DMS AND DMSP PRODUCTION BY MARINE DINOFLAGELLATES

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ABSTRACT

Dimethylsulphoniopropionate (DMSP) is the biogenic precursor of the climate-cooling gas dimethylsulphide (DMS). DMSP is produced by certain phytoplankton groups including dinoflagellates. DMSP is a multifunctional compound potentially acting as an osmolyte, cryoprotectant, antioxidant, methyl donor, grazer deterrent and overflow exudate but its primary biological role and the variation in DMSP content in dinoflagellates remain unclear. As dinoflagellates can be sensitive to agitation, the methods for DMSP measurements in cultures were assessed and the measurement of total DMSP as a surrogate for intracellular DMSP was retained for most experiments. I collected all the published DMSP data in dinoflagellates and measured DMSP in 9 species. The obtained dataset proposed a summary of DMSP concentrations in dinoflagellates which spread over 6 orders of magnitude with an average of 242 mM (n=61) and include the widest and highest DMSP concentrations reported in phytoplankton. The DMSP content was analysed in relation with various biological criteria that determine the wide diversity of the dinoflagellate group. Bioluminescent species and species holding haptophyte-like plastids have significantly lower DMSP concentrations whereas higher concentrations were found in Mediterranean species. The role of DMSP was further investigated in the heterotrophic dinoflagellate *Crypthecodinium cohnii* by testing the effect of abiotic parameters on its DMSP content. DMSP acted as an osmolyte with a short-term response to hyperosmotic shock and long-term response to hypoosmotic shock. Concentrations also increased with growth as a result of glucose depletion suggesting that DMSP might replace glucose-derived osmolytes. The DMSP adjustment of cultures transferred into glucose-depleted or repleted media appeared to be exceptionally fast (<1 min). Nitrogen limitation also increased DMSP concentrations, possibly due to DMSP acting as an overflow metabolite for excess sulphur or as an antioxidant under starvation stress. Overall the results strongly support the contribution of the dinoflagellate group to the DMSP production.

RÉSUMÉ (French version of the abstract)

Le diméthylsulfoniopropionate (DMSP) est le précurseur biogène du diméthylsulfure (DMS), gaz à effet de refroidissement sur le climat. Le DMSP est produit par certains groupes de phytoplancton dont les dinoflagellés. Le DMSP peut avoir de multiples fonctions telles que: osmolyte, cryoprotecteur, antioxydant, donneur de méthyle, molécule de défense contre les brouteurs et exsudat de surcharges métaboliques en conditions de croissance déséquilibrée. Cependant, le rôle biologique majeur du DMSP ainsi que la variabilité du contenu en DMSP chez les dinoflagellés demeure à ce jour incertain. Comme les dinoflagellés peuvent être sensibles à l'agitation, des méthodes de mesure du DMSP dans les cultures ont été évaluées et la mesure du DMSP total en tant qu'estimateur du DMSP intracellulaire a été retenue pour la majeure partie des expériences. J'ai regroupé toutes les valeurs publiées de concentration en DMSP mesurées chez les dinoflagellés et ajouté mes propres mesures effectuées au laboratoire sur 9 espèces. La base de données obtenue propose une synthèse des valeurs de concentration en DMSP exprimées par volume cellulaire qui s'étalent sur 6 ordres de grandeur avec une moyenne de 242 mM (n=61) et qui incluent les valeurs les plus extrêmes et les plus élevées obtenues parmi tous les groupes de phytoplancton. Le contenu en DMSP a été analysé en fonction de plusieurs critères biologiques à l'origine de la grande diversité des dinoflagellés. Les espèces bioluminescentes et les espèces qui contiennent des plastes d'haptophyte ont des concentrations en DMSP significativement plus faibles alors que des concentrations plus fortes sont observées dans les espèces méditerranéennes. Le rôle biologique du DMSP a été plus particulièrement étudié pour le dinoflagellé hétérotrophe *Crypthecodinium cohnii*. Le DMSP agit en tant qu'osmolyte avec une réponse à court terme suite a un choc hyperosmotique et à long terme après un choc hypoosmotique. De plus, la concentration en DMSP augmentait avec la croissance de la culture en réponse à une carence en glucose, laquelle suggérait que le DMSP pourrait remplacer d'autres osmolytes dérivés du glucose. La réponse d'ajustement du DMSP pour une culture transférée dans un milieu carencé ou supplémenté en glucose s'effectuait de manière exceptionnellement rapide (<1 min). La limitation en azote a également affecté la concentration en DMSP chez *C. cohnii* en augmentant sa concentration, soit agissant

potentiellement en tant que métabolite de surcharge lors d'excès de sulfates, soit en tant qu'antioxydant en conditions de stress nutritif. Dans l'ensemble, ces résultats confirment l'importance de la contribution des dinoflagellés dans la production de DMSP.

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LIST OF ABBREVIATIONS

AZP	Azaspiracid shellfish poisoning
CAMR	Central american coastal province
CLAW	Hypothesis proposed by Charlson, Lovelock, Andreae and Warren.
CCMP	Center for culture of marine phytoplankton, Boothbay Harbor USA
CCN	Cloud condensation nuclei
CFP	Ciguatera fish poisoning
Chl	Chlorophyll
CV	Cell volume
DHA	Docosahexanoic acid
DLA	DMSP lyase activity
DMS	Dimethylsulphide
DMSHB	4-dimethylsulphonio-2-hydroxybutyrate
DMSO	Dimethylsulphoxide
DMSP	Dimethylsulphoniopropionate
$DMSP_d$	Dissolved dimethylsulphoniopropionate
$DMSP_p$	Particulate dimethylsulphoniopropionate
$DMSP_T$	Total dimethylsulphoniopropionate ($DMSP_p$ + $DMSP_d$ +DMS)
DSP	Diarrehic shellfish poisoning
FPD	Flame photometric detector
GC	Gas chromatography
HAB	Harmful algal bloom
MMPA	3-methiolpropionate
MSA	Methyl sulfonic acid
MSIA	Methylsulphinic acid
NSP	Neurolytic shellfish poisoning
PFT	Plankton functional types
PSP	Paralytic shellfish poisoning
PUFA	Polyunsaturated fatty acids
PTFE	Polytetrafluoroethylene
ROS	Reactive oxygen species
RSD	Relative standard deviation
RT	Retention time
SD	Standard deviation
SQRT PA	Square root of the peak area
S-S-PT system	System including the succession of a syringe pump, sample loop and purge and trap equipment.
TCD	Thermal conductivity detector

To my mother Maryvonne,

Nature is a universal and reliable teacher
for the one who observes it.

La nature est un professeur universel et sûr
pour celui qui l'observe.

Carlo Goldoni 1707-1793

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Chapter 1 Introduction

This thesis is concerned with the production of dimethylsulphoniopropionate (DMSP) and dimethylsulphide (DMS) by dinoflagellates. This fascinating group of phytoplankton is considered to be highly significant for the production of DMS in marine systems, but the database behind this is scant and dispersed. In this introductory chapter, I will start by discussing the relevance of DMS for the sulphur cycle, the chemical reactions that determine the fate of DMS in the atmosphere, the impact of this atmospheric DMS on climate and the current data on the global distribution of DMS. Then, I will consider the biological origin of DMS, the precursor DMSP, its potential biological functions, its fate in the marine environment including conversion to DMS. Subsequently I will introduce the reader to dinoflagellates and review their main characteristics including aspects that have attracted research interest. Finally, I will present the objectives of this study.

1.1. Dimethylsulphide - DMS

1.1.1. DMS and the sulphur cycle

DMS is a biogenic sulphur gas present in aquatic systems, able to volatilise to the atmosphere and contributing to the sulphurous smell of the sea and sometimes products of the sea (chemical structure detailed in Figure 1.1). It is distinct from the smell of stagnant waters in ponds and lagoons which is often attributable to hydrogen sulphide (H_2S). As reported by Paul Haas, Prof. Challenger was the first to propose methyl sulphide as the odoriferous compound released by seaweeds and Haas identified

this compound in *Polysiphonia fastigiata*, a red marine macroalga, in 1935 (Haas, 1935). This unattractive smell may also be released from shellfish and fish, causing a symptom known as blackberry feed, reducing fish quality and resulting in financial loss (Levasseur et al., 1994).

Figure 1.1 Structural formula of DMS. (Left) 2D and (right) 3D representations (Wikipedia contributors, 2010).

Interest in DMS has grown rapidly since it was suggested (Lovelock et al., 1972) that this gas was responsible for the main transfer of reduced sulphur from the ocean to the atmosphere. Many other authors have since provided further evidence for this role of DMS (Cline and Bates, ; Nguyen et al., 1978; Barnard et al., 1982; Andreae and Raemdonck, 1983; Nguyen et al., 1983). Previously it had been assumed that H_2S balanced the global sulphur budget (Kellogg et al., 1972).

DMS is the major volatile sulphur compound in the ocean. It is more abundant than the other volatile sulphur compounds which are carbonyl sulphide (usually abbreviated COS), carbon disulphide (CS_2) and H_2S. Based on flux estimations and modelling studies, 24 % (Bates et al., 1992) to 30 % (Simo, 2001) of the global sulphur emission to the atmosphere (detailed in Table 1.1) results from natural inputs including marine algae and seaweeds, land vegetation and volcanoes. As DMS is estimated to represent 21 % (Simo, 2001) of the global sulphur flux, it is the major natural sulphur input. This DMS flux represents a significant input in the global sulphur cycle (detailed in Figure 1.2) of 15 $\times 10^{12}$ to 33 $\times 10^{12}$ g S yr^{-1} (Kettle and Andreae, 2000) and accounts for more than 38 % of the global sulphate burden of the atmosphere (Simo, 2001). For comparison anthropogenic emissions were estimated to generate a flux between 55 and 68 $\times 10^{12}$ g S yr^{-1} in 2000 (Stern, 2005).

Table 1.1 Global sulphur emissions and sources (Bates et al., 1992; Malin, 1996). Anthropogenic emissions include biomass burning. Natural emissions include biogenic (marine and land) and volcanic sources.

	G moles S yr^{-1}	Anthropogenic %	Natural %	Marine %	Land %	Volcanic %
Northern hemisphere	2655	84.3	15.7	7.5	0.3	7.9
Southern hemisphere	638	42.5	57.5	43.9	0.6	13
Global	3293	76.2	23.8	14.6	0.3	8.9

Sulphur is a vital element required by all organisms. It is found in the amino acids methionine, cysteine and homocysteine and all the polypeptides, proteins and enzymes that contain them (Giordano et al., 2005). Cysteine and methionine are the precursors of all biological sulphur compounds and cysteine, which contains a reactive sulphydryl group (-SH), forms disulphide bridges that are critical for the tri-dimensional structure of proteins. Sulphur is also a constituent of a variety of compounds including vitamins (B1, B7), polysaccharides, sulpholipids, the antioxidant compound glutathione and its derivatives, various hormones (steroids), the bile constituent taurine and some bacterial transfer-ribonucleic acid (Jocelyn, 1972). Sulphur is present in cofactors such as coenzyme A and iron-sulphur clusters. Iron-sulphur clusters are essential cofactors acting as electron donors or acceptors in many biological reactions including photosystem I and mitochondrial respiratory complexes, acting as enzyme catalyses and as a sensor of intra- and extra-cellular conditions for regulation of gene expression (Lill, 2009). Sulphur is also a constituent of many secondary compounds involved in plant defence such as glucosinolates, alliins and thiopenes (Dahl et al., 2006). In some algae, most cellular sulphur may be accumulated in the metabolite dimethylsulphoniopropionate (Matrai and Keller, 1994).

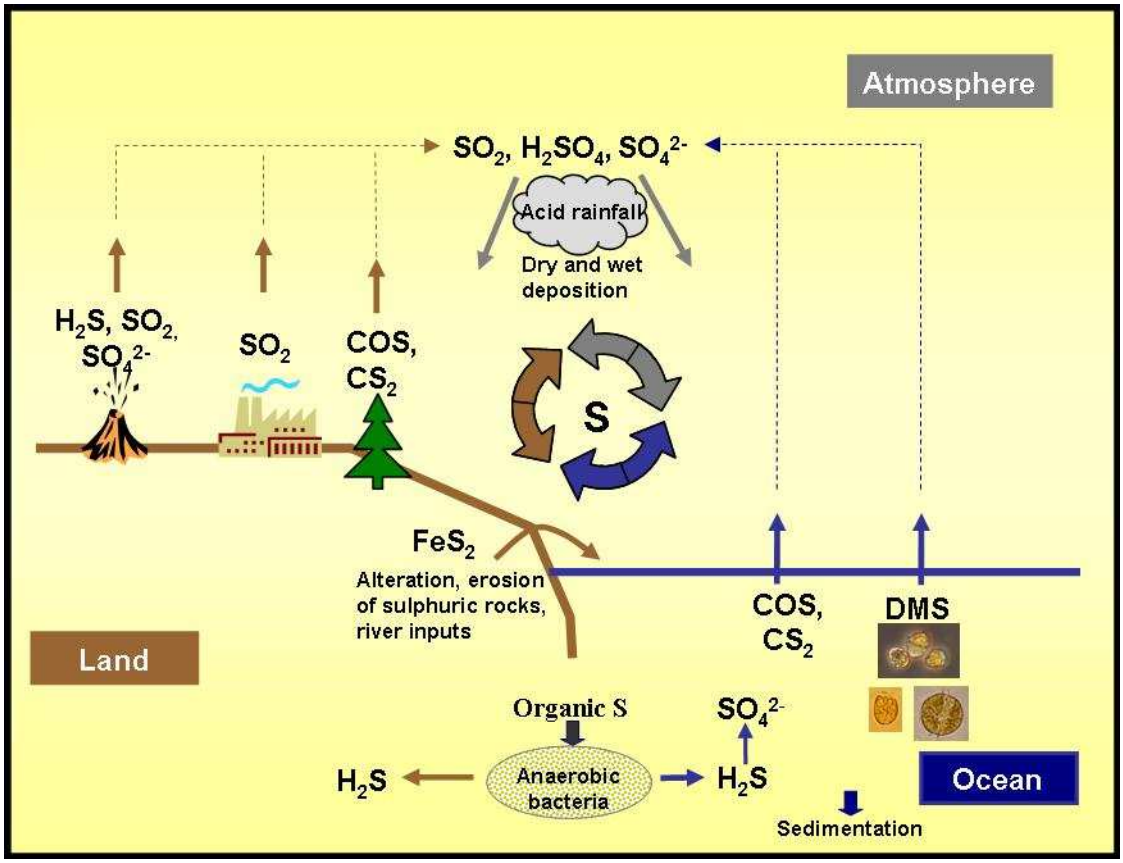

Figure 1.2 A schematic of the global biogeochemical sulphur cycle. S was initially present in sedimentary rocks, weathered and dissolved in the ocean as SO_4^{2-}. Marine organisms assimilate S into sulphur-containing amino-acids, incorporate sulphur into a range of organic cell components and release it as DMS, COS, and CS_2. Other S compounds enter the cycle from land-based natural and anthropogenic sources. All these compounds are oxidised in the atmosphere and return to the earth surface by wet or dry deposition. Anaerobic bacteria mineralise the organic sulphur compounds.

1.1.2. Atmospheric chemistry

Following emission to the troposphere, DMS has a short residence time of 20 to 28 h because its rapid oxidation is promoted principally by hydroxyl radicals (OH) during the day and nitrate radicals (NO_3) at night (Koga and Tanaka, 1993). OH is also called "the detergent of the troposphere" as this major oxidiser makes many trace components water-soluble and they are subsequently rained out of the atmosphere (Comes, 1994). At night, and especially in polluted areas, NO_3 becomes the dominant oxidiser and acts similarly to OH (Winer et al., 1984).

DMS is oxidized by OH, NO_3, BrO and Cl to form, via two main pathways, sulphate (SO_4^{2-}) and methylsulphonic acid (MSA) as shown in Figure 1.3. DMS may react with OH or BrO to add an O atom (addition pathway) and form dimethylsulphoxide (DMSO) which produces dimethylsulphone ($DMSO_2$), methylsulphinic acid (MSIA) and MSA. All these products may be taken up by particles especially if clouds are present, limiting the occurrence of the whole pathway. MSIA may also lead to the production of sulphur dioxide (SO_2) and join the other pathway. DMS may react with OH, NO_3 or Cl to abstract an H atom (abstraction pathway) and forming after successive reactions, SO_2 and sulphuric acid (H_2SO_4). Only sulphuric acid leads to the formation of new aerosol particles which increase the number of cloud condensation nuclei (CCN); the other particulate sulphur products condense on and enlarge existing particles that increase the size and the hygroscopicity of CCN. Depending on which pathway is favoured by the conditions of temperature and cloud presence, an increase in CCN number would increase the albedo whereas an increase in CCN size would reduce the albedo (von Glasow and Crutzen, 2004).

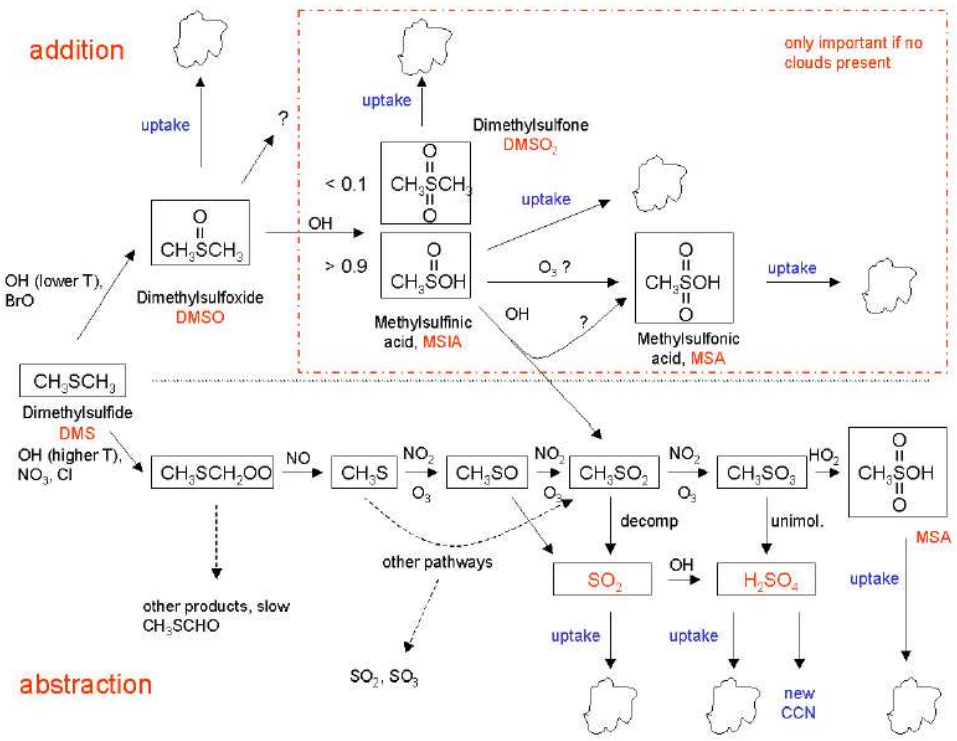

Figure 1.3 Schematic of the atmospheric oxidation pathways of DMS in the marine boundary layer. From von Glasow and Crutzen 2004. Irregular shapes represent clouds or particles that can uptake sulphur compounds.

1.1.3. Impact on climate

The oxidation of atmospheric DMS leads to the formation of sulphate aerosols (SO_4^{2-}) which influence climate. Sulphate aerosol particles alter the Earth's radiation budget by absorbing and refracting solar radiation (Shaw, 1983). Moreover sulphate aerosols can act as cloud condensation nuclei (CCN) for water droplet aggregation and contribute to the formation of cloud over remote oceans and these reflect additional solar radiation back into space (Easter and Hobbs, 1974; Charlson et al., 1987). These two processes directly and indirectly increase the planetary albedo and consequently decrease light irradiance and temperature on the Earth's surface. In addition, DMS-derived products such as MSA and H_2SO_4 contribute to the acidification of rain water. For instance, estimates based on measurements at Amsterdam Island in the Southern Indian Ocean suggested that DMS oxidation products are responsible for 40 % of the rain acidity (Nguyen et al., 1992).

The biological origin of DMS gives the organisms involved in its production a potential role in climate regulation. This theory was first introduced by Lovelock (1972) whilst describing the "Gaïa hypothesis" in which biosphere interacts with and alters its environment. Then Shaw (1983) emphasised this idea by invoking the sulphur cycle as a mean whereby organisms can influence atmosphere. Subsequently, the "CLAW hypothesis" proposed by Charlson, Lovelock, Andreae and Warren (1987) pointed out DMS as the biogenic sulphur gas able to influence climate. This famous hypothesis goes even further by postulating a feedback control of climate on organisms. The CLAW loop (illustrated in Figure 1.4) includes (1) a positive effect of solar radiation on temperature and marine phytoplankton growth, (2) phytoplankton produce DMS which is emitted to the atmosphere leading to formation of sulphate aerosols and clouds reducing the solar radiation, (3) this final negative effect would reduce the temperature and lead to the decrease in phytoplankton growth.

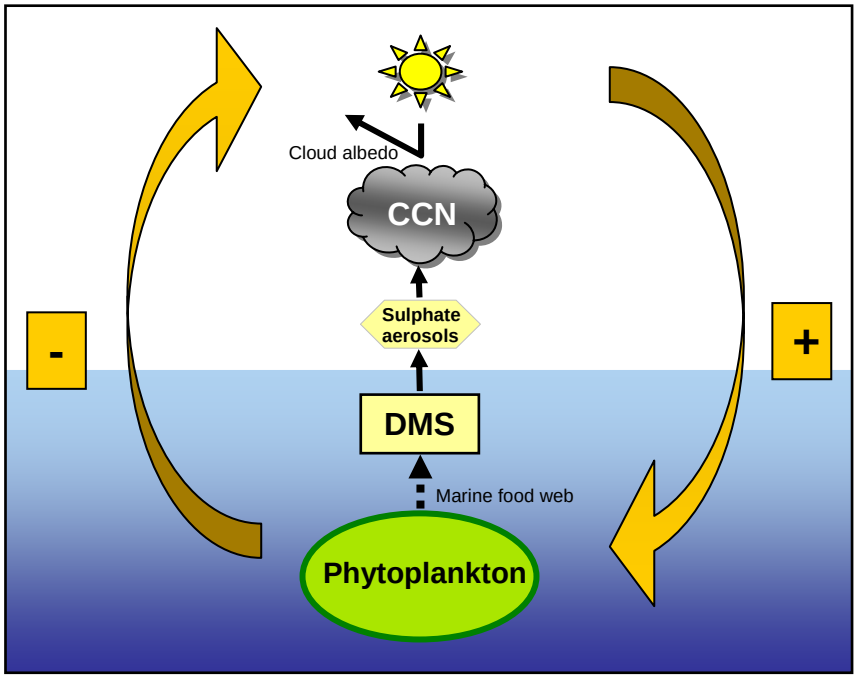

Figure 1.4 Scheme for the "CLAW hypothesis" which describes a potential feedback control of phytoplankton on climate through DMS emissions.

More than twenty years after the publication of the "CLAW hypothesis", research to date has confirmed some connections within the CLAW loop but only at a regional level (Ayers and Cainey, 2007). Many studies have established a connection between oceanic phytoplankton and DMS emissions though this linkage is generally complex being dependant on phytoplankton physiology and the food web (Archer,

2007). There is evidence that oxidation products of DMS regulate the formation of cloud condensation nuclei over the ocean (Raes, 1995; Cainey and Harvey, 2002) and several models seem to support the negative feedback of cooling climate through increased DMS emissions (Bopp et al., 2003; Gunson et al., 2006; Cropp et al., 2007). The elucidation of the CLAW hypothesis has been further complicated by results suggesting that volatile iodinated compounds that are also produced biogenically also lead to aerosols and CCN production (Whitehead et al., 2010).

The current warming effect of the climate (I.P.C.C., 2007) on DMS emissions is unclear. According to the "CLAW hypothesis" the temperature increase would enhance phytoplankton growth and DMS emissions would potentially counteract the warming effect. This theory is supported by the results of the model of Vallina et al. (2007) which showed DMS increase in conditions of global warming. However, the warming climate might also generate a positive feedback with less algal biomass due to intensified water stratification and consequently less DMS emissions (Lovelock, 1997).

1.1.4. Distribution

DMS is found in the atmosphere and marine and freshwater systems. In the oceans DMS is ubiquitous and concentrations are highly variable in spatial and seasonal terms. Coastal waters with high levels of biological activity tend to have higher DMS concentrations than open ocean waters, and surface waters hold more DMS than deeper waters. Mapped sea surface DMS measurements from numerous cruises displays the highest concentration values in coastal North Atlantic, North Pacific and Southern Oceans including Ross Sea, Drake Passage and the South Indian Ocean (Kettle et al., 1999; NOAA-PMEL, 2004). For instance, DMS values can reach 94 nM in the North Atlantic (Malin et al., 1993), 290 nM in the Southern Ocean (Gibson et al., 1990) and similar concentration have been observed in Maizuru Bay in Japan Sea (Uchida et al., 1992a). Exceptional DMS concentrations of 18665 nM have been observed in coral mucus (Broadbent and Jones, 2004). Lowest coverage of the map is found in the Indian

and South Pacific Oceans. High DMS concentrations also occur in upwelling areas (North Africa, Peru, Angola and the Equatorial Pacific Ocean).

In seawater, the global vertical pattern of DMS is characterised by a maximum concentration at or just below the sea-surface and a sharp decrease with depth through the euphotic zone (Andreae and Barnard, 1984). Below the light penetration limit (<1% of sea surface light) very low DMS concentrations can be measured. In the Atlantic Ocean below 250 m a range of 0.02 to 0.31 nM (0.8 - 10 ng S L^{-1}) was measured by Andreae and Barnard in 1980 and 1981 whereas higher values from 1.8 to 8 nM (58 - 257 ng S L^{-1}, Andreae and Barnard, 1984) were found in surface waters. Due to its biological origin, the vertical distribution of DMS tends to follow that of primary production. DMS is significantly correlated to chlorophyll *a* (Chl *a*) in its vertical pattern but Chl *a* maximum is often not clearly associated with DMS maximum. Sea surface DMS concentrations show a diurnal variation in which DMS increases in the day probably due to the diurnal behaviour of phytoplankton (Andreae and Barnard, 1984).

Seawater DMS concentrations fluctuate following a seasonal cycle at mid and high latitudes, with summer maximum and winter minimum in both the northern and the southern hemispheres (Turner et al., 1988; Leck et al., 1990; Kettle et al., 1999). Both hemispheres display a similar DMS annual cycle with a six-month interval. For example, Turner et al. (1988) measured DMS in seawater around the United Kingdom and found a mean concentration of 6.9 nM (220 ng S L^{-1}) in the summer and 0.1 nM (4 ng S L^{-1}) in the winter. Seasonal cycles also appeared to occur in Antarctic waters with higher DMS concentrations in the summer season (Turner et al., 1995).

The seasonal variation in DMS concentrations generally follows the seasonal phytoplankton bloom but it remains difficult to establish correlation between Chl *a* and DMS field measurements (Kettle et al., 1999). On one hand, this correlation occurs during bloom events, for example: Uchida et al. (1992a) obtained good correlation between DMS and Chl *a* concentrations in a dinoflagellate bloom in Japanese waters ($r>0.9$); Smith and DiTullio (1995) found a significant correlation in diatom-dominated Antarctic waters and similar observations have resulted from many field studies (Locarnini et al., 1998; Walker et al., 2000; Yang et al., 2005; Yang et al., 2006). On the

other hand, many other field studies are lacking this correlation, for example: Holligan et al (1987) found no correlation in surface waters of the English Channel and similar results were reported by Uchida et al. (1992b), Watanabe et al. (1995), Townsend and Keller (1996), Locarnini et al. (1998) and Uher et al. (2000). These divergent observations may result from the indirect relationship between DMS concentration and phytoplankton biomass (Watanabe et al., 1995) due to the effect of phytoplankton speciation and physiology on DMS production (Andreae and Raemdonck, 1983). For example a good correlation may be obtained where the phytoplankton is dominated by a strong DMSP producer but not where there is a mixed phytoplankton population. Also, cruises that cover many different hydrographic regions tend to obtain low correlation.

1.2. Dimethylsulphoniopropionate - DMSP

In this section I will discuss how the DMS is produced starting with the biosynthesis of DMSP in algae, the functional reasons for its production and the transformation of DMSP to DMS in the ocean.

1.2.1. Precursor of DMS

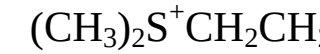

Figure 1.5 The structural formula for DMSP

DMS comes from the breakdown of dimethylsulphoniopropionate (DMSP, previously named dimethyl-β-propiothetin, Figure 1.5) produced by algae and a few higher plants (Challenger and Simpson, 1948; Challenger, 1951; Cantoni and Anderson, 1956). *Polysiphonia fastigiata*, a red macroalga, was the first marine alga investigated as a DMS emitter (Haas, 1935) and DMSP producer (Challenger, 1951). DMSP may be cleaved by the DMSP lyase enzyme which releases DMS, acrylate and a proton (Figure 1.6). Such isozymes can be found in micro- and macro-algae, bacteria and fungi (Steinke et al., 1996; Bacic et al., 1998; Yoch, 2002; Todd et al., 2009). Recently a new DMS-producing enzyme was identified that does not lead to acrylate production. The inferred pathway involved the modification of DMSP with acyl coenzyme-A (Todd et al., 2009).

Figure 1.6 Cleavage reaction of DMSP in DMS and acrylate by DMSP lyases.

The ability to synthesise this DMS precursor is restricted to certain groups of macroalgae and microalgae. In 1989, Keller described a generalised, “rule of thumb”

relationship between the DMSP content and the taxonomic position of these DMSP producers (Keller et al., 1989a). The main producers can be divided into two groups depending on their pigment content: (1) chlorophyll *a* and *c* species including dinophyceae, prymnesiophyceae, and chromophyte algae, (2) chlorophyll *a* and *b* species only represented by the prasinophytes. Macroalgae exhibit the opposite pattern: chlorophyll *a* and *b* species are the main producers (Malin and Kirst, 1997). This relationship between DMSP producers and typical pigment groups suggests a possible link between the plastid type and the ability for DMSP synthesis. Moreover, studies on the DMSP synthesis pathway have suggested that the chloroplast is involved in DMSP synthesis in higher plants (Trossat et al., 1996; Stefels, 2000).

DMSP is a zwitterion (ion with both positive and negative charge) that is soluble at high concentration and unable to permeate through membranes. Thus, it requires a specific transporter (Groene, 1995; Kiene et al., 1998).

1.2.2. Biosynthesis pathways of DMSP

The synthesis pathway of DMSP has been established for 2 land plants and 1 marine macroalga (Stefels, 2000). The pathway has not been elucidated for microalgae, although a theoretical pathway was proposed for the heterotrophic dinoflagellate *Crypthecodinium cohnii* (Uchida et al., 1996). For the 2 land plants, *Wollastonia biflora* (Compositae) and *Spartina alterniflora* (Gramineae), two different pathways have been established from methionine. Both include the production of S-methylmethionine in the cytosol which is then transported to the chloroplast (Trossat et al., 1996) where the subsequent DMSP synthesis reactions take place.

For marine algae, the DMSP pathway has been determined from experiments with the green macroalgae *Enteromorpha intestinalis* (Gage et al., 1997). This pathway involves the formation of the intermediate 2-oxo acid 4-methylthio-2-oxobutyrate (MTOB), its reduction to 4-methylthio-2-hydroxybutyrate (MTHB), methylation to produce 4-dimethylsulphonio-2-hydroxybutyrate (DMSHB), and finally oxidation of DMSHB to DMSP. DMSHB has also been found in several groups of microalgae: the

prasinophyte *Tetraselmis*, the prymnesiophyte *Emiliana huxleyi* and the diatom *Melosira nummuloides* (Gage et al., 1997). As they are also able to convert DMSHB into DMSP, they seem to use the same pathway as *E. intestinalis*. In contrast, the pathway proposed for the heterotrophic dinoflagellate *Crypthecodinium cohnii* has methylthiopropylamine (MTPA) and methylmercaptopropionic acid (MMPPA) as intermediates (Uchida et al., 1996). The cellular compartmentalisation of DMSP biosynthesis in marine algae is still unknown. Figure 1.7 illustrates these pathways which start with the precursor methionine.

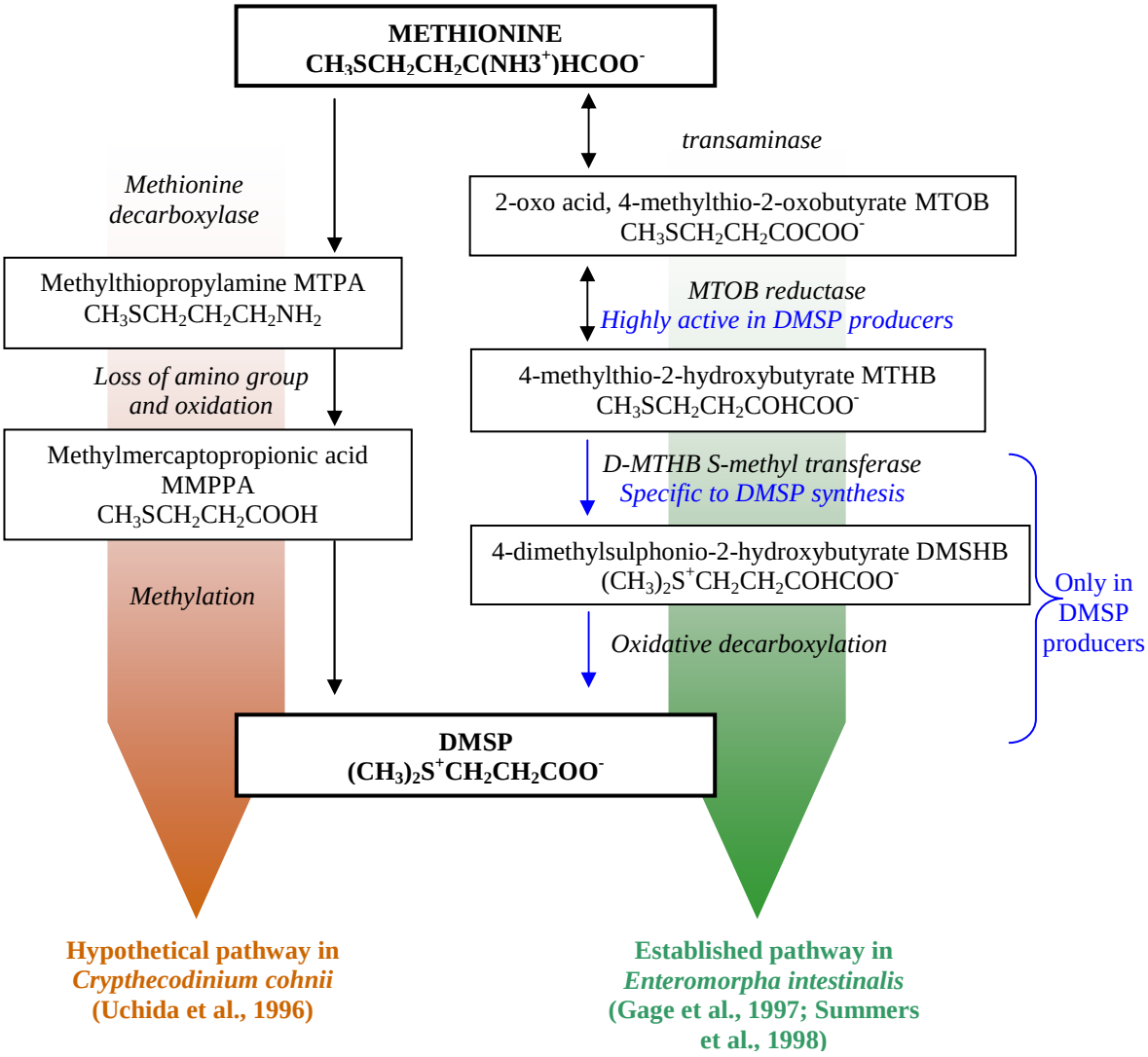

Figure 1.7 Biosynthesis pathway of DMSP in marine algae. (Left) Theoretical pathway in the heterotrophic dinoflagellate *Crypthecodinium cohnii*, (Right) Established pathway in the green macroalgae *Enteromorpha intestinalis*.

The plastid organelle has a central role in sulphur assimilation and organic sulphur production (illustrated in Figure 1.8). As observed in the chlorophyte *Chlamydomonas reinhardtii*, sulphate is imported into the cell by membrane sulphate transporters and then transported to the plastids. It is transformed to 5'adenyl sulphate (APS) and then in turn to sulphide, sulphite and cysteine. Cysteine is the precursor of methionine and all other cellular sulphur compounds (Giordano et al., 2005). In higher plants, cysteine is also synthesised in the cystosol and in the mitochondria and methionine may be synthesised in the plastid and in the cytosol (Wirtz and Droux, 2005). DMSP seems to be synthesised in the chloroplast of higher plants (Trossat et al., 1996) and whilst no evidence has been reported for algal cells, the possible DMSP synthesis in the plastid would require the synthesis or the transport of its precursor methionine into the plastid.

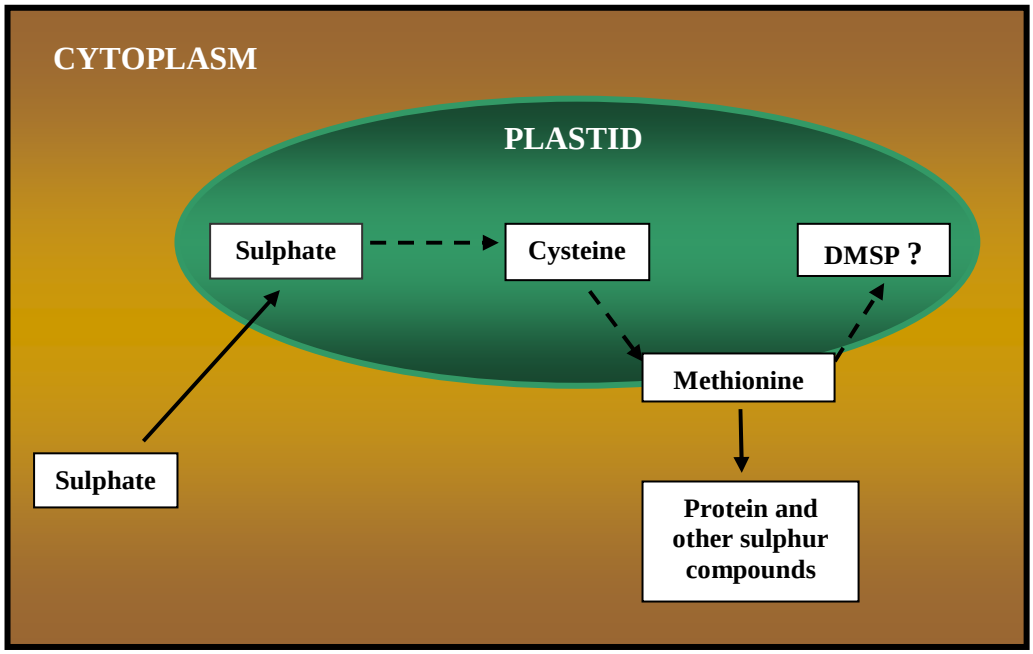

Figure 1.8 Central role of the plastid in sulphur metabolism in a schematic algal cell represented by the outer black box.

1.2.3. Biological functions of DMSP

DMSP is described as a multifunctional compound with many potential roles but no universal one. At a cellular scale, it may be an osmolyte, a cryoprotectant, an antioxidant, an overflow metabolite for sulphur excess, a methyl donor in metabolic reactions and the precursor of a compound involved in a circadian rhythm. At the ecosystem scale, it may be used as a defence chemical against grazers and can also be involved in signalling food location for higher trophic level predators. These functions are described in more detail below.

DMSP is generally recognised as having a primary function as an osmolyte, osmoprotectant or osmoregulator, which means a compound used by cells to regulate osmotic pressure and control turgor in response to salinity fluctuation. This osmoregulation function has been observed in green macroalgae (Dickson et al., 1980) and microalgae such as haptophytes (Vairavamurthy et al., 1985) and prasinophytes (Dickson and Kirst, 1986). DMSP is a tertiary sulphonium compound and it is often compared to glycine betaine which is a quaternary ammonium compound used as an osmolyte by some algae including prasinophytes, cyanobacteria, bacteria and terrestrial plants including salt marsh plants (Dickson and Kirst, 1986; Mulholland and Otte, 2000; Stefels, 2000; Welsh, 2000). Algal cells may contain a variety of osmolyte compounds including ions and organic solutes (Kirst, 1989). DMSP can act as an organic osmolyte for marine algae, but it is not present in all marine algae (Keller et al., 1989a). It is also found in a few freshwater species including for instance, *Peridinium gatunense* (Ginzburg et al., 1998) and *Peridinium bipes* (Uchida et al., 1992b).

DMSP is additionally considered as a compatible solute that has no enzyme inhibitor properties when accumulated in the cell or that gives a protective role for enzyme activity against changes in ionic strength due to salinity or low-temperature stresses (Brown, 1976; Gröne and Kirst, 1991). With its compatible properties, DMSP may act as an osmolyte and also as a cryoprotectant. Therefore, the accumulation of higher DMSP concentrations in polar algae, such as green macroalgae from Antarctica (*Ulothrix implexa, Ulothrix suflaccida, Enteromorpha bulbosa, Acrosiphonia arcta*), when grown at 0 º C and in comparison with algae grown at 10º C supports a potential

role of cryoprotectant for DMSP (Karsten et al., 1992). The prymnesiophyte *Phaeocystis pouchetii* was associated with high DMSP measurements in the Weddell Sea, Antarctica (Kirst et al., 1991). A cryoprotectant may protect proteins against damage from freeze-thawing or may help in maintaining a liquid cytoplasm at temperatures below 0º C but this has not been proven for DMSP yet (Carpenter and Crowe, 1988; Kirst et al., 1991).

It has also been suggested that DMSP can act as an antioxidant (Sunda et al., 2002). All aerobic organisms suffer oxidative stress when the production of reactive oxygen species (ROS: superoxide radical $^{\cdot}O_2^-$, singlet oxygen $^1O_2$, hydrogen peroxide H_2O_2 and hydroxyl radical $^{\cdot}OH$) exceeds the organism's ability to detoxify them and repair the damage they cause. The chloroplast is one cellular site of ROS production due to incomplete reduction of oxygen by cytochromes (Parent et al., 2008). The formation of ROS can cause severe cellular damage such as DNA mutation, DNA breakage, enzyme inactivation and membrane destabilization by lipid scavenging (Lesser, 2006). Cellular defence systems ensure limitation of free radical formation. Antioxidant enzymes such as superoxide dismutases, catalases and peroxidases catalyse ROS reduction to stable species such as H_2O and O_2. Antioxidant compounds such as ascorbic acid, glutathione, tocopherols and carotenoids are also able to quench ROS. Additionally, Sunda et al. (2002) showed that DMSP accumulates under a variety of oxidative stressors such as limitation of Fe, CO_2, UV radiation, H_2O_2 and high concentrations of copper. They hypothesised that DMSP, DMSO, and more efficiently, DMS and acrylate are reactive with $^{\cdot}OH$ and also that DMS is able to diffuse through lipids including photosynthetic membranes to act as an antioxidant. Bucciarelli and Sunda (2003) also observed an increase in intracellular DMSP concentrations of nutrient-limited cultures of diatoms that could be due to an oxidative stress induced by nutrient limitation. Several studies reported an increase in DMSP concentrations in some Antarctic macro- and microalgae exposed to high light conditions (Karsten et al., 1990; Karsten et al., 1992; Stefels and van Leeuwe, 1998) which can cause oxidative stress for photosynthetic organisms (Hideg et al., 2002; Asada, 2006).

DMSP is potentially released as an overflow metabolite for excess energy, excess sulphate and carbon (Stefels, 2000). DMSP would be involved in the nitrate/sulphate balance, both required for protein synthesis. In conditions of nitrogen

deficiency, excess cysteine (precursor of proteins and methionine) could be converted in DMSP and excreted as an overflow mechanism. The excretion of DMSP into the external medium would in theory maintain a constant intracellular level of cysteine and methionine similar to the excretion of glutathione and hydrogen sulphide in higher plants (Rennenberg, 1989; Stefels, 2000). Several studies report the accumulation of DMSP in response to N limitation as expected by this hypothesis (Turner et al., 1988; Gröne and Kirst, 1992). Stefels (2000) also suggested that DMSP production could be involved in protein turnover by re-distributing the N released from methionine during DMSP synthesis to other amino acids. The protein turnover mechanism seems essential in plants to adapt to changing conditions of the environment (Stefels, 2000).

Two earlier studies (Maw and Duvigneaud, 1948; Ishida and Kadota, 1968) proposed that DMSP might be used as methyl donor in metabolic reactions. They show that DMSP may transfer its methyl group to homocysteine and may produce methionine and methylthiopropionic acid. In that case, DMSP would recycle its precursor methionine. No other studies have further investigated this potential function.

DMSP is also involved in the circadian rhythm of the photosynthetic dinoflagellate *Lingulodinium polyedrum* (previously named *Gonyaulax polyedra*) in that it is the precursor of gonyauline, a substance that shortens the circadian clock period (Nakamura, 1990; Roenneberg et al., 1991; Nakamura et al., 1997). Roenneberg et al (1991) observed a shortening of the period of the circadian rhythm of bioluminescence under increased concentrations of gonyauline. Many biological functions including photosynthesis, cell division, bioluminescence and migration are dependant on circadian rhythms that are maintained by light sensors and chemicals. However, to date, gonyauline has only been found in *L. polyedrum*.

DMSP and its by-products have revealed biochemical properties whereby they can act as infochemicals in trophic relationships (Steinke et al., 2002b). Acrylate and DMS have been proposed to act as deterrents against the dinoflagellate *Oxyrrhis marina* when it grazes on *Emiliania huxleyi* (Wolfe et al., 1997) and thus as an algal defence mechanism. Microzooplankton grazers selectively ingested *E. huxleyi* strains with low-DMSP lyase activity suggesting that the concentrations of DMSP by-products played a role in the interaction. Thirdly, DMS may act as a cue in a tritrophic interaction (Steinke

et al., 2002b) whereby it is released by phytoplankton when they are grazed upon by microzooplankton in order to warn mesozooplankton about its prey location. Increasing predation on the microzooplankton would decrease the grazing pressure on phytoplankton and improve fitness of DMS producing strains. At higher trophic levels DMS may influence the migration behaviour of zooplankton to assist them in locating their prey and even provide a signal for areas of high biological activity for predators such as seabirds and marine mammals (Nevitt and Bonadonna, 2005; Bonadonna et al., 2006). Kowalewsky et al., (2006) showed that harbour seals have extraordinarily high olfactory sensitivity for DMS that could enable them to identify and move to profitable feeding grounds.

1.2.4. Fate of oceanic DMSP and DMS

From the biogenic synthesis of DMSP from phytoplankton to DMS emission to the air, there are many bio-physico-chemical processes that influence the transformation, assimilation and degradation of these compounds. Figure 1.9 illustrates these marine DMS source and sink processes and pathways. Moreover, the indirect connection between DMSP and DMS caused by the complexity of the microbial food web (phytoplankton, zooplankton, bacteria, fungi and viruses) explains the lack of correlation between Chl *a* and DMS data (mentioned in section 1.1.4) and complicates prediction of DMS emissions.

The DMSP released by algae in the ocean may be enzymatically cleaved to release DMS or assimilated by marine organisms. Particulate DMSP ($DMSP_p$) is released by algal cells through exudation or lysis due to senescence, grazing and viral infection (Yoch, 2002; Stefels et al., 2007). Dissolved DMSP ($DMSP_d$) becomes available for assimilation by other algal cells or organisms such as bacteria and fungi as a sulphur and/or carbon source (Bacic et al., 1998; Kiene et al., 1999; Zubkov et al., 2001; Van Bergeijk et al., 2003; Vila-Costa et al., 2006). It is also ingested by phytoplankton grazers with a portion usually being eliminated as $DMSP_d$ or through faecal pellet excretion and sedimentation.

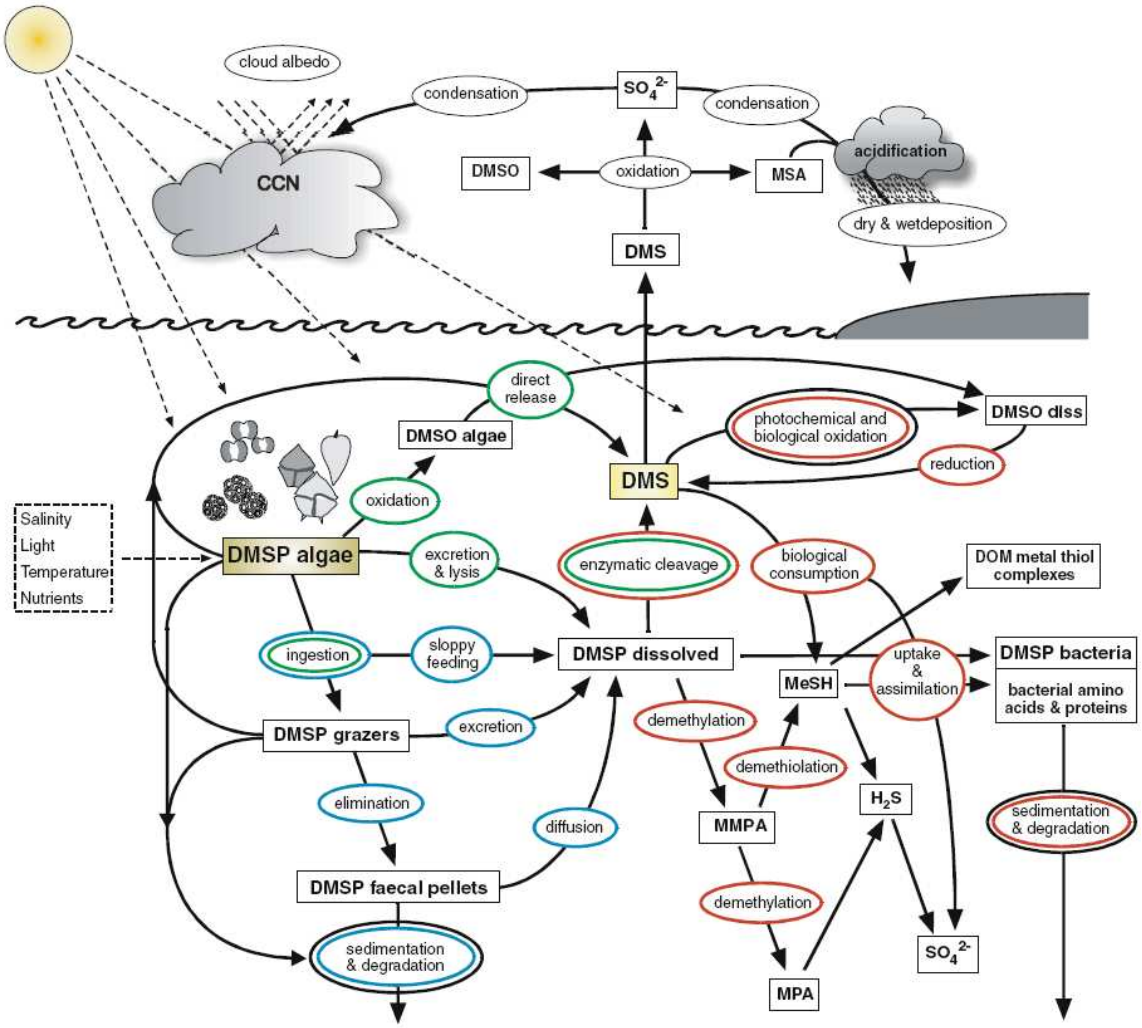

Figure 1.9 Schematic to show the evolution and cycling of DMSP and DMS in the oceanic and atmospheric environments (from Stefels et al. 2007). In the oceanic compartment, transformation processes are performed by biological groups: green for phytoplankton, blue for zooplankton and red for bacteria. The abiotic factors salinity, light temperature and nutrients affect algal physiology, growth and DMSP content. CCN: cloud condensation nuclei; MSA: methylsulphonic acid; SO_4^{2-}: sulphate; DMSO dimethylsulphoxide; DOM: dissolved organic matter; MeSH: methanethiol; H_2S: hydrogen sulphide; MMPA: 3-methiolpropionate; MPA: 3-mercaptopropionate.

$DMSP_d$ may be enzymatically cleaved to DMS by algal and bacterial lyases and then emitted to the atmosphere or oxidised to DMSO. DMS is a volatile compound that can transfer to the atmosphere. However, DMS may be oxidized to DMSO by photochemical or bacterial oxidation (Lee and de Mora, 1999). Del Valle et al. (2007), observed a biological DMSO production rate of 0.07-0.33 nM day^{-1} in the Sargasso Sea. Thus, this non-volatile compound represents an oceanic sink for DMS. DMSO may also be produced by microalgae and then released into the environment as dissolved DMSO (Simo et al., 1998; Hatton and Wilson, 2007). In that case, DMSO could be produced from intracellular oxidation of DMS or DMSP (Hatton et al., 2005). Reduction of DMSO to DMS also exists in some aerobic and anaerobic bacteria and some cyanobacteria (Lee and de Mora, 1999).

The bacterial consumption of DMSP is a DMS source and sink. Various bacteria consume DMSP: free-living bacteria in waters and in sediments, epiphytes or in the "phycosphere" that surrounds algal cells, attached to zooplankton or associated with marine animal guts (Yoch, 2002). The degradation of DMSP is either a cleavage to DMS and acrylate that both may be subsequently degraded or either sequentially demethylated to 3-methiolpropionate (MMPA) and 3-mercaptopropionate (3-MPA); (Taylor and Gilchrist, 1991) or MMPA can be demethiolated to methanethiol (MeSH); (Kiene and Visscher, 1987; Kiene and Taylor, 1988; Kiene, 1996). Kiene et al (1999) showed that the whole range of 13 bacterial strains in culture as well as field samples tested for MeSH utilisation was incorporating 49 to 100% of the added MeSH suggesting that this DMSP degradation product is a major sulphur source for marine bacteria. Four genes involved in the bacterial degradation of DMSP have recently been identified: *dmdA* encoding for a DMSP demethylase (Howard et al., 2006), *dddL* encoding for an enzyme that produce DMS and acrylate from DMSP (Johnston et al., 2008), *dddD* which encodes for an enzyme that degrades DMSP by addition of a coenzyme A releasing DMS and acryloyl CoA (Todd et al., 2007) and *dddP* encoding for another DMS-producing enzyme (Todd et al., 2009).

1.3. Dinoflagellates

In this section, I present the main features of the dinoflagellate group including their classification, biology with anatomic and morphologic description, plastid types and origin, reproduction, distribution, ecology, toxicity, and their scientific applications in palaeogeology and the food and pharmaceutical industries, DMSP content and DMSP lyase activities (DLA) and the challenge of culturing dinoflagellates.

1.3.1. Generalities

Dinoflagellates are eukaryotic aquatic organisms of the plankton. They are also called Dinophyta, from "*dinos*" in greek meaning a whirling, rotation and referring to their characteristic movement (section 1.3.3.). They were previously known as Pyrrhophyta from "*pyrrhos*" meaning fire due to their bioluminescent properties. The dinoflagellate group has more than 2000 living species and the fossil records suggest that there were as many as 4500 species in the past (Gomez, 2005; Taylor et al., 2008). The dinoflagellates already existed at the Triassic (about 240 million years ago) as evidenced by relict resting cells with carbonaceous walls. These walls contain a diagnostic and resistant constituent, dinosporin, which keeps the cell well preserved in marine sediments and allows their identification (Falkowski et al., 2004). In the ocean today, dinoflagellates are generally microscopic with a cell size between 20 and 200 μm (Taylor and Pollingher, 1987) though the diversity of dinoflagellates ranges in cell size from picoplanktonic species < 3 μm (Lopez-Garcia et al., 2001; Moon-van der Staay et al., 2001; Lin et al., 2006) to 2 mm size mesoplanktonic cells like *Noctiluca sp* (Taylor and Pollingher, 1987). They are mostly unicellular but some rare species are filamentous (*Dinothrix sp., Dinoclonium sp*.) or able to form chains (*Alexandrium catenella*, *A. affine*, *A. monilatum*; Hsia et al., 2006).

Dinoflagellates are generally considered as algae but were previously segregated into phytoplankton and zooplankton by reason of their mixed trophic behaviour. Indeed,

all nutritional modes are represented within dinoflagellates. About 50 % of the group are autotrophic (using light, CO_2 and inorganic nutrients), others are heterotrophic (using organic nutrients) and some mixotrophic species can be both. The non-photosynthetic trophic modes are exemplified by species that can be osmotrophs, grazers, symbionts or parasites. For instance, most common corals are a symbiosis between a benthic Cnidaria and a dinoflagellate from the genus *Symbiodinium* (Goodson et al., 2001). Non-photosynthetic species that are predators have developed special organelles to capture their prey including the peduncle (for prey attachment), stomopod (injects lytic substances), a pseudopod extending in a feeding veil or pallium to envelop and digest the prey, tentacles and food vacuoles (Gaines and Taylor, 1984; Gaines and Elbrachter, 1987).

1.3.2. Classification

The traditional classification for dinoflagellates was established by Fensome and co-workers (1993). However, with constant progress in molecular techniques and phylogenetic methods the classification of dinoflagellates is under continuous revision. The website algaebase (Guiry and Guiry, 2009) gives updated classification of algae including dinoflagellates (http://www.algaebase.org/ consulted on the 29/06/2010). In this classification, the class of Dinophyceae belongs to the empire of Eukaryota, the kingdom of Protozoa, the subkingdom of Biciliata, the infrakingdom of Alveolata and the Myzozoa phylum. This class contains 18 orders as detailed in Table 1.2.

Table 1.2 Taxonomic Orders of the Class of Dinophyceae as described in Algaebase website (http://www.algaebase.org/ consulted on the 29/06/2010, Guiry and Guiri, 2009)

Order	Authority	Species
Actiniscales	Sournia	6
Blastodiniales	Chatton	16
Coccidiniales		11
Dinamoebales	Loeblich III	1
Dinophyceae incertae sedis		14
Dinophysiales	Kofoid	334
Gonyaulacales	F.J.R. Taylor	392
Gymnodiniales	Apstein	565
Lophodiniales	J.D. Dodge	14
Noctilucales	Haeckel	19
Oxyrrhinales	Cavalier-Smith	1
Peridiniales	Haeckel	669
Peridiniphycidae incertae sedis		3
Phytodiniales	T. Christensen	19
Prorocentrales	Lemmermann	75
Pyrocystales	Apstein	18
Suessiales	R.A. Fensome et al.	16
Thoracosphaerales	Tangen	12

1.3.3. Morphology

On the basis of external morphology there are 2 groups of dinoflagellates: the armoured and the naked dinoflagellates. The armoured or thecate dinoflagellates have polygonal cellulosic plates under the membrane, located either inside a unique or distributed into several vesicles which form the theca. These thecal plates give a rigid structure to the cell. The number and disposition of the plates is used for the morphologically-based classification. The naked or athecate dinoflagellates have some empty vesicles under the membrane. The outermost layers of the cell composed of the theca (the plasma membrane and subjacent vesicles, either empty or containing plates) and microtubules are collectively called the amphiesma.

Diverse cell shapes are seen in the dinoflagellates, they can be round, oval, polygonal or with elongated expansions like a fork or an anchor as illustrated in Figure 1.10. A motile dinoflagellate bears 2 dissimilar flagella, perpendicularly inserted into the flagella pore and other non-motile dinoflagellates are non-flagellated, ameboid,

coccoid, palmelloid, or filamentous (Barsanti and Gualtieri, 2006). For motile dinoflagellates, the longitudinal flagellum ensures propulsion and the transversal, helical flagellum allows rotation. The resulting motion is helical but directional. Dinoflagellates "swim" from 200 to 500 µm s^{-1} and are the fastest motile algae with the exception of *Mesodinium*, a symbiotic association of a ciliate and a cryptophyte (Raven and Richardson, 1984). Their mobility allows them to perform diel vertical migrations in relatively calm waters. This confers an advantage compared to non-motile species in that they might migrate to the water surface during the day in order to efficiently capture the light and then move down to 5 to 10 meters where nutrients may be more available at night (Raven and Richardson, 1984).

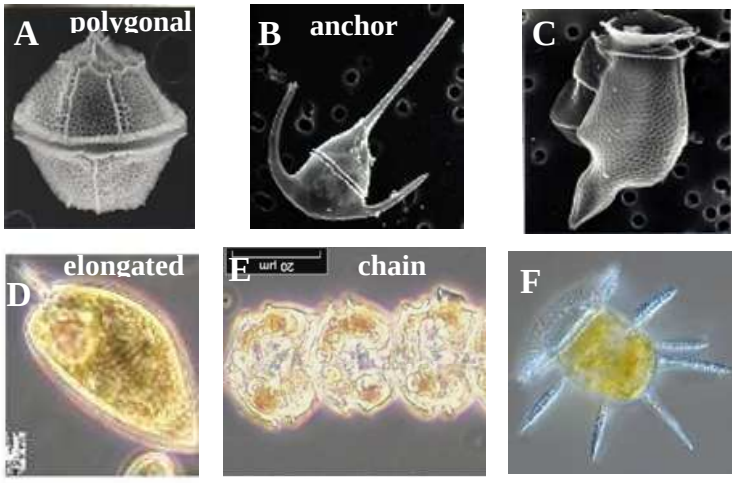

Figure 1.10 Examples of dinoflagellate cell shapes. Scanning electron micrographs of (A) *Lingulodinium polyedrum* (B) *Ceratium tripos* (C) *Dinophysis caudata* (Asma Benouna, Plankton*Net Data Provider). Optical microscopy photographs of (D) *Prorocentrum micans* (E) *Perdiniella catenella* (Regina Hansen and Susan Busch, Plankton*Net Data Provider) and (F) *Ceratocorys horrida* (Alexandra, Plankton*Net Data Provider).

