mass spectral evidence, it has been reported that DMSP may replace glycine betaine, an ammonium analog of DMSP, involved in osmoregulation in some higher plants (Paquet et al., 1994a). In 1986, Andreae proposed that when nitrate levels are low, marine phytoplankton may have the ability to increase the biosynthesis of DMSP rather than glycine betaine. Subsequently, in culture studies, it was also found that excess nitrogen causes the decreased accumulation of DMSP (Turner et al., 1988).

The effects of extreme hyper- or hypo-osmotic stresses in algae were studied by Kirst in 1989 (Kirst, 1989). The results showed that severe osmotic stress can inhibit the photosynthesis and respiration of algae and cause death. Organisms grown near the limit of salinity tolerance could however survive in the short term by osmotic adjustment.

DMSP content and inorganic ion concentration changes had been recorded in the marine green alga *Ulva lactuca* in response to salinity (Dickson, 1980). It was found that under hypo-osmotic stress the DMSP concentration increased linearly with increasing salinity from 30% to 100% of seawater salinity. A stable accumulation of K^+ and Cl^- ions appeared in the hyper-osmotic condition and an accompanying synthesis of DMSP with a time lag of three hours was found following the transient accumulation of Na^+ ions. Some other organic compounds were also examined,

including free amino acids, proline and sugars. Only DMSP responded to the external changes in a way that suggested that it was involved in osmoregulation. The time lag of three hours might be due to the higher energy needed for DMSP synthesis than for the accumulation of free ions (Dickson & Kirst, 1986, Reed, 1983).

The same species of algae collected from different habitats may behave differently to external water stress. Reed demonstrated that *Polysiphonia lanosa* collected from an estuarine site could survive over a greater range of salinities than those collected from a marine site (Reed, 1983).

1.2.4.3 The effect of light on DMSP content

It can be expected that the light intensity and day length would influence the accumulation of DMSP if its synthesis is involved in the photosynthetic cycle. In 1990, Karsten et al. examined five Antarctic green algae (*Urospora penicilliformis, Ulothrix implexa,, Ulothrix subflacida, Enteromorpha bulbosa and Acrosiphnia arcta*) grown under different light intensities and fluctuating day lengths and compared them with algae grown under natural conditions (Karsten et al., 1990b). It was found that the DMSP content of all species decreased with increasing day length. These algae are also affected by the light intensity, DMSP content increasing with increasing photon flux. The *Urospora penicilliformis* appears as a 3.5 fold increase in DMSP concentration in summer than that measured in winter.

In a further study of light response, Antarctic macroalgae was compared with algae from temperate and sub-temperate regions (Karsten et al., 1991b). It was found that maximal growth occurred at lower irradiation levels with Antarctic samples compared with those from more temperate regions. It was suggested that the Antarctic species might adapt to low photo flux rates better than other samples. Whilst most macroalgae maintained the DMSP content in the dark, the DMSP concentration of *U. peniciliformis* and *A. arcta* decreased dramatically after four weeks. The reason for

this decrease might be due to metabolic degradation of DMSP as the release of DMSP into the incubation media was not observed. Karsten et al. proposed that the DMSP biosynthesis is controlled by light dependent enzymes rather than driven by photosynthetically produced NADPH and ATP, which were obviously not used for the DMSP accumulation in the dark (Karsten et al., 1992). Further evidence supporting this argument is that several enzymes participating in amino acid metabolism are light stimulated in algae and methionine is a precursor in the DMSP biosynthesis (Tischner and Huttermann 1980). Enzymes involved in the assimilatory sulphate reduction are also often activated by light.

1.2.4.4 The effect of nitrogen deficiency on DMSP content

It has been hypothesised that DMSP can substitute for other compatible solutes in marine phytoplankton when they are under nitrogen deficient conditions. Preliminary experiments with *Emiliania huxleyi* cultures by Turner et al. showed that less internal DMSP was produced in the nitrate complemented media (Turner et al., 1988). Also, if nitrate was added into a nutrient depleted culture, the cells could decrease their DMSP concentration in 24 hours. These results demonstrated that excess nitrogen resulted in a decreased accumulation of DMSP.

Further experiments performed using the unicellular alga *Tetraselemis subcordiformis* showed that nitrogen deficiency can result in an increase in DMSP accumulation (Grone & Kirst, 1992). The increased DMSP concentration occurred in two stages. Firstly, DMSP levels increased during the first 24 hours from 20 to 35 μmole DMSP per mg Chlorophyll 'a' and then a further increase to 44 μmole DMSP per mg Chlorophyll 'a' appeared after 14 days. If algae were grown in a complete medium under identical conditions, the DMSP concentration was normally maintained in the range 20 to 27 μmole DMSP per mg Chlorophyll 'a'. Nitrogen deficiency affected the growth of the algae. It decreased during the first four days and then stopped under nitrogen deficient conditions.

1.2.4.5 DMSP as a methyl donor

The suggestion that DMSP was a methyl donor was first put forward by du Vigneaud et al. in 1948. Dimethylthetins including DMSP, DMSAcet and sulphobetaine were fed to rats under methyl-free diet conditions (du Vigneaud et al., 1948, Maw & du Vigneaud, 1948). It was found that these dimethylthetins and the methyl donating compound choline and betaine were all able to increase the growth in methyl-free diet rats. It was therefore suggested that these methyl sulphonium compounds could provide biologically released methyl groups and that DMSAcet and homocysteine could efficiently replace methionine in those methyl-free diet rats. In further studies Maw and du Vigneaud found that DMSP and DMSAcet could increase the growth of rats on a methionine-free diet in *vivo* (Maw & du Vigneaud, 1948). Also in *vitro* experiments, employing enzymes found in the kidney and liver of rats and guinea pigs, showed that DMSPAcet is twenty times as active as betaine in methionine formation (Ishida & Kadota, 1968). DMSP was also highly active.

1.2.5 The breakdown of DMSP

The breakdown of DMSP from aquatic flora could be brought about in a number of ways: base hydrolysis, microbial activity (primarily bacterial) or enzymatic cleavage. The stoichiometric cleavage of DMSP to acrylic acid and DMS was reported as a specific reaction of this sulphonium compound by Challenger et al. (Challenger & Hayward, 1954). The reaction carried out by base hydrolysis is extremely slow at the pH of seawater (~8), the half-life being about 8 years at 10°C (Dacey & Blough, 1987). Thus, it is evident that biological processes play a major role in the breakdown of DMSP.

Such biological processes could occur intra- or extracellularly. In seawater, the dissolved DMSP (from zooplankton grazing of phytoplankton or direct excretion by algae) or particulate DMSP (from decaying algae and faecal pellets) is broken down

by micro-organisms. There are two main DMSP metabolism pathways, DMSP cleavage and demethylation (Dacey & Wakeham, 1986). A variety of marine bacteria have been found to cleave DMSP to DMS and acrylate. However, based on the mass balance of the total DMSP consumption with DMS production, less than 30% of the DMSP was found to be converted to DMS by this route. The other major pathway is demethylation of DMSP to give 3-mercaptopropionate with 3-S-methylmercaptopropionate as a possible intermediate (Taylor & Gilchrist, 1991).

1.2.5.1 DMSP lyase in algae

When Challenger identified DMSP from *Polysiphonia fastigata* and suggested that DMSP is likely to be a precursor of DMS, he also presumed that there might be an enzyme catalysing DMSP cleavage in algae. In 1956 DMSP lyase activity was found in crude extracts of the red alga *Polysiphonia lanosa* by Cantoni and Anderson (Cantoni & Anderson, 1956). They suggested that this enzyme is bound to protoplasmic particles and requires sulfhydryl compounds for activity and the pH optimum is around 5.1. This enzyme cleaved DMSP to DMS and acrylate and showed relatively narrow substrate specificity. A further enzyme was found in a cell free extract of the unicellular marine algae *Gyrodinium cohnii*. This is also a sulfhydryl enzyme, but the pH optimum is around 6.2 and it is activated by anions at high concentrations (Kadota & Ishida, 1972). Based on the results observed they suggested that salts alter enzyme activity by changing the organised structures of the protein molecule. This enzyme activity was inhibited by KCN, iodoacetamide (IAA) and *p*-chromercuribenzoate (*p*CMB).

Based on above findings and the high levels of DMSP commonly observed in marine algae, it was suggested that DMSP lysis occurs intracellularly and that the enzyme might be involved in the removal and accumulation of DMSP in response to natural changes in salinity, temperature, seasonal water stress and light.

Recent studies have described the properties of DMSP lyases in crude and partially purified extracts of macro- and micro-algae. These results showed that the widely distributed algal DMSP lyases have fairly different enzyme activities or functions, even in closely-related species (Steinke et al., 1996, Steinke & Kirst, 1996). For example, a DMSP lyase in a microalga *Phaeocystis sp.* showed that its pH optimum was 10.5 or higher, which was spectacularly high compared with available data for the enzyme from other organisms (Stefels & Dijkhuizen, 1996). Based on their experimental results, it was concluded that this DMSP lyase is membrane bound and located extracellularly. The requirement of reduced thiol groups in extracts and the inhibition by *p*-chloromercuribenzenesulfonic acid (*p*-CMBS) suggested cysteine residues to be at the active site of the enzyme. Production and removal of DMSP did not show any significant association with the short term regulation of osmotic potential of cells by changing salinity.

The first isolation and purification of a DMSP lyase from algae was reported in 1995 (Nishiguchi & Goff, 1995). There are two proteins with enzyme activity found in the red alga *Polysiphonia paniculata* differing in their specific enzyme activity, the Michaelis-Menten constants (K_m), isoelectric points (pI) and molecular weights. This enzyme is membrane-bound but the exact location in the algal cells is still unclear. Addition of either magnesium or calcium increased the DMSP lyase activity at concentrations ranging between 20 and 40μM but had only little effect above that range. In contrast, the addition of the divalent metal ion chelators ethylenebis (oxyethylenenitrilo) tetraacetic acid and ethylenediaminetetraacetate dropped the enzyme activity. The activity could be recovered when either the chelators were removed or calcium or magnesium were added to the enzyme medium.

DMSP lyase has also been purified and characterised from the green macroalga *Ulva curvata* (Desouza et al., 1996). The DMSP lyase activity was found in one soluble and three membrane-bound proteins which all cross-reacted with the polyclonal antibody obtained from the bacterial (*Alcaligenes* strain M3A) DMSP lyase. All of these enzymes had an identical molecular weight of 78 KDa and the pH optimum (pH8) is similar to that of seawater. There was no significant influence on the activity

of soluble DMSP lyase in response to changing NaCl concentration. Based on *in vivo* experiments it was found that the DMSP lyase activity increased during the first 24 hours after collection and then decreased with decreasing endogenous DMSP content and with culture aging. The supplemented DMSP could maintain the DMSP lyase activity even when the culture was five days old.

1.2.5.2 DMSP lyase in bacteria

DMS is rapidly emitted from sea and estuarine water samples by adding DMSP (Kiene, 1990, Kiene, 1991, Turner et al., 1988, Wakeham et al., 1987). This activity remains, to some extent, after sterile filtration using 0.2 μm membrane filters. This implies that the production of DMS takes place in both living biomass and as a result of soluble DMSP lyase enzymes present in the water samples either naturally or as a result of cell rupture caused by filtration. It was also found that after 10 hours the DMS production rate increased again in the same filtered samples suggesting that the DMS generation could be due to the regrowth of bacteria in the filtrate (Kiene, 1990). Autoclaving completely stopped the evolution of DMS. Addition of antibiotics had little effect on the production of DMS over the first few hours of incubation, but significantly inhibited subsequent production. Chloroform had no effect on the conversion of DMS over a range of concentrations (0.25 to 1.25 mM). The potential for microbial conversion of DMSP to DMS in marine water samples is therefore significant.

Several marine bacteria that are able to metabolise DMSP have been isolated from seawater (Dacey & Blough, 1987, Diaz et al., 1992, Kiene, 1990, Kirst et al., 1991, Ledyard & Dacey, 1994, Ledyard et al., 1993). A bacterium isolated from the Sargaso Sea can cleave DMSP to DMS and acrylate. It can be grown on acrylate as a substrate but does not metabolise DMS (Ledyard et al., 1993). Further studies found that DMSP appeared to be transported across the cell membrane and accumulated in the bacterial cells (Ledyard & Dacey, 1994). Subsequently, DMS production was initiated. This result implied that there might be an intracellular DMSP lyase present; therefore, the

uptake of DMSP into the cells does precede the cleavage to DMS in this bacterium. The utilisation of acrylate might be another reason for the DMSP degradation to DMS and acrylate by the bacteria so that the acrylate can be used as an energy source.

Recently, a DMSP lyase (dimethylpropio-dethiomethylase), which catalyses the cleavage of DMSP, was purified and characterised from the bacterium *Alcaligenes* species (M3A) by De Souza (De Souza & Yoch, 1995b). This enzyme is inducible by its substrate, DMSP; its product, acrylate; and several acrylate analogs. The K_m values for DMSP determined by cell culture and study of the pure enzyme are similar to the values found for microbes in aerobic seawater and aerobic sediment. It was suggested that the microbes in these ecosystems might have similar enzymes to the one purified from *Alcaligenes*. Furthermore, based on the results of kinetic inhibitor studies, De Souza proposed that this DMSP lyase may be located in the periplasmic space or loosely associated with the outer cell membrane (De Souza & Yoch, 1996). Subsequently, the polyclonal antibody was prepared from the *Alcaligenes* DMSP lyase and it was found that this antibody was equally reactive against the *Alcaligenes* and other marine bacterial DMSP lyases. Therefore, due to the finding of cross-reaction with polyclonal antibodies, it may be possible to employ the bacterial antibody as a probe to identify and quantify DMSP lyase in marine environments.

In 1996 an anaerobic marine DMSP cleavage bacterium (strain W218) was isolated from anoxic intertidal sediment (van der Maarel, 1996). This bacterium can reduce sulphate and acrylate. Based on its characteristics and the 16S rRNA gene sequence, strain W218 was classified to be a *Desulfovibrio* species and named *Desulfovibrio acrylicus*. Subsequently, a DMSP lyase was purified from this bacterium and its enzyme activity can be induced by DMSP and acrylate (van der Maarel et al., 1996b). It was found that DMSP was the only substrate of this enzyme and the K_m and V_{max} for DMSP were 0.45 mM and 2590 μmole min^{-1} mg $protein^{-1}$, respectively at 30 °C. The molecular weight of this enzyme was 49 kDa according to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme activity was not affected by 100 mM NaCl.

1.2.5.3 DMSP lyase in fungi

It has been reported that salt mash ecosystems emit more DMS per unit area than any other ecosystem (Steudler & Peterson, 1985). DMS emissions occur mainly over the areas populated by cordgrass *Spartina alterniflora* containing between 80 and 280 μmole per gram (dry weight) of DMSP (Dacey et al., 1994, Otte & Morris, 1994). Studies also showed an increase of DMS emission during senescence and decay of this grass. Furthermore, it was estimated that fungus is the major contributor of cordgrass shoot decomposition (Newell 1996). Since then, considerable attention has been given to the probability that fungi play an important role in the natural sulphur cycle.

Recently it has been discovered that a DMSP lyase from the fungus *Fusarium lateritium* can cleave DMSP and release DMS (Bacic & Yoch, 1998). This fungus has been isolated from seawater and a salt marsh based on its characteristic utilisation of DMSP as the sole carbon source. This enzyme was inducible by choline and glycine as well as DMSP; however, the enzyme activity increased with time and then decreased dramatically during the induction period. The decrease of enzyme activity could be hindered by amending with DMSP or choline (but not glycine betaine). Based on inhibition studies with nystatin and cyanide, it was suggested that the DMSP breakdown process was energy dependent *in vivo*. Furthermore, the addition of the thiol binding reagent *p*-CMBS (0.3 mM) caused the DMSP uptake to drop to 50%. The low sensitivity to *p*-CMBS suggested that the DMSP lyase in *Fusarium lateritium* is not exposed to the surface and could be cytosolic. The V_{max} of DMSP lyase was 34.7 munit. mg of protein^{-1}.

More species of fungi have since been obtained from marsh grasses and examined for DMSP lyase activity (Bacic et al., 1998). A strong correlation was found between fungi containing DMSP lyase activity and their host plants producing DMSP. Among the various decomposers tested on *Spartina alterniflora*, only two isolates of *Lachnum apartinae* strain I and II showed no DMSP lyase activity. There are only three fungal

isolates containing DMSP lyase activity which live in non DMSP producing plants and different fungi had remarkably different enzyme activities.

1.2.5.4 Demethylation

Recent experimental evidence has suggested that only a relatively small portion (<30%) of the DMSP undergoing degradation in seawater is converted to DMS (Belviso et al., 1993, Kiene, 1991, Kiene, 1992). These low yields of DMS indicated that there might be an alternative route which degrades the majority of the DMSP. Further experiments with anoxic sediment samples by Kiene et al. showed that DMS and acrylate accumulated after DMSP additions (Kiene et al., 1999, Kiene et al., 1998). At the same time the concentrations of methanethiol (MeSH) and 3-methyl mercaptopropionate (3-MPA) also increased. Additions of DMS to the slurries resulting in greatly increased MeSH production implying that the MeSH had arisen from the microbial metabolism of DMS. Due to the occurrence of MPA, they speculated that some of the DMSP was broken down by demethylation. Though 3-mercaptopropionate (MMPA) was not observed in the slurries, it is still a possible intermediate in the sequential demethylation of DMSP. Further experiments with additions of MMPA to the slurries instead of DMSP showed that the production rate of MPA was similar to that from DMSP. It was suggested that the sequential demethylation of DMSP does exist. The low emission of MeSH from MMPA indicated that a similar conversion of DMSP to DMS was not occurring. Two distinct routes for DMSP degradation, either by cleavage or demethylation have therefore been established.

In 1993 an anaerobic bacterium *Desulfobacterium* strain PM4, which could demethylate DMSP to MMPA, was isolated by van der Maarel et al. (van der Maarel et al., 1993). Later they isolated another bacterium grown on MMPA (van der Maarel et al., 1995). This bacterium could convert MMPA stoichiometrically to MPA and methane. So far no single anaerobic microorganism has been isolated that can demethylate DMSP directly to MPA. Recently, the demethylation of DMSP to

MMPA by two other *Desulfobacterium* strains has been described (van der Maarel et al., 1996a).

As for aerobic marine environments, several bacteria grown on DMSP and MMPA have been isolated by Taylor and Gilchrist (Taylor & Gilchrist, 1991). It was found that the bacteria cultured on MMPA could aerobically demethylate MMPA to MPA and demethiolate MMPA to MeSH. Some could also demethylate DMSP as well. Other bactera isolated with DMSP cleaved DMSP to DMS.

A bacterium has also been isolated from a marine intertidal mud sample which, when grown aerobically on DMSP (Visscher & Taylor, 1994), could quantitatively demethylate DMSP and MMPA to MPA.

1.3 Fates of DMSP and DMS in the Marine ecosystem

1.3.1 The fate of DMSP

In the marine ecosystem, DMSP is mainly produced by algae and some higher plants. Whilst the intracellular DMSP could be degraded to DMS in healthy living algal cells in response to environmental stress, free dissolved DMSP is also found in plankton culture filtrates, implying that DMSP might be released from the cells (Ackmam et al., 1966). Further phytoplankton culture experiments showed that little DMS and dissolved DMSP were released by plankton cells during the logarithmic growth period and the stationary period (Stefels & Vanboekel, 1993, Turner et al., 1988, Wolfe & Kiene, 1993). The dissolved DMSP and DMS concentrations, however, began to rise in the medium when the growth rate declined. It was therefore suggested that the dissolved DMSP and DMS were leached or liberated from the decaying or dead cells or that the DMSP was subsequently cleaved to DMS extracellularly by microbial activity. Recent experimental results have shown that the DMS release rate

from phytoplankton was markedly increased by zooplankton grazing (Dacey & Wakeham, 1986, Wolfe & Kiene, 1993). The mechanism of this DMS emission is still however uncertain. It might be that DMSP from the prey is converted to DMS when lysis of ingested prey cells initiated the mixing of algal DMSP and DMSP lyase intracellularly or extracellularly. The DMSP could also be digested by zooplankton without the production of DMS or be degraded in the faecal materials by microbial activity. Zooplankton grazing on algae demonstrated another alternative process or result of the dissolved DMSP and DMS release.

Once DMSP has been deposited into the sediments, the breakdown of DMSP can take place by biological cleavage and demethylation, but which of these processes is predominant depends on the type of sediment. When DMSP consumption was compared for three different types of intertidal sediment, a carbonate sediment, a cyanobacterial mat and a diatom covered tidal mud flat (Visscher et al., 1994), it was found that the DMSP consumption was fastest in the cyanobacterial mat slurries. In each sediment both DMS and MPA were observed simultaneously indicating that cleavage and demethylation occurred at the same time. The DMSP-utilising bacterial population of each sediment was classified into cleavers and demethylators. There were found to be 90% cleavers and 10% demethylators present in the cyanobacterial mat, 20% cleavers and 80% demethylators in the carbonate sediment, and 23% cleavers and 77% demethylators in the diatom mat. The presence of a large population of demethylating bacteria and DMSP lyase suggested that the demethylation pathway and cleavage contribute significantly to DMSP consumption in the marine ecosystem.

As DMS was found to be evolved from the tissues of a variety of fish from various environments, the transfer of DMSP into the natural food chain through fish has been given considerable attention (Ackmam et al., 1972, Dacey et al., 1994, Iida & Tokunaga, 1986). Much of the early interest in DMSP arose because it was found to be responsible for certain problems in fish flavour. The 'blackberry-problem' on the Labrador coast of Canada and the 'gunpowder flavour' experenced in Greenland are both associated with the presence of a DMSP containing pteropod, *Limacina helicina*

in fish guts (Ackmam et al., 1966, Ackmam et al., 1967). It was also found that the Pacific chum salmon *Oncorhynchus Keta* contained significant levels of DMSP which was degraded to DMS during the canning process, giving rise to a 'petroleum odour' (Motohiro, 1962). The DMSP degradation in fish was thought to be related to transmethylation in the liver.

The stability of DMSP accumulation in fish and shellfish varied between species. Oysters maintained constant levels of DMSP for up to two to three months (Ackmam & Hingley, 1968); whereas, cod removed almost all ingested DMSP within two days (Ackmam et al., 1966). The presence of acrylate, the other cleavage product of DMSP in some fish tissues suggested that DMSP degradation could occur (Dacey, 1994). In reef fish the acrylate concentration is about 50% of the DMSP concentration, indicating that some DMSP is decomposed and the remainder is excreted. This finding is consistent with observations that fish faeces liberate DMSP probably from residual, undegraded DMSP.

1.3.2 The fate of DMS

Whilst the main means of production of DMS in the marine ecosystem is believed to be from its biogenic precursor DMSP, another possibility is that it can result from the methylation with inorganic sulphide. A thiol S-methyl transferase capable of the sequential methylation of hydrogen sulphide to DMS via methanethiol has been discovered in yeasts, aerobic bacteria and algae (Taylor & Kiene, 1989). The impact of grazing on algal and bacterial populations must also be included in considerations of DMS production. Dacey and Wakeham have reported that an important pathway for DMS production is likely to be through organisms which graze DMSP producing algae, and those that either metabolise DMSP themselves or release dissolved DMSP (Dacey & Wakeham, 1986).

In solution the majority of the DMS is lost by microbial consumption, with the rate being more than ten-fold quicker than its efflux to the atmosphere (Kiene & Bates, 1990). The formation, interconversion and sinks of DMS in the marine are summarised in the Figure 1.7.

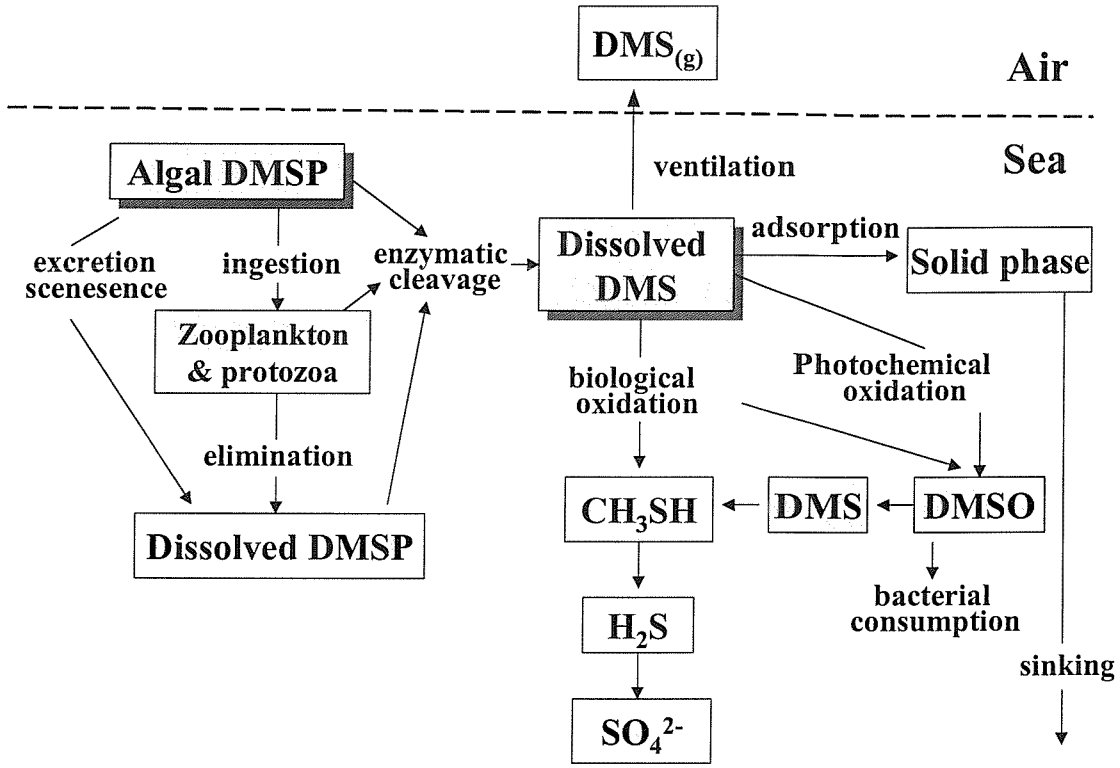


Figure 1.7 Diagram illustrating the known source and sinks for DMS in the surface water (modified from (Kiene & Bates, 1990)).

The DMS consumption rate in estuarine waters is dependent on the DMS concentration and incubation temperature, and metabolism is significantly inhibited by chloroform and azide (Kiene, 1992, Kiene & Service, 1991). Hence, it is clear that the removal of DMS is biologically mediated. The aerobic metabolism of DMS could possibly be by its oxidation to DMSO or use by methylotrophs and chemolithotrophs. Suylen et al. found a methylotroph (Hyphomicrobium EG) and Kanagawa and Kelly identified a chemotroph *Thiobacillus thioparus* in seawater (Kanagawa & Kelly, 1986, Suylen et al., 1986). Both of these microorganisms initially demethylate DMS

to MeSH. The MeSH is subsequently demethylated to HS^{-1} (Kanagawa & Kelly, 1986). In addition, Visscher and Taylor isolated a bacterium grown as a denitrifier on alkyl sulphide including DMS from a marine microbial mat that could stoichiometrically convert DMS to sulphate (Visscher & Taylor, 1993). A bacterium grown aerobically on DMSP and DMS was also isolated from an intertidal mud sample by Visscher and Taylor (Visscher & Taylor, 1994). This bacterium could demethylate DMSP.

DMS has been shown to be rapidly consumed in anoxic sediments. Two types of bacteria have been shown to be involved, sulphate reducers producing MeSH and CO_2 and methanogens producing MeSH and CH_4 (Kiene & Taylor, 1987, Kiene & Taylor, 1988, Zinder & Brock, 1978). Further studies on a stratified coastal salt pond found high levels of bacterial chlorophyll to be present in the H_2S rich waters under the H_2S and O_2 interface and the depth profiles of DMS and H_2S indicated the probability that an anaerobic phototropic bacteria might be able to consume H_2S and DMS producing H_2S , CO_2 and CH_4 (Wakeham et al., 1987). DMS was not decomposed in control sediment slurries incubated in the absence of light or under sterile conditions, suggesting that the metabolism of DMS was a light dependant microbial process. Subsequently, a phototropic anaerobic bacteria (*Thiocystis* sp.) was isolated which could oxidise DMS to dimethylsulphoxide (DMSO) (Zeyer et al., 1987). Once DMSO was produced, it could be reduced chemically by sulphide in marine sediment or by biological reduction under anaerobic conditions (Taylor & Kiene, 1989). A DMSO reductase has been isolated from the bacterium *Rhodobacter capsulatus* by Hatton et al. (Hatton et al., 1994).

The predominant oxidation product of DMS is DMSO and it occurs at relatively high concentrations in surface ocean waters but it is rarely found below the euphotic zone (Andreae, 1980). Apart from biological oxidation, DMS could also be photooxygenated by photosensitisers such as methyl blue or humic acid (Brimblecombe et al., 1987, Taylor & Kiene, 1989) and slowly autoxidised by molecular oxygen in saline solution (Shooter & Brimblecombe, 1989).

1.3.3 Sulphur species in sediment

Due to the anoxic conditions prevailing just beneath the sediment surface, sulphate reduction represents a major pathway for the oxidation of organic matter in sediment (Hines et al., 1997) and the products of the associated reactions significantly influence the composition of the sediment. The ability of sulphur atoms to form a variety of chemical compounds and to undergo changes in oxidation state over an eight electron shift results in this element being involved in a great number of biochemical reactions. The bacterial reduction of sulphate ions releases energy for metabolic processes and results in the production of hydrogen sulphide. The diffusion of the sulphide ions to more oxidising regions of the sediment may be followed by incomplete oxidation intermediates such as polysulphide ions, elemental sulphur and thiosulphate (Jorgensen & Bak, 1991, Troelsen & Jorgensen, 1982) or complete oxidation regenerating sulphate (Elsgaard & Jorgensen, 1992). Also the chemical cycling of metal ions and sulphur species may result in the presence of a suite of possible metal sulphur species.

In the organic sulphur cycle, DMS is the major volatile reduced organic sulphur compound released from the ocean to the atmosphere (Andreae & Raemdonck, 1983, Wakeham et al., 1984). It is believed that the most likely biogenic precursor of DMS in the ocean is the algal natural product, DMSP (Reed, 1983, Vairavamurthy et al., 1985, White, 1982). In oxic and anoxic environments DMSP and DMS can undergo biological conversion (Kiene, 1991, Kiene & Capone, 1988, Kiene & Taylor, 1988, Taylor & Gilchrist, 1991). In marine sediments, DMSP is enzymatically cleaved to DMS and acrylate or is degraded by demethylation to MMPA and subsequently to 3-MPA (Kiene & Taylor, 1987, Kiene & Taylor, 1988, Vandermaarel et al., 1995). MMPA is also degraded to methanethiol and acrylate in anoxic sediments (Kiene & Taylor, 1988). One chemical mechanism for the formation of MPA has been identified as the abiotic addition of H_2S to the double bond of acrylate (Vairavamurthy & Mopper, 1987). However, the chemical formation of MPA was slow in comparison to the biological production from DMSP (Kiene & Taylor, 1988).

The identification of sulphur species in sediments is therefore critical in understanding the bioavailability and fate of sulphur.

1.4 Conclusions

DMSP is present in many macro- and microalgae and is believed to be associated with osmoregulation and cryoprotection. DMSP breaks down to release DMS to the atmosphere where it is rapidly photooxidised to methanesulphonic acid, sulphur dioxide and sulphate. These oxidised compounds in turn contribute to the acidity of precipitation and rainfall and can potentially alter climate through the formation of cloud aerosols. DMS has been identified as the most abundant volatile sulphur compound and DMSP as the most common precursor of DMS from the marine ecosystem. Studies of the distribution and dynamics of DMS and DMSP in the marine environment have enhanced our understanding of the mechanisms controlling the global sulphur cycle.

1.5 Objectives

DMSP is present in many macro- and microalgae and for osmoregulation and cryoprotection. Its release and subsequent breakdown through the decomposition of algae contributes a number of sulphur compounds to estuarine sediments. Studies of biogeochemical properties of DMSP in the estuarine environment will enhance our understanding of some of the mechanisms controlling the global sulphur cycle. To achieve this goal, a number of approaches have been taken that are described in this thesis:

- To develop a number of novel analytical techniques for the measurement of DMSP, DMS and other sulphur species that commonly occur in estuarine environments.
- To purify and characterise a DMSP lyase that cleaves DMSP to DMS and acrylate from the green macroalga *Entermorpha intestinalis*; this alga dominates many estuaries in southern England.
- To investigate the distributions of DMSP and DMSP lyase in sediments to assess the contributions of algal material to the estuarine sediments.
- To study the distributions of a number of other sulphur species in the sediments of the Tamar estuary.

CHAPTER TWO

Analytical Method Development

This chapter describes the development of instrumentation for the measurement of sulphur species that commonly occur in the marine environment. Particular attention is focused on dissolved sulphide, DMS, DMS precursors, DMSP, elemental sulphur and DMSP lyase activity.

2.1 A flow injection system with flame photometric detection for the measurement of sulphide

2.1.1 Introduction

Hydrogen sulphide (H_2S) is generally found in anoxic waters such as the deep water of stagnant basins, sediment pore waters and low oxygen layers of the open sea. Hydrogen sulphide is produced by sulphate-reducing bacteria acting on organic matter and sulphate under anaerobic conditions. The life-time of H_2S in the water is short, probably due to the sulphide being volatilised by aeration or being oxidised by the dissolved oxygen. Hence, the concentration of sulphide in seawater is fairly low and is usually expressed in $\mu mol \cdot dm^{-3}$ H_2S (Grasshoff et al., 1983).

In this section, an alternative approach to the measurement of low concentrations of dissolved sulphide is presented using flow injection analysis (FIA). The flame photometric detector is a highly sensitive sulphur-specific detector which is widely employed in gas chromatography for the measurement of volatile species. It is

normally, however, incompatible with the direct measurement of aqueous samples. The instrumentation described in this section employs a flame photometric detector in a flow injection system. It utilises a continuous-flow reaction stage to generate hydrogen sulphide, which is then transferred to the gas phase, where it can be monitored with a sulphur-specific flame photometric detection system. This novel FIA approach offers a rapid, simple and sensitive method for the measurement of dissolved sulphide and requires minimal sample pre-treatment. The system has been used to evaluate alternative methods for preserving sulphide in samples prior to their analysis.

2.1.2 Experimental

2.1.2.1 Reagents and Standards

All reagents and acids were of analytical reagent grade unless otherwise stated. Deionized (DI) water, with a specific resistance in the range 15 to 18 MΩ. cm, was employed in all experiments. The deionized water was de-oxygenated by bubbling nitrogen through it for 30 min before use.

Sulphide stock solution ($0.1 mol \cdot dm^{-3}$). Analytical reagent grade sodium sulphide ($Na_2S.9H_2O$, Sigma, Poole, U.K.) was washed quickly with DI water to remove surface impurities and immediately dried with tissue paper. Ca. 2.4 g of sodium sulphide was accurately weighed and dissolved in oxygen-free DI water ($0.1\ dm^{-3}$) to give a $0.1\ mol.dm^{-3}$ standard sulphide stock solution. A series of standard solutions was prepared from the stock solution by dilution with de-oxygenated DI water.

Interference test solutions (10^{-2} $mol.dm^{-3}$). $Na_2S_2O_3.5\ H_2O$ (BDH, Poole, U.K.), $Na_2SO_3.\ H_2O$ (BDH, Poole, U.K) and $K_2S_2O_8$ (BDH, Poole, U.K.) were prepared from analytical reagent grade salts.

Alkaline pyrogallol solution was prepared by dissolving 15 g of pyrogallol (Sigma, Poole, U.K.) and 30 g potassium hydroxide (Sigma, Poole, U.K.) in 1dm^3 of de-oxygenated water.

Vitamin E solution. 1 g (±)α-tocopherol (Sigma, Poole, U.K.) was dissolved in 50 ml of ethanol to give a 1.16 x 10^{-3} $mol.dm^{-3}$ stock solution.

Zinc acetate solution. 2.088 g zinc acetate (BDH, Poole, U.K.) was dissolved in 200 ml DI water containing 0.2 g gelatine (BDH, Poole, U.K.) to make a 4.76 x 10^{-2} $mol.dm^{-3}$ zinc acetate/gelatine stock solution.

After preparation all stock solutions were sealed under a nitrogen atmosphere.

2.1.2.2 Instrumentation

(a) Introduction

The FIA system consisted of three main sections: a continuous flow reaction system, a gas/liquid separator and a custom-built flame photometric detection (FPD) system (Fig. 2.1). The sample was injected into a stream of degassed water and then mixed with sulphuric acid before being passed through a PTFE reaction coil. After the addition of nitrogen, the mixture was divided into gaseous and liquid phases in a gas/liquid separator. The liquid then travelled to waste and the volatile H_2S was swept into the FPD system. A more detailed description of the components of the apparatus is to be found in subsequent sections.

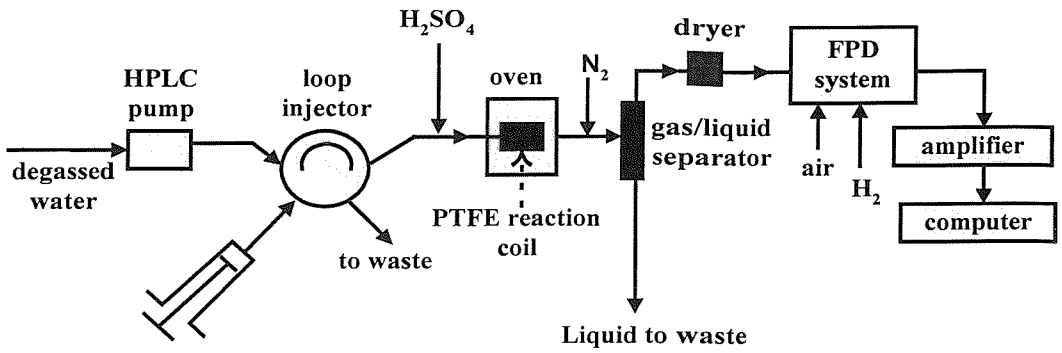


Figure 2.1 Flow injection analysis system for the measurement of sulphide.

(b) The flame photometric detection system

The FPD system was custom built by Howard and Russell (Howard & Russell, 1995). In order to obtain a more sensitive and stable detector, the air-hydrogen flame emission source was housed in an insulated box and heated by a 300W cartridge heater to 250 °C to prevent condensation (Fig. 2.2). Flame emission was monitored by a photomultiplier tube (PMT, EMI 6256B) viewing the flame via a quartz light pipe through a wide-bandpass glass filter (Oriel BG12, Leatherhead, U.K.). The optimum flame gas flows were air (150 ml/ min) and hydrogen (130 ml/min). Finally, the signal from the PMT was converted to a voltage, amplified (by a factor of 1, 10, 100, or 1000) and then analysed using an Axxiom 717 chromatographic integrator and output on a chart recorder.

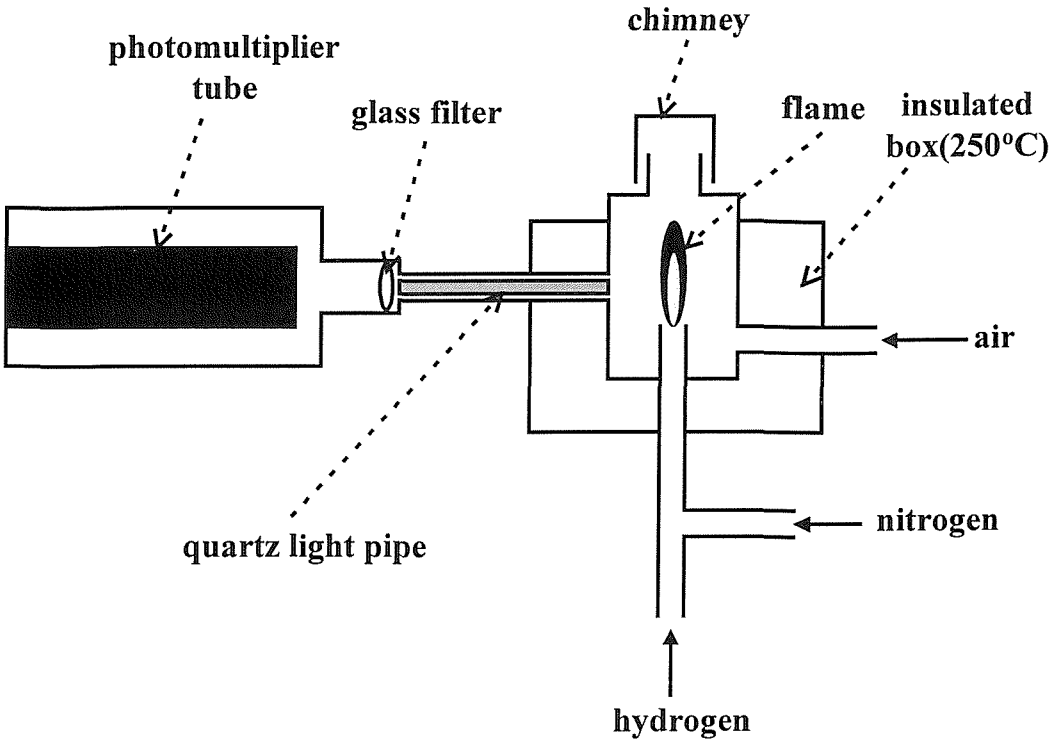


Figure 2.2. Schematic representation of the flame photometric detector (FPD) system (not to scale).

(c) Operation

200 µl samples were injected using a Rheodyne 7010 injection valve into a flowing stream (1 ml/min) of degassed water. The constant water flow was delivered by an HPLC pump (Gilson model 303).

A stream of 0.1 mol.dm^{-3} sulphuric acid (1.5 ml/min) was then mixed into the sample stream using a peristaltic pump and the mixture was passed through a PTFE reaction coil (30 cm x 0.74 mm i.d.) held at room temperature. In the reaction coil volatile H_2S was produced and subsequently purged from solution by nitrogen gas (35 ml/min). Longer reaction coils resulted in increased peak broadening and a need for an increased time interval between injections.

The nitrogen and H_2S were separated from the liquid stream by a gas/liquid separator (Fig. 2.3) and the gas stream was dried before it passed into the FPD detection system.

Two types of dryer were compared, namely a chemical drying agent of anhydrous magnesium perchlorate (14–25 mesh) and a Nafion dryer (size 12″, 0.64″ i.d., held in 0.3 μm molecular sieves).

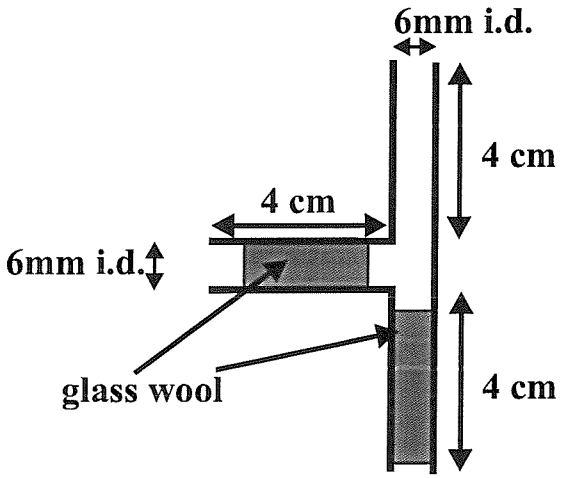


Figure 2.3 Gas/liquid separator

2.1.2.3 Interference tests

10^{-2} mol.dm^{-3} solutions of NaS_2O_3, Na_2SO_3 and $K_2S_2O_8$ were analysed to investigate whether they produced interfering signals.

2.1.2.4 Solution storage

The stability of sulphide solutions is poor due to their sensitivity to light and oxygen. In order to limit this degradation, all sample vials were covered with foil and kept in the dark to minimise photolysis. Moreover, a variety of antioxidants and protection agents were added to prevent oxidation. Oxygen was also excluded from the sample vial using nitrogen gas.

A number of approaches were evaluated as methods which could be employed to protect the sulphide samples from oxidation.

(a) Anti-oxidants

Oxygen is the most common oxidant to affect the stability of sulphide in solution. Its solubility in water is ca. 9.1 mg O_2 / dm^3 H_2O, corresponding to 2.84 x 10^{-4} $mol.dm^{-3}$. A number of reducing agents were therefore evaluated as anti-oxidants for the preservation of sulphide solutions.

10^{-2} $mol.dm^{-3}$ Na_2SO_3, 3 x 10^{-4} $mol.dm^{-3}$ alkaline pyrogallol or various concentrations of Vitamin E, ranging from 10^{-3} to 7 x 10^{-4} $mol.dm^{-3}$, were added to degassed water to remove oxygen. 2.52 x 10^{-5} $mol.dm^{-3}$ Na_2S solutions were then prepared in these solutions and the flasks were immediately stoppered. The effectiveness of the anti-oxidant was assessed by comparing the sulphide contents of treated samples with new sulphide standards.

(c) Chemical fixation of sulphide

Sodium sulphide standards were transferred to100 ml volumetric flasks. To some was added 5 ml of zinc acetate stock solution while to others was added 5 ml of the zinc acetate/gelatine stock solution. All sample vials were stoppered by silicone rubber seals and the flasks were flushed with nitrogen gas for 1.5 min through stainless steel hypodermic needles fitted with three-way stopcocks. With the stopcocks closed to isolate the samples from the atmosphere, these samples can be analysed by FIA after different storage times and compared to open flask samples at room temperature.

Ten 1.04 x 10^{-5} $mol.dm^{-3}$ sodium sulphide solutions were prepared with added zinc acetate/gelatine solution and these were sealed under nitrogen to test long term storage (1 week). All sample vials were kept in the dark at room temperature.

2.1.2.5 Calibration

A series of standard sodium sulphide solutions was analysed and a log (concentration) vs log (peak height) plot was constructed.

2.1.3 Results and discussion

2.1.3.1 Instrument performance

The system performance using a Nafion dryer was compared to that obtained using chemical drying with magnesium perchlorate. The former method gave rise to greater sensitivity; the magnesium perchlorate drying system giving rise to a signal only 66% of that which was obtained using the Nafion dryer. This may be due to the chemical agent absorbing not only water vapour but also water soluble H_2S, leading to the formation of MgS.

In initial experiments, the sensitivity of the FIA system to sulphide was found to be inconsistent. Improved peak height reproducibility was achieved by using a fixed time interval between injections. In addition, it was noted that the sensitivity decreased as the time interval between injections increased (Fig. 2.4). When the time interval between injections was below 1.2 min, the resolution of signal peaks was poor. This may be due to the transport of water droplets carrying sulphide through the Nafion membrane. This membrane material is a non-porous ion-exchange membrane which allows small polar molecules to pass through and which has a high permeability to H_2, He and H_2O. Hence, when the sulphide samples were regularly passed through the Nafion tube, a steady state could be set up. Consequently, the signal would be related to the concentration of sulphide reaching the FPD. Based on the desire to obtain a good separation between peaks and to obtain maximum sensitivity, 1.6 min was adopted as the optimum time interval between injections (Fig. 2.5).

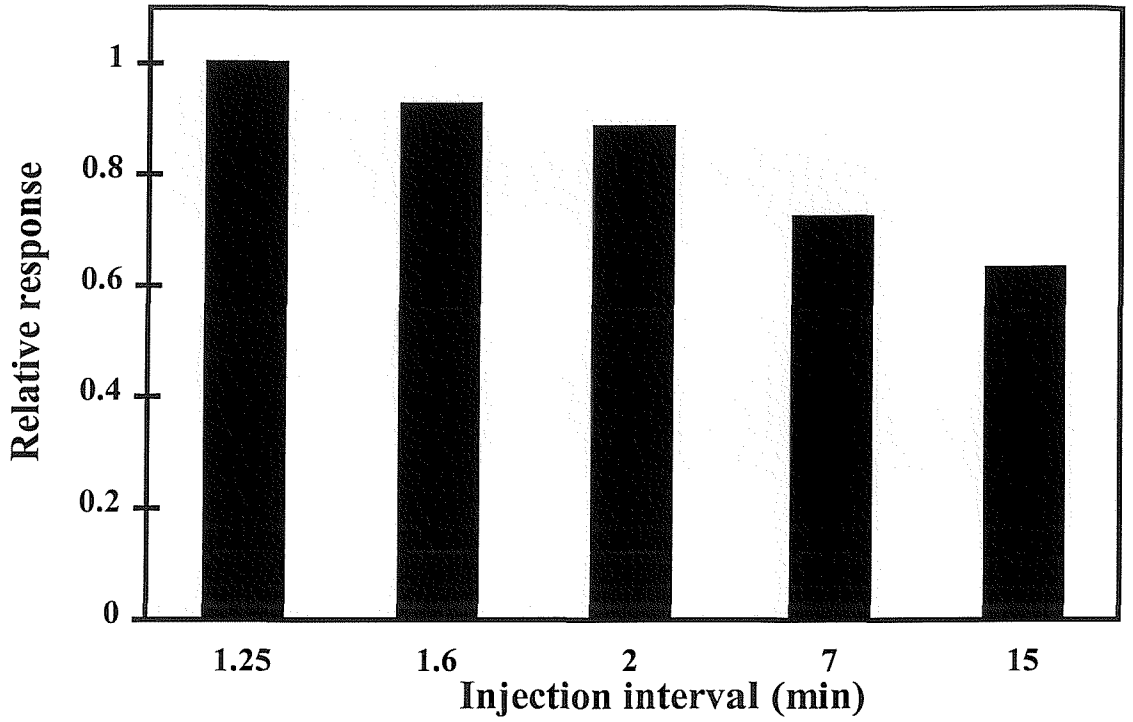


Figure 2.4 Relative response from a 10^{-5} mol.dm^{-3} sodium sulphide solution varying the time interval between injections. [Peak heights measured relative to 1.25 min injection.]

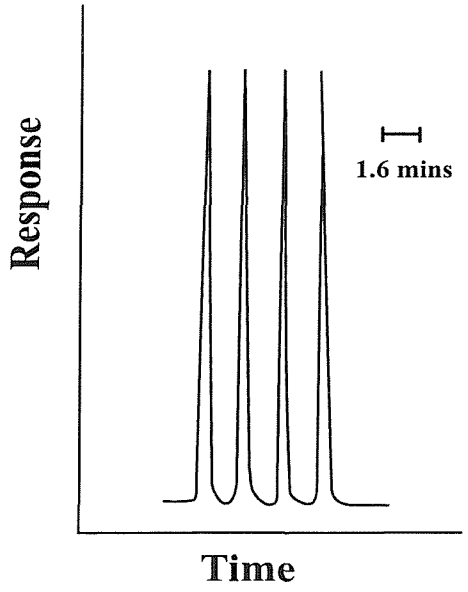


Figure 2.5 Response from 2.1 x 10^{-5} mol.dm^{-3} sodium sulphide standard solutions (Nafion dryer at room temperature, 1.6 min injection time intervals.)

The effect of temperature on the generation rate of H_2S was tested by putting the reaction coil in an oven at different temperatures between 25 and 70 °C. There was no significant improvement (data not shown). Thus, all experiments were carried out at room temperature.

The FIA system was calibrated by using peak height measurements obtained from a series of standard solutions. In order to account for the typically non-linear instrument response resulting from the S_2 emission, a $\log_{10}$ (response) vs $\log_{10}$ (concentration) plot calibration was employed, giving a gradient of ca 2 (Fig. 2.6).

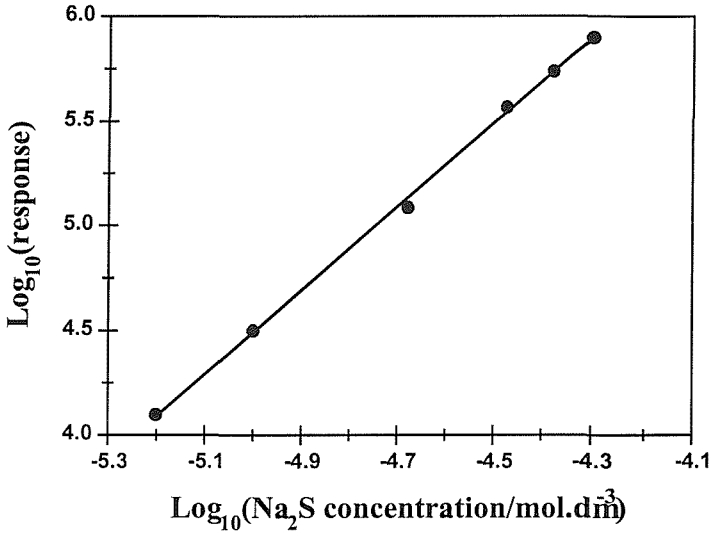


Figure 2.6 Typical calibration line for Na_2S, ranging from 6.3 x 10^{-6} to 5 x 10^{-5} mol.dm^{-3} (gradient 1.998).

Ten replicate analyses of different standard solutions of sulphide were made to test the reproducibility of the technique. The relative standard deviation of the mean was 2.56%. The detection limit of the system, defined as the concentration of sulphide which produced a signal twice the standard deviation for a signal close to the blank, was 2.1x 10^{-6} mol.dm^{-3} of sodium sulphide. This corresponds to 4.1 x 10^{-10} mole or 12.7 ng absolute of injected sulphide.

High concentration (10^{-2} mol.dm^{-3}) solutions of NaS_2O_3, Na_2SO_3 and $K_2S_2O_8$ were also injected into the FIA system but no measurable signal was obtained.

2.1.3.2 Storage of sulphide solutions

The instability of sulphide solutions is evident from the fact that after two hours, the response generally dropped to ca. 80% of the initial peak height. Thus, one should be careful to keep sample bottles and standard solutions as air–free as possible and to run standard solutions before and after the analysis to ensure accurate calibration.

(a) Antioxidants

In an attempt to resolve the oxidation problem, 10^{-2} mol.dm^{-3} Na_2SO_3 was added to each sample solution. It was however found that the baseline shifted up with the injection and the peak had a long tail. Decreasing the concentration of Na_2SO_3 did not improve the situation significantly.

The addition of alkaline pyrogallol caused the loss of sulphide. After 1.5 hours the response dropped to ca. 21%. Moreover, signal reproducibility was not improved by employing a longer reaction coil and stronger acid (1 mol.dm^{-3} sulphuric acid). In contrast, it caused peak broadening. From the results obtained above, it seems that alkaline pyrogallol reacted with sulphide in the sample during storage.

An assessment of the antioxidant efficiency of Vitamin E was carried out by comparing solutions containing Vitamin E with those without Vitamin E. The results of this experiment are shown in Table 1. All data was calibrated using a fresh sodium sulphide standard solution. After 6 hours storage in an open flask at room temperature, all solutions had dropped to about 70% or below. Maximum losses were obtained using the high concentration of Vitamin E. It was also found when the

sample flasks were opened frequently, the oxidation of sulphide was dramatically increased.

	0 hrs	3 hrs	6 hrs
2.52×10^{-5} mol.dm^{-3} Na_2S	100 ± 0.8	98 ± 2.1	71.5 ± 2.8
Na_2S/ 7×10^{-4} mol.dm^{-3} Vitamin E	113 ± 3.6	93.5 ± 1.7	70 ± 2.8
Na_2S/ 1.2×10^{-3} mol.dm^{-3} Vitamin E	109 ± 1.6	80.6 ± 1.4	57 ± 1.2

Table 2.1. The effect of the addition of Vitamin E on sample stability (% recovery vs fresh sulphide standard)

In order to further assess the effects of Vitamin E and exposure times, six samples were assessed over a period of 3 days (Table 2.2). After 3 days, the responses from sulphide samples had dramatically decreased. Some samples were undetectable. The addition of Vitamin E seems to stabilise the sulphide but significant losses still occurred.

Day	0	1	2	3
2.52×10^{-5} mol.dm^{-3} Na_2S	100 ± 4.9	85.5 ± 2.3	60.7 ± 1.9	<40
Na_2S / 10^{-3} mol.dm^{-3} Vitamin E	102 ± 4.5	82.6 ± 2.2	54.7 ± 4.8	66.3 ± 4.9

Table 2.2. Recovery (%) of sulphide during long-term storage.

(b) Chemical fixation of sulphide

Zinc acetate was used to preserve sulphide by the production of either a zinc sulphide precipitate or, if the zinc acetate is mixed with gelatine, the generation of colloidal ZnS. This latter approach prevents sedimentation of the zinc sulphide or adhesion of the precipitate onto the container walls. Standards prepared in open flasks and under a nitrogen atmosphere were also compared and the results are shown in Table 2.3. Sulfide was found to be more stable in zinc acetate/gelatine solution. Losses of sulphide from the zinc acetate solution are believed to be due to precipitation of ZnS, causing the adherence of precipitated material on the nucleation sites of the container walls.

	Open to atmosphere	inert atmosphere
2.52×10^{-5} mol.dm^{-3} Na_2S	18.1 ± 10.5	31.3 ± 2.9
Na_2S / zinc acetate	69.1 ± 1.5	93.8 ± 2.9
Na_2S / zinc acetate with gelatine	92.4 ± 2.6	101.4 ± 1.0

Table 2.3. Recovery (%) of sulphide after storage for 24 hour in open flasks and under an inert atmosphere systems.

The stability of the sulphide was significantly improved by storage under nitrogen. A long term storage experiment was therefore carried out (Fig.2.7). After 2 days, the response of the sulphide sample had dramatically dropped by ca. 75% and it was undetectable on the 4th day. Sulfide samples in zinc acetate solution showed a dramatic decrease after 2 days. When the flask was shaken before injection, however, the signal could be recovered back to ca. 72%. It is evident that ZnS precipitate had been produced and sunk to the bottom of the flask. Furthermore, sulphide in zinc acetate/gelatine solution was very stable even after 6 days storage. This approach is suitable for the storage of samples when immediate analysis can not be carried out.

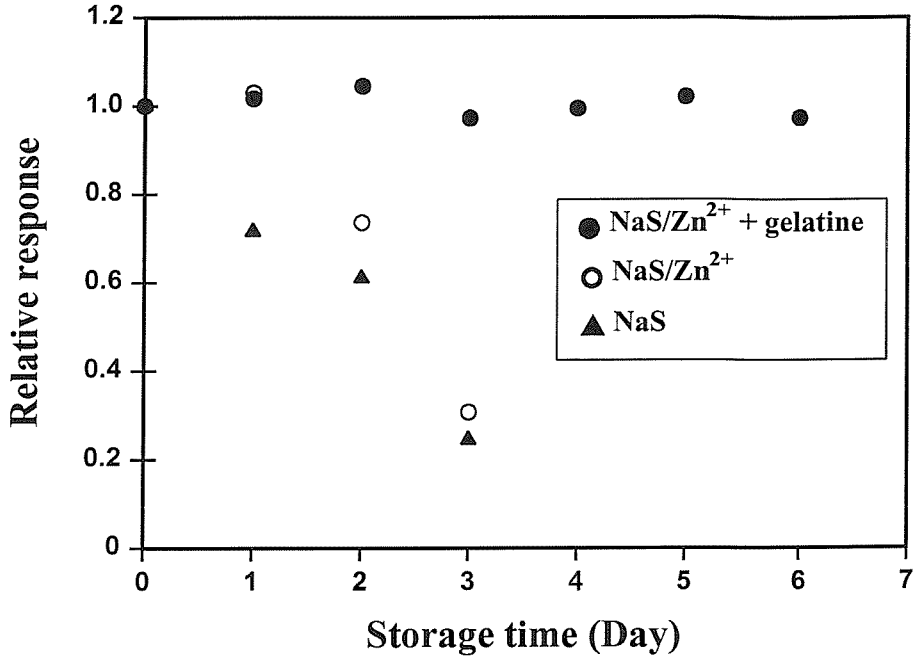


Figure 2.7 Storage under a nitrogen atmosphere.

In order to confirm the effectiveness of the zinc acetate/gelatine solution, a further long term storage experiment was carried out. Ten replicates of 1.04 $x10^{-5}$ $mol.dm^{-3}$ sodium sulphide with zinc acetate/gelatine solution were measured after 7 days storage. The result was 9.89 x 10^{-6} $mol.dm^{-3}$ ± 4.94 x 10^{-7} $mol.dm^{-3}$ sodium sulphide. This represented a 95.1% ± 4.7% recovery of sulphide. The storage loss was less than 5 % and the relative standard deviation of mean was also under 5%. From the above results it is evident that a zinc acetate/gelatine solution can be successfully employed to stabilise sulphide samples under a nitrogen atmosphere.

2.1.4 Conclusion

The FIA sulphide determination method is specific, rapid and easy to use. It is capable of a turnover of ca. 10 samples per hour with a precision of better than 5% and is

suitable for the routine analyses of environmental and industrial samples. In addition it requires only a few ml of sample and minimal sample treatment is required.

Interference tests failed to show any response from other common sulphur-containing chemicals. For storage it is recommended that a zinc acetate/gelatine solution should be added and that the sample should be kept under a nitrogen atmosphere. If this is done a sulphide recovery of 95.1% ± 4.8% (n=10) can be achieved after a week in storage.