Two basic cell shapes exist in the dinoflagellates: desmokont cells which are composed of 2 large plates and 2 dissimilar flagella inserted apically and dinokont cells with 2 dissimilar flagella inserted ventrally (as illustrated by Figure 1.11). A dinokont cell is divided into two parts, an epicone and a hypocone separated by a groove named the cingulum or girdle and can be thecate or athecate (Lee, 1999). This is illustrated on Figure 1.12.

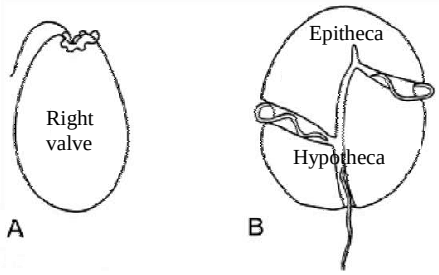

Figure 1.11 Cell morphologies (A) desmokont, (B) dinokont (Smithsonian Institution, 2010).

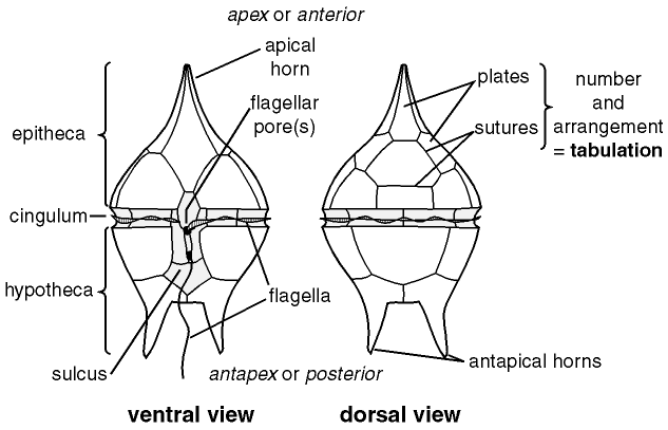

Figure 1.12 Detailed morphology of dinokont cells. (MacRae, 2010).

1.3.4. Anatomy

The molecular phylogeny of the dinoflagellates strongly confirms their eukaryotic position (de la Espina et al., 2005) but their atypical nucleus or dinokaryon (previously the mesocaryon) presents a unique genome organisation with some non-eukaryotic features. Dinoflagellates are haploid and the genome size is between 1.5 to 225 pg DNA cell^{-1} (LaJeunesse et al., 2005) which is a huge amount given that eukaryotes in general have a range of 0.04 to 3 pg DNA cell^{-1} and up to 40 pg DNA cell^{-1} in plants (de la Espina et al., 2005). Even the lowest values seen in the Genus *Symbiodinium* (1.5-4.8 pg DNA cell^{-1}) are roughly equivalent to the human genome (3.5 pg cell^{-1}; Gregory et al., 2007; www.genomesize.com) and DNA content is even higher in *Prorocentrum micans* and *Karenia mikimotoi* (LaJeunesse et al., 2005). On the other hand, the recently discovered picodinoflagellates have the potential to display smaller genomes (Lin, 2006). Such high levels of DNA can be explained by a redundancy up to

60 % of repetitive sequences (de la Espina et al., 2005) and by gene transfers from endosymbiont nuclei (see section 1.3.5). Dinoflagellate DNA has a high G + C ratio and exceptional bases such as 5-hydroxymethyluracil. Additionally, dinoflagellates are unique in the eukaryotic group because they have no DNA-associated histones and consequently the DNA is not packaged in nucleosomes in the chromosomes. They do have unusual basic histone-like proteins associated with the DNA, but these are more similar to bacterial DNA-binding proteins than eukaryotic histones. Dinoflagellate chromosomes are permanently condensed even during interphase. The number of chromosomes is highly variable between species from 4 to 8 in parasitic species and from 20 to 270 in free-living species. However, the mitotic cycle is mostly similar to eukaryotes except that the nuclear membrane does not split (de la Espina et al., 2005).

Photosynthetic dinoflagellates display a diverse range of 5 types of chloroplasts or plastids (detailed in section 1.3.5). Peridinin plastids are the most common in existant dinoflagellates but 4 other types have been identified: the prasinophyte-like plastid, haptophyte-like plastid, cryptophyte-like, and diatom-like plastids according to the affiliation of their plastidial endosymbiont. Pigment content differs between them (Table 1.3): chlorophylls *a* and *c2* occur in all of them except the prasinophyte-like plastid which contains chlorophyll *a* and *b*. Chlorophyll *c1* is additionally present in haptophyte-like and diatom-like plastids. The carotenoid group also differs with each plastid type containing representative carotenoids (listed in Table 1.3). Diatom-like plastids are differentiated by astaxanthin whilst fucoxanthin is the major carotenoid. The exceptional freshwater dinoflagellate *Tovellia sanguinea* contains a special carotenoid assemblage of a diatom-like plastid but with more astaxanthin than peridinin associated with dinoxanthin, diadinoxanthin and β -carotene (Frassanito et al., 2006). In general, a dinoflagellate species will hold only one kind of chloroplast, but there can be several of these generally distributed in the periphery of the cell or under the surface, or, occasionally plastids are aggregated in bands or on one side of the cell. Plastids usually have an individual or several internal pyrenoids which are centres for CO_2 fixation (de Salas et al., 2008).

Table 1.3 The pigment composition for dinoflagellates of different plastid types. The typical carotenoids for each group are shown in bold.

Plastid	Chlorophyll	Carotenoids
Peridinin	a, c2	β-carotene, **peridinin** diadinoxanthin, diathoxanthin, dinoxanthin (Lee, 1999)
Haptophyte-like	a, c1, c2	**19'-hexanoyloxyfucoxanthin** and or **19'-butanoyloxyfucoxanthin,** fucoxanthin, β,ε-carotene, β, β -carotene, diatoxanthin, diadinoxanthin, 19-hexanoyloxyparacentrone 3-acetate, in some species the diester of gyroxanthin (Hansen et al., 2000; Bjornland et al., 2003; de Salas et al., 2005).
Cryptophyte-like	a, c2	**Alloxanthin** (Meyer-Harms and Pollehne, 1998)
Diatom-like	a, c1, c2	Fucoxanthin, diadinoxanthin, diatoxanthin, an unidentified fucoxanthin-like xanthophyll, β -carotene, γ-carotene and **astaxanthin** (Withers et al., 1977).
Prasinophyte-like	a, b	**Prasinoxanthin**, β –carotene (Watanabe et al., 1987; Hackett et al., 2004a)

Dinoflagellate chloroplasts can be surrounded by several layers of membranes. These provide evidence of endosymbiotic events in that they result from phagocytosis, but the number of layers can also reduce with evolution's time. The endosymbiont components vary between plastid types depending on their evolutionary history (Table 1.4). The peridinin plastid is thought to have a red algal origin (Zhang et al., 1999; details in section 1.3.5). This plastid type is bounded by 3 membranes and its genetic information is concentrated on plasmid-like minicircles, each containing 0-3 genes and a total of genes up to 14 (Zhang et al., 1999; Howe et al., 2008). Koumandou and Howe (2007) observed in experiments with *Amphidinium operculatum* that whilst the copy number of minicircles can show little difference (up to 4-fold) between different minicircles at the same growth stage, the copy number of minicircles dramatically increases (1-2 orders of magnitude) during the latter stages of growth. The haptophyte-like plastid is also delimited by 3 membranes but no endosymbiont nucleus has been identified. The cryptophyte-like plastid is enclosed by 2 membranes and the nucleus

appears also absent. The diatom-like plastid is bordered by only 1 membrane and the nucleus and several organelles such as mitochondria, ribosomes, and even plastids of the endosymbiont are present suggesting a more recent endosymbiosis (Schnepf and Elbrachter, 1988; Hackett et al., 2003; Hackett et al., 2004a).

Table 1.4 Anatomic features of different plastid types among dinoflagellate species.

Plastid type	Membranes	Nucleus	Organelles
Peridinin	3	1-3 minicircle genes	
Haptophyte-like	3	absent	3 thylakoids (Steidinger, 1998)
Cryptophyte-like	2	absent	
Diatom-like	1	nucleus	Mitochondria, ribosomes, plastids
Prasinophyte-like	2	absent	Ribosomes

The dinoflagellate cytoplasm contains other organelles including mitochondria, Golgi apparatus, endoplasmic reticulum and atypical structures may be found in some species such as trichocysts, a pusule and an eyespot (Lee, 1999). The pusule is a saclike element that opens into the flagellar canal and is thought to act as an osmoregulator. The eyespot, which is responsible for phototaxis, is composed of lipid globules with or without a plastid remnant structure and occasionally has a more complex structure (Lee, 1999). The trichocysts are rod-shaped organelles assumed to excrete material for defence (Ukeles and Sweeney, 1969; Lee, 1999; Tillmann and Reckermann, 2002). Grains of starch and oil can be accumulated as metabolic storage in the cytoplasm (Dodge and Crawford, 1971; Lee, 1999).

1.3.5. Plastid evolution in plants and dinoflagellates

It is clear that different plastid types exist within terrestrial and marine plants. Given that the plastid is involved in sulphur metabolism and potentially involved in DMSP synthesis (as described in section 1.2.2) as well as photosynthesis, an organism's physiology might be strongly influenced by its plastid type. For these reasons, in this section I will describe plastid evolution in plants and dinoflagellates.

The insertion of the plastid in the eukaryotic lineage was the result of the phagocytosis of a prokaryotic alga without digestion and with its subsequent conservation as an endosymbiont (Keeling, 2004). In general, endosymbiotic events were infrequent through plant and algal evolution, but dinoflagellates are exceptional in their ability to gain, lose or replace their plastids (Saldarriaga et al., 2001). Some dinoflagellates are known to be kleptoplastidic (Lewitus et al., 1999; Gast et al., 2007) whereby they exhibit an intermediate stage between the endosymbiont and its full incorporation as a plastid. For this reason, dinoflagellates have attracted research attention as a key stage for understanding the acquisition of plastids in eukaryote evolution. The use of new molecular techniques has allowed comparison of the plastid and nuclear genomes of different organisms and the ongoing readjustment of the classification of dinoflagellates.

It is now established that the plastids present in the photosynthetic eukaryotes today came from a first endosymbiotic event between a eukaryote and a cyanobacterium-like cell (McFadden, 2001; Keeling, 2004). This ancestral alga gave rise to three further groups depending on their pigment diversification: glaucophytes, green algae and red algae (Illustrated by Figure 1.13). The glaucophyte group is still present nowadays (Bhattacharya and Schmidt, 1997). The green algal group, characterised by chlorophyll *a* and *b*, is at the origin of land plants and a secondary endosymbiosis event led to the green aquatic lineage. Depending on the eukaryotic host, different clades compose this lineage: euglenoids, chlorarachniophytes and dinoflagellates with a "green plastid" (Falkowski et al., 2004). The red algal group, defined with chlorophyll *a* and *c*, is at the origin of red macroalga and a second

endosymbiosis resulted in the red lineage which includes diatoms, haptophytes, cryptophytes, dinoflagellates and brown macroalgae. These endosymbiotic events have ensured the transfer of photosynthetic ability from prokaryotic to eukaryotic organisms, the spread of autotrophic behaviour and the expansion of aquatic and terrestrial primary producers.

Focusing on dinoflagellates, the red plastid or peridinin dinoflagellates are the most common today. A tertiary endosymbiotic event allowed the acquisition of other types of plastid (illustrated by Figure 1.13). Therefore, for example, the endosymbiosis of a haptophyte led to a dinoflagellate with a haptophyte-like plastid. The peridinin plastid from the previous endosymbiosis may be lost as in haptophyte-like plastid containing dinoflagellates or may be kept as in diatom-like plastid containing dinoflagellates. The origin of dinoflagellates with green or prasinophyte-like plastid was proposed by Keeling (2004) to have come from a serial secondary endosymbiosis after the loss of the red plastid and not from the green lineage.

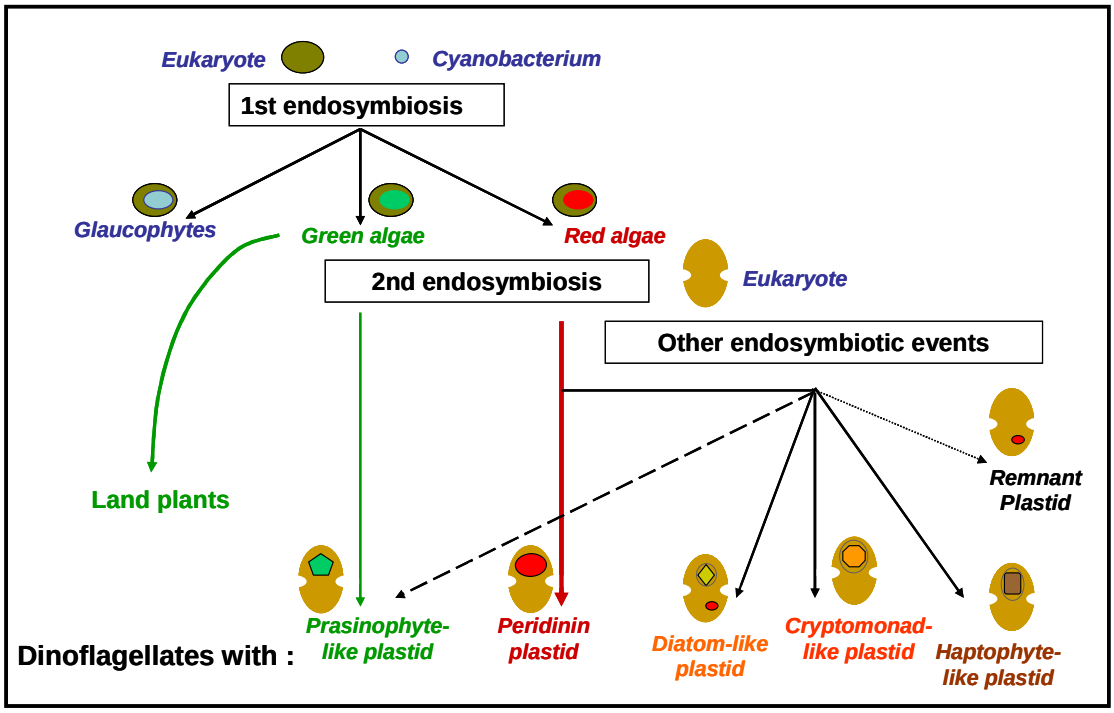

Figure 1.13 Plastid evolution and diversity in the dinoflagellate group. Illustration modified from Keeling (2004).

The identification of dinoflagellate species with non-peridinin plastids is recent and only about 20 such species are known - a low number in comparison with the estimated 1000 photosynthetic species (Table 1.5).

Table 1.5 Examples of species with non-peridinin plastid types.

Plastid types	Species
Prasinophyte-like plastids	*Lepidodinium viride, Gymnodinium chlorophorum*
Haptophyte-like plastids	*Karenia brevis, Karenia mikimotoi, Karenia umbella, Karlodinium micrum (K. veneficum), Karlodinium armiger, Takayama tasmanica, Takayama helix, Gyrodinium aureolum (K. mikimotoi), Karlodinium australe.*
Diatom-like plastids	*Durinskia baltica, Durinskia capensis, Galeidinium rugatum, Kryptoperidinium foliaceum, Peridinium quinquecorne.*
Cryptophyte-like plastids	*Dinophysis acuminata, Dinophysis acuta, Dinophysis norvegica, Gymnodinium acidotum*

During the endosymbiotic process the endosymbiont undergoes several changes to become the slave of the host (Keeling, 2004) and host control is enabled by a massive gene transfer from the endosymbiont nucleus to the host genome (Hackett et al., 2004b). In most cases, the endosymbiont has undergone some changes as the degeneration or loss of its nucleus, cytoplasm and some membranes from the second and tertiary endosymbiosis. Several stages of the endosymbiont conversion to a plastid are found in the different plastid types of dinoflagellates.

The peridinin plastid genome of dinoflagellates is extremely reduced to minicircles containing up to 14 genes (Koumandou et al., 2004; Leung and Wong, 2009) whereas plant and algal plastid genomes contain 130 to 200 genes (Zhang et al., 1999). The minicircle genes encode the core subunits of the photosystems and rRNA, other plastidic genes have been transferred to the nucleus (Hackett et al., 2004b). Dinoflagellates have certainly developed a particular machinery of gene-transfer which explains massive gene flows.

The haptophyte-like plastid dinoflagellates have gained some new haptophyte nuclear-encoded plastid genes including RuBisCO form I instead of form II which is of proteobacterial origin and found in peridinin plastids, one photosystem gene encoding oxygen-evolving enhancer protein 1 (*psbO*), a new plastid-targeted glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene closely related to the haptophytes and unlike

the cytosolic form encoded by a GAPDH gene closely related to peridinin plastids (Takishita et al., 2004; Yoon et al., 2005). However, some of the previous peridinin genes are missing in the nuclear genome of the haptophyte-like plastid dinoflagellates. For example a sequence of 48 nuclear encoded genes has not been found (Yoon et al., 2005).

Dinoflagellates with a diatom-like plastid suggest an endosymbiont that is not completely "submissive". This intermediate plastid is surrounded by only a single membrane and it retains a cytoplasm with a nucleus, ribosomes, mitochondria and chloroplasts (Schnepf and Elbrachter, 1999; Horiguchi and Takano, 2006). Moreover, the host still bears a remnant peridinin plastid surrounded by three membranes but which is deprived of pigments. This is also called the stigma or eyespot (Dodge and Crawford, 1969).

Some heterotrophic dinoflagellates retain a remnant plastid. For instance, the osmotrophs *Crypthecodinium cohnii* and *Oxyrrhis marina* contains some plastid targeting N-terminal peptides (Sanchez-Puerta et al., 2007; Slamovits and Keeling, 2008) and the parasite *Perkinsus atlanticus* bears some remnant plastids with four membranes (Teles-Grilo et al., 2007).

1.3.6. Reproduction

Asexual and sexual reproduction is used by dinoflagellates. Asexual reproduction is commonly used for population growth. Haploid vegetative cells divide by binary fission and mitotic division of the nucleus. Some types of sexual reproduction may be induced by nutrient deficiency (Figueroa and Bravo, 2005) and starts with the production of gametes that may be of different sexual types (+/-). The fusion of gametes leads to the production of diploid hypnozygotes or planozygotes. The hypnozygote is a resting non-motile stage, morphologically dissimilar to vegetative cells and planozygotes are motile cells, morphologically similar to vegetative cells.

1.3.7. Distribution

Dinoflagellates are globally distributed and have colonised all aquatic environments. Mainly marine, they generally develop in coastal waters (e.g. *Alexandrium spp, Karlodinium spp. Karenia spp, Gymnodinium spp*), in estuaries (e.g. *Heterocapsa triquetra, Alexandrium tamarense, Dinophysis acuminata*), in brackish waters (*Durinskia baltica*), open oceans (e.g. *Gambierdiscus toxicus, Akashiwo sanguinea* in the Sargasso Sea) and in freshwaters (e.g. *Gymnodinium fuscum, Peridiniopsis berolinense, Peridinium bipes, Peridinium gatunense*). New dinoflagellate species are regularly isolated and identified (de Salas et al., 2003; de Salas et al., 2004a; de Salas et al., 2004b; de Salas et al., 2005; Mason et al., 2007). It is difficult to determine whether they are emergent species or that new species identification results from improved scientific techniques and monitoring.

Dinoflagellates proliferate in blooms of up to 10^5 cells ml^{-1} (Hall et al., 2008) at all latitudes and all coasts. They are often called “red tides” because of the deep reddish-brown colouration of the water due to their plastidial pigments. Several factors affect the formation and expansion of dinoflagellate blooms. Some dinoflagellate species show a geographic expansion that may have resulted from vectors such as transport with organisms for aquaculture, transport in ballast water of ocean vessels, natural migration with water currents or migratory birds (Hoppenrath et al., 2007). Moreover, eutrophication and changing climate may favour or influence the occurrence of dinoflagellate blooms (Livingston, 2007; Mohamed and Mesaad, 2007). The abundance of some dinoflagellate species has increased with sea surface temperature (Edwards et al., 2006). In the NW Atlantic *Ceratium arcticum* formed denser winter populations in the 1990s compared to the 1960s (Johns et al., 2003). In the Central North Sea *Ceratium fusus* has bloomed earlier in the season and reached higher abundance in the period 1981-2002 compared to the period 1958-1980 (Edwards and Richardson, 2004). In the Mediterranean Sea dinoflagellate abundance increased relative to that of diatoms from 1997 to 2002 (Mercado et al., 2007). Finally, dinoflagellate blooms are more frequently observed due to an increased effort in water monitoring because of toxin production (see section 1.3.9).

1.3.8. Ecology

Dinoflagellates are a key component of marine ecosystems. Heterotrophic dinoflagellates constitute 1/3rd of the microzooplankton biomass (Buitenhuis et al., *in press*) that actively graze on diatoms and other marine phytoplankton (Sherr and Sherr, 2007), bacterial biomass, marine snow, other dinoflagellates, copepod eggs and early naupliar stages of zooplankton (Jeong, 1999). In turn they are eaten by other dinoflagellates (with existence of reciprocal predation and cannibalism), other protists and copepods (Jeong et al., 2007). Photosynthetic dinoflagellates are also grazed by microzooplankton such as heterotrophic dinoflagellates and mesozooplankton such as copepods (Calbet et al., 2003; Johnson et al., 2003). Generally photosynthetic dinoflagellates are in competition with diatoms for the same niche space including light and nutrients (Smayda, 2002). Many dinoflagellates tend to prefer calmer waters (Berdalet, 1992; Barlow et al., 1995) though certain species appear adapted to more turbulent habitats (Smayda and Reynolds, 2001; Smayda, 2002). Some dinoflagellate species have several advantages which lead to their dominance such as (1) the ability to migrate towards the illuminated surface or deeper rich nutrient zone, (2) mixotrophic feeding that allows them to grow on organic matter (Stoecker, 1999), (3) cyst production that ensures annual bloom events and also emigration to and colonisation of new areas (Smayda, 2007) and (4) production of chemical defences including allelopathic compounds (Tillmann et al., 2008), toxins, bioluminescent compounds and DMSP that may reduce grazing (Verity and Smetacek, 1996; Steinke et al., 2002b; Wendler et al., 2002). Dinoflagellate populations may be limited by virus attacks. Several viruses have been identified and their role in controlling dinoflagellate blooms is under investigation (Nagasaki et al., 2006)

1.3.9. Toxins and diseases

Toxic species of dinoflagellates represent a minority of dinoflagellate diversity with less than 60 species out of 2000 living species (Steidinger and Tangen, 1997), but they can sometimes form Harmful Algal Blooms (HAB). The production of toxins can

sometimes have dramatic consequences on the ecosystem, public health and the economics of seafood products (Van Dolah, 2000; Paul et al., 2007; Wang, 2008). Dinoflagellates produce a large variety of toxins including neurotoxins and toxins that affect the digestive system. Toxins that are associated with human poisoning syndromes are detailed below.

Paralytic Shellfish Poisoning (PSP) is caused by the ingestion of seafood contaminated with saxitoxins or gonyautoxins which create neurologic and gastrointestinal disorders. These toxins may block neuronal activity by binding to voltage-dependant Na channels. Depending on the amount of toxin ingested, syndromes can range from numbness to drowsiness and through fatal respiratory paralysis. Causative species are members of the genera *Alexandrium, Gymnodinium, Pyrodinium*.(Van Dolah, 2000; Wang, 2008). A gonyautoxin analogue has been isolated from *Alexandrium minutum* but its mode of action has not been investigated yet (Lim et al., 2007).

Neurolytic Shellfish Poisoning (NSP) is induced by assimilation of brevetoxins produced by *Karenia brevis*. These toxins selectively bind to voltage-dependant Na channels altering Na ion fluxes and also trigger neural Ca influx. Symptoms such as nausea, loss of motor control, muscular ache, and unconsciousness appear after brevetoxin exposure. Massive fish kills have been caused by these toxins but no human mortality has been reported (Flewelling et al., 2005; Wang, 2008).

Diarrhetic Shellfish Poisoning (DSP) is created by toxins such as okadaic acid produced by *Prorocentrum lima, Prorocentrum belizeanum* and *Dinophysis* toxins. These toxins inhibit the activity of protein phosphatases involved in cellular metabolism and the cell cycle which affects the function of intestinal epithelia inducing gastrointestinal problems including vomiting and diarrhoea. After chronic exposure they may act as tumor promotors (Van Dolah, 2000; Wang, 2008; Blunt et al., 2009).

Pectenotoxins are produced by several *Dinophysis sp* (Wilkins et al., 2006) and accumulate in the hepatopancreas of shellfish (Espina et al., 2010). These toxins are associated with DSP toxins and their mode of action is under investigation. The organ

target seems to be the liver given that direct intraperitoneal injection in mice causes liver congestion and bleeding (Espina et al., 2010).

Ciguatera Fish Poisoning (CFP) is an intoxication syndrome of the Ciguatera disease. It is caused by the maitotoxins which are initially produced by *Gambierdiscus toxicus* and then biotransformed and bioaccumulated along the food chain, ending in the lipidic tissues of fish. Ciguatera symptoms include a series of gastrointestinal, neurologic and cardiovascular disturbances (numbness, reversal of temperature perception, tachycardia, hypertension, blurred vision, paralysis). In rare cases it may be fatal or cause chronic symptoms for months or years. The ciguatoxins bind to Na channels like brevetoxins but with a higher toxic potency. Ciguatera is a world health problem for tropical and subtropical areas and is estimated to affect 10000 - 50000 persons per year (Withers, 1982; Durand-Clement, 1987; Lewis, 2006; Friedman et al., 2008).

Azaspiracid Shellfish Poisoning (AZP) is caused by the assimilation of azaspiracids produced by the recently identified dinoflagellate *Azadinium spinosum* (Tillmann et al., 2009). Symptoms include gastrointestinal and neurologic troubles, necrosis of organ tissues and after chronic exposure lung tumors may be initiated. These toxins appear to trigger Ca channels (Wang, 2008).

Estuary-associated syndromes are induced by assimilation of toxins associated with blooms of the heterotrophic dinoflagellate *Pfiesteria* and cause headache, skin lesions and respiratory irritation (Engelhaupt, 2007).

Yessotoxins produced by *Protoceratium reticulatum, Lingulodinium polyedrum* and *Gonyaulax spinifera* have recently been isolated from dinoflagellates (Wang, 2008; Blunt et al., 2009). They are potent neurotoxins that might interact with Ca and Na channels. Palytoxins and ovatoxin-a (Ciminiello et al., 2008) have been identified from the benthic species *Ostreopsis spp*. Palytoxins may cause human illness and death with symptoms of ataxia, drowsiness, weakness of limbs (Wang, 2008), respiratory difficulties, conjunctivitis and skin irritation (Gallitelli et al., 2005).

1.3.10. Palaeogeological interest

In common with phytoplanktonic foraminifera, dinoflagellates are used as tracers for palaeoclimatic and palaeoceanographic studies (de Vernal et al., 1997; de Vernal et al., 2007). Assemblages of dinoflagellate cysts in sediments are identified and provide a proxy for conditions of temperature, salinity, ice cover and seasonality that allow reconstitution of past climatic and oceanographic conditions. Cysts result from the fusion of gametes and are a dormant stage that sediments and resists degradation due to a resistant cell wall that contains dinosporin. Not all dinoflagellates are able to form cysts; 13 to 16 % of living dinoflagellates are estimated to produce cysts (Taylor et al., 2008). Some cysts may also contain silicate or calcium carbonate and have contributed to fossil limestone and chalk deposition (Wendler et al., 2002). In addition to providing information about the environment in the past, calcareous dinoflagellates also contribute to knowledge of the global calcium cycle and also provide information of the past environment (Gussone et al., 2010).

1.3.11. Food and pharmaceutical industries

Food interest in Crypthecodinium cohnii

The omega-3 polyunsaturated fatty acids (PUFA) are required by the human body as a membrane constituent because they cannot be synthesized directly (Richard et al., 2008). Docosahexanoic acid (DHA, 22:6) is one of the most important ω-3 PUFA along with eicosapentaenoic acid (EPA, 20:5) and α-linolenic acid (18:3). DHA accumulates in membranes of several human tissues including reproductive, nervous and visual tissues and the grey matter of the brain. They are considered essential in infant brain development and nutrition. DHA is also involved in the anticholesterolaemic and anti-inflammatory process and reported to contribute to the prevention and treatment of diseases such as arterioscleriosis, arthritis, thrombosis and variety of cancers. Food sources of DHA are fish and fish oils which are a limited

resource. Microalgae are considered a promising source and particularly non-light limited heterotrophic species such as *Crypthecodinium cohnii* (Mendes et al., 2009). Furthermore, this species possesses high fatty acid content (> 20 %) with predominance of DHA (30 – 50 %) which makes it easier for DHA purification for food supplements and infant food manufacture. *C. cohnii* cultures can be produced profitably in industrial scale bioreactors with a low price carbon source like carob pulp (Mendes et al., 2007).

Pharmacologically bioactive compounds in dinoflagellates

Because of their chemical diversity, dinoflagellates are a rich source of bioactive natural compounds with application in pharmacology. The Ciguatera-responsible maitotoxin produced by *Gambierdiscus toxicus* causes platelet aggregation (Nakahata et al., 1999). An extracellular polysaccharide produced by *Gymnodinium sp* is a potential anti-cancer agent due to its topoisomerase DNA inhibition activities (Umemura et al., 2003) and also induces apoptosis of human myeloid leukemia K562 cells. Other dinoflagellate chemicals have been found with cytotoxic activity (Mayer and Gustafson, 2006): amphidinoli X (Tsuda et al., 2003a), amphidinoli Y (Tsuda et al., 2003b) and the polyketide lingshuiol (Huang et al., 2004) produced from *Amphidinium sp*; protoceratin II-IV is a polyether glycoside from *Protoceratium cf. reticulatum* (Konishi et al., 2004) and zooxanthelactone is a fatty acid produced by *Symbiodinium sp*. (Onodera et al., 2004).

Several other new molecules of interest have been recently identified (Blunt et al., 2009) including several iriomoteolides and amphidinolides extracted from some *Amphidinium sp* which have a cytotoxic activity to human B lymphocytes. Some amphidinolactones also extracted from species of *Amphidinium* have cytotoxic activity against some leukemia cells and epidermoid carcinoma cells. New zooxanthellamide and symbiodinolide have been extracted from some *Symbiodinium* species and have special cytotoxic and enzyme (cyclooxygenases which are involved in inflammatory response) inhibition activities respectively. A complex spirolide has been found to be produced by *Alexandrium ostenfeldii*.

All these new molecules testify to the chemical richness of dinoflagellates and their unknown potential. These important findings provide promise for the development of new drugs and research into diseases including cancer.

1.3.12. Challenge of culturing dinoflagellates

Many scientific observations led to the conclusion that some dinoflagellates are very sensitive to turbulence and they are considered amongst the most fragile algal groups (Thomas and Gibson, 1990). Attempts to grow dinoflagellates can fail if the cultures are aerated, bubbled or rotary shaken. In general, this sensitivity can be a limiting factor in studying dinoflagellates and has inevitably resulted in a selective approach towards the easily-grown strains. Berdalet and Estrada (2006) summarised the effect of turbulence on 44 dinoflagellate strains: the growth of 5 strains was increased, 7 strains showed no effect, the growth of 29 strains was reduced and cell death occurred for 3 strains including *Crypthecodinium cohnii*.

Water motion may cause physical and physiological damage such as loss of flagella, cell lysis or growth inhibition by transient arrest of the cell cycle (Yeung and Wong, 2003; Yeung et al., 2006; Camacho et al., 2007b; Llaveria et al., 2009b). Cells are even more susceptible to damage during the dark phase when they are in the division process (Camacho et al., 2007b). Turbulence may also affect more specific physiological processes including an increase of toxin production (Juhl et al., 2001), bioluminescence and DMSP cleavage (Wolfe et al., 2002).

On the other hand, the recent commercial interest in *C. cohnii* cultures for omega-3 fatty acid production led to the development of fast-growing dense cultures bubbled with oxygen (de la Jara et al., 2003), in flasks rotated by an orbital shaker (Lopes Da Silva et al., 2006) and also in stirred bioreactors (Mendes et al., 2007). Therefore this species seems to be more robust than previously described by Berdalet and Estrada (2006). Hu *et al.* (2007) confirmed that *C. cohnii* ATCC30772 was able to

resist a certain amount of hydrodynamic force though with an associated loss of flagella. The example of *C. cohnii* suggests that the culture of dinoflagellates can become feasible with perseverance and the acquisition of more knowledge.

1.3.13. DMSP and DLA in dinoflagellates

Various studies reported the DMSP content of dinoflagellates in cultures and showed the contribution of dinoflagellates in DMSP and DMS pool in the field (detailed in Chapter 5). In 2007, Stefels and co-workers recommended the inclusion of the dinoflagellate group amongst the 6 phytoplankton groups recognised as DMS producers for inclusion in future global ocean climate models. To date, about 60 species and strains including 5 heterotrophic species have been analysed for their DMSP content. DMSP concentrations are widely variable within the group with 6 orders of magnitude difference observed in DMSP concentrations per cell volume (Chapter 5). Only the heterotroph *Oxyrrhis marina* has been reported with no DMSP (Keller et al., 1989a). The underlying reasons for this wide variation in intracellular DMSP concentration have not been investigated.

Several field studies reported some potential DLA associated with dinoflagellate blooms and subsequent laboratory studies began to investigate DLA in dinoflagellate cultures. Only fifteen dinoflagellate species and strains have been tested and reported with associated DLA (Chapter 5).

1.4. The major objectives of this study

The research work that I conducted for this thesis can be divided into 3 parts focusing on 3 major objectives: (1) the adaptation of methods for DMSP and DMS measurement for dinoflagellate cultures (2) the addition of new DMSP and DLA data for dinoflagellate cultures and the compilation and analysis of all published DMSP and DLA data (3) the investigation of factors affecting DMSP content in the heterotrophic dinoflagellate *Crypthecodinium cohnii*.

Methods for DMS and DMSP measurements have been developed primarily for field studies and large volumes of sample and require assessment of precision and optimisation for small volumes of cultures in laboratory studies. Moreover, Kiene and Slezak (2006) recently criticised some methods of DMSP measurements. They compared various filtration procedures and concluded that filtration may damage cells and transfer particulate DMSP to the dissolved fraction. They recommended the use of gentle filtration and especially gravity filtration when possible. Given that dinoflagellate cells are known to be sensitive to turbulence and agitation that may break cells, they may be prone to damage during handling. The first challenge of this study was to adapt the method of DMSP and DMS measurements specifically to dinoflagellate cultures. I began by assessing the analytical uncertainty and investigated the factors that may affect results such as the sample processing time and sample volume.

The dinoflagellates are currently considered as one of the major DMSP-producing phytoplanktonic groups (Keller et al., 1989a; Stefels et al., 2007) but this is despite studies being rather sporadic and the large diversity within the group which encompasses species with high concentrations of DMSP and some that have little or no DMSP. In addition, the release of DMS from DMSP to the oceanic pool depends in part on the activity of algal DMSP-lyase enzyme (DLA) and yet very few studies have reported the presence of such enzymes in dinoflagellates. The compilation of all the published DMSP and DLA data for dinoflagellates was warranted to summarise and analyse the available knowledge and identify the main knowledge gaps. I also collected

additional data of DMSP and DLA from dinoflagellate cultures to enable a more in-depth assessment of the DMSP production ability of this phytoplankton group and its contribution to the DMS pool and sulphur cycle.

Various studies show that DMSP has multiple biological roles. Only a few of these investigations have involved dinoflagellates and even less heterotrophic dinoflagellates. Thus, I performed a series of experiments with the model heterotrophic dinoflagellate *Crypthecodinium cohnii* to determine how external factors may affect the DMSP content of this species.

Chapter 2 General methodology

In this chapter I describe the general methodology applied for the investigation of my thesis topic including the culturing techniques for micro-algae and the instruments regularly used for monitoring culture growth and DMS and DMSP concentrations. I present the dinoflagellate species selected, their conditions of culture and the challenge that culturing represents in this fragile phytoplankton group. Then I describe the instruments I used, their principles and their applications to my study. The assessment and optimisation of methods for measuring DMS and DMSP in dinoflagellate cultures is presented in the following chapter (Chapter 3).

2.1. Culturing techniques for dinoflagellates

2.1.1. Algal culturing conditions

Algal strains were ordered from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP; Boothbay Harbor, USA), and one strain was kindly given by Dr Debora Iglesias-Rodriguez (National Oceanographic Centre of Southampton, UK). These strains have diverse geographic origins such as the North Sea, East Atlantic, West Atlantic, East Pacific and Antarctic Oceans and consequently require different environmental conditions for growth. A list of the dinoflagellate strains used and their culture conditions is presented in Table 2.1.

Table 2.1 Dinoflagellate species investigated. Details include their full names, synonyms, strain codes, origin, toxicity and axenicity of the culture, and the medium, light intensity (LI in µmol photons m^{-2} s^{-1}) and temperature (T °C) used in this study.

Latin name	Synonyms	Strain code	Collection site	Medium	LI	T °C
[T] *Alexandrium minutum*	*Alexandrium ibericum*	CCMP113	Ria de Vigo, Spain	L1	156	15
[A] *Amphidinium carterae*		CCMP1314	Falmouth, USA	f/2	204	22
[A] *Crypthecodinium cohnii*	*Glenodinium cohnii*	CCMP316	Unknown	f/2 + NPM	127	22
[A] *Heterocapsa triquetra*	*Peridinium triquetra*	CCMP449	St Lawrence Estuary, Canada	f/2	120	15
[T] *Karlodinium veneficum*	*Karlodinium micrum*	CCMP415	Norway, North Sea	f/2	122	15
Kryptoperidinium foliaceum	*Peridinium foliaceum, Glenodinium foliaceum*	CCMP1326	La Jolla, California Bight, USA	L1	204	22
[PT] *Lingulodinium polyedrum*	*Gonyaulax polyedra*	LP2810	San Diego, California, USA	f/2	204	22
Polarella glacialis		CCMP1138	McMurdo Sound, Antarctica	Provasoli	101	4
[A] *Scrippsiella trochoidea*	*Peridinium trochoideum*	CCMP1599	Falmouth, USA	f/2	102	15

[A] Axenic, [T] Toxic and [PT] Potentially toxic

The phytoplankton strains were grown in batch culture. Erlenmeyer flasks of 100 ml or 250 ml volume were filled up to one third of the volume with enriched seawater media (different media are mentioned in Table 2.1) and capped with a cotton-filled muslin bung covered with a piece of foil. Cultures were set in incubators (MLR-351 Plant Growth Chamber, Sanyo, Loughborough, UK) under controlled conditions of light intensity, photoperiod and temperature that ensured algal growth (Figure 2.1). The photoperiod was 14:10 light:dark cycle with an incubator level 3/5 of maximum output of light intensity supplying 150 ± 50 µmol photons m^{-2} s^{-1}. The phytoplankton species were divided into 2 groups depending on their tolerable range of temperature. The "cold group" was placed in an incubator at 15 ºC and the "warm group" was placed in another incubator at 22 ºC. These temperatures fall in the range of temperatures recommended by the algal supplier but I have not tested if they are optimal.

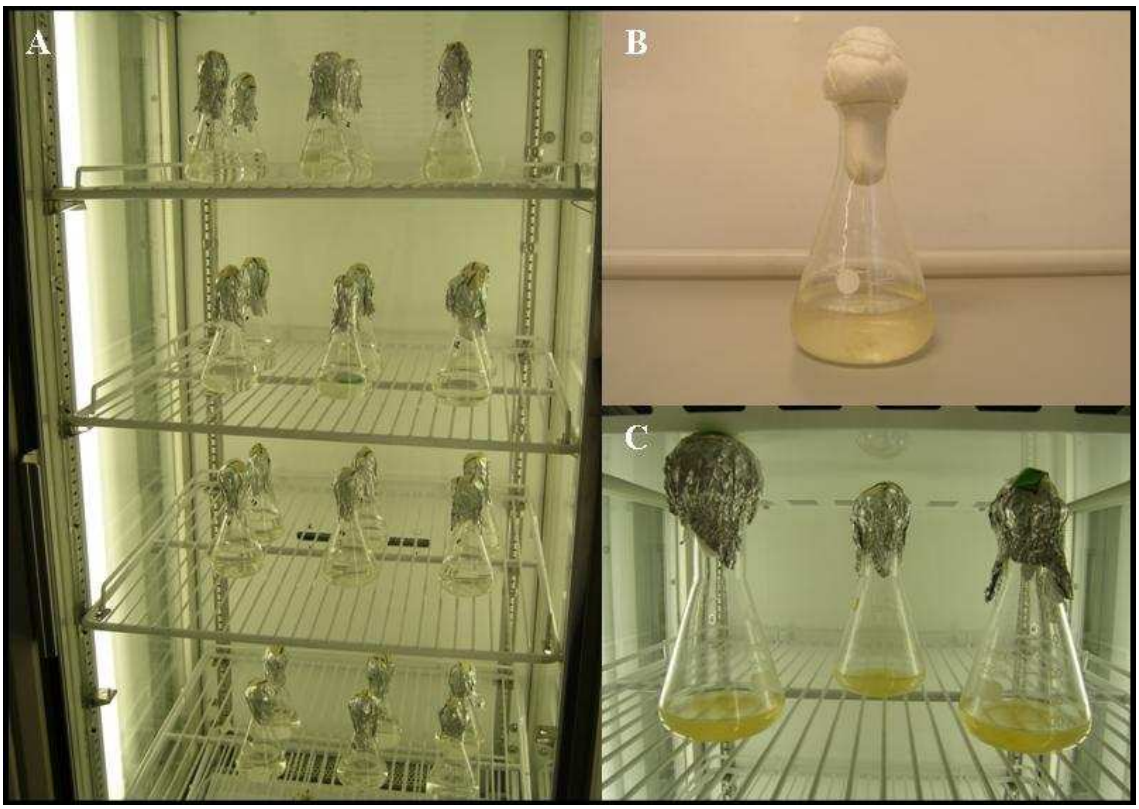

Figure 2.1 Example of batch cultures of algae. (A) Several cultures of *Crypthecodinium cohnii* inside an incubator. (B) Erlenmeyer flask capped with a cotton bung. This culture of the heterotrophic *C. cohnii* displays a pale colour due to the lack of chlorophyll. (C) Example of 3 replicate cultures of the photosynthetic species *Heterocapsa triquetra* in stationary phase.

Manipulation of algal cultures requires a clean and sterile environment to avoid contamination by other organisms (bacteria, fungi, other algae). In this regard, all

manipulations were conducted inside a laminar flow cabinet (Class II, Walker). This cabinet and all items going inside were wiped with 70 % ethanol before use. Glassware and culture media were autoclaved at 120 ºC for 20 minutes. Those cultures that were axenic were checked regularly for axenicity by microscopic observation of samples stained with DAPI (see section 2.1.3 for more details).

2.1.2. Medium preparation

All different media were enriched seawater. They were prepared with natural seawater collected either from the open ocean, during oceanic cruises, or from coastal waters pumped out from the North Sea by the CEFAS institute (Lowestoft, UK). This water was stored in the dark at 12 ºC. The water was then filtered through a 0.2 µm pore size cellulose acetate filter to eliminate potential organisms and particles. According to the recommendations of Mclachlan (1973) to avoid precipitation in the medium, the seawater was diluted with distilled water (2.5 %), and one drop of concentrated hydrochloric acid (HCl) was added before autoclaving. HCl is used to compensate for the increase of pH due to the loss of CO_2 during the heating process (120 ºC during 30 minutes). Also, the nutrients were added aseptically several hours after cooling down. Several media were made up for the panel of species grown: f/2, L1, Provasoli, f/2 + NPM. All these medium recipes are based on the CCMP recipes (Wilson, 2009).

2.1.2.1. f/2 medium

The f/2 formulation (Table 2.2) is commonly used for phytoplankton (Guillard and Ryther, 1962; Guillard, 1975). The denomination f/2 means that concentrations are divided by 2 compared to the original formula. This formula contains macronutrients including nitrogen and phosphorus and micronutrients such as a trace metals and vitamins at concentrations that ensure algal growth. Silicate is a component of f/2 but was omitted since it is not required for dinoflagellate growth.

Table 2.2 Components of f/2 medium without silicate. The proportions are listed for the stock solution preparation, the volume of the solution used and the final concentrations obtained in the medium (dH_2O means distilled water).

Compounds	**Stock solution g/L dH_2O**	**Quantity in 1 L of medium**	**Medium molar concentration M**
f/2 Medium			
$NaNO_3$	75	1 ml	8.83×10^{-4}
NaH_2PO_4 H_2O	5	1 ml	3.63×10^{-5}
Trace metals solution		1 ml	
Vitamin solution		1 ml	
Trace metals			
Fe/EDTA	6.25		
$CuSO_4$ $7H_2O$	9.8	1 ml	4×10^{-8}
Na_2MoO_4 $2H_2O$	6.3	1 ml	3×10^{-8}
$ZnSO_4$ $7H_2O$	22	1 ml	8×10^{-8}
$CoCl_2$ $6H_2O$	10	1 ml	5×10^{-8}
$MnCl_2$ $4H_2O$	180	1 ml	9×10^{-7}
Vitamin solution			
Vitamin B12 (cyanocobalamin)	1	1 ml	2×10^{-10}
Biotin	0.1	10 ml	4×10^{-9}
Thiamine HCl		200 mg	6×10^{-7}

Trace metals include iron, copper, molybdenum, zinc, cobalt and manganese. These elements are essential for algae by participating in enzymatic and cytochrome reactions but become toxic if introduced above a *trace* concentration (Reynolds, 2006). Vitamins consist of cobalamin (vitamin B12), biotin and thiamine. Some algae from all lineages (including dinoflagellates) are not able to synthesize some of these vitamins and are called auxotrophs. Because bacteria may supply these vitamins it was initially difficult to develop axenic cultures (Croft et al., 2005; Reynolds, 2006).

2.1.2.2. L1 medium

L1 medium (Table 2.3) differs from the f/2 formulation by the addition of trace elements. Selenium, nickel, vanadium and chromium are added to the f formulation. These metals are metabolised by certain microorganisms and used as enzyme components (Madigan et al., 2003). Some organisms that have a higher demand of nutrients should be grown in this richer medium rather than f/2.

Table 2.3 Trace metals of L1 medium

Compounds	Stock solution g/L dH_2O	Quantity in 1 L of trace metal solution	Medium molar concentration M
$FeCl_3 \cdot 6H_2O$		3.15 g	1.17×10^{-5}
$Na_2EDTA\ 2H_2O$		4.36 g	1.17×10^{-5}
$CuSO_4 \cdot 5H_2O$	2.45	0.25 mL	1×10^{-8}
$Na_2MoO_4 \cdot 2H_2O$	19.9	3 mL	9×10^{-8}
$ZnSO_4 \cdot 7H_2O$	22	1 mL	8×10^{-8}
$CoCl_2 \cdot 6H_2O$	10	1 mL	5×10^{-8}
$MnCl_2 \cdot 4H_2O$	180	1 mL	9×10^{-7}
H_2SeO_3	1.3	1 mL	1×10^{-8}
$NiSO_4 \cdot 6H_2O$	2.7	1 mL	1×10^{-8}
Na_3VO_4	1.84	1 mL	1×10^{-8}
K_2CrO_4	1.94	1 mL	1×10^{-9}

2.1.2.3. Provasoli medium

Provasoli medium is derived from f/2 with addition of 15 ml L^{-1} of an alkaline soil extract (Provasoli et al., 1957). Soil extract was prepared with soil collected in the grounds of the University in an area containing no fertilizer or pesticides. This soil was diluted with two parts of distilled water and this solution was made alkaline with NaOH (2 - 3 g L^{-1}) before autoclaving for 2 hours. After cooling down, the mixture was filtered and the extract was 50–fold diluted with distilled water. The soil extract supplies organic substances which allows the culture of some algae unable to grow in only mineral enriched medium such as the Antarctic dinoflagellate *Polarella glacialis*. However, the key active compounds remain unknown due to the complexity of the soil chemistry.

2.1.2.4. f/2 + NPM medium

The derivative f/2 + NPM medium (Table 2.4) contains an extra organic solution necessary for the culture of heterotrophic or mixotrophic organisms (Guillard, 1960). I used this medium for culturing the heterotrophic dinoflagellate *Crypthecodinium cohnii*. f/2 components supply a mineral source of nutrients available for mixotrophs and also metals and vitamins for mixotrophs and heterotrophs. The organic solution contains an organic source of carbon and nitrogen. Acetate, succinate and glucose are potential carbon sources. Peptone, bacto-tryptone (enzymatic digest of casein) and yeast extract are potential nitrogen sources, supplying peptides and amino-acids.

Table 2.4 Organic solution of f/2 + NPM. This solution was added as 100 ml in 900 ml of f/2 medium.

Compounds	Quantity in 1L of organic solution
Sodium acetate	1 g
Glucose	6 g
(di-) sodium succinate $6H_2O$	3 g
Peptone	4 g
Bacto-tryptone	1 g
Yeast extract	0.1 g

2.1.3. DAPI staining

DAPI staining is a simple method to check algal culture axenicity. DAPI (4',6-diamidino-2-phenylindole, Figure 2.2) is a fluorochrome commonly used to stain DNA. This fluorochrome binds to minor grooves of double-stranded DNA and intercalates between adenosine and thymine bases (Kapuscinski, 1995). When excited with UV light (360 nm), this stable complex fluoresces by emitting blue light (450 nm). Observation of stained DNA by fluorescence microscopy gives a clear view of bacterial presence or absence among algal cells (Porter and Feig, 1980).

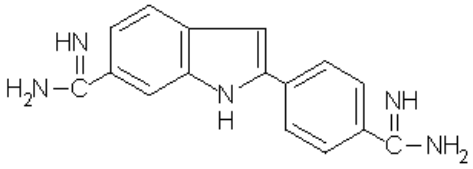

Figure 2.2 DAPI structure (http:// sigmaaldrich.com)

To avoid bacterial contamination during the staining process, I used 0.2 µm filtered reagents and rinsing seawater. Also, I performed all manipulations wearing protective clothes in a fume cupboard to prevent hand from the carcinogenic properties of this dye.

The culture sample (1-5 ml) was fixed with a reactive mixture of lugol (3 µl ml^{-1} of aqueous KI 10 % w/v and iodine 5 % w/v) and formalin (50 µl/ml of 20 % aqueous formaldehyde with 100 g L^{-1} hexamine) that keeps cells more intact than lugol only (Sherr et al., 1993; Sherr and Sherr, 1993). Then, sodium thiosulphate (1 µl/ml of a 3 % solution stored in the fridge) was added to discolour the iodine colouration that would reduce the DAPI fluorescence and finally, DAPI (10 µl ml^{-1} of 1mg ml^{-1} solution, Sigma-Aldrich, D9542) was added. The culture sample was left to react with the chemicals for at least 5 minutes. The stained sample was filtered by means of a water vacuum pump through 2 filters: a 0.2 µm pore black polycarbonate filter of 25 mm diameter supported by a 0.45 µm pore nitrate cellulose backing filter. The filters were then rinsed with sterile seawater. The black filter was collected and deposited on a slide bearing a drop of immersion oil for adherence. The filter was covered by a second drop of oil immersion and a cover slip. Slides were examined under the microscope (BX40, Olympus, Essex, UK) at 100-fold magnification objective with epifluorescence light

and a UV light filter. The dinoflagellate cells fluoresce with intense blue colour (Figure 2.3). If bacteria are present, the black filter background appears cloudy at low magnification (20-fold) and bacteria are visible with elongated blue shapes at high (100-fold) magnification (illustrated in Figure 2.3).

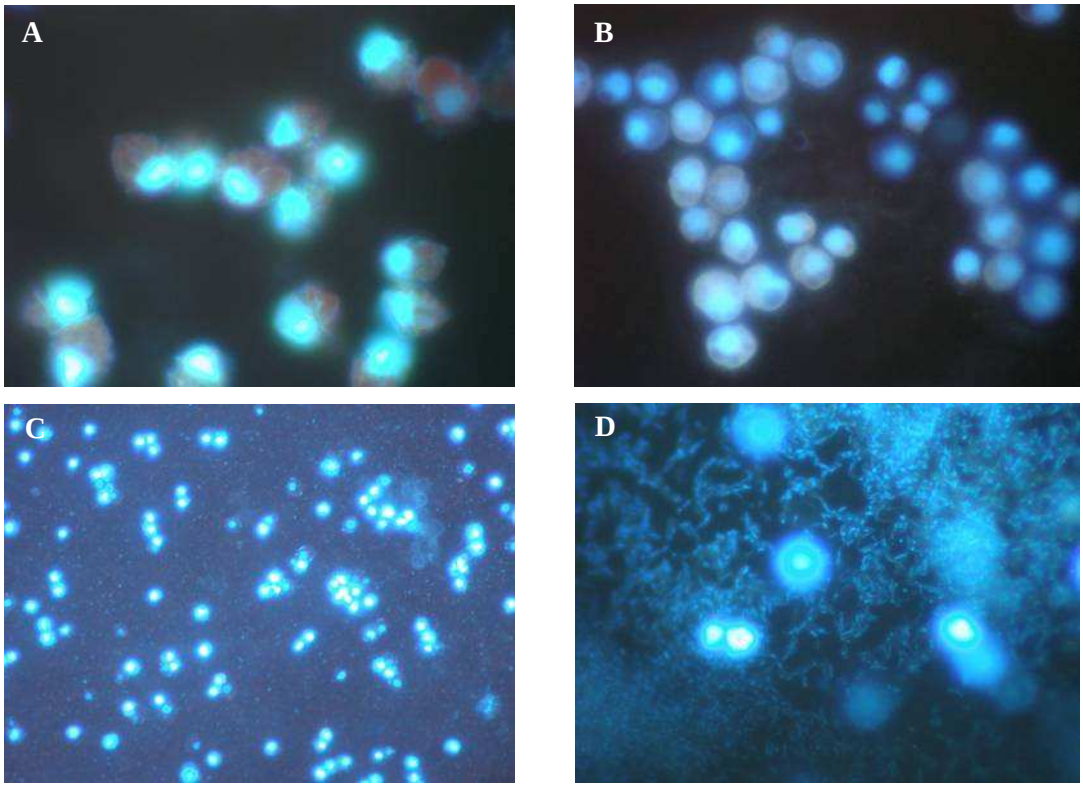

Figure 2.3 Comparison of axenic (A and B) and non-axenic (C and D) cultures of dinoflagellates stained with DAPI and observed with an epifluorescent microscope. Axenic culture of (A) *Crypthecodinium cohnii* and (B) *Heterocapsa triquetra* observed at the 100-fold magnification objective. Samples were taken at the end of the experiments in order to check the axenicity. Only algal cells fluoresce, absence of bacteria is proved by no fluorescing background. Bacterial contamination of *Crypthecodinium cohnii* culture (C) At 20-fold magnification objective, the fluorescent algal cells appear superimposed on a cloudy background. (D) At 100-fold magnification bacteria appear as elongated sticks.

2.1.4. Handling and sampling techniques for dinoflagellate cultures

Dinoflagellates are amongst the most fragile phytoplankton group and are subject to cell breakage when agitated or manipulated in laboratory culture (detailed in section 1.3.12). In this study, special attention was given to the culture handling and sampling techniques to reduce the physiological damage of cells.

To minimise disturbing dinoflagellate physiology, cultures were grown in batch mode without agitation. Only *Crypthecodinium cohnii* cultures were agitated once a day to avoid aggregate formation due to the production of extra-cellular polysaccharides.

Prior to sampling, flasks were manually agitated in order to collect a homogenous sample, representative of the culture. The flasks were gently swirled 2 to 3 times and sampled straight away. For each flask sampling, 1 culture aliquot was taken and used for the measurement of several parameters. The sampling of 1 culture aliquot rather than 1 sample for each parameter measurement reduces the turbulence and stress applied to the culture flask. Cultures were kept outside of the incubator for the shortest time possible to minimise the effect of different environmental conditions of light and temperature. For the polar species *Polarella glacialis*, the culture was carried between the incubator and the safety cabinet using a coolbox containing ice packs.

2.2. Analytical instrumentation and techniques

This section describes the techniques used to characterise the cultures and the parameterisation includes the cell counts, cell volume, chlorophyll *a*, CHN content, DMS and DMSP content.

2.2.1. Particle counter for cell density and cell volume measurements

Cell density may be measured by counting cells on a haemocytometer placed under a microscope or using a particle counter that automatically counts a large number of cells. The use of the particle counter which is less laborious and less time consuming was favoured. However, I compared the 2 methods to assess the use of the particle counter for dinoflagellate cells that have a close to spherical shape.

A particle counter (Coulter Multisizer 3, Beckman, High Wycombe, UK), equipped with a 100 µm aperture tube, was used to monitor the following growth parameters: cell density (cell ml^{-1}), total cell volume (μL_{cell} L^{-1} that is equivalent to 10^6 μm^3 ml^{-1}) and volume per cell (μm^3).

The Coulter particle counter principle is based on the displacement of an electrolyte volume by a particle passing between two electrodes. The changing flow leads to a change in the impedance that is transformed into a pulse proportional to the particle volume. The measurement of several pulses in a given volume is equivalent to a measure of the particle concentration in the sample. The particle volume is automatically converted to particle diameter by calculations set for spherical particles. The change in impedance cannot convey additional information of shape and colours.

In general, I diluted 1 ml of culture sample in 10 ml of filtered seawater as the electrolyte. The dilution avoids saturating the electrodes and could be increased for dense cultures in stationary phase, for instance 0.5 or 0.2 ml of culture sample could be added. The cell density is the integral of the peak obtained on the computer interface (an

example is shown on Figure 2.4). Limits of the peak are taken outside of the peak area and are kept constant for a species. The total cell volume (CV) was read similarly to the cell density. The volume of the cell was derived from the reading of the mean cell size displayed by the statistics of the software then calculating the spherical volume associated. Indeed, the software displays the mean size value derived from the particle volume obtained for an assumed spherical particle. The size value is not representative for dinoflagellate cells which are not exactly round shaped, but the calculation of the volume gives a correct representative dimension independent of the dinoflagellate shape.

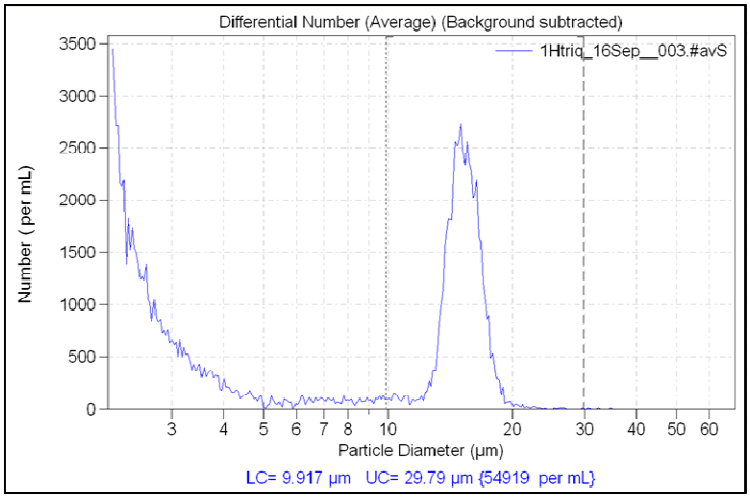

Figure 2.4 Example of cell density result obtained with the particle counter for the measurement of *Heterocapsa triquetra* culture. The reading of the cell density is taken between a lower and upper limit of particle diameter on both sides of the peak. (here, 54919 cell ml^{-1}).

To assess the use of the particle counter for dinoflagellate cultures, I have compared cell density measurements performed with the particle counter and the counting with a haemocytometer under the microscope (BX40, Olympus, Essex, UK). Using the haemocytometer, the method was to count 5 haemocytometer cells on 5 slides and to calculate the average. Four different dilutions of a *Heterocapsa triquetra* culture (25, 50, 75, 100 %) were counted with the 2 methods to assess how the particle counter measurements respond to a range of low to high cell density. The results are shown in Figure 2.5.

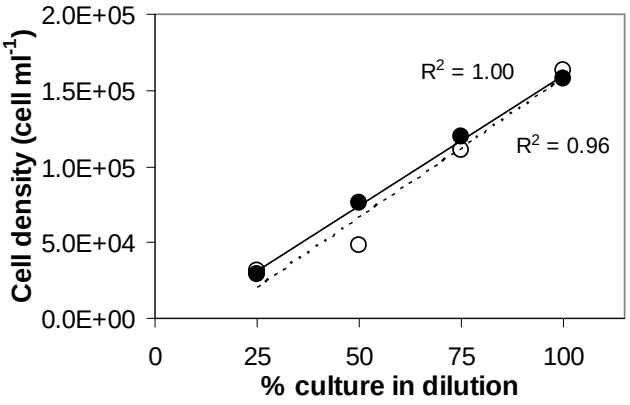

Figure 2.5 Comparison of cell density results obtained using the particle counter (●) and the haemocytometer (○). Four dilutions of *Heterocapsa triquetra* culture sample were determined (25, 50, 75 and 100 % culture). R^2 is the correlation coefficient associated with the linear regression of the culture dilution and the cell density measured with the particle counter.