2.2 The measurement of DMSP

2.2.1 Introduction

DMSP was first extracted and characterised from the red alga *Polysiphonia fastigata* by Challenger and Simpson in 1948 (Challenger & Simpson, 1948) and a presumptive test for DMSP was developed based on the measurement of DMS evolved on treatment with cold alkali. Since that time this indirect method, now often incorporating a cryogenic trap to concentrate DMS prior to detection (Andreae et al., 1983), has been employed almost exclusively for DMSP measurement. DMS preconcentration can be carried out by complexation with mercury, chemisorption on gold wool or palladium foil (Ferek et al., 1986, MacTaggart et al., 1987). However, this method of DMSP measurement may cause some misleading results as other naturally occurring sulphonium compounds may also yield DMS with alkali. Hence, it is clear that more discriminating methods need to be applied to the determination of DMS-precursors in the marine environment.

Recently, various methods of direct DMSP identification have been employed such as thin layer chromatography (TLC) (Gorham et al., 1981), thin layer electrophoresis (TLE)(Gorham et al., 1981), fast atom bombardment mass spectrometry (FABMS)

(Hanson & Gage, 1991), infra-red spectroscopy (Larher et al., 1977), gas chromatography-mass spectrometry (GC-MS) (Hanson et al., 1993) and NMR spectroscopy (Larher & Hamelin, 1979, Paquet et al., 1994b). These are mostly qualitative demonstrations of the presence of DMSP. Even though there are quantitative techniques such as TLC followed by the scanning reflectance densitometry and HPLC with UV, they are still too insensitive and difficult to be used for the low concentrations to be studied in environmental systems (Gorham et al., 1981). An HPLC separation with on-line selective hydrolysis postcolumn and sulphur-specific detection has been developed for the investigations of DMS-precursors (Howard & Russell, 1995).

This section describes the selectivity and performance of an adaptable flow injection system employing various in-line chemical reactions to measure a diverse range of sulphur compounds. As with the sulphide system described previously, the sulphur-volatiles that are generated are then separated from the liquid and swept into a custom-built flame photometric detector (described in section **2.1.2.2)**.

Two methods are described in this section, both of them having been originally developed for the measurement of dimethylsulfonium compounds by Howard and Russell (Howard & Russell, 1995, Howard & Russell, 1997). In order to gain insight into the natural cycle of sulphur in marine ecosystem, these techniques have been evaluated for the determination of a wider range of sulphur compounds in preparation for the screening of environmental extracts such as sea water, sediment, seaweed etc. and the quantitative measurement of samples containing a single known sulphur compound.

2.2.2 Experimental

This FIA system is similar to that which is described in section 2.1.2.2. The only differences are to be found in the choice of reagents and their flow rates. The volatile

sulphur products from samples are then purged from the stream and swept to a FPD (Fig. 2.8)

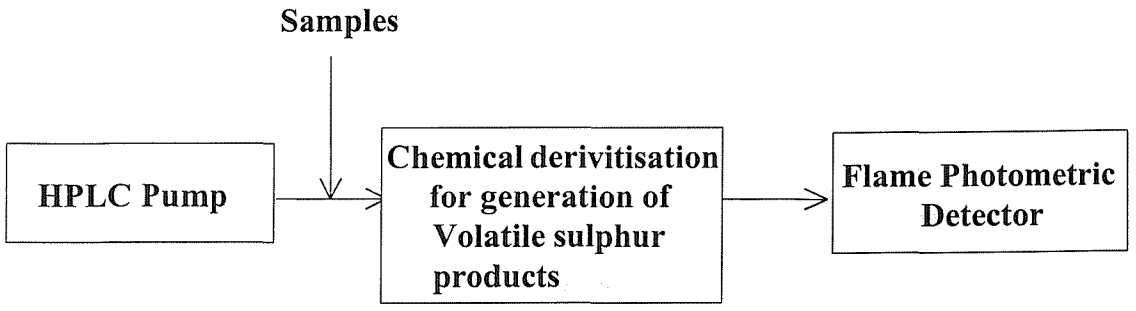


Figure 2.8 Schematic representation of the flow injection system.

2.2.2.1 Reagents and Standards

Standard stock solutions (10^{-2} mol.dm^{-3}).

Analytical reagent grade 3-mercaptopropionic acid (3-MPA, Sigma, Poole, U.K.), methyl-3(methylthio)-propionate (M-3-MTPA, Aldrich, U.K.), dimethyl sulphoxide (DMSO, BDH, Poole, U.K), dimethyl sulphide (DMS, Sigma, Poole, U.K.) and dimethyl disulphide (DMDS, BDH, Poole, U.K.) were dissolved in DI water to give 10^{-2} mol.dm^{-3} standard stock solutions. Series of standard solutions were prepared from the stock solutions by dilution with DI water. All stock solutions were kept under a nitrogen atmosphere.

DMSP standard solutions.

Dimethylsulfoniopropionate (DMSP) was prepared as a 10^{-2} mol.dm^{-3} stock solution in DI water. The pure DMSP was synthesised by Dr. D. Russell (University of Southampton, UK) (Howard & Russell, 1997). For calibration, a series of standard solutions (8-50 μmol.dm^{-3}) was prepared from the stock solution and kept at 4 °C. The DMSP stock solution is stable at 4 °C for 3 months.

Synthesis and characterisation of 3-methylthiopropionate (3-MTPA).

3-methylthiopropionate was synthesised from methyl 3-methylthiopropionate (Sigma, Poole, U.K.) using the procedure described by Wackett et al. (Wackett et al., 1987). 0.1 mole methyl 3-methylthiopropionate was hydrolysed with 30 ml of 4 M NaOH (0.12 mole) for 2 hours at room temperature. The free acid was prepared by the addition of excess HCl solution and two phases were obtained. 50 ml of diethyl ether was added to extract the organic compounds. The organic phase was separated and dried over anhydrous $MgSO_4$ and then evaporated to remove the solvent. The crude product was diluted with 50 ml diethyl ether and washed twice with 50 ml 1M HCl and then twice extracted with 50 ml 1M NaOH. The aqueous phase was acidified and then extracted with diethyl ether (50 ml). The organic phase was dried over $MgSO_4$ and solvent was removed by evaporation. TLC on silica gel plates was used to determine the purity of the product. The solvent system used was diethyl ether : petroleum (1 : 3). After drying, the plates were developed using iodine vapour.

Further characterisation of the 3-methylthiopropionate was carried out by proton nuclear magnetic resonance spectroscopy (300 MHz) and negative ion electrospray mass spectrometry.

2.2.2.2 Selectivity of a base hydrolysis FIA system

The selectivity of the base hydrolysis FIA system for the measurement of DMSP was tested using DMDS, dimethyl sulfoxide (DMSO), 3-methylthiolpropionate (3-MTPA), 3-MPA and methyl 3-methylthiopropionate (M-3-MTPA). 200 µl of 10^{-2} M standard samples were injected into a flowing stream (1 ml/min) of degassed water using a Rheodyne 7010 injection valve. The continuous eluent was delivered by an HPLC pump (Gilson model 303). An in-line base hydrolysis was carried out by mixing the sample stream with a pumped stream of 4M sodium hydroxide solution at a rate of 1.5 ml/min. The PTFE reaction coil (3.2 m x 0.74 mm i.d.) was held in a

thermostatted oven at 85°C to enhance the rate of hydrolysis. After the hydrolysis step, the liquid stream was mixed with a nitrogen gas stream at a flow rate of 43 ml/min. The volatile sulphur species that had been produced were purged from the liquid stream in a liquid-gas separator and the remaining liquid was pumped to waste. The gas stream was then passed through a dryer consisting of two glass vials connected in series. The first part is an empty 4 ml glass vial used for the condensation of water vapour. The second vial contained the chemical drying agent anhydrous magnesium perchlorate (14-25 mesh). The resulting gas was passed into the air-hydrogen flame of a custom-built flame photometric detection system as described in section 2.1.2.2 (b). The optimum flame gas flows were air (100 ml/min) and hydrogen (95 ml/min) respectively.

2.2.2.3 Selectivity of a borohydride-based FIA system

The selectivity of a FIA system combining an in-line acidic borohydride reaction for the generation of volatile sulphur species from sulphur-containing compounds was also tested using the sulphur compounds described in section 2.2.2.2. The basic instrumental configuration was the same as described in section 2.2.2.2. The only one exception was that a 10% (V/V) HCl solution was employed instead of the NaOH and that a 2M $NaBH_4$ solution was pumped into the acidified sample stream (1ml/min) at a rate of 1.4ml/min by using a peristaltic pump. The volatile sulphur species that were produced were subsequently purged from the liquid stream with nitrogen gas (43 ml/min) and then swept into the FPD detection system. The flow rate of air and nitrogen gas were unchanged, but the hydrogen flow rate was reduced from 120 ml/min to around 100 ml/min due to the extra hydrogen produced by the decomposition of the borohydride. The fluctuating production of hydrogen gas also increased the FPD background noise. The problem can be solved by adding a pellet of NaOH to stabilise the borohydride solution.

2.2.2.4 Calibration

The two instruments described above were each calibrated by using peak area measurements obtained from a series of DMSP standard solutions. In theory, the response of each sulphur compound in FPD is proportional to the square of the sulphur concentration. The slope of log (response) vs log (concentration) plot should be 2 but the experimental value is usually in a range 1.6 to 2. The selectivities of these two instruments toward sulphur compounds were assessed using the sensitivity relative to that of DMSP.

2.2.3 Results and discussion

2.2.3.1 Synthesis and characterisation of 3-MTPA

The crude product showed by TLC the presence of starting material (methyl 3-(methylthio)-propionate). The 3-methylthiopropionate was therefore further purified by washing with 1M NaOH, acidifying back to free acid and re-extracting the product into diethyl ether. 3.757 g of oily yellow liquid product was produced after solvent removal. The end product was stored under nitrogen and in the dark to prevent oxidation.

Characterisation of the product by ^{1}H NMR (300MHz, $CDCl_3$) δ 2.66 (m, 4H), δ 2.06 (s, 3H); LMES$^-$ mass spectroscopy, m / z 232.8 ($MTPA^-$), 154.7 (M^-Cl) and the presence of only one spot on the TLC plates (Rf values of the product were 0.209 and 0.679 respectively using ether : petroleum (1:3) and absolute ethanol) identified the product as 3-methylthiopropionate.

2.2.3.2 Selectivity of a base hydrolysis FIA system

Only DMSP generated volatile sulphur species. No response was obtained from DMSO, DMDS, 3-MTPA, 3-MPA and M-3-MTPA at concentrations as high as 10^{-2} M. The method therefore appears, based on their limited collection of compounds, to be specific for the measurement of DMSP. The detection limit was 6×10^{-6} M of DMSP.

2.2.3.3 Selectivity of a borohydride-based FIA system

In this section, DMSP was used as a standard reference compound. The response of the system is dependent approximately on the square of the concentration and the comparison was made by assessing the concentration of each compound required to produce a fixed instrument response. The relative concentration is therefore the concentration of a compound that produces the same peak area response as 10^{-6} M of DMSP. The results obtained from various sulphur species are shown in Table 2.4.

	Equal response concentration relative to DMSP
DMSP	1
3-MTPA	1.85×10^{2}
DMSO	1.55
M-3-MTPA	1.8×10^{3}
DMDS	0.54
3-MPA	No signal

Table 2.4 The concentration of various sulphur compounds required to produce the same response as DMSP in the borohydride FIA system.

Unfortunately, 3-MPA did not produce a response in the borohydride FIA system. The signal from 3-MTPA was 4 orders of magnitude less than that obtained from DMSP. It was therefore necessary to check whether this small signal was generated by 3-MTPA or by other impurities present in the product. A three week old 10^{-2} M solution of 3-MTPA did not produce a measurable signal. It therefore appears that 3-MTPA does not produce a measurable signal and that the original signal may have been generated from a volatile or unstable sulphur impurity. Another method was therefore required for the measurement of 3-MTPA and 3-MPA.

For M-3-MPTA, the instrument was 1800 times less sensitive than DMSP. As for DMSO and DMDS, the responses are the same order of magnitude as DMSP.

2.2.4 Conclusion

The base hydrolyse FIA system is more specific than the borohydride variant.

2.3 Headspace gas solid chromatography for the measurement of DMS and DMS precursors

2.3.1 Introduction

A number of GC detectors have been employed for the measurement of DMS in environmental analysis. These include the flame photometric detector (FPD), the flame ionisation detector (FID), the electron capture detector (ECD), the dual flame modification of the FPD (DFPD) and the pulsed flame photometric detector (PFPD) (Cheskis et al., 1993, Patterson et al., 1978). When particularly low levels of DMS

have to be measured, a preconcentration process may be required prior to its detection. Andreae et al. have developed a glass cryogenic trap filled with 15% OV3 on Chromosorb W which could not only effectively concentrate DMS but also separate different compounds (Andreae & Barnard, 1983).

The measurement of DMSP based on its cold alkaline hydrolysis to DMS and acrylate was first described by Challenger et al. in 1957 (Challenger et al., 1957). The reaction is completed in an hour (White, 1982). Since some other natural sulfonium compounds, such as S-methylmethionine (SMM), also release DMS after treatment with strong alkali, other DMS precursors (DMS-pr) might interfere in their analysis (Howard & Russell, 1996). The DMS released by treatment with strong alkali is therefore inferred DMS precursors. This section describes a headspace analysis technique using gas solid chromatography with flame photometric detection for the measurement of DMS precursors.

2.3.2 Experimental

2.3.2.1 Gas solid chromatography apparatus

The gas solid chromatography (GSC) system consisted of Pye Unicam GC oven equipped with a PTFE column (3.2 m x 1/8 " i.d.) containing Chromosorb 101 (60-80 mesh) and a flame photometric detector (EMI 6256B). For the measurement of DMS, the oven was operated under isothermal conditions at 150 °C with a nitrogen gas carrier gas flow rate of 25 ml/min.

2.3.2.2 Headspace analysis

DMSP standard solutions (1 – 20 mM) were placed in 4 ml screw-capped glass vials and made up to 1 ml with deionized water. 1 ml of a 10 $mol \cdot dm^{-3}$ NaOH solution was

then added to each vial to give a series of DMSP standard solutions. The vials were immediately sealed with a septum cap containing a PTFE–faced butyl rubber septum. All these DMSP standard solutions were incubated in a boiling water bath for 10 min and then cooled to room temperature. 50 μl of the headspace gas was taken with a gas tight syringe and injected into the GC system. A log (response) vs log (concentration) calibration curve was constructed. As for real samples, the sample reactions were carried out at room temperature and then the headspace gas was injected into the GC system as described above.

2.3.3 Results and discussion

Strong alkaline hydrolysis of DMSP gives DMS and acrylate and the released DMS in the headspace can be determined by GSC. The retention time of DMS is approximately 3.2 mins in this system. The GSC system was calibrated by using peak area measurements obtained from a series of standard DMSP solutions (Fig, 2.9) covering the range from 2×10^{-6} to 3×10^{-4} mol.dm^{-3}. Each measurement was made four times.

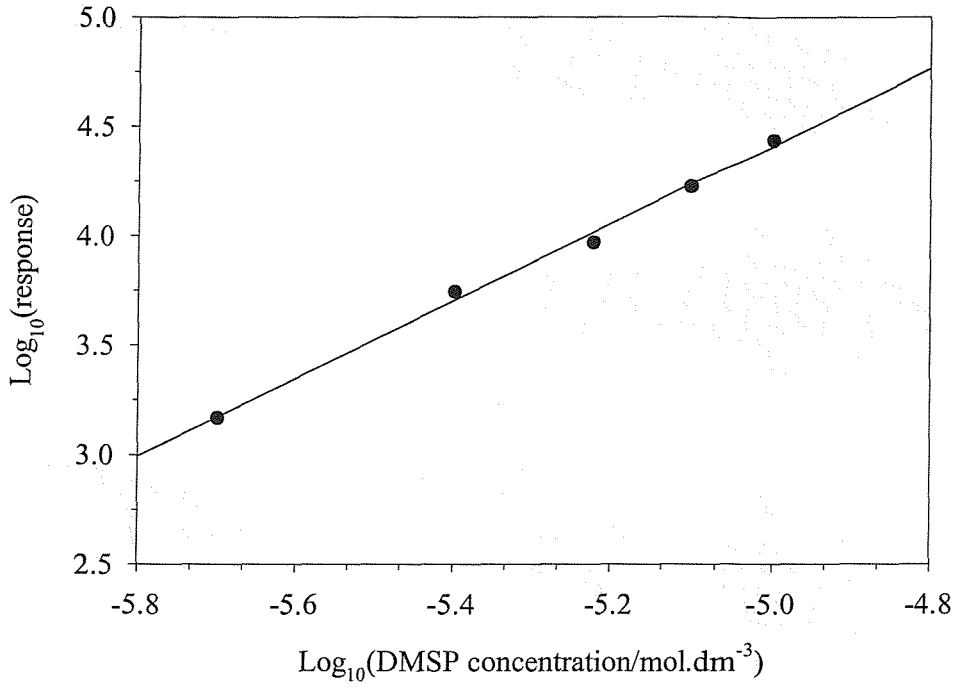


Figure 2.9 Typical calibration line for DMSP ranging from 2 x 10^{-6} to 10^{-5} mol.dm^{-3} (gradient 1.753).

2.3.4 Conclusion

The headspace procedure offers a very simple, sensitive and fast method for the measurement of DMS that can also be applied to the measurement of DMS-precursors in marine samples following their cold alkaline hydrolysis.

2.4 DMSP lyase assay

2.4.1 Introduction

DMSP is degraded intracellularly in some algae by an enzymatic cleavage to produce DMS and acrylate (Cantoni & Anderson, 1956, Ishida & Kadota, 1968). In later extensive studies it became evident that bacterial cleavage of dissolved, extracellular DMSP is also an important alternative route of DMS production (Dacey & Blough, 1987, Desouza & Yoch, 1995a, Kiene, 1990, Kiene, 1991, Ledyard & Dacey, 1994). The enzyme that converts DMSP into DMS and acrylate is called DMSP lyase. The DMSP lyase activity has been studied in seawater (Kiene, 1990, Kiene, 1992, Kiene & Service, 1991, Kiene & Taylor, 1987) and salt marsh sediment samples (Dacey & Blough, 1987, Kiene & Taylor, 1987, Kiene & Taylor, 1988, Taylor & Gilchrist, 1991), where bacteria were assumed to be the primary source of the enzyme. A number of marine algae also contain the enzyme (Desouza et al., 1996, Nishiguchi & Goff, 1995, Steinke et al., 1996).

The DMSP lyase activity of samples is commonly determined by measuring the amount of DMS emitted from added DMSP over time. The evolved DMS is subsequently analysed by gas chromatography (Bacic & Yoch, 1998, Desouza & Yoch, 1995b, Ledyard et al., 1993, Stefels & Dijkhuizen, 1996). This section reports the development of such a method for measuring DMSP lyase activity. The DMSP lyase activity is calculated from the rate of DMS production with time under specified conditions.

2.4.2 Experimental

1 ml of sample was put into a 4 ml vial and left at room temperature for 10 hours to digest endogenous DMSP. Then 1 ml of neutralised $2x10^{-2}$ M DMSP solution and 0.5 ml of 100mM potassium sulphate buffer solution were added to the vial and immediately sealed with a screw cap containing a PTFE-faced butyl rubber septum.

The mixture was shaken for homogenisation and DMSP lyase reaction started. The DMS liberated into the headspace was sampled with a gas tight syringe (50 µl gas) and then injected into the GSC system as described in Section 2.3.2.1. Repeated measurements were made over a 5 to 10 hours period dependant on the activity of DMSP lyase. DMSP lyase activity was calculated from the rate of DMS evolution by this enzymatic cleavage. A calibration curve was prepared from a series of DMSP standard solutions to which NaOH had been added (see section 2.3.2.2).

2.4.3 Discussion

In the measurement of DMSP lyase activity, DMSP is present in considerable excess and the enzyme activity is therefore directly proportional to the DMS production rate. For the purposes of this work one unit of the enzyme activity was defined as being the quantity of enzyme that will liberate one µmole of DMS per minute at a specified temperature. More details of the DMSP lyase will be presented in the next chapter.

2.5 Capillary gas chromatography of elemental sulphur

2.5.1 Introduction

Elemental sulphur is an intermediate product in the biogeochemical cycling of sulphur in marine sediments. The measurement of elemental sulphur can be very complicated and time consuming as it normally has to be derivatized prior to its determination by colorimetry or polarography (Berner, 1964, Cutter & Krahforst, 1988). The precision and accuracy of such methods are poor and sensitivity is also relatively low. A new analytical method for the measurement elemental sulphur has been reported using gas chromatography-mass spectrometry (Chen et al., 1997). This technique was found to be simple, fast, sensitive and interference free. The absolute detection limit of this

method was 96 pg S^{o}. This method has been applied to the determination of elemental sulphur in lake sediments. In this section, capillary gas chromatography with flame photometric detection was examined as a direct means of measuring elemental sulphur.

2.5.2 Experimental

2.5.2.1 Reagents and standards

Standard stock solutions (1000 ng/μl). 0.1 g of elemental sulphur (S^{o}, Sigma) was accurately weighed and dissolved in reagent grade anhydrous diethyl ether (Sigma 100 ml) to give a 1000 ng/μl stock solution. A series of standard solutions was prepared daily from the stock solution by dilution with anhydrous diethyl ether. All standard solutions were stored in ground glass stoppered volumetric flasks and kept at room temperature.

2.5.2.2 Instrumentation

Capillary gas chromatography was performed using a Perkin-Elmer model 8500 gas chromatograph equipped with a flame photometric detector and fitted with a 30 m fused silica capillary column (Alltech Econo-cap 0.25mm i.d.) coated with a medium polarity bonded phase (SE-54, 0.25 μm film thickness). The injection port temperature and the detector were maintained at 170 °C and 250 °C, respectively. Nitrogen was used as a carrier gas at a flow rate of 1 ml/min. A 1 μl sample volume was injected using the splitless injection method. The GC temperature program was optimised to provide good separation of the standard elemental sulphur (Table 2.5).

Start temperature (°C)	End temperature (°C)	Rate (°C / min)	Time (min)
40	40	0	3
40	320	15	18.67
320	320	0	5

Table 2.5 The optimised GC column conditions.

2.5.2.3 The extraction of elemental sulphur compound into diethyl ether from sediments

Sediment samples were collected from the estuary of the River Test in Totton, using syringe cores which could be sealed with rubber stoppers. The samples were kept in an ice box after sampling and returned to the laboratory within an hour. These cores were stored at -20°C until they could be analysed. To avoid the influence of oxidation, sample preparation was carried out as quickly as possible. Approximately 15 g of defrosted sediment, dried with anhydrous magnesium sulphate, was extracted using 15 ml of diethyl ether and sonicated for 12 hours. 1 µl of the organic phase was injected into the chromatograph for the determination of elemental sulphur.

2.5.3 Results and discussion

2.5.3.1 Instrument performance

Elemental sulphur resulted in a major peak at 19.19 min (presumed to be S_8) together with two small peaks (16.06 and 17.65 min) that were assigned to different forms of elemental sulphur. For calibration, a series of the standard elemental sulphur solutions in diethyl ether, ranging between 5 to 40 ng /µl, was employed. A calibration line was generated from peak area measurements (Fig. 2.10). The detection limit was determined by injecting 1 µl of individual standard solutions until a signal was

observed that was twice the standard deviation of a signal close to the blank. The absolute detection limit for the elemental sulphur determination by capillary GC was ca. 1 ng of sulphur injected.

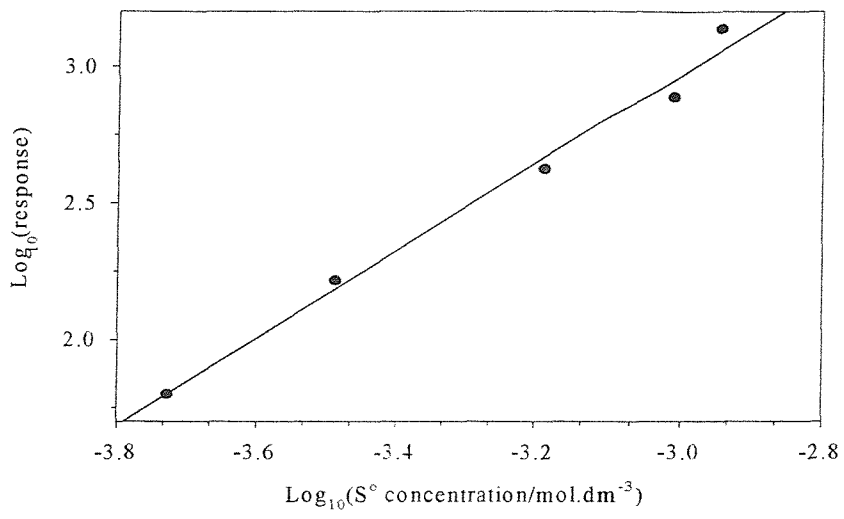


Figure 2.10 Typical calibration line for elemental sulphur ranging from 6 to 36.4 ng/µl (gradient 1.603).

2.5.3.2 The extraction of elemental sulphur from sediments

In all extraction experiments that were carried out using dry sediment, only one major peak was evident at 19.19 min. That was as element sulphur. Two minor peaks also

accompanied the S_8 sulphur peak at 16.05 and 17.64 min as same as the standard solution. These two peaks were assigned to different forms of elemental sulphur and need further identification. For quantitation, the sample extract was diluted to fit in the range of the calibration line.

2.5.4 Conclusion

The capillary gas chromatography with flame photometric detector instrumentation is successful for the determination elemental sulphur. The absolute detection limit of this method is 1 ng and elemental sulphur can be well identified through a combination of temperature programs.

2.6 Conclusion

This chapter has described the development of a number of analytical methods. These have been for the measurement of sulphide, DMSP, DMS precursors and elemental sulphur and for the assay of DMSP lyase activities. Flow injection analysis methods have been developed for the measurement of acid volatile sulphide species and precursors of DMS such as DMSP. The measurements of DMS, DMS precursors and elemental sulphur were carried out by gas chromatography.

The flow injection system differs only in the chemistries employed to generate volatile sulphur species from the analytes. An acid reaction step permits the measurement of sulphide species such as S^{2-}. Stabilisation of sulphide can be successfully achieved by formation of colloidal zinc sulphide. By replacing the acid reaction step with a simple base hydrolysis, a system has been developed that responds strongly to DMSP but not to other tested potential DMS precursors. For the measurement of a wider range of DMS precursor species the base reaction step can be

replaced by $NaBH_4$. Whilst not exhibiting equal sensitivity to all DMS precursors, this technique can provide an invaluable source of information on the presence of other precursors such as S-methylmethionine (SMM) etc. All these variants of the FIA system exhibit detection limits of ca 10^{-6} M and are suitable for use in the study of sulphide species in estuarine sediments.

Gas chromatography has been developed for the separation and identification of DMS and this has been used to assess the DMS evolved from DMS precursors following the base hydrolysis reaction. This technique has been successfully applied to the measurement of total DMS precursors in estuarine sediments and the assay of DMSP lyase activities. The detection limit is ca 10^{-6} M of DMS.

A method based on capillary gas chromatography with flame photometric detection has been developed for the measurement of elemental sulphur. The absolute detection limit of elemental sulphur is 1 ng injected. This technique is suitable for the study of the distribution of elemental sulphur in marine sediments.

CHAPTER THREE

DMSP and DMSP lyase in Algae

This chapter is divided into two parts. Firstly, an investigation of the effect of heating on the apparent DMSP content of algae is described following experiments reported by Karsten in 1994. Secondly, a DMSP lyase that releases DMS from DMSP was isolated from *Enteromorpha intestinalis* and characterised.

3.1 The DMSP content of marine algae

3.1.1 Introduction

DMSP is an important biochemical precursor of atmospheric DMS (Andreae, 1990) and is widely distributed in macro- and microalgae and in some coastal higher plants (Karsten et al., 1994, Karsten et al., 1990a, Reed, 1983, Weber et al., 1991, White, 1982). In general the DMSP content is highest in green and some red algae (Karsten et al., 1990a, Reed, 1983). The methods employed for the measurement of the DMSP content of macroalgae normally involve the treatment of freshly collected algae with strong alkali (NaOH) in a gas-tight vial, followed by gas chromatographic quantification of the emitted DMS (Edwards et al., 1988, Karsten et al., 1990a, Reed, 1983). In the study of DMSP in algae, Karsten found that for some species, oven drying resulted in significantly higher levels of DMSP being measured (Karsten et al., 1994). In view of the non-specific nature of the alkaline hydrolysis method employed to measure DMSP it was therefore proposed to investigate further this apparent DMSP increase resulting from the oven drying of algae prior to their analysis.

3.1.2 Experimental

3.1.2.1 Sample collection and storage

Twelve species of macroalgae were collected from Lepe beach, Hampshire on the 28th September 1997 (ref. SZ 457985). These seaweed samples were returned to the laboratory in unfiltered seawater within an hour of collection and were then transferred into an aerated tank of seawater. The analysis was carried out within 24 hours of sampling. A portion of the fresh algal material was blotted dry using tissue paper and then oven dried at 85 °C for 24 hours. Other samples were analysed without the oven drying stage.

3.1.2.2 Headspace analysis

The fresh samples were rinsed with seawater, blotted dry using tissue paper and weighed (0.2-0.5 g depending on the species) into 4 ml screw-capped glass vials with 1 ml of 10 mol.dm^{-3} NaOH solution. Each vial was immediately sealed with a septum cap containing a PTFE–faced butyl rubber septum. The vial was incubated at room temperature for 24 hours and 50 µl of the headspace gas was taken with a gas-tight syringe and injected into the GC system (as described in Section 2.3.2.1). Oven dried samples were also prepared as described above. All samples were run in four replicates.

3.1.2.3 Calibration

Calibration curves were constructed using the procedure described in Section 2.3.2.2. After alkali treatment, DMSP was cleaved in a 1:1 ratio into DMS and acrylate. Quantitation was obtained by comparing peak areas arising from the released DMS to the calibration curve.

3.1.3 Results and discussion

The DMSP contents of the macroalgae are shown in Table 3.1. The green algae contained higher concentrations of DMSP than the red and brown algae, both in fresh samples and in oven dried samples. Similar results were reported by Bischoff *et al.* and White (Bischoff et al., 1994, White, 1982).

In all species the DMSP contents were higher in the fresh samples than in the oven dried samples. It was possible that some of the DMS precursors decomposed on heating. In contrast, Karsten *et al.* found that oven drying of some green and red algae resulted in significant increases in the reported DMSP levels (Karsten et al., 1994). In order to explain this interesting result, they postulated that the drying process might cause cell wall breakdown and the loss of their ion exchange properties. The evolved DMS was therefore released without delay from the cells. The result obtained by Bischoff *et al.* however showed that oven drying of green and red algae resulted in the lowering of the measured DMSP levels (Bischoff et al., 1994). The significant increase of DMSP on oven drying could not be reproduced by Russell and Howard (Russell, 1995). Based on the results of these and previous studies, the heating procedure seems to affect the condition and structure of DMSP in algae. Thus fresh samples were preferred for the determination of the intracellular DMSP content of algae.

Species	Fresh sample mmole / kg FW.	Water content (%)	Oven dried sample mmole / kg FW.
Ulva lactuca	11.000 ± 1.271	75.23	8.0115 ± 1.807
Enteromorpha intestinalis	6.400 ± 0.96	84.50	4.53 ± 0.520
Enteromorpha linza	17.150 ± 2.44	67.26	5.318 ± 1.550
Rhodophyllis sp.	0.061 ± 0.024	81.20	< 0.031
Seirospora seirosperma	0.163 ± 0.012	81.64	< 0.039
Grateloupia doryphora	0.022 ± 0.003	76.03	< 0.022
Halarachnion ligulatum	< 0.017	79.43	< 0.017
Griffithsia floculosa	0.083 ± 0.026	86.80	< 0.014
Rhodymenia pseudopalmata	0.018 ± 0.004	71.68	< 0.013
Fucus vesiculosus	0.144 ± 0.127	77.61	< 0.013
Dictyota dichotoma	0.257 ± 0.245	82.01	< 0.024
Laminaria saccharina	0.031 ± 0.010	69.40	< 0.012

Table 3.1 DMSP content of macroalgae from fresh samples and oven dried samples (data represented as mean ± standard deviation (n = 4) on the basis of fresh weight (FW)).

3.2 The Isolation and Characterisation of DMSP lyase from *Enteromorpha intestinalis*

3.2.1 Introduction

A DMSP lyase (dimethylpropio-dethiomethylase), which catalyses the cleavage of DMSP, has recently been isolated from the bacterium *Alcaligenes* species (M3A) by De Souza (De Souza & Yoch, 1995b). This enzyme is inducible by its substrate, DMSP; its product, acrylate; and several acrylate analogues. The Michaelis-Menten constant (K_m) values for DMSP determined by cell culture and by the study of the pure enzyme are similar to the values found for microbes in aerobic seawater and

aerobic sediment. It was suggested that the microbes in these ecosystems might have similar enzymes to the one purified from *Alcaligenes*.

Furthermore, based on the use of inhibitors, de Souza proposed that this DMSP lyase may be located in the periplasmic space or loosely associated with the outer cell membrane (De Souza & Yoch, 1995a). Subsequently, the polyclonal antibody was prepared from the *Alcaligenes* DMSP lyase and it was found that this antibody was equally reactive against the *Alcaligenes* and other marine bacterial DMSP lyases. Therefore, due to the finding of cross-reaction with polyclonal antibodies, it may be possible to employ the bacterial antibody as a probe to identify and quantify DMSP lyase in marine environments.

In marine algae, DMSP lyase is believed to be a major mechanism by which DMSP is broken down to DMS. In 1956 DMSP lyase activity was found in crude extracts of the red alga *Polysiphonia lanosa* by Cantoni and Anderson (Cantoni & Anderson, 1956). They suggested that this enzyme is bound to protoplasmic particles and requires sulfhydryl compounds (with SH groups) for activity. The pH optimum was around 5.1 and it showed relatively narrow substrate specificity.

Preliminary studies of DMSP lyases in crude and partially purified extracts of macro- and micro-algae have shown that these enzymes can exhibit different enzyme activities or functions, even in closely related species (Kadota & Ishida, 1968, Stefels & Dijkhuizen, 1996, Steinke et al., 1996, Steinke & Kirst, 1996).

The first purification of DMSP lyase from algae was obtained using the red alga *Polysiphonia paniculata* (Nishiguchi & Goff, 1995). This enzyme activity was found in two different membrane-bound proteins having different isoelectric points (pI), K_m and molecular masses. The addition of cysteine protease inhibitors decreased the DMSP lyase activity by binding to the cysteine residues of the enzyme active site. The addition of magnesium and calcium on the other hand increased the enzyme

activity. Thus, it was tentatively suggested that this DMSP lyase is a metal dependant enzyme and that it may be associated with osmoregulation by changing the intracellular DMSP concentration and ion shifts.

DMSP lyase has also been purified and characterised from the green macroalga *Ulva curvata* (De Souza et al., 1996). The DMSP lyase activity could be detected in one soluble and three membrane-bound proteins which all cross-reacted with the polyclonal antibody raised against the bacterial (*Alcaligenes* strain M3A) DMSP lyase. All of these enzymes had an identical molecular weight of 78 kDa and the optimum pH (pH8.0) is similar to that of seawater. However, altering the NaCl concentration did not significantly affect the activity of the soluble DMSP lyase.

Enteromorpha intestinalis is an euryhaline alga that is one of the first algae to colonise exposed areas because it can tolerate a range of salinities and temperature. It grows in freshwater, tidal pools, estuaries, mud flats and hypersaline environments. The aim of the work reported in this section was to isolate and characterise DMSP lyase from this important alga which colonises many of the estuaries of south England.

3.2.2 Materials and Methods

3.2.2.1 Preparation of a crude enzyme extract

Enteromorpha intestinalis was collected from the mouth of Langston harbour at Eastney, Portsmouth (ref: SZ 688993) from 1996 to 1997. Samples were returned to the laboratory within an hour of collection and kept in a well aerated tank of sea water. The fresh material was briefly rinsed in seawater and blotted dry using tissue paper. This sample was suspended in an extraction buffer which contained 50mM potassium phosphate buffer, 10% glycerol and protease inhibitors (1mM

benzamidine; 5mM β-mercaptoethanol and 0.1mM phenylmethylsulphonylfluoride (PMSF). This mixture was adjusted with NaOH to pH8.0. Other protease inhibitors, such as 5mM EDTA and 5mM dithiothreitol (DTT), were also tested. This extraction buffer will be referred to as the buffer for further isolation steps, but its pH might be adjusted to desired values by adding NaOH or HCl. The extraction buffer and alga were put on an ice bath for 20 min before being mixed together. The mixture was then homogenised 3 times using a high speed homogenizer for 1.5 min. A 7 to 10 min interval between homogenizations allowed the mixture to be kept below 10 °C in an ice bath. The homogenate was then centrifuged at 27,000 g for 30 min at 4°C. The supernatant solution containing the DMSP lyase activity was called the crude extract. The DMSP lyase activity was determined by the measurement of the DMS produced by DMSP lyase acting on added DMSP.

Some alga was blotted dry using tissue paper and frozen at -20 °C on the day of sampling. The frozen alga was defrosted on an ice bath and extracted using the procedure described previously. DMSP lyase activity was still present in the crude extract after freezing for one to two months and enzyme purification could therefore be carried out using frozen material.

3.2.2.2 Protein assays

The protein concentrations in the crude extract and subsequent isolates were measured using the Bradford reagent (Bio-Rad) standardised using bovine serum albumin (Bradford 1976). 200 µl of the reagent was added into an Eppendorf microcentrifuge tube containing 5 µl of protein sample and 795 µl of the buffer solution described above. The reaction was shaken and left for 2 min. For the blank solution, 200 µl of the reagent was mixed with 800 µl of the buffer solution. The solution was then transferred to a spectrophotometer cuvette (1 ml) and total protein was assayed spectrophotometrically at 595 nm.

3.2.2.3 Enzyme assay

2 ml of buffered (pH8.0) enzyme solution was placed in a screw-capped glass vial (volume 4 ml). 1ml of DMSP solution was added from a neutralised stock solution (2 x 10^{-2} M) and the vial was immediately sealed with a septum cap containing a PTFE–faced butyl rubber septum. The reaction vials were held at room temperature for enzyme assay unless mentioned specially. 50 µl of the headspace gas was then analysed for DMS by gas chromatography using flame photometric detection (GC-FPD) (Section 2.4). A unit of DMSP lyase activity is defined as the amount of enzyme required to produce 1 µmol of DMS in 1 min at room temperature (19 °C). Specific enzyme activity is the DMSP lyase activity per gram of protein. Specific enzyme activity is used as an index of purification efficiency at each separation step.

3.2.2.4 Purification of soluble DMSP lyase

The buffer used in subsequent procedures and assays had the same composition as that used in the extraction step, but its pH was adjusted to desired values by adding NaOH or HCl. It will simply be referred to as the buffer.

(a) Optimisation of conditions for protein precipitation

A crude extract was prepared by the extraction process described earlier. Ammonium sulphate was added slowly in 4 steps to the crude extract, producing concentrations of 35, 55, 65 and 75 % saturation. All of these mixtures were stirred for 30 min at 4 °C and then centrifuged at 25,000 g for 15 min. The resulting protein precipitates were separately dissolved in the minimum quantity of extraction buffer and dialysed in the same buffer overnight. Finally, all of the supernatants and dissolved precipitate

fractions were measured for DMSP lyase activity and total protein. Fractions were collected for further purification procedures based on these measurements.

(b) Column chromatography trials

In order to further purify the DMSP lyase, a number of chromatography columns were examined: GFC Sephacryl S-300 HR, the anion exchange material diethylaminoethyl Sepharose CL-6B, the cation exchange material CM Sepharose CL-6B and the hydrophobic chromatography medium Phenyl Superose (Pharmacia).

(1) Hydrophobic chromatography

Following a 40 % ammonium sulphate protein precipitation step, the supernatant was loaded onto a Phenyl Superose fast protein liquid chromatography (FPLC) column (Pharmacia, 5 x 50 mm) which had been equilibrated with buffer (pH8.0) containing ammonium sulphate at 40 % saturation. The column was eluted with a gradient of the buffer solution (pH8.0) containing from 40 to 25 % ammonium sulphate saturation and was then eluted with the pure buffer (pH8.0). The flow rate was 0.1 ml/min. Fractions were collected and assayed for activity.

(2) Ion exchange chromatography

Based on the observation that enzyme activity could be maintained in frozen algae for at least a month, a bulk concentrated enzyme fraction, prepared from a 35% to 75 % ammonium sulphate saturation cut, was kept at –20 ^{o}C. This frozen concentrated enzyme fraction was used in the following chromatography trials.

An aliquot of the frozen concentrated enzyme was defrosted, dialysed in the buffer (pH6.5) overnight and then loaded onto a 5 ml CM Sepharose Cl-6B syringe column equilibrated with 50ml of the buffer (pH6.5). The column was washed with 10 ml of

the buffer (pH6.5) and then eluted with 10 ml of the buffer (pH6.5) containing 0.5 M KCl. 1ml fractions were collected and assayed for lyase activity.

Some of the frozen concentrated enzyme was also dialysed in the buffer (pH8.0) and then loaded onto a 5 ml DEAE Sepharose CL-6B syringe column equilibrated with 50 ml the buffer (pH8.0). The column was washed with 10 ml of the buffer (pH8.0) and then eluted with 10 ml of the buffer (pH8.0) containing 0.5 M KCl. 1ml fractions were collected and assayed for lyase activity.

(3) Gel filtration chromatography

A portion of the concentrated enzyme protein was defrosted and directly passed through a GFC Sephacryl S-300 column using buffer (pH7.0) as eluent. The first portion containing enzyme activity was filtered with a Nuclepore membrane (pore size 0.1 μm, 47mm i.d.). The enzyme activity in the unfiltered fraction was determined and compared with that of the filtrate.

(c) Separation by sequential column chromatography

The protein fraction obtained from 35% to 75% ammonium sulphate precipitation was redissolved in the buffer (pH8.0) and dialysed in 2 L of the same buffer solution overnight with one change of the buffer. The sample was ultracentrifuged at 170 kg for one hour to remove solid material and then loaded onto a DEAE Sepharose CL-6B column (50 x 85 mm) which had been pre-equilibrated with the buffer (pH8.0). The pellet from the centrifugation was resuspended in the buffer and assayed for enzyme activity. The column was eluted with 400 ml of buffer (pH8.0) and then with a linear gradient of 520 ml of a buffer containing KCl from 0.3 to 0.6 M at a flow rate of 50

ml/hr. The fractions containing DMSP lyase activity were pooled and concentrated with a 10 kDa membrane fitted in an Amicon stirred cell (50ml) at 4 °C.

The concentrated sample was dialysed overnight against buffer (pH8.0) with one buffer change. Ammonium sulphate was added slowly to the sample solution to give a final concentration of 35% saturation. The mixture was stirred for 30 min at 4 °C and then centrifuged. The supernatant was filtered with a Nuclepore membrane (pore size 0.22 μm) before being applied to a hydrophobic interaction chromatography (HIC) Sepharose CL-4B column (50 x 60 mm) which had been preequilibrated with the buffer (pH8.0) containing 35% saturation ammonium sulphate. The column was eluted with 250 ml of 35% saturation ammonium sulphate buffer (pH8.0) and then with a linear gradient of 510 ml of a buffer (pH8.0) containing ammonium sulphate from 35% to 0 saturation, at a flow rate of 50 ml/hr. The fractions containing DMSP lyase activity were pooled, concentrated with a 10 kDa membrane fitted in an Amicon stirred cell (50ml) and then with a Centricon 10 filter (Amicon Corp) to 1 ml at 4 °C.

The 1 ml sample was loaded onto a Hiload 16/60 Superdex 200 preparative grade gel filtration column (Pharmacia Biotech) in an FPC system. The column was equilibrated with the buffer (pH8.0) and eluted with the same buffer (0.5 ml/min). The fractions having enzyme activity were mixed and concentrated again to 1ml for separation using a Hiload 16/60 Superdex 75 preparative grade gel filtration column. This second column was also equilibrated with the buffer (pH8.0) and eluted with the same buffer (0.5 ml/min).

(d) Enzyme purity

The purity of each fraction was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Cromassie blue and silver stains. Molecular mass and subunit composition of DMSP lyase were determined by calibrating migration distances using commercially available molecular weight marker proteins (Sigma). Nondissociating discontinuous electrophoresis was performed on 8% and

10% SDS-polyacrylamide gel (8x10 cm). The stacking gel was loaded at 80 V and then switched to 120 V at room temperature until the elution front was 1~ 2 cm above the bottom of the gel. In addition, a Pharmacia PhastSystem electrophoresis (SDS-PAGE) was also employed. Electrophoresis was carried out on an 8-25% acrylamide gradient gel (PhastGel 8-25) using a PhastGel SDS buffer strip using a constant current of 20 mA at 15 °C.

(e) Determination of molecular weight

The molecular weight of the soluble DMSP lyase was determined by calibrating Superdex 200 and Superdex 75 columns with standard proteins. The subunit molecular weight of DMSP lyase was obtained from the SDS-PAGE with a series of protein standards.

3.2.2.5 Isolation of detergent-released DMSP lyase

The above experiments indicated that membrane-bound DMSP lyase might be present in *Enteromopha intestinalis*. The isolation of membrane-bound DMSP lyase from *Enteromopha intestinalis* was therefore carried out by treating with detergent. The experiment was started with the pellet that had been separated from the crude enzyme extract described above (Section 3.2.2.1). A 300 g pellet was suspended in 300 ml of buffer (pH8.0) containing 1.5% Triton X-100 and 0.025% sodium azide and stirred at 4 °C for 24 hours. The mixture was centrifuged at 25,000 g for 40 min. Ammonium sulphate was added to the supernatant to give a final concentration of 35% saturation. The mixture was then stirred for an hour and ultracentrifuged at 140,000 g for 40 min. The floating thick lipid layer was removed and filtered through a Whatman No1 filter. The clear solution was re-filtered through a Nuclepore membrane (pore size 0.22 μm) before being loaded onto a Sepharose CL-4B (50 x 60 mm) HIC column which had been equilibrated with the buffer (pH8.0) containing ammonium sulphate at 35 % saturation. The column was eluted with 500 ml of the buffer solution (pH8.0) containing a gradient from 35% to 0 saturation ammonium sulphate, followed by a

washing step with 430 ml of the buffer (pH8.0). The flow rate was 50 ml/hr. Fractions were collected and assayed for lyase activity.

The three portions with enzyme activity were pooled individually and concentrated using a Centricon 10 filter (Amicon Corp) to 1ml. These were then loaded onto Superdex 75 columns (Pharmacia). The column was eluted with buffer (pH8.0) at 0.5 ml/min. Fractions were collected and assayed for lyase activity.

3.2.2.6 Determination of the optimum temperature and pH of the soluble DMSP lyase activity

DMSP lyase activities were tested at 3 different temperatures, namely room temperature (ca. 19 °C), 30 °C and 37 °C. Before the addition of the DMSP solution, the reaction mixtures were preincubated at the test temperature for 10 min. For the determination of the pH optimum, the reaction mixtures were adjusted with NaOH and HCl to a range of pH values between 5.3 and 8.3. Enzyme activities were assayed as described above (Section 3.2.2.3). All of these experiments were carried out with 100 µl of the frozen concentrated enzyme fraction as described in Section 3.2.2.4(b)(2).

3.2.2.7 Characterisations of the soluble DMSP lyase

(a) The effects of cofactors and metal chelators on the activity of DMSP lyase

The effects of some substances which can act as enzyme cofactors were examined. Sodium chloride and reduced glutathione (R-GHS) were added to the crude extract to give final concentrations of 0.5M NaCl and 0.0128 g R-GHS per ml, respectively. To measure whether metal chelators inhibited lyase activity, 5 mM and 10 mM EDTA were added to the crude extract and incubated at 37 °C for 10 min. Subsequently, magnesium chloride (Sigma) and calcium chloride (Sigma), at concentrations ranging from 5mM to 100 mM, were examined to assess their effects on the activity of the

DMSP lyase. After each incubation the activity of the enzyme was measured after the addition of neutralised DMSP stock solution. Untreated samples served as controls.

(b) The substrate specificity of DMSP lyase

A variety of sulphonium compounds including DMSP, S-methyl methionine (SMM), dimethylsulphonio-2-propionate (DMS-2-MP), dimethylsulphonioacetate (DMSAcet) and dimethylsulphoniopentanoate (DMSPent) were prepared as 150mM stock solutions. These compounds had been synthesised by Dr. D. Russell (University of Southampton, UK)(Howard & Russell, 1997). The test of substrate specificity was carried out by individually adding the sulphonium stock solutions to reaction vials containing partially purified soluble DMSP lyase (after HIC column separation) and 50mM phosphate buffer (pH8.0). After 2 hours, the enzyme activity was measured.

The inhibition effects of DMSP analogues were examined by incubating the above sulphonium compounds (DMSP excluded) at the concentrations between 3 and 4 mM, with partially purified soluble DMSP lyase in 50mM phosphate buffer (pH8.0) for 10 min. The DMSP substrate was then added to the reaction vials and the enzyme activity was measured and compared with that of the untreated sample.

3.2.2.8 Determination of the kinetic constants for soluble DMSP lyase

The K_m and maximum velocity (V_{max}) of the soluble DMSP lyase were determined by measuring DMS evolution from 41.3 μg of partially purified DMSP lyase reacting with DMSP at concentrations between 20 to 8000 μM and calculated by Lineweaver-Burke plots. The velocity is reported as the amount of DMS produced per 41.3 μg enzyme per min. These reactions were all carried out in 4ml gastight vials at room temperature and the DMS product was removed from the headspace and immediately injected into the GC-FPD system as described for the enzyme assay (Section 2.4).

3.2.3 Results and Discussion

3.2.3.1 Preparation of a crude enzyme extract

Storage of alga at -20 °C demonstrated that lyase activity was still to be found in frozen material within 2 months of collection. In the crude extract from this frozen material the specific enzyme activity was ca. 0.6 to 1 μmol DMS·min^{-1}· mg^{-1} protein. However, the specific activity after overnight dialysis dropped to about 50% of the specific activity of the crude extract. This may be due to the depletion of the natural co-extracted DMSP concentration during the dialysis process and natural decay. Similar results have been reported by Steinke who showed that the specific activities of three *Enteromorpha* species in crude cell extracts ranged from 7 to over 100 nmole DMS·min^{-1}· mg^{-1} protein (Steinke et al., 1996).

The crude extract preparation required the addition of protease inhibitors to maintain the DMSP lyase activity. It was found that without these protease inhibitors the enzyme activity dramatically decreased to 0 in further purification steps. A number of protease inhibitors: 1mM benzamidine, 0.1mM PMSF and 5mM β-mercaptoethanol were chosen for further experiments. Dithiothreitol (DTT) was however an inhibitor of DMSP lyase. The relative enzyme activity decreased dramatically when DTT was added into the reaction mixture at a concentration of ca. 1 mM (Table 3.2). The enzyme activity dropped to less than 10% with the addition of 5 mM DTT.

Dithiothreitol concentration (mM)	Relative enzyme activity (%)[a]
0	100
1	17.3
5	6.8

Table 3.2 The effect of dithiothreitol on DMSP lyase activity

[a] The enzyme activity of untreated sample served as control (100% activity).

3.2.3.2 Purification of the soluble DMSP lyase

(a) Optimisation of conditions for protein precipitation

In order to obtain optimum conditions for the enzyme precipitation, 4 ammonium sulphate saturation cuts were employed. Even though the protein fraction in the 0 to 35 % cut showed the highest enzyme specific activity, over 95 % of the total DMSP lyase remained in the supernatant (Fig. 3.1). The protein precipitate in the 35 to 65 % cut was the optimum fraction, containing almost 80% of the total DMSP lyase. Above the 75% cut, DMSP lyase activity was rarely measured in the supernatant. A similar result was reported for *Ulva currata* (Desouza et al., 1996) and some bacterial DMSP lyases. In contrast, the protein fraction in the 0 to 35 % ammonium sulphate cut contained almost 90% of the total enzyme activity in *Enteromorpha clathrata* (Steinke & Kirst, 1996).

The precipitate from the 35% to 65% ammonium sulphate cut was dissolved and dialysed to reduce the ammonium sulphate concentration. The specific enzyme activity was found to have increased 7 fold compared with the crude extract and the protein precipitation step was therefore a useful purification procedure.

Due to residual particles, it was necessary to ultracentrifuge the concentrated protein before loading it onto the column. Enzyme activity was however found in the resuspended pellet and it is therefore suggested that there are water soluble as well as membrane-bound DMSP lyases existing in *Enteromorpha intestinalis.* It has been reported that DMSP lyase is membrane-bound in the macroalgae Rhodophyte *Polysiphonia lanosa* (cantoni & anderson, 1955), *P. paniculata* (Nishiguchi & Goff, 1995), the Chlorophyte *Enteromorpha clathrata* (Steinke & Kirst, 1996) and *Ulva currata* (Desouza et al., 1996).

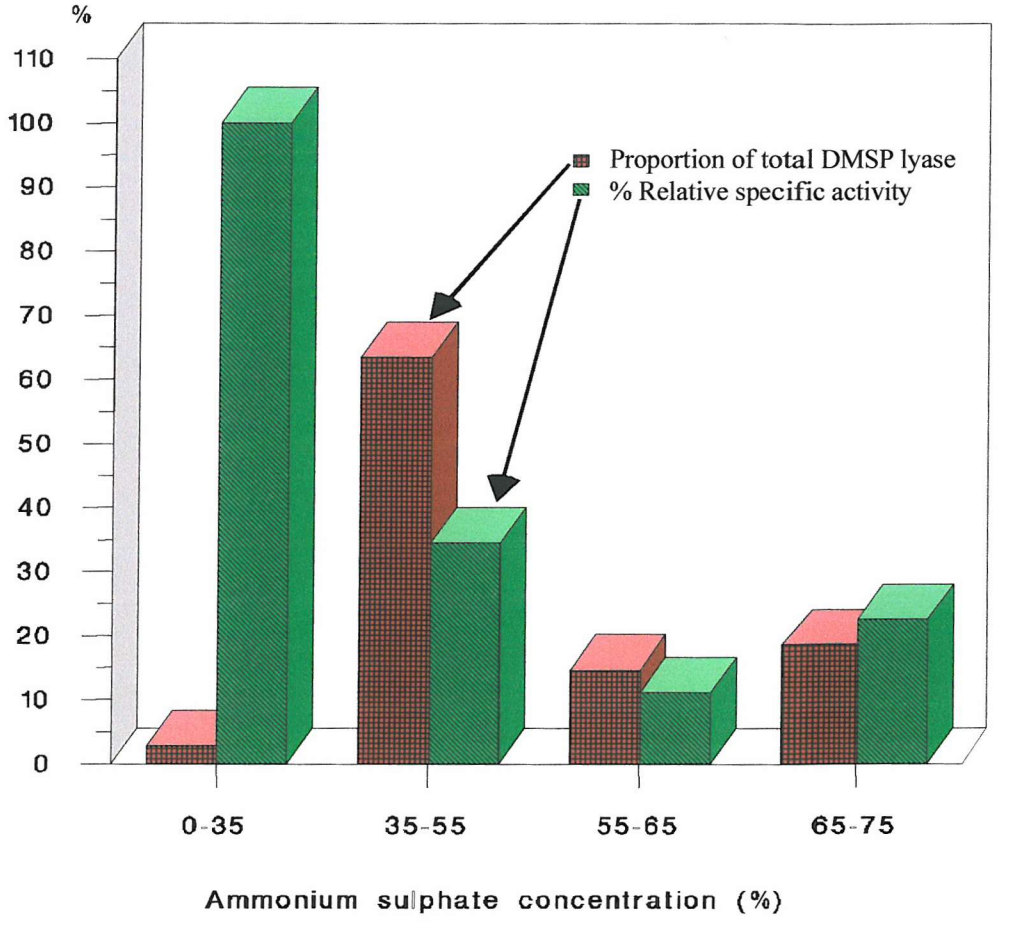


Figure 3.1 The amount of DMSP lyase and its relative specific enzyme activity in each ammonium sulphate cut precipitate fraction. A unit of DMSP lyase activity is defined as the amount of enzyme required to produce 1 μmol of DMS in 1 min at room temperature. 100% relative specific activity was 0.685 units/mg protein and total DMSP lyase activity in the precipitate was 14.6 units.

(b) Column chromatography trials

The DMSP lyase bound tightly to the Phenyl Superose FPLC column, showing a great hydrophobic affinity. Whilst about one-third of the total protein passed straight out of the column during loading, all the DMSP lyase was bound in the column. The lyase was however released during the gradient elution. Thus, the hydrophobic column seems to be a valuable potential separation procedure for further purifications of DMSP lyase.

The DMSP lyase passed through the CM Sepharose CL-6B syringe column; however, it was bound in the DEAE Sepharose CL-6B syringe column and was released with the buffer (pH8) containing 0.5 M KCl. Therefore, the DEAE Sepharose CL-6B column was chosen as a purification step.

Unfortunately, DMSP lyase passed through the GFC Sephacryl S-300 HR columns without any separation. However, when the first fractions from the GFC column were pooled and filtered with a 0.1 μm membrane filter, the enzyme activity in the filtrate dropped by 30-40 % compared with the unfiltered sample. This observation provides evidence that the protein aggregates during the dialysis procedure. In order to avoid the clogging of columns by this particulate matter it is necessary to centrifuge the samples before loading the column. In addition, the probability of column overloading must be considered as an explanation of why the column separation performance was poor. Gel filtration was therefore used for the last step of the enzyme purification.

Finally, another approach was tried. The concentrated extract was loaded onto a DEAE Sepharose CL-6B column, the fractions containing enzyme activity were collected and loaded onto a GFC Sephacryl S-300 HR column. The activity in the water soluble fraction bound tightly to DEAE-Sepharose CL-6B at pH8.0. The

enzyme eluted with a high salt concentration and only a small protein peak coincided with the DMSP lyase activity peak (Fig. 3.2). The following GFC Sephacryl S-300 HR column showed a sharp enzyme activity peak (Fig. 3.3) and its V/V_0 value was about 1.5. This was therefore considered to be a successful purification procedure.

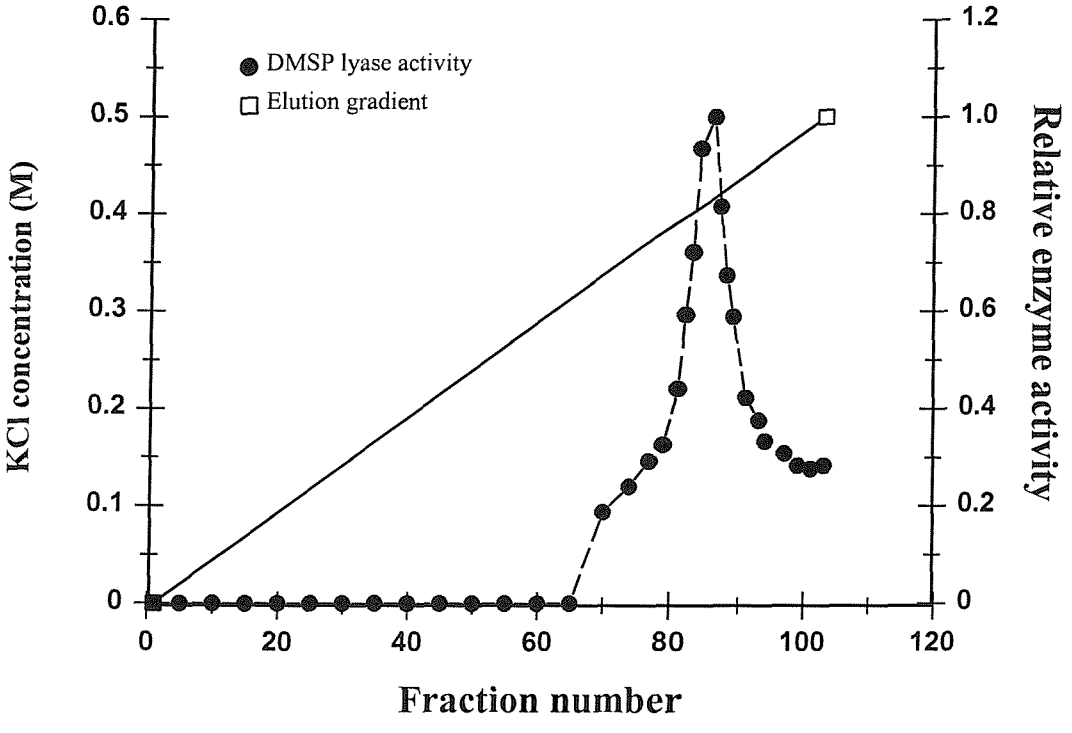


Figure 3.2 Anion-exchange chromatography of DMSP lyase showing its elution from a DEAE-Sepharose C-L 6B column (26 by 177 mm).