The use of particle counter and haemocytometer gave very similar values of cell density. The linear response of the particle counter was better than the haemocytometer. The use of the particle counter was therefore satisfactory for dinoflagellates with a similar shape to *Heterocapsa triquetra* (almost spherical shape, like a diamond) and allowed fast measurements of total cell volume and volume per cell. All other dinoflagellate species investigated in this study have a similar shape to *H. triquetra*, round to oval shape. Given that many experiments have been performed with *Crypthecodinium cohnii*, which also has a similar shape to *H. triquetra*, I tested *C. cohnii* for linearity of response using the particle counter. In Figure 2.6, 3 samples of a dense *C. cohnii* culture were prepared at 3 dilutions (100, 50 and 20 %) and show satisfactory linearity of the measurement with this species.

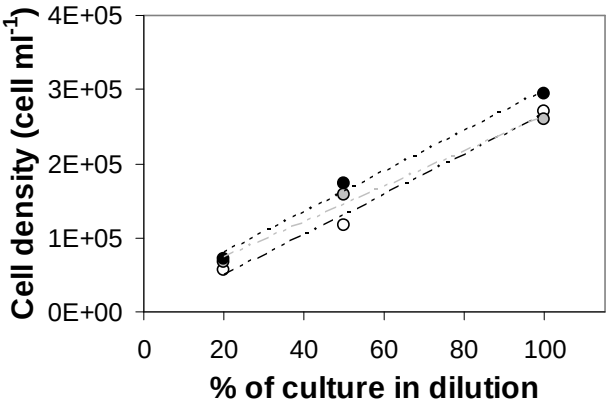

Figure 2.6 Linearity response of cell density measurements performed with the particle counter using different dilutions (20, 50 and 100 %) of 3 samples of a dense *Crypthecodinium cohnii* culture. For the 3 samples, the linear regression of the culture dilution and the cell density measured using the particle counter gave a correlation coefficient R^2=0.99.

To assess the precision of the particle counter, I took 5 measurements of a *H. triquetra* culture sample in exponential growth phase and calculated the uncertainties (Table 2.5, formulas are detailed in section 2.2.5). I obtained a RSD of 2.2 and 2.3 % for cell density and total cell volume (CV) respectively and a precision p_x of 2.7 and 3.9 %. These values showed that the uncertainty of the measurement with the particle counter was low.

Table 2.5 Calculation of uncertainties (σ_x and p_x) for the measurement of cell density and total cell volume (CV) based on 5 replicate measurements of a *H triquetra* sample.

Replicates of measurement	Cell density (cell ml^{-1})	Total CV ($\mu L_{cell}\ L^{-1}$)
1	35559	89.3
2	37092	93.0
3	36579	92.1
4	35523	88.8
5	37114	93.3
Average	36373	91.3
σ_x	789.6	2.10
p_x	980.2	3.54

2.2.2. Gas Chromatography for DMS and DMSP measurements

Gas chromatography is a technique of analytical chemistry used to separate, identify and quantify gaseous compounds. A gas chromatograph (Schimadzu GC-2010, Milton Keynes, UK) was used for DMS and DMSP measurements. It was equipped with a fused-silica capillary column 30 m × 0.53 mm CP-SIL 5CB (Varian, Oxford, UK). According to the supplier, this column is appropriate for a large panel of compounds including sulphur compounds and separates mainly by boiling point. A flame photometric detector (FPD) was used due to its sensitivity to sulphur compounds. A mix of hydrogen (60 ml min^{-1}) and air (70 ml min^{-1}) supplied the flame which burned the sulphur compounds subsequently emitting a light signal. Helium was used as neutral carrier gas (28 or 35 ml min^{-1} for DMS and DMSP methods, respectively) to convey the sample injected through the column.

When DMS is released by the column at a specific time called the retention time (RT), DMS is perceived by the FPD which emits a signal amplified by photomultiplier tubes and transmitted to the computer. A peak appears on the computer interface and

when this peak is higher than the background noise of the chromatograph, the peak is automatically integrated by the software. An example of peak obtained is shown on Figure 2.7. The RT and the shape of the peak (sharp and narrow with maximum possible height) are dependent on the gas chromatoghraph (GC) settings including column temperature, gas flow rate and temperature. The GC settings used in this study are described for each method (purge and trap and headspace analyses) in the following section 2.2.3.1. In the optimum range of the detector, the response is non-linear but approximates a square root function. Therefore, the square roots of peak areas (SQRT PA) are used for the DMS quantification by comparison with a concentration range of standards. The chromatograph was calibrated using commercially available DMSP that is converted to DMS by cold hydrolysis (Centre for Analysis, Spectroscopy and Synthesis, University of Groningen laboratories, The Netherlands). The linear range of the FPD for sulphur compounds is limited to 3 orders of magnitude.

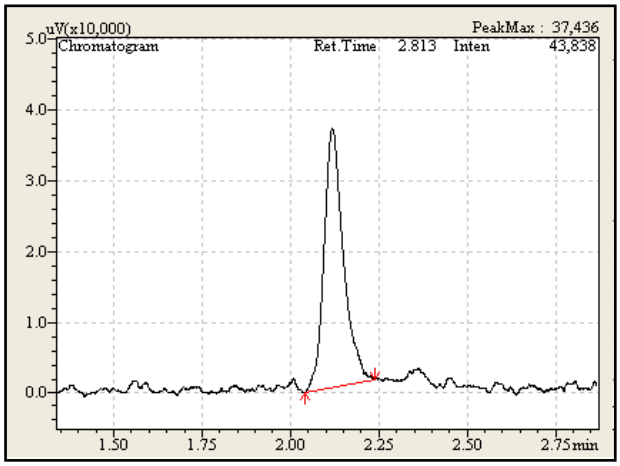

Figure 2.7 Example of GC chromatogram for a DMSP sample. The peak has a Retention Time (RT) of 2.05 min and the peak area is automatically integrated by the software (delimited by the red line and arrows).

DMSP (not volatile) needs to be converted to DMS prior to measurement and total DMSP is about 100-fold higher in concentration in cultures than DMS. Thus, there are considerations of sample preparation, sample size and calibration range. To measure DMS in cultures, I used a “purge-and-trap” method which involves extraction of almost all the gas from a liquid sample and cryogenic concentration in a sample loop. For DMSP measurements in cultures, I performed the conversion to DMS by chemical cleavage of DMSP to DMS and acrylate with a strong base (NaOH). DMSP was measured by gas chromatography using the headspace method. The calculation of

DMSP concentration was based on the 1:1 ratio of this chemical conversion (equation 1); (Challenger and Simpson, 1948; Cantoni and Anderson, 1956; Ackman et al., 1966).

$$\underset{\text{DMSP}}{(CH_3)_2S^+CH_2CH_2COO^-} \rightarrow \underset{\text{DMS}}{CH_3SCH_3} + \underset{\text{Acrylate}}{CH_2{=}CHCOO^-} + H^+ \quad (1)$$

These methods of sample preparations are detailed in the following section.

2.2.2.1. Purge-and-trap method

This method was based on that developed by Turner *et al.*, (1990) to allow measurement of DMS at a low concentration level, initially in seawater field samples. As indicated in the name, the first step consists of *purging* a liquid sample with a purging gas in order to extract, in a second step, DMS that concentrates in a cryogenic *trap* before injecting it into the GC. Figure 2.8 illustrates the purge-and-trap system.

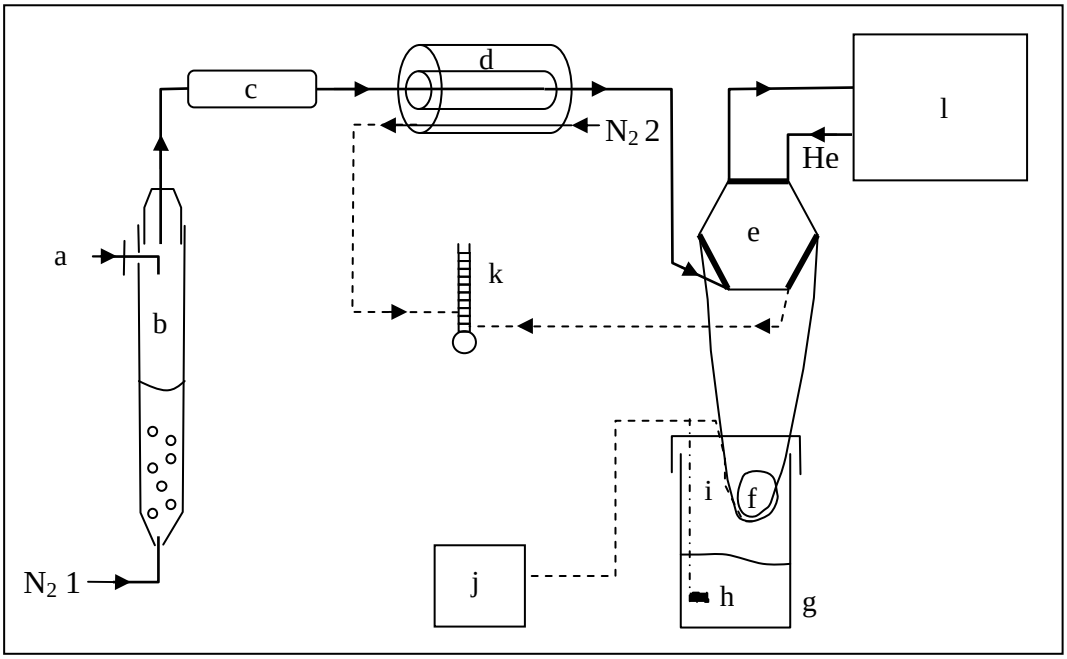

Figure 2.8 Diagram of the purge-and-trap system. It is composed of (a) sample injection port, (b) glass purge tube, (c) glass wool-containing water trap, (d) counter flow Perma Pure gas drier, (e) manual six-port sample stream switching valve, (f) in-house cold trap consisting of: (g) Dewar flask with liquid nitrogen, (h) heating resistor and (i) temperature probe, (j) trap temperature controller, (k) flow meter, (l) GC. Two nitrogen inputs are coming in the system: N_2 1 is the purging gas that carries DMS along the system; N_2 2 is the drier flow of the Perma Pure gas drier. The rates of these two flows are checked by the flow meter.

The procedure was to introduce the filtered culture sample through the injection port (a) into the purge tube (b) which received the purging gas (nitrogen, oxygen-free grade, purified through an activated charcoal filter) through a fine glass frit which generated streams of small bubbles. Using an elongated purge tube increased the path length for nitrogen bubbles and enhanced the purging efficiency. The top of the purge tube was connected by 1/8-inch-o.d. PTFE (polytetrafluoroethylene) tubing to a series of water traps in order to remove water vapour from the gas extract, thus avoiding ice formation and blockages in the cryogenic trap (f). The first water trap (c) was a glass wool-containing glass tube followed by a gas drier (d, MD-050, Perma Pure) as a second trap. The gas drier was composed of a Nafion membrane of 72 inches length which was permeable to water vapour and not DMS: the water vapour was rapidly removed by a counterflow of N_2 (N_2 2, 150 ml min^{-1}). The sample gas extract passed through the cold trap (f, 25 cm $1/8^{th}$ inch PTFE tubing wound in a double loop maintained at – 150 ºC ± 5 ºC) where DMS was accumulated and N_2 passed to the flow meter (k). The cold trap was suspended in the headspace of a Dewar flask containing liquid nitrogen and the temperature was regulated by a simple feed-back system consisting of a temperature sensor (i) attached to the trap, a resistor (h) immersed in the liquid N_2, and an electronic control box (j, designed and built at UEA). If the temperature of the headspace rose above -150 ºC, the resistor generated heat and increased vapour pressure of the liquid N_2 until the headspace achieved the target temperature. This technique was used to avoid the risk of trapping oxygen at lower temperature which, if injected into the detector, can change the flame characteristics and sensitivity. The sample purging lasted for several minutes to allow the accumulation of DMS in the cold trap. I determined that 10 minutes of purging were required for a 5 ml filtered sample with a purge flow rate of 60 ml min^{-1} (detailed in chapter 3, section 3.5.3.1). The cold loop was promptly heated in boiling water while the sample was flushed by the He carrier gas into the GC (l) by switching a six-port valve (e). The gas flow settings of the purge and trap system and the GC settings associated with this method are detailed in Table 2.6.

Table 2.6 GC and flow settings used for the purge and trap system.

GC components	Temperature (°C)	Gas	Flow (ml min^{-1})	Pressure
Injector	200	Helium	Total flow 28.4 Purge flow 3	45.6 kPa
Column	60			
Detector	250	Hydrogen	60	
		Air	70	
Purge and Trap components				
Purging flow		Nitrogen	60	
Drying flow (Nafion drier)		Nitrogen	150	

Standards were prepared in 5 ml gas tight glass vials closed with a screw cap and a Teflon septum. DMSP stocks were prepared by dilution of purchased DMSP in distilled water to a concentration of 0.8 µg S ml^{-1} and stored at -20 °C. The stocks were then defrosted and diluted again with distilled water to solutions of concentrations ranging from 0.004 to 0.016 µg S ml^{-1}. Several DMSP standards were prepared by adding DMSP solutions, distilled water up to 4 ml and 1 ml of 10 M NaOH. The mixture was left overnight in the dark to react. Each standard concentration was prepared in duplicate and all standards covered a concentration range from 0.002 - 0.294 µmol L^{-1} that was broader than the span of sample concentrations. The detection limit was 0.002 µmol L^{-1} based on the lowest concentration used in my calibration. Concentrations measured were well above this detection limit. Standards were injected into the purge tube using a 5 ml disposable syringe.

The method was assessed by checking the linearity of the calibration curve and by calculating the uncertainty of 5 standard replicates. I obtained excellent linearity using this method as shown in Figure 2.9 (R^2=1). Also I obtained very good precision with 5 standards replicates (RSD = 3.2 % and p_x= 3.9 %).

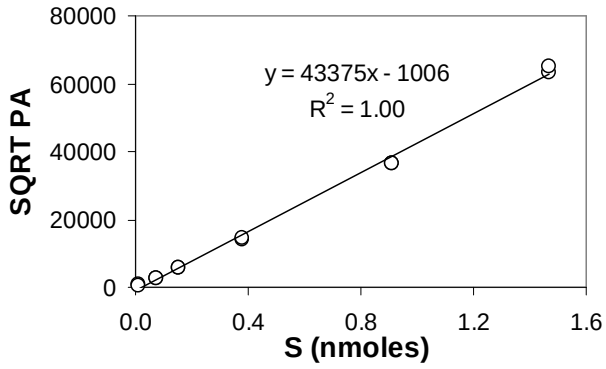

Figure 2.9 Example of calibration curve obtained with the purge and trap method. (SQRT PA = square root of the peak area) for duplicate DMS standards ranging from 0.01 to 1.47 nmoles. The linear regression curve is shown with its correlation coefficient R^2.

DMS was measured in the aqueous fraction of cultures. A sample preparation system for small volumes (< 2ml) was built. The S-S-PT system (Syringe pump - Sample loop - Purge and Trap, Figure 2.10) was used to filter and introduce the culture sample into the purge and trap system. The aim was to introduce precise and reproducible sample volumes.

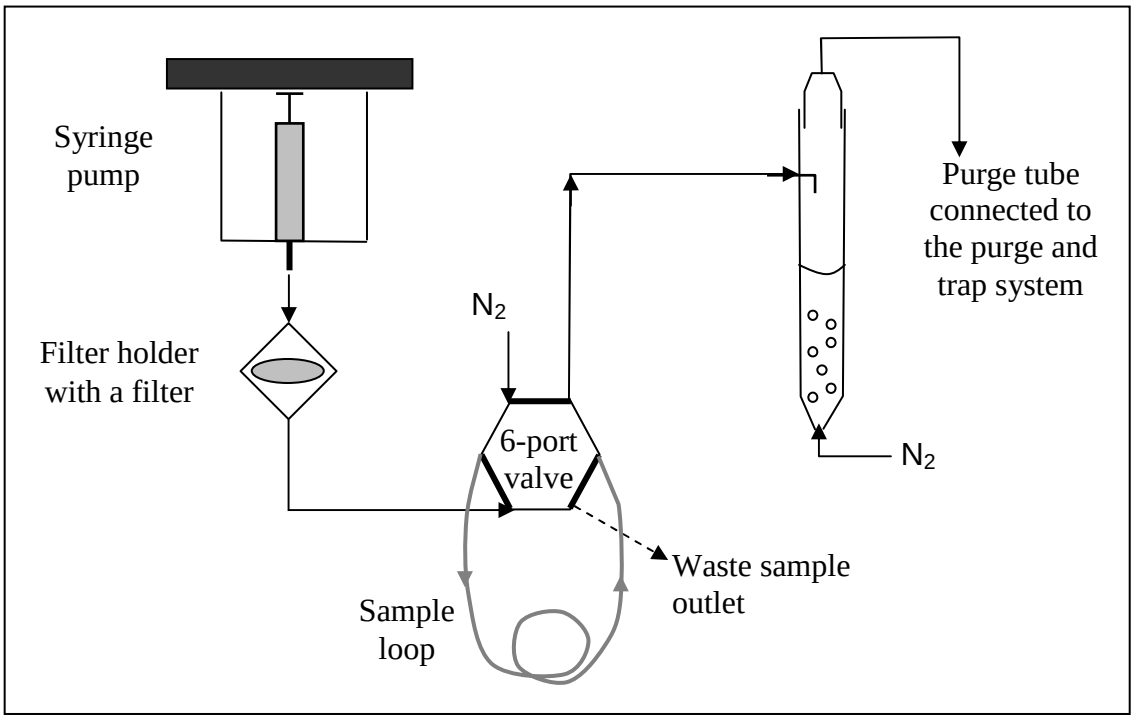

Figure 2.10 S-S-PT system: Syringe pump - Sample loop - Purge and trap system used for sample preparation (filtration and injection into purge tube) before DMS measurement.

From a culture aliquot, 5 ml of sample was taken up with a 5 ml disposable syringe fitted with a stopcock and placed on the syringe pump (KDS220, Linton Instrumentation, Diss, United Kingdom). The pump was set to a filtration speed of 14 ml min^{-1} (maximum speed available for this syringe volume) as I observed that rapid filtration minimised the conversion of DMSP to DMS (chapter 3, section 3.5.1.2.3 and 3.5.3.2). Only 4.7 ml of the sample was filtered to avoid stressing the cells as I observed that a gradient of DMS was generated over the filtration (chapter 3, section 3.5.3.2). The filtration was through a filter holder (Swinnex, Millipore, Watord, UK) containing a 25 mm diameter glass-fibre filter (Whatman, GF/F). This filter holder was connected to a sample loop which ensured accurate delivery of small sample volumes. The sample loop was made out of $1/8^{th}$ inch of PTFE tubing and was connected to a six-port valve.

After loading, the sample was flushed into the purge tube. Loops of different volumes were used (0.2, 0.5 and 2 ml).

2.2.2.2. Headspace method for DMSP

This method has been developed by Steinke et al., (2000) to measure DMS production by DMSP lyase and has been adapted in this study for measuring DMSP. This technique allows measurement of elevated concentrations of DMSP (DMS up to 80 µmol L^{-1}) in a liquid sample made alkaline by NaOH addition. Liquid samples are equilibrated with a headspace of air and the partition coefficient for DMS is controlled by temperature.

For total DMSP ($DMSP_T$= particulate + dissolved DMSP + DMS), 1 ml of culture was directly introduced into a 5 ml vial containing 2 ml of 0.75 M NaOH. The vial was immediately closed with a screw cap and a Teflon septum, then agitated. Samples were left in the dark to react (> 12 h) and could be stored at that stage in the dark for two weeks. Prior to the analyses, samples and standards were put overnight (12 h) on a heating block set at 30 ºC to allow DMS to equilibrate between the liquid and gas phase. Eighty µl of the headspace was sampled and injected into the gas chromatograph by means of an autosampler (Multipurpose sampler MPS, Gerstel, Mülheim an der Ruhr, Germany) composed of an automatic arm equipped with a 100 µl heated glass syringe. The GC settings applied for this method are described in Table 2.7.

Table 2.7 Description of the GC settings associated with the headspace method

GC components	Temperature (ºC)	Gas	Flow (ml min^{-1})	Pressure
Injector	200	Helium	Total flow 34.7	68.5 kPa
			Purge flow 3	
Column	120			
Detector	250	Hydrogen	60	
		Air	70	

A concentration range of duplicate DMSP standards was used to calibrate the GC and calculate the DMSP concentration of the sample. The standards were prepared as described in Steinke et al. (2000) by adding a drop of DMSP stock solution into the cap of the vial. The vial, containing 3 ml of 0.5 M NaOH, was closed and agitated. The samples and the standards had the same headspace volume and final NaOH concentration. An example of a calibration curve is shown in Figure 2.11.

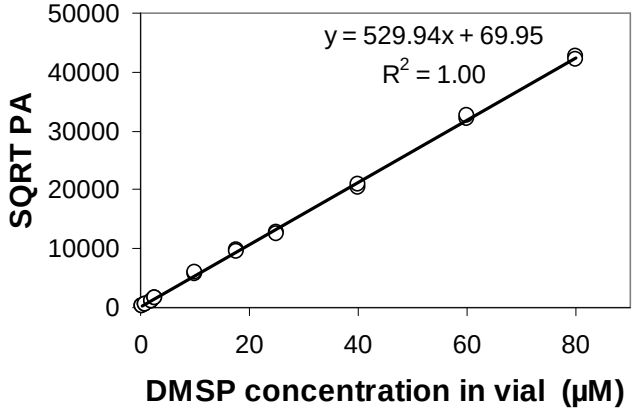

Figure 2.11 Example of calibration obtained for headspace method of DMSP measurement by gas chromatography. (SQRT PA= square root of the peak area).

The uncertainty of this method was RSD= 1.8 % and p_x = 2.2 % for standards (n=5) and RSD= 1.3 % and p_x = 1.6 % for *H. triquetra* samples (n=5). When normalising DMSP to cell number or total CV, the uncertainty was RSD= 2.5 % and 2.6 % respectively and p_x=1.6 %.

2.2.2.3. Headspace method for *in vitro* DMSP lyase activities

The method was based on the description from Steinke et al., (2000). One hundred ml of dinoflagellate culture was sampled in mid to late exponential phase. The culture sample was filtered through a 2 µm pore size polycarbonate filter of 47 mm diameter. The speed of filtration was gently improved by means of a hand vacuum pump (< 15 cm Hg). The filter was folded 3 times and cut into pieces of a suitable size to fit into a 1.8 ml sterile cryogenic vial (cryotube, Thermo Scientific Nunc) containing 1.7 ml of 300 mM tris buffer in 500 mM NaCl and adjusted to pH 8.2 with concentrated HCl. The sample was sonicated (sonicator Microson XL2000, Misonix, Farmingdale,

USA) at 5 W, 5 pulses of 5 s with 10 s intervals to ensure cell extraction without heating and enzyme damage. The cryogenic vial containing the enzyme extract was snap-frozen in liquid nitrogen and stored at – 80 ºC for later analysis. This cold storage after extraction does not affect the DMSP lyase activity (Steinke et al., 2000).

Prior to measurement of DMSP lyase activity, the enzyme extract was defrosted and kept cold in ice. Then, 295 µl of enzyme extract was introduced into 2 ml glass vials closed with a screw cap and a Teflon septum. The vials were incubated at 30 ºC on a heating block in agreement with the standard temperature recommended to perform an enzyme assay (International Union of Biochemistry, 1965). Fifteen µl of the vial headspace was auto-sampled at 10 to 15 minute intervals before and after the addition of 5 µl 1.2 mM DMSP stock solution. Two samples were taken before the addition of DMSP to obtain the baseline activity. Then, DMSP (previously purged for 30 minutes to reduce the amount of free DMS) was added by rapidly injecting the DMSP with a Gilson pipette and immediately closing and agitating the vial. DMS production was then monitored over time by performing 4 sequential measurements. To determine the DMSP lyase activity, DMS production was compared between vials containing enzyme extract and vials containing tris buffer (295 µl), both receiving DMSP addition. An example of the data obtained is shown in Figure 2.12.

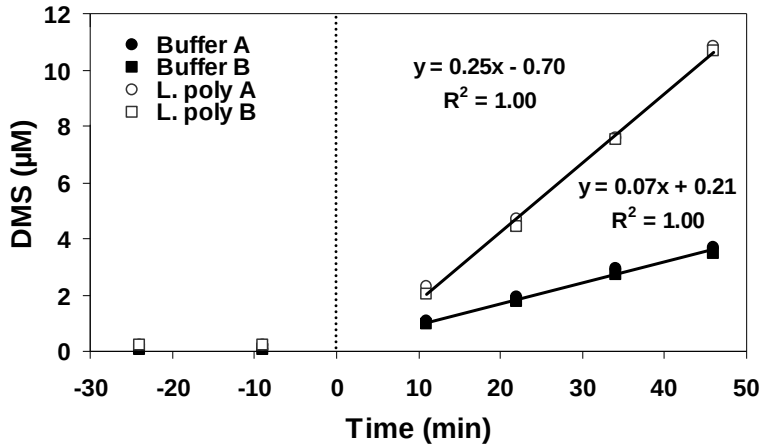

Figure 2.12 Example of DMS production measured in *Lingulodinium polyedrum* extract samples (L. poly) and in tris buffer (Buffer). A and B are duplicate samples of the algal cell extract. The dotted line shows the addition of DMS at time 0.

DMS production rate was calculated from the slope generated by all the sampling points after DMSP addition, corrected by a loss rate (+ 6 %) due to the DMS loss of the pierced septum of the vial and corrected by the DMS production measured in the buffer vials that represent the DMSP abiotic cleavage as applied by Steinke et al. (2000). The gas chromatography system was calibrated using duplicate standards prepared by mixing 300 µl NaOH with a drop of DMSP stock solution in the 2 ml vials and covering a concentration range of 0.10 - 30 µmol L^{-1}. An example of a calibration curve is shown in Figure 2.13. The detection limit of the assay was based on the average DMS production detected in buffers of 7.34 nM h^{-1}.

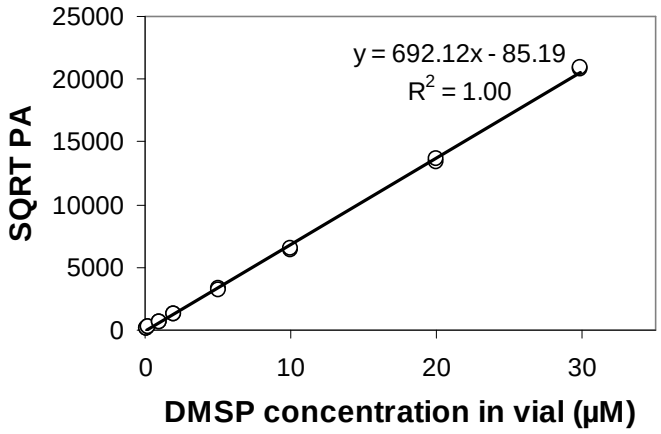

Figure 2.13 Example of calibration curve obtained by gas chromatography for DMSP lyase measurements for duplicate DMSP standars ranging from 0.1 to 30 µmol L^{-1}. The linear regression curve is shown with its correlation coefficient R^2. (SQRT PA= square root of the peak area)

2.2.3. Fluorometer and *in vitro* determination of chlorophyll *a* by acetone extraction and acidification method

A fluorometer measures the fluorescence emitted by pigments such as chlorophyll. When excited with low wavelengths of high energy light (blue light at ~ 450 nm) the chlorophyll fluoresces by emitting longer wavelengths of lower energy light (red light at 680 nm). The fluorescence is generally proportional to the chlorophyll *a* (Chl *a*) concentration, if no other pigment interferes with the signal. The AU-10 Field Turner Designs fluorometer was equipped with a 10-045 lamp, an excitation filter of 340 - 500 nm and an emission filter > 650 nm.

The method of chlorophyll extraction from cells by acetone and acidification was applied, according to the methods described by Parsons et al (1984). Phaeophytin is a natural degradation product of Chl *a* (i.e. loss of the Mg ion), present in variable amounts and can interfere with the fluorescence signal of Chl *a* (structure of Chl *a* is shown in Figure 2.14). In order to correct for this background fluorescence, the sample is acidified, to convert any Chl *a* to phaeophytin. The equations (4 and 5 detailed later) used for calculating the Chl *a* concentrations incorporate correction for the original phaeophytin contribution.

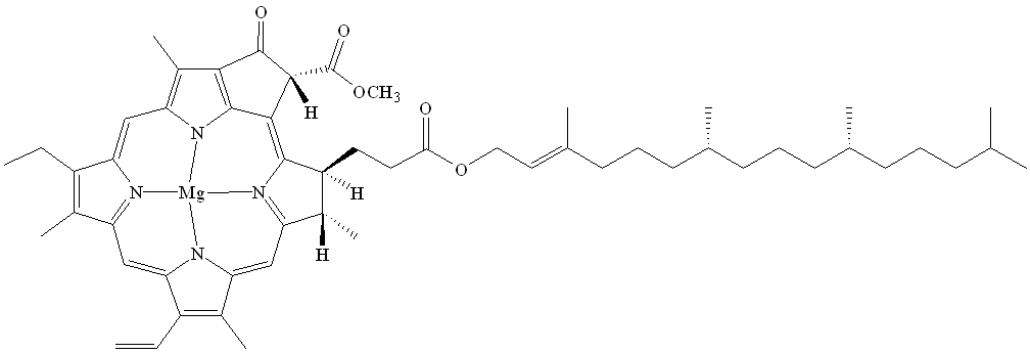

Figure 2.14 Chemical structure of chlorophyll *a*.

For the sample preparation, 5 ml of culture were filtered by gravity through a GF/F filter of 25 mm of diameter on a glass filtration unit. The use of a filter holder without a fritted glass filter support allowed fast gravity filtration of a couple of

minutes. The filter was folded in half, wrapped in foil and snap-frozen in liquid nitrogen prior to storage in a freezer at – 80 ºC. This storage prevents the degradation by light and heat without significant chlorophyll loss for up to a month, as recommended by the Method 445.0 Revision 1.2 from the U.S. Environmental Protection Agency (Arar E. J. and Collins G. B., 1997)

The following steps of Chl *a* extraction from cells were performed in a low light environment to prevent chlorophyll degradation. The filter was introduced into a 20 ml glass vial, closed with a screw cap, then 10 ml of 90 % acetone (prepared by mixing analytical grade acetone with distilled water) were added. These steps were repeated for each filter and the set of vials was covered with foil and placed in a fridge at 4 ºC in the dark to achieve the chlorophyll extraction during 20 to 24 hours. During this period, vials were manually agitated several times to improve the extraction. About 9 ml of extract were poured into a borosilicate tube for fluorescence reading. The fluorescence was measured before and after acidification with 3 drops of 8 % HCl. The chlorophyll *a* concentration was calculated according to equation 2 (Parsons T.R. et al., 1984):

$$\text{Chlorophyll } a \text{ in mg/L} = F_D \times (Y/Y\text{-}1)\,(U - A)\,v / V \qquad (2)$$

Where,

F_D = mean (Chl*a* concentration / U) for chlorophyll standards

Y = mean (U / A) for chlorophyll standards

U = fluorescence before acidification

A = fluorescence after acidification

v = volume of extract (ml)

V = volume of culture (ml)

The fluorometer was calibrated with a range of pure Chl *a* solutions of known concentrations (0.006 - 0.227 mg L^{-1}) on the day of the sample analyses. These standard solutions were prepared from a stock solution of 1 mg L^{-1} of Chl *a* extracted from *Anacystis nidulans,* Cyanophyceae (Sigma-Aldrich, C6144) and diluted in 90 % acetone. Prior to the standard preparation, the concentration of the stock solution was checked with a spectrophotometer (Lambda 25, Perkin Elmer) and the average value of

the results obtained from calculations of the Scor (U.N.E.S.C.O., 1966) and Lorenzen methods (Lorenzen, 1967), equations 3 and 4, was used.

Scor Chl *a* = 11.6(U665) – 0.14(U630) – 1.31(U645)	(3)
Lorenzen Chl *a* = 26.7(U665-A665)	(4)

Where U is the absorbance of the solution and A is the absorbance after acidification with 2 drops of 8 % concentrated HCl (which is equivalent to 2.88 % chloride).

An example of fluorometer calibration is illustrated in Figure 2.15. The fluorometer has a linear response to a range of Chl *a* standards measured before and after acidification. The average ratio U/A is used in the calculation as detailed in equation 2. The fluorescence measurements on dinoflagellate species showed a RSD that ranged between 14 and 55 % (n=9); (see chapter 4 for more details).

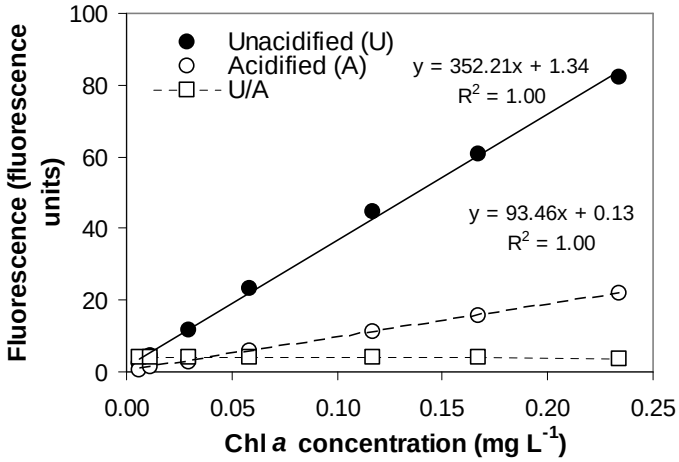

Figure 2.15 Example of linear response of the fluorometer for fluorescence measurements of non-acidified and acidified Chl *a* standards. The average ratio U/A is used in the calculation of sample Chl *a* concentrations.

2.2.4. Elemental analyser and CHN measurement

The elemental analyser (CE440, Exeter Analytical, Coventry, UK) was used to determine the C and N composition of a culture sample. I was interested in using the C and N content as another culture characteristic (Matrai and Keller, 1994; Menden-Deuer and Lessard, 2000) and to express DMSP concentration per C units. These data are particularly useful for modelling studies.

The analysis was performed on cells harvested by filtration and dried. In this regard, 2 ml of culture were filtered through a filter holder (Swinnex, Millipore, Watord, UK) containing a 13 mm diameter GF/F filter (Whatman, GF/F), previously combusted at 450 ºC for 4 hours in a muffle furnace. The gentle filtration was performed using a hand vacuum pump (< 5 cm Hg). The filter was folded in half, wrapped in foil and rapidly cooled in liquid nitrogen prior to storage at – 80 ºC. The samples were stored for less than a month before analysing a set of samples. Two days before analysis, the samples and blank filters were dried at 35 ºC for 48 hours then placed in nickel capsules, previously combusted at 1000 ºC for 1 h.

Figure 2.16 is a schematic diagram of the components of the instrument. To start the analyses, the samples were placed into an autosampler where they were purged continuously with helium which is used as a carrier gas through the analytical system. The first sample fell into a combustion chamber injected with pure oxygen, inside a high temperature furnace at 928 ºC. These conditions lead to the combustion of the sample into elements (C, H, N). The gas mixture was carried through oxidation reagents that transform C into CO_2, H to H_2O and N to N_xO_y. The oxidation reagents also removed other unwanted elements like phosphorus, halogens and sulphur. Then the gas sample passed in a copper column which reduced the nitrogen oxides to nitrogen and eliminated the surplus of oxygen.

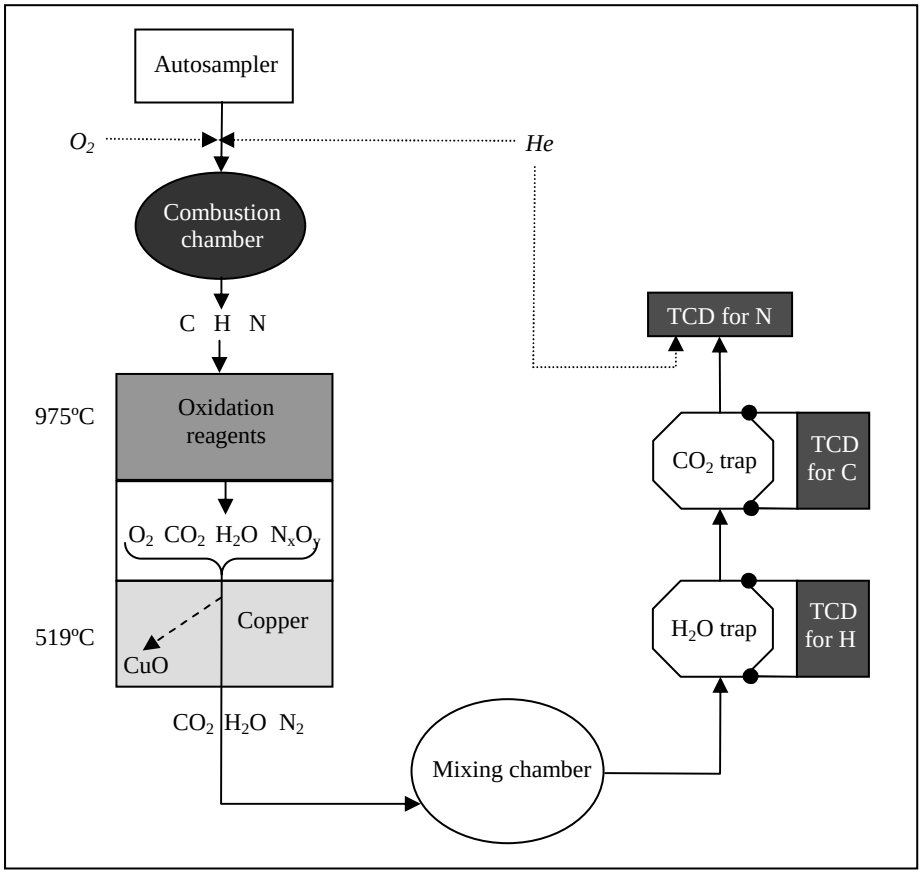

Figure 2.16 Schematic diagram of the CHN elemental analyser (CE440, Exeter Analytical).

The gas sample was pulsed into the mixing chamber that ensured constant pressure, temperature and homogeneity of the sample. The gas stream then flowed through a series of 3 thermal conductivity detectors (TCD). First, a water absorption trap was connected to a TCD which detected the water content of the gas stream before and after it passed through the water trap. Similarly, a CO_2 trap connected to another TCD resulted in C detection. Finally N was detected by a TCD which compared the gas stream containing N and He with another flow of pure He as reference. The detection signal is proportional to the concentration of components in the sample and this concentration is calculated from standards. The analyser was adjusted with 10 standards of 2 mg of acetanilide (C_8H_9NO) which were measured at the beginning of the run and at intervals between samples. The sample measurements were blank corrected with analysis of pre-combusted, blank filters. The RSD (σ_x) obtained for the C and N measurements ranged between 7 and 32 % (n=9); (see Chapter 4 for details).

2.2.5. Uncertainty analyses

For the analytical methods that were used commonly through my study (particle counter, purge-and-trap and GC , headspace analyses and GC), I have assessed the precision of the measurements using 5 replicates of standards or of *Heterocapsa triquetra* culture samples in exponential phase. The uncertainty was calculated in two ways (equation 1): the standard deviation of the sample (σ_x) also reported as the relative standard deviation (RSD) and the precision (p_x) obtained by using the *t*-statistic derivation for small numbers (< 30) of samples (equation 5, Beckwith T.G. et al. 1993).

$x \pm p_x$	$p_x = t_{0.025,\,\nu} (\sigma_x / \sqrt{n})$	(5)

Where x is the average of the result, *t* is the value read out from the student distribution for a probability of 0.025 or 95% confidence and a degree of freedom ν equivalent to n – 1 where n is the number of samples.

The uncertainty of a result involving the measurement of 2 parameters was calculated with the formula of error propagation (equation 6, Lichten 1999). For instance, the DMSP value (z) expressed per cell volume includes the errors of measurement for both DMSP (x) and cell volume (y).

$z = x / y$	$p_z = p_{(x/y)} = \sqrt{[(x/y)^2 ((p_x/x)^2 + (p_y/y)^2)]}$	(6)

Chapter 3 Testing and optimising the methodology for DMSP and DMS measurement in dinoflagellate cultures

Abstract

I have performed a series of experiments on *Heterocapsa triquetra* batch cultures to optimise the measurement of DMSP and DMS in dinoflagellate cultures. I found that DMSP concentrations were increasing during the photoperiod and at the end of the exponential growth phase. The sampling time for further experiments was, therefore, established to be at the mid-time of the photoperiod and at mid-exponential phase to enable the determination of the effect of the experimental treatment. I assessed the mass balance of measurements of total DMSP ($DMSP_T$) and the sum of its individual fractions ($DMSP_p$, $DMSP_d$ and DMS) and found that measurement of $DMSP_T$ gave 25 % more than the sum of the parts. As $DMSP_p$ was the major fraction, I investigated the method of $DMSP_p$ measurement with a special focus on the filtration methods. Overall, the use of a hand vacuum pump, an automated syringe pump and gravity filtration did not show significant difference in $DMSP_p$ concentration. When using the syringe pump, $DMSP_p$ concentrations were increased and the analytical uncertainty reduced by setting the highest delivery speed. $DMSP_T$ measurements showed a better analytical uncertainty than $DMSP_p$ measurements and no significant difference with $DMSP_p$ concentrations, which supports the use of $DMSP_T$ as a surrogate of intracellular DMSP. DMS measurement was also optimised with the use of the syringe pump at high delivery speed and a sample loop to inject the constant volume of samples. The analytical uncertainty of culture samples were rather higher than the standards but the study showed that shortening the filtrations and using a small volume resulted in lower DMS concentrations.

3.1. Introduction

Prior to investigating scientific questions on DMSP and DMS production by dinoflagellates, the first issue was to assess and optimise the methods applied for the measurement of these parameters. Intracellular DMSP is also called particulate DMSP ($DMSP_p$). The fraction of DMSP released into the culture medium by cell lysis and exudation is called dissolved DMSP ($DMSP_d$). It can then lead to the DMS formation by enzymatic cleavage. Some DMS may also potentially be contained by cells: it was initially thought that the DMSP lyase was segregated from its substrate DMSP in the cell (Wolfe and Steinke, 1996) but more recently, Sunda et al. (2002) have hypothesised that this enzyme could produce intracellular DMS. Here, total DMSP ($DMSP_T$) includes $DMSP_p$, $DMSP_d$ and DMS (Figure 3.1) and differs from the notation $DMSP_t$ that can be found in other studies and which excludes DMS.

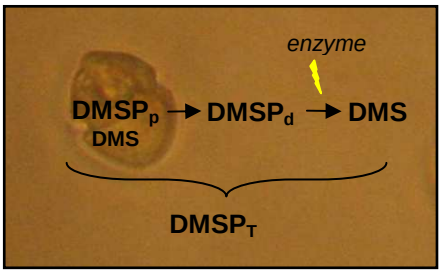

Figure 3.1 Illustration of the $DMSP_T$ fractions in a phytoplankton culture.

The methods used to measure these parameters were tested and optimised for dinoflagellate cultures. Several sources of variation may influence the result such as the biology of the organism and sample preparation.

The biology of the organism involves changes in the metabolic processes throughout the diel cycle and the life time of the organism and the population. The diel cycle determines a circadian rhythm in photosynthetic dinoflagellates such as *Lingulodinium polyedrum* (Roenneberg and Merrow, 2001) with growth occurring during the light period and cell division at the end of the dark period (Wong and Kwok, 2005). Diel vertical migrations (Raven and Richardson, 1984) are also performed by

dinoflagellates. To test whether the diel cycle would affect the DMSP content of the photosynthetic dinoflagellate *Heterocapsa triquetra*, $DMSP_p$ concentrations were monitored over 14 h of the photoperiod. Over the life time of a phytoplankton population, the physiology of the organism may change with ageing and nutrient limitation. To determine whether the growth stages of the population would affect the DMSP content of dinoflagellates, $DMSP_p$ concentrations were monitored in *H. triquetra* cultures over 12 days. By identifying these potential biological variations in DMSP concentrations, I aimed to determine the sampling conditions that would limit these variations when comparing different experimental treatments.

Dinoflagellates are fastidious organisms known to be sensitive to agitation and prone to cell damage (Berdalet and Estrada, 2006). The consequences on the measurement of compounds such as DMSP and DMS is not well known and need to be considered. Kiene and Slezak (2006) pointed out that the effect of filtration pressure on $DMSP_d$ release due to cell breakage could lead to overestimation of this parameter. This finding implies that $DMSP_p$ would be lost by the cell and transferred to the dissolved fraction. Therefore, $DMSP_p$ would potentially be underestimated. On the other hand, mechanical stress such as turbulence may trigger an increase in intracellular DMSP concentration in dinoflagellates (Llaveria et al., 2009a). Therefore, the filtration process may affect DMSP measurement by increasing or lowering its result. For these reasons several filtration systems and protocols were investigated for DMSP and DMS measurements.

I have conducted a series of experiments on batch cultures of the dinoflagellate species *Heterocapsa triquetra* to determine the potential sources of variation and imprecision in DMSP and DMS measurements, with the aim of optimising method assay for their measurement.

3.2. *Heterocapsa triquetra* as a laboratory model

Heterocapsa triquetra is a photosynthetic and bloom-forming dinoflagellate commonly found in the world in estuaries and coastal waters (Litaker et al., 2002). *H. triquetra* is easily cultured and has become one of the most investigated dinoflagellates in laboratory work, especially in molecular studies (McEwan et al., 2008). This species is included in a genome sequencing project in Canada. *H. triquetra* may be considered as a laboratory model for photosynthetic dinoflagellates due to its growing ability in laboratory cultures, geographic distribution and the available knowledge. It is a thecate species with a basic dinokont shape like a diamond (Figure 3.2).

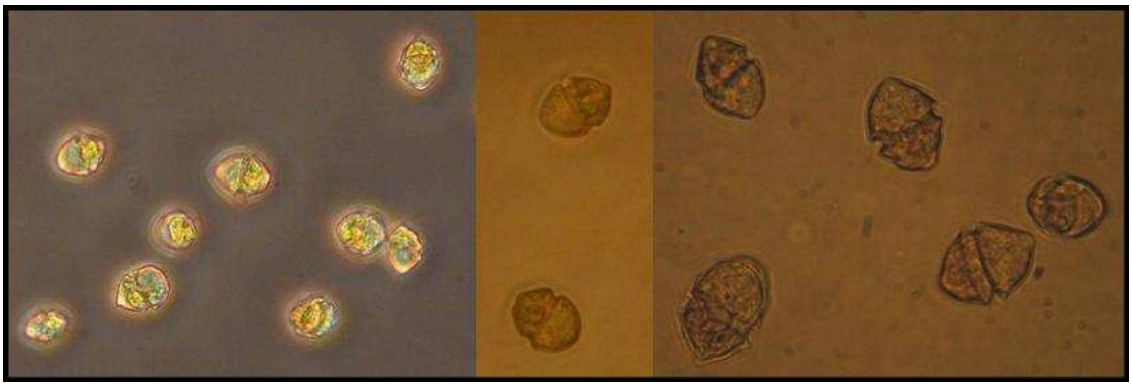

Figure 3.2 *Heterocapsa triquetra* cells in exponential growth phase observed under the microscope at several magnification objectives 40-fold and 100-fold and phase contrast; the cell length was 22 µm according to microscope measurements.

The following experiments presented in this chapter have been conducted with *H. triquetra* as an example of dinoflagellate behaviour and response to the sample preparation and analytical techniques used for DMS and DMSP measurements. *Heterocapsa triquetra* CCMP 449 was kept axenically and was grown in batch cultures at 15 ºC under 150 ± 50 µmol photons m^{-2} s^{-1} with a 14:10 light:dark cycle.

3.3. Statistical analyses

Statistical analyses were conducted to compare and assess the use of several techniques applied to DMSP and DMS measurements. Given that I observed that DMSP values are not normally distributed, I used non-parametric tests.

The non-parametric Mann-Whitney *U* test (SPSS package, version 16.0) was performed to determine if 2 datasets were statistically different ($P<0.05$) or similar ($P>0.05$). This test was applied to compare the DMSP values obtained with 2 different methods such as 2 different volumes of culture or 2 methods of filtration. The Mann-Whitney *U* test was also performed for comparing 2 methods of standard preparation for $DMSP_T$ measurements and for comparing $DMSP_p$ and $DMSP_T$ values.

The non-parametric Kruskal-Wallis test (SPSS package, version 16.0) was conducted for determination of the significant difference between a group of several datasets and identifying the effect of a variable factor. In this study, this test was applied for assessing the effect of growth on $DMSP_p$ concentrations and also for the comparison of $DMSP_p$ data obtained at various delivery speeds of the syringe pump.

The Spearman test (SPSS package, version 16.0) was performed to establish or exclude a potential correlation between 2 parameters such as DMSP concentrations and the duration of the photoperiod.

The analytical uncertainty of a method was represented by the relative standard deviation (RSD) associated with the average of several replicates. The comparison of RSD obtained for different methods was applied to assess the method that gives the best reproducibility (lowest RSD).

3.4. Biological variation of DMSP concentrations

In order to enable comparison of DMSP concentrations during an experiment and between experiments, it was important to establish if there was variability associated with different stages of the light cycle and growth. The results of the following experiments were used to determine the optimum times of sampling.

3.4.1. Variation of DMSP concentration over the photoperiod

To investigate the diel pattern of DMSP concentration in *H. triquetra*, the $DMSP_p$ concentration was monitored during the 14 hours of photoperiod. The first sample was taken just before the start of the light cycle. The second sample was 1 hour later and the following samples were taken every two hours. The following parameters were monitored: $DMSP_p$ and total cell volume. $DMSP_p$ measurements were performed by gently filtering 2 ml of culture (the selection of the sample volume is detailed in section 3.5.1.2.1) with a hand vacuum pump (<5 cm Hg). The $DMSP_p$ per cell volume concentrations, which are an equivalent of concentration per biomass, are shown in Figure 3.3. The $DMSP_p$ samples are measured using the headspace method and analysed by gas chromatography using a range of DMSP standards (section 2.2.2.2).

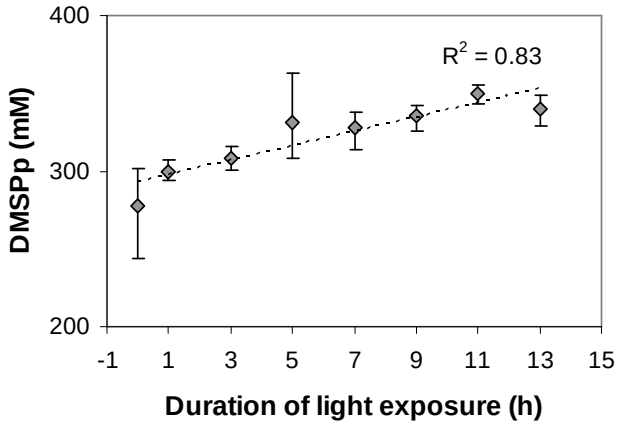

Figure 3.3 Effect of 14 h light exposure on $DMSP_p$ concentration per cell volume (in mM equivalent to mmol L_{cell}^{-1}) in a *Heterocapsa triquetra* culture. Averages are derived from 3 replicate samples and range bars show minimum and maximum values. The dashed line is the linear regression of these 2 parameters and the correlation coefficient R^2 is shown.

The $DMSP_p$ concentration showed an increase (277 to 350 mM at maximum, 27 % increase) with increasing duration of light exposure and these 2 parameters were significantly correlated ($P<0.01$, Spearman test). In agreement with previous observations and hypothesis of Merzouk et al., (2004), the $DMSP_p$ concentrations of photosynthetic dinoflagellates showed a variation over the photoperiod. Potentially acting as an antioxidant, DMSP could be involved during the light cycle to protect cell damage from the oxidative photosynthetic products (hydroxyl radicals, singlet oxygen and other reactive oxygen species; Sunda et al., 2002). However, whilst Merzouk et al., (2004) found maximum $DMSP_p$ concentrations occurring at midday in field samples, I observed here, that DMSP seemed to accumulate over the photoperiod. The difference between field and laboratory observations might be due to the fact that light intensity is constant in the laboratory incubator whereas in natural conditions the light intensity reaches its maximum at midday. On the other hand, another photo-protective compound, UV absorbent mycosporin-like amino acid (MAA) accumulates in culture of the dinoflagellate *Scrippsiella sweeneyae* with a maximum concentration in the middle of the light period (Taira et al., 2004). In cultures of the coccolithophore *Emiliania huxleyi,* DMSP concentrations also show a diel cycle with maximum occurring at the end of the dark period and decreasing over the light period (Bucciarelli et al., 2007). The diel cycle of DMSP appear to differ among phytoplankton species.

According to these results, experiments which involve, for instance, one sample per day over several days should have a sampling time that remains constant (at the same time for each day) to limit any effect of diel variability. Therefore, I established, for the following experiments, to sample the cultures at the mid-time of the photoperiod, at 7 hours after the start of the photoperiod.

3.4.2. Variation of DMSP concentration over growth stages

The variation of $DMSP_p$ concentrations was also investigated during the growth of *H. triquetra*. The culture was sampled every three days, during 12 days. The $DMSP_p$, total cell volume and cell density were monitored. $DMSP_p$ measurements were

performed as previously described. Figure 3.4 shows data for the growth parameters and DMSP concentrations.

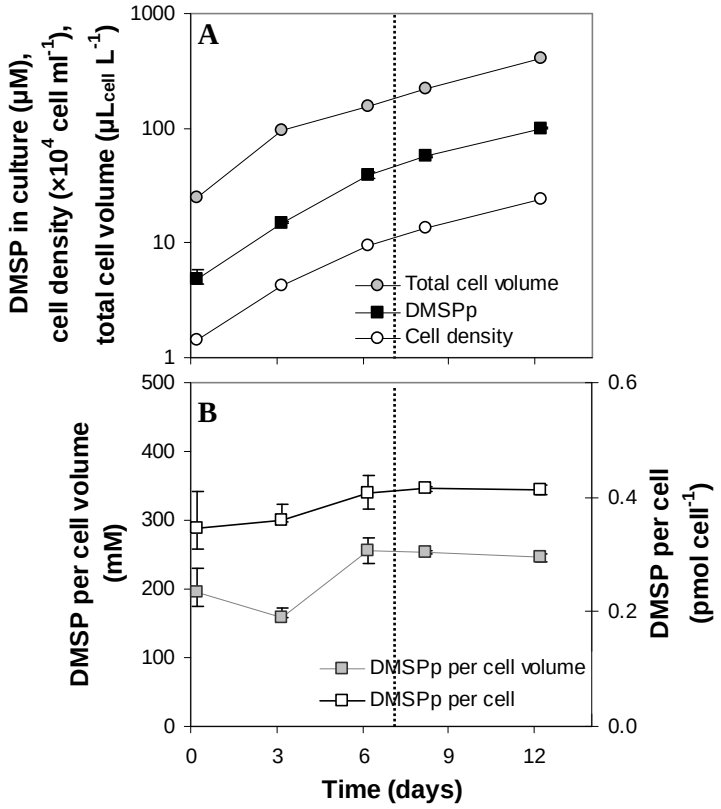

Figure 3.4 Growth and $DMSP_p$ content of *Heterocapsa triquetra* over 12 days. (A) Total cell volume, cell density and $DMSP_p$ concentrations in culture. (B) $DMSP_p$ concentrations per cell volume and per cell. Cell density and total cell volume were measured from 1 culture aliquot sampled once every 3 days. $DMSP_p$ values are averages derived from 3 replicate samples; range bars show minimum and maximum values. The dashed line separates the exponential growth phase from the onset of the stationary growth phase based on the growth rate reduction.

H. triquetra culture grew exponentially during the first 6 days from 1.4×10^4 to 9.6×10^4 cell ml^{-1} (25 to 153 μL_{cell} L^{-1}) then the growth slowed down towards the stationary phase to reach a maximum cell density of 2.4×10^5 cell ml^{-1} (407 μL_{cell} L^{-1}) after 12 days (Figure 3.4 A). $DMSP_p$ concentrations in the culture increased similarly to the total cell volume and the cell density. The $DMSP_p$ per cell volume values appeared to increase at the end of the exponential phase (Figure 3.4 B). A significant difference was observed between the DMSP concentration per cell volume at the different sampling times from 0 to 12 days ($P<0.05$, Kruskal-Wallis test) and hence, there is a significant effect of the growth on the $DMSP_p$ concentration per cell volume (from 159 to 256 mM so 61 % increase over the growth). The $DMSP_p$ per cell seems to slightly increase from 0 to 6 days during the exponential growth phase and significant difference

was obtained for the $DMSP_p$ concentrations per cell between the exponential growth phase (0 - 6 days) and the onset of the stationary growth phase (9 - 12 days); ($P<0.05$, Mann-Whitney *U* test).

Additionally, other experiments on the heterotrophic dinoflagellate *Crypthecodinium cohnii* have shown some substantial variations of the DMSP concentrations over the growth stages (presented in Chapter 6). Given that DMSP concentrations vary over growth stages, I decided to perform the sampling of the culture at the same growth stage to allow comparison between experiments. I choose the mid-exponential phase of growth as it is usually used to avoid the effect of nutrient limitation and ageing population, as for example to compare the DMSP content of several species.

3.5. Effect of the methods of sample preparation on DMSP and DMS concentrations

At the beginning of my laboratory work with dinoflagellates, I measured the total DMSP in cultures ($DMSP_T$) and all the fractions $DMSP_p$, $DMSP_d$ and DMS to assess if there was a mass balance. At that time, DMS measurements were performed on the filtrate collected from 2 ml of culture passed through a 25 mm diameter glass-fibre filter (Whatman, GF/F) by using a hand vacuum pump (not exceeding <5 cm Hg) and directly poured into the purge tube of the purge and trap system. For $DMSP_d$ +DMS measurements, 1 ml of NaOH was added to another filtrate of sample obtained similarly to the DMS sample and the $DMSP_d$ value was calculated by subtraction of the DMS value. The $DMSP_p$ and $DMSP_T$ measurements were done as previously described (section 2.2.2.2 and 3.4.1). Figure 3.5 gives a general overview of the DMSP pool and fractions and the analytical methods applied for their measurements.

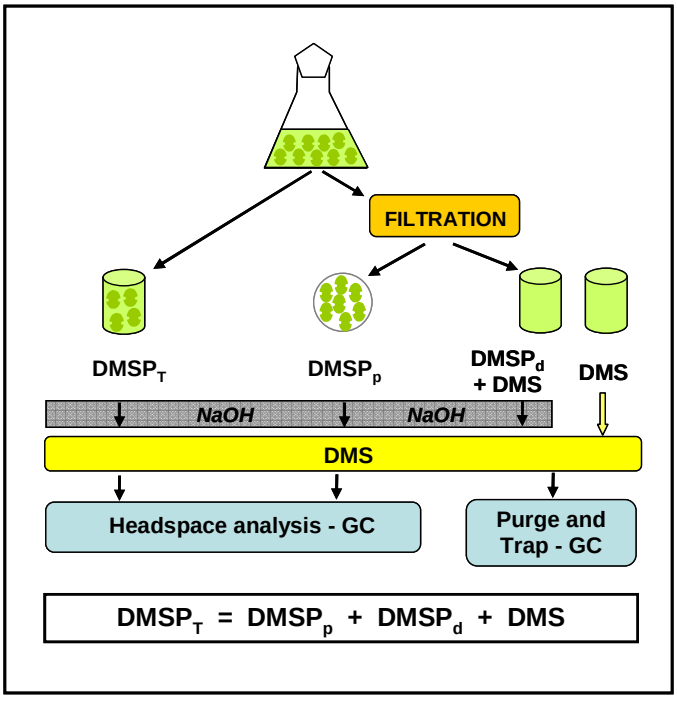

Figure 3.5 Summary of the techniques applied to the measurement of $DMSP_T$ and its fractions. GC indicates the use of gas chromatography. $DMSP_T$ is measured on the liquid culture sample, $DMSP_p$ is analysed from the filter which has collected the cells and $DMSP_d$ and DMS are measured in the filtrate.

On 2 successive days, I performed sampling of a culture of *Heterocapsa triquetra* in exponential phase. Each day I took 3 samples from this culture and measured the 4 parameters $DMSP_T$, $DMSP_p$, $DMSP_d$ and DMS in each of the 3 samples. Figure 3.6 shows the data normalised to cell volume.

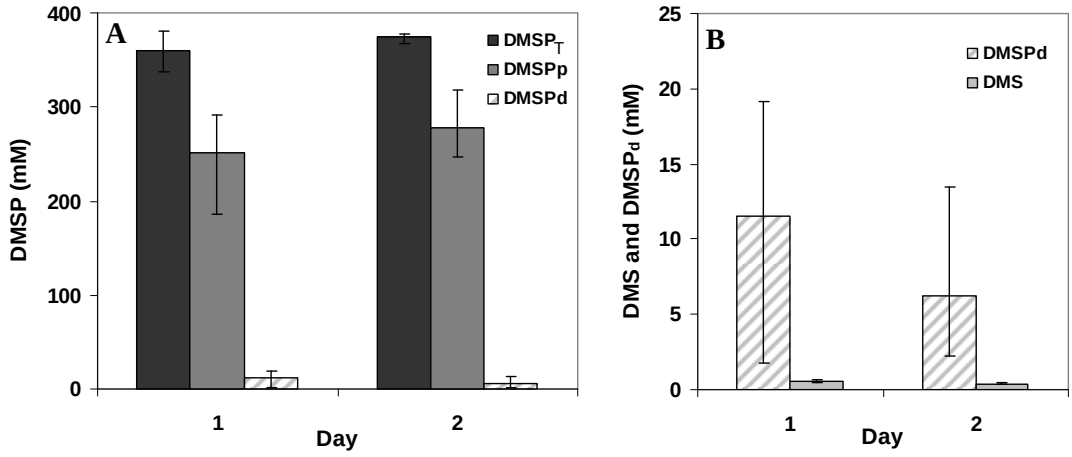

Figure 3.6 Concentrations per cell volume of $DMSP_T$ and its fractions $DMSP_p$, $DMSP_d$ and DMS measured in a sample of *Heterocapsa triquetra* culture in exponential phase. Results are presented on 2 panels because of the broad span of the data (A) $DMSP_T$, $DMSP_p$, $DMSP_d$. (B) $DMSP_d$ and DMS. Data are average values from 3 replicate samples and range bars show minimum and maximum values.