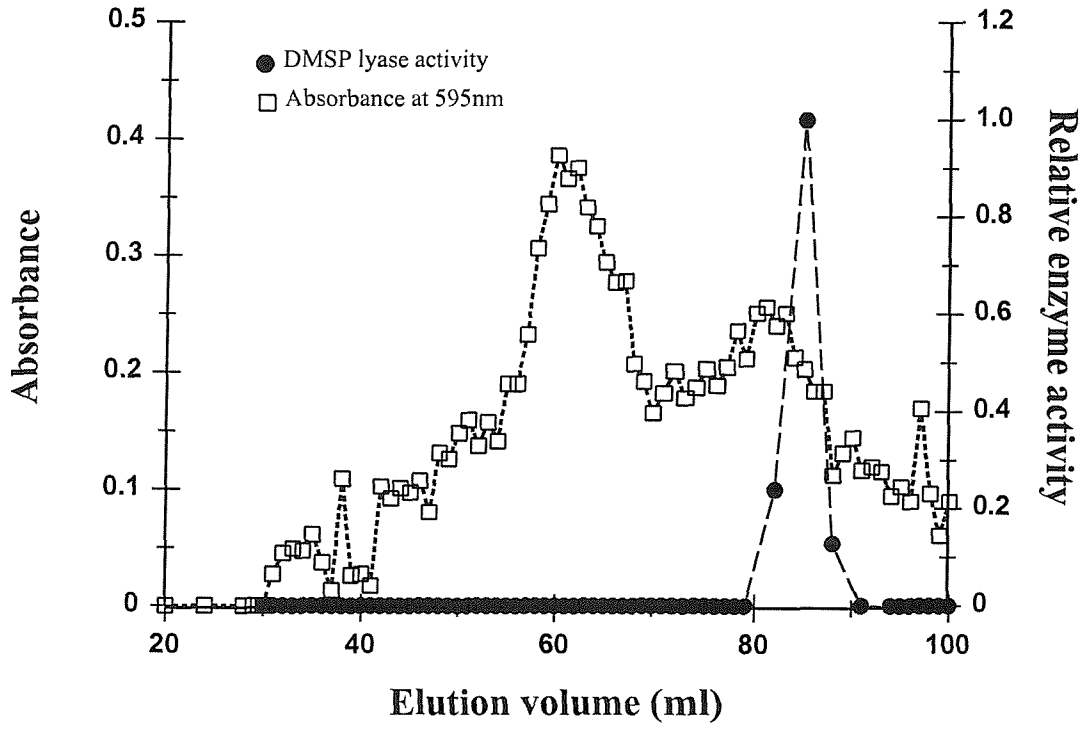


Figure 3.3 Gel filtration chromatography of DMSP lyase showing its elution from a Sephacryl S300 HR column (3 by 90 cm). (●) Absorbance of fractions at 595 nm were measured 2 min after adding the Bradford reagent to generate colour.

(c) Separation by sequential column chromatography

As a compromise between recovery of activity and the degree of purification, 35% and 70% ammonium sulphate cuts were collected and dialysed with buffer (pH8.0) for subsequent purification. This soluble enzyme fraction was purified by anion-exchange chromatography followed by hydrophobic interaction chromatography and then by gel filtration. The enzyme in the 35% to 70% ammonium sulphate fraction was tightly bound to the DEAE Sepharose CL-6B column and released with a gradient elution from 0.3 M to 0.6 M KCl and then the buffer (pH8.0) containing 0.6 M KCl until all activity eluted (Fig. 3.4).

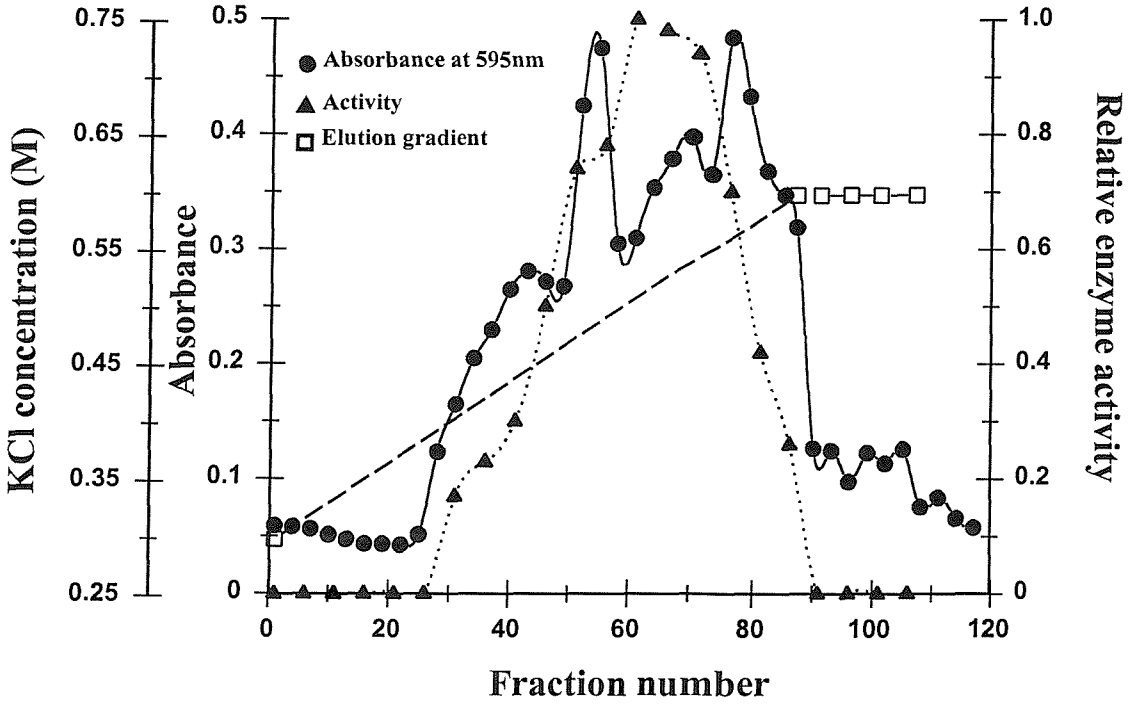


Figure 3.4 Elution of the soluble *Enteromopha intestinalis* DMSP lyase from the DEAE Sepharose CL-6B column (50 by 85 mm). (●) Absorbance of fractions at 595 nm were measured 2 min after adding the Bradford reagent. (▲) Relative enzyme activity was measured in each fraction with GC-FPD of headspace DMS converted from DMSP. (□) The concentration of KCl in the elution buffer.

After ion-exchange chromatography, the fraction with enzyme activity was applied to a Sepharose CL-4B column for further purification. From Fig. 3.5, it can be seen that a small protein peak coincided with DMSP lyase activity.

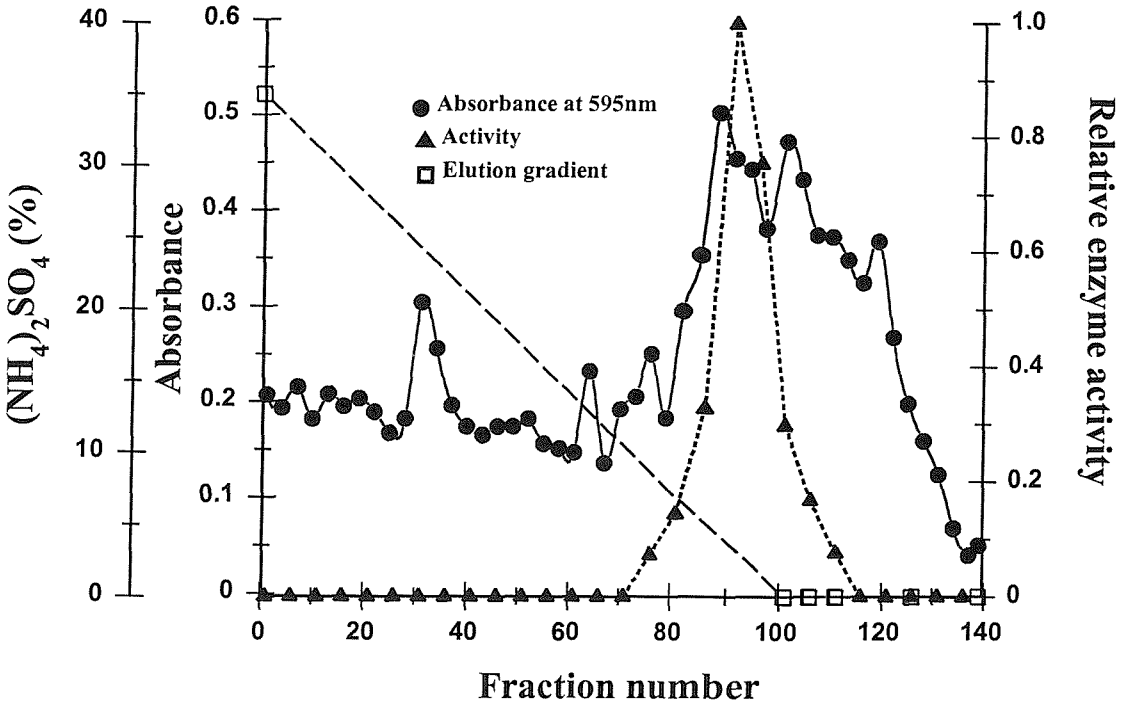


FIG. 3.5 Hydrophobic interaction chromatography of the soluble DMSP lyase showing its elution from the Sepharose CL-4B column (50 by 60 mm). (●) Absorbance of fractions at 595 nm were measured 2 min after adding the Bradford reagent.

The remaining enzyme in the Sepharose fractions was collected and purified using a Superdex 200 column (Fig.3.6). The peak containing DMSP lyase activity eluted late from the column and therefore a Superdex 75 column was chosen for the isolation of the low molecular weight material (Fig. 3.7). The 70 to 80 ml fraction containing DMSP lyase activity coincided with a symmetrical peak of protein showing that the enzyme was probably quite pure. The front tail of the enzyme peak that overlapped with another massive peak of protein was therefore discarded.

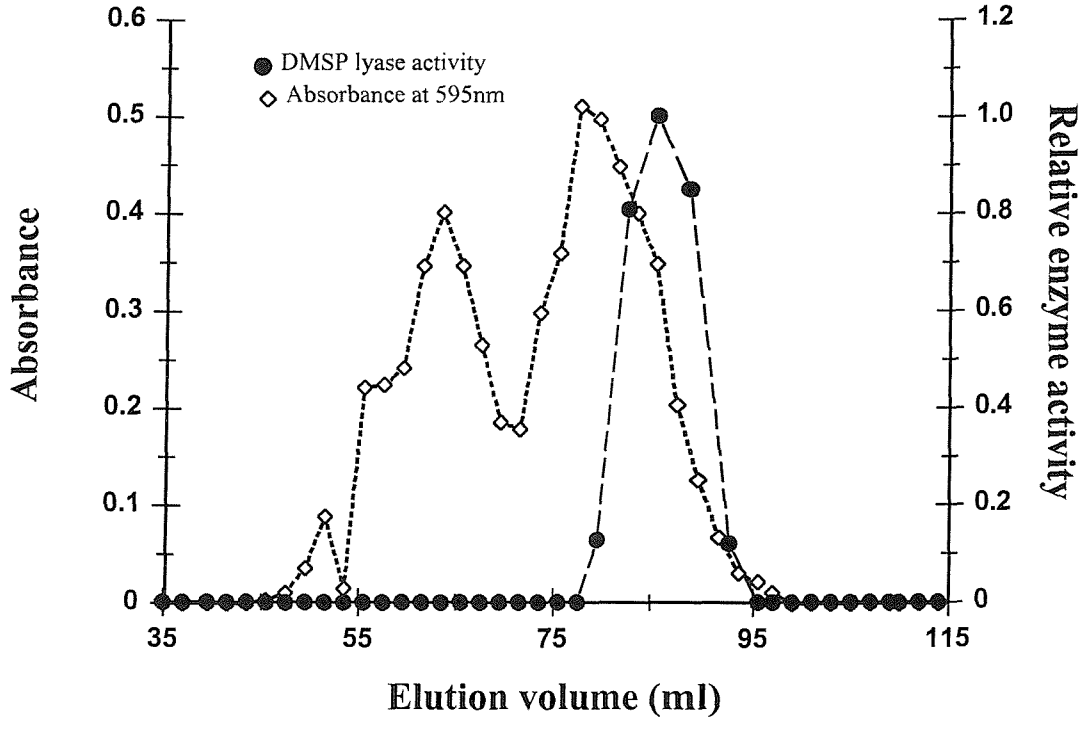


Figure 3.6 Elution of the soluble DMSP lyase from the Superdex 200 gel filtration column (Pharmacia, 16/60). The void volume of the column was approx. 40 ml. (●) Absorbance of fractions at 595 nm were measured 2 min after adding the Bradford reagent.

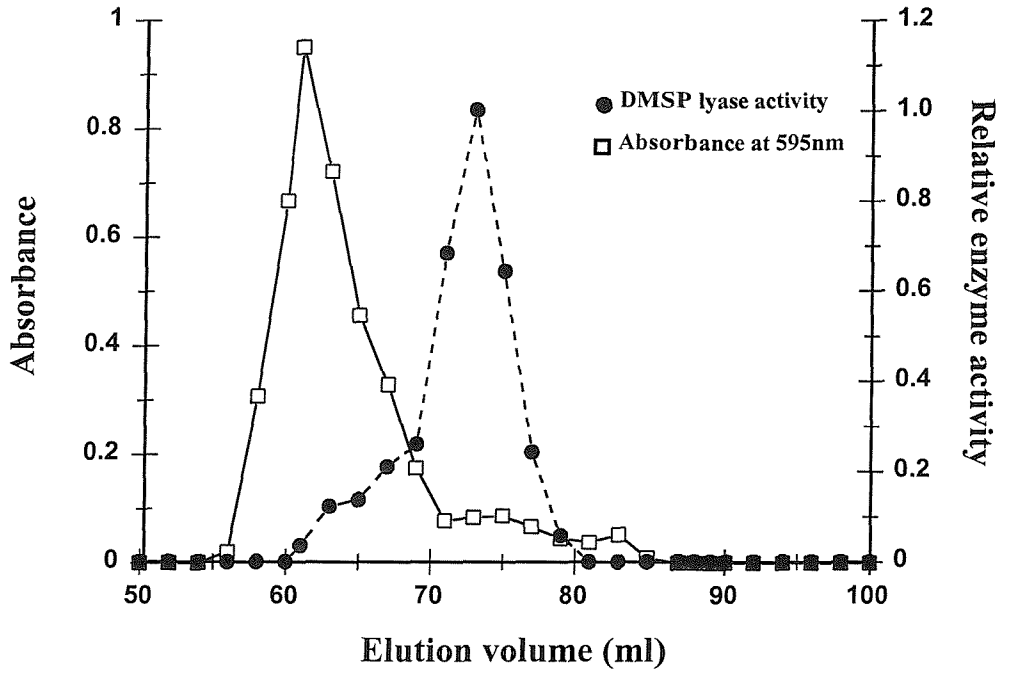


Figure 3.7 Elution of the soluble DMSP lyase from the Superdex 75 gel filtration column (Pharmacia, 16/60). (●) Absorbance of fractions at 595 nm were measured 2 min after adding the Bradford reagent.

(d) Enzyme purity

The total protein concentration and enzyme activity were measured at every purification step and the results are shown in Table 3.3. The specific enzyme activity increased ca. 178 fold from protein precipitation to the final gel filtration steps. With each purification step however more enzyme was lost and some was used in the enzyme assay.

Step	Total protein (mg)	Total activity (mUnits)[a]	Specific activity (mU/mg)[b]	Purification (fold)
35-65% $(NH_4)_2SO_4$ precipitate	243.8	1972.6	8.1	1.0
DEAE-Sepharose CL-6B	31.9	2129.5	66.2	8.3
Phenyl-Sepharose CL-4B	9.3	543.6	58.4	7.2
Superdex 200	1.59	280.6	176.5	21.8
Superdex 75	0.13	181.4	1439.3	178

Table 3.3 Purification of the soluble DMSP lyase from *Enteromorpha intestinalis*.
[a] A unit of DMSP-lyase activity is defined as the amount of enzyme required to produce 1 μmole of DMS per min at ambient temperature (ca. 20 °C).
[b] The purification factor of DMSP-lyase is expressed as the amount of enzyme relative to the total protein extracted in each $(NH_4)_2SO_4$ cut.

The fraction collected from the Superdex 75 column was separated by SDS-PAGE gel (10%) electrophoresis and then silver stained followed by treatment with Cromassie blue. Two strong bands and two faint bands appeared on the gel indicating some impurity remaining in the last purification fraction. The molecular weights of these bands are determined below.

(e) Determination of molecular weight

The apparent molecular weight of the soluble protein was determined, using molecular weight standards, on gel filtration chromatography to be 25.2 kDa. Based on protein standard applied to the SDS-PAGE gel (Fig. 3.8), four fragment bands were observed with apparent molecular weights ranging from 25 to 50 kDa (Table 3.4). The 25.5.kDa molecular weight component was similar to the result obtained by

gel filtration chromatography. It was therefore suggested that the soluble DMSP lyase might be a monomer protein with a molecular weight of approx. 25.5 kDa.

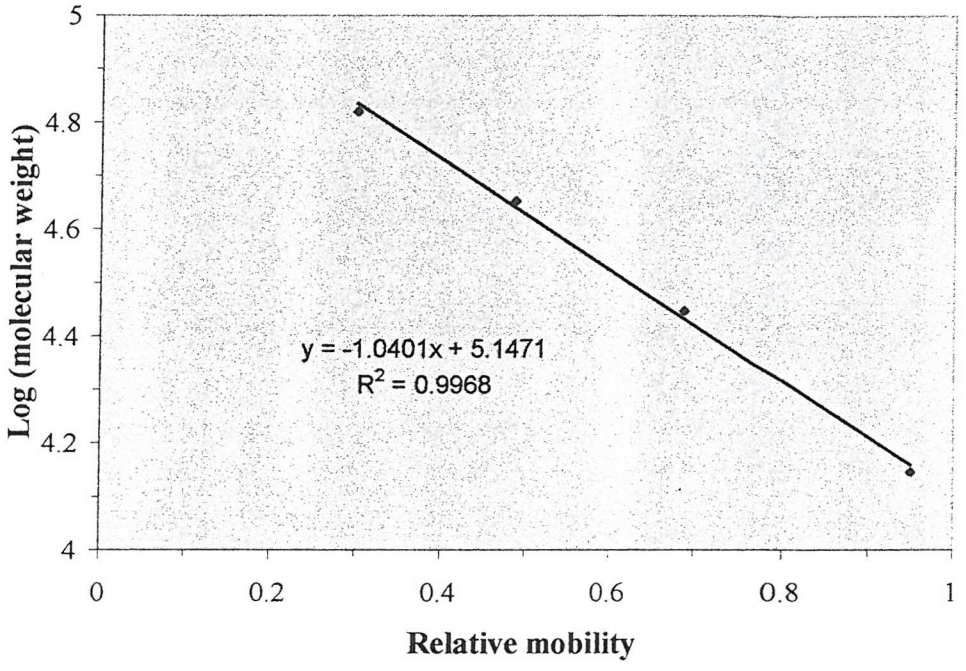


Figure 3.8 Calibration curve of SDS-PAGE obtained with standard proteins from the MW-SDS-70L kit (Sigma) and carried out by running on a 10 % gel.

	Relative mobility (R_f)	Molecular weight (kDa)
Band 1	0.475	45.0
Band 2	0.575	35.4
Band 3	0.625	31.4
Band 4	0.713	25.5

Table 3.4 The four fragment bands obtained from the SDS-PAGE (10%) gel of the purified enzyme.

In their study of DMSP lyase from *U. currat* de Souza *et al.* (1996) found one soluble and three membrane-bound isozymes of DMSP lyase, all of which had apparent molecular weights of 78 kDa (Desouza et al., 1996). The relationship between the soluble and membrane-bound isozymes is still unknown. Two different membrane-

bound DMSP lyases purified and characterized from *P. paniculata,* had molecular weights of 92.6 and 36.5 kDa respectively and different kinetic constants and characteristics (Nishiguchi & Goff, 1995).

3.2.3.3 Isolation of detergent-released DMSP lyase

The detergent extract of *Enteromorpha intestinalis* tissue was loaded onto the Sepharose CL-4B column. Three hydrophobic variant forms were obtained which eluted with a gradient of ammonium sulphate from 40% to 0 saturation (Fig. 3.9). The fractions of each peak containing DMSP lyase activity were pooled and loaded onto the Superdex 200 column individually for further purification. Unfortunately enzyme activity came out in every fraction without any separation through gel filtration column. This was the end of the isolation of detergent released DMSP lyase. The reason for their independent migration on the hydrophobic interaction column is still unknown.

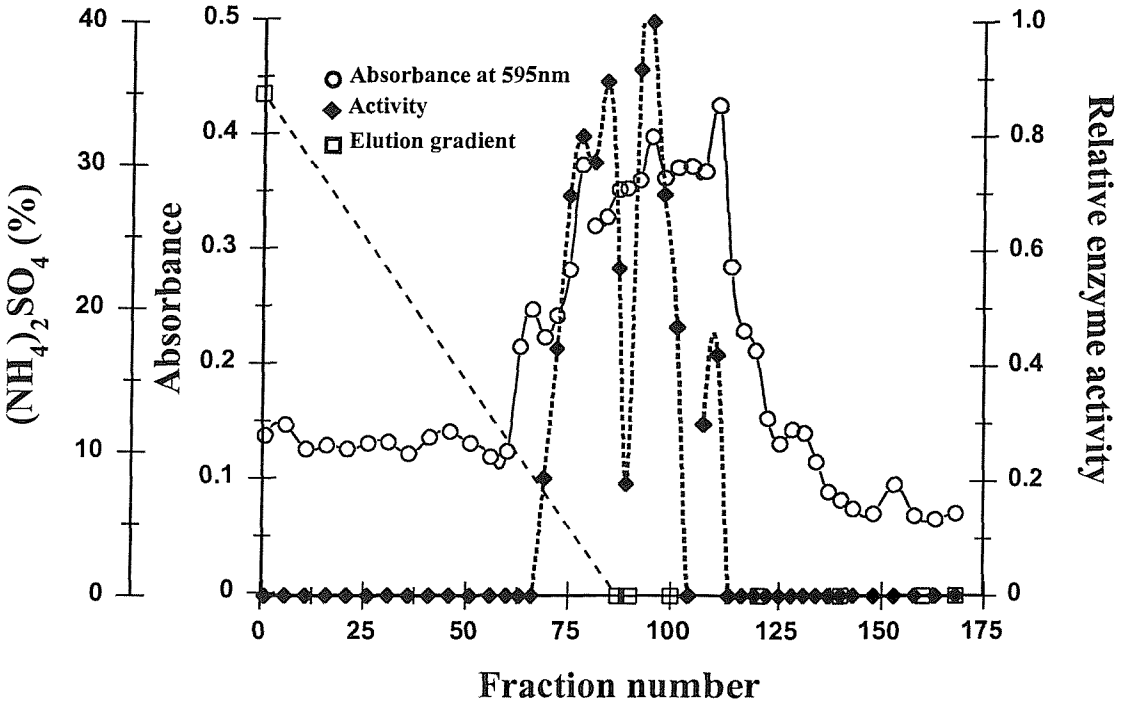


Figure 3.9 Hydrophobic interaction chromatography of the membrane bound DMSP lyase showing its elution from the Sepharose CL-4B column (50 by 60 mm). (●) Absorbance of fractions at 595 nm were measured after adding the Bradford reagent to generate colour for 2 min.

3.2.3.4 Determination of the optimum temperature and pH of soluble DMSP lyase activity

DMSP lyase activities varied with pH (Fig. 3.10). The enzyme activity steadily increased with the increased pH values until pH8.0. In order to avoid the interference of base hydrolysis of DMSP, pH8.0 was adopted as the optimum pH condition for enzyme assay. The *Enteromorpha intestinalis* DMSP lyase had an optimum pH of 8.0 which is similar to that reported for *Ulva lactuca* (Diaz & Taloor, 1994) and *Ulva currata* (Desouza et al., 1996) but unlike that observed from *Enteromorpha clatharta* that had an optimum pH of 6.2 to 6.4 (Steinke & Kirst, 1996).

The enzyme activity increased with temperature (Fig. 3.11), the highest enzyme activity being observed at 37 °C. The differences in enzyme characteristics are suggested as being due to species and habitat differences.

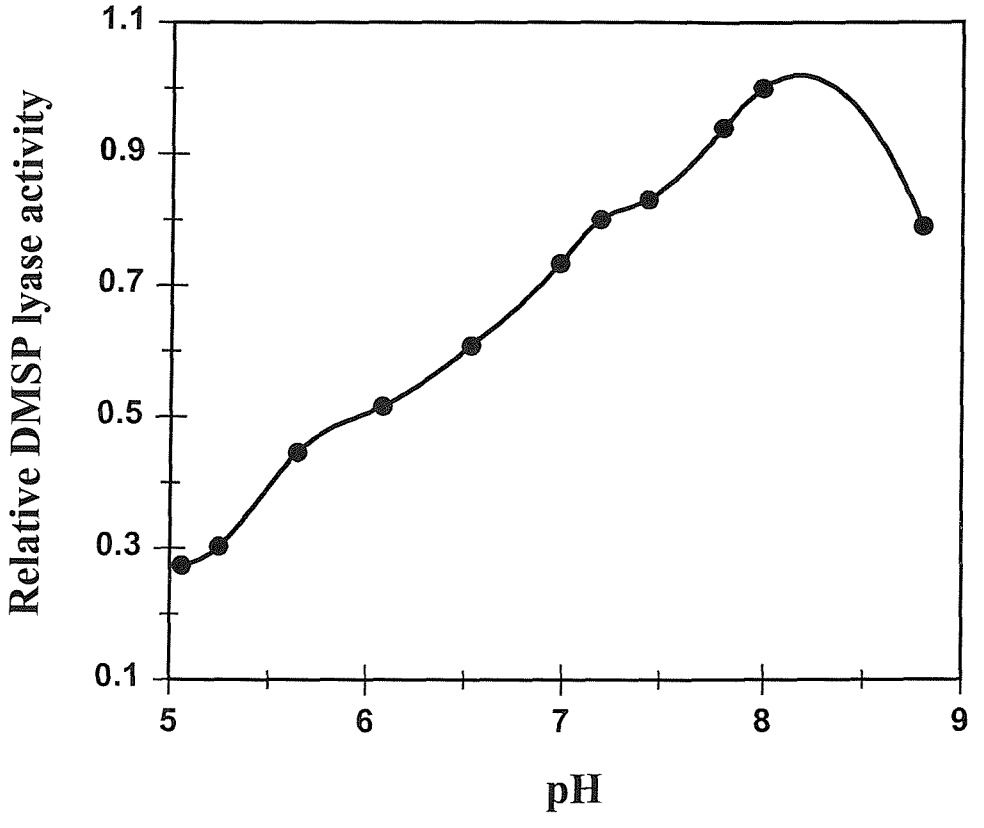


Figure 3.10 Effect of pH on the soluble DMSP lyase activity.

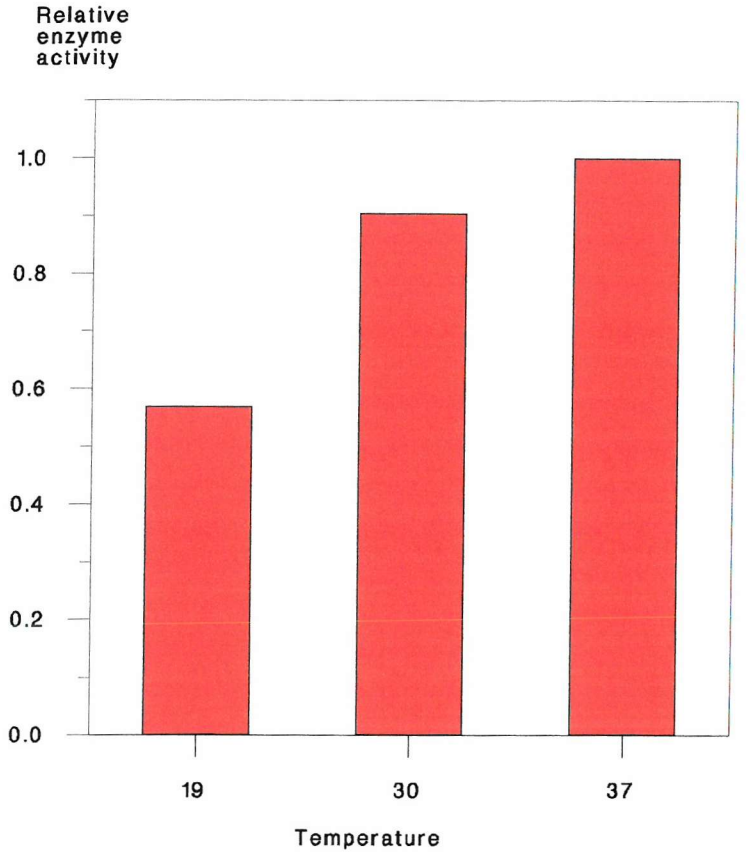


Figure 3.11 Effect of temperature on the soluble DMSP lyase activity.

3.2.3.5 Characteristics of the soluble DMSP lyase

(a) The effects of cofactors and metal chelator on the activity of DMSP lyase

The enzyme activity was neither inhibited nor activated by 500 mM NaCl (Table 3.5). This has been shown to be the case for *Enteromorpha clathrata* (Steinke & Kirst, 1996), *Ulva currata* (Desouza et al., 1996) and *P. doudoroffii* (Desouza & Yoch, 1995a). The addition of reduced glutathione (R-GHS) did not affect the enzyme activity, suggesting that the DMSP lyase of *Enteromorpha intestinalis* was not a thiol-based enzyme, unlike that of *Polysiphonia lanosa* (Cantoni & Anderson, 1956).

	Concentration (mM)	Relative enzyme activity (%)[a]
NaCl	500	98
Reduced glutathione (R-GHS)	42	95

Table 3.5 Effects of NaCl and R-GHS on the soluble DMSP lyase from *Enteromorpha intestinalis*.

[a] The enzyme activity of an untreated sample served as control (100% activity).

The addition of the metal chelating compound EDTA reduced the enzyme activity (Fig. 3.12). It is therefore evident that this DMSP lyase is probably a metal-dependent enzyme. Subsequently $MgCl_2$ and $CaCl_2$ were added to the reaction vials to see their effects. It was found that the enzyme activity was dramatically increased with $MgCl_2$ at a concentration of 5 mM and then slightly enhanced with $MgCl_2$ up to 100 mM. A similar result has been reported by Nishiguchi and Goff (Nishiguchi & Goff, 1995). In contrast, the DMSP lyase activity steadily decreased with $CaCl_2$ concentration (Fig. 3.12).

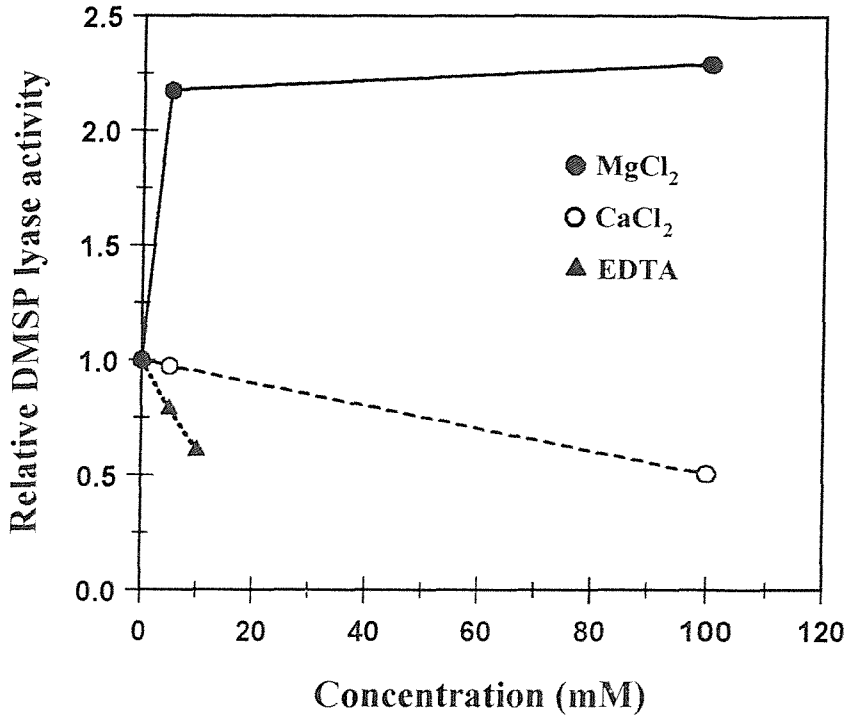


Figure 3.12 The effect of magnesium chloride, calcium chloride and EDTA on the soluble DMSP lyase activity. The untreated sample served as a control and its enzyme activity was defined to be 1.

(b) Substrate specificity and the effects of sulphonium compounds on the DMSP lyase

Amongst the sulphonium compounds tested, DMSP is the only substrate of the enzyme. A number of DMSP analogues were examined for the effects on the soluble DMSP lyase (Table 3.6). It was found that SMM was neither a substrate nor an inhibitor of the enzyme. DMSPent was the most potent inhibitor; the enzyme activity dropped to approx. 46% compared with the untreated sample. DMS-2-MP and DMSAcet however weakly inhibited the enzyme activity (by 18% and 15% respectively).

	Concentration (mM)	Relative enzyme activity (%)[a]
S-methyl methionine (SMM)	3.5	104.5
Dimethylsulfonio-2-propionate (DMS-2-MP)	3	81.4
Dimethylsulfonio acetate (DMSAcet)	4	85.5
Dimethylsulfonio pentanoate (DMSPent)	4	46.1

Table 3.6 Effects of DMSP analogues on the soluble DMSP lyase from *Enteromorpha intestinalis.*

[a] The enzyme activity of an untreated sample served as control (100% activity).

3.2.3.6 Determination of the kinetic constants of the soluble DMSP lyase

The K_m is defined as the substrate concentration when the enzymatic reaction rate is half of V_{max}, and the V_{max} is the maximum velocity of the enzyme when it is saturated with substrate. The Michaelis-Menten type kinetics of DMSP lyase from *Enteromorpha intestinalis* for DMSP are shown in Fig. 3.13 and Fig. 3.14. When concentrations of DMSP ranging from 20 to 8000 μM were tested, this partially purified soluble DMSP lyase had a K_m for DMSP of 0.286 mM and V_{max} of 21.9 nmole DMS/min/mg protein at room temperature (19 °C).

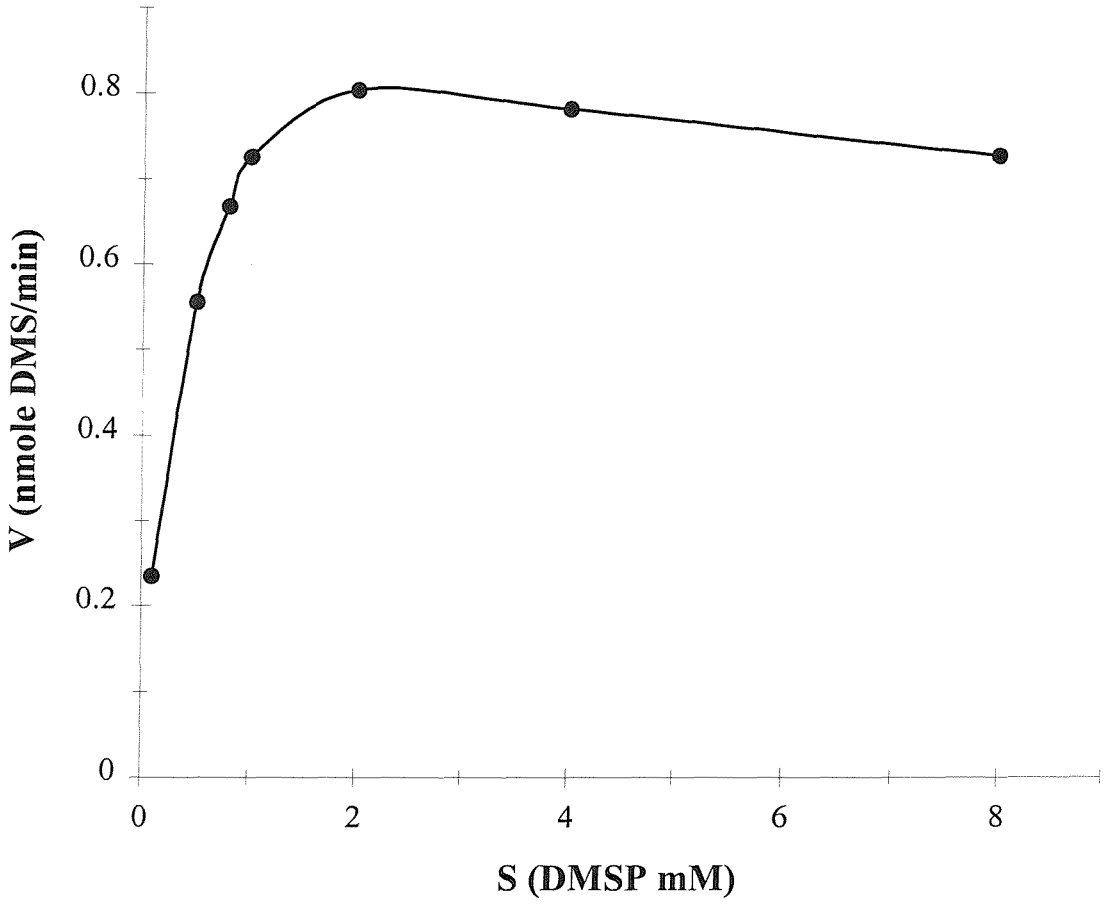


Figure 3.13 The effect of DMSP concentration on the soluble DMSP lyase activity. S = substrate concentration (mM). V = nM of DMS converted from substrate DMSP per minute per 41.3 μg of partially purified protein.

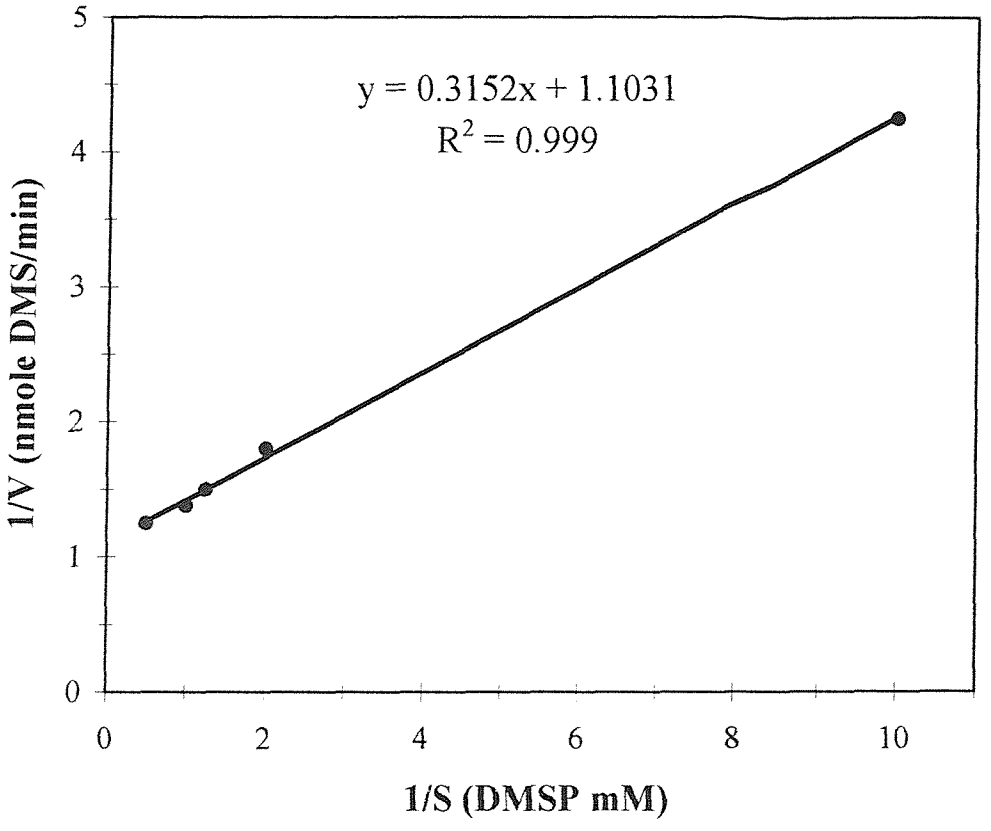


Figure 3.14 Lineweaver-Burke plot (R^2 =0.999) for the soluble DMSP lyase in *Enteromorpha intestinalis*. The K_m and V_{max} derived from this plot were 0.286 mM and 21.9 nmole DMS/min/mg protein at room temperature (19 °C), respectively.

3.2.4 Conclusion

The DMSP lyase has been partially purified from the green macroalga, *Enteromorpha intestinalis* collected from the mouth of Langston harbour at Eastney. The enzyme activity was still found in the frozen alga stored at –20 °C for two months. In the crude extract from this frozen material, enzyme specific activity was ca. 1 μmol DMS·min^{-1}· mg^{-1} protein. However, the specific activity after overnight dialysis dropped to about 50% of the specific activity of the crude extract. The purification process employed a 35% to 70 % saturation ammonium sulphate cut followed by ion-exchange, hydrophobic interaction and gel filtration chromatography. Four bands

obtained from SDS-PAGE electrophoresis suggested that there were some impurities still present after the last purification step.

This DMSP lyase is fairly sensitive to pH. The enzyme activity steadily increased with increasing pH until pH8.0. In order to avoid the interference of base hydrolysis of DMSP, pH8.0 was adopted as the optimum pH condition for the enzyme assay. The highest measured enzyme activity was observed at 37 °C. However, the influence of NaCl concentration is insignificant. Based on the results of inhibition studies employing R-GHS and EDTA, it is suggested that this DMSP lyase is a metal dependant enzyme but not a thiol-based enzyme. The enzyme activity was increased with $MgCl_2$ concentration but decreased with $CaCl_2$ concentration.

The DMSP lyase is located both as a soluble and detergent-released protein in the green macroalga, *Enteromorpha intestinalis*. The molecular weight of the soluble protein was determined by gel filtration chromatography to be approximately 25.2 kDa. Three hydrophobic variant forms were isolated from the detergent-solubilized activity. This DMSP lyase could possibly be loosely bound membrane protein according to its high affinity to the hydrophobic column. Some proteins with activity sites were released or broken down during homogenization as the water soluble enzyme. Other proteins were further released by the detergent. The location of the enzyme *in vivo* is still unknown. The relationship between the soluble and detergent-released enzyme also needs further investigation.

Amongst the sulphonium compounds tested, only DMSP was converted to DMS by this DMSP lyase. The results indicate the highly specific substrate characteristic of DMSP lyase. SMM was neither a substrate nor an inhibitor of the enzyme and DMSPent was the most potent inhibitor. Additionally DMS-2-MP and DMSAcet weakly inhibited the DMSP lyase activity.

The K_m and V_{max} of the soluble DMSP lyase from *Enteromorpha intestinalis* derived from a Lineweaver-Burke plot, were 0.286 mM and 21.9 nmole DMS/min/mg protein at room temperature, respectively.

Although a number of DMSP lyases have very recently been purified from marine bacteria and algae, these enzymes have quite different characteristics and biological functions. These differences between DMSP lyases are believed to be due to species and habitat differences.

3.3 Conclusion

The DMSP contents of oven dried algae were lower than those obtained from fresh algae and the measurement of DMSP content should therefore be carried out using fresh samples.

The DMSP lyase was partially purified from *Enteromorpha intestinalis*. Based on the results above, this enzyme was present as both water-soluble and detergent-released forms. The location and structure of the detergent–released DMSP lyase in the alga remains unknown and needs further investigation. As for the water-soluble form, it was a metal dependant but not a thiol-based enzyme. The optimum pH value was at pH8.0 and the enzyme activity increased with increasing temperature. Also this enzyme was extremely specific to its substrate DMSP. The molecular weight of the soluble enzyme was estimated by gel filtration chromatography to be approx. 25.2 kDa. There were still some impurities present in the enzyme product even after the last purification step. The NaCl concentration had no significant effect on enzyme activity *Enteromorpha intestinalis* to colonise a wide range of habitats differing in their salinities.

Based on these properties of the DMSP lyase, it appears that *Enteromorpha intestinalis* may be well suited to operation in an estuarine environment. During high tide, its response to changing external osmotic potential might arise from either transferring inorganic ions into cells or producing organic solutes in cells. It has been reported that inorganic ions, proline and sucrose are involved in short term osmoregulation in *Enteromorpha intestinalis* but DMSP is not (Edwards et al., 1988). The DMSP lyase will be stimulated by increased Mg^{2+} thereby reducing the

intracellular DMSP level. It has however been found that the intracellular DMSP level remains constant during short term hyperosmotic conditions. It seems that the production mechanism of DMSP should also be considered. Steinke and Kirst found that the DMSP lyase is stimulated in the *Enteromorpha clathrata* by high salinities after 72 hours incubation and the intracellular DMSP level increased 1.7 fold. An increased NaCl concentration did not however stimulate DMSP lyase activity *in vitro* (Steinke & Kirst, 1996). The mechanism of DMSP cleavage *in vivo* is very complicated due to a number of osmolyte compounds participating in the intracellular osmotic adjustment.

CHAPTER FOUR

Sulphur Species and DMSP lyase Activity in Tamar Estuary Sediments

This chapter describes the measurement of sulphur species and DMSP lyase activities within several estuarine sediment cores from the Tamar estuary, South West England. In this study the concentration profiles of various sulphur species were investigated at two sites (Calstock and Halton Quay) in the upper reaches of the Tamar Estuary. Additionally, a comparison of DMSP lyase activities in surface sediments from Halton Quay was also carried out.

4.1 Experimental methods

4.1.1 Sampling

4.1.1.1 Sites

The Tamar Estuary, located in the south west of England, can be said to extend 31km from a weir at Gunnislake to its mouth at Plymouth Sound (Fig. 4.1). It is a partially mixed and flood dominant estuary with a salinity structure dependent on the rate and volume of fresh water inflow, the strength of tidal currents and depth (Dyer, 1973). It has been the site of extensive mining activity (Embrey & Synes, 1987). Large areas of intertidal mudflats or mudbanks exist along the length of the river. In the lower estuary the intertidal mudflats are typical of estuaries that have a shallow slope and

that are relatively firm resulting from long term deposition (Bale, 1985). In contrast, the mudbank sediment within the upper estuary demonstrates marked seasonal migration (Uncles et al., 1994). The sediment there is less extensive; however, it is much steeper and the consistency of the superficial mud is more fluid.

Field sampling was carried out at Calstock (8 km from Weir Head) and Halton Quay (13.6 km from Weir Head)(Fig. 4.1). Whilst the Calstock site is subject to human activity that could provide some anthropogenic influences, the estuary at Halton Quay is open and bounded by a salt mash (*Spartina* species). The site is little influenced by human activity.

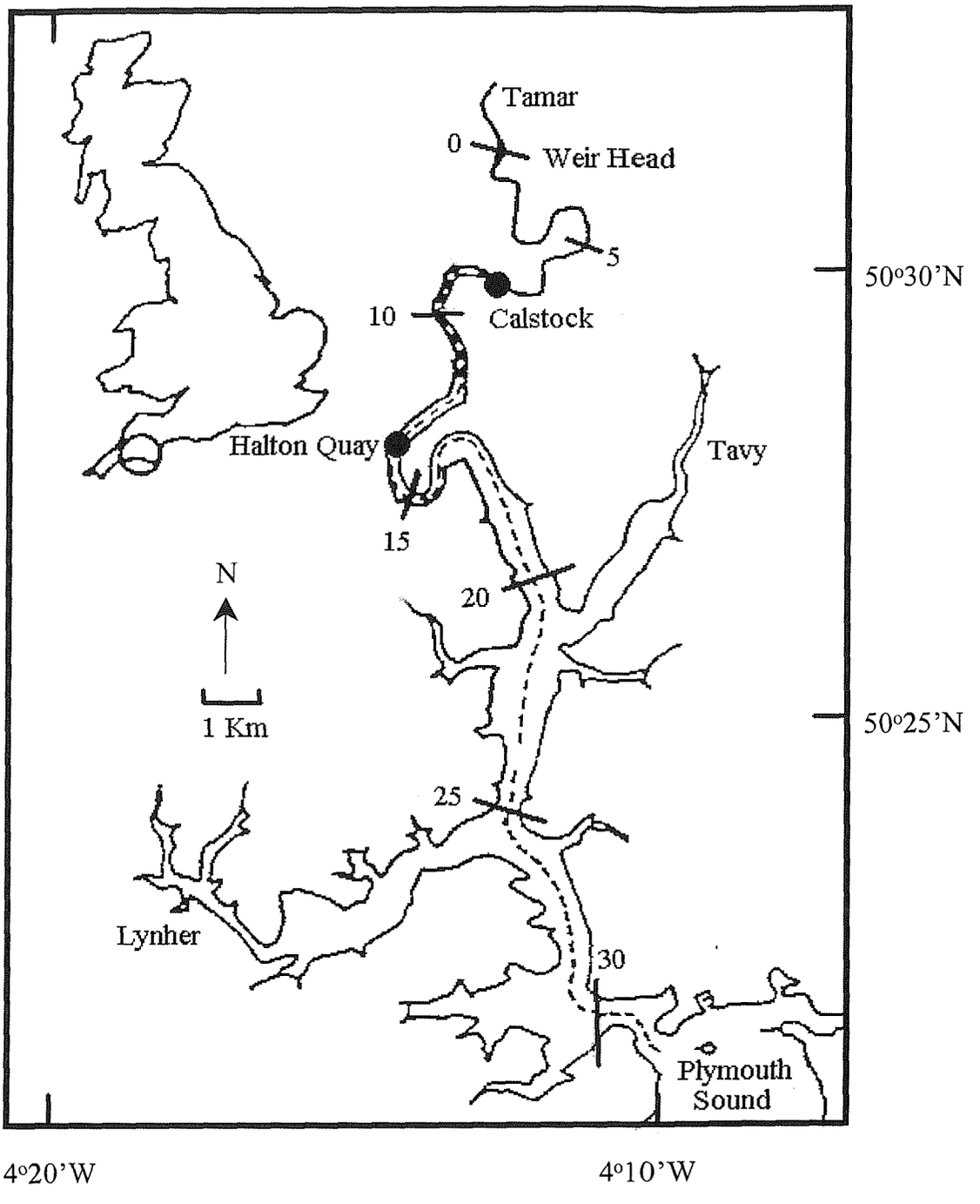


Figure 4.1 Sketch map of the Tamar Estuary in south west England, showing distances (in kilometres) from Weir Head and the location of the Calstock and Halton Quay sampling sites.

4.1.1.2 Sampling procedure

Sediment samples were collected from Calstock and Halton Quay using syringe corers (50 ml) and a Perspex sediment corer (10 cm diameter, 50 cm length) in June and September 1998. The syringe cores were sealed with rubber stoppers and kept in individual plastic bags. The large sediment cores were sectioned in the field and then stored under a nitrogen atmosphere in small polyethylene bags. The structures of all the sediment cores consisted of a brown oxic zone, an intermediate mixed zone and a black zone that was assumed to be anoxic. Samples were stored in an ice box until their return to the laboratory when they were stored at -20°C awaiting analysis. To minimise the influence of oxidation, sample preparation was carried out as quickly as possible after collection.

4.1.2 Sample analysis

4.1.2.1 Water content

The water content of each sediment was assessed by heating a weighed portion of the sediment at 110°C overnight until constant weight. Three replicate measurements were made.

4.1.2.2 Major and minor elements analysis

The major and minor element compositions of the sediments were determined by X-ray fluorescence spectroscopy using a Philips PW1400 automatic sequential wavelength-dispersive X-ray spectrometer.

Individual sediment samples were weighed wet, freeze dried in an open plastic bag, weighed again and then ground to a powder using a pestle and mortar. These dried sample powders were used for the major and minor element analyses.

Major elements

Major elements were determined in a fused bead using a borate flux. Approximately 2 g of the dried sediment sample was weighed into a dried crucible, ignited at 970°C for 20 mins, cooled down and weighed again. The sample was weighed prior to and after ignition to permit determination of the loss on ignition (LOI). 0.8 g of the ignited powder was accurately weighed and was dissolved in a eutectic flux (Spectroflux 100B, ICPH, composed of 4 parts of lithium metaborate and 1 part of lithium tetraborate) at 1200 °C to make a fused bead. Absorption and enhancement matrix effects were corrected by using the influence coefficients supplied by Philips. For elements well above their detection limit the precision is typically less than 1 % relative standard deviation. The detection limit here is the concentration limit below which a signal cannot be distinguished from the background.

Trace elements

Trace elements were determined on non-ignited powder pellets. Approximate 10 g of the dried sediment sample was subjected to a pressure of 4.72 tonnes per cm to form a 40 mm diameter pellet. Standard reference materials were included in the sample measurement to assess accuracy. The precision of the determination is typically about 5 % relative standard deviation and the lower limit of detection is nominally 1-5 ppm for trace elements.

4.1.2.3 Measurement of water-extractable sulphide

Approx. 20 g of each sediment sample was extracted by homogenisation with 7 g of oxygen-free DI water (sparged with nitrogen gas for 30 mins), adjusted to pH8 by the addition of NaOH, in centrifuge tubes filled with nitrogen. All sediment slurries were homogenised using a vortex mixer for 2 mins and then centrifuged at 5000 g for 10 mins. The supernatant fluid was then separated and filtered through a Whatman GF/C

filter. The solution was then analysed using the FIA system described in Section 2.1 for the measurement of dissolved sulphide.

4.1.2.4 Measurement of DMSP

Approx. 20 g of sediment was placed into a centrifuge tube filled with nitrogen. 5-6 ml of 1% (w/v) HCl solution was added and immediately sealed with a screw cap after being flushed with nitrogen. The mixture was homogenised with a vortex mixer for 2 mins and then centrifuged at 5000 g for 10 mins. The supernatant fluid was then separated and filtered through a Whatman GF/C filter. The solution was analysed using the base hydrolysis FIA system described in Section 2.2.2.2 for the measurement of DMSP.

4.1.2.5 Measurement of DMS precursors

Approx. 1.5 g of sediment sample was weighed into a 4 ml glass vial and 1 ml of 10 M NaOH solution was added. The vial was sealed immediately and the mixture was homogenised using a vortex mixer. The base hydrolysis was carried out at room temperature for 24 hours. The concentration of DMS precursors were determined by headspace analysis as described in Section 2.3.2.2.

4.1.2.6 Measurement of elemental sulphur

Approx. 1 g of sediment was weighed into a 25 ml glass vial and dried with anhydrous magnesium sulphate. 15 ml of diethyl ether was added into the dried sediment and the vial sealed. The glass vial was shaken on a wheel mixer at 9 rpm for 24 hours at room temperature. The liquid phase was poured into a 100 ml volumetric flask. The pellet was washed with a further 20 ml of diethyl ether and the washings were added to the volumetric flask. The sample was made up to volume with diethyl

ether. The resulting sample was analysed by capillary gas chromatography (Section 2.5).

4.1.2.7 DMSP lyase assay

Approx. 1.5 g of sediment was weighed into a 4 ml vial and left uncapped at room temperature for 10 hours to digest endogenous DMSP. Sediment was stirred to allow complete release of the DMS formed to the atmosphere. Then 1 ml of neutralised $2x10^{-2}$ M DMSP solution and 0.5 ml of 100mM pH8 sulphate buffer solution were added into the vial and immediately sealed with a screw cap containing a PTFE-faced butyl rubber septum. The mixture was then shaken. DMSP lyase activity was determined by headspace analysis as described previously (Section 2.4).

4.2 Spa tial variation of DMSP and DMSP lyase in surface sediments

4.2.1 Sampling sites

Details of the sampling area of Halton Quay are shown in Figure 4.2. The surface sediment at Halton Quay is composed of various brown and yellow muds, patches of green algae cover the sediment. In this area salt marsh sediments support marsh grasses. *Rumex rupestis* and *Spartina* sp are identified. Eight sampling sites (1 to 8) were investigated to assess the spatial variability of the surface sediments.

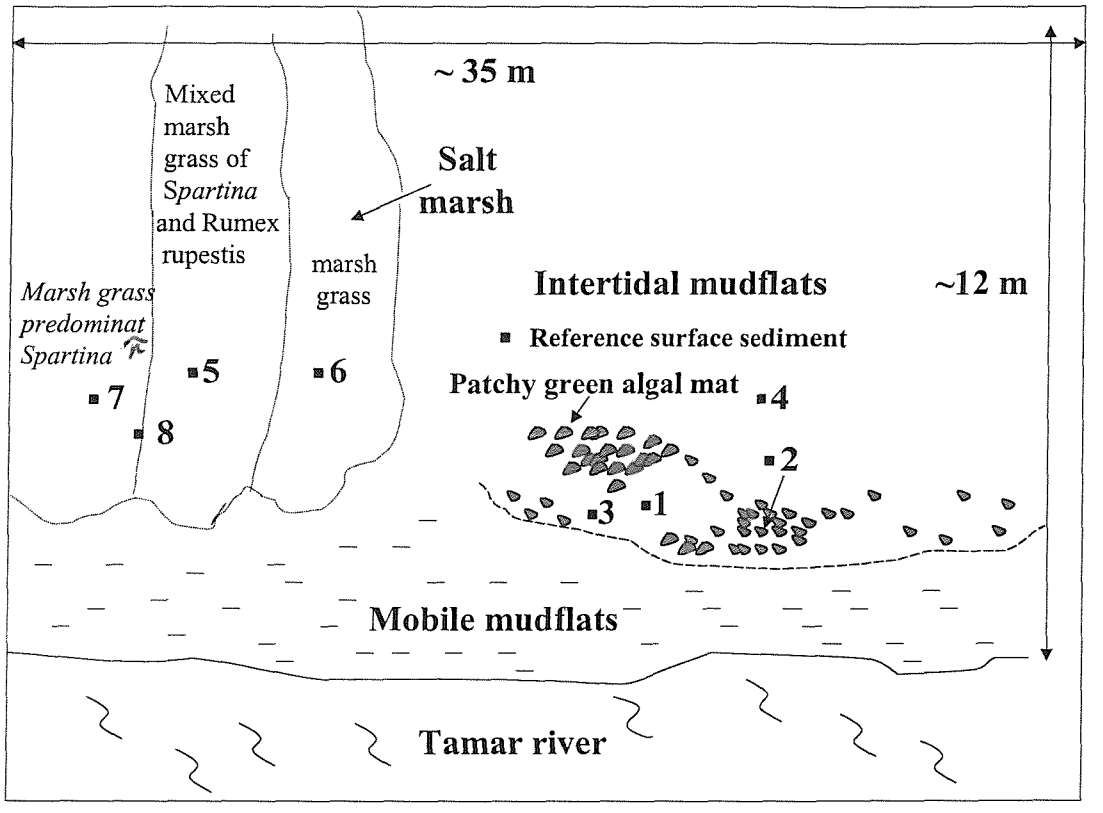


Figure 4.2 Sketch diagram of sampling sites of surface sediments at Halton Quay.

4.2.2 Description of samples

Halton Quay provides a range of different sampling sites and is therefore a good place for investigating the levels of DMSP and DMSP lyase in surface sediment. Eight syringe cores (ca 10 cm max) were collected at different locations (Fig. 4.2) on the 11th of September 1998. For comparison, one surface sample without any significant organic matter was collected with a plastic spatula. This sample consisted of

approximate 80 g of intertidal surface sediment collected from the top 2 cm at a sample site located 2.5 meters above the low water level. The sample was stored in a polyethylene container. Sample details were listed in Table 4.1. All samples were stored at -20 °C within two hours of sampling. Prior to analysis, syringe cores were defrosted. The top 2 cm of the sediment was used for measurement of DMSP and DMSP lyase activity.

Reference surface sediment:	Fine grained mud. Sediment to 2 cm depth.
Core1:	3 cm in length . Yellow and brown.
Core2:	4 cm in length. Green seaweed on the surface.
Core3 :	2.3 cm in length. Light brown.
Core4:	2 cm in length. Very fluid. Light brown.
Core5:	4.6 cm in length. Sediment contained mixed marsh grasses, predominantly *Spartina* sp.
Core6:	4 cm in length. Sediment contained marsh grass.
Core7:	6 cm in length. Salt marsh sediment contained large woody roots and had a sulphurous smell.
Core8:	4 cm in length. Salt marsh sediment, highly fluid.

Table 4.1 Descriptions of surface sediment cores.
(For locations of the 8 cores see Fig. 4.2)

4.2.3 Results and discussion

The results of comparisons of different surface sediments are shown in Table 4.2. Reference surface sample was used as a standard for intertidal surface sediment due to no significant visual or physical difference over the whole sampling area. In general, the DMSP lyase activities are approx. 0.3 munits g^{-1} wet sediment in the surface sediment without vegetative growth (such as at site 4, 8 and Reference surface sediment). Sample 6, which was collected from salt marsh sediments was not significantly different from the Reference surface sediment. Sample 5 and 7 had notably high DMSP lyase activities and were the two samples characterised by the presence of *Spartina* sp..

As for the DMSP content, the Reference surface sediment sample contained 4.29 nmole / g wet sediment. All other samples except sample 2 contained similar levels. Sample 2 exhibited a high DMSP concentration (15.5 nmole g^{-1} wet sediment) due to the presence of a green algae which other evidence would suggest would contain high levels of DMSP. Surprisingly the salt marsh sediments colonised by *Spartina* sp. did not contain high levels of DMSP. The high lyase activities in these sediments may however be responsible for the low measured levels of DMSP, even when a high flux of DMSP into the sediments may be expected due to the presence of *Spartina* DMSP.