According to these results, it appeared that the sum of the fractions was not equal to but lower than the $DMSP_T$ measurements and that a fraction of about 24 to 27 % was missing. Given that $DMSP_p$ was 70 - 74% of $DMSP_T$ for the *H. triquetra* culture in exponential phase whereas $DMSP_d$ and DMS were only 2 – 3 % and 0.1 - 0.2 % of $DMSP_T$, respectively, it was more likely that the missing fraction would come from the $DMSP_p$ measurement.

In this regard, I first focussed my attention on testing and improving the $DMSP_p$ measurement and essentially in the filtration process which was the critical step in the sample preparation that could produce underestimation (Kiene and Slezak, 2006) and imprecision. Therefore, I investigated several parameters influencing the filtration pressure such as: the sample volume of culture, the methods of filtration such as the hand vacuum pump, the syringe pump with a range of delivery speeds and filtration by gravity.

3.5.1. Optimisation of the $DMSP_p$ measurement

3.5.1.1. $DMSP_p$ measurement of standards

Given that culture samples contained a filter I tested if the presence of a filter in the standards was affecting the $DMSP_p$ concentrations. Some duplicate standards were prepared by adding the DMSP stock solution on the filter, folding it and introducing it in the vial containing NaOH similar to sample preparation. The comparison of these 2 methods is presented in Figure 3.7.

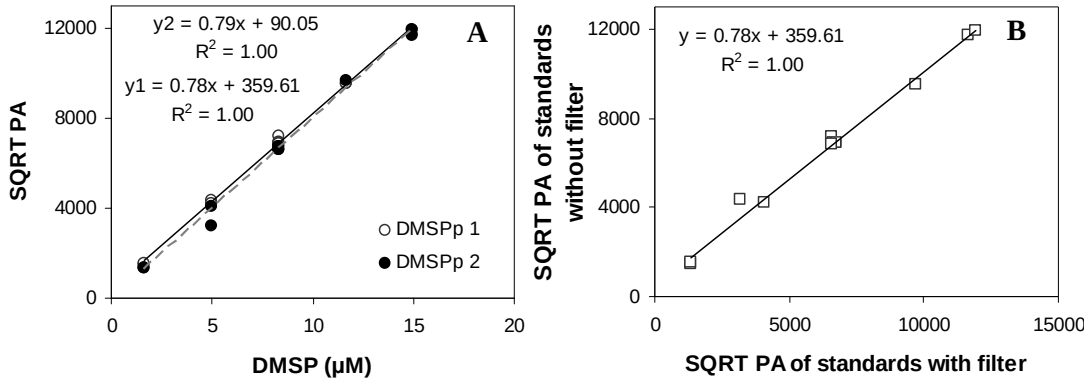

Figure 3.7 Comparison between $DMSP_p$ standards without filter ($DMSP_p$ 1) and containing a filter ($DMSP_p$ 2). (A) Calibration curves for the 2 sets of standards, both curves are superimposed. (B) Least-squares regression obtained for these 2 sets of standards. SQRT PA means the square root of the peak area obtained on the chromatogram.

The results showed similar values obtained for the calibration curve with the 2 methods tested ($P>0.05$, Mann-Whitney *U* test). The filters had therefore no effect on the DMSP measurement and the use of filters was unnecessary for further $DMSP_p$ standard preparation.

3.5.1.2. $DMSP_p$ measurement of culture samples

For preparation of culture samples, the sample was filtered using a hand vacuum pump not exceeding 5 cm Hg. The filter was folded in 4 and put in the top part of the

vial with no direct contact with NaOH to avoid DMS release in the open vial. The vial was closed with a gas tight cap, agitated and stored as previously described, for later analyses.

3.5.1.2.1. Effect of the sample volume when filtering by means of a hand vacuum pump on $DMSP_p$ concentrations

To assess the effect of the sample volume I filtered 1 ml, 2 ml, 3 ml, 4 ml and 5 ml of culture using the hand vacuum pump. Each condition was repeated 3 times. DMSP concentrations obtained for each sample volume are shown in Figure 3.8.

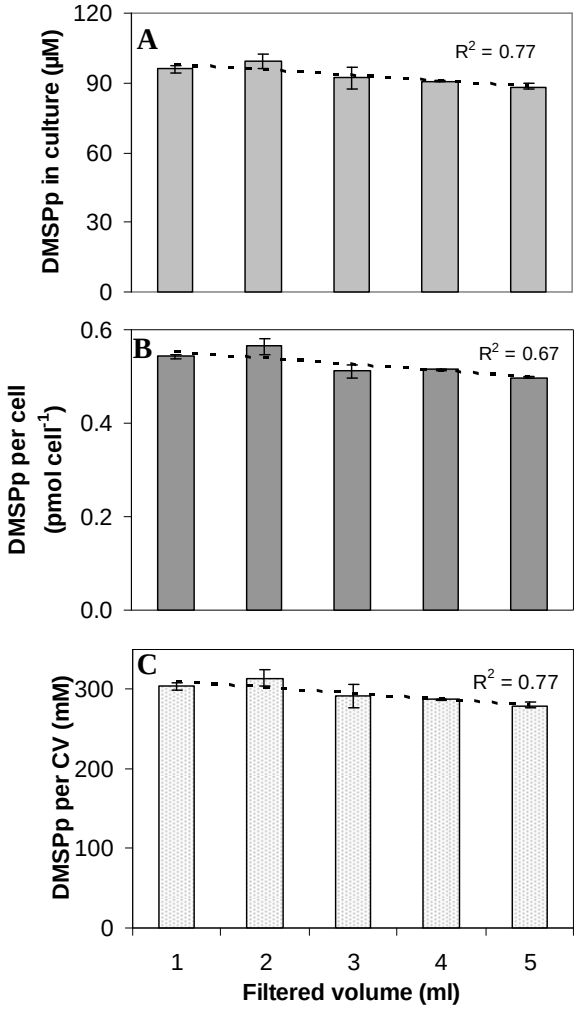

Figure 3.8 Effect of the volume of filtered culture on $DMSP_p$ measurement in *Heterocapsa triquetra* culture (A) $DMSP_p$ concentration in the culture, (B) per cell, (C) per cell volume (CV). The column height represents the average (n=3 except n=2 for the filtered volume of 2 ml) and range bars display the minimum and maximum values. The dashed line shows the least -squares regression of the $DMSP_p$ concentrations to the volume of sample filtered and the correlation coefficient R^2 is shown.

The filtration duration lasted between 1 and 3 minutes, with longer periods for larger volumes. There was a significant correlation between the $DMSP_p$ concentration in the culture and the volume of the sample filtered ($P<0.01$, Spearman test). Similar correlation was obtained for $DMSP_p$ normalised per cell and per cell volume. The $DMSP_p$ values decrease with increasing volume of culture filtered. The larger volumes (4 - 5 ml) showed significant difference with the smallest volume of 1 ml (6 and 8 % respectively, $P=0.05$, Mann-Whitney *U* test) and suggestive difference with a volume of 2 ml (9 and 11 % respectively, $P<0.10$; Table 3.1).

Table 3.1 Probability (P) of significant difference between $DMSP_p$ per cell values obtained for 2 volumes of filtration obtained with the non-parametric Mann-Whitney *U* test. $P\leq 0.05$ indicates significantly different $DMSP_p$ values.

Volume compared (ml)	1 - 2	2 - 3	2 - 4	2- 5	1 - 4	1 - 5
Probability (P)	0.564	0.248	0.083	0.083	0.05	0.05

The volume of culture filtered had an effect on DMSP concentration. The load of numerous cells aggregating on the filter may cause blockage and more pressure is required to allow the filtration. A larger volume seems to reduce the DMSP value probably due to a DMSP loss (i.e. if cells are damaged, they may release DMSP into the dissolved fraction) as suggested by Kiene and Slezak (2006). I, therefore, favoured the use of smaller volumes and selected the volume of 2 ml for the following experiments in this project rather than 1 ml to allow detectable measurement of low density samples or potentially low DMSP producing-species.

In this experiment the use of the hand vacuum pump gave a good reproducibility of $DMSP_p$ measurement with a relative standard deviation (RSD) between 0.4 and 5 % (n=3). However, these values vary within and across experiments (in another experiment shown previously in Figure 3.3, the RSD spread between 2 and 11 %). I tested other filtration systems to see how it would affect DMSP concentrations.

3.5.1.2.2. Comparison of filtration using an electrical pump and a hand vacuum pump

The effect of the filtration system was tested by comparing $DMSP_p$ concentrations obtained using the hand vacuum pump and an electrical pump set to a comparable low pressure (5 cm Hg). For each filtration method, 3 replicates of culture sample were filtered and analysed for $DMSP_p$ concentrations. Results are shown in Figure 3.9.

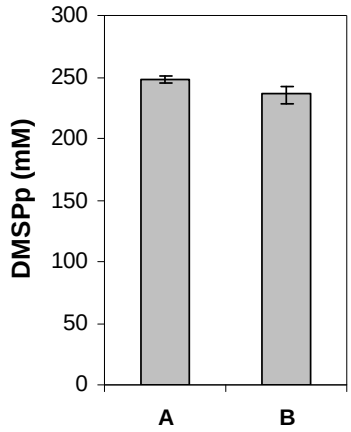

Figure 3.9 Comparison of $DMSP_p$ concentrations per cell volume obtained using 2 different filtration methods (A) hand vacuum pump and (B) an electrical pump at a similar pressure for a sample volume of 2 ml of *Heterocapsa triquetra* culture. Averages (n=3) are shown by the column height and error bars show minimum and maximum values.

The DMSP value obtained with the electrical pump was significantly lower than the value obtained with the hand vacuum pump (5 % difference, P= 0.05, Mann-Whitney *U* test). In this experiment the $DMSP_p$ measurement gave a RSD of 1.3 % when using a hand vacuum pump and 2.9 % when using the electrical pump. According to these results, the hand vacuum pump gave a higher DMSP concentration with better precision whereas the use of the electrical pump had no benefit.

3.5.1.2.3. Comparison of filtration with a syringe pump using various speeds of filtration

An automated syringe pump (KDS220, Linton Instrumentation, Diss, United Kingdom) allows consistent delivery speed and consequently identical filtration pressure on each culture replicate. I tested 5 delivery speeds of 0.5, 1, 2, 4 and 7 ml min^{-1} on 2 ml aliquots of culture using 2.5 ml plastic syringes. The culture sample, in late exponential growth phase at a cell density of 8.7×10^4 cell ml^{-1}, was directly sucked up with the syringe up to 2 ml and placed on the syringe pump. The effect of the syringe pump on the DMSP values was compared with the use of the hand vacuum pump which filters at a speed of 1 to 2 ml min^{-1}. The DMSP concentrations obtained are shown in Figure 3.10.

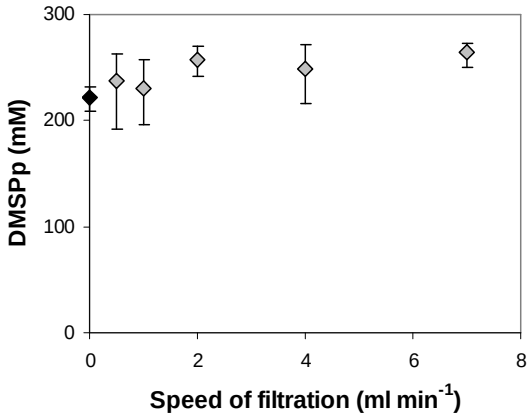

Figure 3.10 Effect of delivery speeds (from 0.5 to 7 ml min^{-1}) of the syringe pump (grey symbols) compared with the use of the hand vacuum pump (black symbol on the Y axis) on the $DMSP_p$ values. The volume of *Heterocapsa triquetra* culture sample was 2 ml. Syringes of 2.5 ml volume were used with the syringe pump. Shown are average values with range of data (n=3).

Delivery speeds between 0.5 and 7 ml min^{-1} showed similar $DMSP_p$ values ($P>0.05$, Kruskal-Wallis test). The variation between replicate samples (RSD 4 – 17 %) appeared in some cases larger than the variation between the delivery speeds (RSD 13.9 %). Therefore, the use of the syringe pump at delivery speeds between 0.5 and 7 ml min^{-1} did not reduce the variation between replicate samples. However, a significantly higher $DMSP_p$ value (16 % difference, $P=0.05$, Mann-Whitney *U* test) was obtained using the syringe pump at the highest delivery speed of 7 ml min^{-1} compared to the result obtained using the hand vacuum pump and which suggests that less DMSP may have been lost in the dissolved fraction.

To test if the use of higher delivery speeds (> 7 ml min^{-1}) had any effect on $DMSP_p$ values, larger syringe volumes needed to be used since the speed range of the pump was dependant on the syringe volume. Plastic syringes of 10 ml volume were used to investigate the delivery speeds between 2.5 and 20 ml min^{-1}. To obtain an accurate volume in the syringe, the sample of culture in exponential growth phase at 3.0×10^4 cell ml^{-1} was taken up with a Gilson pipette and injected into the syringe blocked with a stopcock. The samples were filtered and analysed for $DMSP_p$ concentrations which are shown in Figure 3.11.

Figure 3.11 Effect of 8 delivery speeds of the syringe pump on the $DMSP_p$ values using the 10 ml syringes and 2 ml culture sample of *Heterocapsa triquetra* culture at at 3×10^4 cell ml^{-1}. Shown are average values with ranges of data (n=3 except n=2 for 2.5 and 5 ml min^{-1}).

Again in this experiment, no significant differences were obtained between the delivery speeds tested (P>0.05, Kruskal-Wallis test; Figure 3.11) on a culture sample of *Heterocapsa triquetra* culture. The RSD obtained was between 6 and 19 % with the smallest RSD for a delivery speed of 20 ml min^{-1}.

A similar test was repeated for 4 delivery speeds using a higher cell density culture, in late exponential phase at 10.5×10^4 cell ml^{-1}, to test how more elevated biomass would have an effect on the filtration and $DMSP_p$ measurement (Figure 3.12).

Figure 3.12 Effect of 4 delivery speeds of the syringe pump on DMSPp per cell volume for 2 ml of a culture at a cell density of 10.5×10^4 cell ml^{-1}. Shown are average values with ranges of data (n=3).

At a cell density of 10.5×10^4 cell ml^{-1}, the same variability was obtained for sample replicates (RSD of 4 - 20 %) with the smallest variability found at 20 ml min^{-1}. The $DMSP_p$ concentration increased with the increase of the delivery speed used for the filtration (significant correlation, $P<0.05$, Spearman test).

I compared the $DMSP_p$ concentrations obtained for the delivery speeds 5, 10, 15, 20 ml min^{-1} at low and high cell density of *H. triquetra* culture (Figure 3.11 and Figure 3.12, respectively). No significant difference was obtained for the delivery speeds of 5, 10 and 15 ml min^{-1} ($P>0.05$ Mann-Whitney *U* tests for each speed), however, significant difference was observed for the delivery speed of 20 ml min^{-1} ($P<0.05$ Mann-Whitney *U* test). Therefore the $DMSP_p$ concentration per cell volume was higher in *H. triquetra* culture of 10.5×10^4 cell ml^{-1} (194 mM, 11 % higher) than in a culture of 3.0×10^4 cell ml^{-1} (174 mM) when the samples were filtered at 20 ml min^{-1}. However, it was only possible to observe this difference if the variation between replicate samples was minimised as appeared to be the case at 20 ml min^{-1} of delivery speed.

I tested, in a different way, how elevated biomass would have an effect on the filtration and $DMSP_p$ measurement by increasing the sample volume to 5 ml. The effect of 4 different delivery speeds on $DMSP_p$ values was tested for a 5 ml sample of culture at a cell density of 3.5×10^4 cell ml^{-1}. The results are shown in Figure 3.13.

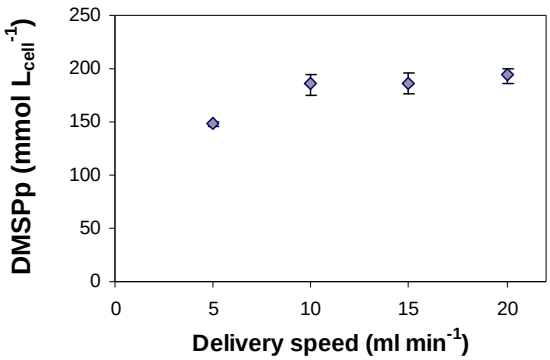

Figure 3.13 Effect of 4 delivery speeds of the syringe pump on the $DMSP_p$ concentrations per cell volume for 5 ml of culture at a cell density of 3.5×10^4 cell ml^{-1}.

Similar concentrations were obtained for 10, 15 and 20 ml min^{-1} ($P>0.05$, Kruskal-Wallis test) and significantly lower DMSP concentrations were obtained at 5 ml min^{-1} ($P<0.01$, Mann-Whitney *U* test). The $DMSP_p$ concentration and the delivery

speed are again positively correlated ($P<0.05$, Spearman test). The analytical uncertainty was from 4 to 12 % with the smallest one being at 5 ml min^{-1}.

When comparing all the results obtained for the 4 delivery speeds from 5 to 20 ml min^{-1} in the 3 previous experiments (Figures 3.11, 3.12 and 3.13), no significant difference in $DMSP_p$ concentrations was obtained between experiment ($P>0.05$, Mann-Whitney *U* test) and no difference appeared between the 4 delivery speeds tested ($P>0.05$, Kruskal-Wallis test). However, significant correlation was found between the $DMSP_p$ concentrations per cell volume and the delivery speed of the filtration ($P<0.05$, Spearman test).

With the use of the syringe pump, $DMSP_p$ concentrations increased with increase in delivery speed. For a culture at higher cell density (8 - 10 $\times 10^4$ cell ml^{-1}), the lowest variability between replicates was obtained at the highest delivery speed of 20 ml min^{-1}. This delivery speed also allowed the observation of significantly higher $DMSP_p$ concentration in cultures of higher cell density than cultures of lower cell density and which was hidden by the large variability of replicates when using the hand vacuum pump. The use of a higher sample volume such as 5 ml did not show any benefit in comparison with the experiment using a 2 ml sample and supported the idea that low delivery speeds such as 5 ml min^{-1} should be avoided given that it resulted in significantly lower $DMSP_p$ concentrations.

In conclusion, higher delivery speeds needed to be favoured when using the syringe pump for $DMSP_p$ measurements since the $DMSP_p$ concentrations were increased and a better precision of the measurement was generally observed (4 - 9 % at 20 ml min^{-1}). Therefore, I decided to set the syringe pump at the maximum available speed depending on the syringe type which was 14 ml min^{-1} for a 5 ml syringe and 7 ml min^{-1} for a 2.5 ml syringe.

3.5.1.2.4.Comparison of filtration by gravity with other filtration methods

Gravity filtration was recommended by Kiene and Slezak (2006) to avoid potential breakage of cells and DMSP transfer from the particulate to the dissolved fraction of the culture. Initially, gravity filtration did not work since there was too much back pressure from the glass frit, supporting the filter. I finally used a glass filter holder without a glass frit which enabled the filtration by gravity. Other processes of filtration were performed including filtration using the syringe pump (with a 2.5 ml syringe at 7 ml min^{-1}) and the hand vacuum pump. Two cell densities of culture (4.6×10^4 cell ml^{-1} and 25×10^4 cell ml^{-1}) were tested for this experiment in order to assess *H. triquetra* responses to the different filtration methods applied. For each cell density, 3 replicates of 2 ml culture sample were filtered and analysed. The results of the experiment are given in Figure 3.14.

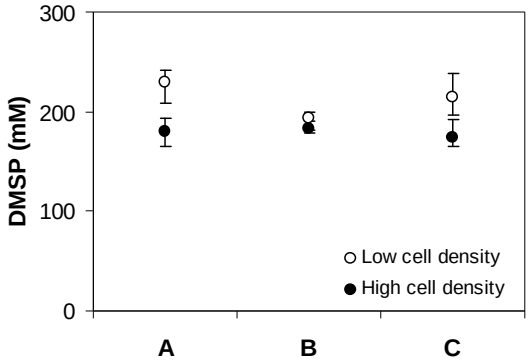

Figure 3.14 Effect of 3 filtration systems on $DMSP_p$ concentrations per cell volume of culture samples at low and high cell densities (4.6×10^4 cell ml^{-1} and 25×10^4 cell ml^{-1} respectively). (A) Filtration by gravity, (B) syringe pump at 7 ml min^{-1}, (C) hand vacuum pump.

The syringe pump gave the best reproducibility of the measurements, with the smallest RSD of 4 - 5 % at the 2 cell densities in comparison with 8 - 9 % and 9 – 10 % for the gravity filtration method and the use of the hand vacuum pump, respectively. The 3 filtration methods showed no difference in $DMSP_p$ concentrations at high cell density ($P>0.05$, Kruskal-Wallis test). At low cell density gravity filtration showed significantly higher DMSP than when using the syringe pump ($P=0.05$). However there was no significant difference between the gravity filtration method and the use of the hand vacuum pump ($P>0.05$, Mann-Whitney *U* test) and between the use of the hand vacuum pump and the syringe pump ($P>0.05$, Mann-Whitney *U* test). Therefore, the benefit of using the gravity filtration method was not obvious and did not need to be

especially used for $DMSP_p$ measurements in dinoflagellates as might have been expected according to the recommendations of Kiene and Slezak (2006). In this experiment, the use of the syringe pump did not lead to a higher DMSP concentration than the use of the vacuum pump in contrast to the result observed in Figure 3.10.

The difference between $DMSP_p$ values obtained for low and high cell density might be due to different physiological conditions. However, the response of *H. triquetra* cells to the filtration methods, in terms of analytical uncertainty, is similar for the 2 different growth stages (i.e. similar RSD at low and high cell densities). Therefore, the growth stage of the culture had no effect on the precision of $DMSP_p$ measurement but could affect the value of DMSP concentration. On the other hand, the choice of filtration method affected the precision of the measurement.

3.5.1.2.5. Conclusion on the effect of the filtration methods on $DMSP_p$ measurement

Several filtration methods were tested with the aim of selecting the method that would produce the smallest analytical uncertainty and the highest $DMSP_p$ value. Between the 3 methods, including the hand vacuum pump, the syringe pump and gravity filtration, no general significant difference was obtained for $DMSP_p$ concentration. The $DMSP_p$ measurement was thus not increased by the use of any of the filtration methods and the analytical uncertainty appeared lower in some cases for the use of the syringe pump.

As I observed that $DMSP_p$ concentration varied over growth stages I compared the effect of the use of the hand vacuum pump and the syringe pump on the $DMSP_p$ concentrations in culture of similar cell densities (low cell density in exponential phase). Table 3.2 shows the summary of the results obtained across several experiments presented previously in this chapter.

Table 3.2 Comparison of $DMSP_p$ concentrations and RSD obtained between filtration using the syringe pump and the hand vacuum pump for a low cell density culture of *Heterocapsa triquetra*.

Filtration method	Experiment	Culture cell density $\times 10^4$ cell ml^{-1}	Average $DMSP_p$ mM (n=3)	RSD (n=3)
Syringe pump (2.5-20 ml min^{-1})	Fig 3.11	3	160 - 184	6 - 19
Syringe pump 20 ml min^{-1}	Fig 3.11	3	174	6
Syringe pump (5-20 ml min^{-1})	Fig 3.13	3.5	148 - 195	4 - 12
Syringe pump 20 ml min^{-1}	Fig 3.13	3.5	195	9.1
Syringe pump (7 ml min^{-1})	Fig 3.14	4.6	193	4
Hand vacuum pump (growth stages)	Fig 3.4 B	4.2	159	1.2
Hand vacuum pump	Fig 3.14	4.6	214	9
SUMMARY				
Hand vacuum pump		**4.2 ; 4.6**	**159 ; 214**	**1.2 - 9**
Syringe pump		**3 - 4.6**	**148 - 195**	**4 - 19**
Syringe pump 20 ml min^{-1}		**3 ; 3.5**	**174 ; 195**	**6 ; 9**

Both filtration methods produced similar range of $DMSP_p$ concentrations: 159 – 214, 148 - 195 mM for the hand vacuum pump and the syringe pump, respectively. The analytical uncertainty appeared in these cases less important with the hand vacuum pump (1.2 - 9 %) than the syringe pump (4 – 19 %). The same conclusion is true for all experiments presented in this chapter (RSD of 0.4 - 11 % for the hand vacuum pump and 4 - 20 % with the syringe pump). The rather large range of variability has the potential to hide crucial differences in $DMSP_p$ concentrations as for example, over growth stages. The variation between replicates tended to be reduced by using the highest delivery speeds available on the syringe pump. At delivery speed of 20 ml min^{-1} the range of DMSP concentration obtained and the analytical uncertainty were reduced (174 - 195 mM, 6 - 9 % RSD) and the analytical uncertainty was in the same range obtained using the hand vacuum pump.

Finally, given that the $DMSP_p$ measurement was not obviously successfully improved by using other filtration methods, I investigated the $DMSP_T$ measurement as a surrogate of the intracellular DMSP. Previous measurements showed that $DMSP_p$ was the major fraction of $DMSP_T$ (70 – 74 %, Figure 3.6) and that the analytical uncertainty for $DMSP_T$ measurement was 1.3 % (n=5) for *H. triquetra* samples of culture in exponential phase (3×10^4 cell ml^{-1}; see chapter 2 section 2.2.2.2). Therefore, the analytical uncertainty for $DMSP_T$ measurement was better than the uncertainty obtained for $DMSP_p$ measurement. The absence of a filtration step in the $DMSP_T$ measurement ensured a better replication of samples. Reducing the analytical uncertainty was crucial

to improve the likelihood of determining variation of DMSP in different experimental conditions. The absence of filtration also reduced the time of sample preparation and enabled successive sampling at short intervals, that would not be possible for $DMSP_p$ measurements (as performed in chapter 6, section 6.2.5). Moreover, $DMSP_T$ represented the total production of DMSP and derivatives by phytoplankton cells in the culture. The $DMSP_T$ measurement was also used by Keller et al. (Keller, 1988/1989; Keller et al., 1989a; Keller et al., 1989b) for measuring the DMSP content of 123 phytoplankton strains and by Yost et al. (2009), who observed no significant difference between $DMSP_T$ and $DMSP_p$ concentrations in 5 strains of the dinoflagellate *Symbiodinium microadriaticum* in exponential growth phase.

3.5.2. Adaptation of the headspace method for $DMSP_T$ measurement

3.5.2.1. $DMSP_T$ measurement for standards

The method for measuring $DMSP_T$ ($DMSP_p$ + $DMSP_d$ + DMS) was adapted from the headspace method similar to the protocol for $DMSP_p$ measurement but without filtering the sample. This method was therefore convenient for $DMSP_T$ analyses since the concentrations fall in a similar range to $DMSP_p$ and DLA measurements (Steinke et al., 2000). As previously described (chapter 2 section 2.2.2.2) the standards for calibrating the gas chromatograph were made up by introducing 3 ml of 0.5 M NaOH in a 5 ml glass vial, then adding a drop of DMSP (2 - 15 µl) solution under the cap of the vial and closing and agitating the vial. $DMSP_T$ samples were prepared in order to obtain the same final NaOH concentration. In this way, 1 ml of culture was rapidly added to 2 ml of 0.75 M NaOH in a 5 ml glass vial and immediately closed with a gas tight cap to avoid DMS loss. The samples and standards were left to react in the dark for later analyses.

Similar to the standards, the $DMSP_T$ samples were prepared to end up with a final concentration of 0.5 M NaOH, a total liquid volume of 3 ml and an identical headspace volume of 2 ml. However, $DMSP_T$ samples contained 1 ml of phytoplankton culture grown in enriched seawater which might alter the pH of the sample and which contained salts that might also alter the transfer of DMS to the gaseous phase in comparison with the distilled water used to prepare the standards. Henry's law constant (Hc= concentration in the air/ concentration in water) of DMS is higher in seawater than in distilled water, for the same conditions of temperature (0.069 and 0.056, respectively, measured by Wong and Wang, 1997), i.e. DMS is less soluble in seawater than in distilled water.

For testing if the addition of 1 ml of culture grown in seawater was affecting the DMSP cleavage into DMS or the DMS partitioning in the air and liquid phases, I prepared a range of standards by adding 1 ml of seawater to 2 ml of 0.75 M NaOH in the 5 ml glass vial before adding a drop of DMSP solution under the cap. I compared

those standards to the standards made up by introducing 3 ml of 0.5 M NaOH and the DMSP solution as described above. Each standard was prepared in duplicate. Results are shown in Figure 3.15.

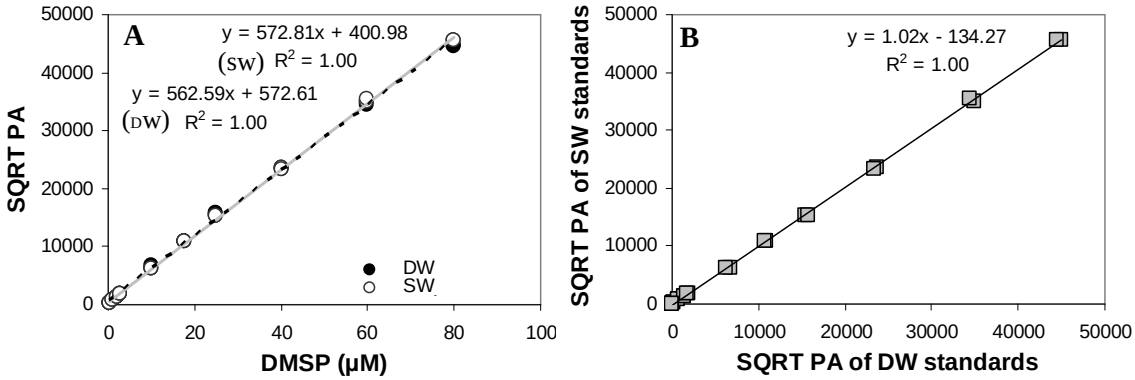

Figure 3.15 Comparison of standards prepared with NaOH diluted in distilled water (DW) and prepared with one third of seawater (SW) to simulate the culture sample preparation. SQRT PA means the square root of the peak area obtained on the chromatogram. (A) Calibration curves for standards prepared with DW and SW, both curves are superimposed. (B) Regression of the 2 sets of standards (DW, SW).

The standards made up with a third of seawater in the NaOH solution led to comparable peak areas to those obtained for standards made up with NaOH diluted in distilled water ($P>0.05$, Mann-Whitney *U* test, and R^2 of 1.0 for the least squares regression of peak areas for both standards preparation, Figure 3.15 B). This difference in the preparation did not alter the chemical cleavage of DMSP by NaOH and did not affect the DMS transfer to the air phase of the analytical vial. However, a white precipitate appeared when the seawater was added.

The standards used for calibrating the GC are prepared by adding a drop of DMSP solution under the cap of the vial, subsequently closed and agitated. This method avoided mixing the DMSP solution and the NaOH while the vial was open and prevented any DMS loss. For $DMSP_T$ samples, the culture was directly added in the vial containing NaOH and rapidly closed. To test if the sample preparation for $DMSP_T$ measurement produced a DMS loss I prepared 5 standards by adding 1 ml of DMSP solution in 2 ml of 0.75 M NaOH (called DMSP 2). I compared these standards with standards prepared by putting a drop of DMSP solution under the cap and closing the

vial containing the NaOH solution (DMSP 1). Each standard was made in duplicate. Results are given in Figure 3.16.

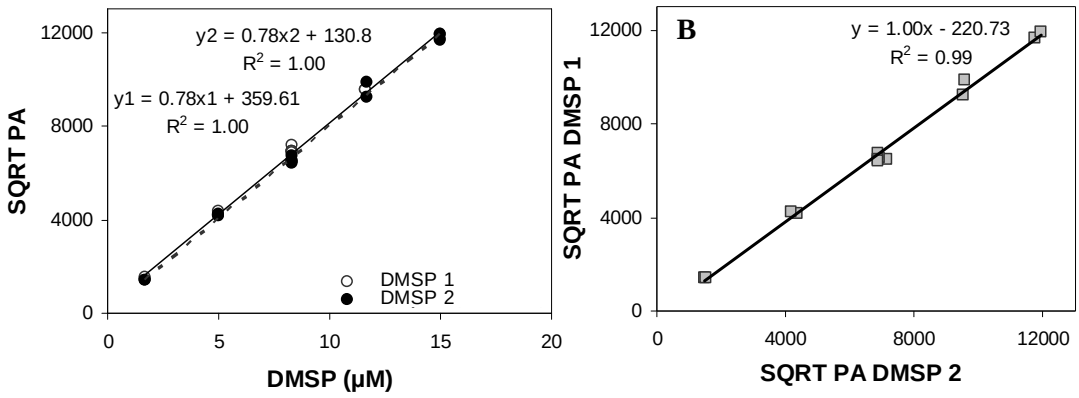

Figure 3.16 Comparison of standards prepared by putting a drop of DMSP solution under the cap of the vial containing the NaOH solution and closing the vial (DMSP 1) and standards prepared by addition of 1 ml of DMSP solution in 2 ml of 0.75 M NaOH similarly to the culture sample (DMSP 2). The square root of the peak area (SQRT PA) is the equivalent DMSP value read on the chromatogram.

The 2 sets of standards gave similar values of peak areas for the range of DMSP concentrations prepared ($P>0.05$, Mann-Whitney *U* test). Moreover, the least-squares regression of the peak areas obtained with both methods of standard preparation gave a correlation coefficient of 0.99 (Figure 3.16 B) which supported the result that these 2 methods (DMSP 1 and 2) gave similar results. Therefore, given that culture samples are prepared similarly to the standards DMSP 2, no significant DMS loss appeared to occur when using this method.

The analytical uncertainty of the $DMSP_T$ measurements was 1.8 % (n=5) for standards and 1.3 % (n=5) for *H. triquetra* samples in exponential growth phase.

3.5.2.2. $DMSP_T$ measurement for culture samples

Given that $DMSP_p$ concentrations vary over the growth I investigated how $DMSP_T$ and $DMSP_p$ both varied over the growth of *Heterocapsa triquetra* to test if $DMSP_T$ was a good surrogate of intracellular DMSP (Figure 3.17). The culture was sampled every 3 days, over 12 days. Culture aliquots of 2 ml were filtered using a hand vacuum pump and analysed for $DMSP_p$ and 1 ml aliquots were directly mixed with

NaOH and analysed for $DMSP_T$. Results obtained for the measurements of these 2 parameters are presented in Figure 3.17.

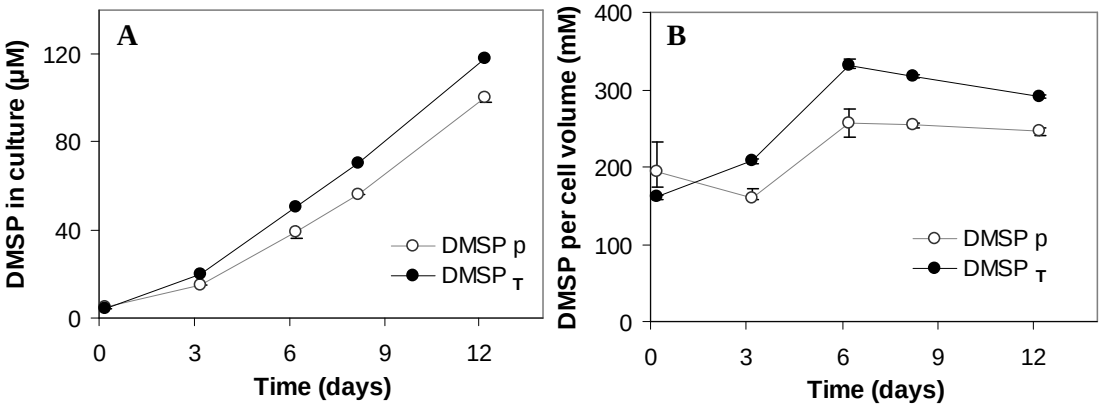

Figure 3.17 $DMSP_T$ and $DMSP_p$ concentrations in *Heterocapsa triquetra* culture over time. (A) DMSP per litre of culture. (B) DMSP per cell volume. Shown are average values and range bars give minimum and maximum values (n=3, except n=2 for $DMSP_T$ data at t=0 and t=12). When not visible, range bars are smaller than symbol size.

$DMSP_p$ and $DMSP_T$ concentrations in the culture were not significantly different ($P>0.05$, Mann-Whitney *U* test) and showed similar increases over time. $DMSP_p$ and $DMSP_T$ concentrations per cell volume showed the same general trend over time with an increase between days 3 and 6 and a slight decrease after day 6. No significant difference was observed between the $DMSP_p$ production and the $DMSP_T$ production over time ($P>0.05$, Mann-Whitney *U* test; the productions were calculated as the difference of DMSP concentrations between one data point and the next one over the period of time). Between 3 and 12 days, $DMSP_p$ was 77 to 85 % of the $DMSP_T$ fraction. The RSD obtained for $DMSP_T$ measurements (0.6 - 5.2 %) were smaller than those obtained for $DMSP_p$ measurements (1.1 - 16.4 %). With a better analytical uncertainty and not significantly different values, $DMSP_T$ seems to be a good surrogate of the intracellular DMSP concentration.

3.5.3. Optimisation of DMS measurement

3.5.3.1. DMS measurement of standards

The purge and trap method was applied to DMS measurement as it was initially developed (details in Chapter 2 section 2.2.2.1). The purge efficiency (i.e. the amount of DMS extracted) depends on the volume of the liquid, the purge flow rate and the time of purging. The volume was fixed at 5 ml, to fall in the sensitivity range of the GC, according to preliminary tests. The time required for purging DMS out of the solution with a purge flow rate of 60 ml min^{-1} was tested.

A 5 ml solution was introduced in the purge tube by successively adding 1 ml of DMSP solution, 3 ml of distilled water, 1 ml of 10 M NaOH and then rapidly closing the purge tube. In order to determine the time necessary for purging, the amount of DMS purged out was monitored over 45 minutes by stop-start purging the same sample. The square root of the peak area (SQRT PA) shows the value of the DMS peak obtained by the GC for a defined period of purging (Figure 3.18). The percentage of extracted DMS is calculated as the cumulative SQRT PA at t_x over the cumulative SQRT PA at 45 minutes of purging.

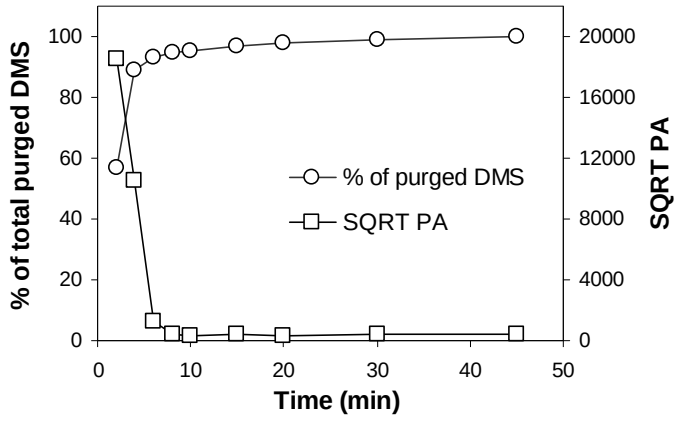

Figure 3.18 Purge efficiency of the purge and trap system containing a total liquid volume of 5 ml that includes 1 ml of DMSP standard solution (16.24 ng S ml^{-1}) and monitored during 45 minutes. The symbol (○) indicates the extracted DMS expressed as a cumulative percentage of the final DMS peak areas at 45 minutes and the symbol (□) shows the square root of the peak area read on the chromatogram.

The SQRT PA decreased abruptly up to 6 minutes of purging, as the DMS concentration decreased in the purge tube. The cumulative percentage increased to 89 % after 4 minutes of purging, 93 % after 6 minutes and 95 % after 10 minutes. I thus, decided to establish 10 minutes as purging protocol for a 5 ml solution of both standards and samples.

Given that DMS is less soluble in seawater than distilled water, I tested whether there was a difference between standards prepared with 3 ml of distilled water and 3 ml of seawater. Results are given in Figure 3.19.

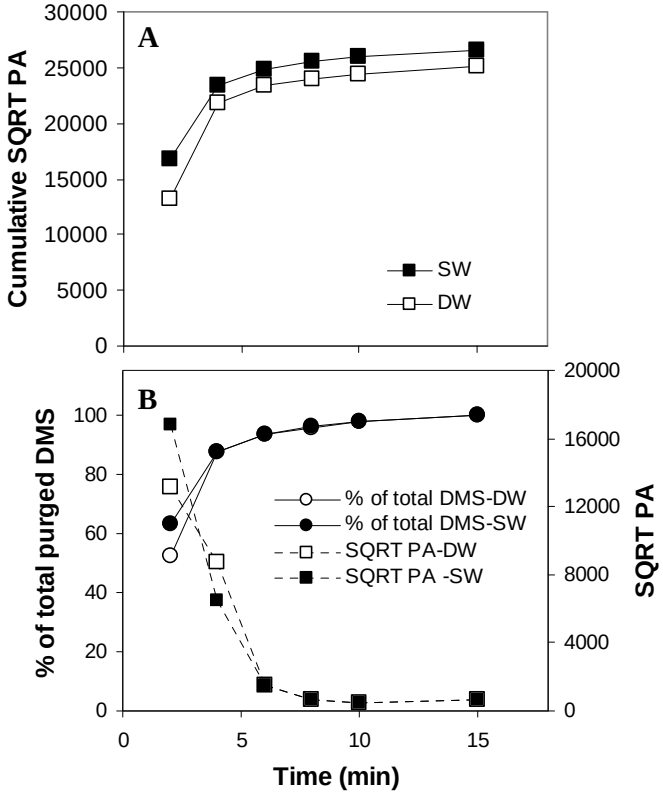

Figure 3.19 Comparison of the purge efficiency using distilled water (DW) and using seawater (SW) in the standard preparation. (A) Cumulative square root of the peak areas (SQRT PA) obtained after 6 periods of purging. (B) The symbols (○●) indicate the extracted DMS expressed as a cumulative percentage of the final DMS amount at 15 minutes and the symbols (□■) show the square root of the peak area read on the chromatogram.

The use of seawater led to slightly higher DMS values (Figure 3.19 A) compared to those obtained with distilled water but no significant difference was observed ($P>0.05$, Mann-Whitney *U* test). The seawater may contain a DMS background that could explain the slightly higher DMS values observed or the purging

in seawater may yield more DMS at the beginning. However, at 10 minutes of purging the cumulative percentage of DMS appeared similar with distilled water or seawater (Figure 3.19 B). Because distilled water is always available in the laboratory and is less likely to contain DMS background, I decided to use distilled water in the DMS standards and in filtered samples to make up the volume to 5 ml.

Given that for $DMSP_T$ measurements DMSP is left for 24 hours to react with NaOH, I decided to do the same with the DMS standards. I prepared DMS standards in 5 ml glass vials closed with a screw cap and a Teflon septa and left them 24 hours to react in the dark before performing DMS measurements (as described in Chapter 2 section 2.2.2.1). The analytical uncertainty for DMS standards was 3.2 % and was slightly higher than the analytical uncertainty of $DMSP_T$ standards (1.3 %). This difference is likely to result from the coupling of the purge and trap system and the gas chromatography for DMS standard analyses whereas only gas chromatography is used for analyses of $DMSP_T$ standards.

3.5.3.2. DMS measurements of culture samples

The sample preparation for DMS measurement in culture samples differs from that of standards, by the addition of a filtration step and a successive transfer into the purge tube. Because the syringe pump showed a better analytical uncertainty in several experiments for filtration of replicate samples in $DMSP_p$ measurement, I used this system for DMS measurements. The syringe, placed on the pump, was connected to a filter holder which was connected to a sample loop that dispensed the exact volume of filtrate into the purge and trap system (S-S-PT system: Syringe pump - Sample loop - Purge and Trap, described in 2.2.2.1). I determined the analytical uncertainty of this system by performing DMS measurements of replicate standards and *H. triquetra* replicate samples. Table 3.3 shows the average and the RSD of the square root of the peak areas.

Table 3.3 DMS measurement in standards and *Heterocapsa triquetra* samples using the S-S-PT system (Syringe pump-Sample loop-Purge and trap). Average of the square root of the peak area is shown as the result of the measurement and the relative standard deviation (RSD) represents the analytical uncertainty of the method for (n) measurements.

S-S-PT system	Test	Average of SQRT PA	RSD %	n
Standards (2 ml at 0.73 ng S ml^{-1})		1948	2	3
Heterocapsa triquetra sample	1	715	25	3
(2 ml analysed)	2	874	33	3

The analytical uncertainty of the S-S-PT system was low for standards (2 %, n=3), however, the uncertainty for DMS measurement in *Heterocapsa triquetra* samples was rather high (25 and 33 %). Several factors might produce this variability: the culture flask agitation before sampling, the filtration process (e.g. delivery speed of the pump, duration of filtration, filter, cell density, sample volume) and the degree of potential DMS production from dissolved DMSP by DMSP lyase activity given that this enzyme may be produced by *H. triquetra* (Niki et al., 2000).

To test if there was any production of DMS in the filtrate of culture sample, I filtered a large volume of culture (20 ml) from a plastic syringe (20 and 60 ml) through a filter holder (Swinnex, Millipore, Watord, UK) containing a 25 mm diameter glass-fibre filter (Whatman, GF/F) connected to a glass syringe (20 and 50 ml) using the syringe pump set at 14 ml min^{-1}. I eliminated the air in the glass syringe and introduced a 2 ml aliquot of filtrate in the sample loop. The sample was then purged and analysed for DMS. Then, the measurement was repeated twice more at 15 minute intervals to see if there was any change in DMS value and potential DMS production in the filtrate over time. This experiment was performed once with an aliquot of a culture not agitated before sampling and then repeated with the same culture (18 ml) agitated before each sampling to see if DMS measurement was affected by the swirling of the flask (i.e. if the DMS transfer to the air was increased by manual agitation). The experiment was repeated for a larger volume of culture (38 ml) to determine if the sample volume has an effect on DMS measurement. Table 3.4 shows the average and the RSD of SQRT PA obtained and Figure 3.20 shows the DMS values obtained over time.

Table 3.4 Tests of filtrate stability, agitation effect and sample volume on DMS measurement in *H. triquetra* sample. Average of the square root of the peak area is shown as the result of the measurement and the relative standard deviation (RSD) represents the analytical uncertainty of the method for (n) measurements.

Large filtrate kept in a glass syringe. For each filtrate, 3 aliquots of 2 ml were analysed	**Average of SQRT PA**	**RSD %**	**n**
20 ml filtrate (Flask was not swirled before sampling.)	4238	2	3
18 ml filtrate. (Usual swirl of the flask).	5426	6	3
38 ml filtrate. (Usual swirl of the flask).	10166	19	3

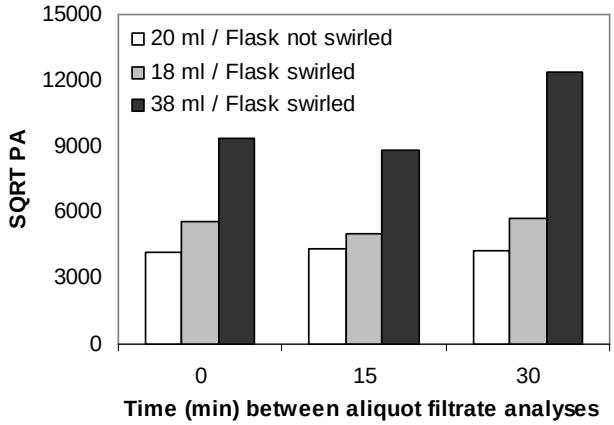

Figure 3.20 Effect of resting time of the filtrate, volume of the filtrate and swirl of the flask on DMS measurement from *Heterocapsa triquetra* culture samples. For each filtrate (18, 20 and 38 ml) 3 aliquots of 2 ml were injected in the purge tube and analysed at about 15 minute intervals. Only the first filtrate (20 ml) was obtained from a non-swirled culture.

For a 20 ml or 18 ml filtrate, whether the flask was agitated or not, the RSD of the DMS measurement was low (2 and 6 %) and no trend appeared over time for the 3 replicates of filtrate aliquot. These results suggest that the DMS concentration remained stable in the filtrate between the measurements of the 3 aliquots of culture and that no DMS appeared to be produced after the filtration of the sample. Consequently, the variability between replicates in DMS measurement could not come from DMS production in the filtrate. Therefore, the variability in DMS measurement is more likely to come from the filtration step rather than from post-filtration processes acting on the filtrate. The averages of DMS values were significantly different (P=0.05, Mann-Whitney *U* test) with higher DMS values for swirled flask (5426 for 18 ml filtrate) than non-swirled flask (4238 for 20 ml filtrate). Therefore the agitation of the flask seemed to increase (28 %) the DMS value. For a filtrate of double volume (38 ml), larger RSD

of 19 % and double DMS value of SQRT PA (square root of the peak area) was obtained even if only 2 ml aliquot of filtrate was analysed ($P<0.05$, Mann-Whitney *U* test). In this case, the result implies that the DMS value obtained was affected by the volume of the filtrate (as found for $DMSP_p$ measurement in section 3.5.1.2.1) or the time of filtration which increased with the sample volume (discussed below).

To test if the filtration process was generating a gradient of DMS production as cells accumulated on the filter, I stop-started filtering a 20 ml culture sample (in stationary phase). This provided 3 aliquots of culture sample (2 ml), at different stages of the filtration, for DMS analysis. For comparison, I measured the DMS content of another 20 ml sample of the same culture, filtering 17 ml in one go and taking the ending aliquot of filtration. Table 3.5 shows the SQRT PA obtained for each filtrate.

Table 3.5 DMS measurements performed on two 20 ml samples of *H. triquetra*, one sample filtered in 3 parts by stop-start filtering the culture and 1 sample filtered entirely in one go.

	SQRT PA	**RSD %**	**n**
1st sample, 20 ml culture sample, 4.7 ml filtered 3 times	13502	33	3
Filtrate 1	8787		
Filtrate 2	13975		
Filtrate 3	17743		
2nd sample, 20 ml culture sample, 17 ml filtered continuously	21981		1

The SQRT PA obtained for each of the successive filtration aliquots (Filtrate 1, 2 and 3) of the 1st culture sample increased (33 % RSD). This result indicated that DMS was not homogenous in the filtrate and that the more the cells accumulated on the filter, the more DMS was released in the filtrate. The filtration of the second culture sample (17 ml) which was filtered continuously gave a higher value (21981) than all the DMS values obtained for the 1st sample. Therefore, a gradient of DMS appeared over the filtration of the sample, i.e. the filtration pressure triggered DMS release from cells. Consequently, the time that cells are left in the syringe during filtration may also affect the DMS release. I, hence, decided to perform DMS measurements with only small sample volumes (4.7 ml when using the syringe pump) and high delivery speed of the syringe.

To investigate the effect of the filtration method on DMS measurement, the gravity filtration was also tested. Two ml of culture were filtered through a 3.0 μm pore cellulose nitrate filter of 25 mm of diameter (I noticed that these filters became wet faster than GF/F filters and could shorten the filtration) on a glass filtration unit. The filtrate was collected in a 2 ml disposable tube and 1 ml of filtrate was analysed for DMS measurement. Table 3.6 shows the average of the SQRT PA and RSD for samples taken without agitating the flask and after agitation.

Table 3.6 DMS measurements performed after gravity filtration of 2 ml of *H. triquetra* sample. Three replicate samples were taken without swirling the flasks and 3 others were taken after swirling the flask. The duration of filtration is shown for each replicate.

Gravity filtration of 2ml sample. Analysis of 1 ml filtrate.	**Duration of filtration**	**SQRT PA**	**RSD %**	**n**
Without swirling flask	04:00	4352		
	05:15	5838		
	02:30	1294		
	Average	*3828*	61	3
Usual swirl of the flask	04:00	3973		
	03:10	2974		
	02:10	1547		
	Average	*2831*	43	3

The analytical uncertainty was not improved by this method (RSD of 43 - 61 %). The DMS values obtained for non-agitated samples were higher than the values of the agitated samples (26 % difference) but no significant difference was observed ($P>0.05$, Mann-Whitney *U* test). By contrast with previous observation of a significant increase of DMS value when the flask was agitated (Table 3.4), in this experiment the agitation of the flask appeared to reduce the DMS value and did not lead to a significant difference. The effect of the agitation of the flask on DMS measurement is rather complex and cannot be predicted with the results obtained here. Nevertheless, the agitation of the flask should be generally limited for dinoflagellate culture and sampling.

However, the duration of filtration for these replicates was recorded (Table 3.6) and the duration and DMS values were significantly correlated ($P<0.01$, Spearman test) and the least-squares regression of these 2 parameters gave a high correlation coefficient $R^2=0.97$ (Figure 3.21). The DMS values thus, increased with the time of filtration.

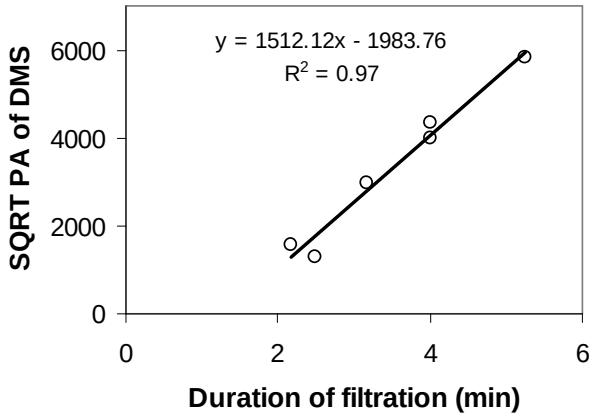

Figure 3.21 Regression of DMS values (SQRT PA for square root of the peak area obtained from the chromatogram) and the duration of filtration.

The duration of filtration is the main parameter affecting the variability of the DMS measurement in the method of gravity filtration. To allow comparable measurements during experiments, similar volumes of culture samples will be used for filtration (5 ml). The syringe pump will be set at high speed (14 ml min^{-1} for 5 ml syringe) to shorten the filtration step and avoid increasing DMS value. Moreover, the sample loop ensures injecting the same portion of the filtrate for all replicates.

3.6. Conclusion

Similar to other phytoplankton species such as *Emiliania huxleyi*, the intracellular DMSP concentration of photosynthetic dinoflagellates varies with the diel cycle. In this study, I showed for the first time that the $DMSP_p$ concentration varied over the light cycle in the dinoflagellate species *Heterocapsa triquetra* grown in batch cultures and that $DMSP_p$ accumulated over the photoperiod. I also determined that the $DMSP_p$ concentration fluctuates over the growth of the batch culture, with an increase at the end of the exponential growth phase. For comparison within an experiment or between several experiments, I decided to perform sampling of the cultures at the mid-time of the photoperiod and at mid-exponential phase to exclude these potential biological variations.

It is the first time that the accuracy of the methods for DMSP and DMS measurements was assessed by measuring all the fractions of the DMSP pool ($DMSP_T$, $DMSP_p$, $DMSP_d$ and DMS). For the model species of dinoflagellate, *Heterocapsa triquetra*, I found that a fraction of about a quarter of $DMSP_T$ was missing. Given that $DMSP_p$ was the major fraction of $DMSP_T$, I investigated the method for $DMSP_p$ measurement by testing several methods of filtration. In one experiment, the use of the automated syringe pump lead to higher $DMSP_p$ concentrations than the hand vacuum pump but in another experiment, no difference appeared when using the syringe pump, the hand vacuum pump and the filtration by gravity. However, the method of filtration using the syringe pump was optimised by using the highest delivery speed available given that I observed that this speed was positively correlated with the DMSP concentration obtained and reduced the analytical uncertainty. Moreover this method was also applied for DMS measurement.

Given that $DMSP_p$ measurement was not obviously improved by the use of different filtration methods, I favoured the use of $DMSP_T$ as a surrogate of intracellular DMSP. Therefore, I assessed the method of $DMSP_T$ measurement and determined that the direct addition of 1ml of phytoplankton culture in 2 ml of 0.75 NaOH was not affecting the DMSP cleavage or the DMS partitioning in the air and liquid phases in comparison with the method of standard preparation. Moreover the variation of DMSP

over the growth was similar for $DMSP_p$ and $DMSP_T$ and the analytical uncertainty was lower for $DMSP_T$ than $DMSP_p$ measurements.

I also investigated the method for DMS measurement and obtained an analytical uncertainty of 3.2 % (n=5) for the method of standard preparation. I used a SSPT system (syringe pump, sample loop and purge and trap) for optimising the analytical uncertainty of the culture samples, however, the RSD was much higher (33 %) than for the standards (2 %) . I found that DMS concentrations can be affected by swirling the flask and were reduced by shortening the time of filtration and decreasing the sample volume. Consequently, I decided to limit as much as possible the flask agitation before the sampling and to use small culture volume (5 ml) and high delivery speed of the syringe (14 ml min^{-1}) for DMS measurement.

Chapter 4 DMSP content and DLA in batch cultures of nine dinoflagellate species

Abstract

Dinoflagellates are recognised as one of the major phytoplankton groups producing DMSP, the precursor of the sulphur trace gas dimethylsulphide (DMS) which has the potential to cool the climate. In order to increase the DMSP data base for this group I measured DMSP content, DMSP lyase activity (DLA), carbon, nitrogen and Chl *a* in 9 dinoflagellate cultures (8 phototrophic and 1 heterotrophic strain). Culture growth rates ranged from 0.11 to 1.82 day^{-1} with the highest value being observed for the heterotroph. Cell volume varied between 454 and 18439 μm^3 and C and N content were proportional to the cell volume. However, DMSP content did not correlate with cell volume and I observed 2 orders of magnitude variability in DMSP content and detected DLA in 5 of the 9 species. The dataset includes the first DMSP measurements for a dinoflagellate from the Antarctic and a species with diatom-like plastids. Lower DMSP concentrations were found in athecate species and a dinoflagellate that harbours haptophyte-like plastids. These data should help towards including the dinoflagellates as a separate group in future global climate models including those that explicitly include DMSP production.

4.1. Introduction

Dimethylsulphide (DMS) is derived from the degradation of the phytoplankton metabolite dimethylsulphoniopropionate (DMSP). It is transferred from the ocean to the atmosphere by sea-to-air exchange and rapidly oxidises to form sulphate aerosols that can serve as cloud condensation nuclei (CCN). These aerosols and CCN increase the albedo and reduce the amount of solar radiation reaching the Earth's surface. These effects are more substantial in areas remote from anthropogenic influence where industrial aerosols are dominant. Charlson and co-workers, (1987) proposed the "CLAW hypothesis", a potential control feed back by algae on climate through the sulphur cycle as initially suggested by Shaw (1983). In this conceptual model they suggested that in an ocean warming scenario algae would reduce solar radiation via increased DMS emissions, thus counteracting the initial warming. The validity of the CLAW hypothesis continues to be a topic that attracts considerable debate (e.g. Chapter 1, section 1.1.3).

Modelling DMS production and its effect on climate is a real challenge due to the complexity of the biological and physical processes involved (Stefels et al., 2007). The biological processes are initiated with DMSP production by various marine phytoplankton, the primary issues being which species and groups synthesise DMSP, in what amounts and under what conditions. Subsequently, DMSP is released to the environment by cell lysis due to senescence, grazing and viral infection. Much of this dissolved DMSP is assimilated or catabolised by bacteria without the production of DMS or exported via sedimentation of grazer faecal pellets. Nonetheless, a portion of the DMSP is converted to DMS by the action of bacterial and algal enzymes.

Here, we focus on marine dinoflagellates which are recognised as one of the major DMSP-producing phytoplankton groups (Stefels et al., 2007). Dinoflagellates are common in aquatic ecosystems, benthic environments and sea-ice. They are a very diverse group with free-living, symbiotic and parasitic representatives. It is thought that about 50 % of the 2000 or so living dinoflagellate species are photosynthetic, whilst others are heterotrophic and some have both nutritional modes. Some of the earliest DMSP and DMS production studies focussed on cultures of the osmotrophic

dinoflagellate *Gyrodinium cohnii* which is now known as *Crypthecodinium cohnii* (Ishida, 1968). The later work of Keller et al. (1988/1989; 1989a; 1989b) highlighted the wide variability in DMSP concentration between dinoflagellates. To date about 40 dinoflagellate species have been tested for DMSP content, 9 species have also been assessed for DMSP lyase activity and data from various field studies suggests that dinoflagellates may make a significant contribution to marine DMS production (Gibson et al., 1996; Steinke et al., 2002a; Franklin et al., 2009).

Here, I present DMSP content and DMSP lyase activity data for 9 diverse dinoflagellate species with associated C, N and Chl *a* measurements. My overall aim was to increase the database available for describing and modelling the contribution of this important phytoplankton group to DMSP and DMS production.

4.2. Materials and Methods

4.2.1. Dinoflagellate cultures

Within the constraints of the range of cultures readily obtainable from culture collections, I selected 9 diverse dinoflagellate species for this study. Eight cultures were purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences, Boothbay Harbor, USA) and *Lingulodinium polyedrum* LP2810 was kindly provided by Dr Débora Iglesias-Rodríguez (National Oceanography Centre, Southampton, UK). Among the 9 dinoflagellate species, 4 photosynthetic species (*Heterocapsa triquetra* CCMP449, *Scrippsiella trochoidea* CCMP1599, *Amphidinium carterae* CCMP1314 and *Lingulodinium polyedrum* LP2810) and 1 heterotrophic species (*Crypthecodinium cohnii* CCMP316) were chosen because they are relatively common species and allow for some comparison with published values for DMSP content and DMSP lyase activity. In addition, I selected *Alexandrium minutum* (CCMP113) a photosynthetic species which has been studied only in field samples and a representative from polar waters *Polarella glacialis* (CCMP1138). The dinoflagellate group is unique in having species that harbour a range of different plastid types, so to cover this I also used *Kryptoperidinium foliaceum* (CCMP1326) which harbours diatom-like plastids and *Karlodinium veneficum* (CCMP4151) with its haptophyte-like plastids. Dinoflagellate cells are also diverse in their morphology with some cells having an armour of cellulosic plates or being naked. Among these species, 3 are naked or athecate (*A. carterae*, *P. glacialis* and *K. veneficum*) and others are armoured or thecate.

4.2.2. Culture conditions

Basic information for the 9 dinoflagellate species and the growth conditions used are given in Table 4.1. Enriched seawater media were prepared as recommended

by the culture collection or originator. Growth conditions were at or close to their recommendations and were not optimised for maximum growth. For all experiments, triplicate batch cultures were grown in incubators (MLR-351 Plant Growth Chamber, Sanyo, Loughborough, UK) with a light: dark cycle of 14:10 hours. Four of the species were axenic and axenicity was checked at the end of each experiment by microscopic observation of samples stained with DAPI (detailed in section 2.1.3).

Table 4.1 A summary of the dinoflagellate species investigated. Names, origin, toxicity, axenicity and culture conditions (LI is light intensity in µmol photons m^{-2} s^{-1} and T is temperature in °C) are described. Three species are athecate (*A. carterae, P. glacialis and K. veneficum*), others are thecate. *K. foliaceum* contains diatom-like plastids, *K. veneficum* has haptophyte-like plastids and others have peridinin plastids except the heterotroph *C. cohnii* which is thought to have a remnant plastid.

Latin name	Synonyms	Strain code	Collection site	Medium*	LI	T °C
[T] *Alexandrium minutum*	*Alexandrium ibericum*	CCMP113	Ria de Vigo, Spain	L1	156	15
[A] *Amphidinium carterae*		CCMP1314	Falmouth, MA, USA	f/2	204	22
[A] *Crypthecodinium cohnii*	*Glenodinium cohnii* *Gyrodinium cohnii*	CCMP316	Unknown	f/2 + NPM	127	22
[A] *Heterocapsa triquetra*	*Peridinium triquetra*	CCMP449	St Lawrence Estuary, Canada	f/2	120	15
[T] *Karlodinium veneficum*	*Karlodinium micrum*	CCMP415	Norway, North Sea	f/2	122	15
Kryptoperidinium foliaceum	*Peridinium foliaceum,* *Glenodinium foliaceum*	CCMP1326	La Jolla, California Bight, USA	L1	204	22
[PT] *Lingulodinium polyedrum*	*Gonyaulax polyedra*	LP2810	San Diego, California, USA	f/2	204	22
Polarella glacialis		CCMP1138	McMurdo Sound, Antarctica	Provasoli	101	4
[A] *Scrippsiella trochoidea*	*Peridinium trochoideum*	CCMP1599	Falmouth, MA, USA	f/2	102	15

* Media were prepared as described in chapter 2

[A] Axenic, [T] Toxic and [PT] Potentially toxic

4.2.3. Experimental approach

Three replicate cultures were grown for each species. Triplicate samples were taken for DMSP, C, N and Chl *a* analyses in early to mid-exponential phase based on cell density increase and in a range of total cell volume between 20 and 60 $\mu L_{cell}\ L^{-1}$ given that cell volume varied substantially between species. Average and relative standard deviation (RSD) from all samples (n=9) are given for each parameter in the results. All parameters were measured following the methods described in Chapter 2. The total DMSP ($DMSP_T$) which includes particulate and dissolved DMSP plus DMS, was measured as an approximation of intracellular DMSP (Keller et al., 1989a; Yost and Mitchelmore, 2009).

For *in vitro* DMSP lyase activity (DLA) measurements, the cultures were sampled in mid-to late exponential phase at a total cell volume between 40 and 100 $\mu L_{cell}\ L^{-1}$. These higher cell densities were more favourable for detection of enzyme activity. The culture was concentrated by gently filtering 100 ml through a 2.0 µm polycarbonate filter and collecting the filter (detailed in section 2.2.2.3.). Culture extracts were prepared from the filter by sonicating in buffer and duplicate measurements were performed on each culture extract (detailed in section 2.2.2.3.). The average and standard deviation of 3 replicate cultures are shown in the results. The detection limit of the assay was based on the average DMS production detected in buffers of 7.34 nM h^{-1}.

For monitoring culture growth, cell density (cell ml^{-1}), total cell volume ($\mu L_{cell}\ L^{-1}$ equivalent to $10^6\ \mu m^3\ ml^{-1}$) and the mean cell volume (μm^3) were monitored daily, or less frequently depending upon the growth rate of each individual species. The growth rate was calculated using the formula $[\mu = (\ln N_2/\ln N_1)/t_2\text{-}t_1]$ where N_1 and N_2 were the cell densities recorded at times t_1 and t_2. For the main sampling time where additional measurements of DMSP were performed, the growth rate was calculated as an average of two growth rates calculated for the period preceding (t_1-t_0) and following (t_2-t_1) the main sampling time (t_1).

4.2.4. Data analysis

The normality of the data was assessed by performing Kolmogorov-Smirnov and Shapiro-Wilk tests (using SPSS, version 16.0). Some data were normalised by $\log_{10}$ transformation prior to regression analysis. The carbon to volume relationship and nitrogen to volume relationship were determined by model I regression as described by Menden-Deuer and Lessard (2000). The least-squares regression analyses were performed using Microsoft Office Excel, version 2003 and the statistical significance of regressions were tested by ANOVA. The correlation of two variables was tested with Pearson tests for normal variables and the non-parametric Spearman test for non-normal variables (SPSS, version 16.0). The non-parametric Mann-Whitney *U* test was also applied for the comparison of two independent groups of non-normal data (SPSS, version 16.0).

4.3. Results

Growth curves for the nine dinoflagellate species are shown in Figure 4.1. Growth rates varied between 0.11 and 1.82 day^{-1} with an average RSD of 8 %. The average growth rate for phototrophic species was 0.32 day^{-1} (Table 4.2). The polar species *P. glacialis* grew the slowest at 0.11 day^{-1} and *A. carterae* was the fastest growing phototrophic species at 0.72 day^{-1}. The heterotrophic species *C. cohnii* grew faster than any of the phototrophic species at 1.82 day^{-1}. These growth rates are comparable to dinoflagellate growth rates previously reported in the literature (e.g. 0.16 - 1.28 day^{-1}, Tang 1996).

The mean cell volume was extremely variable between species (Table 4.2) with a 4-fold difference between the smallest (454 μm^3 for *A. carterae*) and the biggest species (18439 μm^3 for *L. polyedrum*). The 3 athecate species (*A. carterae*, *P. glacialis* and *K. veneficum*) were the smallest of the group (454-680 μm^3). These cell volumes fall in the range reported for dinoflagellates by Menden-Deuer and Lessard (2000). The average 6 % RSD for the cell volume measurements I measured with a Coulter Multisizer 3 particle counter compares favourably with 22-56 % RSD values derived from microscopy data (Verity et al., 1992; Menden-Deuer and Lessard, 2000).

The dinoflagellate cells contained on average 1233 ± 1801.3 pg C $cell^{-1}$and 277 ± 445.2 pg N $cell^{-1}$ (Table 4.2). The average concentrations on a cell volume basis were 0.330 ± 0.2515 pg C μm^{-3} and 0.067 ± 0.0410 pg N μm^{-3}. The RSD of the C and N measurements per cell and per cell volume were 19 % and 20 % respectively. *C. cohnii* contained higher C and N concentrations than photosynthetic species. The C and N concentrations per cell volume were compared for the thecate and athecate dinoflagellates, but no significant difference was found (Mann-Whitney *U* test, $P>0.05$). The C: N ratio varied between 3.6 and 6.3 (Table 4.2).

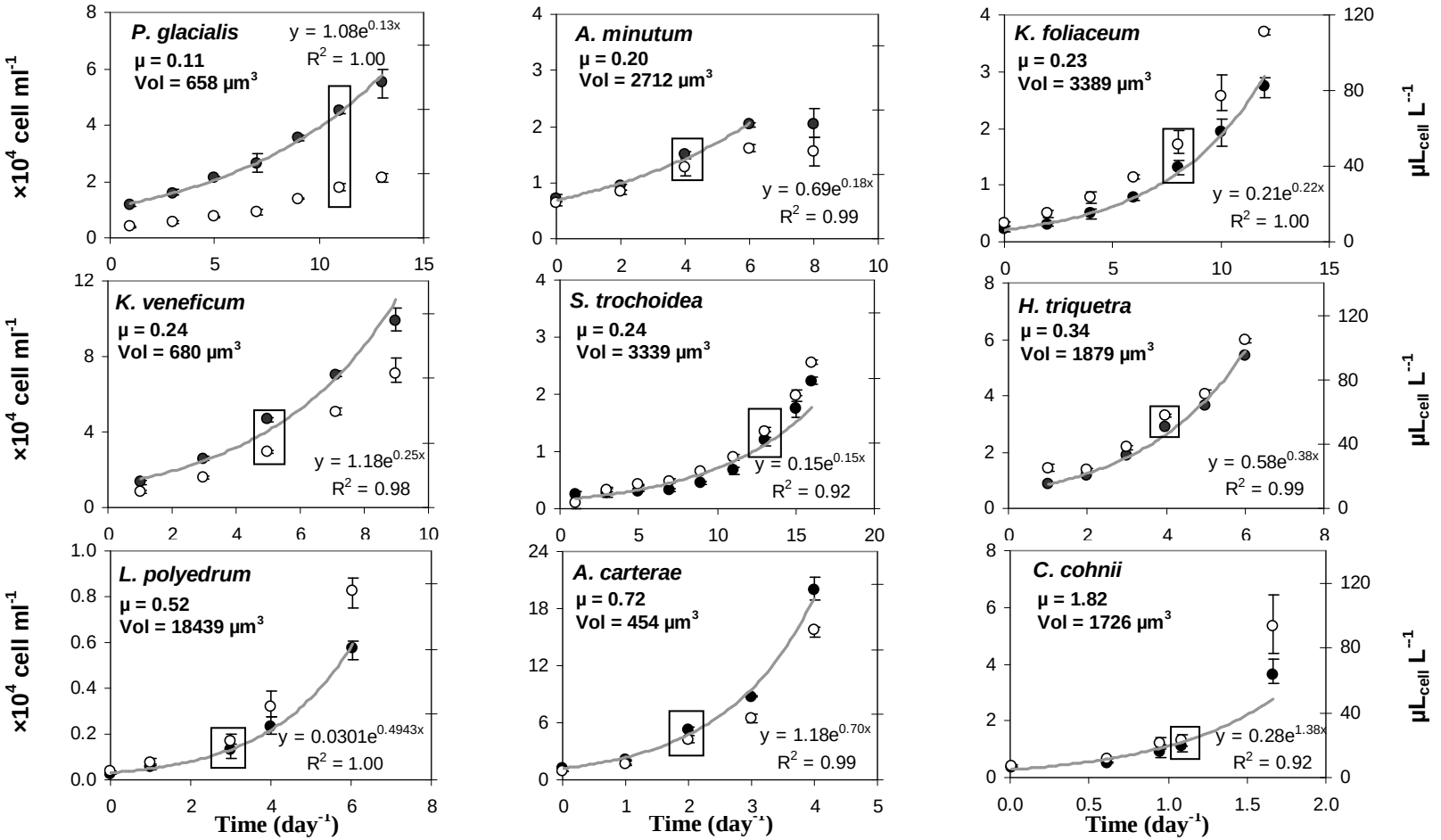

Figure 4.1 Growth curves for 9 dinoflagellate species. The data are presented in terms of cell density in cell ml^{-1} (●) and cell volume in μm^3 ml^{-1}(○). The average value for 3 replicate cultures is given with range bars to show the minimum and maximum values. Exponential trendlines are shown for the cell density curves with correlation coefficients to support how the data points follow an exponential increase. The last data points may be excluded from the trendline when its incorporation reduces the correlation coefficient due to the ending of exponential phase. Samples were taken for analysis during the exponential phase as indicated by the rectangles and the growth rate (μ in day^{-1}) and cell volume (Vol) for this sampling point are indicated. Species are ordered from left to right in increasing growth rate order.

Table 4.2 Growth rate, mean cell volume, C, N and Chl *a* concentrations measured in 9 dinoflagellate species. The C:N ratio was calculated with C and N expressed per litre of culture. Averages (± relative standard deviation) obtained from 9 culture samples are given. *Crypthecodinium cohnii* is a heterotrophic species so Chl *a* measurements were not possible and the average values for the phototrophs exclude this species.

Species	Growth rate (day^{-1})	Volume (μm^3)	C ($pg\ cell^{-1}$)	C ($pg\ \mu m^{-3}$)	N ($pg\ cell^{-1}$)	N ($pg\ \mu m^{-3}$)	C : N ratio	Chl *a* ($fg\ cell^{-1}$)	Chl *a* ($fg\ \mu m^{-3}$)
H. triquetra	0.34 (0.11)	1879 (0.03)	344 (0.14)	0.173 (0.13)	55 (0.10)	0.028 (0.09)	6.3 (0.15)	2873 (0.31)	1.45 (0.30)
S. trochoidea	0.24 (0.06)	3339 (0.11)	649 (0.23)	0.163 (0.19)	153 (0.31)	0.038 (0.28)	4.3 (0.30)	2424 (0.52)	0.61 (0.50)
A. carterae	0.72 (0.02)	454 (0.04)	139 (0.18)	0.301 (0.20)	31 (0.12)	0.066 (0.14)	4.5 (0.19)	2311 (0.16)	4.94 (0.18)
A. minutum	0.20 (0.07)	2712 (0.07)	745 (0.11)	0.251 (0.21)	160 (0.11)	0.054 (0.20)	4.7 (0.07)	3309 (0.17)	1.11 (0.24)
C. cohnii	1.82 (0.09)	1726 (0.05)	2051 (0.22)	0.987 (0.24)	352 (0.23)	0.169 (0.24)	5.8 (0.15)	- -	-
P. glacialis	0.11 (0.16)	658 (0.11)	199 (0.18)	0.285 (0.21)	37 (0.12)	0.052 (0.17)	5.5 (0.21)	1005 (0.54)	1.43 (0.55)
K. foliaceum	0.23 (0.08)	3389 (0.05)	1037 (0.17)	0.260 (0.18)	225 (0.14)	0.057 (0.15)	4.6 (0.17)	14771 (0.14)	3.71 (0.15)
L. polyedrum	0.52 (0.08)	18439 (0.04)	5760 (0.32)	0.308 (0.30)	1429 (0.29)	0.076 (0.27)	4.0 (0.25)	57352 (0.37)	3.07 (0.35)
K. veneficum	0.24 (0.07)	680 (0.01)	176 (0.18)	0.244 (0.13)	48 (0.27)	0.067 (0.27)	3.6 (0.32)	1879 (0.18)	2.60 (0.18)
Averages									
Phototrophs	0.32 (0.62)	3944 (1.52)	1131 (1.68)	0.248 (0.22)	267 (1.78)	0.055 (0.29)	4.7 (0.18)	10740 (1.80)	2.37 (0.63)
All species	0.49 (1.09)	3697 (1.53)	1233 (1.46)	0.330 (0.76)	277 (1.61)	0.067 (0.61)	4.8 (0.18)	- -	- -

Least-squares regression analyses of log_{10} transformed C per cell amounts to the mean cell volume (Figure 4.2) showed a significant relationship ($P<0.01$, Table 4.3) between these variables. The correlation was also supported by the Pearson test ($P<0.01$). The N per cell values showed similar relationships with cell volume. Therefore the C and N content may be predicted from the equations listed in Table 4.3.

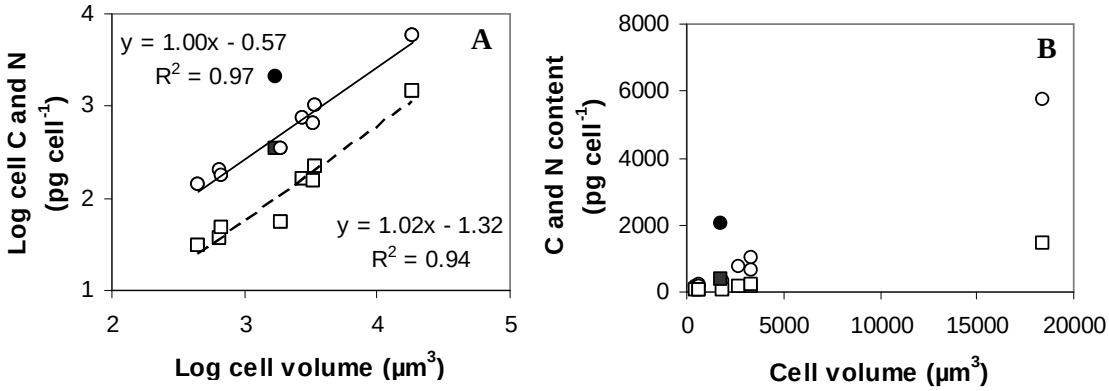

Figure 4.2 (A) Least-squares regression of log_{10}-transformed cell carbon (○) and cell nitrogen (□) to cell volume for the 8 autotrophic dinoflagellates. Data for the heterotroph *C. cohnii* are shown by the black symbols (●■) and inclusion or exclusion of these leads to the regression equations shown in Table 4.3. (B) Visualisation of the same data without log_{10} transformation.

Table 4.3 Regression analyses for log_{10}-transformed carbon and nitrogen to cell volume data. For each analysis, the intercept (a) and the slope (b) with their 95% confidence intervals (CI) of the resulting equation [Log_{10} C (pg cell^{-1}) =a + (b × Log_{10} Vol (µm^3))] are listed with the square of the correlation coefficient (r^2), the number of data points (n) and the probability (P) associated with the regression.

	a	95%CI	b	95%CI	r^2	n	P
Log_{10} C per cell							
Autotrophs	-0.572	1.182	0.997	0.355	0.97	8	<0.01
All species	-0.445	2.847	0.981	0.858	0.81	9	<0.001
Log_{10} N per cell							
Autotrophs	-1.321	1.743	1.023	0.524	0.94	8	<0.001
All species	-1.213	2.729	1.009	0.822	0.81	9	<0.001

The Chl *a* content ranged from 1005 fg cell^{-1} in *P. glacialis* to 57352 fg cell^{-1} in *L. polyedrum* with an average of 10740 ± 19344.5 fg cell^{-1}. These values are close to the 190 - 23000 fg cell^{-1} range reported for 35 dinoflagellate species (Tang, 1996). Expressed on a per cell volume basis Chl *a* concentrations ranged from 0.61 fg µm^{-3} in *S. trochoidea* to 4.94 pg µm^{-3} in *A. carterae* with an average of 2.37 ± 1.481 pg µm^{-3}. Chlorophyll data showed an RSD between replicates of 30 % which was larger than for other parameters but of the same order observed in other phytoplankton culture studies (e.g. Steinke et al., 1998).

I adopted the method of $DMSP_T$ used here because I found the model dinoflagellate *Heterocapsa triquetra* was quite sensitive to filtration and this technique gave more consistent data. With this method, the average RSD obtained here for 9 dinoflagellate species was 14 % per cell and 16 % per cell volume on average (Table 4.4) which is lower than the RSD of 52 % given for $DMSP_T$ analysis by Yost et al (2009). $DMSP_T$ concentrations per cell volume ranged from 11 mM in *K. veneficum* to 364 mM in *H. triquetra* and the average was 174 ± 142.8 mM (Table 4.4). Two groups of species can be distinguished: (1) *A. minutum, A. carterae, S. trochoidea* and *H. triquetra* had DMSP concentrations between 290 and 364 mM strongly above the average; (2) *K. veneficum, L. polyedrum, K. foliaceum, P. glacialis* and *C. cohnii* had medium to low DMSP concentrations ranging from 11 to 103 mM. I also found that thecate species contained significantly higher DMSP concentrations per cell volume than athecate species (Mann-Whitney *U* test, $P<0.05$). Given that the athecate species had a smaller cell volume than thecate species I tested the correlation between the cell volume and the DMSP content but no significant correlation was obtained between the cell volume and DMSP concentration per cell volume (Spearman test, $P>0.05$). For correlation between cell volume and DMSP per cell opposite conclusions were obtained with different statistical tests. These two parameters were significantly correlated ($P<0.05$) with a Spearman's test, not significantly correlated ($P>0.05$) but suggestively correlated ($P=0.097$ <0.10) with Pearson's test using data normalised by $\log_{10}$ transformation and the least-squares regression was not significant ($P>0.05$; regression illustrated in Figure 4.3).

Table 4.4 DMSP data for 9 dinoflagellate species. $DMSP_T$ is expressed per total cell volume (CV), per cell, per carbon, per nitrogen and per chlorophyll *a*. (C)DMSP:C indicates the DMSP-carbon to C ratio and (S)DMSP:N indicates the DMSP-sulphur to N ratio. Values are ranged in decreasing order of $DMSP_T$ per cell volume. Averages (± relative standard deviation) obtained from 9 culture samples are given. Phototrophs include all species except the heterotroph *Crypthecodinium cohnii*.

Species	$DMSP_T$:CV (mM)	$DMSP_T$:cell (pmol $cell^{-1}$)	$DMSP_T$:C (mmol mol^{-1})	$DMSP_T$:N (mmol mol^{-1})	$DMSP_T$:Chl*a* (mmol g^{-1})	(C)DMSP:C (%)	(S)DMSP:N g g^{-1}
H. triquetra	364 (0.06)	0.719 (0.07)	25.1 (0.13)	184.5 (0.09)	250 (0.30)	12.6 (0.16)	0.422 (0.13)
S. trochoidea	326 (0.13)	1.300 (0.19)	24.0 (0.16)	119.3 (0.26)	536 (0.49)	12.0 (0.30)	0.273 (0.37)
A. carterae	300 (0.12)	0.140 (0.09)	12.1 (0.18)	63.8 (0.11)	61 (0.16)	6.0 (0.20)	0.146 (0.15)
A. minutum	290 (0.20)	0.861 (0.10)	13.9 (0.06)	75.4 (0.05)	260 (0.14)	6.9 (0.15)	0.173 (0.15)
C. cohnii	103 (0.24)	0.213 (0.22)	1.2 (0.13)	8.5 (0.14)	- -	0.6 (0.31)	0.019 (0.32)
P. glacialis	94 (0.12)	0.066 (0.05)	4.0 (0.18)	25.3 (0.12)	66 (0.54)	2.0 (0.19)	0.058 (0.13)
K. foliaceum	56 (0.13)	0.223 (0.12)	2.6 (0.15)	13.8 (0.12)	15 (0.12)	1.3 (0.21)	0.032 (0.19)
L. polyedrum	23 (0.32)	0.426 (0.33)	0.9 (0.30)	4.2 (0.27)	7 (0.36)	0.4 (0.46)	0.010 (0.44)
K. veneficum	11 (0.09)	0.008 (0.09)	0.5 (0.19)	2.3 (0.27)	4 (0.19)	0.3 (0.20)	0.005 (0.28)
Averages							
Phototrophs	183 (0.82)	0.468 (0.97)	10.4 (0.97)	61.1 (1.05)	150 (1.25)	5.2 (0.97)	0.14 (1.05)
All species	174 (0.82)	0.440 (0.99)	9.4 (1.05)	55.2 (1.14)	- -	4.7 (1.05)	0.13 (1.14)

$DMSP_T$ per cell varied between 0.008 pmol $cell^{-1}$ in *K. veneficum* and 1.3 pmol $cell^{-1}$ in *S. trochoidea* with an average of 0.44 ± 0.434 pmol $cell^{-1}$. Because the cell volume was highly variable between species, the $DMSP_T$ values expressed per cell volume and per cell did not give the same species order.

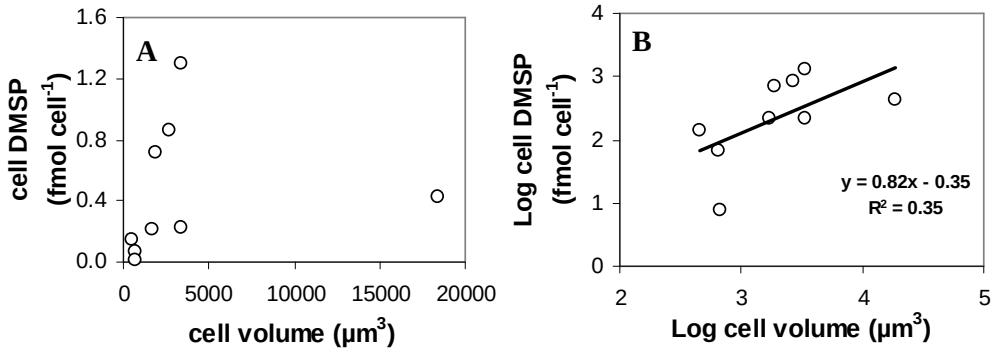

Figure 4.3 Least squares regression of (A) DMSP per cell to cell volume (B) log_{10} transformed data.

The $DMSP_T$ per C and per N values followed a similar pattern to $DMSP_T$ per cell volume values because the C and N contents were positively correlated with the cell volume. The $DMSP_T$ per C quotas ranged from 0.5 to 25.1 mmol mol^{-1} with an average of 9.4 ± 9.89 mmol mol^{-1} (Table 4.4). The $DMSP_T$ per N values ranged from 2.3 to 184.5 mmol mol^{-1} with an average of 55.2 ± 62.75 mmol mol^{-1}. Using the $DMSP_T$ values as an approximation of the intracellular DMSP, the C in DMSP would represent between 0.3 and 12.6 % of the total cell C across this group of dinoflagellates. The sulphur in DMSP to N ratio (S(DMSP):N, Table 4.4), which is a key parameter for DMS models, ranged from 0.005 to 0.42 g g^{-1} with an average of 0.13 ± 0.144 g g^{-1}.

$DMSP_T$: Chl *a* ranged from 4 mmol g^{-1} in *K. veneficum* to 536 mmol g^{-1} in *S. trochoidea* with an average of 150 ± 187.6 mmol g^{-1}. The $DMSP_T$:Chl *a* ratio showed a similar species order to the $DMSP_T$ per cell volume apart from *A. carterae* and *S. trochoidea* which had high and low Chl *a* concentration per cell volume respectively.

Five of the 9 dinoflagellate species presented detectable DMSP lyase activity with DMS production ranging from 15.7 to 1180.5 nM h^{-1} in culture extracts (Table 4.5). The highest DMSP lyase activity was found in the *A. minutum* culture (1180.5 nM h^{-1}). This activity was about twice that observed for the *A. carterae* culture, 5 and 7-fold

higher than in *C. cohnii* and *L. polyedrum* cultures. DMSP lyase activity was barely detectable in *S. trochoidea* (15.7 nM h^{-1}) slightly above the detection limit (7.3 nM h^{-1}) and no activity was detected with this assay method in *H. triquetra, P.glacialis, K. foliaceum and K. veneficum.*

Table 4.5 *In vitro* DMSP lyase activity (DLA) for 9 dinoflagellate species. DMS production is expressed per litre of culture, per cell and per total cell volume (CV). Averages (± relative standard deviation) obtained from 3 replicate cultures are listed. BDL means Below Detection Limit.

Species	DLA (nM h^{-1})	DLA: cell (fmol $cell^{-1}$ h^{-1})	DLA: CV (mmol L_{cell}^{-1} h^{-1})
H. triquetra	BDL	BDL	BDL
S. trochoidea	15.7 (0.30)	0.61 (0.37)	0.15 (0.34)
A. carterae	613.0 (0.02)	3.07 (0.08)	6.70 (0.06)
A. minutum	1180.5 (0.07)	59.73 (0.07)	22.56 (0.10)
C. cohnii	239.8 (0.17)	7.27 (0.27)	2.86 (0.33)
P. glacialis	BDL	BDL	BDL
K. foliaceum	BDL	BDL	BDL
L. polyedrum	164.8 (0.10)	0.80 (0.16)	1.75 (0.14)
K. veneficum	BDL	BDL	BDL
Averages			
Phototrophs	493.5 (1.06)	16.05 (1.81)	7.79 (1.31)
All species	442.8 (1.06)	14.30 (1.79)	6.80 (1.34)

The variability among the 9 species obtained for each parameter is summarised and visualised in the box and whisker plots shown in Figure 4.4 and Figure 4.5. The C:N ratio showed little variation between species (Figure 4.4). The C and N concentrations showed little variation per cell volume and variation per cell over 2 orders of magnitude. This difference arises from the variation in cell volume that also spreads over 2 orders of magnitude. The variation in DMSP concentrations expressed per cell volume, per cell, per C, N and Chl *a* extended over 2 orders of magnitude (Figure 4.5). The DMSP lyase activity showed same order of variability per cell and slightly less per cell volume.

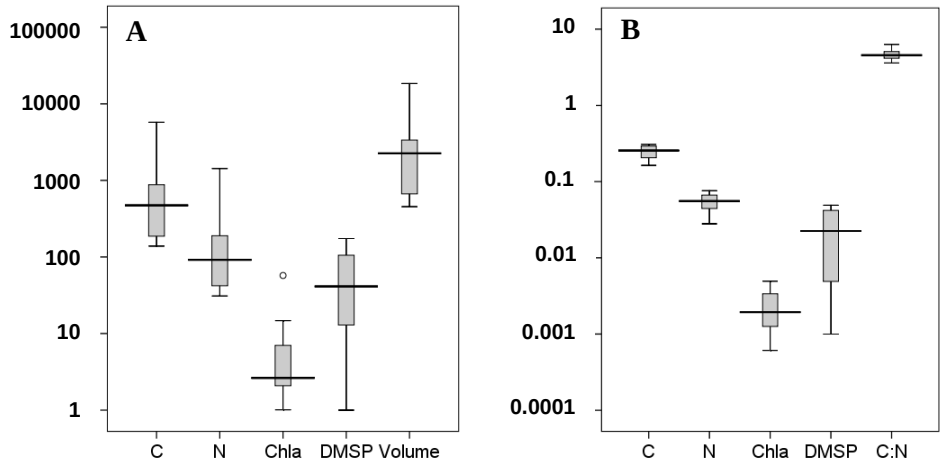

Figure 4.4 Box and whisker plots showing the spread of the data (error bar), the 1st and 3rd quartile range (box) and the median value (horizontal bar) obtained for each parameter measured for 9 dinoflagellate species. The symbol (○) denotes excluded outlier data referring to *Lingulodinium polyedrum*. (A) Cellular contents of C, N, Chl *a*, DMSP, expressed in pg cell^{-1} and the volume of the cell in µm^{3}. (B) Concentrations of C, N, Chl *a*, DMSP per cell volume in pg µm^{-3} and the C:N ratio.

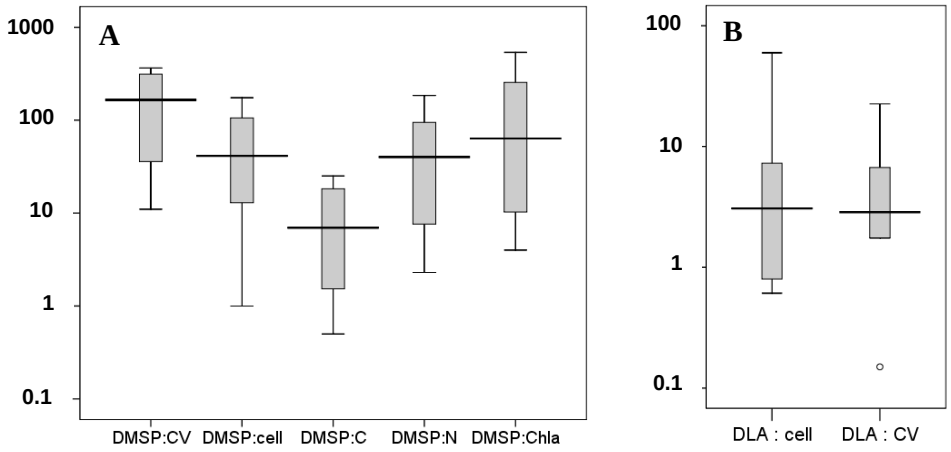

Figure 4.5 Box and whisker plots showing the spread of the data (error bar), the 1st and 3rd quartile range (box) and the median value (horizontal bar) obtained for (A) DMSP and (B) DMSP lyase activity (DLA) measured for 9 dinoflagellate species. The symbol (○) denotes excluded outlier data referring to *Scrippsiella trochoidea*. DMSP is expressed per cell volume (CV) in mM, per cell in pmol cell^{-1}, per carbon in mmol mol^{-1}, per nitrogen in mmol mol^{-1} and per Chl *a* in mmol g^{-1}. DLA is expressed per cell in fmol cell^{-1} h^{-1} and per total CV in mmol L_{cell}^{-1} h^{-1}.

4.4. Discussion

In order to compare the DMSP levels found in different phytoplankton groups and with a view to providing values for inclusion in ecosystem and global climate models, it is necessary to express DMSP data in terms of C, N and Chl *a* (Stefels et al., 2007). Menden-Deuer and Lessard (2000) developed an equation to estimate carbon content from cellular volume using a database of 34 dinoflagellate species. DMSP values expressed per C often come from estimates derived from such equations and only rarely from real measurements. DMSP values from field studies are sometimes expressed in terms of readily available Chl *a* data. Whilst it is difficult to apportion Chl *a* to specific phytoplankton in the field, it is an attractive parameter for modelling studies as a proxy for phytoplankton biomass and because of the ready availability of satellite Chl *a* data. In this study, my approach was to measure these parameters directly.

4.4.1. C and N content

The C and N contents as well as the C:N ratio (3.6 - 6.3) obtained in my study are close to the 3.44 - 6.45 range for 20 dinoflagellates published by Menden-Deuer and Lessard (2000). Verity et al., (1992) obtained a higher range of C:N values from 5.1 to 8.8 for 3 phototrophic dinoflagellates. Meksumpun et al. (1994) obtained a C:N ratio of 6.8 and 7.6 in *Alexandrium catenella* and *Scrippsiella trochoidea* respectively. They also noticed that this ratio varied with growth stage by increasing at the early exponential phase, then decreasing and increasing again at the stationary phase in *A. catenella* and increasing during the exponential phase and decreasing at the stationary phase in *S. trochoidea*. These variations might explain some of the variability between studies. Turning to the basic Redfield ratio which defines the elemental composition of marine phytoplankton as 106:16:1 for C:N:P, the C:N ratio would be 6.625. I found that the photosynthetic species showed a C:N ratio of 4.7 ± 0.84, slightly lower than the Redfield ratio.

Whilst *C. cohnii* showed a higher C concentration per cell volume (0.987 pg μm^{-3}) than the phototrophic species measured here (average of 0.248 ± 0.0539), its carbon quota of 2051 pg C $cell^{-1}$ fits well within the range of 223 - 35349 pg $cell^{-1}$ published for other heterotrophic species (Menden-Deuer and Lessard, 2000). The N content of this species (352 pg $cell^{-1}$) was also higher compared to that of the photosynthetic species and higher than values obtained in another study (48.43; 89.09 pg $cell^{-1}$, Menden-Deuer and Lessard, 2000) but I am not aware of any further data for comparison.

The C content measured in *L. polyedrum* (5760 pg $cell^{-1}$) was 6 times higher than the 990 pg $cell^{-1}$ value reported by Mullin et al. (1966) despite similar cell volumes of 18439 μm^3 and 16800 μm^3 respectively. It is not clear whether these differences reflect the potential for variation between the many strains of this species.

In agreement with the results of Menden-Deuer and Lessard (2000), I observed a significant positive relationship between cell volume and C and N content. I obtained an equation to predict the C content from the mean cell volume [Log_{10} C = -0.445 + (0.981 × Log_{10} Vol)] which is similar to the equation that they calculated [Log_{10} C = -0.353 + (0.864 × Log_{10} Vol)] on the basis of all previous published values. Moreover in agreement with their findings, I found no significant difference in C and N concentrations between thecate and athecate dinoflagellates.

4.4.2. DMSP and DMSP lyase activity

All the dinoflagellates included in this study produced DMSP. However, in common with a previous study by Keller et al., (1989a) considerable variability was observed. In contrast with coccolithophores where a significant correlation between cell volume and DMSP cell quotas has been found (Franklin et al., 2010), no obvious correlation was found for these 9 dinoflagellate species. The significant correlation obtained with non parametric test between cell volume and DMSP cell quotas for the 9 dinoflagellate species was not confirmed by the parametric test using log_{10}-transformed data. However, thecate species contained significantly higher DMSP concentrations

than athecate species. No relationship between the presence or absence of cellulosic plates and DMSP content has been described previously. The collection of more data is warranted to clarify these relationships.

H. triquetra, S. trochoidea, A. carterae and *A. minutum* showed high DMSP concentrations (290 - 364 mM) that are comparable with or higher than those reported for the well-studied prymnesiophytes *Emiliania huxleyi* (50 - 304 mM from for example Franklin et al., 2010; Harada, 2007; Keller, 1988/1989; Keller et al., 1989a; Steinke et al., 1998; van Rijssel and Gieskes, 2002; Wolfe et al., 1997) and *Phaeocystis sp.* (71 - 260 mM from Keller et al., 1989a and Stefels and van Boekel 1993). These dinoflagellates are common bloom-forming species that may act as strong sources of DMS in coastal waters especially during bloom events.

I compared the DMSP and DMSP lyase activity data obtained in this study with data available in the literature and the values are summarised in Table 4.6. In the following section I consider each of the species investigated in turn.

Heterocapsa triquetra is found in estuaries and coastal waters worldwide (Tomas, 1997; Litaker et al., 2002) and has developed as a model photosynthetic dinoflagellate species for laboratory experiments. I obtained similar DMSP content values for *H. triquetra* CCMP449 to those for strain NIES (Niki et al., 2000; Table 4.6). These strains were isolated from different geographic zones (Japan Bay for NIES and the Canadian St Lawrence Estuary for CCMP449) and it is not clear whether they were cultured in different temperature and light conditions, though the same medium was used. Niki et al. (2000) detected substantial DMSP lyase activity in this species suggesting that it would also directly contribute to the DMS pool in the sea, but I was unable to detect any DLA in CCMP449.

Table 4.6 Comparison of DMSP and DMSP lyase activity (DLA) results from this study and published data. Data are given for DMSP per cell volume (CV), DMSP per cell, DLA per cell and DLA per CV. DMSP values expressed in pg cell^{-1} were converted to mol cell^{-1} units using a DMSP molecular weight of 134.2 g. (ND) indicates that DLA was not detected. (NT) indicates that DLA was not tested.

Species /Strain	Data source	DMSP : CV (mM)	DMSP : cell (pmol cell^{-1})	DLA: cell (fmol cell^{-1} h^{-1})	DLA : CV (mmol L_{cell}^{-1} h^{-1})
H. triquetra					
CCMP449	This study	364	0.719	ND	ND
NIES-7	Niki et al., (2000)	300	0.6	30	20
S. trochoidea					
CCMP1599	This study	326	1.3	0.61	0.15
CCAP1134/1	Hatton and Wilson (2007)	169	0.329	NT	NT
CCMP1131	Keller et al., (1989a)	350	2.861	NT	NT
NIES-369	Niki et al., (2000)	600	1.3	18	8
A. carterae					
CCMP1314	This study	300	0.140	3.07	6.70
CCAP1102/1	Hatton and Wilson (2007)	57	0.0147	NT	NT
CCMP1314	Spiese et al. (2009)	109	-	NT	NT
X	Harada (2007)	288	0.0977	4488	13260
CCMP1314	Harada (2007)	326	0.133	1.48	3.61
CCMP1314	Keller (1988/1989)	377	0.144	NT	NT
A. minutum					
CCMP113	This study	290	0.861	59.73	22.56
Field sample	Jean et al., (2005)	3388	14.2	NT	NT
C. cohnii					
CCMP316	This study	103	0.213	7.27	2.86
CCMP316	Keller et al., (1989a)	377	0.341	NT	NT
L. polyedrum					
Lp2810	This study	23	0.435	0.80	1.75
CCMP1738	Harada (2007)	13	0.119	102.6	11.52
CCAP1121/2	Hatton and Wilson (2007)	5	0.0545	NT	NT

Scrippsiella trochoidea is a common, cosmopolitan neritic and estuarine species (Tomas, 1997). The DMSP content of *S. trochoidea* has been measured in several studies and shows intra-specific variation in the range 169 - 600 mM (Table 4.6). Here, DMSP in *S. trochoidea* CCMP1599 (326 mM, grown at 15 º C) is in the middle of the range previously observed and close to the value reported for CCMP1131 (350 mM) which was grown at 20º C. I detected DLA of 0.61 fmol cell^{-1} h^{-1}in *S. trochoidea* culture extracts which was lower than the 18 fmol cell^{-1} h^{-1} obtained by direct measurement of *in vivo* assay on a culture sample of the other strain NIES-369 performed by Niki et al., (2000). This difference is likely to result from the use of different analytical methods but does not exclude a potential intra-specific variability between strains of *S. trochoidea*.

Amphidinium carterae is a common species in temperate and tropical waters (Tomas, 1997). Published values for *A. carterae* include 3 other studies on CCMP314 and the data suggest intra-specific variation in DMSP content in the range of 57 – 377 mM (Table 4.6). *A. carterae* CCMP1314 is also a potential DMS producer due to the presence of DMSP lyase activity. I obtained DLA of 3.07 fmol cell^{-1} h^{-1} which is rather close to the 1.48 fmol cell^{-1} h^{-1} value for *in vitro* activity for the same strain reported by Harada (2007). DLA values for what we assumed to be a different strain of *A. carterae* (strain X, 4488 fmol cell^{-1} h^{-1}; Harada, 2007) suggests wide intra-specific variation in DLA.

Alexandrium minutum is a toxic species that can cause paralytic shellfish poisoning (PSP) after consumption of contaminated seafood. It is globally distributed due to a recent expansion and blooms have been more frequent in recent decades (McCauley et al., 2009). Jean et al. (2005) isolated a small number of *A. minutum* cells (<80) in a field sample from the Mediterranean Sea and found the DMSP content to be 3388 mM and 14.2 pmol cell^{-1} (Table 4.6). This is the highest DMSP level reported for any phytoplankton. Using *A. minutum* CCMP113 originally isolated on the Atlantic coast of Spain, I found 10-fold lower DMSP but nevertheless this is a substantial DMSP concentration. Moreover the DMSP lyase activity (59.73 fmol cell^{-1} h^{-1}) associated with this species makes it a potential DMS producer.

Crypthecodinium cohnii is a brackish to marine heterotrophic dinoflagellate that is usually associated with decomposing temperate and tropical seaweeds (Tomas, 1997; Mendes et al., 2009). *C. cohnii* CCMP316 showed a lower DMSP content than previous results (Table 4.6). However, I have seen considerable variation in DMSP content during different growth phases and depending on nutrient availability (reported in Chapter 6 and Chapter 7). *C. cohnii* is a fast growing species and I took care to monitor growth and perform analysis during the exponential phase. This might account for the higher values reported by Keller and co-workers (1989a). The DMSP lyase activity of *C. cohnii* was previously detected by Kadota and Ishida (1968) but was not quantified. In this study, I reported for the first time *in vitro* DLA measurements in *C. cohnii* culture extracts. The heterotrophic species *C. cohnii* synthesized a medium range concentration of DMSP and DMSP lyase activity and thus, is a potential DMS producer.

Polarella glacialis is a bipolar species found in Antarctic sea-ice, saline lakes and Canadian Arctic seawater (Montresor et al., 1999; Montresor et al., 2003; Thomson et al., 2004; Rengefors et al., 2008). Dinoflagellates have been suggested to be a major source of DMSP in Antarctic coastal waters (Gibson et al., 1996) and this species was reported as dominant in the phytoplankton community in Antarctic fast ice (Thomson et al., 2006). *P. glacialis* is the first polar dinoflagellate tested for DMSP content. A medium DMSP concentration was obtained in this species (94 mM and 0.066 pmol cell^{-1}) and no DMSP lyase activity was detected.

Kryptoperidinium foliaceum is an estuarine species that holds a diatom endosymbiont (Withers and Haxo, 1975; Chesnick et al., 1997) and the first dinoflagellate harbouring diatom-like plastids assessed for DMSP content. A medium to low DMSP concentration was contained by this species (56 mM and 0.223 pmol cell^{-1}) and no DMSP lyase activity was detected.

Lingulodinium polyedrum is a neritic species present in warm waters from temperate to tropical regions and is generally considered as a non-toxic dinoflagellate (Lewis and Hallett, 1997; Tomas, 1997). Sulfonium compounds other than DMSP such as gonyauline and gonyol have been identified in this species and may contribute to the total DMSP pool thereby overestimating DMSP concentration (Nakamura et al., 1996). *L. polyedrum* LP2810 showed a lower DMSP content (23 mM) than other species but

slightly higher than value obtained for other strains (5 - 13 mM, Table 4.6). *L. polyedrum* LP2810 showed some DMSP lyase activity but with lower DMS production compared with the strain CCMP1738 (Harada, 2007).

Karlodinium veneficum is an ichtyotoxic species that is common in temperate estuarine waters (Bachvaroff et al., 2009) and a representative of dinoflagellates that have haptophyte-like plastids. This species displayed a low DMSP content and no DLA was detected with my assay conditions. A few dinoflagellates with haptophyte-like plastids have been tested for DMSP and DMSP lyase activity and they also showed low DMSP content (18 mM in *Karenia brevis* from Harada, 2007, 0.65 and 0.36 mM in *Karenia mikimotoi* from Keller et al., 1989a). Harada (2007) also detected DLA in *Karenia brevis*.

DMSP-carbon represented a variable fraction of the total cellular carbon. I found that in species containing significant amounts of DMSP (*H. triquetra, S. trochoidea, A. carterae, A. minutum*), DMSP was a major component of the total cellular carbon (6 - 13 %). These results are in agreement with the calculations of Matrai and Keller (1994) which showed 12 – 20 % values for 2 dinoflagellates, with the calculations of Stefels et al. (2007) based on 32 dinoflagellate species (11 %) and with the value of 8 % calculated by Archer et al., (2002a) from previous published data and used in a model predicting the DMSP conversion into DMS in a phytoplankton bloom. In species that produce smaller amounts of DMSP (*K. veneficum, L. polyedrum, K. foliaceum P. glacialis* and *C. cohnii*), DMSP contributed less to the cellular carbon pool (0.3 - 2%). The DMSP to C ratio was also variable between species. I obtained a DMSP:C average value of 0.0094 mol mol^{-1} (n=9) that was lower than the 0.022 mol mol^{-1} calculated by Stefels et al., (2007) from 32 DMSP published values converted to C units using the Menden-Deuer and Lessard (2000) equation. In general, the range of dinoflagellate species used here appear to have lower DMSP contents than the 32 values considered by Stefels et al. (2007) and thus, lower DMSP:C quotas.

The DMSP:Chl *a* ratio is often quoted in field data but seldom given alongside culture measurements. The DMSP:Chl *a* average obtained here was 150 mmol g^{-1} (Table 4.4) and is provided to allow comparison with field data and provide values for modellers. It is in agreement with the 111 mmol g^{-1} calculated by Stefels et al (2007) for

32 dinoflagellate species. The DMSP:Chl *a* range of 4 to 536 mmol g^{-1} (Table 4.4) I obtained in this study was a little broader than the values reported for mixed field populations (40 - 280 mmol g^{-1} in Belviso et al., 2000 and Lee et al., 2009). On the other hand, dinoflagellate cultures have a higher DMSP:Chl *a* ratio than other taxonomic groups like diatoms (2 – 97 mmol g^{-1}) and *Phaeocystis pouchetii* (11 – 134 mmol g^{-1}) measured in their natural environment (field data reviewed by Matrai and Vernet, 1997). For comparison with laboratory data, dinoflagellate cultures also have a higher DMSP:Chl *a* ratio than *Emiliania huxleyi* cultures (40 and 85 mol mol^{-1} which is equivalent to 45 and 95 mmol g^{-1}; Bucciarelli et al., 2007). The higher DMSP:Chl *a* ratio of dinoflagellates may result from their lower Chl *a* content. Tang et al., (1996) found a 55 % lower Chl *a* content per C unit than in other taxa (diatoms, prymnesiophytes and chrysophytes). In this context, methods are being developed to allow identification of some phytoplankton groups from satellite data (Alvain et al., 2005; Alvain et al., 2008), so this might allow the prediction of phytoplankton-associated DMSP concentrations in the field in the future.

The DMSP lyase activity measured in these dinoflagellate cultures varied from 0.61 - 59.73 fmol $cell^{-1}$ h^{-1} (Table 4.5), which is comparable with the range observed for strains of *Emiliania huxleyi* (0.12 - 750 fmol $cell^{-1}$ h^{-1} ; Steinke et al., 1998). However, I measured DLA at pH 8.2 which is close to the natural environment of phytoplankton, but I did not optimise the pH for maximal enzyme activity as Steinke et al., (1998) did. pH optima of 6 to 6.5 and 6.0 have been reported for DLA in *C. cohnii* and *Alexandrium* cultures respectively (Kadota and Ishida, 1968; Wolfe et al., 2002). On the other hand, Yost et al., (2009) found that a pH of 8.0 was best for DLA measurements with the dinoflagellate *Symbiodinium microadriaticum* and Harada (2007) obtained stronger DLA with a pH of 8 to 8.5 for dinoflagellate cultures. Given the comparison of DLA from different studies may be distorted by the use of different pH conditions, I recommend the first use of a close to seawater pH for future phytoplankton screening studies that would represent the activity of external lyases. Little is known on intracellular activity and sub-cellular localisation of DMSP lyases and dinoflagellate cells generally have a pH similar to that of the seawater of 8 (Dason and Colman, 2004) whereas other phytoplankton species may have a more acidic intracellular pH from 5 to 8 (Raven and Smith, 1980; Wheeler and Hellebust, 1981; M. J. Merrett et al., 1996).

Among the 5 species which displayed DMSP lyase activity, 3 were axenic. The presence of bacteria in *A. minutum* and *K. foliaceum* cultures means that I cannot exclude the potential for bacterial DMSP lyase activity in these cultures. For DLA measurement in this study, the preparation of the cellular extract started with a filtration (2.0 µm pore filter) which should exclude most unattached bacteria and reduce the bacterial population. Attached bacteria represent only a small fraction of the total bacterial population associated with healthy phytoplankton (Smith et al., 1995) and are unlikely to be the major source of DMSP in the assay. Moreover, Wolfe et al. (2002) observed similar DMSP lyase activities in axenic and non-axenic strains of *Alexandrium*. Ideally, confirmation of the origin of the enzymatic activity would necessitate testing axenic cultures of the same strains. However, in the field, DLA will always be associated with the whole microbial community and not the dinoflagellates alone.

4.4.3. Input for models

Various scientists have used diagnostic (empirical) models to simulate global DMS emission based on remote sensing data such as chlorophyll *a*, mixed layer depth and solar radiation (Simo and Dachs, 2002; Vallina and Simo, 2007). They have been successful in realistically predicting the DMS emission in comparison with observed DMS concentrations and have even reproduced the DMS seasonality (Simo and Dachs, 2002; Halloran et al., 2010) and particular DMS patterns including the DMS summer paradox of high DMS and low Chl *a* concentrations in low latitudes (Vallina and Simo, 2007). Moreover a strong positive correlation between the solar radiation dose and the DMS concentrations in the upper mixed layer at a global scale has been established (Vallina and Simo, 2007). However, these diagnostic models appear limited in simulating DMS predictions in areas such as shelf-seas, equatorial and Antarctic regions (Halloran et al., 2010). This is not surprising given that Chl *a* and DMS are not directly connected (see section 1.1.4) and that there is generally a lack of correlation between these two parameters in field data (Holligan et al., 1987; Watanabe et al., 1995; Uher et al., 2000).

Prognostic (mechanistic) models have been developed to improve the description of the underlying ecosystem processes that control DMSP and DMS production. The approach has generally been to couple existing ecological models which implement the nutrient-phytoplankton-zooplankton food chain to DMSP and DMS production. In Gabric et al. (1993; 1999) and Cropp et al. (2007), the phytoplankton component is described as a whole and the DMSP production is parameterised by the γ factor (γ = S(DMSP):N). The γ factors of 0.3 (Gabric et al., 1993) and 0.358 (Cropp et al., 2007) were based on limited data for a few phytoplankton species or measurements of a specified Arctic phytoplankton population (γ=0.04 and 0.2, Gabric et al., 1999). For the better prediction of DMSP concentrations for different phytoplankton populations and areas γ factors are needed for bigger set of culture and field measurements. Here, I report additional values for this factor (average γ = 0.13) for the 9 dinoflagellate species studied (Table 4.4). As yet these types of model do not take the taxonomic variability in phytoplankton cell DMSP content into account, but this would be necessary to represent the phytoplankton diversity and succession.

The phytoplankton module of DMS models has progressed by implementing DMSP quotas for different phytoplankton groups. In 1996, van den Berg and co-workers succeeded in representing the DMS production and variation in the southern North Sea by including 6 phytoplankton groups: 3 of which produced DMSP: diatoms, non-grazed flagellates (*Phaeocystsis*) and grazed flagellates. The other parameters needed for gradual improvement of models are the description of the conversion of DMSP to DMS, exudation rates (Laroche et al., 1999), DMSP lyase activity (van den Berg et al., 1996), bacterial cleavage and microzooplankton grazing (Archer et al., 2002a). Then, in 2005 Le Quéré and co-workers proposed a model which compartmentalises the phytoplankton into 10 Plankton Functional Types (PFT) including phytoplankton and zooplankton classified according to their biogeochemical roles (e.g. coccolithophores as calcifiers, diatoms as silicifiers). In this scheme autotrophic dinoflagellates are placed in the "mixed phytoplankton" PFT group. The parameterisation of this group is based on a DMSP:C production value of 12 mmol mol^{-1} which is the same as that used for the "DMS producers" group. My measurement for 8 phototrophic dinoflagellates gave an average of 10.4 ± 10.06 mmol mol^{-1} which supports their estimation. On the other hand, the autotrophic dinoflagellates are

considered in this PFT as inefficient DMS producers lacking DMSP lyase activity, having no direct impact on the S cycle and no defined biogeochemical role. However, 5 of the 9 species tested here showed substantial DMSP lyase activity. Moreover some dinoflagellate species may contain DMSP in comparable and higher concentrations than *Phaeocystis* which they included in the "DMS producers" group or "*Emiliania*" which is not included but recognised as affecting the DMS cycle. In view of my results, I suggest that dinoflagellates should be described as efficient DMSP and DMS producers. The phytoplankton groups implemented in models should include a separate dinoflagellate group or include a defined group of dinoflagellate species (which contain substantial amounts of DMSP) inside a DMS producer group. Moreover, DMS cleavage by DMSP lyase could be further implemented for each phytoplankton group. Similar to mechanistic models that have been developed for toxin production by dinoflagellates (John and Flynn, 2002), simple models could be developed for DMSP and DMS production by dinoflagellate species in coastal areas where dinoflagellates periodically form blooms.

A DMS model has been tested by Vogt et al., (2010) with the implementation of 5 PFT from Le Quéré et al., (2005) including coccolithophores, diatoms, nanoflagellates (N_2-fixers, DMS producers represented mainly by *Phaeocystis* and mixed phytoplankton represented by autotrophic dinoflagellates and chrysophytes) micro- and meso-zooplankton for the prediction of DMSP production. This model was successful in predicting mean DMS concentrations and seasonality at mid and high latitudes comparable to observed data. The model-data agreement is reasonably good with correlations obtained of 0.47 and 0.62 for the Kettle and Andreae (2000) database and the Global Surface Sea database (http://saga.pmel.noaa.gov/dms/). However, this model underestimated chlorophyll concentrations in various areas including the Arctic, North Atlantic and most upwelling regions, and underestimated DMS concentrations in the Southern Ocean, equatorial Pacific and Northern North Atlantic. These limitations might be reduced by further developing the biological component of the DMS model with all the phytoplankton groups described by Stefels et al. (2007) as main DMSP-containing species. This will be possible by increasing the database of DMSP:C data of the key phytoplankton groups and the other species-specific data needed for the various parameters of the model, for example: grazing, cleavage by free DMSP-lyase and photolysis (as described in Vogt et al., 2010).

Heterotrophic dinoflagellates are thought to represent about 50 % of the living dinoflagellates i.e. about 1000 species. A few heterotrophs have been shown to contain DMSP including *Crypthecodinium cohnii*, *Pfiesteria piscicida, Pfiesteria shumwayae* (Miller and Belas, 2004) and *Protoperidinium pellucidum* (Jean et al., 2005). I found that the DMSP:C ratio of *C. cohnii* was substantially lower than for photosynthetic species due to its higher C content (Table 4.2and Table 4.4). The capacity of DMSP production in heterotrophic dinoflagellates warrants further investigation with a bigger set of species because they may be a large source of DMSP and DMS which needs to be better defined and potentially parameterised in models.

4.5. Conclusion

I have reported here supplementary data for C, N, Chl *a*, DMSP content and DMSP lyase activity of 9 dinoflagellate species. I address these data to modellers in order to implement the dinoflagellate group in future DMS model investigations.

I confirmed that dinoflagellates have a low growth rate as previously described except in the case of *Crypthecodinium cohnii*, an osmotrophic species that grows rapidly on a medium containing glucose as the dominant C source. I also verified the relationship between cell volume and C and N content proposed for dinoflagellates by adding new species to the available dataset.

Wide variability in cell volume and Chl *a* content was evident among the 9 species. DMSP content varied over 2 orders of magnitude with some species containing similar DMSP concentrations to those observed in the well-studied DMSP-producers *Phaeocystis* and *Emiliania*. Athecate dinoflagellates and species that harbour haptophyte-like plastids tended to have lower DMSP concentrations than other dinoflagellate species. Comparison with published data suggests that there is a potential for intra- and inter-specific variation in DMSP concentrations and DMSP lyase activity. It is possible that contrasting dinoflagellate species, with for example 12 % or 0.3 % of total carbon content as DMSP, may not be using this metabolite in the same way. Further research to elucidate the multifunctional role of this compound for dinoflagellates and other types of phytoplankton is warranted and might explain some of the inter-specific variability observed.

Dinoflagellates are a complex group to parameterise in DMSP and DMS models due to their extreme biological diversity and the large variability in DMSP concentrations. A compilation of all available data on DMSP content and DMSP lyase activity in dinoflagellates is necessary to define the contribution of this plankton group to DMS production and the S cycle more precisely. This database (Chapter 5) is aimed to implement the dinoflagellates in the phytoplankton ecosystem of climate models. The dinoflagellate group is generally divided into the 2 autotrophs and heterotrophs sub

groups, but it is not yet clear that division is warranted on their DMSP and DMS contribution level.

I measured several new dinoflagellate species and the effort should be sustained to increase our knowledge of the capacity of dinoflagellates to produce DMSP and DMSP lyase. In this regard, the heterotrophic dinoflagellates deserve particular attention. More data will lead scientists to a better definition of this group and a more accurate prediction of DMS emissions. Dinoflagellate populations are being affected by global warming: as evidenced by an increase in abundance in the Mediterranean and North Seas (Edwards et al., 2006; Mercado et al., 2007) and earlier seasonal blooming (Edwards and Richardson, 2004). Moreover, we are currently lacking knowledge on the future of polar species in the context of melting sea ice in polar areas. Polar species such as *Polarella glacialis* may be a DMSP source. The consequences for DMS emissions and climate need to be addressed. With more species-specific data implemented in mechanistic models it would be possible to predict the likely consequences of the proliferation or extinction of one or more species. Finally, given that dinoflagellates are also more carbon dense than diatoms (Menden-Deuer and Lessard, 2000), their proliferation could have a valuable positive effect on ocean carbon sequestration.

Chapter 5 A re-appraisal of the variability in DMSP content and DLA in dinoflagellates

Abstract

Twenty years ago Maureen Keller and co-workers published a seminal study that identified dinoflagellates as an important marine phytoplankton group with respect to the production of DMSP. Here, I present an updated synthesis and analysis of all the DMSP and DLA measurements currently available for dinoflagellates. The dataset covers 66 species and strains and reveals over 6 orders of magnitude variability in intracellular DMSP concentration per cell volume and substantial variation in DLA. Inter-specific variability was explored with reference to biological characteristics. Neither toxicity nor the presence of a theca appeared to be related to DMSP concentration. Bioluminescent species produced significantly lower concentrations ($P<0.05$) than non-bioluminescent ones. Dinoflagellates with a haptophyte-like plastid contained lower amounts of DMSP than those with peridinin plastids ($P<0.01$), whereas those containing cryptomonad-like plastids tended to have higher DMSP concentrations. The only clear phylogenetic relationship seen was lower DMSP concentrations in bioluminescent species of the Pyrocystales. Heterotrophic dinoflagellates were also considered given their potential importance in the field. They are the only heterotrophs known to synthesise DMSP and this ability may support the theory of their photosynthetic origin. However, the heterotrophic species investigated to date showed wide variability in DMSP content and the species *Oxyrrhis marina* had no detectable DMSP. Over the whole data set there was no apparent relationship with the oceanic province of origin, with the exception of isolates from the Mediterranean province which had significantly higher DMSP content than those from other areas ($P<0.05$). This data compilation and analysis supports the notion that DMSP-containing dinoflagellates are an important potential source of DMS to the global atmosphere and highlights current gaps in knowledge.