	DMSP lyase (munits / g)	DMSP (nmole / g)
Reference surface sediment	0.28	4.29
1	0.52	5.47
2	0.50	15.49
3	0.37	6.68
4	0.29	<3.1
5	1.39	3.4
6	0.32	5.09
7	0.63	3.26
8	0.31	3.96

Table 4.2 DMSP lyase activities and DMSP concentration analysed in a variety of surface sediments. (Data based on wet weight)

4.3 Vertical distribution of sulphur species in sediments of the Tamar estuary

4.3.1 Sampling sites

Three large cores (HQC1, HQC2 and HQC3) were collected from salt marsh sediments in Halton Quay where *Spartina* is the predominant marsh grass during two sampling trips. The locations of these cores were shown in Figure 4.3. In addition, one large core CsC was collected from Calstock on the 24th June 1998. In Calstock there was no marsh grass population.

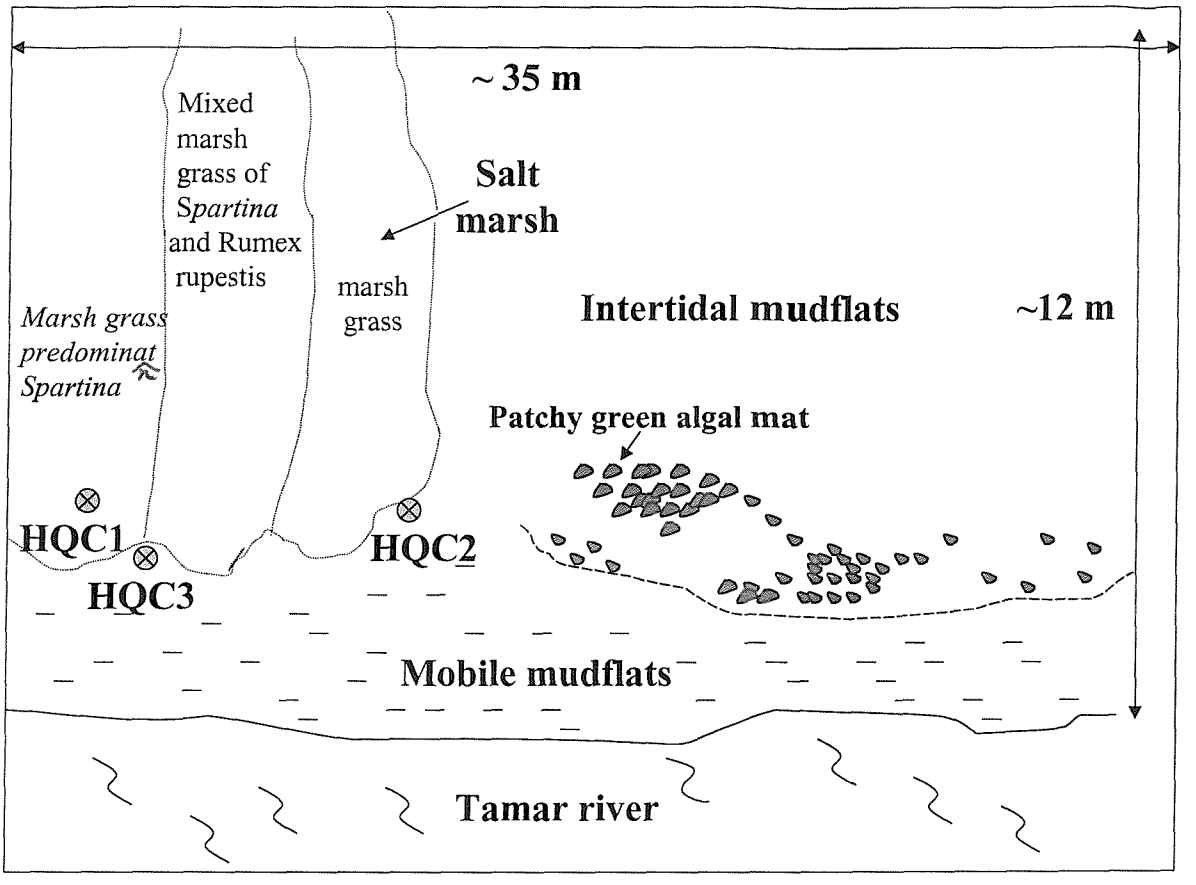


Figure 4.3 Sketch diagram of sampling sites of sediment cores at Halton Quay.

4.3.2 Description of samples

4.3.2.1 Visual description of sediment cores

A brief summary of the principal attributes of each core is given in Table 4.3 and Figure 4.4.

Core HQC1:	26 cm long (7 sections); June 24th, 1998.
Location:	One and half meters above the low water level.
Analyses:	Water content. Dissolved sulphide. Elemental sulphur. DMSP lyase activity.
Core HQC2:	11.5 cm long (4 sections); September 11th, 1998.
Location:	About two meters above the low water level and the border of the salt marsh.
Analyses:	Water content. Dissolved sulphide. DMSP lyase activity. DMSP. Elemental sulphur. Major and trace elements.
Core HQC3:	19 cm long (7 sections); September 11th, 1998.
Location:	Front of the salt marsh and 1 meter above the low water level.
Analyses:	Water content. Dissolved sulphide. DMSP lyase activity. DMSP. Elemental sulphur. Major and trace elements.
Core CsC:	19.5 cm long (5 sections); June 24th, 1998.
Location:	Under the bridge and one and half meters below the high water level.
Analyses:	Water content. Dissolved sulphide. DMSP lyase activity. DMSP.

Table 4.3 Sediment core locations and parameters measured at Halton Quay and Calstock.

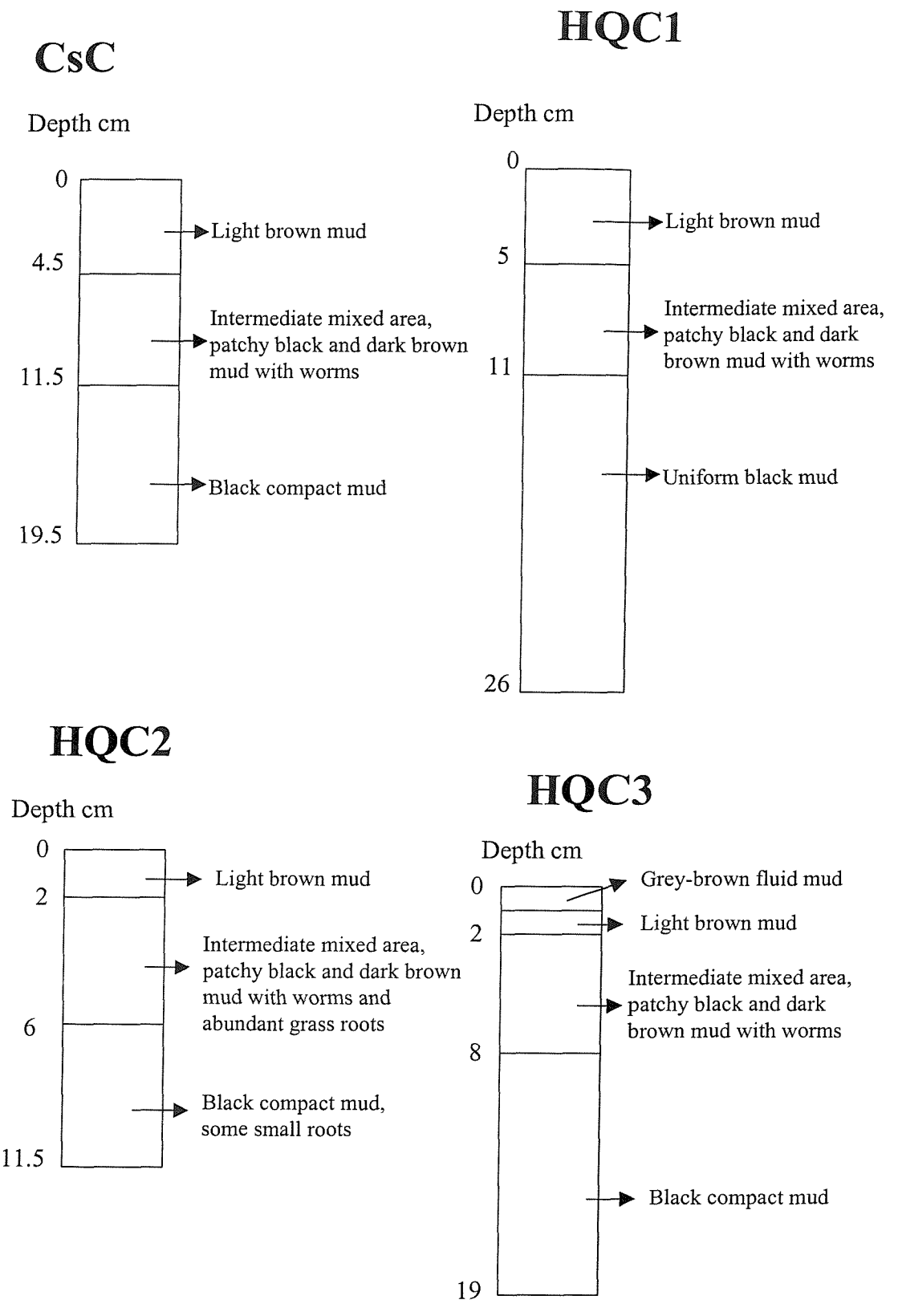


Figure 4.4 Visual logs of sedimentary faces in split sediment cores from Halton Quay and Calstock.

4.3.2.2 Water content

The water content profiles of sediment cores were similar from Calstock and Halton Quay. The water content was high in the surface sediment ranging between 62% and 70% and then decreased with depth to ca. 50% approximately 15 cm below the surface. After that the water content was relatively constant until the end of the core.

4.3.2.3 Major and minor elements

The data obtained from XRF in this section are all represented on a sediment dry weight basis.

(a) Major elements

The existence of silicon, titanium, potassium, chromium and aluminium in recent sediment is primarily due to their incorporation into the crystal structure of allogeneic mineral particles (Shotyk et al., 1990). In such lattice-bound sites these elements appear to exhibit inert geochemical behaviour and so slight variations in their concentration profiles usually originate from changes in the composition of the sedimentary material. The compositional variations throughout the core are partly due to large variations in organic carbon content. This problem has been eliminated by normalising the data to aluminium. The normalised profiles of Si, Ti, K and Cr (Fig. 4.5 (a)(b)) show that these element distributions conform to one another very closely.

The constancy of the Si/Al, Ti/Al, K/Al and Cr/Al ratios throughout the two cores show the composition of the mineral component to be consistent over time at each sampling site (Fig 4.5). These values do not show any significant difference between the two sites (Table 4.4). From the above results, the bulk of the mineral fraction appears to be relatively uniform across the sample area.

Core	Si/Al	Ti/Al	K/Al	Cr/Al
HQC2	3.07 ± 0.01	0.059 ± 0.001	0.287 ± 0.004	12.59 ± 0.40
HQC3	3.12 ± 0.05	0.061 ± 0.001	0.283 ± 0.002	12.81 ± 0.38

Table 4.4 Element to aluminium ratios in Halton Quay sediment cores.

Loss on ignition (LOI) was determined for all core samples during preparation for the measurement of trace elements. There is a strong correlation between LOI and organic carbon (Lewis, 1997). Therefore, it is permitted to extrapolate the LOI data into organic carbon data with a high degree of confidence. LOI decreased with increasing depth in the core HQC3 (Fig. 4.5 (b)). This may be due to a combination of microbial decomposition and the lack of a replenishing carbon source throughout the core. In the case of the core HQC2 (Fig. 4.5 (a)), a constant LOI profile is obtained from the surface down through the core. The visual inspection of HQC2 core (Fig. 4.4) showed abundant organic marsh grass roots within the intermediate mixed zone. It is therefore suggested that the marsh vegetation may constitute the main carbon source to the sub-surface communities.

The cycling of manganese, sulphur and iron compounds in sediment involves a complex suite of geochemical interactions. The distribution of these elements throughout the cores may enable the identification of different zones within the sediments where different oxidation and reduction reactions happen. The redox zonation is assigned to sequential utilisation of different chemical species as electron acceptors for organic decomposition and it is a useful scheme with which to interpret the observed element profiles (Froelich et al., 1979). Assessment of two sedimentary manganese profiles reveals that similar characteristics exist in each (Fig. 4.6). Solid phase of manganese (reported to be insoluble Mn (III) oxides) decreases sharply over the upper few centimetres and then slightly increases with increasing depth within the deeper sediment. This is indicative of manganese reduction to soluble manganese (II) increasing with depth until approx. 3 cm beneath the surface.

Unlike manganese, there is not a significant boundary between the oxic and sub-oxic zones evident in the concentration profile of iron (Fig. 4.6). In HQC2 iron is relatively constant; whereas, the level of iron in HQC3 increases slightly with depth over the upper few cm to a maximum value of approximately 4.95 wt. %. Its concentration is then relatively constant throughout the rest of the core. Also there is no significant visual change in these sediment cores. The above results suggest that a series of reactions may take place within these sediments without any predominant reaction causing a significant change in the iron concentration.

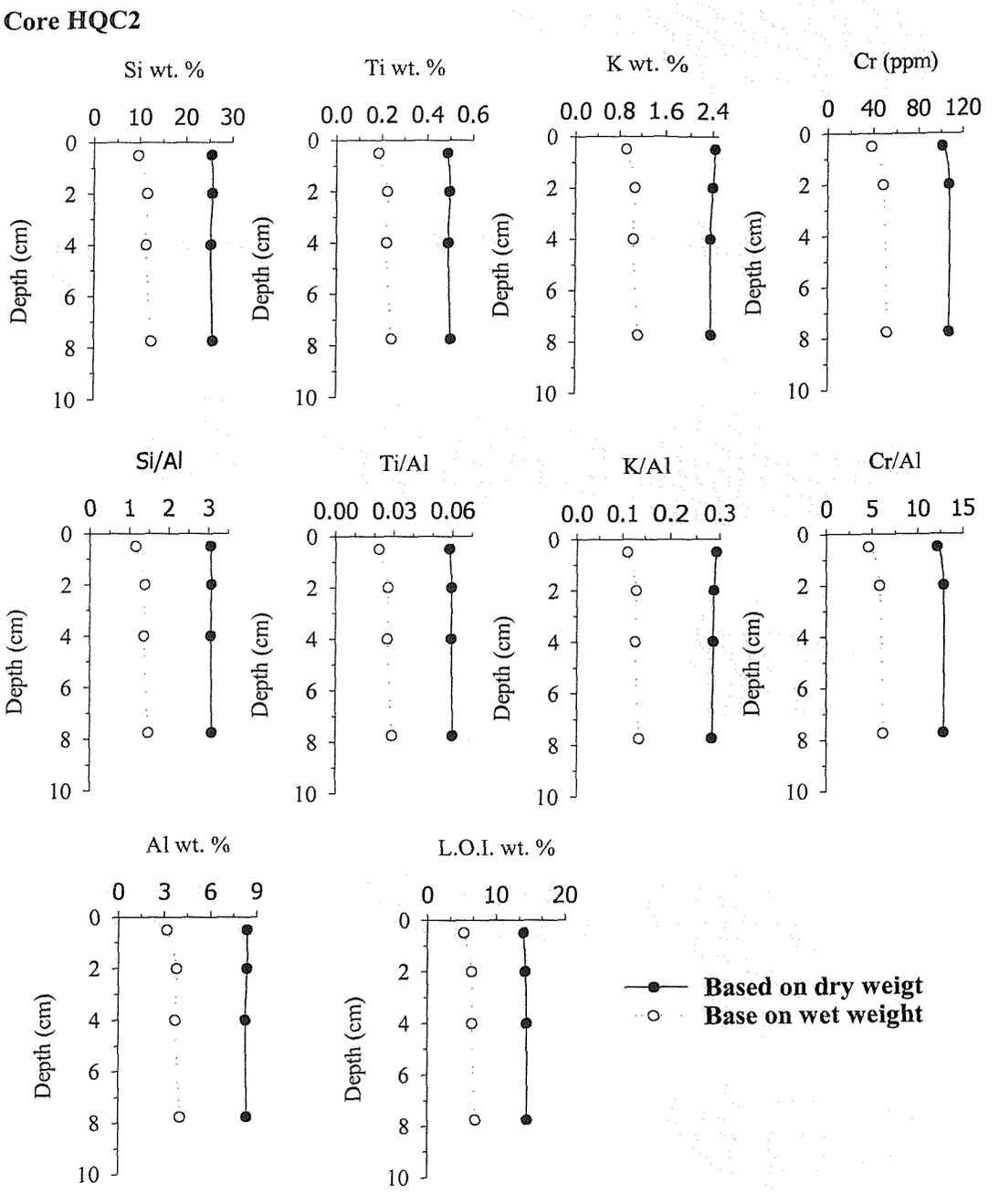


Figure 4.5 (a) Concentration profiles and normalised ratios of elements controlled by mineralogical variations. Loss on ignition also shown in the core HQC2. (wt is expressed as dry weight of sample)

Core HQC3

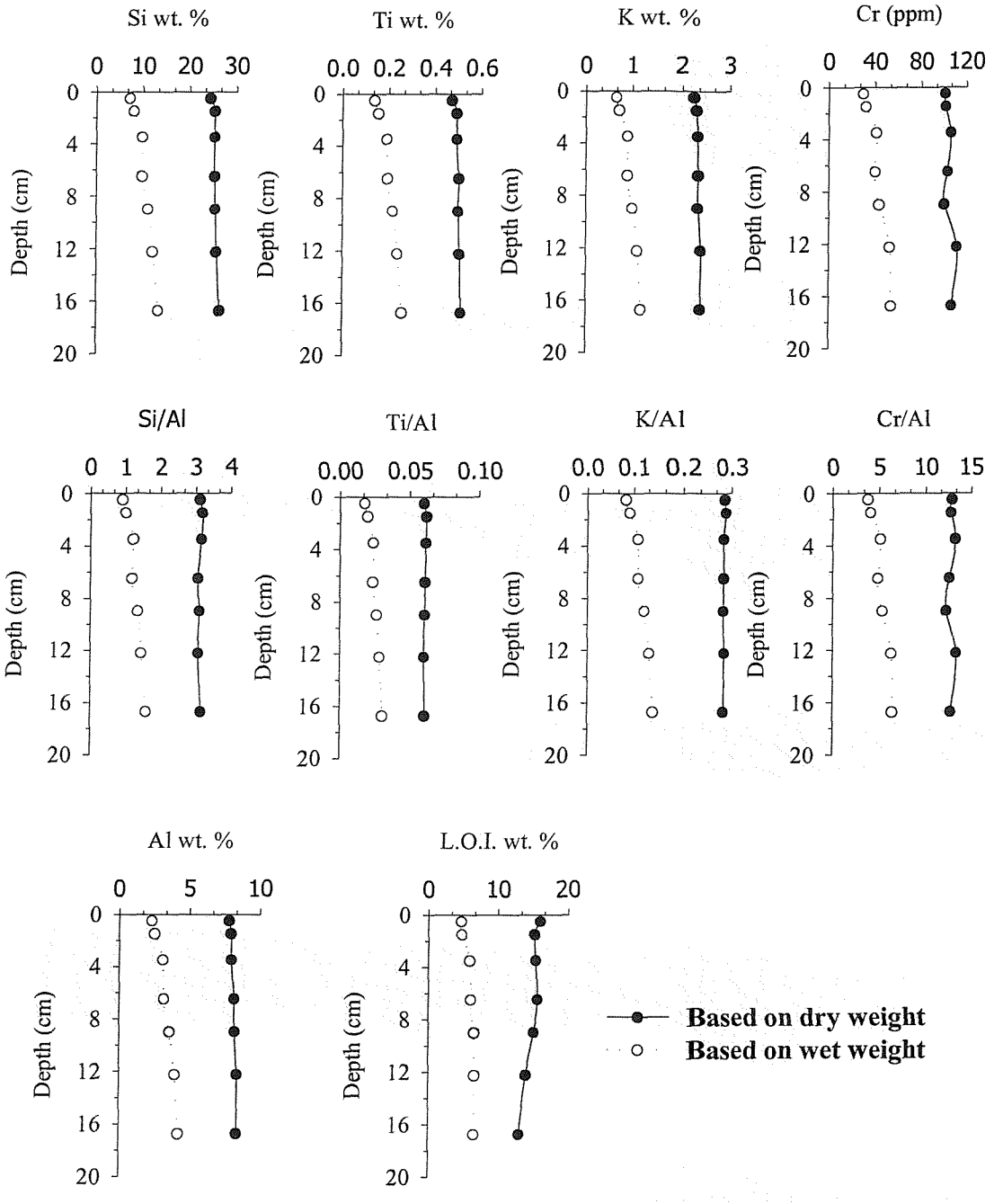
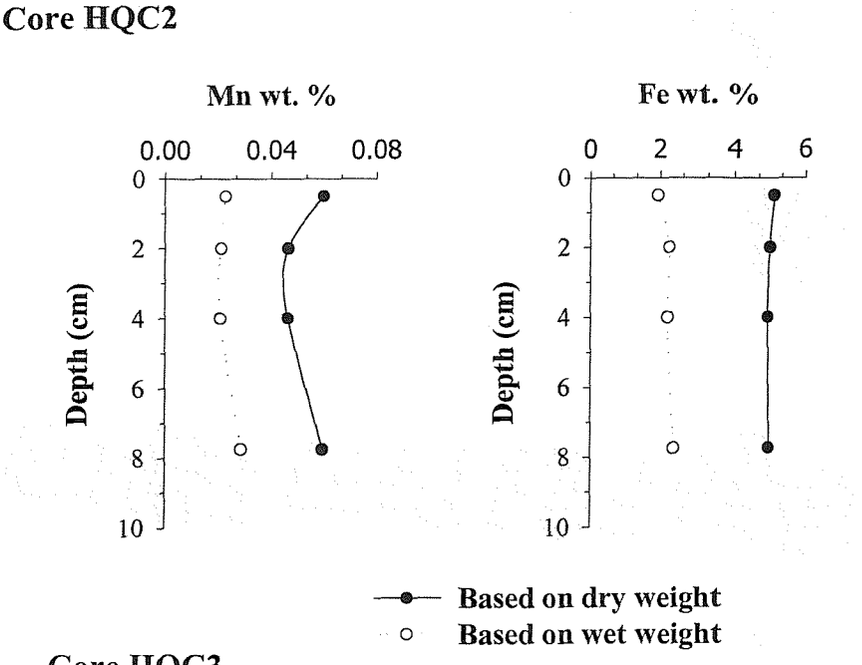


Figure 4.5 (b) Concentration profiles and normalised ratios of elements controlled by mineralogical variations in the core HQC3. Loss on ignition also shown.



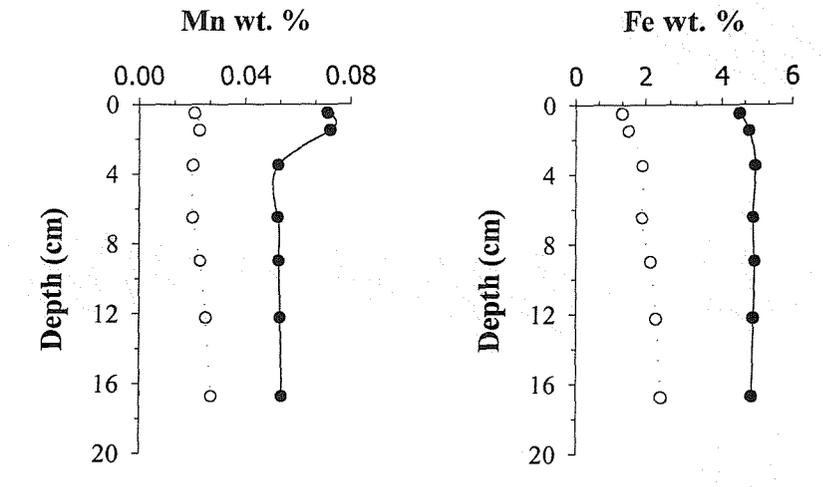


Figure 4.6 Concentration profiles of Mn and Fe in the core HQC2 and HQC3.

(b) Trace elements

The concentrations of As, Zn and Pb have been determined in two cores from Halton Quay. The USGS standard shale sample (MAG1) was used as a standard. The catchment area of the Tamar river is abundant in tin, copper, lead, silver and zinc mines. They also yield large quantities of pyrite and arsenopyrite, and wolframite is found widely associated with tin-bearing zones. Due to intensive mining activities in the past few decades and natural deposits in this area, the concentrations of As, Zn and Pb are relatively high in the estuarine sediments. A significant elevation of the arsenic levels has also been found in local vegetation, soil, waters and sediments (Aston, 1975, Howard et al., 1988).

The concentration profiles of As, Zn and Pb show that the levels of these elements significantly increase with increasing depth throughout each core and those profiles are relatively similar to each other (Fig. 4.7). It is suggested that long term deposition of sediment containing high concentrations of these trace elements will have resulted from the intensive mining activities in the past and followed by redeposition further down the estuary. The new deposit of upper sediments contains less trace elements, but the concentrations are still higher than those obtained from other sediments. That is because the geology here contains high concentrations of these minerals.

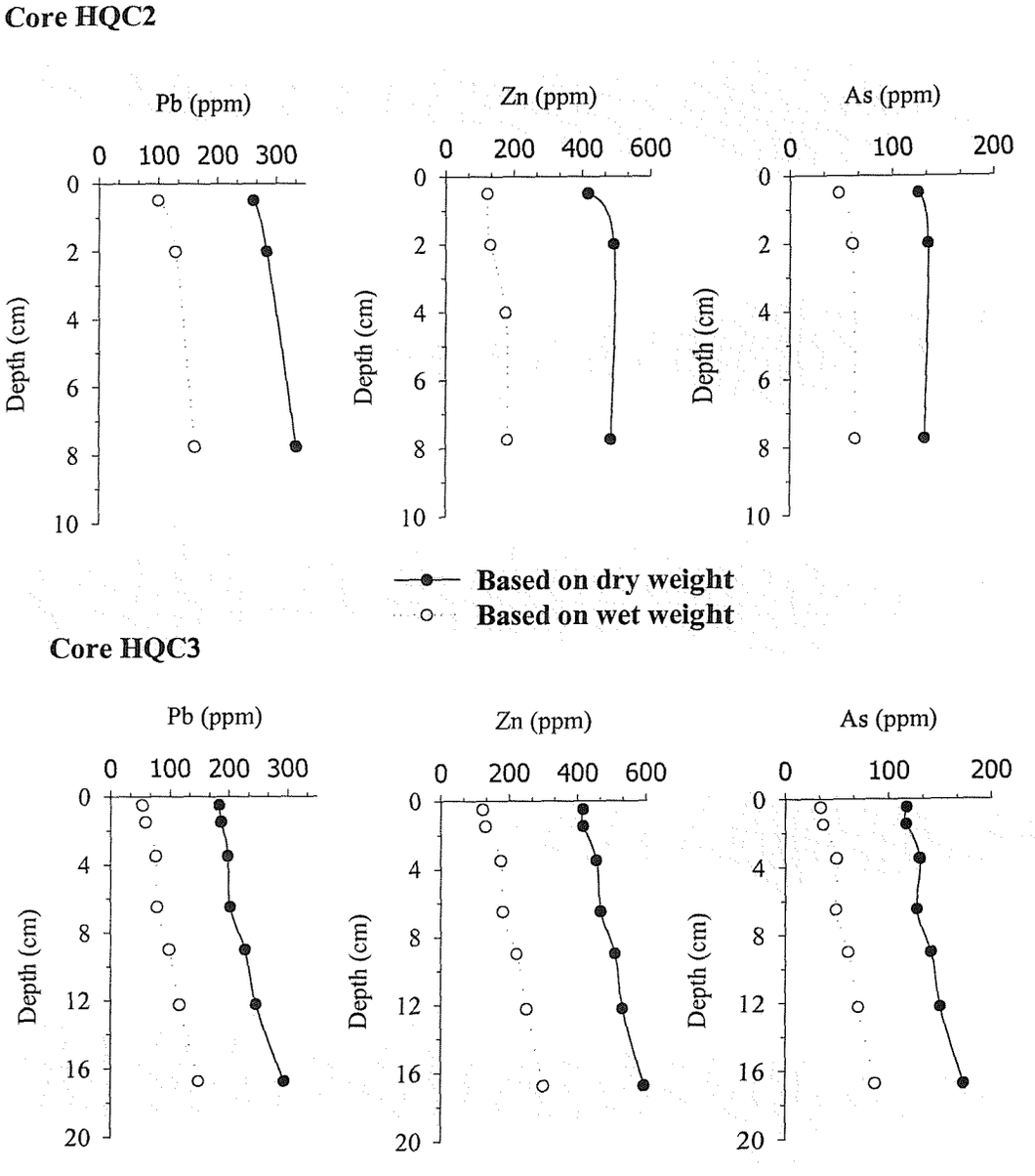


Figure 4.7 Plot of Pb, Zn and As concentration as a function of depth in the core HQC2 and HQC3.

4.3.3 Sulphur species in sediments

4.3.3.1 DMSP and DMSP lyase

Based on the dry sediment weight, the profiles of DMSP through the sediment cores all indicated that the DMSP concentration decreases with increasing depth until about 3 cm beneath the surface sediment (Fig. 4.8). In general, the highest concentration of DMSP occurred in the top surface sediments due to the presence of algae and some higher plants, the major producers of DMSP.

The DMSP lyase activities are high in the upper surface of the cores but decreased sharply with depth (Fig. 4.8). The DMSP lyase activity in the HQC3 core was approx. 3 fold higher than that in the HQC2 core.