5.1. Introduction

Various dinoflagellate species were highlighted as dimethylsulphoniopropionate (DMSP) and dimethylsulphide (DMS) producers in some of the earliest DMSP research in the 1960's. *Amphidinium carterae* was the first dinoflagellate shown to produce DMS (Ackman et al., 1966). The heterotrophic dinoflagellate *Crypthecodinium cohnii* (previously *Gyrodinium cohnii*) produces substantial amounts of DMSP and was used by Ishida and co-workers as a model species for a series of laboratory studies (see Ref Ishida, 1968). They looked at the biochemical properties of the enzyme responsible for the cleavage of DMSP to DMS; now known as DMSP-lyase. They also investigated the metabolic pathway of DMSP in this species (reviewed by Uchida et al., 1996). An extensive DMSP screening of 123 clonal phytoplankton cultures was done by Keller et al. (Keller, 1988/1989; Keller et al., 1989a; Keller et al., 1989b). On the basis of analyses on 25 dinoflagellate strains, they concluded that dinoflagellates are significant DMSP producers. More recently, Yoch (2002) reviewed and emphasised the contribution of small dinoflagellates (< 10 µm) in DMSP production. Furthermore, Stefels et al. (2007) recommended that dinoflagellates should be included in the 6 phytoplankton groups recognised as DMS producers for inclusion in future global climate models.

It is now generally accepted that dinoflagellates are one of the most significant phytoplankton groups in terms of production of DMSP, the precursor of DMS. However this generalisation hides the variability apparent in the Keller et al., (1989a) data in that they found some dinoflagellate species to have very high intracellular DMSP concentrations whereas others produced little or none. The underlying causes of this variability are unknown. Moreover, it is fundamental to know which species are strong DMSP and DMS sources in order to better predict their potential impact on climate, especially when considering bloom-forming species. In the twenty years since Keller's survey, several scientists have measured the DMSP content of dinoflagellate cells in laboratory experiments and provided evidence of their contribution to DMS and DMSP production in the field. In this chapter, I review and analyse the available literature in order to summarise and reconsider the knowledge and progress achieved in this research

field, and to highlight the issues where further investigation is warranted. The initial approach was therefore to re-assess the diversity in DMSP production ability amongst dinoflagellate species and strains. Secondly, this diversity was explored against biological criteria such as ability to produce toxins and bioluminescent compounds, plastid types, taxonomic orders and phylogenetic links, and oceanic provinces of origin. Finally, given the apparent multifunctional roles that have been suggested for DMSP (see Chapter 1, section 1.2.3), I will consider what this diversity could imply in terms of the functional roles of DMSP for the dinoflagellates that produce it.

5.2. Dinoflagellates associated with DMSP production in the field

The relevance of DMS for the S cycle and climate has led scientists to put a lot of effort into surveying DMS and DMSP concentrations in the field. Phytoplankton can be monitored by different analyses e.g. chlorophyll *a*, detailed pigment composition, taxonomic and abundance determinations. Dinoflagellates are common to abundant in most aquatic systems worldwide (Taylor et al., 2008) and the group includes both photosynthetic and heterotrophic species. In today's ocean most of the photosynthetic species contain peridinin and so can be identified as belonging to the dinoflagellate group by the presence of this typical pigment. However, a smaller sub-set of dinoflagellate species lack this pigment or contain additional pigments due to having gained another type of endosymbiont (more details in section 1.3.5).

In some field surveys, DMSP concentrations have been correlated with dinoflagellate pigments like the carotenoids peridinin and diadinoxanthin. In water samples from the Caribbean Belize lagoon, Sunda et al. (2005) obtained a positive correlation based on regression analysis between these pigments and DMSP concentrations (r^2=0.92, P<0.001 with peridinin and r^2=0.90, P<0.001 with diadinoxanthin). Belviso et al. (2001) found a weak correlation with peridinin (r^2=0.22, n=201, not statistically significant) in data for surface water samples from a range of diverse oceanic regions. Furthermore, since a few dinoflagellate species do not contain peridinin, surveying for this pigment in bloom areas where there are non-peridinin

species can lead to an underestimate of the dinoflagellate biomass (Belviso et al., 2000) and thereby misrepresent its correlation with DMSP concentrations.

In other field campaigns, dinoflagellate abundance has been correlated with DMSP concentration in various marine areas. In the northwest Atlantic, Levasseur et al., (1994) and Yang et al. (2009) obtained significant correlations (r^2=0.45, P≤0.01 and r^2=0.60, P<0.001 respectively). In Mediterranean coastal waters, Jean et al. (2006) found significant correlation (r^2=0.58, P<0.05) as did Gibson et al. (1996) in Antarctic coastal waters (r=0.875, P≤ 0.01). In the western English channel, Turner et al. (1988) found a good correlation of one dominant species *Gyrodinium aureolum* with DMS concentrations (r=0.79, n=25). In phytoplankton blooms dominated by one or more dinoflagellate species, DMSP concentrations were associated with the prevailing dinoflagellate species. So far, 13 dinoflagellate species have been reported (Table 5.1, 1st column). Some other dinoflagellate species have been reported to be present during bloom events associated with DMS production but their lesser abundance did not correlate with DMS or DMSP concentrations (Table 5.1, 3rd column). From these potential DMSP-containing species, 4 species (*Prorocentrum micans, Dinophysis acuminata, Heterocapsa triquetra and Prorocentrum arcuatum*) have been confirmed to contain DMSP by direct measurements (see section 5.3.).

Symbiodinium spp, the dinoflagellate symbionts or zooxanthellae in corals, are a substantial source of DMSP and DMS in the Great Barrier reef where some of the highest concentrations (18665 nM) reported for any marine environment have been found (Broadbent and Jones, 2004). Other dinoflagellate symbionts in giant clams, jellyfish and anemones also potentially contain DMSP (as shown in in Table 5.2 and reported by Van Alstyne et al., 2006).

Table 5.1 Dinoflagellate species associated with DMSP concentrations in the field. Species that were dominant in the bloom or that showed a correlation with DMSP concentrations are listed in the 1st column; other species that were less abundant but were still a potential DMSP source are listed in the 3rd column. The field area and the reference of the publication are detailed.

Dominant species associated with DMSP in the field	Correlations and comments	Other potential DMSP producers present	Field area	Reference
Gyrodinium aureolum (now *Karenia mikimotoi*)	Abundance correlated with DMS concentrations r=0.79, n=25		Western English Channel	Turner et al. (1988)
Prorocentrum sp, Gymnodinium sp.	Major DMSP contributors in size fraction >10 µm		Northwestern mediterranean coastal waters	Belviso et al (1990)
Katodinium sp.	$DMSP_T$ measured in bloom sample from 104 to 314 nmol L^{-1}	*Gymnodinium sp, Alexandrium sp, Scrippsiella sp, Dinophysis sp.*	Gulf of Maine, northwest Atlantic	Townsend and Keller (1996)
Gyrodinium impudicum	Diel cycle in DMSP and cell numbers	*Prorocentrum micans, Prorocentrum triestinum, Scrippsiella sp, Amphidinium sp.*	Mediterranean Sea	Belviso et al (2000)
Prorocentrum minimum	Estimated as contributing to up to 35% of the DMSP concentration.	*Dinophysis acuminata, Dinophysis norvegica, Diplopsalis spp, Gonyaulax spp.*	Northern North Sea	Archer et al. (2001) Archer et al., (2002b)
Gyrodinium flagellare			Northwest Atlantic	Scarratt et al., (2002)
Alexandrium tamarense *Scrippsiella trochoidea*	r^2=0.95, P<0.01 r^2=0.68, P≤ 0.05	*Heterocapsa triquetra*	St Lawrence Estuary, Canada	Merzouk et al., (2004)
Heterocapsa rotundata *Protoperidinium ovatum*	Most abundant species Abundance follows the same pattern as DMSP concentrations	*Alexandrium tamarense, Dinophysis acuminata, Gymnodinium sp, Scrippsiella trochoidea*	St Lawrence Estuary, Canada	Michaud et al., (2007)
Alexandrium minutum	Correlated at P<0.05	*Ceratium furca* (r=0.56 P=0.052) *Dinophysis acuminata* (r=0.55, P=0.055) *Prorocentrun arcuatum* (r=0.54, P=0.059)	Mediterranean coastal waters.	Jean et al., (2005)
Prorocentrum compressum	r=0.70, P<0.05	*Gymnodinium sp.*	Mediterranean coastal waters.	Jean et al., (2009)

5.3. The variability in DMSP content in dinoflagellates

To investigate the variability in the DMSP content of dinoflagellates I searched published data and found 17 papers that report DMSP values. The data are listed in Table 5.2 and include my data from chapter 4. Particulate DMSP ($DMSP_p$) and total DMSP values ($DMSP_T$ = $DMSP_p$ + dissolved DMSP + DMS) were taken to be equivalent values for estimation of DMSP content on the assumption that this is the main fraction of $DMSP_T$ (Keller et al., 1989a; Yost and Mitchelmore, 2009). Keller's survey (1988/1989; 1989a; 1989b) remains the richest work done so far and provides 24 of the 61 DMSP concentrations per cell volume values available for dinoflagellate strains. Nonetheless, other publications have brought some new values for DMSP content in this phytoplankton group and this review aims to bring all these data together. Fifty five of these values result from laboratory culture measurements, with only 6 values derived from field samples (Belviso et al., 2000; Jean et al., 2005). Hence, the data are as they were published except that I converted all the data into the same units for comparison. DMSP values are plotted in 2 graphs: one is expressed per cell (pmol $cell^{-1}$, Figure 5.1), the other per cell volume (mM, equivalent to mmol L_{cell}^{-1}, Figure 5.2). Since some data are available only per cell or per cell volume, the 2 graphs do not contain the same number of data points. Figure 5.1 and 5.2 also visualise some of the biological criteria that are discussed in section 5.6.

Table 5.2 DMSP content for dinoflagellates arranged in decreasing order of DMSP concentration per cell volume (CV). DMSP concentration per cell are also shown. For some species the concentration is only available in 1 of the 2 units. The data were collected from the literature as shown. References are given in the table footer. The abbreviation ND indicates that DMSP was not detected. Additional details for the species such as clone designations and synonyms, presence of theca, oceanic provinces of origin and taxonomic orders are provided. DMSP values expressed in pg cell^{-1} were transformed to mol cell^{-1} using a DMSP molecular weight of 134.2 g mol^{-1}.

Original species and clone names	Synonyms	DMSP concentrations per CV (mM)	DMSP concentrations per cell (pmol cell^{-1})	Data source	Thecate/ Athecate	Longhurst oceanic provinces	Taxonomic orders (Algaebase)
Alexandrium minutum		3388	14.2	1	T	MEDI	Gonyaulacales
Prorocentrum sp. IIB2B1		1082	0.122	2	T	Unknown	Prorocentrales
Gyrodinium impudicum		820	-	3	A	MEDI	Gymnodiniales
Scrippsiella trochoidea NIES-369		600	1.3	4	T	KURO	Peridiniales
Dinophysis acuminata		477	14.7	1	T	MEDI	Dinophysales
Heterocapsa pygmaea GYMNO	CCMP1322	451	0.145	2	T	CARB	Peridiniales
Prorocentrum arcuatum		442	13.5	1	T	MEDI	Prorocentrales
Amphidinium carterae AMPHI	CCMP1314	377	0.1438	5	A	NWCS	Gymnodiniales
Crypthecodinium cohnii CCOHNII	CCMP316	377	0.341	2	T	Unknown	Gonyaulacales
Heterocapsa triquetra CCMP449		364	0.7019	6	T	NWCS	Peridiniales
Scrippsiella trochoidea PERI	CCMP1331	350	2.86	2	T	Unknown	Peridiniales
Symbiodinium microadriaticum HIPP	CCMP827	345	0.1803	2	T	Unknown	Suessiales
Amphidinium carterae CCMP1314		326	0.133	7	A	NWCS	Gymnodiniales
Scrippsiella trochoidea CCMP1599		326	1.3	6	T	NWCS	Peridiniales
Amphidinium operculatum CCAP1102/6		312	0.299	8	A	Unknown	Gymnodiniales
Heterocapsa triquetra NIES-7		300	0.6	4	T	KURO	Peridiniales
Amphidinium carterae CCMP1314		300	0.14	6	A	NWCS	Gymnodiniales
Alexandrium minutum CCMP113		290	0.861	6	T	NAST(E)	Gonyaulacales
Amphidinium carterae		288	0.0977	7	A	Unknown	Gymnodiniales
Gymodnium nelsoni		280	-	9	A	Unknown	Gymnodiniales
Alexandrium tamarense CCMP115		235	-	10	T	NECS	Gonyaulacales

Table 5.2 continued

Original species and clone names	Synonyms	DMSP concentrations per CV (mM)	DMSP concentrations per cell (pmol cell^{-1})	Data source	Thecate/ Athecate	Longhurst oceanic provinces	Taxonomic orders (Algaebase)
Alexandrium tamarense CCMP116		205	-	10	T	NAST(E)	Gonyaulacales
Alexandrium tamarense CCMP1771		196	-	10	T	NECS	Gonyaulacales
Thoracosphaera heimii L603	CCMP1071	194	0.198	2	Unknown	GEST	Thoracosphaerales
Cachonina niei CACH	*Heterocapsa niei*	193	0.3204	2	T	Unknown	Peridiniales
Prorocentrum micans M12-11	CCMP691	190	4.418	2	T	Unknown	Prorocentrales
Heterocapsa sp. GT23	CCMP450	190	0.582	2	T	NWCS	Peridiniales
Alexandrium fundyense CCMP1719		183	0	10	T	NWCS	Peridiniales
Scrippsiella trochoidea CCAP1134/1		169	0.329	8	T	NECS	Peridiniales
Prorocentrum minimum CCMP1329		167	0	11	T	NWCS	Prorocentrales
Alexandrium fundyense GT429	CCMP1846	140	1.975	12	T	NWCS	Gonyaulacales
Protoperidinium pellucidum		134	14.7	1	T	MEDI	Peridiniales
Gymnodinium sp. 94GYR		125	0.1788	2	A	NWCS	Gymnodiniales
Prorocemtrum minimum EXUV	CCMP1329	111	0.159	5	T	NWCS	Prorocentrales
Amphidinium carterae CCMP1314		109	0	11	A	NWCS	Gymnodiniales
Crypthecodinium cohnii CCMP316		103	0.213	6	T	Unknown	Gonyaulacales
Polarella glacialis CCMP1138		94	0.066	6	A	APLR	Suessiales
Prorocentrum micans SB1		87	0.083	8	T	Unknown	Prorocentrales
Amphidinum carterae CCAP1102/1		57	0.0147	8	A	NECS	Gymnodiniales
Kryptoperidinium foliaceum CCMP1326		56	0.223	6	T	CCAL	Peridiniales
Gonyaulax spinifera LY11363		48	0.155	8	T	NECS	Gonyaulacales
Gymnodinium simplex WT8	*Protodinium sp* CCMP419	46	0.238	2	A	PNEC	Gymnodiniales
Ceratium furca		38	9.8	1	T	MEDI	Gonyaulacales
Gymnodinium simplex CCAP1117/3	*Protodinium sp* CCMP 418	35	0.0009	8	A	NECS	Gymnodiniales
Gymnodinium nelsoni GSBL	*Akashiwo sanguinea*	30	1.818	2	A	Unknown	Gymnodiniales

Table 5.2 continued

Original species and clone names	Synonyms	DMSP concentrations per CV (mM)	DMSP concentrations per cell (pmol cell^{-1})	Data source	Thecate/ Athecate	Longhurst oceanic provinces	Taxonomic orders (Algaebase)
Lingulodinium polyedrum LP2810		23	0.426	6	T	CCAL	Gonyaulacales
Alexandrium tamarense CCAP1119/1	CCMP115	20	0.083	8	T	NECS	Gonyaulacales
Karenia brevis CCMP2281		18	0.00011	7	A	CARB	Gymnodiniales
Gonyaulax spinifera W1	CCMP409	16	1.08	2	T	NWCS	Gonyaulacales
Lingulodinium polyedrum CCMP1738		13	0.119	7	T	CARB	Gonyaulacales
Karlodinium veneficum CCMP415	*Karlodinium micrum*	11	0.008	6	A	NECS	Gymnodiniales
Gambierdiscus toxicus GT200A		10	1.192	2	T	Unknown	Gonyaulacales
Lingulodinium polyedrum CCAP1121/2		5.09	0.0545	8	T	NECS	Gonyaulacales
Gonyaulax polyedra GP60e	*Lingulodinium polyedrum* CCMP405	4.01	0.134	2	T	CAMR	Gonyaulacales
Dissodinium lunula L823	*Pyrocystis lunula* CCMP731	1.94	0.864	2	Unknown	GEST	Pyrocystales
Gyrodinium aureolum KT3	*Karenia mikimotoi* CCMP430	0.65	0.005	2	A	Unknown	Gymnodiniales
Gyrodinium aureolum PLY497A	*Karenia mikimotoi* CCMP429	0.36	0.005	2	A	NECS	Gymnodiniales
Ceratium longipes 090201		0.23	0.0149	12	T	NWCS	Gonyaulacales
Pyrocystis noctiluca CCMP4	CCMP732	0.01	0.045	2	Unknown	CAMR	Pyrocystales
Pfiesteria shumwayae CCMP2089		0.00425	0.003	13	T	NWCS	Peridiniales
Pfiesteria piscicida CCMP1830		0.00344	0.003	13	T	NWCS	Peridiniales
Symbiodinium microadriaticum CCMP1633		-	0.3299	14	T	NPTG	Suessiales
Symbiodinium sp isolated from *Tridacna gigas* (giant clam)		-	0.310	15		Unknown	Suessiales
Symbiodinium sp isolated from *Lobophytum compactum* (coral)		-	0.285	16		AUSE	Suessiales
Symbiodinium microadriaticum CCMP421		-	0.201	14	T	NEWZ	Suessiales
Symbiodinium pilosum isolated from *Zoanthus sociatus* (zoanthid)		-	0.164	16		AUSE	Suessiales
Symbiodinium microadriaticum CCMP828		-	0.123	14	T	CARB	Suessiales

Table 5.2 continued

Original species and clone names	Synonyms	DMSP concentrations		Data source	Thecate/ Athecate	Longhurst oceanic provinces	Taxonomic orders (Algaebase)
		per CV (mM)	per cell (pmol cell^{-1})				
Symbiodinium sp A3 isolated from *Tridacna squamosa* (giant clam)		-	0.116	16		AUSE	Suessiales
Symbiodinium microadriaticum CCMP829		-	0.0814	14	T	AUSE	Suessiales
Symbiodinium sp isolated from *Montipora verrucosa* (coral)			0.0635	17		Unknown	Suessiales
Symbiodinium sp isolated from *Cassiopeia xamachina* (polyp stage of the jellyfish)		-	0.048	16		AUSE	Suessiales
Symbiodinium sp isolated from *Pocillopora damicornis* (coral)			0.048	17		Unknown	Suessiales
Peridinium gatunense		-	0.036	18	T	(Lake)	Peridiniales
Symbiodinium microadriaticum CCMP830		-	0.0338	14	T	NAST(W)	Suessiales
Oxyrrhis marina		ND	ND	2	A	Unknown	Oxyrrhinales

Note: Data are from the following sources: [1] Jean et al. (2005), [2] Keller et al. (1989a), [3] Belviso et al. (2000), [4] Niki et al. (2000), [5] Keller (1988/1989), [6] data from chapter 4, [7] Harada (2007), [8] Hatton and Wilson (2007), [9] Dacey and Wakeham (1986), [10] Wolfe et al. (2002), [11], Spiese et al. (2009), [12] Keller et al. (1989b), [13] Miller and Belas (2004), [14] Yost and Mitchelmore (2009) [15] Jones et al. (1994), [16] Broadbent et al. (2002), [17] Hill et al. (1995), [18] Ginzburg et al. (1998).

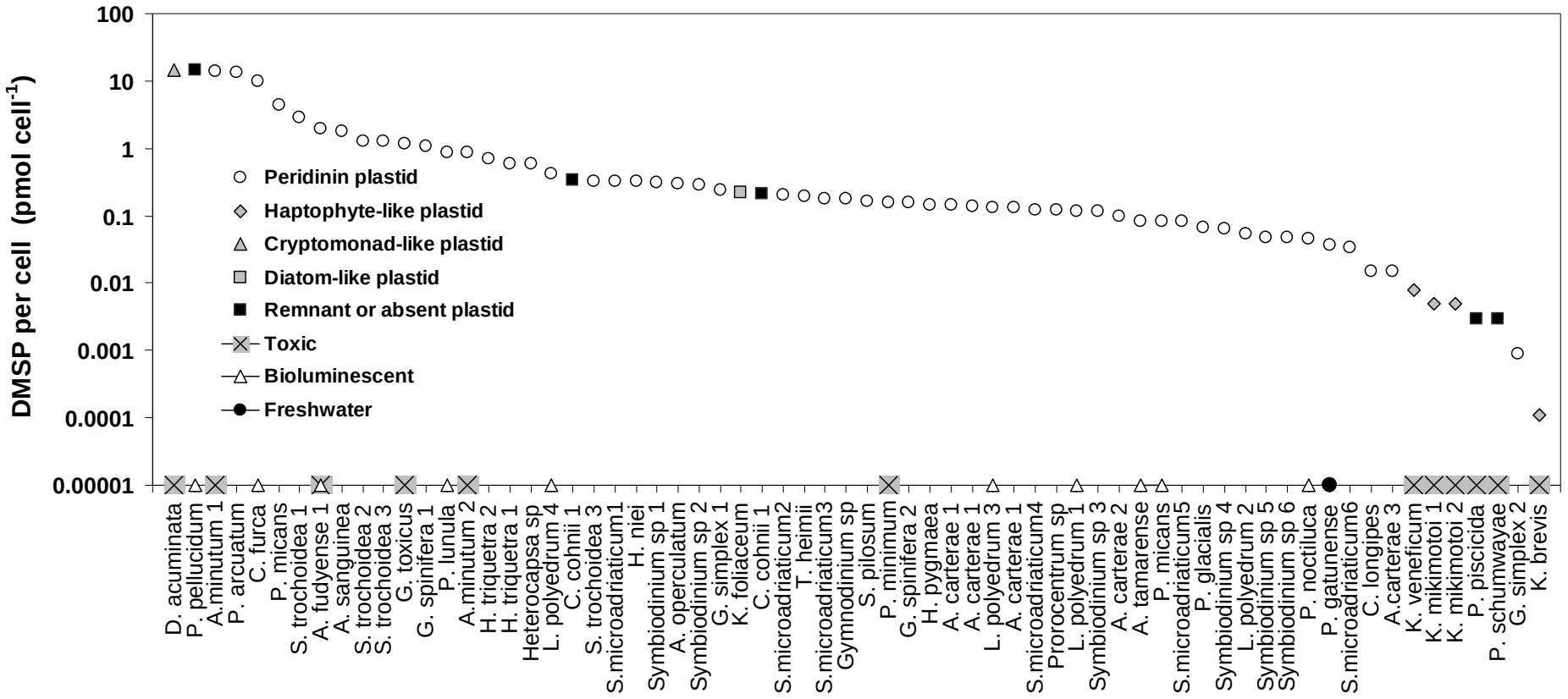

Figure 5.1 DMSP concentrations per cell in dinoflagellate species ranged in decreasing order. Several biological criteria are indicated on the graph as shown in the figure legend. Heterotrophic species have a remnant plastid or no plastid and all but one of the species are of marine origin. Due to space limitations the clone number is not indicated but different strains are designated by a number (1 - 6).

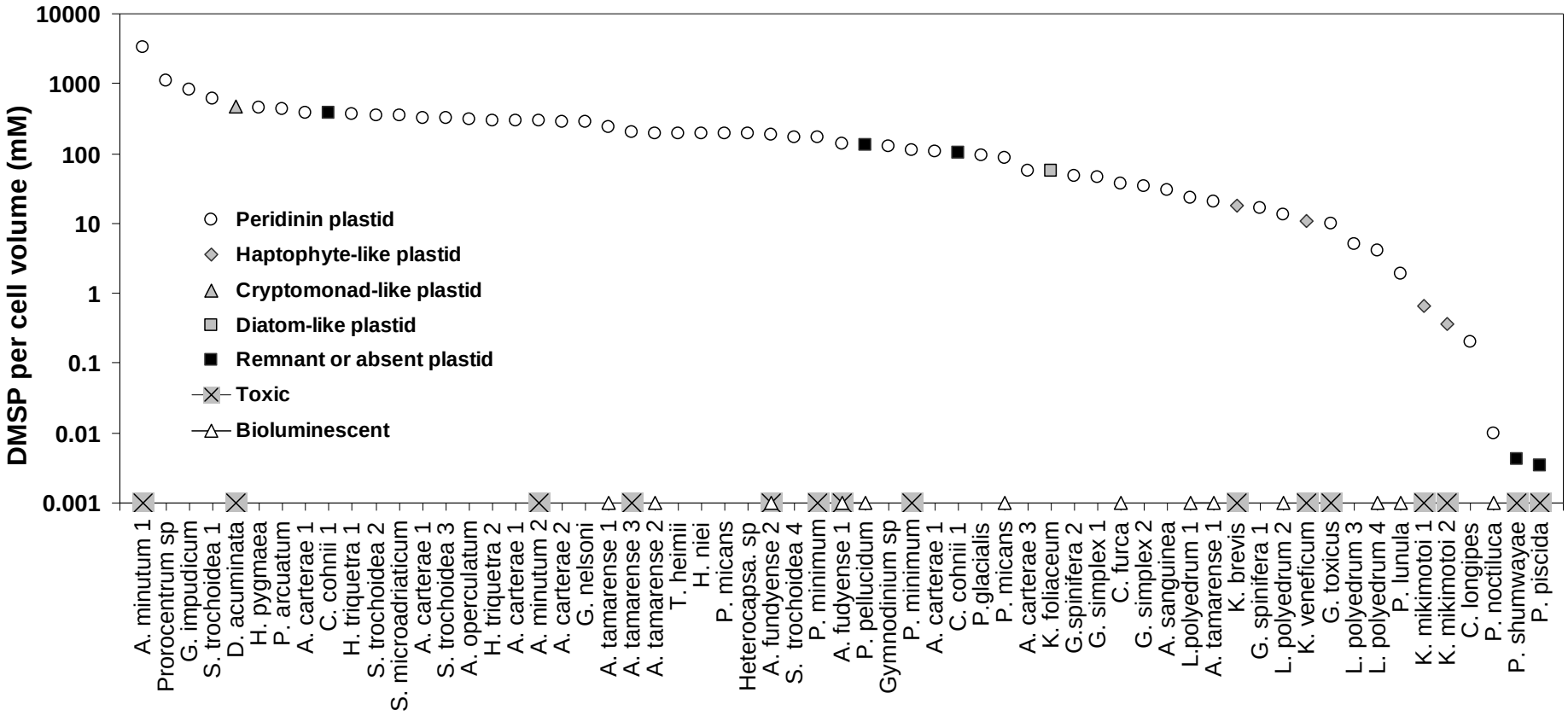

Figure 5.2 DMSP concentrations per cell volume in dinoflagellates ranged in decreasing order. Several biological criteria are illustrated on the graph as shown in the figure legend. Heterotrophic species have a remnant plastid or no plastid. Due to space limitations the clone number is not indicated but different strains are designated by a number (1 - 4).

In all there are 66 values of DMSP concentration per cell describing 48 species and 63 strains, and 61 values of DMSP concentration per cell volume describing 40 species and 56 strains. There is more than 1 value for some species and strains. For example, there are data for 4 different *L. polyedrum* strains and 3 *A. carterae* strains and one of these have 4 published values. This may give some indication of the intra-specific variability in DMSP content observed; this will be discussed in section 5.4. Overall Figure 5.3 visualises the high variability in DMSP concentrations reported for dinoflagellates. The whole range of DMSP concentration per cell spreads over 5 orders of magnitude from a minimum of 0.00011 pmol $cell^{-1}$ in *Karenia brevis* to a maximum of 14.7 pmol $cell^{-1}$ in *Dinophysis acuminata*. The mean concentration is 1.409 pmol $cell^{-1}$ (± 3.565 as standard deviation) and the median is 0.171 pmol $cell^{-1}$. DMSP measurements done in marine invertebrates that harbour zooxanthallae that were not included in this analysis, show DMSP in the range of 0.021 - 3.831 pmol $cell^{-1}$ (Van Alstyne et al., 2006), which easily falls within the overall range presented here. The range in DMSP concentrations per cell volume covers 6 orders of magnitude from a minimum of 0.0034 mM in *Pfiesteria piscicida* to a maximum of 3388 mM in *Alexandrium minutum*. The average concentration is 242 mM (± 459 as standard deviation) and the median is 140 mM. When outlier and extreme values are excluded, the range of DMSP concentration covers 4 orders of magnitude in both units (Figure 5.3).

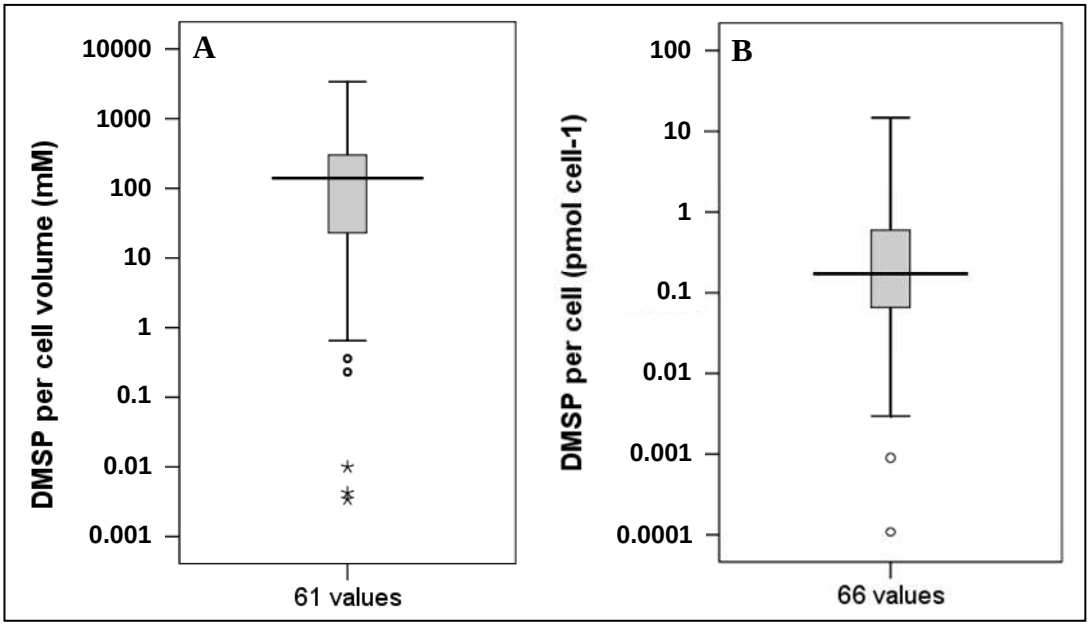

Figure 5.3 Box and whisker plots to show the spread of the DMSP concentration data for dinoflagellate strains (error bar), the upper and lower quartile range (box) and the median value (horizontal bar). (A) DMSP per cell volume (B) DMSP per cell data. The symbols denote excluded outlier data (○ between 1.5 and 3 box length from the upper and lower edge of the box) and extreme cases (✶ > 3 box length from the upper or lower edge of the box).

The values of DMSP per cell volume provide some insight into the potential biological role of DMSP as an osmolyte. A unicellular alga living in seawater with a salinity of 35 containing approximately 0.6 M NaCl and having an osmolarity of 1200 mOsm L^{-1} should in theory produce intracellular osmolytes to the equivalent osmolarity of 1200 mOsm L^{-1}. Cells generally have more than one compound with osmotic properties including ions, carbohydrate derivatives and amino acid derivative compounds (Kirst, 1989; Welsh, 2000). Kirst (1989) estimated the contribution of organic osmolytes to be 25 – 60 % or 300 – 700 mOsm L^{-1} of the osmotic potential of microalgae characterised by a high cytoplasm to vacuole ratio, as is the case for most dinoflagellates (Taylor, 1987). According to the range of values listed in Table 5.2 for dinoflagellates (0.0034 - 3388 mM), I propose the division of the species into 4 groups according to DMSP concentration (Table 5.3).

Table 5.3 Groups of dinoflagellate species sorted according to level of DMSP content. These levels give indication of the potential role of DMSP as an osmolyte.

Groups	DMSP per cell volume	Expected role for DMSP	Species
1	> 500 mM	Major osmolyte and/or other roles	*Alexandrium minutum, Prorocentrum sp., Gyrodinium impudicum, Scrippsiella trochoidea*
2	300-500 mM	Osmolyte	*Dinophysis acuminata, Heterocapsa pygmaea, Prorocentrum arcuatum, Amphidinium carterae, Crypthecodinium cohnii, Heterocapsa triquetra, Scrippsiella trochoidea, Symbiodinium microadriatcum, Amphidinium operculatum.*
3	10-300 mM	Medium to minor osmolyte	*Alexandrium minutum, Gymnodinium nelsoni, Alexandrium tamarense, Thoracosphaera heimii, Heterocapsa niei, Prorocentrum micans, Heterocapsa sp., Alexandrium fundyense, Scrippsiella trochoidea, Prorocentrum minimum, Protoperidinium pellucidum, Gymnodinium sp, Amphidinium carterae, Crypthecodinium cohnii, Gymnodninium simplex, Kryptoperidinium foliaceum, Gonyaulax spinifera, Ceratium furca, Akashiwo sanguinea, Lingulodinium polyedrum, Karenia brevis, Karlodinium veneficum.*
4	< 10 mM	Unlikely to be a significant osmolyte	*Gambierdiscus toxicus, Lingulodinium polyedrum, Pyrocystis lunula, Karlodonium mikimotoi, Ceratium longipes, Pyrocystis noctiluca, Pfiesteria shumwayae, Pfiesteria piscicida.*

Species containing more than 300 mM of DMSP (groups 1 and 2) are likely to use DMSP as an osmolyte. A concentration greater than 500 mM (group 1) would make DMSP the major osmolyte in the cell. Values above 500 mM are rare; only 4 are found in the dataset. These concentration values result from field samples (Jean et al., 2005), one value is estimated (Belviso et al., 2000) and all of them originate from studies done in the Mediterranean Sea. Three of these values are above 700 mM and exceed the contribution of organic osmolytes estimated by Kirst (1989). *Alexandrium minutum* produces 3388 mM of DMSP (Jean et al., 2005) which is far above the osmolarity of the seawater. However, these extremely high values might imbalance the osmolar pressure of the cell and might indicate additional roles of DMSP. Some dinoflagellates are very sensitive to turbulence and shear stress (Camacho et al., 2007a) and it has been shown that this can cause transient cell cycle arrest within a day, mortality in several days (Llaveria et al., 2009b) and also increases in DMSP production and content on a time-scale of days in *Alexandrium minutum* cells (Llaveria et al., 2009a). During the sampling process in the field or in the lab, stressed cells may thus increase their DMSP

concentration. These elevated values may also result from an overestimation of DMSP due to the potential cleavage of other DMS precursors by the cold alkali hydrolysis generally used for analysis (Spielmeyer and Pohnert, 2009). Various studies have shown that algae may contain other compounds with a DMS group (dimethylsulphoniomethoxybutyrate, dimethylsulphoniomethoxypropanoate isolated from red algae by Patti et al., 1993, and gonyol and gonyauline found in the dinoflagellate *Lingulodinium polyedrum* by Nakamura et al., 1997). Nonetheless, it is not known whether these compounds release DMS under cold alkali treatment.

A concentration below 300 mM (group 3) does not exclude a contribution of DMSP to the osmotic balance of the cell but positions it as a medium to minor osmolyte. Finally, a concentration of less than 10 mM (group 4) is unlikely to support a significant role as an osmolyte, though its presence might imply some benefits for the organism. The freshwater *Peridinium gatunense* isolated from the Lake of Galilee is in this group. This species is unlikely to use DMSP as an osmolyte in its natural environment. Nonetheless, it is produced at a concentration that falls in the range seen in other marine dinoflagellates (0.036 pmol cell^{-1}) and Ginzburg and co-workers (1998) showed that *P. gatunense* increases its DMSP content when transferred to a saline environment. Further investigations on the biogeographic expansion of this species are warranted to determine if its ability to produce DMSP could be a relic from its evolution in a saline environment.

5.4. Intra-specific variability in DMSP content of dinoflagellates

This dataset for DMSP content also illustrates the intra-specific variability because some of the species included have been analysed between 2 and 6 times (Figure 5.4, Table 5.4). These data include measurements on different strains and multiple analyses on the same strain. The intra-specific variability was assessed by calculating the relative standard deviation (RSD in %) and varied between 19 and 119 % for DMSP measurements expressed per cell volume and between 0 and 140 % for DMSP measurements expressed per cell. Whilst a few species show rather small variability

between studies with 14 - 28 % for the DMSP measurements per cell volume and 0 - 32 % for the DMSP measurements per cell, a more noticeable intra-specific variability is obtained for most of the species studied several times with a RSD of 41 - 119 % for measurements of DMSP per cell volume (Figure 5.4 A) and 51 - 140 % for measurements of DMSP per cell (Figure 5.4 B).

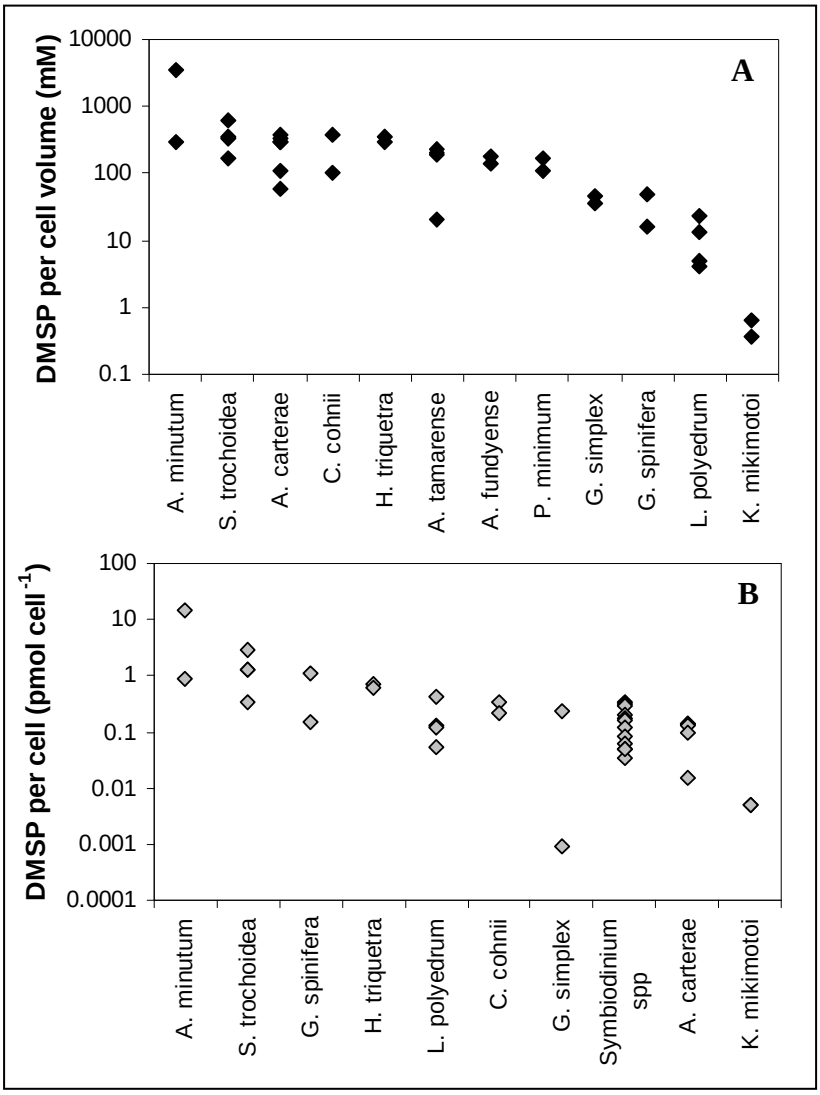

Figure 5.4 Intra-specific variability in DMSP measurements (A) per cell volume and (B) per cell.

Table 5.4 List of the standard deviation (SD) and the relative standard deviation (RSD) calculated from the database presented in table 5.2. The number of data available for each species is given (n).

Species	DMSP per cell volume (mM)				DMSP per cell (pmol cell^{-1})			
	n	Mean	SD	RSD %	n	Mean	SD	RSD%
A. minutum	2	1839	2190.6	119	2	7.531	9.4321	125
S. trochoidea	4	361	178.3	49	4	1.448	1.0476	72
A. carterae	6	243	128.5	53	5	0.106	0.0541	51
C. cohnii	2	240	193.7	81	2	0.277	0.0898	32
H. triquetra	2	332	45.3	14	2	0.660	0.0841	13
A. tamarense	4	164	97.2	59	-	-	-	-
A. fundyense	2	161	30.7	19	-	-	-	-
P. minimum	2	139	39.6	28	-	-	-	-
G. simplex	2	41	7.8	19	2	0.119	0.1677	140
G. spinifera	2	32	22.6	71	2	0.618	0.6541	106
L. polyedrum	4	11	8.8	78	4	0.183	0.1654	90
K. mikimotoi	2	0.5	0.21	41	2	0.005	0	0
S. microadriaticum	-	-	-	-	6	0.158	0.1043	66
Symbiodinium spp	-	-	-	-	13	0.245	0.0718	29

Several values are reported for *Symbiodinium spp* isolated from diverse marine invertebrates. These species have not been identified and given that the original species *Symbiodinium microadriaticum* that was thought to be the common symbiont is now considered to include several species (Coffroth and Santos, 2005), I decided to show also the variability between all 13 values for *Symbiodinium spp,* which is 29 % (Table 5.4).

One source of intra-specific variability comes from the use of diverse strains which could imply diverse genotypes and/or phenotypes leading to different capacities for DMSP synthesis. For instance, the strains NIES369, CCMP1331, CCMP1599 and CCAP1134/1 of *Scrippsiella trochoidea* contain DMSP in concentrations of 600, 350, 326, 169 mM respectively, which gives a RSD of 49%. Similarly, 6 strains of *S. microadriaticum* show an intra-specific variability of 66% RSD for DMSP content per cell. However, this is not the only source of variability given that the strain *A. carterae* CCMP1314 produce a wide range of DMSP concentrations between 4 studies (109 mM in Spiese et al., 2009, 300 mM in Chapter 4, 326 mM in Harada, 2007 and 377 mM in Keller, 1988/1989). The evolution of the strain maintained in laboratory for several years may explain a part of the variability obtained for the same strain as it has been shown that 50 % of the DNA of the diatom *Thalassiosira weissflogii* diverged from another isolate in 7 years (von Dassow et al., 2008). Intra-specific variability in

DMSP has also been observed in other phytoplankton groups. Steinke et al. (1998) measured the DMSP content in 6 strains of *Emiliania huxleyi* and obtained a RSD of 39 %.

Intra-specific variability might also result from different environmental conditions in the field or laboratory culture at the sampling time leading to various physiological and metabolic responses in the organism or population being studied (e.g. different culture growth phases, photosynthetic efficiencies, nutrient availability and uptake). Five strains of *S. microadriaticum*, originating from 5 different oceanic provinces and measured successively in the same laboratory show large intra-specific variability (75 % RSD). On the other hand, variation in *Crypthecodinium cohnii* CCMP316 DMSP content during different batch culture growth phases (as shown in Chapter 6) could immediately explain the variability in the range of 103 to 377 mM reported in different studies (Table 5.2).

The axenicity of the culture might also affect the DMSP result. Bacteria may consume dissolved DMSP as a carbon and/or sulphur source leading to a slight decrease in $DMSP_T$ given that the dissolved fraction is a minor fraction of $DMSP_T$ in healthy cultures (Yost and Mitchelmore, 2009). Moreover, the presence of bacteria in the phytoplankton cultures, as under the natural conditions of phytoplankton cells in the sea, alters the environmental conditions by using some nutrients, re-mineralising others and supplying the vitamin B12 required by some algal species (Croft et al., 2005). In this way the presence of bacteria could affect the physiology of the algae and potentially the DMSP content. However, contrary to these ideas, Wolfe et al. (2002) measured the DMSP content of 3 strains of *Alexandrium tamarense* and obtained a similar value for the axenic strain CCMP1771 (196 mM) and for the non-axenic strains CCMP115 and CCMP116 (235 and 205 mM). In this case, the presence of bacteria does not seem to alter the DMSP content of dinoflagellate cultures substantially.

Whilst the intra-specific variability observed in the dataset presented here could result from a combination of the potential reasons outlined above, in most cases it is hard to explain the main cause of the variability. For instance the species *Alexandrium tamarense* CCMP115 has been used in 2 studies with a concentration of 235 mM reported in one of these (Wolfe et al., 2002) and 20 mM in the other (Hatton and

Wilson, 2007). In these studies the cultures were maintained in similar conditions of temperature (14 - 15ºC), light (70 - 80 µmol photons m^{-2} s^{-1}) and photoperiod (14:10 L:D cycle), though the medium (F/2 or Kmin+L1) and the growth phases sampled (exponential phase or early exponential phase) were slightly different. Differences in sample preparation (e.g. sampling method, sample volume, type of filtration for DMSP or no filtration for $DMSP_T$ measurement) and analytical methods could perhaps also explain some of the variability. Therefore, a general method for DMSP measurement in phytoplankton cultures (e.g. use of the $DMSP_T$ measurement as in chapter 4, sampling in exponential phase) should be established to limit the variation attributable to a range of analytical methods. Finally to be more realistic, the DMSP content of a species should be described by a range of values which could cover those for different strains and those for strains in various environmental conditions.

5.5. Comparison with other taxonomic groups

Discussions on DMSP production often emphasise the 2 prymnesiophyte representatives *Emiliania huxleyi* and *Phaeocystis spp* (Liss et al., 1993; Malin and Steinke, 2004; Stefels et al., 2007). These two species are important DMSP producers and they have been investigated in-depth due to their capacity to form large-scale blooms in e.g. the North Atlantic Ocean and the Antarctic Ocean. In consequence, a range of data has been published from laboratory studies on these 2 species. Focussing on DMSP content per cell volume, various strains of *E. huxleyi* have been reported to contain between 50 and 304 mM (For example Franklin et al., 2010; Harada, 2007; Keller, 1988/1989; Keller et al., 1989a; Steinke et al., 1998; van Rijssel and Gieskes, 2002; Wolfe et al., 1997) and some *Phaeocystis* species contain 71 to 260 mM (For example Keller et al., 1989a; Stefels and van Boekel, 1993). These data provide good evidence of a wide intra-specific variation for *E. huxleyi* and inter-specific variation for *Phaeocystis sp*. Moreover, the variation in DMSP content may result from different physiological conditions of the species since Stefels and van Boekel (1993) observed an increase in DMSP content over the growth from 71 in exponential growth phase to 150 mM in stationary growth phase.

However, a taxonomic comparison using the larger 29 values dataset for DMSP content for a range of prymnesiophytes from the Keller et al. surveys gives a range of 3 to 413 mM with an average of 155 mM (Keller, 1988/1989; Keller et al., 1989a; Keller et al., 1989b). Moreover, a recent study performed by Franklin et al., (*in press.*) on 10 species of coccolithophores (included in the prymnesiophytes) reports DMSP content in the range 174 mM (*Gephyrocapsa oceanica*) to 715 mM (*Umbilicosphaera hulburtiana*). According to these values the dinoflagellates, where the data for DMSP concentrations span from 0 to 3388 mM with an average of 242 mM (Table 5.2), could be described as the taxonomic group that produces the widest range of intracellular DMSP concentrations (per cell volume) as well as the highest values seen to date.

The comparison between DMSP content data for dinoflagellates and other phytoplankton types presented here, suggests that it would be worthwhile to do more monitoring of dinoflagellate blooms and further investigation of individual species with respect to DMS and DMSP production capacity. The need for such research is compelling given the potential contribution of DMS to climate cooling and because dinoflagellate populations appear to be changing in response to the changing climate. Dinoflagellate abundance has increased recently in some areas including the NW Atlantic where *Ceratium arcticum* formed denser winter blooms in the 1990s compared to the 1960s (Johns et al., 2003); the Central North Sea where *Ceratium fusus* has bloomed earlier in the season and reached higher abundance in the period 1981 - 2002 compared to 1958 - 1980 (Edwards and Richardson, 2004); and in the Mediterranean Sea where the abundance of dinoflagellates relative to diatoms increased between 1997 and 2002 (Mercado et al., 2007).

5.6. Biological criteria as a source of DMSP variability

The 5 to 6 orders of magnitude variability in DMSP content among dinoflagellate species and strains revealed here could certainly be a reflection of the huge diversity of this group and its long evolutionary history since the Triassic (-240 million years ago). This phytoplankton group is currently thought to contain more than 2000 benthic, planktonic and symbiotic species distributed ubiquitously in aquatic habitats across the globe in marine, brackish, freshwater, snow and sea ice environments. The diversity is also substantial in terms of biogeography, trophic behaviour (e.g. autotroph, heterotroph, mixotroph), ecological interactions (primary producers, predators, symbionts, parasites), morphology, plastid types and production of toxins and bioluminescent compounds (Taylor, 1987; Lee, 1999). Among these biological criteria, I now consider the available data for toxicity, bioluminescence, plastid types, taxonomic orders, phylogeny and oceanic provinces in relation to the variability in DMSP content in dinoflagellates. Given that there is wide variation in cell size among dinoflagellate species (Chapter 4, section 4.3), more attention is focussed on DMSP per cell volume data rather than DMSP values per cell.

5.6.1. Toxicity

About 60 of the 2000 dinoflagellate species are able to produce toxins (Steidinger and Tangen, 1997) and form Harmful Algal Blooms (HAB). These toxins can accumulate in seafood and impact human health. A variety of toxic molecules, including saxitoxin, brevetoxin, ciguatoxin, maitotoxin, okadaic acid, yessotoxin, azaspiracid, have been isolated from dinoflagellates (see details in Chapter 1 section 1.3.9). These toxins have diverse chemical structures, mechanism of actions and cause syndromes such as paralytic, neurotoxic, diarrheic and azaspiracid shellfish poisoning, ciguatera disease and estuary associated syndrome (Hackett et al., 2004a). The ecological role of toxins is not clearly defined but the consequences for marine ecosystems can be dramatic in cases of massive kills of fish, birds or mammals (Paul et al., 2007). Generally, toxins are thought to act in chemical defence as grazer deterrents

(Ianora et al., 2006). Allelopathic properties have been shown for karlotoxin which is produced by *Karlodinium veneficum* (also called *K. micrum*) and inhibits the growth of some other dinoflagellates (Adolf et al., 2006). However, allelopathic activity in most dinoflagellates has been associated with compounds other than toxins (Ianora et al., 2006) which are still under research investigation and not yet identified (Tillmann and John, 2002; Ma et al., 2009).

There is a commonality here between toxins and DMSP because DMSP may also be involved in predator-prey interactions. DMSP and its by-products acrylate and DMS may act as grazer deterrents and signal prey location as a chemical cue for higher trophic levels such as fish, birds and marine mammals (Wolfe et al., 1997; Steinke et al., 2002b; Strom et al., 2003; Nevitt and Bonadonna, 2005; Bonadonna et al., 2006; DeBose et al., 2010). No published studies have investigated the relationship between these two defence metabolites (i.e. the favoured metabolite production in species able to produce both metabolites). In Figure 5.5 I compare the distribution of DMSP concentrations for toxic and non-toxic species. *L. polyedrum* was considered here as a non-toxic species because no information was available for these particular strains whereas other strains are reported to be potentially toxic (Bruno et al., 1990; Armstrong and Kudela, 2006). From the 61 data available for DMSP concentrations per cell volume Figure 5.5 A), 15 dinoflagellate strains are toxic and produce DMSP across the whole range of values (0.003 - 3388 mM). The lack of any obvious relationship was confirmed using the non-parametric Mann-Whitney *U* test which showed no significant difference between toxic and non-toxic species in terms of DMSP content ($P>0.05$, SPSS, version 16.0).

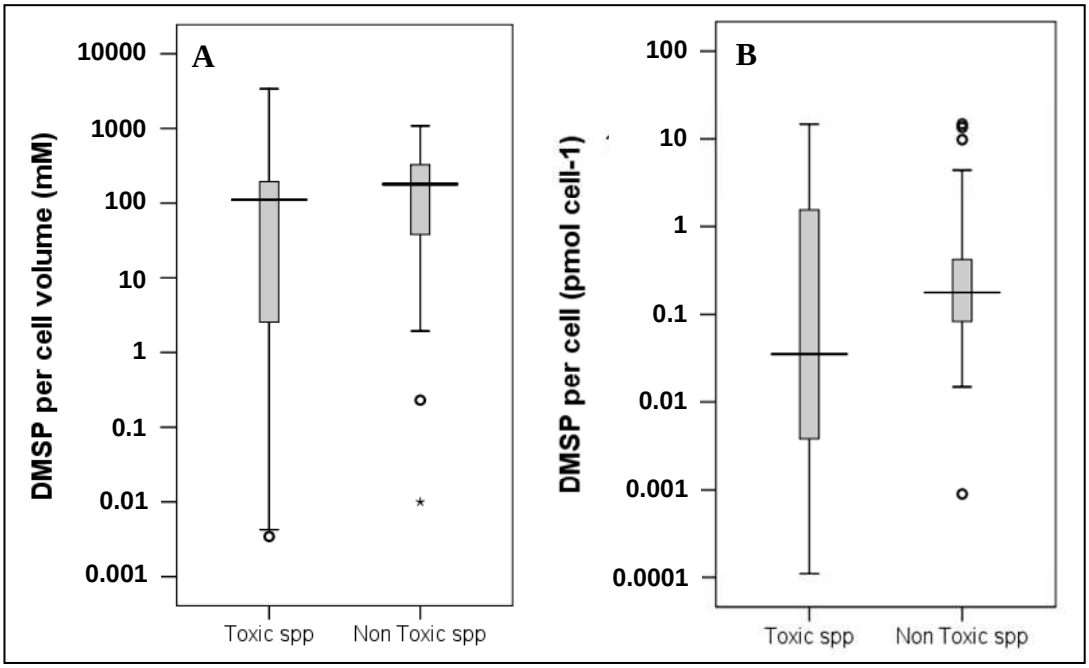

Figure 5.5 Box and whisker plots showing the distribution of DMSP concentration (A) per cell volume for toxic (n=15) and non toxic (n=46) strains and (B) per cell for toxic (n=12) and non-toxic (n=54) strains. A general description for box and whisker plots is given in Figure 5.3.

Clearly the dataset is limited to just a few toxic species and some of these may have been growing in conditions that stimulated or inhibited toxin production in some way. In this regard it is interesting to note that nitrogen may affect toxin and DMSP biosynthesis in opposite ways. Saxitoxins contain 33 % nitrogen and hence production depends on nitrogen availability: Anderson and co-workers (1990) observed that *Alexandrium fundyense* cultures produced less saxitoxin in low nitrogen medium. Because DMSP does not contain any nitrogen, it has been hypothesised that synthesis ought to be favoured over that of nitrogen-containing osmolytes in cases of nitrogen deficiency (Andreae, 1986) but such experiments have not been done with dinoflagellates. Whilst only a small fraction of dinoflagellates are toxic, it would be interesting to assess the relationship between the production of these DMSP and N-containing toxins across variable nutrient conditions.

5.6.2. Bioluminescence

Some marine dinoflagellate species emit a light flash under water turbulence called bioluminescence. This light is a chemical reaction of the enzyme luciferase acting on its substrate luciferin that occurs in vesicles called scintillons and results in an emission of photon (Lee, 1999). For most species represented in the dataset, I collected bioluminescence data from the CCMP website (Wilson, 2009) and personal communication from scientists. Several species were classified as non-bioluminescent when no direct data was available and when no record was found on potential bioluminescence. Over these species, *Ceratium furca*, *Prorocentrum micans* SB1 and *Protoperidinium pellucidum* are considered here as bioluminescent due to previous listing as bioluminescent species (Tett, 1971; Poupin et al., 1999), although no specific data are available for the strains present in the dataset. Bioluminescent species are rather weakly represented in the dataset: 13 and 10 species in the datasets for DMSP per cell volume and per cell respectively (as shown in Figure 5.1 and 5.2 and appendix II). They contain significantly lower DMSP concentrations per cell volume (0.003 - 235 mM, Figure 5.6) than the non-bioluminescent ones ($P<0.05$, Mann-Whitney *U* test). Bioluminescent species contain a higher range of DMSP concentration per cell (0.045 – 14.7 pmol cell^{-1}) compared to other dinoflagellates.

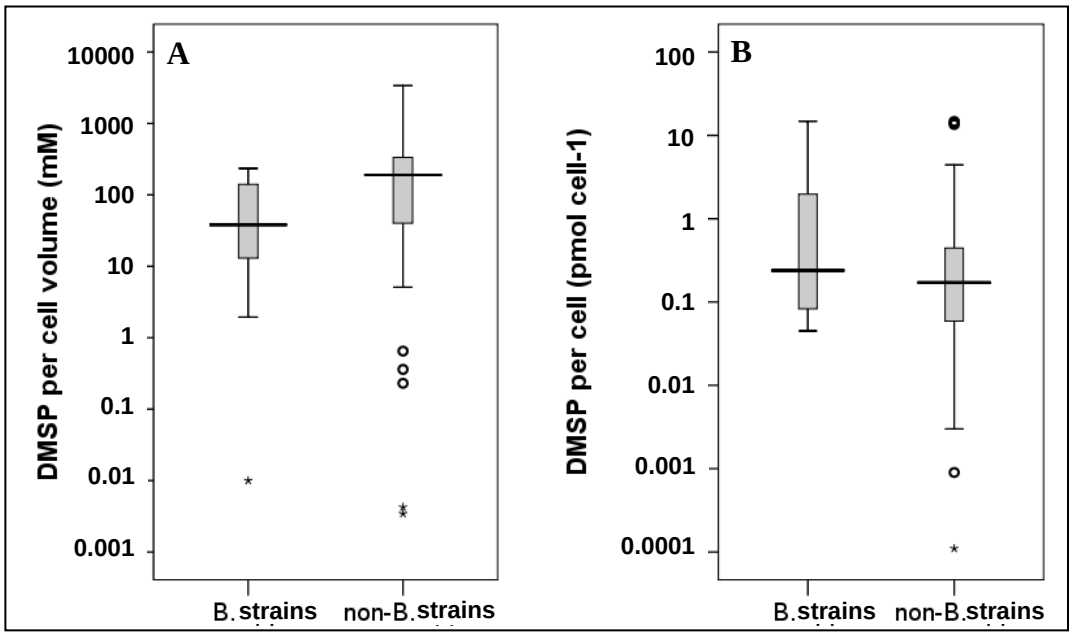

Figure 5.6 Box and whisker plots to show the distribution of DMSP concentration (A) per cell volume for bioluminescent (B., n=13) and non-bioluminescent strains (non-B., n=48) and (B) per cell for bioluminescent (n=10) and non-bioluminescent strains (n=56). A general description for box and whisker plots is given in Figure 5.3.

The most intensively studied bioluminescent species such as *Pyrocystis spp* and *Lingulodinium polyedrum* produce very low DMSP concentration per cell volume (0.01 to 23 mM). On the other hand, the non-bioluminescent strain *Alexandrium tamarense* CCMP116 produces similar DMSP concentration per cell volume (205 mM) to the bioluminescent strains CCMP115 and CCMP1171 (235 and 196 mM respectively).

Theories on the benefit gained from bioluminescence by dinoflagellates include descriptions of a nocturnal grazer deterrent role (Lee, 1999) whereby the light "frightens" the predator (Buskey et al., 1983) or a signal that indicates the presence of grazer to higher trophic level organisms (Abrahams and Townsend, 1993). The latter idea has also been suggested for the lysis by-product of DMSP (Wolfe et al., 1997; Steinke et al., 2002b). As yet, no research has been done on the potential inter-play between bioluminescence and DMSP-based grazer deterrence.

5.6.3. Plastid types

Dinoflagellates are a unique phytoplankton group in that they contain representatives that harbour different plastid types (detailed in chapter 1, section 1.3.5) On the evolutionary time-scale, dinoflagellates have acquired and replaced plastids through several endosymbiosis events (Falkowski et al., 2004; Keeling, 2004). So far, 6 plastid types have been identified among dinoflagellate species including the peridinin plastid, haptophyte-like plastid, diatom-like plastid, cryptomonad-like plastid, prasinophyte-like plastid and the possible remnant but non-functional plastid as suggested for the heterotrophic species *Crypthecodinium cohnii* (Sanchez-Puerta et al., 2007).

Trossat et al., (1996) have shown that the coastal terrestrial plant *Wollastonia biflora* synthesises DMSP in the chloroplast, and whilst there is no direct evidence, it is generally assumed that the same is true for phytoplankton. Trossat and co-workers showed that the chloroplast was the site of one of the steps in the DMSP biosynthesis pathway leading to the DMSP precursor DMSP-aldehyde. They also isolated a chloroplastidic enzyme that oxidises this precursor and estimated after measurement

that half of the DMSP content is located in chloroplasts (Trossat et al., 1998). Such findings support the assumption of the chloroplast as the location of part of the DMSP synthesis pathway, at least in higher plants.

I was interested to see if the variability in the plastids of dinoflagellates (equivalent to the chloroplast of land plants) may account for some of the wide variability in DMSP concentrations seen in this group. Among the 6 existing plastid types in dinoflagellate species, only 5 of them are represented in the dataset of DMSP values I assembled including the peridinin plastid, haptophyte-like plastid, diatom-like plastid, cryptomonad-like plastid and the remnant plastid found in heterotrophic species (Table 5.5). No measurements for DMSP concentration appear to have been carried out on species harbouring prasinophyte-like plastids and I was unable to obtain an example to test. I assume here that all the heterotrophic species included in the dataset have a remnant plastid or no plastid but acknowledge that direct evidence is not available for all of these species. Nonetheless this is in line with recent findings for a few additional heterotrophic species that were shown to have a remnant plastid (Sanchez-Puerta et al., 2007; Slamovits and Keeling, 2008). Dinoflagellates with the 4 functional plastid types (Figure 5.7) produce DMSP in a smaller concentration range (3 orders of magnitude for species with peridinin plastids, 2 for species with haptophyte-like plastids, 1 value available for species with cryptomonad and diatom-like plastids) than the heterotrophic dinoflagellates whose DMSP concentration spread over 4 to 5 orders of magnitude.

Table 5.5 Plastid types harboured by dinoflagellate species listed in this study.

Plastid types	Species
Haptophyte-like	*Karenia mikimoitoi* *Karenia brevis* *Karlodinium veneficum*
Cryptomonad-like	*Dinophysis acuminata*
Diatom-like	*Kryptoperidinium foliaceum*
Remnant or absent	*Prorocentrum pellucidum* *Crypthecodinium cohnii* *Pfiesteria piscicida* *Pfiesteria schumwayae*
Peridinin plastid	All other species listed in table 5.2 are assumed to have peridinin plastids.

Species with peridinin plastids are well represented in the dataset which is not surprising as they are the most common plastids found in dinoflagellates in the ocean today. These 50 values mainly range over 3 orders of magnitude from 10 to 3388 mM for DMSP per cell volume (with values between 0.01 and 10 mM considered as outlier and extreme values) and 4 orders of magnitude from 0.0009 to 14.2 pmol cell^{-1} for DMSP per cell data (with 1 outlier value excluded).

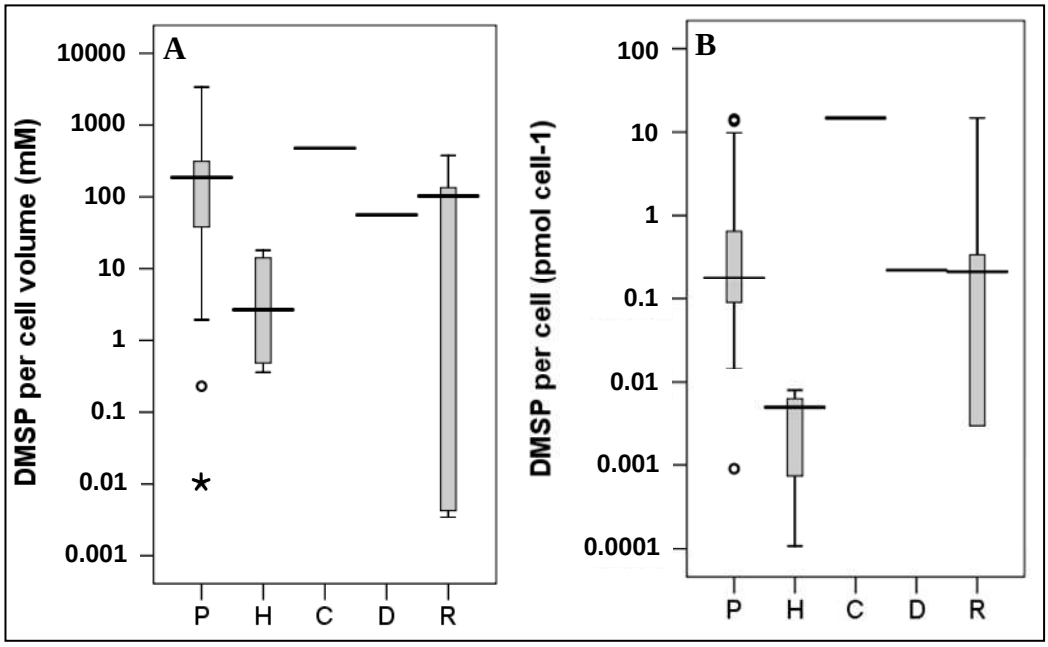

Figure 5.7 Box and whisker plots to show the distribution of DMSP concentration depending on plastid types (P: Peridinin plastid; H: Haptophyte-like plastid, C: Cryptomonad-like plastid, D: diatom-like plastid, R: Remnant or absent plastid) and (A) expressed per cell volume (n=50, 4, 1, 1, 5 from left to right boxes) and (B) per cell (n=55, 4, 1, 1, 5 from left to right boxes). For cryptomonad- and diatom-like plastids only one value is available and in each case this is represented by a horizontal line without box. A general description for box and whisker plots is given in Figure 5.3.

Only single values are available for the cryptomonad-like and diatom-like plastid groups and both fall in the range of the peridinin-plastid species. The high value of 477 mM obtained for *Dinophysis acuminata* which has cryptomonad-like plastids comes from the field work study of Jean et al. (2005) mentioned previously. It is possible to grow this species in culture (Park et al., 2006) so laboratory measurements should now be possible. Unfortunately no other species containing this plastid type are available in culture. The single value for the diatom-like plastid species comes from this thesis (Chapter 4).

Four strains harbouring haptophyte-like plastids (2 strains of *Karenia mikimotoi*, 1 strain of *Karenia brevis*, 1 strain of *Karlodinium veneficum*) produce 0.36 - 17.7 mM

and 0.00011 - 0.008 pmol cell^{-1} DMSP. This is significantly lower than the DMSP concentration per cell volume of the peridinin plastid containing-dinoflagellates ($P<0.01$, Mann-Whitney *U* test). As mentioned earlier, a lot of research has focussed on DMSP in the haptophytes *E. huxleyi* and *Phaeocystis sp* but in contrast the 4 dinoflagellates with haptophyte-like plastids produce rather low amounts of DMSP. I can only conclude that the available data do not support the idea that DMSP concentration in dinoflagellates is related to the plastid type. It seems more likely that the ability to produce DMSP depends on the conservation or loss of genes through evolution and potentially the regulation of gene expression.

It has been shown that *Karenia brevis* blooms have become more frequent and reached 20-fold higher biomass at the end of the 20th century compared to the 1950s. This might be due to the increase of nutrients released by human activities (Brand and Compton, 2007). Cell numbers can reach in excess of 750 000 cell L^{-1} so whilst *K. brevis* has a medium to low DMSP concentration, its capacity to form such dense blooms makes it an important potential DMSP and DMS source.

It is clear that the ability to produce DMSP in dinoflagellates exists across the whole range of plastid types and in the absence of functional plastids. Heterotrophic dinoflagellates have a large variability in DMSP content that spread from 0.003 to 376.9 mM and from 0.003 to 14.7 pmol cell^{-1}. DMSP production by heterotrophic species does not exclude a plastid origin of the responsible genes. According to the plastid evolution theory, the acquisition of a new plastid is followed by the transfer of some plastid genes to the dinoflagellate nucleus (Keeling, 2004). This would allow heterotrophic species to synthesise DMSP if these genes were initially present in the functional plastid of a photosynthetic ancestor and transferred to the nucleus. This could explain why these dinoflagellates are the only heterotrophic organisms capable of DMSP synthesis. It would also be coherent with the hypothesis of a common photosynthetic ancestor for dinoflagellates (McFadden, 2001). For instance, the heterotrophic species *Crypthecodinium cohnii* is thought to contain a relic plastid (Sanchez-Puerta et al., 2007) and produces a considerable amount of DMSP (Table 5.2). It is unlikely that DMSP synthesis occurs in a relic plastid and where plastids are absent synthesis would require an alternative subcellular location. This might possibly account for the DMSP metabolic pathway in *Crypthecodinium cohnii* (Uchida et al., 1996)

which is different to that described for algae (Gage et al., 1997). By contrast, the heterotrophic *Oxyrrhis marina* does not produce DMSP (Keller et al., 1989a). It is an early-branching dinoflagellate with plastid-derived genes (Slamovits and Keeling, 2008) that may have a cryptic plastid. Genes or their function might have been lost in this species.

5.6.4. Thecate and athecate species

Some dinoflagellate cells are covered by an external rigid layer of cellulose plates known as theca or armour, whereas naked dinoflagellates are called athecate or unarmoured. In the previous chapter (Chapter 4) I found a significant difference in DMSP content between thecate and athecate species. Here, I tested this difference with a bigger dataset for 40 thecate species and 18 athecate, plus 3 non-defined species. The range of DMSP concentrations per cell volume for athecate species is within that for the thecate species (Figure 5.8). The non-parametric Mann-Whitney *U* test obtained no significant correlation (P>0.05). Therefore the difference observed in Chapter 4 could be coincidental due to the small sample size (as observed and discussed in section 4.3 and 4.4.2).

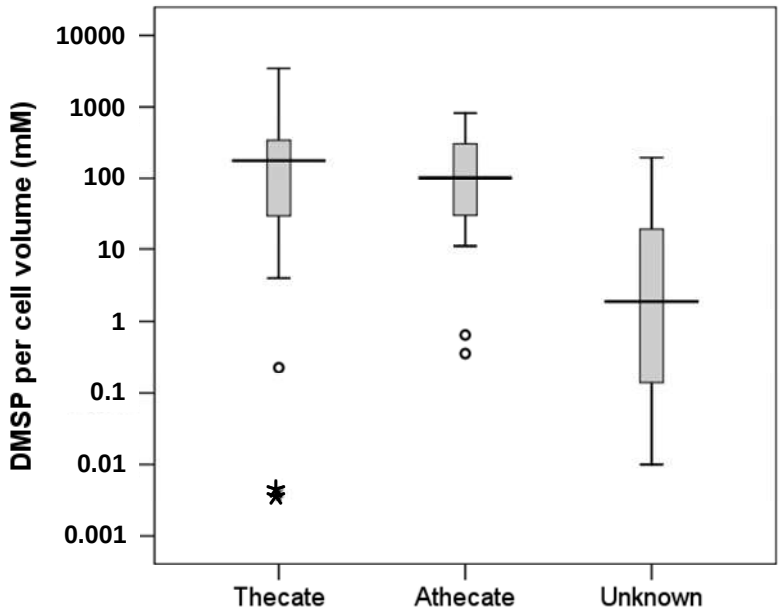

Figure 5.8 Box and whisker plots showing the distribution of DMSP concentration per cell volume for thecate (n=40) and athecate (n= 18) strains. Three species are not defined (unknown category). A general description for box and whisker plots is given in Figure 5.3.

5.6.5. Taxonomic orders and phylogeny

The diversity of dinoflagellates which results from the long evolutionary history of this taxa can be represented by phylogenetic trees or by clustering species in taxonomic orders. Here, I test whether taxonomic orders and phylogeny may account for a part of the inter-specific variability in DMSP content observed for dinoflagellates.

Given the ongoing progress in phylogenetic studies and molecular techniques, the classification for dinoflagellates is frequently revised. The updated and publically accessible classification of the online database "Algaebase" (Guiry and Guiry, 2009) was used here to identify the taxonomic order of each species of interest. The class Dinophyceae contains 18 orders (Table 5.6) of which 9 are covered by the dataset presented here (Table 5.2, Figure 5.9). An older classification for dinoflagellates published by Fensome and co-workers in 1993 grouped living and fossil dinoflagellates. However, this classification is less advantageous in that the genera *Pfiesteria* and *Polarella* are missing because they were isolated after it was published. This "Algaebase classification" differs from the Fensome classification by the presence of the Pyrocystales order whose representatives were previously placed in the Gonyaulacales order.

Table 5.6 : Taxonomic Orders of the Class of Dinophyceae as described in Algaebase. The orders marked with an asterisk are represented in the dataset. *Incertae sedis* means of uncertain taxonomic position.

Order	Authority	Species
Actiniscales	Sournia	6
Blastodiniales	Chatton	16
Coccidiniales		11
Dinamoebales	Loeblich III	1
Dinophyceae incertae sedi		14
Dinophysiales*	Kofoid	334
Gonyaulacales*	F.J.R. Taylor	392
Gymnodiniales*	Apstein	565
Lophodiniales	J.D. Dodge	14
Noctilucales	Haeckel	19
Oxyrrhinales*	Cavalier-Smith	1
Peridiniales*	Haeckel	669
Peridiniphycidae incertae sedis		3
Phytodiniales	T. Christensen	19
Prorocentrales*	Lemmermann	75
Pyrocystales*	Apstein	18
Suessiales*	R.A. Fensome et al.	16
Thoracosphaerales*	Tangen	12

The Gonyaulacales, which is one of the best represented orders in the database in terms of number of species belonging to it (Figure 5.9), shows the largest range of DMSP concentrations per cell volume (0.23 - 3388 mM). However, the order of Gymnodiniales shows the largest range of DMSP concentrations per cell (0.0001 - 1.8 pmol $cell^{-1}$). Comparing the medians of DMSP content in most taxonomic orders, a similar range of DMSP concentrations per cell volume (194 - 346 mM) is observed. The Pyrocystales produce the lowest median value of DMSP (0.97 mM, 2 values only) and the Dinophysiales produce the highest median value of DMSP (477 mM, 1 fieldwork value only). The 2 representatives of the Pyrocystales are bioluminescent species (section 5.6.2).

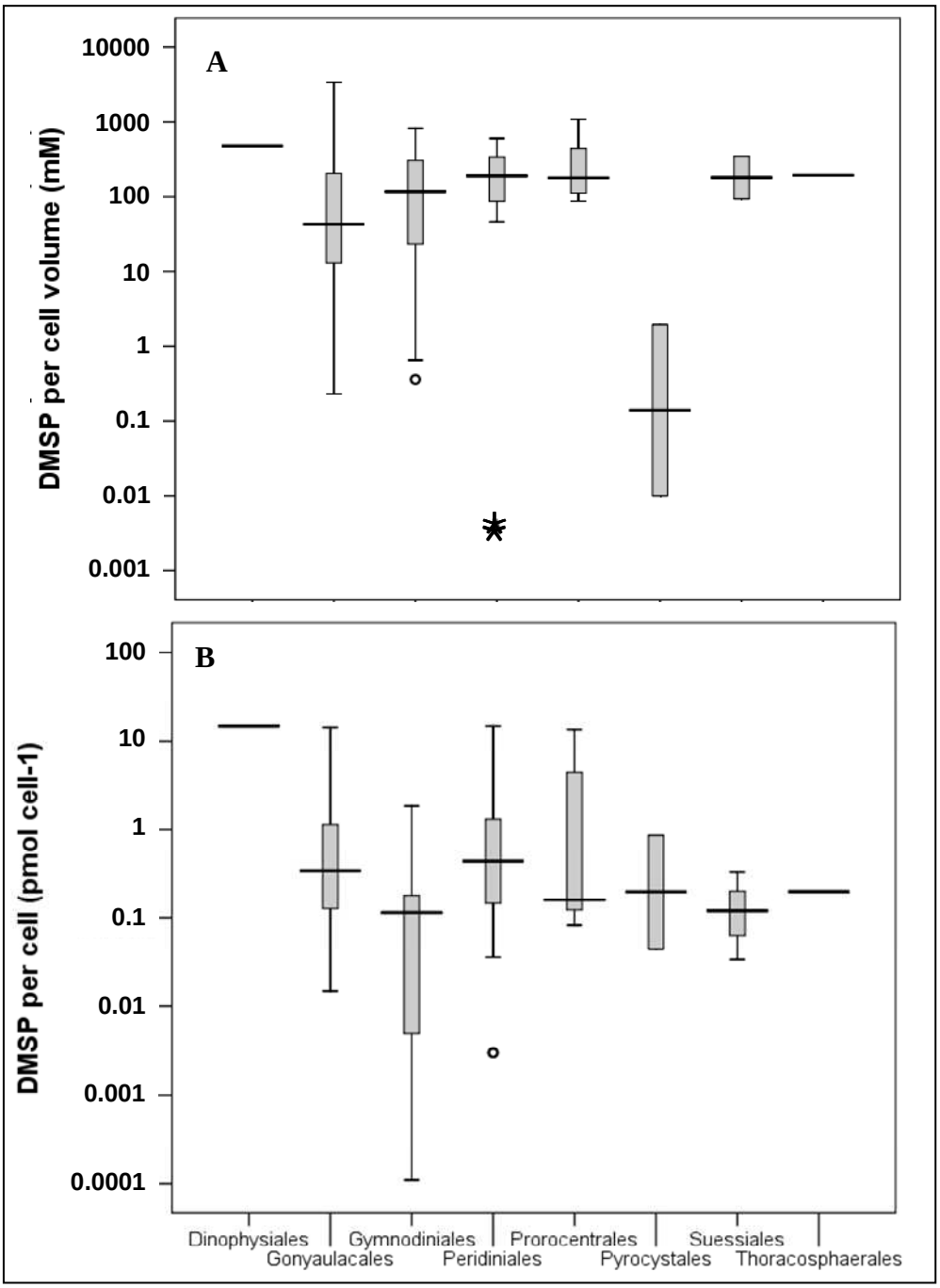

Figure 5.9 Box and whisker plots showing the distribution of DMSP concentration (A) per cell volume (n= 1, 18, 16, 15, 6, 2, 2, 1 from left to right boxes) and (B) per cell (n= 1, 15, 14, 14, 5, 2, 14, 1 from left to right boxes) sorted by taxonomic orders. Taxonomic orders are ranged in alphabetic order. The Dinophysiales and Thoracosphaerales are represented by only one value illustrated by a horizontal line without box. A general description for box and whisker plots is given in Figure 5.3.

Given these results I can conclude that there is no substantial variability between most of the taxonomic orders (Gonyaulacales, Gymnodiniales, Peridiniales, Prororcentrales, Suessiales, Thoracosphaerales). The Pyrocystales and the Dinophysiales stand out a little more by having lower and higher DMSP contents

respectively, though in these cases very few data are available. More measurements might enable a firm conclusion to be drawn.

Dinoflagellate diversity can be illustrated with phylogenetic trees which show the evolutionary separation between species, so I built a tree based on the observed range in DMSP content among species (Table 5.2). The tree was generated from 37 small subunit rDNA sequences available on the Nucleotide database of the National Center for Biotechnology Information (NCBI,Table 5.7). These sequences are for 37 alveolates including 35 dinoflagellates and 2 species, *Perkinsus marinus* and *Toxoplasma gondii*, which share a common ancestor with dinoflagellates (Fast et al., 2001; Stelter et al., 2007) are used to root the tree. Sequences of 1703 nucleotides were aligned using Bioedit software and optimised manually. The tree was constructed using MrBayes software using the evolutionary model GTR + Γ + COV as described in Shalchian-Tabrizi et al. (2006). This model was compared with another model (GTR + invΓ) and the model with the highest harmonic mean was favoured (GTR + Γ + COV).

The phylogenetic tree obtained (Figure 5.10) is colour coded according to DMSP concentrations. It shows similarities in species positions with trees based on SSU rDNA obtained in other studies (Saldarriaga et al., 2001; Saldarriaga, 2004; Shalchian-Tabrizi et al., 2006). For instance *Polarella glacialis*, *Gymnodinium simplex* and *Symbiodinium microadriaticum* form a cluster as observed by Saldarriaga et al. (2001). *Alexandrium*, *Pyrocystis*, *Ceratium* and *Crypthecodinium cohnii* are grouped in the most recently diverging species as seen in other studies (Saldarriaga et al., 2001; Saldarriaga, 2004; Murray et al., 2005; Shalchian-Tabrizi et al., 2006). *Gymnodinium impudicum* was also obtained as an early diverging species as in Saldarriaga et al. (2004).

Table 5.7 List of the strains analysed for the phylogenetic tree and their Genbank accession number from the Nucleotide database of NCBI. Strains are ranged in alphabetic order.

Species	Strain number	Genbank accession number
Akashiwo sanguinea	CCMP1740	EF492486
Alexandrium fundyense	CCMP1719	DQ444290
Alexandrium minutum	none	U27499
Alexandrium tamarense	MUCC99	AF022191
Amphidinium carterae	CCMP1314	AF274251
Amphidinium carterae	ACWGTNZ CAWD10	AF009217
Amphidinium operculatum	CCMP123	EF057406
Ceratium furca	none	AJ276699
Ceratium longipes	CCMP1770	EU927566
Crypthecodinium cohnii	ATCC 30336	FJ821501
Dinophysis acuminata	none	EU130569
Gambierdiscus toxicus	HIT91_D8_1	EF202890
Gonyaulax polyedra (Lingulodinium polyedrum)	CCMP1738	EF492507
Gonyaulax spinifera	CCMP409	AF022155
Gymnodinium galatheanum (Karlodinium veneficum)	GE-2	AF272050
Gymnodinium impudicum	CCMP1678	DQ785884
Gymnodinium simplex	CCMP419	U41086
Heterocapsa niei	CCMP447	AF274265
Heterocapsa pygmaea	CCMP1322	EF492500
Heterocapsa triquetra	MUCC285	AF022198
Karena brevis	CCMP 718	AF274259
Karenia mikimotoi	CCMP429	FJ587220
Peridinium foliaceum (Kryptoperidinium foliaceum)	none	AF231804
Peridinium gatunense	none	DQ166208
Perkinsus marinus	P1	AF126013
Pfiesteria piscicida	none	AF077055
Pfiesteria shumwayae	Noga-S, VIMS 1049	AY245694
Polarella glacialis	CCMP 2088	EF434275
Prorocentrum micans	Isolate 1	AY803739
Prorocentrum minimum	CCMP1329	DQ336060
Protoperidinium pellucidum	none	AY443022
Pyrocystis lunula	CCCM 517	AF274274
Pyrocystis noctiluca	CCMP732	AF022156
Scrippsiella trochoidea	CCCM 602	AF274277
Symbiodinium microadriaticum	CCMP830	AY456111
Thoracosphaera heimii	CCCM670	AF274278
Toxoplasma gondii	ME49	L37415

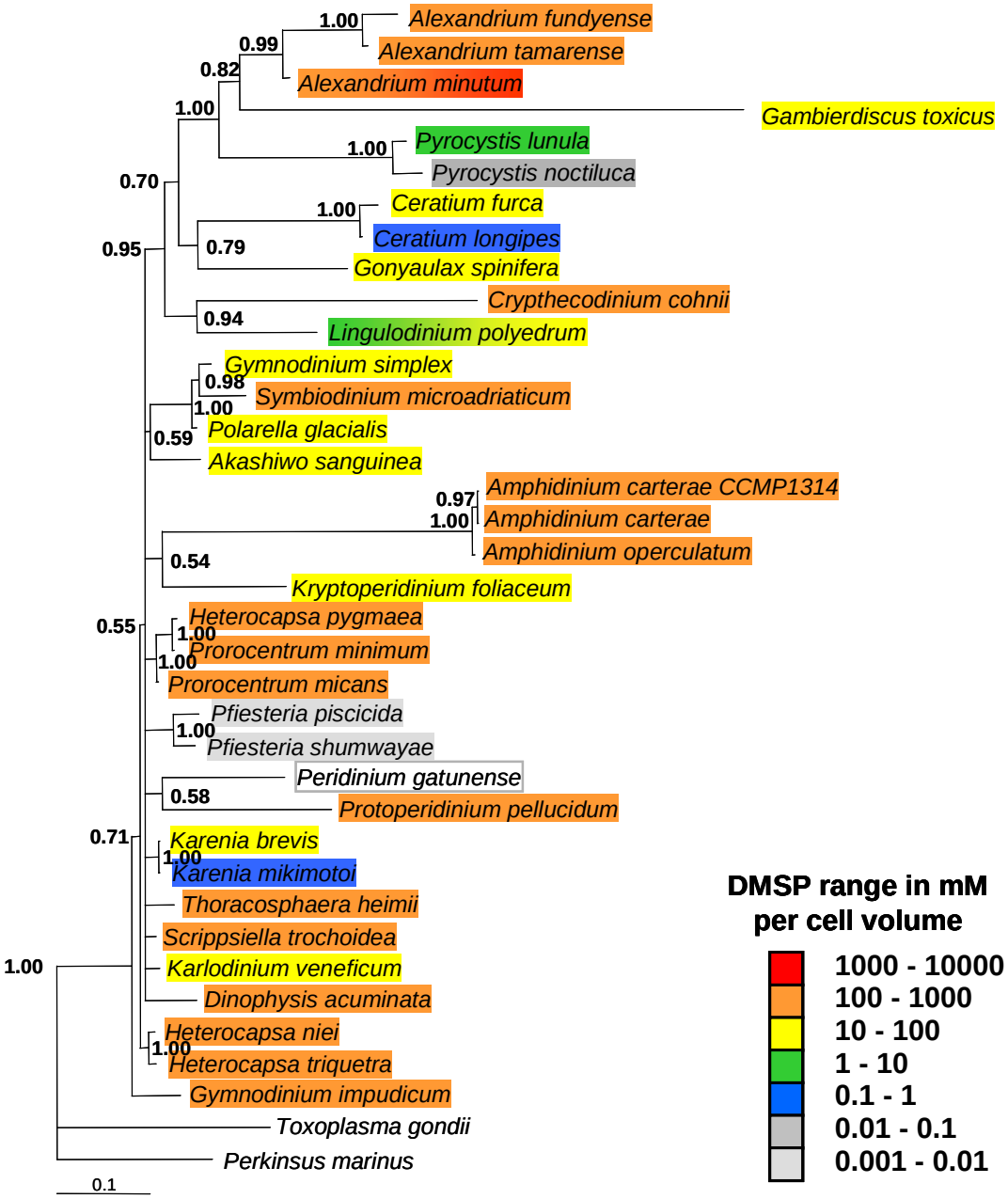

Figure 5.10 Comparison of DMSP content among dinoflagellate species and their phylogenetic relationships. Tree of small subunit rDNA sequences from 37 alveolates including 35 dinoflagellates, inferred with Bayesian posterior probabilities using an evolutionary model GTR+Γ+COV. All bootstrap values are shown (0.57 - 1.00). Cold and warm colours illustrate the range in DMSP concentration. *Peridinium gatunense* contains 0.036 pmol cell^{-1} of DMSP but its range in terms of DMSP per cell volume is unknown.

The phylogenetic tree shows that some closely related species vary substantially in DMSP concentrations. For instance *Ceratium furca* and *Ceratium longipes* produce 38 mM and 0.2 mM respectively. Similarly, *Pyrocystis noctiluca* and *Pyrocystis lunula* produce 0.01 and 0.65 mM. *Lingulodinium polyedrum* (photosynthetic species) and *Crypthecodinium cohnii* (heterotrophic species) appear as sister groups in some phylogenetic trees (Saldarriaga et al., 2001; Saldarriaga, 2004) but produce DMSP at very different concentrations (5, 13, 23 mM for *L. polyedrum* and 103, 377 mM for *C. cohnii*). A lot of variability in DMSP content is also observed between closely-related species such as *Alexandrium*, *Gambierdiscus*, *Pyrocystis* and *Ceratium*. The recently diverging species *Alexandrium minutum* falls in the highest DMSP concentration range. By contrast, early diverging species such as *Gymnodinium impudicum*, *Heterocapsa triquetra* and *H. niei* contain rather high amount of DMSP which suggests that this character might be ancient and tend to be conserved through dinoflagellate evolution but with nuances between species. I conclude that DMSP-producing ability does not show a coherent phylogenetic pattern.

5.6.6. Oceanic provinces

Dinoflagellates are globally distributed in the ocean (Taylor, 2008) and more abundant in coastal areas. Longhurst (2007) has defined 57 biogeochemical oceanic provinces based on physical forcing and algal ecology (Figure 5.11). By identifying the province of origin for each species in the database (Table 5.2), I investigated the environmental influence on the DMSP synthesis capacity of dinoflagellates. Moreover, I attempted to assess oceanic regions with DMSP-rich dinoflagellate populations or with populations of low DMSP content.

With 11 to 14 of the 57 oceanic provinces covered by the DMSP per cell volume and per cell data respectively (Figure 5.12), only a very small part of the ocean is illustrated so far in the dinoflagellate species database. Unfortunately, the strain collection site is not known for 23 % (14 of 61) and 24 % (16 of 66) of the strains for DMSP concentrations per cell volume and per cell respectively. The missing data weaken the analyses. Furthermore, there is currently no data for the North Pole, South Atlantic and Indian Ocean, and only 8 values are available for the southern hemisphere including the Austral Polar province, the New Zealand Coastal province and the East Coastal Australian province.

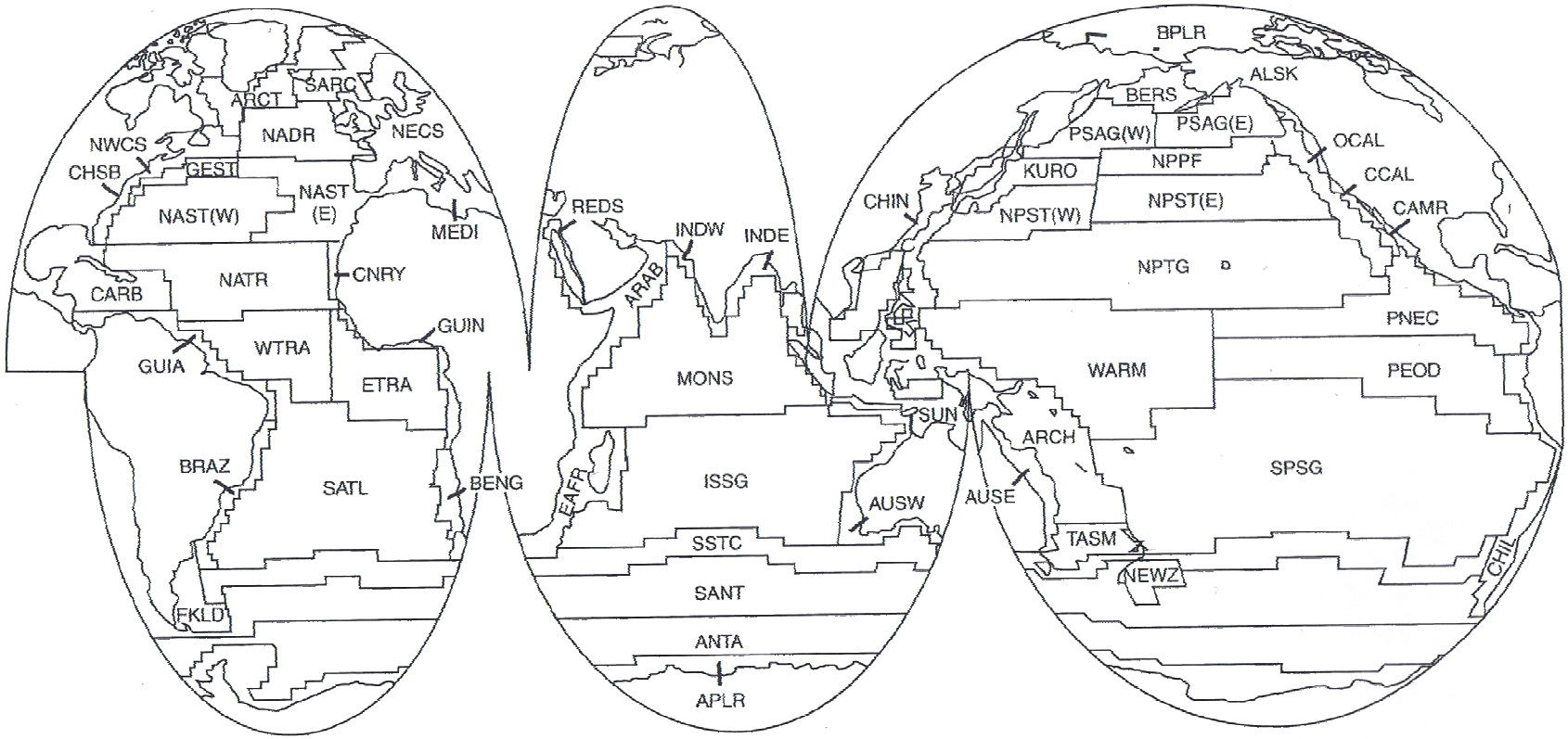

Figure 5.11 Division of the ocean in 57 biogeochemical provinces by Longhurst (2007).

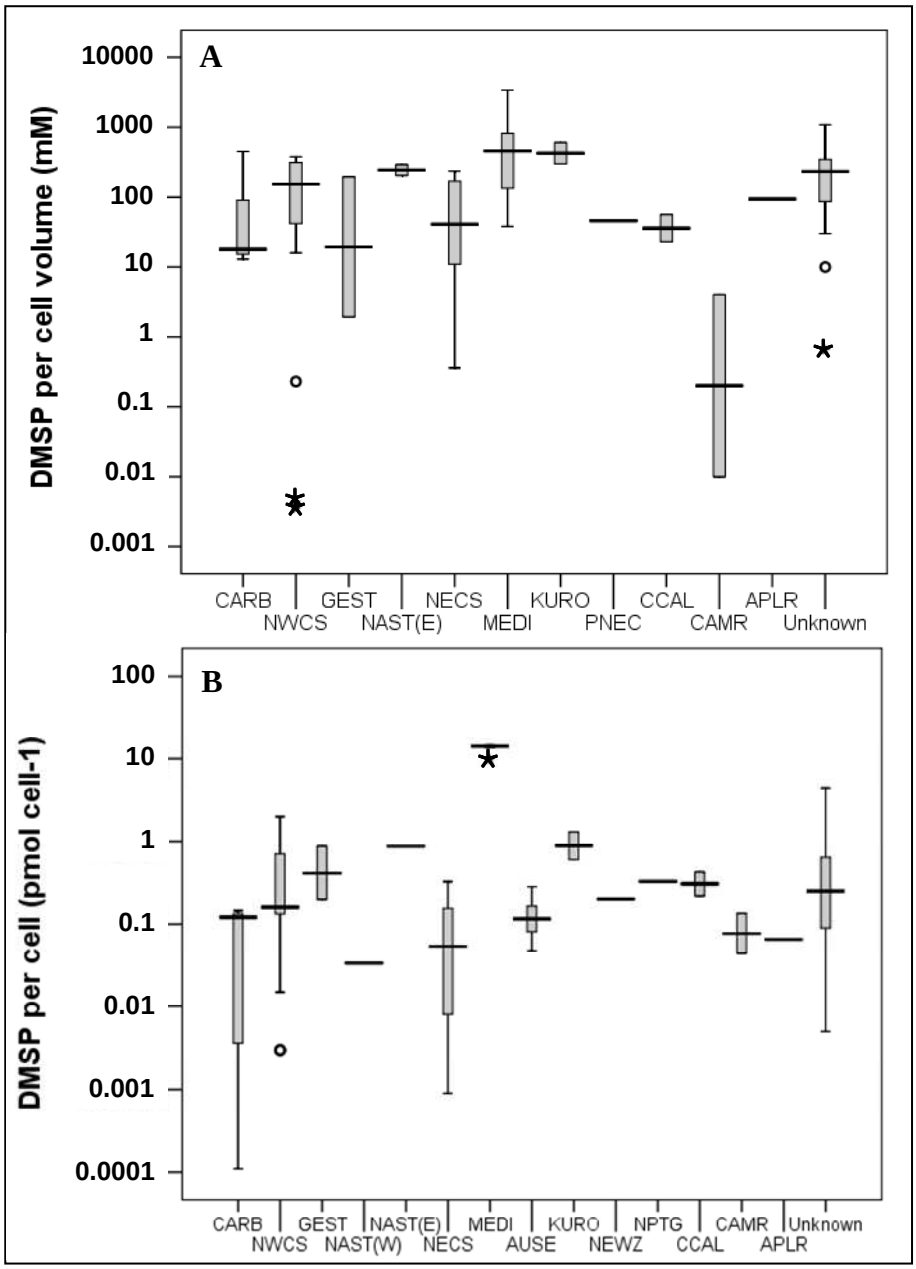

Figure 5.12 Box and whisker plots to show the distribution of DMSP concentration (A) per cell volume (n=3, 16, 2, 2, 10, 6, 2, 1, 2, 2, 1, 14 from left to right boxes) and (B) per cell (n=4, 13, 2, 1, 1, 9, 5, 5, 2, 1, 1, 2, 2, 1, 16 from left to right boxes) depending on oceanic provinces. Provinces are ranged from West to East from CARB to CCAL based on the representation of Longhurst (2007) and APLR is positioned at the end as it means the Austral Polar front. Some provinces are represented by only one value which is indicated by a horizontal line without box. A general description for box and whisker plots is given in Figure 5.3.

Comparing the median of the DMSP concentrations per cell volume within oceanic provinces, they vary over 2 orders of magnitude when excluding the CAMR province (2 – 459 mM, Figure 5.12). The species examined from the CAMR province in the East Pacific were bioluminescent (*Pyrocystis noctiluca* and *Lingulodinium*

polyedrum) and consequently significantly lower DMSP productions per cell volume ($P<0.05$, Mann-Whitney *U* test) were seen (see section 5.6.2). The same variability separates the median of the DMSP concentrations per cell which vary over more than 2 orders of magnitude (0.054 - 14.2 pmol $cell^{-1}$). However, large variability may also be observed inside an oceanic province as is the case of the Northeast Atlantic Shelf province (NECS) which is represented by values that cover 3 orders of magnitude (0.36 - 235 mM) or in the Central American Coastal province (CAMR) with values from 0.01 - 4.01 mM.

Interestingly, the Mediterranean province (MEDI), which has slightly higher salinity and temperature than other provinces (Black and MacDougall, 2002) shows DMSP values that are significantly higher than for other provinces ($P<0.05$, Mann-Whitney *U* test). This is interesting in that DMSP acts as an osmolyte (Dickson et al., 1980; Vairavamurthy et al., 1985; Dickson and Kirst, 1986). However, the Mediterranean Sea has a salinity of 38 which is equivalent to 0.65 M NaCl or an osmolarity of 1300 mOsm L^{-1} which is barely higher than the average osmolarity of the ocean (1200 mOsm L^{-1}). Hence, it is unlikely that salinity could explain more than a small part of the higher DMSP content observed in some Mediterranean species. Furthermore, all DMSP values for Mediterranean species come from fieldwork studies (Belviso et al., 2000; Jean et al., 2005) so there is a need for measurements to be done under controlled laboratory conditions. The Kuroshio Current province (KURO) in the North West Pacific also shows higher DMSP values (mM) than other provinces with the presence of *Scrippsiella trochoidea* and *Heterocapsa triquetra*. Such species have been reported with elevated DMSP concentrations (Table 5.2).

Different oceanic provinces provide different environmental conditions (e.g. water current, temperature, turbidity, nutrients and biota) affecting phytoplankton distribution, physiology and metabolism, and subsequently DMSP production. This analysis highlights the scarcity of data for some provinces. A substantial, and probably unrealistic, effort would be required to increase the number of DMSP measurements for dinoflagellate species to the point of good global coverage. Improvement in our understanding of how the environmental conditions affect the DMSP production by this group should perhaps focus on lab studies in the short-term.

5.7. DMSP lyase activities in dinoflagellates

The contribution of dinoflagellates to DMS production results first from their capacity to produce DMSP and secondly from their ability to synthesize DMSP lyase which might transform DMSP into DMS and acrylate before or just after its release from the cell. Less investigation has been done so far into DMSP lyase activity in dinoflagellates compared to the DMSP content. Nonetheless, Gibson et al. (1996) have observed high acrylate concentrations in Antarctic seawater that correlated with a bloom of dinoflagellates and cryptophytes and might suggest DMSP lyase activity. Additionally, Steinke et al. (2002a) noted the significant contribution of dinoflagellates to *in vitro* DMSP lyase activity levels in a phytoplankton bloom during a North Atlantic field campaign.

A few laboratory studies have confirmed the ability of dinoflagellates in producing this enzyme but with a noticeable inter- and intra-specific variability (Table 5.8). *In vitro* DMSP lyase activity (DLA) varies between species from 0.15 to 13260 mmol L_{cell}^{-1} h^{-1} and from 0.61 to 13.3×10^{10} fmol $cell^{-1}$ h^{-1}. *Amphidinium carterae* shows a very large variability in DLA between strains including values from 3.61 to 13260 mmol L_{cell}^{-1} h^{-1}. *Scrippsiella trochoidea* and *Lingulodinium polyedrum* also show substantial variability over 1 to 2 orders of magnitude among studies (depending on the unit per cell or per CV). DLA has also been detected in 5 strains of *Symdiodinium microadriaticum* with extremely high DMS production (Yost and Mitchelmore, 2009). The extremely high per cell DLA values for the coral symbiont *S. microadriaticum* could explain why some of the highest DMS concentrations reported in the marine environment are for mucus rope samples from coral reefs (Broadbent and Jones, 2004).

Table 5.8 DMSP lyase activities (DLA) measured in dinoflagellate cultures ranged in decreasing order per cell volume. Strain X has no identification number. All DLA values result from *in vitro* measurements, except data from source (3) which are *in vivo* measurements. NA indicates not available data. ND indicates that DLA was not detected.

Species	Strains	DLA		Data source
		per cell volume (mmol L_{cell}^{-1} h^{-1})	per cell (fmol $cell^{-1}$ h^{-1})	
Amphidinium carterae	X	13260	4488	1
Alexandrium fundyense	CCMP1719	1620	NA	2
Alexandrium tamarense	CCMP115	438	NA	2
Alexandrium tamarense	CCMP116	336	NA	2
Alexandrium tamarense	CCMP1771	234	NA	2
Karenia brevis	CCMP2281	166.8	924	1
Alexandrium minutum	CCMP113	22.56	59.73	4
Heterocapsa triquetra	NIES7	20	30	3
Lingulodinium polyedrum	CCMP1738	11.52	102.6	1
Scrippsiella trochoidea	NIES369	8	18	3
Amphidinium carterae	CCMP1314	6.70	3.07	4
Amphidinium carterae	CCMP1314	3.61	1.48	1
Crypthecodinium cohnii	CCMP316	2.86	7.27	4
Lingulodinium polyedrum	LP2810	1.75	0.80	4
Scrippsiella trochoidea	CCMP1599	0.15	0.61	4
Symbiodinium microadriaticum	CCMP829	NA	13.3×10^{10}	5
Symbiodinium microadriaticum	CCMP1633	NA	12.9×10^{10}	5
Symbiodinium microadriaticum	CCMP421	NA	3.86×10^{10}	5
Symbiodinium microadriaticum	CCMP828	NA	3.46×10^{10}	5
Symbiodinium microadriaticum	CCMP830	ND	ND	5
Heterocapsa triquetra	CCMP449	ND	ND	4
Polarella glacialis	CCMP1138	ND	ND	4
Kryptoperidinium foliaceum	CCMP1326	ND	ND	4
Karlodinium veneficum	CCMP415	ND	ND	4

Note
The data shown originate from the following sources: (1) Harada, 2007, (2) Wolfe et al., 2002 , (3) Niki et al., 2000 , (4) data from Chapter 4, (5) Yost and Mitchelmore, 2009.

Only 2 values result from *in vivo* measurements (data 3 in table 5.8, Niki et al. 2000) whereas all the other values are from *in vitro* measurements (data 1, 2, 4, 5 in Table 5.8, Harada 2007, Wolfe et al. 2002, data from Chapter 4, Yost and Mitchelmore 2009). For *in vitro* measurements, the pH optimum for enzyme activity differs among species. For instance, DLA from *Crypthecodinium cohnii* (Ishida, 1968) and *Alexandrium* (Wolfe et al., 2002) were highest at a pH around 6 whereas DMSP lyase activities from *Amphidinium carterae* and *Karenia brevis* were maximal at a more basic pH of 8 - 8.5 (Harada, 2007). Hence, one source of the variability observed could be the different pH conditions applied while analysing DLA. Some DLA values come from

measurements performed on axenic cultures and others from non-axenic cultures. In *S. microadriaticum*, 1 to 15 % of DLA was from bacterial origin (Yost and Mitchelmore, 2009). Nonetheless, in their natural environment, dinoflagellates are always associated with bacteria. Overall the DLA values observed in dinoflagellates (n=15) which cover 11 orders of magnitude for DLA per cell (n=24) or 4 orders of magnitude (n=15) when excluding *S. microadriaticum* data. This range is wider than that observed for 6 strains of the coccolithophore *Emiliania huxleyi* which spanned nearly 3 orders of magnitude from 1.2 to 750 fmol $cell^{-1}$ h^{-1} (Steinke et al., 1998).

5.8. Conclusions

The database for about 60 values of DMSP in dinoflagellates assembled here reveals 5 to 6 orders of magnitude variation per cell and per cell volume respectively between species. The dinoflagellates produce an average 1.409 pmol $cell^{-1}$ DMSP or 242 mM per cell volume DMSP. The values (0 - 3388 mM) are the widest and the highest DMSP concentrations seen to date in phytoplankton, so the initial assertion that dinoflagellates can be a significant potential DMSP source in the field is correct. DLA measurements in dinoflagellates are limited to the data set from a very small number of studies. And these already show a large inter- and intra-specific variation amongst dinoflagellate species. I therefore recommend increased monitoring of dinoflagellate blooms and their evolution with respect to global change, with a special focus on DMSP and DMS production via DMSP lyase activity.

I examined whether the large inter-specific variability in DMSP concentration observed could relate to underlying biological characteristics of the different species and strains. There is no apparent relationship with toxicity, the presence of theca, phylogenetic relationships or the oceanic province of origin. However, bioluminescent species appear to produce significantly lower DMSP concentrations than non-bioluminescent ones and consequently so do the Pyrocystales order and the CAMR province which are represented in the dataset by bioluminescent species. This result should motivate further research on the benefit and interaction of DMSP and bioluminescence production given that they have a potential common role in deterring grazers. Among the plastid types represented, the species that harbour haptophyte-like plastids had significantly lower DMSP content whereas species with cryptomonad-like plastids seemed to have higher DMSP content than the other plastid types. The identification of the genes involved in DMSP synthesis could help in understanding this difference. Also, it is unclear whether heterotrophic species could synthesise DMSP in a remnant plastid or in a different sub-cellular location. However, their unique ability to synthesise DMSP as heterotrophs gives support to the theory of their photosynthetic origin. Among other oceanic provinces represented, only the Mediterranean appears to contain species with higher DMSP content. The intra-specific variability observed for

some species is as large as or larger than in other phytoplankton species such as *Emiliania huxleyi*.

The analyses done on the assembled dataset highlight several gaps in knowledge. Overall, no clear relationship was found between DMSP concentration and plastid type but the dataset is strongly dominated by species with peridinin plastids which are the most common type in the oceans. Rather few examples of the other plastid types are available in culture. An improved analysis would necessitate more data for non-peridinin plastid types in order to obtain a better analysis on the role of the plastid type. There is also a considerable bias in the geographic origin of the dinoflagellates available in culture. Most data relate to species collected in the northern hemisphere and only about a quarter of the oceanic provinces is covered by the current dataset. Few data are available so far from the southern hemisphere or the poles, although new species of dinoflagellates have been identified during the last decade in South African and South Australian waters and in the Southern Ocean and most of these have haptophyte-like plastids (de Salas et al., 2003; de Salas et al., 2004a; de Salas et al., 2004b; de Salas et al., 2005; de Salas et al., 2008). Also dinoflagellates may be a dominant population of the fast ice protist community as *Polarella glacialis* was in East Antarctica in spring 1996 and 1997 (Thomson et al., 2006). There have been rather few field studies focussing on DMSP concentrations and DMSP lyase activities in dinoflagellates so our overall understanding of how environmental conditions affect them is quite poor. Due to toxicity issues, many coastal areas around the world are monitored for dinoflagellates and recurring dinoflagellate blooms e.g. *Karenia brevis* blooms in the Gulf of Mexico but these investigations have never been extended to DMSP and DMS production.

Most data compiled here are based on measurements done at a single time point and rather few data indicate how DMSP content varies with growth conditions, age of culture and physiological conditions (e.g. the variation of abiotic factors such as salinity, light, nutrient availability, temperature and the presence of competitive species, predators and viruses). This sulphur metabolite could be variously involved in many processes such as osmotic balance of the cell, antioxidative mechanisms and prey-predator interactions (see Chapter 1 section 1.2.3) and hence, is certainly affected by dinoflagellate physiology. In-depth investigations on the effect of dinoflagellate

physiology on DMSP content and DMSP lyase activity would give us a better insight into the biological role of DMSP for this phytoplankton group. Nonetheless, from the dataset compiled here, it seems unlikely that the primary function of DMSP could be that of an osmolyte where DMSP concentrations are very low (<10 mM). Another function suggested for DMSP and its by-products as anti-grazing compounds has not yet been shown for dinoflagellates, though they are well known for the production of toxins or luciferin (in the case of bioluminescence). Interestingly, *Alexandrium tamarense* is able to produce these 3 categories of compounds. Grazing experimentations with this species might help to clarify the role of these compounds, especially under N-limited conditions which may affect the saxitoxin and DMSP production in opposite ways.

This review summarises the available data on DMSP content in dinoflagellates. This major phytoplankton group is highly diverse in terms of its geographic distribution and biological characteristics and this is reflected in the wide range of DMSP concentrations and DMSP lyase activities seen. The data support the overall conclusion that dinoflagellates are one of the major DMSP-producing phytoplankton groups with potential to contribute to DMSP production and its flux to the atmosphere. A number of gaps in knowledge are highlighted that I hope will stimulate further DMSP-related research on this fascinating protist group.

Chapter 6 Effects of growth phases, salinity and nutrient variation on the DMSP content of the heterotrophic dinoflagellate *Crypthecodinium cohnii*- evidence for an osmotic role of DMSP

Abstract

Although dimethylsulphoniopropionate (DMSP) is known to be primarily produced by photosynthetic organisms, some heterotrophic dinoflagellates are able to produce DMSP in relatively high concentrations. I investigated the biological role of DMSP for heterotrophic species by testing the physiological response of *Crypthecodinium cohnii* on the cellular DMSP concentration over growth stages and under various salinity and nutrient conditions. DMSP appeared to act as an osmolyte under salinity treatments with long-term response after hypoosmotic shock and short-term response after hyperosmotic shock. I also found evidence for a 4-fold increase in DMSP concentration per cell volume at the end of exponential growth phase in response to glucose depletion in the medium. I suggested that DMSP might replace glucose-derived osmolytes in glucose-deprived medium for this species. The adjustment of DMSP content to the external glucose concentration was performed extremely fast within seconds and likely involves similar mechanisms of sensing and molecular processes that were recently described in yeasts. These results suggest that DMSP may be used as an osmolyte not only by photosynthetic macro and microalgae but also by heterotrophic dinoflagellates and that such function is regulated by an exceptionally fast response to environmental stimuli.

6.1. Introduction

Crypthecodinium cohnii is a heterotrophic protist belonging to the large and diverse dinoflagellate group. This species is distributed in temperate and marine waters living in marine to brackish habitat and usually found on decaying seaweeds (Tomas, 1997; Mendes et al., 2009). Heterotrophic dinoflagellates represent about half of living dinoflagellates (Taylor, 1987) and the dinoflagellate group contains 2183 species (Guiry and Guiry, 2009, http://www.algaebase.org/ consulted on the 29/07/2010). Heterotrophic dinoflagellates also account for $1/3^{rd}$ of the microzooplankton biomass (Buitenhuis et al., *in press*) and actively graze on diatoms (Sherr and Sherr, 2007), hence, provide a crucial component of the planktonic foodweb. This species is also of recent interest for biotechnological applications since it is intensively studied for the production of the ω-3 fatty acid, docosahexaenoic acid DHA that is known to reduce cardio-vascular diseases (Mendes et al., 2009).

In laboratory cultures, *C. cohnii* can be grown as an osmotroph, because it is able to actively take up dissolved organic matter through membranes. However, *C. cohnii* may not be restricted to osmotrophic feeding given that it showed ability to prey on small flagellates (Taylor, 1987; Ucko et al., 1997). Other phagotrophic heterotrophic dinoflagellates including *Oxyrrhis marina*, *Noctiluca scintillans* and *Gyrodinium lebourae* are facultative osmotrophs and some heterotrophic species are suspected to be obligate osmotrophs, for example, species of the genera *Histioneis*, *Ornithocercus*, *Protoperidinium* and *Triposolenia* (Taylor, 1987).

Ishida (1968) was the first to identify the presence of dimethylsulphoniopropionate (DMSP), the precursor of the climate active gas dimethylsulphide (DMS) in *C. cohnii*. The intracellular DMSP content was quantified in a range from 103 mM (see chapter 4) to 377 mM (Keller et al., 1989a). Additionally, Kadota and Ishida (1968) identified the presence of an enzyme responsible for DMSP cleavage to DMS in *C. cohnii*, that possibly belongs to the group of DMSP lyase isozymes. I previously quantified the *in vitro* activity of this enzyme at 2.86 mmol L_{cell}^{-1}

h^{-1} (see chapter 4). Nevertheless, other enzymes for DMS production exist in bacteria and fungi but these have not been investigated in protists (Todd et al., 2009).

DMSP and DMSP lyase enzymes are both synthesised in many but not all dinoflagellates (listed in chapter 5). Heterotrophic dinoflagellates are remarkable given they are the only heterotrophic organisms having this capacity. Moreover, the metabolic pathway of DMSP in *C. cohnii* (Uchida et al., 1996) differs from the one established for macroalgae (Gage et al., 1997); (see Chapter 1 section 1.2.2). The benefit of the conservation of DMSP synthesis in heterotrophic organisms is unclear. Research on marine algae suggests several biological roles for DMSP (compatible solute, methyl donor, anti-oxidant, overflow mechanism, predator deterrent; see Chapter 1 section 1.2.3. for more details) and it is possible that it is "multifunctional", hence serves various physiological roles. Its role in heterotrophic dinoflagellates in general and *C. cohnii* specifically is unknown but my own measurements and published values of DMSP concentrations in *C. cohnii* suggest that it is present at sufficient levels to involve DMSP in a potential osmolyte role (discussed in Chapter 5, section 5.3).

In the study presented here I investigated the physiological conditions that affect the DMSP content of *Crypthecodinium cohnii* with the aim of elucidating the function of this sulphur metabolite in this heterotrophic dinoflagellate. To achieve this aim, I quantified DMSP at different culture growth stages and under different salinity and nutrient conditions. The data provide a first insight into the contribution of DMSP and DMS from the ecologically important group of heterotrophic dinoflagellates.

6.2. Material and methods

6.2.1. Culture conditions

CCMP316, an axenic strain of *Crypthecodinium cohnii* (Seligo) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Boothbay Harbor, USA). Batch cultures were grown in 100 to 500 ml Erlenmeyer flasks with f/2 + NPM medium (modified from Guillard 1960) at 22°C and 100 μmol photons m^{-2} s^{-1} with a 14:10 h light:dark cycle. This heterotrophic species does not require light for growth, nonetheless, for consistency sampling was usually done in the middle of the photoperiod. The f/2 nutrient enrichment contained inorganic nitrogen, phosphorus, trace metals and vitamins. The NPM organic solution contained organic sources of carbon and nitrogen (Table 6.1). At the start of each experiment, experimental flasks were inoculated from a *C. cohnii* stock culture in late exponential growth phase to obtain initial cell density of 10^4 cells ml^{-1}. The inoculation marked the starting time of the experiments. The axenicity of the cultures was checked at the end of each experiment by DAPI staining (Sherr et al., 1993). The organic rich f/2 + NPM medium is organically rich and highly favourable to bacterial development and where contamination occurred it was obvious by visual inspection. Contamination of cultures with bacteria occurred rarely and these cultures were discarded immediately.

Table 6.1 NPM organics. Concentrations shown are per litre of final medium

NPM organics	Compounds	g per L
Organic carbon (org C)	sodium acetate	0.1
	glucose	0.6
	(di)-sodium succinate	0.3
Organic nitrogen (org N)	peptone	0.4
	bacto-tryptone	0.1
	yeast extract	0.01

6.2.2. Salinity treatments

Fifteen flasks (100 ml of volume) containing f/2 + NPM medium (medium recipe described in section 2.1.2.4) were inoculated with a stock culture to obtain 24 ml of starting batch cultures at 10^4 cells ml^{-1}. All flasks were sampled 6 and 24 h after the inoculation before diluting the cultures with fresh medium at 24h to alter the salinity. This salinity change marked the start of the experiment (t=0). Four salinity treatments were tested (9, 20, 45 and 58, measured using the practical salinity scale) and compared with a control of unchanged salinity (salinity of 31). Each of the 5 conditions was performed in 3 culture replicates. The dilution was done with 60 ml of f/2 + NPM medium prepared at various salinities to obtain the final salinities described above. Medium used for dilutions were prepared as follows: (1) for hypoosmotic shock, filtered seawater was diluted with distilled water to lower salinities. (2) for hyperosmotic shock, filtered seawater was heated below its boiling point to evaporate water and increase salinity. To limit the pH variation, the pH was adjusted with HCl and NaOH solutions before autoclaving medium. Autoclaved medium was enriched with sterile f/2 + NPM solutions and added to the cultures. Final pH ranged from 6.8 to 7.4. The cultures were monitored for up to 78 h after treatment but my investigations focus on the period of exponential growth during the first 36 h after change of salinity.

6.2.3. Nutrient supplementation treatments

Eighteen flasks (100 ml of volume) containing f/2 + NPM medium were inoculated with a stock culture to obtain 77 ml of starting batch cultures at 9 - 10×10^3 cells ml^{-1}. All flasks were sampled at 6, 30, 36 and 48 h. Immediately after the fourth sampling and as the cultures entered the stationary growth phase, nutrients were added in 15 flasks. Four treatments of nutrient additions were tested and compared with a control (0) that received no addition and a control (1) that received addition of all nutrients as indicated in Table 6.2. I prepared 3 organic stock solutions (NPM = org C + org N; org C; org N) and for each treatment I added one of these solutions at the same volume and concentration as the organic solution added in control 1 (6.8 ml). For 3 treatments, the f/2 enrichment was added at the same volume and concentration as in

control 1 (0.68 ml). Each of the six conditions was performed in 3 culture replicates. For comparison of growth between all conditions, total cell volume was corrected according to the dilution applied to the culture that was not applied to the control 0.

Table 6.2 List of nutrient supplementations. The f/2 enrichment comprises sodium nitrate, sodium phosphate, trace metals and vitamins (Guillard and Ryther, 1962; Guillard, 1975). Glc means glucose.

Controls and treatments	Nutrients added
Control 0	no addition
Control 1 = all nutrients	f/2 enrichments + org C + org N
Control 1 – org N	f/2 enrichments + org C
Control 1 – org C	f/2 enrichments + org N
Control 1 – Glc	f/2 enrichments + org N + sodium acetate + sodium succinate
Control 1 – F/2	org C + org N

6.2.4. Carbon availability treatment

Five media of different organic C concentrations were prepared in triplicate in 15 flasks of 100 ml volume. The organic C concentrations were 5, 10, 20, 50 and 100 % of original recipe (Table1). A stock culture of *C. cohnii* was grown in normal f/2 + NPM medium and used as inoculum in the 15 flasks. Batch cultures were sampled after few minutes, 3, 24, 30, 36 and 54 h to measure $DMSP_T$ per cell volume (CV).

6.2.5. Time-response of DMSP adjustment

Three media were prepared with different types and concentrations of organic C: (1) 100 % D-Glc according to the normal recipe for f/2 + NPM, (2) 5 % D-Glc, (3) 5 % D-Glc + 95 % L-Glc. Each medium was prepared in triplicate and dispensed in a total of 9 flasks. Each flask was inoculated with a stock culture of *C. cohnii* and immediately sampled after 30 seconds for $DMSP_T$ per CV measurements and every 30 seconds up to 5 min. At 10 min a last sample was taken for $DMSP_T$ per CV and total CV measurements.

6.2.6. DMSP and DMS production

Production of DMS and $DMSP_T$ were calculated by subtracting the DMS/P value from one sample point to the next one and dividing by the period between the two sampling points (equation 1).

$$\text{DMSP production} = \frac{DMSP_2 - DMSP_1}{t_2 - t_1} \qquad (1)$$

$$\text{DMS production} = \frac{DMS_2 - DMS_1}{t_2 - t_1} \qquad (2)$$

6.3. Results

6.3.1. Growth, DMSP and DMS concentrations in batch cultures

The growth of *Crypthecodinium cohnii* comprised 3 successive stages, the relatively short exponential and stationary growth phases and decline phase over a period of about 174 h (Figure 6.1 A). The exponential phase lasted for approximately 54 h during which total cell density and cell volume increased to reach a maximum of 4.2×10^5 cells ml^{-1} and 452 μL_{cell} L^{-1}. However, whilst growth rate remained constant up to 54 h the volume per cell started to decrease earlier and indicated continued cell division while accumulation of biomass was reduced (Figure 6.1 B). The stationary phase followed and lasted for about 24 h, though this shows up best in total cell volume (CV) data (Figure 6.1 A). Between 54 and 78h cell density and total cell volume remained fairly stable, but growth rate declined and volume per cell reduced by 54 % from 2100 to 970 μm^3. Finally the following decline phase was characterised by a reduction in cell density and total cell volume and a negative growth rate.