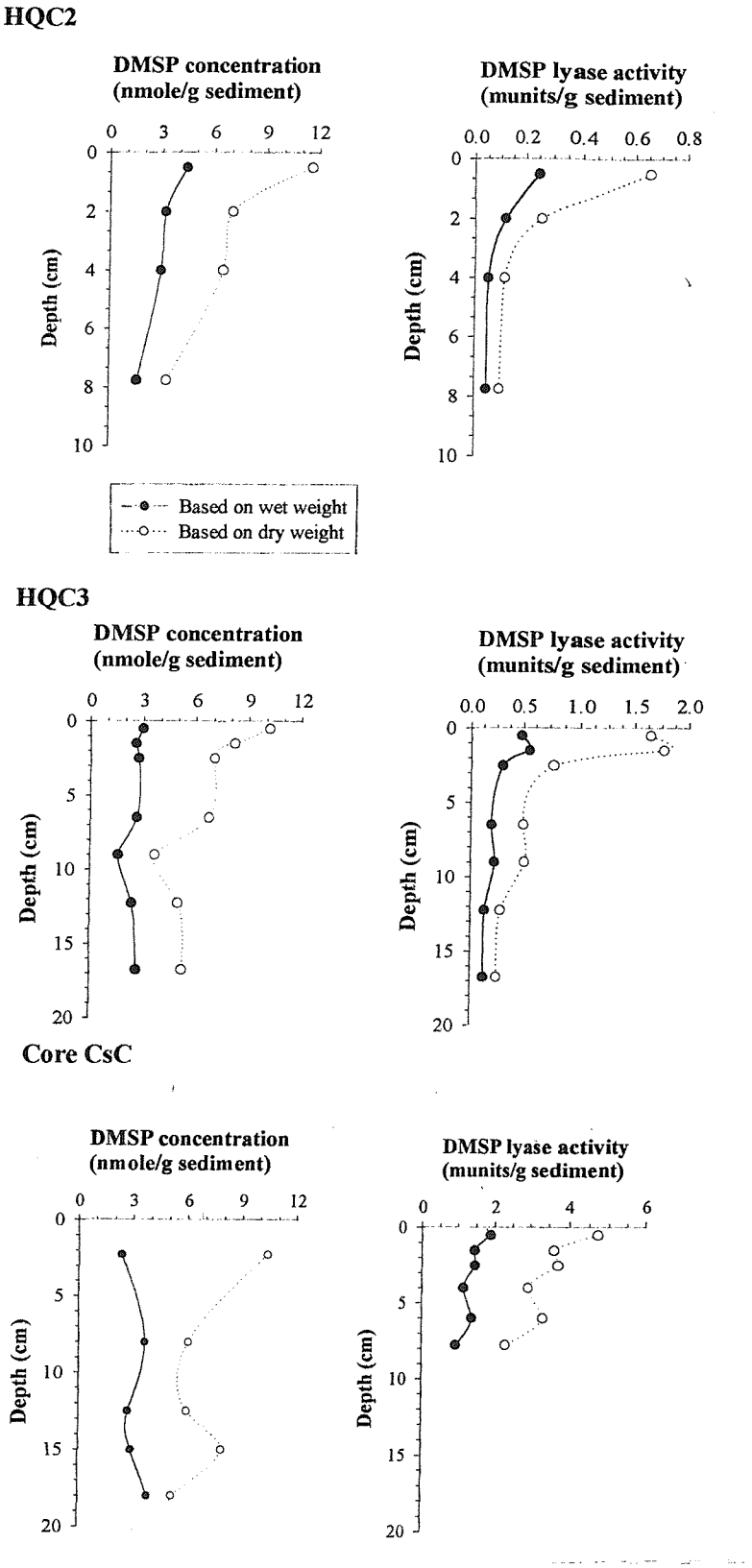
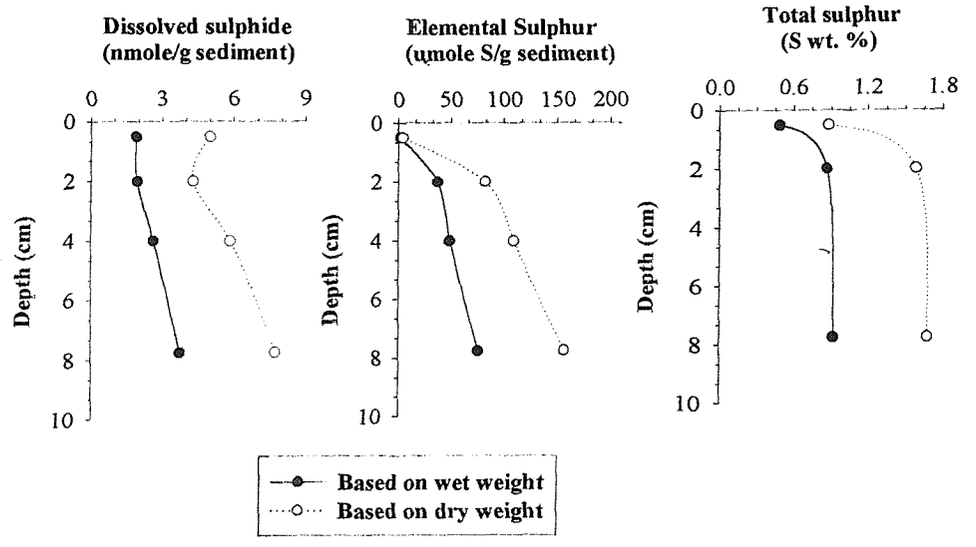


Figure 4.8 Depth profile of dissolved DMSP extracted from interstitial water and DMSP lyase activity.

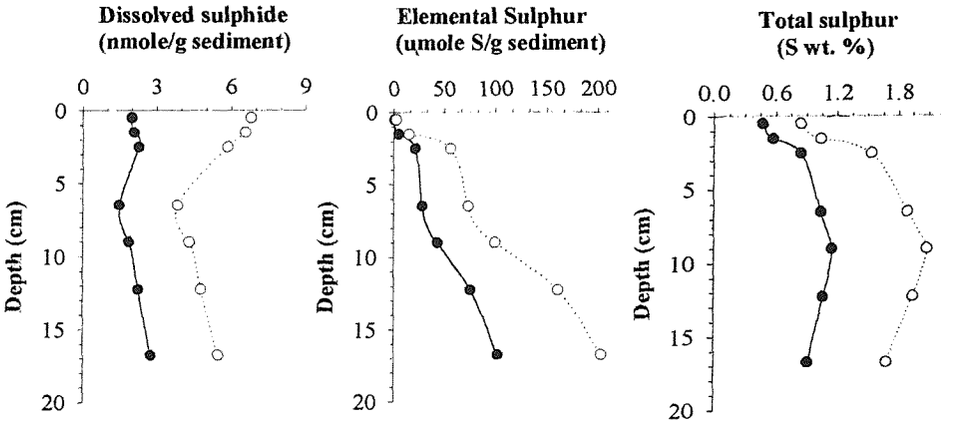
4.3.3.2 Dissolved sulphide, elemental sulphur and total sulphur

The distributions of dissolved sulphide, elemental sulphur and total sulphur in sediments are shown in Figure 4.9.

HQC2



HQC3



Core CsC

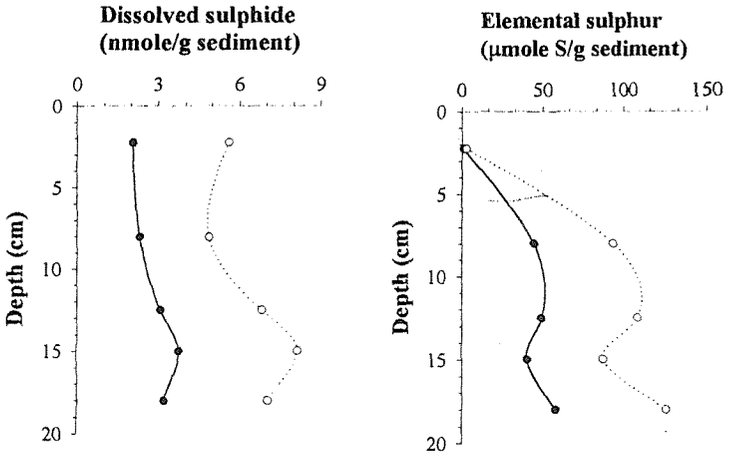


Figure 4.9 Depth profile of dissolved sulphide extracted from interstitial water, elemental sulphur and total sulphur.

4.3.4 Discussion

4.3.4.1 DMSP and DMSP lyase

The relationship between the DMSP contents and DMSP lyase activities of the HQC2 and HQC3 cores are shown in Figure 4.10. From this graph it may appears that high concentrations of DMSP are accompanied by high DMSP lyase activities. As the DMSP level drops with depth through the sediment cores, the DMSP lyase activities decreased as well (Fig. 4.8). This apparent association between DMSP and DMSP lyase activities needs further investigation to confirm and identify the nature of the correlation. Abundant *Spartina* roots were observed in the upper sediment of HQC3 and it is in this region that high DMSP lyase activities are found. The association of *Spartina* with high DMSP lyase activities was also found in the study of surface sediments (section 4.3.3). It has been reported that salt marshes dominated by *Spartina alterniflora* exhibit a high emission rate of DMS (Steudler, 1985) and that the salt marsh grass *Spartina alterniflora* contains a high concentration of DMSP (Pakulski & Kiene, 1992). The finding of high enzyme activities in HQC3 was therefore possibly due to the intracellular DMSP lyase of *Spartina* and DMSP lyase-containing microbes which populate *Spartina* sp.. Whilst in the deeper sediments DMSP lyase activities were low, they were still significant (Fig 4.8). The presence of DMSP lyase activities is not solely dependent on algae or plants. A DMSP-cleaving anaerobe (strain W218), for example, has been isolated by van der Maarel et al. 1996 from anoxic intertidal sediment (Vandermaarel et al., 1996b). It is therefore probable that the DMSP lyase activities found in deeper sediments may be due to bacterial activity.

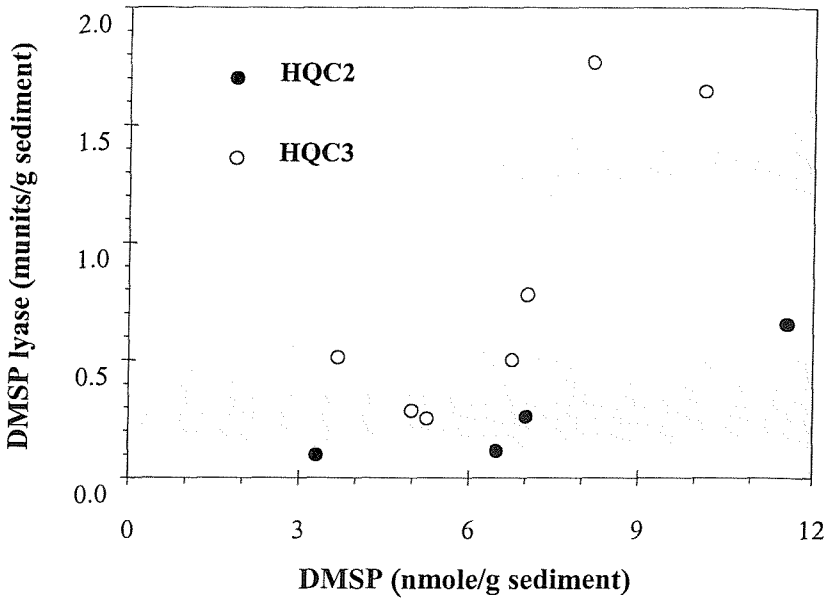


Figure 4.10 The relationship between DMSP content and DMSP lyase activity in cores HQC2 and HQC3.

Even higher DMSP lyase activities were measured at Calstock yet these sediments did not contain any obvious plant material. Calstock differs from the Halton Quay site in having intensive human activity. This may have resulted in the presence of different bacterial populations capable of releasing DMS from the added DMSP. These DMSP consuming bacteria do not necessarily employ a DMSP lyase to cleave DMSP to DMS and acrylate and might utilise alternative DMSP breakdown mechanisms to produce DMS and other products. High DMSP lyase activities would therefore be reported because of the dependence of the assay on the measurement of DMS production. It is also possible that the DMSP lyases present at the two sites differ in their optimal conditions. The optimum pH condition that was employed to assess the lyase activities was taken from the study of *Enteromorpha intestinalis*. It has been reported that the various DMSP lyases found in algae, bacteria, fungi and phytoplankton differ significantly, requiring different pH conditions with their activities being dependent on the locations, habitats and species involved (Wolfe & Steinke, 1996). If *Spartina* DMSP lyase differs significantly in the conditions required for optimal activity then the conditions derived from the *Enteromorpha* study may not be appropriate, leading to depressed levels measured at Halton Quay.

Whist the lyase activities in the sediment cores from the two sampling areas were significantly different, the DMSP levels were of the same magnitude in all cores. At both areas the DMSP concentrations were still significant at depth even though no photosynthesis could be occurring to produce DMSP. There are a number of explanations as to how this penetration of DMSP into deep sediments may occur. If the DMSP originates from photosynthetic organisms it may be carried to the deeper sediment by bioturbation. Alternatively this DMSP may originate from currently unidentified non-photosynthetic organisms. It is also possible that other DMS precursors are present contributing to the evolved DMS employed as the basis for DMSP measurements. More specific methods would be required to assess this possibility. It is interesting to note that despite the absence of macroalgae or *Spartina* at Calstock significant levels of DMSP were found in the surface sediments. This may be due to the generation of DMSP by microalgae inhabiting the surface sediments.

4.3.4.2 Sulphur species

The total sulphur content in the sediments of Tamar estuary generally increased with depth, averaging 0.7 wt. % in the surface sediment and ca 1.8 wt. % of dry weight sediment below 3 cm (Fig. 4.9). Elemental sulphur is the major sulphur compound in sediments accounting for about 30 to 40 % of total sulphur at Halton Quay (Fig. 4.9). The concentration of elemental sulphur in the sediment increased with increasing depth in both the cores that were studied. Similar results of elemental sulphur distributions have been reported (Berner, 1964, Chen et al., 1997). However the elemental sulphur concentration at Calstock was very low in surface sediments probably due to tidal and anthropogenic mixing and oxygenation of these sediments. The sulphur levels then increased significantly with depth reaching levels similar to those recorded at Halton Quay within the anoxic region.

A large number of inorganic and biologically-mediated oxidative processes can lead to the formation of elemental sulphur. The oxidation of H_2S can be catalysed by dissolved trace metals, metal oxide phases (such as amorphous iron oxides and MnO_2) and microbial activity with the main end products normally being S_8 and SO_4^{2-} (Jorgensen, 1977, Pyzik & Sommer, 1981, Stumm & Morgan, 1981). Alternatively, sulphate reduction can also lead to the production of elemental sulphur. Once elemental sulphur is formed in the sediment, elemental sulphur is likely to be involved in further oxidation, reduction or disproportionation that will depend on where it is present in the sediment (Canfield & Thamdrup, 1996).

The concentration of DMSP found in surface sediments is very low compared with the total sulphur. It is therefore unlikely that DMSP was a major contributor of sulphur compounds to the sediments (Fig. 4.8).

In general the level of dissolved sulphide extracted by water from the top surface sediments was 2-3 nmol per g of wet sediment. Whilst there was some indication of a small increase with depth no major change was observed (Fig 4.8). The concentration of dissolved sulphide measured in this study was much higher than the theoretical solubility of S^{2-} in a water system containing highly insoluble heavy metal sulphides. This high level of dissolved sulphide may be due to the analytical method used in this study which can measure both the dissolved and colloidal metal sulphide. With the high metal loading in the Tamar estuary, S^{2-} would rapidly combine with metal ions resulting in the formation of dissolved, colloidal and insoluble metal sulphides. Dissolved and colloidal metal sulphides can pass through the 0.45 μm filter paper and consequently be determined by the acid-based flow injection system (Section 2.1). Therefore, the concentration of dissolved sulphide in these cores remained essentially constant, fixed by its low solubility formation and release of colloidal metal sulphides.

From the visual logs (Fig. 4.4), the sediment appeared dark black with a strong sulphur smell between 7 and 11 cm below the surface. A high concentration of

sulphide in deep sediment was expected. The dark black sediment may be due to a high concentration of precipitated pyrite (Libes, 1992). In addition, the concentration of iron was ca 4.6 wt. % (Fig. 4.6) which is about 2 to 3 fold higher than that of total sulphur. Pyrite is therefore likely to be responsible for the remaining 50% to 60 % of the total sulphur present in the sediments.

4.4 Conclusions

4.4.1 Spatial variation in surface sediment

The spatial variations in intertidal surface sediment were investigated at Halton Quay. In general, the whole sampling area of surface sediment without vegetative growth contained similar levels of DMSP and DMSP lyase activities. The surface sediment covered with a green algal mat was found to contain ca. 3 fold more DMSP than that measured in the Reference surface sediment, showing the contribution of green algae to the measured levels. Little DMSP was however found in the salt marsh sediment from *Spartina* sp. colonised areas despite reports of high DMSP being present in *Spartina* itself. When considered together with the DMSP lyase activities, the high lyase activities observed in the *Spartina* colonised areas may be responsible for the low measured levels of DMSP, even when a high flux of DMSP into the sediments from *Spartina* may be expected. A strong sulphurous smell was also observed in this salt marsh area which may be due to the released DMS from DMSP. It was therefore suggested that DMSP lyase was responsible for the relatively low DMSP contents of the salt mash sediments colonised by *Spartina*.

4.4.2 Vertical variation

Extensive past mining activity and the natural geology of the region causes Tamar estuary sediments to contain significantly elevated concentrations of trace metals such as As, Zn and Pb. The distribution of the mineral components in the studied cores has

shown that the sediments are relatively uniform within the sampling area. The boundary between oxic and sub-oxic regions of the sediment cores is shown in the manganese depth profiles but not in the iron levels which are swamped by the presence of iron minerals. The increased concentration of Mn in the surface sediments indicates that manganese oxidation increases within the top sediment generating the insoluble MnO_2 which can play a key oxidant role in the cycling of dissolved species such as arsenite.

Elemental sulphur is between 30 to 40 % of total sulphur. Due to its very low concentration DMSP is unlikely a major source of sulphur in the sediments. The concentration of DMSP is high in surface sediment due to the presence of algae and some higher plants and then decreases with depth. The penetration of DMSP into the deep sediment may be due to intensive bioturbation or tidal movement. It is also possible that other DMS precursors contributed to the evolved DMS that was the basis for the DMSP measurements. Colloidal metal sulphide may be responsible for the high level of 'dissolved' sulphide observed in this study. Based on the visual observation and the major element measurement, pyrite is suggested to be the major form of sulphur in the sediments.

The DMSP concentrations and DMSP lyase activities are all high in surface sediments supporting extensive vegetation growth. It is believed that algae and some higher plants are the major producers of both DMSP and DMSP lyases. The DMSP lyase activity decreased with decreasing DMSP content throughout the sediment depth indicating that there was a strong relationship between them.

CHAPTER FIVE

General discussion and conclusions

5.1 Introduction

The green macroalga *Enteromorpha intestinalis* is one of the major sources of DMSP in intertidal sediments of estuaries in southern England. DMSP lyases were isolated and characterised from this alga. Such algae, together with marsh grasses, release DMSP and its lyase into the sediments. This thesis has covered work carried out to investigate the fate of these compounds in estuarine sediments.

5.2 The DMSP content of algae

Since its initial identification in a number of macroalgae, DMSP has been recognised as a major source of DMS in the marine environment. This thesis has described a base hydrolysis flow injection analysis (FIA) system for the specific detection and quantification of DMSP. A borohydride based FIA system was however found to widen the range of DMS precursors that could be investigated, but with variable selectivity.

The DMSP content of algae was found to be inconsistent. This variability is believed to be due to the instability of DMSP during sample preparation that can be attributed to the presence of DMSP lyase. Any environmental stress can affect the DMSP lyase

activity which in turn would influence the original concentration of DMSP. A previous report that oven drying of algal samples at 80 °C significantly increased the measured content of DMSP in some samples (Karsten et al., 1994) could not be confirmed. In the twelve species of algae studied, rather than increasing the levels found, the measured DMSP concentrations were lowered by oven drying. This heating process appears to reduce the DMSP levels, presumably by thermal decomposition. Fresh samples were therefore subsequently employed for the determination of the DMSP content of algae.

5.3 DMSP lyase from *Enteromorpha intestinalis*

Green algae such as *Enteromorpha intestinalis* colonise many coastal areas of the United Kingdom and are found throughout the estuaries of southern England. The DMSP content of this alga was found to be ca 6.4 ± 0.96 mmole kg^{-1} FW, a value that compares well with previously reported values (Karsten et al., 1994, Russell, 1995). The DMSP concentration can be expected to vary with time and situation, as the alga responds to changing environmental conditions and stress. To achieve this, DMSP must be rapidly synthesised and eliminated in response to environmental changes to be an effective osmolyte. Intracellular DMSP content of algae may be regulated by their DMSP lyase in response to changing external conditions.

DMSP lyases which cleave DMSP to DMS and acrylate have been isolated and characterised from the green macroalga *Enteromorpha intestinalis*. The *Enteromorpha* lyases reported in this study were found to be located both as a soluble and detergent-released proteins. The molecular weight of the soluble protein was determined to be approximately 25.2 kDa. Three hydrophobic variant forms of detergent-released protein were isolated from the detergent-solubilised fraction. The location of the enzyme *in vivo* is still unknown. Some previous reports had shown that DMSP lyase is membrane bound in the Rhodophytes *Polysiphonia lanosa* (Cantoni & Anderson, 1956)), and *P. paniculata* (Nishiguchi & Goff, 1995) and the Chlorophyte, *Enteromorpha clathrata* (Steinke & Kirst, 1996). The relationship between the soluble and detergent-released enzyme also needs further investigation to confirm

whether this soluble enzyme is a loosely bound membrane protein released during the purification process or not.

Estuarine and intertidal macroalgae are exposed to frequent fluctuations in external salinity. In this study the DMSP lyase from *Enteromorpha intestinalis* has been shown not to be affected by changing NaCl concentration. Being insensitive to the Na^+ concentration *Enteromorpha intestinalis* is tolerant to the external fluctuating salinity. It has previously been reported that the DMSP lyase activity is stimulated in *Enteromorpha clathrata* by high salinities after 72 hours incubation, but that increased NaCl concentrations did not stimulate DMSP lyase activity *in vitro* (Steinke & Kirst, 1996).

The DMSP lyase activity was found to increase with increasing Mg^{2+} concentration. During high tide, the salinity of estuarine water increases and algae are forced to pump out water from cells or to transport more inorganic ions into the cells to balance the osmotic stress. The changing Na^+ concentration will not affect the rate of enzymatic degradation of DMSP but the enhanced Mg^{2+} concentration may stimulate the DMSP lyase activity. This would lead to a decrease of DMPS content in algae causing a reduction in the osmolyte strength. This result seems to conflict with the hypothesis that intracellular DMSP would accumulate during the high tide to balance the increased external stress. How rapidly the DMSP lyase responds to changes in salinity or whether enzyme activity is blocked by other seawater constituents is still unknown. DMSP has been reported not to be involved in short term hyperosmotic regulation in *Enteromorpha intestinalis* whilst inorganic ions, proline and sucrose are (Edwards et al., 1988). It is possible that any change of ion content in the plant cells might be restricted to the vacuolar compartment when changes in cytoplasmic ion levels would lead to metabolic disruption (Brady *et al.*1984). Mechanisms of DMSP cleavage *in vivo* are complicated and the cellular osmotic pressure adjustment is involves various osmolytes participating together.

The functionality of DMSP lyase is dependent not only on the location of the enzyme in the cell but also on the affinity and concentration of substrate. The rates of catalysis

of DMSP depend on the response of the enzyme to changing levels of DMSP. The K_m value indicates the affinity of DMSP lyase for the substrate DMSP. The K_m value of the soluble DMSP lyase from *Enteromorpha intestinalis* was 0.286 mM which was in the same range as the enzyme from *Ulva curvata* (Desouza et al., 1996). High K_m values were normally associated with high DMSP levels in the algae. In addition ,this enzyme activity is very sensitive to the pH. The optimum pH is at pH8 which is the same as the pH of estuarine water. Different optimum pH values have been reported in previous studies of other algae. Also this DMSP lyase is only specific to its substrate DMSP rather than some other DMS precursors or DMSP analogues. Due to these differences in enzyme characteristics and functionality, it has been suggested that the algal DMSP lyases are responsible for the majority of intracellular DMS production in the system. Therefore, the biogenic DMS production is dependent not only on environmental factors influencing DMSP conversion but also on the species specific properties of DMSP lyase from different algae.

5.4 The relationship between the DMSP concentration and DMSP lyase activities of sediments

As a result of die back, in the early stages of the degradation of *Enteromorpha intestinalis* it releases DMSP and DMSP lyase into intertidal sediments. The concentration of DMSP and DMSP lyase activity both decreased with increasing depth in the sediment cores with low concentrations of DMSP being found in deep sediments. Measurable levels of subsurface DMSP and lyase are believed to be due to bioturbation transporting DMSP from the surface, but the possibility of other bacterial sources of DMSP and lyase in these deeper sediments can not currently be excluded. This is supported by the isolation of a bacterial DMSP lyase from anaerobic intertidal sediments (Vandermaarel et al., 1996b). These findings suggest that there is a strong relationship between DMSP content and DMSP lyase activities in sediments.

5.5 DMSP and sulphur cycling in sediments

On and within the intertidal surface sediments DMSP is liberated from decaying algae or by grazing. Once DMSP has been deposited into the sediments, its breakdown can commence (Fig. 5.1). In this study the distribution profiles of various sulphur species were investigated in intertidal sediments of the Tamar estuary. High levels of DMSP were found in the surface sediments. Based on the studies of spatial variations of intertidal surface sediments it was found that surface sediments without vegetative growth exhibit similar significant levels of DMSP and DMSP lyase activities. Green algae were the major contributors of measured DMSP. Little DMSP was however found in the salt marsh sediments from areas colonised by *Spartina* sp. despite reports that high levels of DMSP are present in *Spartina* (Pakulski & Kiene, 1992). When considered together with the DMSP lyase activities, the high lyase activities may be responsible for the low measured levels of DMSP even when a high flux of DMSP into the sediments may be expected due to the presence of *Spartina* DMSP. This breakdown of DMSP was supported by a strong sulphurous smell observed in this salt marsh area which may be due to the release of DMS from DMSP. It has been reported that DMS is one of major gases evolved from North Sea intertidal areas (Harrison et al., 1992). It was therefore suggested that the high DMSP lyase activities could be responsible for the relatively low DMSP contents of the salt marsh sediments colonised by *Spartina*.

The released DMS could either dissolve in the interstial water of sediments or emit into water column and atmosphere. The dissolved DMS could undergo sequential biological demethylation to H_2S. H_2S can be reoxidised to $S_2O_3^{2-}$, elemental sulphur, SO_3^{2-} and SO_4^{2-} or precipitated in the form of metal sulphides and locked up in sediments and sedimentary rocks. Extensive past mining activity and the natural geology of the region has left a legacy of high levels of metals such as As, Zn and Pb in the sediments of the Tamar estuary. These sediments contain high levels of metal

sulphides and the solubility of these sulphides is believed to control the level of sulphide in the interstitial waters.

From the measurement of the profiles of sulphur species through sediment cores it was evident that organic sulphur compounds made up a small proportion of the total sulphur that was present. Elemental sulphur was the major sulphur compound measured in this study ranging from 30 to 40 % of the total sulphur. A slightly increased total sulphur content within the root-rich layer surface sediments could arise from the presence of organic sulphur compounds present in the vegetation.

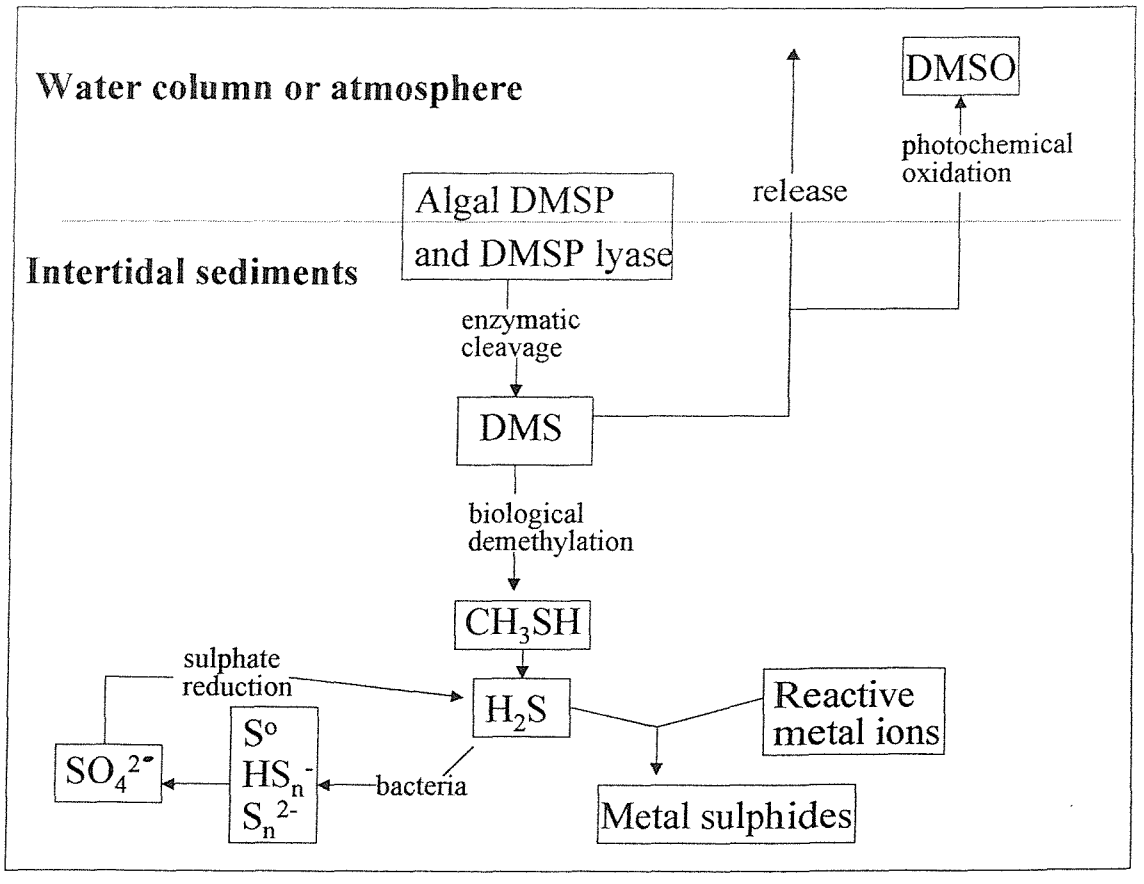


Figure 5.1 Schematic diagram illustrating the breakdown of DMSP and the sulphur cycle in sediments.

The research described in this thesis provided valuable information on the behaviour of DMSP and DMSP lyase in estuarine sediments. As with all research this throws up a number of aspects which require further studies.

5.6 Suggestion for future work

The work reported in this thesis has led to the identification of a number of areas of study that should be carried out:

- The levels of dissolved sulphide in sediment interstitial waters may be high due to the presence of colloidal metal sulphides. This could be investigated by ultrafiltration of interstitial waters.
- A knowledge of the contribution of pyrite to the sediment total sulphur would provide an useful context for the presence of elemental sulphur in the sediments.
- Novel analytical methods are required for the measurement of DMSP demethylation products in sediments.
- The association of DMSP content and DMSP lyase activities in sediments requires further investigation. In particular the presence of both species at depth within the sediment core requires further explanation and secondary DMSP and DMSP lyase generating organisms identified.
- The measurement of lyase activity in sediments depends strongly on the pH at which the measurement is carried out. This pH is not necessarily that of the lyase extracted from *Enteromorpha*. It will therefore be necessary to investigate the optimum pH values of DMSP lyases present in sediments.

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