$DMSP_T$ and $DMSP_p$ concentrations in culture showed similar patterns over the growth which demonstrated that $DMSP_p$ was the major contributor to $DMSP_T$ (Figure 6.1 C). Statistical analyses showed no significant difference between $DMSP_T$ and $DMSP_p$ measurements (Mann-Whitney *U* test, $P=0.566 > 0.05$). $DMSP_T$ concentrations noticeably increased in line with cell growth during the exponential phase and decreased during the following phases.

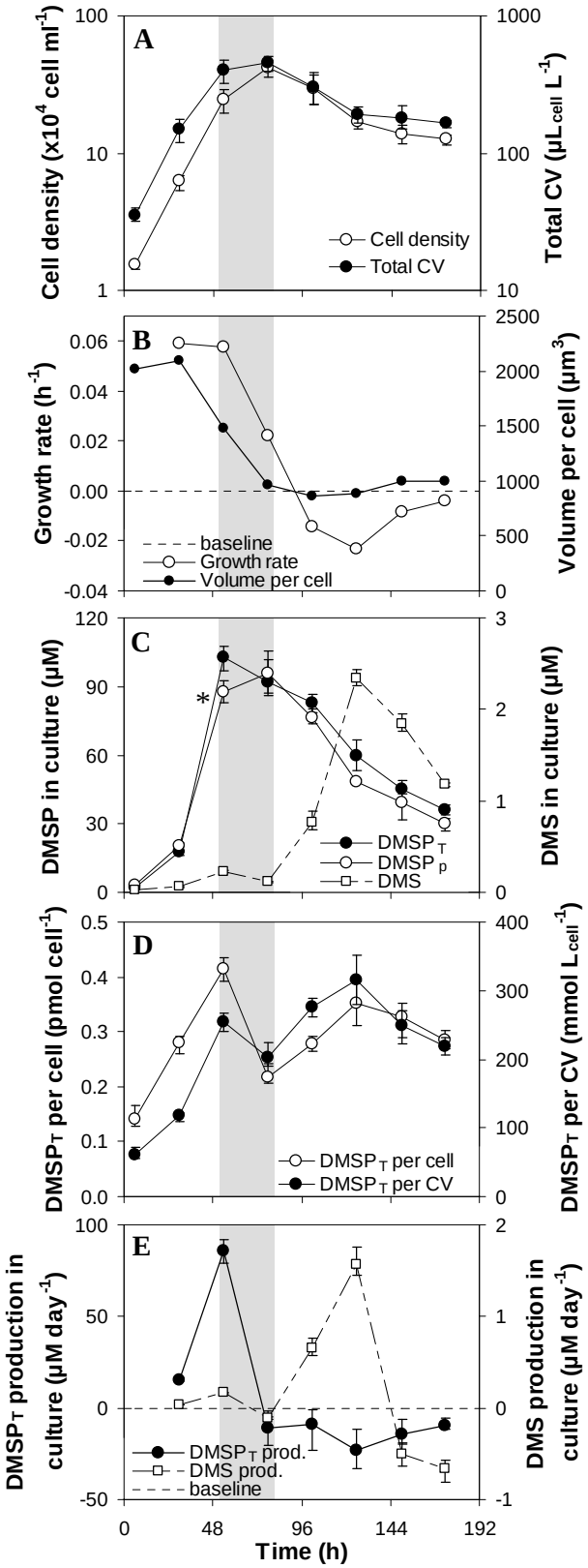

Figure 6.1 Growth and DMSP/DMS parameters in batch cultures of *Crypthecodinium cohnii*. (A) Cell density and total cell volume (CV). (B) Growth rate and volume per cell. (C) $DMSP_T$, $DMSP_p$ and DMS concentrations in the culture. (D) $DMSP_T$ per cell and per CV. (E) Production of $DMSP_T$ and DMS in culture. Shaded areas indicate the stationary phase of growth based on measurements of total CV. Shown are average values with range of data (n=3, except in panel C for $DMSP_p$ concentrations where the asterisk indicates n=2). When no range bars are visible, the range of data was smaller than the symbol size. Baseline of panels B and E show the zero growth and zero production respectively.

Normalised $DMSP_T$ concentrations substantially increased at the end of the exponential phase and decreased during the following phases (Figure 6.1 D). $DMSP_T$ concentrations per cell volume (CV) increased 4-fold from 61 mM (equivalent to mmol L_{cell}^{-1}) at the early exponential phase (6 h) to 255 mM at the beginning of the stationary phase (54 h) and $DMSP_T$ concentrations per cell increased 3-fold from 0.142 to 0.415 pmol $cell^{-1}$. Then, $DMSP_T$ concentrations decreased somewhat during the stationary phase when normalised to cell volume but showed a sharp decrease when normalised to cell density. Indeed, cell volume reduction compensated for the decrease of $DMSP_T$ concentration when it was normalised to CV. During the decline phase $DMSP_T$ concentrations slightly increased and decreased again.

DMS concentration in the culture was low during the exponential and stationary phase (0.023 to 0.229 μM) and increased during the decline phase to a maximum of 2.342 μM (Figure 6.1 C). In mid-exponential phase, DMS concentration was 0.063 μM and represented 0.4 % of $DMSP_T$ (17.6 μM). DMS concentration increased to 0.229 μM at 54 h similar to the increase in total cell volume. Then, DMS concentration increased sharply to 2.342 μM during the decline phase accounting at its maximum for 4 % of $DMSP_T$ concentration. Whilst the major DMSP production appeared to occur at the end of the exponential phase, the major DMS production occurred during the decline phase when growth rate was negative (Figure 6.1 E and B).

6.3.2. Effect of salinity treatments on growth and DMSP content

After 24 hours of exponential growth, batch cultures of *Crypthecodinium cohnii* were diluted to obtain various salinities in the medium. Medium with a salinity of 31 obtained with the normal recipe of the f/2 + NPM medium recipe was used as a control for comparisons with other salinity treatments (9, 20, 45 and 58).

The total CV showed a similar pattern for the different salinities tested except lower values at a salinity of 9 that resulted from lower volume per cell. After dilution with fresh medium, the cultures recovered to similar exponential growth rates during the first 12 h (Mann-Whitney *U* test, P>0.05, Table 6.3) then reduced after 24 h indicating the onset of the stationary growth phase. Moreover, the growth became more erratic at salinities of 45 and 58 after 24 hours of treatment (Figure 6.2 A and B).

The volume per cell was significantly affected by the different salinities (Mann-Whitney *U* test, P<0.05, Table 6.3). The volume per cell reduced with salinities 9 and 20 from 6 h and 12 h respectively up to the end (Figure 6.2 C and D). The volume per cell increased at salinities 45 and 58 from 24h to the end of the experiment.

Table 6.3 Probabilities obtained with Mann-Whitney *U* tests for comparison of growth rate, volume per cell and $DMSP_T$ per CV between salinity treatments (9, 20, 45, 58) and the control (salinity of 31) over a period of time after treatment. Asterisks show significant difference between the treatment and the control.

	Growth rate	**Volume per cell**			**$DMSP_T$ per CV**		
	6-12h	**6-36 h**	**6-12 h**	**12-36 h**	**6-12 h**	**12-36 h**	**36-78 h**
Salinity 9	0.688	≤0.001*	0.002*	≤0.001*	1.000	≤0.001*	≤0.001*
Salinity 20	0.378	0.002*	0.63	≤0.001*	0.004*	0.002*	0.009*
Salinity 45	0.688	0.023*	0.173	0.023*	0.025*	0.453	0.627
Salinity 58	0.337	0.019*	1.000	0.019*	0.004*	0.583	0.310

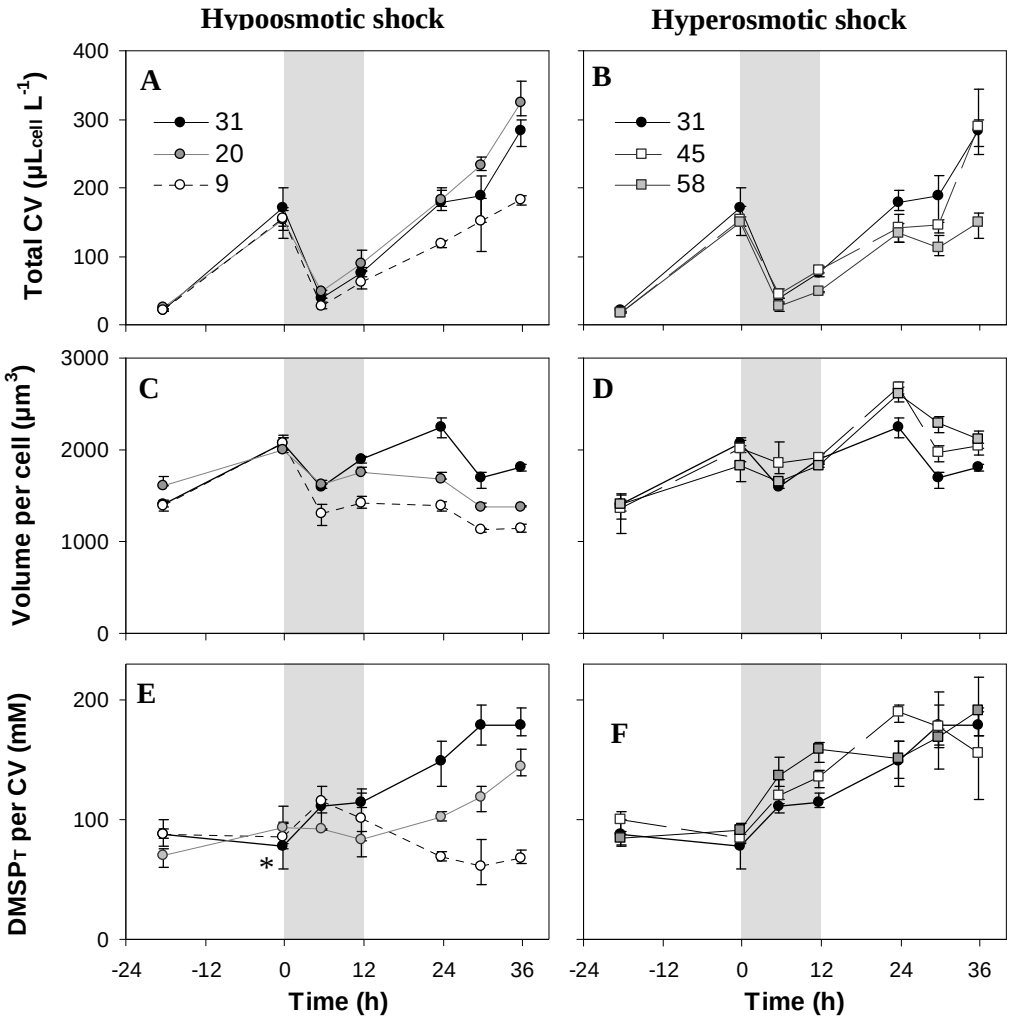

Figure 6.2 Effect of salinity on growth parameters and $DMSP_T$ per CV in batch cultures of *Crypthecodinium cohnii*. For ease of comparison, data are divided into "hypoosmotic shock" (left column, salinities of 9, 20 and 31) and "hyperosmotic shock" (right column, salinities of 31, 45, and 58). (A and B) Total CV. (C and D) Volume per cell. (E and F) $DMSP_T$ per CV. Shaded areas show the first 12 hours of treatment. Shown are average values with range bars of data (n=3, except in panels E and F for $DMSP_T$ per CV at salinity 31 where the asterisk indicates n=2). When no range bars are visible, the range of data is smaller than the symbol size.

The $DMSP_T$ per CV was also significantly affected by the different salinities. For most salinity treatments, the $DMSP_T$ per CV was rapidly adjusted to the external salinities after 6 hours following the medium transfer (Figure 6.2 E and F). Only the treatment of salinity 9 showed a delay in the DMSP adjustment after 12 h. Then, whilst at low salinities the $DMSP_T$ per CV remained adjusted during the whole experiment up to 78 h, at high salinities the $DMSP_T$ per CV shortly returns after 12 h to similar concentrations as in the control. Table 6.3 shows the probabilities obtained for statistical comparisons between treatments and the control at different periods of the experiment. From 6 to 12 h following the salinity treatment, the $DMSP_T$ per CV was significantly different from the control at salinities of 20, 45 and 58. From 12 to 78 h, the $DMSP_T$ per

CV was significantly different from the control at salinities of 9 and 20. Therefore *C. cohnii* cells appeared to be capable of short-term acclimation to hyperosmotic shock by increasing their DMSP to CV ratio but were not able to maintain this metabolic response for long period of time. However, *C. cohnii* cells performed short to long-term acclimation to hypoosmotic shock by decreasing their DMSP to CV ratio and this was a lasting metabolic adaptation (Figure 6.3 visualises the DMSPT per CV for all salinities tested at different sampling times).

Therefore, the DMSP content and the volume of the cell were affected alternately in response to the salinity treatment. At salinity of 9, the volume per cell was first reduced and then the DMSP per CV decreased whereas at high salinities of 45 and 58, the DMSP per CV first increased and then the volume per cell enlarged.

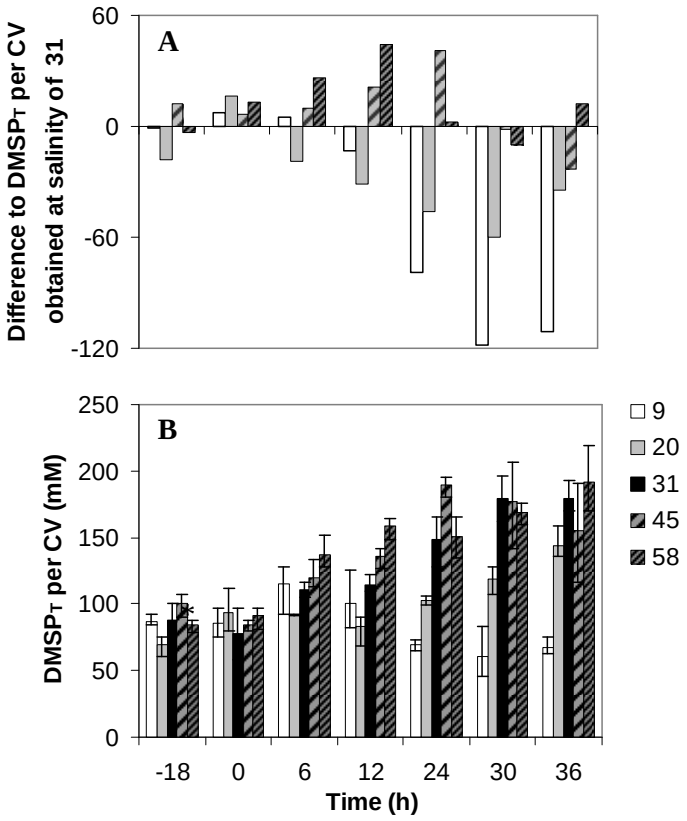

Figure 6.3 Summary of the effect of salinity on $DMSP_T$ concentrations per cell volume (CV). (A) $DMSP_T$ per CV expressed as a difference to $DMSP_T$ per CV obtained at salinity of 31. (B) DMSPT per CV obtained for 5 salinities tested (9, 20, 31, 45 and 58); shown are average values with range of data (n=3, except at time 0 for salinity of 31 where the asterisk indicates n=2).

6.3.3. Influence of nutrients on growth and DMSP content

The increase in $DMSP_T$ per cell and per CV at the end of exponential growth phase in batch cultures (Figure 6.1 C) was further investigated by supplementing cultures with the various components of the f/2 + NPM medium (Table 6.1 and Table 6.2) at the onset of the stationary growth phase.

The growth, volume per cell and $DMSP_T$ per CV of each supplementation treatment was compared with that of a control with addition of all nutrients (Control 1) and a control without nutrient addition (Control 0). All the cultures showed similar growth and $DMSP_T$ per CV patterns until the nutrients were added in late exponential phase (Figure 6.4, shading). As before, *C. cohnii* culture reached the stationary phase after 48 h of growth and over this period $DMSP_T$ per CV increased 4-fold (47 to 184 mM, Figure 6.4 A and K). The volume per cell reduced slightly during the exponential growth phase then reduced more substantially during the following phases (Figure 6.4 F, “Control 0”).

The addition of all nutrients (Control 1: f/2 enrichment nutrients + org C + org N) restarted growth, delayed the reduction of the cell volume and a significant decrease of $DMSP_T$ per CV was observed over the next 6 h (from 184 to 100 mM, Figure 6.4 A, F, K, “Control 1”). Similar results were obtained for the addition of all nutrients – f/2 enrichment (Figure 6.4 B, G, L). For other treatments which lacked some organic components, the growth rates were lower than after complete nutrient addition (Figure 6.4 C, D, E) and the volume per cell decreased, more substantially in treatment lacking org C (H, I, J). The addition of all nutrients – org N (Figure 6.4 M) led to similar DMSP decrease than after complete nutrient addition. However, when all nutrients – org C (Figure 6.4 N) or all nutrients – Glc (Figure 6.4 O) were added the decline in $DMSP_T$ per CV was eliminated. This suggested that org C and especially glucose was required to obtain the drop of $DMSP_T$ per CV. In other words, the carbon depletion was responsible for the increase in $DMSP_T$ per CV at the end of the exponential phase when no nutrients were supplemented (Figure 6.4 K, “Control 0”).

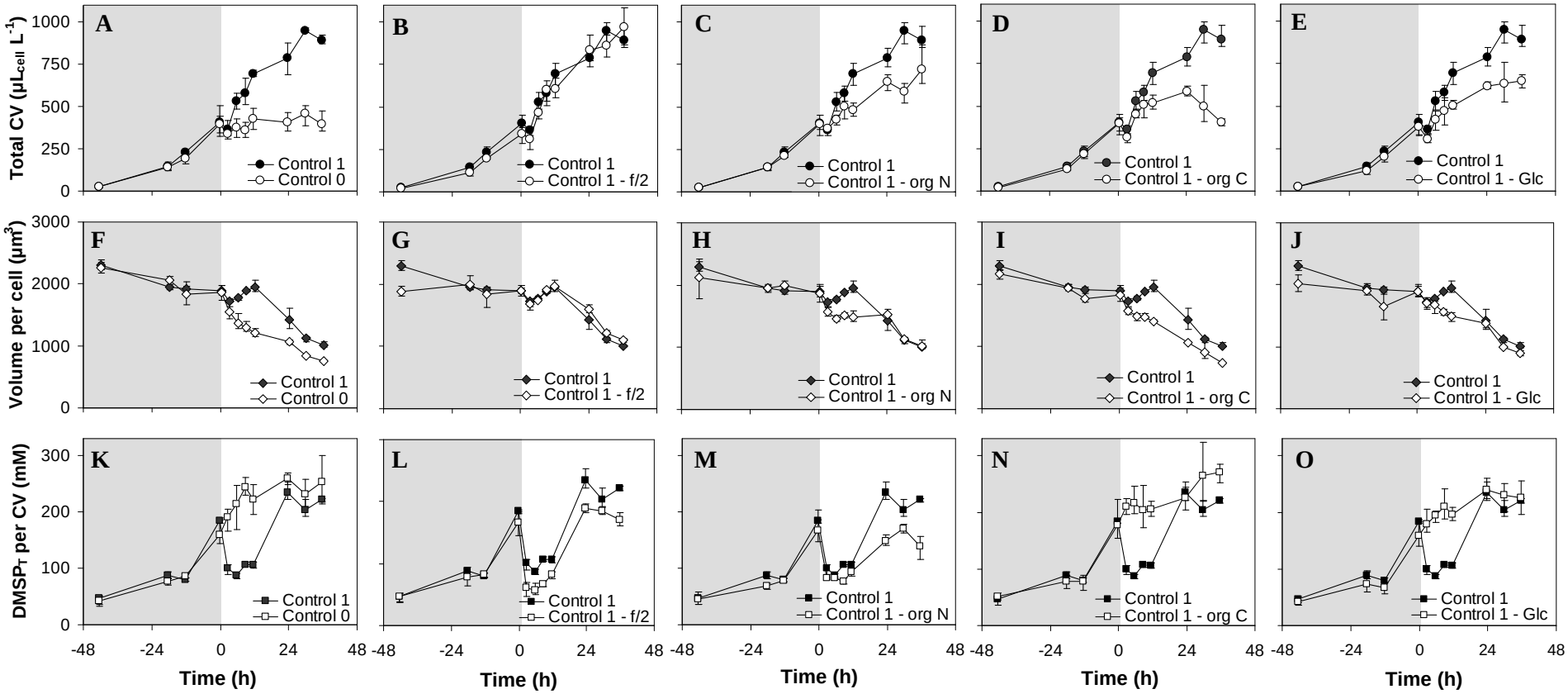

Figure 6.4 Effect of nutrient additions on growth parameters and $DMSP_T$ per CV in batch cultures of *Crypthecodinium cohnii*. (A to E) Total CV. (F to J) Volume per cell. (K to O) $DMSP_T$ per CV. Different nutrient additions are presented on each column. Control 0 has no addition, Control 1receive all nutrients and other treatments receive less nutrients than Control 1. Time 0 indicate time of nutrient addition and shading indicates exponential growth before nutrient addition. Shown are average values with range of data (n=3). When no range bars are visible, the range of data is smaller than the symbol size.

6.3.4. Influence of the C availability on the DMSP content

The depletion of C causing the increase in $DMSP_T$ per CV that I observed in the nutrient supplementation experiment (Figure 6.4 N and O) was further investigated by transferring *C. cohnii* in media with various C concentrations (5, 10, 20, 50 and100 % of organic carbon). Batch cultures were sampled just after inoculation into new medium (<5 min) and over the next hours (3, 24, 30, 36, 54 h) to measure the $DMSP_T$ per CV.

Reducing the available C in the medium resulted in an increase in the $DMSP_T$ per CV (Figure 6.5). There was a significant effect of the available C concentrations on the $DMSP_T$ content (Kruskal-Wallis, $P<0.001$). I also checked that all tested C treatments gave significantly different $DMSP_T$ per CV concentrations in comparison to those of the control with 100 % C (Mann-Whitney *U* test, $P<0.01$ detailed in Table 6.4). The adjustment of DMSP per CV in *C. cohnii* cells to the external C concentration appeared to be very fast in a period of minutes or seconds since the $DMSP_T$ per CV was adjusted at the first sampling a few minutes after the transfer.

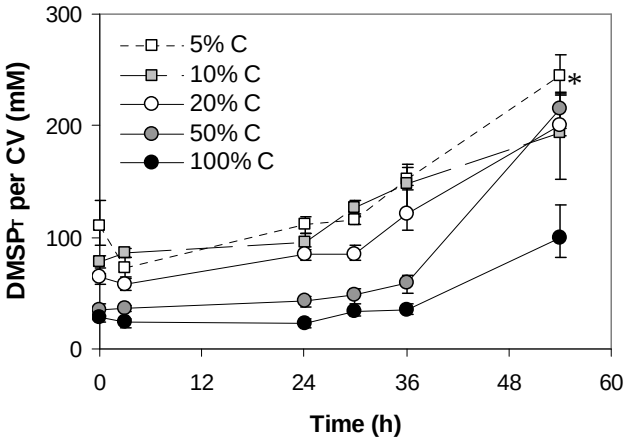

Figure 6.5 Effect of external organic C concentrations on DMSP per cell volume (CV). Cultures were transferred in medium containing 5, 10, 20, 50 and 100 % C. Time 0h illustrates the first sampling performed a few minutes after the inoculation in new medium. Shown are average values with range of data (n=3, except at 5 % C where the asterisk indicates n=2). When no range bars are visible, the range of data is smaller than the symbol size.

Table 6.4 Probabilities (P) obtained with Mann-Whitney *U* tests for comparison of $DMSP_T$ per CV and volume per cell between treatment with low C concentration (5, 10, 20, and 50 %) and control (100 %). Asterisks show significant difference between the treatment and the control.

C concentration	P obtained for $DMSP_T$ per CV	P obtained for volume per cell
5 %	≤0.001*	0.027*
10 %	≤0.001*	0.019*
20 %	≤0.001*	0.129
50 %	0.002*	0.429

The volume per cell appeared to be also affected by the external C concentrations (Figure 6.6). At 5 % and 10 % organic C concentration, the volume per cell was significantly lower than the control (Table 6.4). At 20 and 50 % organic C concentration, no significant difference was observed with the control. Only very low C concentrations affected the volume per cell of *C. cohnii* by reducing it.

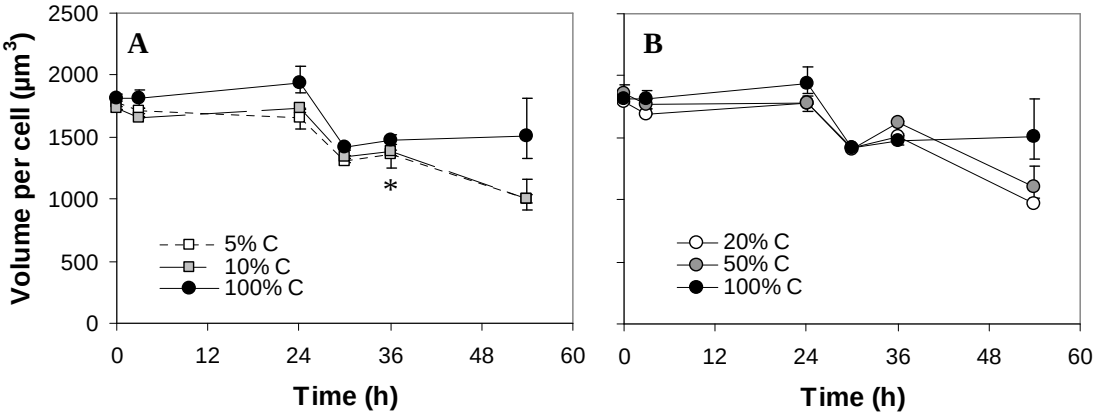

Figure 6.6 Effect of external organic C concentrations on volume per cell. Cultures were transferred in medium containing 5, 10, 20, 50 and 100 % C at time 0 h. The first sampling was performed a few minutes after the inoculation into new medium. (A) Treatments with 5 and 10 % were significantly different from the control at 100 % C. (B) Treatments with 20 and 50 % were not significantly different from the control at 100 % C. Shown are average values with range of data (n=3, except at 5 % C where the asterisk indicates n=2). When no range bars are visible, the range of data is smaller than the symbol size.

6.3.5. Time-response of DMSP content to the external glucose concentration

In order to test the time required by *Crypthecodinium* cells to adjust their DMSP content to the external C concentration of the medium, I monitored the $DMSP_T$ concentrations every 30 seconds during 5 min after the inoculation of *C. cohnii* in a medium depleted in glucose (Glc, 5 % of the normal concentration).

Figure 6.7 A shows that $DMSP_T$ per CV was adjusted in less than 30 sec to the external C concentration. On the first sampling point, the $DMSP_T$ per CV increased by 100 % (from 72 to 149 mM) in cells inoculated in glucose-depleted medium whereas the $DMSP_T$ per CV decreased by 60% (from 87 to 35 mM) in cells inoculated in replete medium.

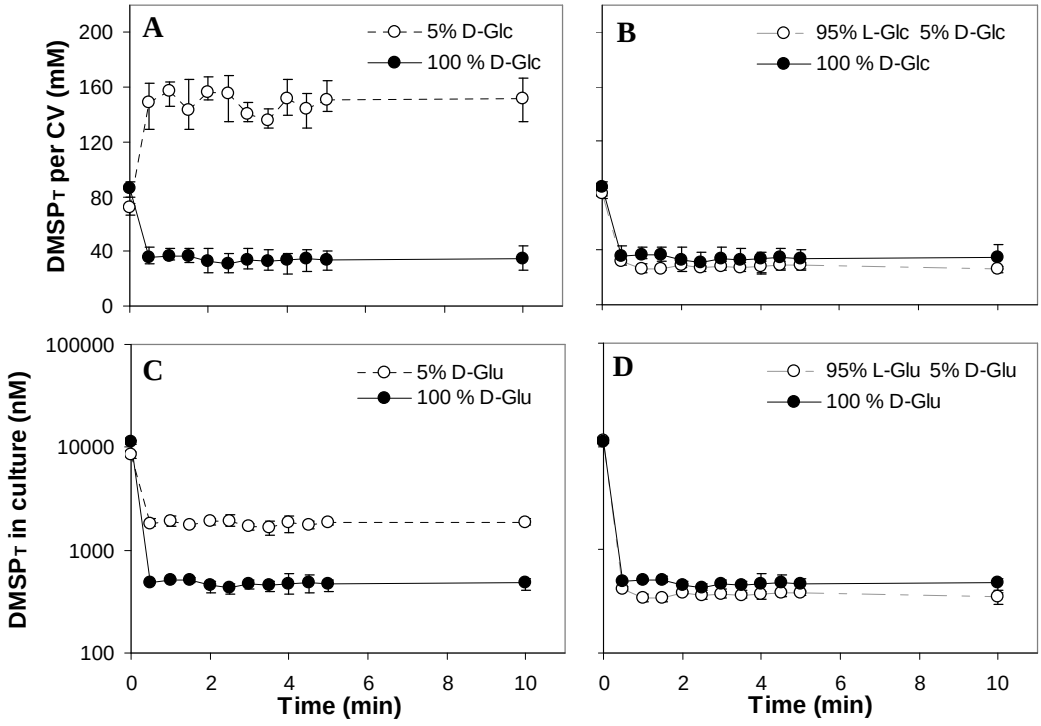

Figure 6.7 Time-response of $DMSP_T$ per CV to (A) D-glucose (Glc) limitation, (B) D-Glc limitation compensated with L-Glc in batch cultures of *Crypthecodinium cohnii* and time-response of $DMSP_T$ per L of culture to (C) D-Glc limitation, (D) D-Glc limitation compensated with L-Glc The first data point indicates DMSP concentration in stock culture before the introduction in new medium. Shown are average values with range of data (n=3). When no range bars are visible, the range of data is smaller than the symbol size.

Given that the medium with 5 % glucose differed from the 100 % glucose medium in osmolar properties, I tested the same glucose limitation treatment with compensation of 95 % L-Glc. In presence of 95 % L-Glc and 5 % D-Glc, $DMSP_T$ per CV was similar to that of the control 100% D-Glc (Figure 6.7 B). This suggested that L-Glc was absorbed by cells similarly to D-Glc or that L-Glc potentially balanced the osmolar pressure of the medium. After 10 min of treatment the mean volume of the cell was similar for the 3 conditions tested: 1787 μm^3 at 100 % D-Glc, 1675 μm^3 at 5 % D-Glc and 1763 μm^3 at 5 % D-Glc compensated with 95 % L-Glc (Mann-whitney *U* tests, $P>0.05$ for each treatment compared with the control, n=6 for each treatment). Measurements of $DMSP_T$ in culture not normalised to cell volume, also showed an obvious difference between the 5 % D-Glc treatment and the other conditions (Figure 6.7 C and D).

6.4. Discussion

I investigated the growth and DMSP content of *Crypthecodinium cohnii* in batch cultures and under different salinity and nutrient conditions in order to identify the possible physiological roles of DMSP in this species.

$DMSP_T$ concentrations were used as an estimation of the intracellular DMSP. Dinoflagellates are very sensitive to shear stress (as described in sections 1.3.12) so that measurements of intracellular DMSP are often affected by filtration artefacts (see section 3.5.1.2). With no significant differences between $DMSP_T$ and $DMSP_p$ measurements, $DMSP_p$ was the major fraction of $DMSP_T$ over the growth. Dissolved DMSP ($DMSP_d$) and DMS might account for a larger fraction during the decline phase when the population became senescent. According to my results, maximum DMS concentrations accounted for 4 % of $DMSP_T$. My own measurements of $DMSP_d$ were highly variable but suggested that $DMSP_d$ accounted for 0.5 to 3 % of $DMSP_T$ over the growth (data not shown).

6.4.1. Effect of salinity on *C. cohnii* batch cultures – evidence for an osmotic role of DMSP

DMSP is a sulphur metabolite described as a potential compatible solute (Stefels et al., 2007) and I found here that its concentration was significantly affected by salinity change and acted certainly as such in *C. cohnii*. DMSP per CV decreased with low salinities and increased with high salinities. This species may often be found associated with decaying seaweeds on beaches, in estuaries or drifting attached on macrophyte branches (Mendes et al., 2009) thereby affected by large osmotic variations due to tidal exposure, evaporation and desiccation, and precipitation. The growth of *C. cohnii* appeared not to be affected by the salinity change in accordance with the adaptation to the brackish and marine natural habitat of this species (Tomas, 1997). However, even if *C. cohnii* survived at high salinity treatments in our study, this species has not been reported in natural hyper-saline environments.

C. cohnii cells respond to external salinity variation by short-term osmoacclimation to high salinities and long-term osmoacclimation to low salinities. At low salinities, DMSP per CV decreased and remained adjusted over a relatively long period of several days. At high salinities, DMSP per CV increased and remained adjusted over a short period of less than 12 h.

The response of DMSP per CV may differ at low and high salinity treatments as it has been observed in the diatom *Cylindrotheca closterium*. The DMSP content of this diatom increases with salinity up to 44 but does not increase further at higher salinities but leads to the increase of other osmolytes than DMSP (Van Bergeijk et al., 2003). The different responses observed in *C. cohnii* suggest the potential production of other osmolytes at high salinities.

The volume per cell of *C. cohnii* was affected by salinity treatments as a potential result of cell division, biomass increase or sexuality but not of cell turgor. After a few seconds of treatment, the volume of wall-less cells or the turgor pressure of walled cells might increase at lower salinity and decrease at higher salinity (Kirst 1989). *C. cohnii* cells are covered by thin thecal plates likely to maintain the cell volume. Moreover the opposite trend of the volume per cell was observed through salinity treatments showing decrease at lower salinity and increase at higher salinities after several hours. This volume change was thus, not directly related to the cell osmotic variation. As growth rates (based on cell number measurements) were similar at medium and low salinities, the smaller volume at low salinities could result from a reduced biomass increase at similar cell division rates. At high salinities the volume increased resulting generally from biomass increase when the growth rate was unchanged and sometimes from lower growth rates (especially at extremely high salinity). The cell size variation may also result from change in gametes and zygotes production. Beam and Himes (1974) observed homothallic sexual reproduction in several strains of *C. cohnii* that lead to the production of gametes and zygotes of various sizes. Zygotes can result from the fusion of 2 or more rarely 3 cells and can generate 4 or 8 cells. The life cycle which influence cell sizes may be affected by external factor such as oxidative stress that induces the activation of sex genes in the multicellular green alga *Volvox carteri* (Nedelcu et al., 2004) or phosphate deficiency that allows

gamete fusion in the dinoflagellate *Lingulodinium polyedrum* (Figueroa and Bravo, 2005).

The osmolyte role for DMSP is not generalised to all algae that produce it. There is evidence that DMSP acts as an osmolyte in some haptophytes (Vairavamurthy et al., 1985; Stefels, 2000), prasinophytes (Dickson and Kirst, 1986) and green macroalgae (Dickson et al., 1980; Karsten et al., 1991). In some other green macroalgae species, DMSP does not respond to salinity variations (Edwards et al., 1987; Van Alstyne et al., 2003). Some dinoflagellates including *Pfiesteria piscicida*, *Ceratium longipes*, *Pyrocystis lunula*, *Gambierdiscus toxicus* contain DMSP in such small amounts that it is unlikely to act as an osmolyte (as concluded in Chapter 5, section 5.3). Additionally, DMSP concentration remains stable under salinity changes in some other organisms such as the terrestrial plant *Spartina anglica* (van Diggelen et al., 1986). Here, I report for the first time in detail that DMSP acts as an osmolyte in a heterotrophic dinoflagellate.

6.4.2. Influence of nutrients on *C. cohnii* batch cultures – antagonism with glucose

In normal conditions (f/2 + NPM medium), *C. cohnii* cultures showed a rapid growth due to the rich organic nutrition, reaching the stationary phase in about 54 h and the DMSP per CV increased substantially at the end of the exponential phase. At this stage, also called the retardation phase, nutrients start to deplete in the culture and affect the physiological state (Barsanti and Gualtieri, 2006). Hence, I have investigated whether the nutrient depletion that occurs at the end of the exponential growth phase could cause the increase in DMSP content.

The nutrient addition experiments in nutrient-depleted batch cultures identified the carbon source and especially glucose as the limiting nutrient triggering the increase of DMSP at the end of the exponential growth phase. DMSP production is also dependant on other nutrients than carbon. In my results the input of organic N and f/2 nutrients led to a greater DMSP production. F/2 nutrients supply trace metals and

vitamins necessary to enzyme activities involved in metabolic processes and DMSP synthesis.

Deschamps et al. (2008) observed that starch synthesis and accumulation in *C. cohnii* occurs during the early to mid-exponential phase then recedes and stops at the onset of the stationary phase when glucose was no longer available. Clearly, glucose assimilation and DMSP production both vary with growth stages and show an antagonistic behaviour. It is possible that DMSP replaces the glucose-derived osmolytes when glucose is limited. In many organisms, glucose is metabolised to carbohydrates that are known to play a role as osmolytes: trehalose in some cyanobacteria, yeasts, bacteria, actinomycetes (Welsh, 2000) and animals (Westh and Ramlov, 1991); sucrose in some microalgae, cyanobacteria and phototrophic bacteria; glycerol in some microalgae and yeasts; glucosylglycerol in cyanobacteria; arabitol and sorbitol in yeasts (Welsh, 2000); mannitol in microalgae, bacteria, fungi and plants (Iwamoto and Shiraiwa, 2005). Therefore, in microalgae including cyanobacteria, sucrose, mannitol, glycerol, glucosylglycerol and trehalose have been reported as glucose-derived osmolytes. *C. cohnii* produces a large variety of glucose-derived compounds as it irregularly excretes polysaccharides containing mainly glucose, galactose and mannose and also in lesser amounts fucose, uronic acid and xylose (de Swaaf et al., 2001). No studies have reported the cell content of glucose-derived compounds inside *C. cohnii* cells. In our experiments, glucose limitation could lead to the lack of glucose-derived osmolytes, the utilisation of most intracellular polysaccharides and the replacement by amino acid-derived osmolytes including DMSP. Similarly some yeasts produce different organic osmolytes during the different growth phases, for example they accumulate glycerol during exponential phase then trehalose during stationary phase when glucose is limiting (Meikle et al., 1988). Indeed, in situations of C limitation for *C. cohnii*, DMSP can be synthesised via the pool of existing methionine or through the degradation of proteins as described by Uchida et al. (1996). This hypothesis moreover explains the requirement of N organic source to produce DMSP as observed in my experiment.

6.4.3. Time-response of DMSP adjustment

The osmotic action of DMSP was described as a rather slow process (Kirst, 1989; Stefels, 2000) but I observed an exceptionally fast DMSP per CV adjustment in response to glucose limitation. Microalgal DMSP responds slowly to hyper- and hypo-osmotic shocks over a period of a few hours compared to non-organic osmolytes including Na^+ and K^+ ions that change within an hour (Kirst, 1989). However, there is the exception of the prasinophyte *Tetraselmis subcordiformis* that adjusts its DMSP content in less than 1 h to hypoosmotic shock and about 3 h to hyperosmotic shock (Dickson and Kirst, 1986). In my salinity experiments, the DMSP per CV changed in less than 6 h in response to most of the hyper- and hypo-osmotic treatments and a longer period of about 12 h was observed at salinity of 9. These periods were the first sampling point of our experiment and the exact duration has not been investigated. However, the DMSP concentration was adjusted within seconds in response to carbon and glucose limitation in the medium. This rapidity in the DMSP metabolism observed in *C. cohnii* might result from the fast general metabolic activity of this species in laboratory cultures. *C. cohnii* is an osmotrophic heterotrophic eukaryote grown on organic source of N and C. It could be compared to yeasts in terms of metabolism and growing conditions.

The physiological response of *C. cohnii* may be similar to other unicellular eukaryotes such as yeasts which are able to sense nutrient changes, especially glucose, and adjust their metabolism within minutes. Wang et al., (2004) observed that starved yeasts change the expression of about 60 % of their genes by more than 2-fold increase or decrease 20 minutes after glucose addition. Zaman et al. (2008) observed similar response for yeasts which change by 2-fold the expression of 40 % of their genes within minutes after glucose addition in culture grown on glycerol. This adjustment occurs in a few minutes and involves complex signalling networks and rapid adjustment of the metabolism. Such system is not known in *C. cohnii* and further research is required to understand how this extremely fast adjustment could occur within seconds.

The fast response of $DMSP_T$ per CV within 30 seconds could more likely result from a physical response of the cell volume change due to a difference in osmolar pressure of the medium. However, the cell volume was unchanged after 10 minutes of

treatment at 5 % D-Glc and the measurement of $DMSP_T$ per litre of culture also showed a large difference between the 5 % D-Glc and the 100 % D-Glc conditions. Some experiments investigating the cell volume change over the 10 minutes period after introduction in the 5 % D-Glc could determine more precisely a potential effect of the different osmolarities of the media. Additionally, the time-response of *C. cohnii* to DMSP adjustment could be tested by adding glucose in a culture entering the stationary phase, as I already observed a sharp decrease in DMSP concentrations. Finally, to support the idea of an extremely rapid DMSP synthesis within the 30 seconds of culture transfer in 5 % D-Glc medium the investigation of the molecular processes involved in DMSP synthesis are necessary, as for example the measurement of an enzyme activity involved in DMSP synthesis in *C. cohnii* such as the hypothetical methionine decarboxylase.

In the experiment with 5 % D-Glc compensated with 95 % L-Glc, *C. cohnii* cells reacted as if they were incubated in 100 % D-Glc showing similar $DMSP_T$ per CV and $DMSP_T$ in culture concentrations. Given that L-Glc cannot be used as a carbon source, the addition of L-Glc was applied here to complete the osmotic potential of glucose depleted medium (95 % L-Glc + 5 % D-Glc). These results suggest that the addition of L-Glc could balance the osmolarity of the medium similarly to the presence of 100 % D-Glc in the medium and eliminating the requirement of intracellular DMSP production or the addition of L-Glc could balance the osmolarity of *C. cohnii* cells by being absorbed similarly to D- Glc, potentially acting as an osmolyte and replacing DMSP. The assimilation of L-Glc has been observed in other organisms such as in cells of rat and hamster, rabbit intestines (Neale and Wiseman, 1968; Bihler et al., 1969; Wood et al., 1989), house sparrows (Chang et al., 2004) and yeasts (Ritchie et al., 1986). Further experiments with radio-labelled (^{14}C) L-Glc could determine whether this sugar is absorbed by *C. cohnii* cells. In conditions of low D-Glc concentrations completed with L-Glc, the medium was carbon-limited and the absence of DMSP increase excluded the effect of starvation stress as a stimulus for DMSP production.

Crypthecodinium cohnii potentially re-metabolised its DMSP stock in conditions of carbon-replete conditions. In conditions of glucose-depleted medium that was repleted by glucose addition, $DMSP_T$ concentration decreased and I observed that the $DMSP_d$ fraction decreased as well (result not shown) suggesting that DMSP was rather

used by cells than released to the medium. Kadota and Ishida (1968) observed that *C. cohnii* was not able to incorporate an external source of DMSP but more recently, Salo et al., (2009) found that the heterotroph *Oxyrrhis marina* was able to incorporate and assimilate dissolved DMSP by osmotrophy. Such ability should be re-assessed for *C. cohnii* cells. Therefore, DMSP appears to be an organic resource not only for bacteria but potentially for heterotrophic dinoflagellates which are able to assimilate and metabolise DMSP. Moreover *C. cohnii* might be able to re-metabolise its intracellular stock of DMSP. Another example of microalga utilising DMSP has been described by Kates and Volcani (1996) who presented DMSP as a theoretical intermediate in the metabolic pathway of the phosphatidylsulfocholine in the diatoms *Nitzschia spp*. Further experiments with *C.cohnii* cultures supplemented with radio-labelled (^{35}S) DMSP could determine whether DMSP is incorporated by *C. cohnii* cells in replete conditions and in glucose-limited conditions.

6.5. Conclusion

I have shown that DMSP acts as an osmolyte for *Crypthecodinium cohnii* in various salinity treatments and suggested that other osmolytes might be produced at high salinity. Moreover, DMSP also seems to be produced as a substitute for glucose-derived osmolytes in conditions of carbon or glucose depletion. Osmotic regulation for turgor pressure is a fundamental process for aquatic species. In this regard and especially for osmotrophic species such as *C. cohnii* are special cases in that uptake of carbon compounds will disturb osmolarity.

DMSP might act as an osmolyte in other heterotrophic dinoflagellates as well. Other heterotrophic dinoflagellates that are known to produce DMSP include *Protoperidinium pellucidum, Pfiesteria schumwayae* and *Pfiesteria pisciscida* and potentially *Protoperidinium ovatum* (see Chapter 5). However, some of these other heterotrophs show different trophic behaviours, including grazing and are likely to use DMSP in different ways. On the other hand, *C. cohnii* strains are adapted to their natural brackish habitat and have developed or maintained strategies such as DMSP

synthesis to survive large salinity variations; other heterotrophs that would live in different environments might not have this ability.

Contrary to slow adjustment of organic osmolytes previously observed in microalgae, the adjustment of DMSP concentration to glucose limitation is exceptionally fast within seconds. Similar processes have been observed in yeasts within minutes involving nutrient availability sensing and response by regulating gene expression. Given that this species is also investigated in biotechnology areas such as ω-3 fatty acid production, extremely fast molecular processes might be of interest.

I observed that DMSP concentration may suddenly increase but also decrease depending on the external glucose concentration and in the latter case the fate of DMSP that could be released or re-metabolised is still unclear. According to our observations DMSP seemed to be re-metabolised but more investigation would help to confirm this first observation.

Only a small number of heterotrophic dinoflagellates have been investigated for their DMSP content in regard to the thousand or so living species that constitute 1/3rd of the microzooplankton biomass. This significant component of the marine ecosystem is a potential source of DMSP and DMS and a potential contributor to the global sulphur cycle. I hope my results are going to encourage further research on the DMS and DMSP production by heterotrophic dinoflagellates.

Further research is warranted to determine the influence of nutrient and salinity variations on DMSP content of *C. cohnii* in conditions close to their natural environment. In its natural habitat, *C. cohnii* likely utilises various sugars and organic molecules and the influence of a natural carbon source might differ from the antagonism behaviour between glucose and DMSP that I observed. Mesocosm cultures of *C. cohnii* could be used to assess the effect of natural disturbance such as precipitations and natural salinity variation on its DMSP content or laboratory cultures with seawater from areas where *C. cohnii* have been identified would represent more realistically the natural DMSP content of this species and its variation over the growth.

Chapter 7 Effects of darkness and nitrogen availability on growth and DMSP content of the heterotrophic dinoflagellate *Crypthecodinium cohnii*

Abstract

Following the description in the previous chapter of an osmolyte function of DMSP in the heterotrophic dinoflagellate *Crypthecodinium cohnii*, I have investigated other potential roles for this secondary metabolite. The possible functions of antioxidant or overflow metabolite were examined by assessing the effect of light and nitrogen limitation on growth and DMSP content of *C. cohnii*. Batch cultures were grown in dark and light conditions. Although *C. cohnii* reached significantly lower cell volume under light exposure (24 % difference in average, $P<0.05$), the DMSP per cell volume was similar (89 - 620 mM in the dark and 95 - 788 mM in the light, $P>0.05$). The presence of photo-protective carotenoids in *C. cohnii* is likely to reduce the requirement of antioxidant and, hence, DMSP may not be utilised as an antioxidant under light exposure. The nitrogen limitation was applied by reducing the concentration of nitrogen-containing compounds in the medium. The results showed that during the exponential growth phase, nitrogen limitation increased the DMSP content (1.54-fold increase for a 20-fold nitrogen dilution) suggesting a potential role for DMSP as an antioxidant in response to N-limitation stress or a possible role of overflow metabolite to remove excess sulphur and recycle amino groups. During the late exponential and stationary growth phases, the DMSP production in response to glucose limitation (as observed in the previous chapter) was clearly dependent on nitrogen availability. These new results highlight the multifunctional properties of DMSP within *C. cohnii* species and show a strong relationship between the nitrogen availability and the DMSP content of heterotrophic dinoflagellates.

7.1. Introduction

In the previous chapters, I described how DMSP concentrations vary in dinoflagellate species to better understand the biological role of DMSP in this important plankton group. In chapter 6, I focussed my study on the frequently used heterotrophic model *Crypthecodinium cohnii* given that whilst heterotrophic dinoflagellates are a potential source of DMSP, few studies have investigated the role of this compound in such organisms.

Previously, I showed that *C. cohnii* utilises DMSP as an osmolyte and as a potential substitute to glucose-derived osmolytes by investigating the effect of some abiotic parameters such as salinity and nutrients on the DMSP content of *C. cohnii*. For nutrient treatments, I examined, in depth, the effect of glucose limitation, demonstrated that this results in increased DMSP concentrations and I also noticed the effect of nitrogen limitation that appeared to restrain the DMSP concentration in conditions of glucose co-limitation. The effect of nitrogen limitation without glucose limitation on DMSP concentration in *C. cohnii* is not known. In other phytoplankton groups, nitrogen limitation has been reported to increase DMSP concentrations, such as in prasinophytes (Gröne and Kirst, 1992) and diatoms (Bucciarelli and Sunda, 2003). In these reports, DMSP was suggested to act as a potential antioxidant in response to nutrient-limitation stress (Bucciarelli and Sunda, 2003) or as an overflow metabolite during conditions of unbalanced growth with excess sulphur and lack of nitrogen (Stefels, 2000). In this chapter, I investigated in more detail the effect of nitrogen limitation on the DMSP concentration in *C. cohnii*.

Some other abiotic parameters such as light exposure or dark conditions might also affect the DMSP concentrations. *C. cohnii* is able to grow in complete darkness as a heterotrophic species. In fact, it is growing with even higher growth rate in the dark in comparison to light. Therefore, light has been suggested to inhibit growth by allocation of resources towards the production of photo-protective pigments such as carotenoids (Tuttle and Loeblich, 1973). Several observations suggest that the presence or absence of light may trigger different metabolic responses in protists: Jakobsen et al. (2004)

have shown that heterotrophic species have a circadian cycle of growth and feeding rates that is maintained by a light cue such as a light irradiance threshold in the case of the heterotrophic dinoflagellate *Oxyrrhis marina*. Strom (2001) reported that the heterotrophic dinoflagellate *Noctiluca scintillans* shows light-aided digestion that could result from the potential photo-oxidation of the intracellular organic matter. Moreover the same study showed that two ciliate species grow with substantially higher rates when exposed to light than in darkness.

As far as I am aware, the effect of light or darkness on the DMSP content of heterotrophic dinoflagellates that are exposed to a light-dark cycle in natural conditions has not been investigated. Important questions that need addressing are: Does growth inhibition in the presence of light also affect the DMSP content? Does light exposure trigger the production of antioxidant compounds such as DMSP?

In this chapter, I investigated the effect of the abiotic parameters light exposure and nitrogen limitation on the DMSP content of *C. cohnii* batch cultures. The aim was to identify other potential roles of DMSP in *C. cohnii* and increase our insight into the factors that are affecting its concentration.

7.2. Materials and Methods

Axenic batch cultures of *Crypthecodinium cohnii* were grown in f/2 + NPM medium at 22 ºC in a controlled environment (MLR-351 Plant Growth Chamber, Sanyo). The treatments tested with the cultures are described below.

7.2.1. Light and dark treatment

A stock culture grown under 100 µmol photons m^{-2} s^{-1} with a 14:10 light:dark cycle in late exponential growth phase was inoculated into 6 Erlenmeyer flasks of 250 ml volume containing 120 ml of medium. Three starting cultures were placed in three 1L Winchester black plastic containers inside the incubator and three starting cultures were placed under light exposure that was similar to the light levels experienced by the stock culture. Growth parameters and total DMSP were monitored for these 2 treatments once a day over growth phases including the exponential, stationary and decline phases of growth in 7 days. All cultures were sampled at the same time that was the mid-time of the photoperiod of cultures in light.

7.2.2. Nitrogen limitation treatment

The compounds that constitute the main sources of organic nitrogen in the f/2 + NPM medium are peptone, bacto-tryptone and yeast extract. As described in chapter 6 (section 6.2.1), other f/2 + NPM components include the organic C sources glucose, sodium acetate and sodium succinate. I investigated N limitation by removing the main sources of N from the medium (Table 7.1).

Fifteen flasks containing 5 different media in triplicate were inoculated at the beginning of the experiment with a stock culture in late exponential growth phase. The media contained various concentrations of organic nitrogen: 5, 10, 20, 50 and 100 % of the original recipe (Table 7.1). Growth parameters and total DMSP were monitored over

the different growth phases to quantify the effect of nitrogen limitation. Three supplementary flasks containing medium with 10 % organic nitrogen were inoculated similarly to the others. After 54 h of growth and as these 3 cultures appear to be in stationary growth phase, the organic nitrogen was added to reach the 100 % of the original recipe in order to check the effect of nitrogen limitation and supplementation on growth and DMSP concentrations.

Table 7.1 Organic sources of nitrogen in original f/2+NPM recipe equivalent to 100% N.

NPM organic nitrogen	Compounds	g per L
Organic nitrogen (org N)	peptone	0.4
	bacto-tryptone	0.1
	yeast extract	0.01

7.2.3. Measurements and analyses

Growth parameters including cell density (cells ml^{-1}) and total cell volume ($\mu L_{cell}\ L^{-1}$) were monitored using a particle counter (Multisizer 3, Beckman).

Total DMSP ($DMSP_T$) measurements were performed as an approximation of particulate DMSP ($DMSP_p$) and were conducted similarly to the methods described in Chapter 2, Chapter 4 and Chapter 6.

The production of total cell volume (CV) was calculated as a difference of CV between one sample point and the previous one over the period of time (equation 1). The same calculation was performed for the production of DMSP over a period of time (equation 2).

$$\text{CV production} = \frac{CV_2 - CV_1}{t_2 - t_1} \quad (1) \qquad \text{DMSP production} = \frac{DMSP_2 - DMSP_1}{t_2 - t_1} \quad (2)$$

Given that data were not normally distributed, non parametric statistical tests (Mann-Whitney *U* and Spearman tests) were carried out with a statistical software package (SPSS, version 16.0). When analysing the effect of a treatment on several parameters over the growth of the culture, I performed statistical analyses on all data collected over the growth to consider the global effect of the treatment and I also performed statistical analyses on parts of the growth to allow observation of a potential effect of the treatment during specific periods of the growth. For the light experiment, I divided the total data into 3 growth phases and performed analyses on the whole growth (6 - 150 h) and on each phase (6 - 54 h, 54 - 102 h, 102 - 154 h).

7.3. Results

7.3.1. Effect of light on growth and DMSP content

Some batch cultures of *Crypthecodinium cohnii* were grown in complete darkness and other batch cultures were exposed to light. Cultures were sampled over the growth phases to investigate the effect of light on growth and DMSP content.

For detailed comparison between treatments, I divided all the data into 3 phases of growth (Figure 7.1, shading): (1) the exponential phase from 6 to 54 h when the cell density increased, (2) the stationary phase from 54 to 102 h when cell density appeared stable (3) the decline phase from 102 to 154 h when the cell density decreased. The $DMSP_T$ in culture followed the same trend as the cell density over growth. The total CV, however, showed a short plateau at the end of phase 1 as cells were dividing without accumulating biomass and an increase at the beginning of phase 2 as cells where not dividing but accumulating biomass.

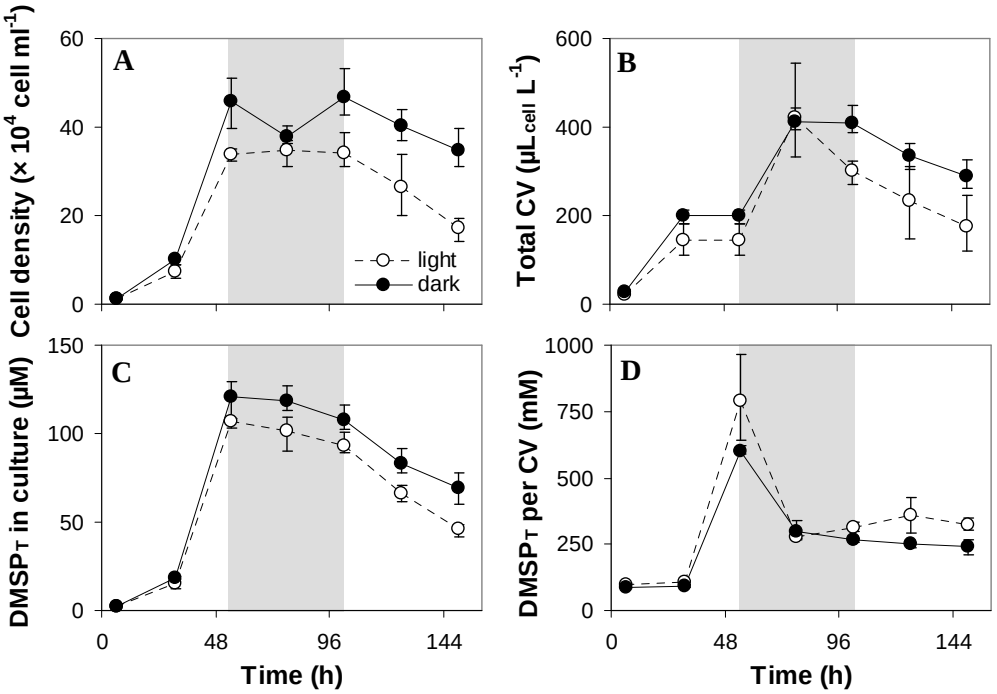

Figure 7.1 Effect of light exposure and darkness on growth and DMSP parameters in batch cultures of *Crypthecodinium cohnii*. (A) Cell density. (B) Total cell volume. (C) $DMSP_T$ in culture. (D) $DMSP_T$ per CV. Shown are average values with range of data (n=3). When no range bars are visible, the range of data was smaller than the symbol size. The growth is divided into 3 phases and the grey-shaded area is the stationary phase.

The growth was significantly affected by light exposure (Figure 7.1 A and B). Cell density and total CV were significantly different over the growth phases between the 2 treatments with lower values in the presence of light (Mann-Whitney *U* test, $P<0.05$, Table 7.2). The total CV of cultures exposed to light represents 24 % less on average than total CV of cultures grown in the dark. Similarly, the light exposure resulted in an average of 25 % reduction of the cell density in comparison with cultures in the dark. However, similar cell density was observed between the 2 treatments during the exponential growth phase and similar total CV was obtained during the stationary phase (Table 7.2).

The effect of light exposure on DMSP concentrations in the culture was insignificant and ranged from 2 to 121 µM in the dark and 2 and 107 µM in the light (Mann-Whitney *U* test, $P>0.05$, Table 7.2) over the whole period of the experiment. However, during the stationary phase only, a significant difference was observed between dark and light treatment ($P<0.05$). After normalising DMSP to total CV, the data ranged from 89 - 620 mM and 95 - 788 mM DMSP per CV in the dark and light respectively and the difference was insignificant for the whole period of the experiment($P>0.05$). During the decline phase only, a significant difference was obtained in DMSP per CV between dark and light treatments ($P<0.01$).

Table 7.2 Statistical probabilities obtained with Mann-Whitney *U* test for comparison of growth and DMSP parameters between cultures exposed to light and in darkness over a period of growth (GPh = Growth phase). Asterisks show significant differences.

	All growth	**Exponential GPh**	**Stationary GPh**	**Decline Ph**
	6 - 150 h	**6 - 54 h**	**54 - 102 h**	**102 - 150 h**
Cell density	0.013*	0.402	0.001*	0.002*
Total CV	0.045*	0.046*	0.171	0.007*
DMSP in culture	0.178	0.402	0.007*	0.07
DMSP per CV	0.054	0.145	0.233	≤0.001*

7.3.2. Effect of nitrogen availability on growth and DMSP content

Given that I previously observed that DMSP concentration in *C. cohnii* was partially dependent on nitrogen availability (Chapter 6), I further investigated the influence of a range of nitrogen concentrations (5, 10, 20, 50, 100 %) on growth and DMSP content.

The cell density and total cell volume (CV) was affected by nitrogen availability and the DMSP content showed an opposite response between exponential and stationary growth phases (Figure 7.2). The maximum cell density and total CV was lower under nitrogen limitations (Figure 7.2 A and B), as for instance the total CV was 617, 398, 242, 159 and 139 µLcell L^{-1} in cultures containing 100, 50, 20, 10 and 5 % nitrogen, respectively. Whilst DMSP per CV showed significant negative correlation with N concentrations during exponential growth phase (6 - 30 h, Spearman test, $P<0.05$) DMSP per CV showed significant positive correlation with N concentrations during stationary growth phase (48 – 78 h, Spearman test, $P<0.01$; Figure 7.2 C). Therefore, during the exponential growth phase, at 6 h after the culture inoculation in nitrogen-limited media, higher DMSP concentrations were observed compared to the nitrogen-enriched medium. For instance, DMSP was increased significantly (Kruskal-Wallis test, $P<0.05$) 1.54-fold in 5 % N compared to 100 % N medium (106 and 69 mM of DMSP respectively), 1.46-fold in 10 % N, 1.39-fold in 20 % N and 1.22-fold in 50 % N (101, 96 and 84 mM of DMSP respectively). During the period of late exponential and stationary phases, the DMSP concentrations were more elevated in cultures with higher N concentrations. During the same period, between 30 and 78 h, the biomass production (total CV production) and the DMSP production were both dependent on nitrogen concentrations (R^2= 0.99 and R^2=0.93 respectively, Figure 7.3).

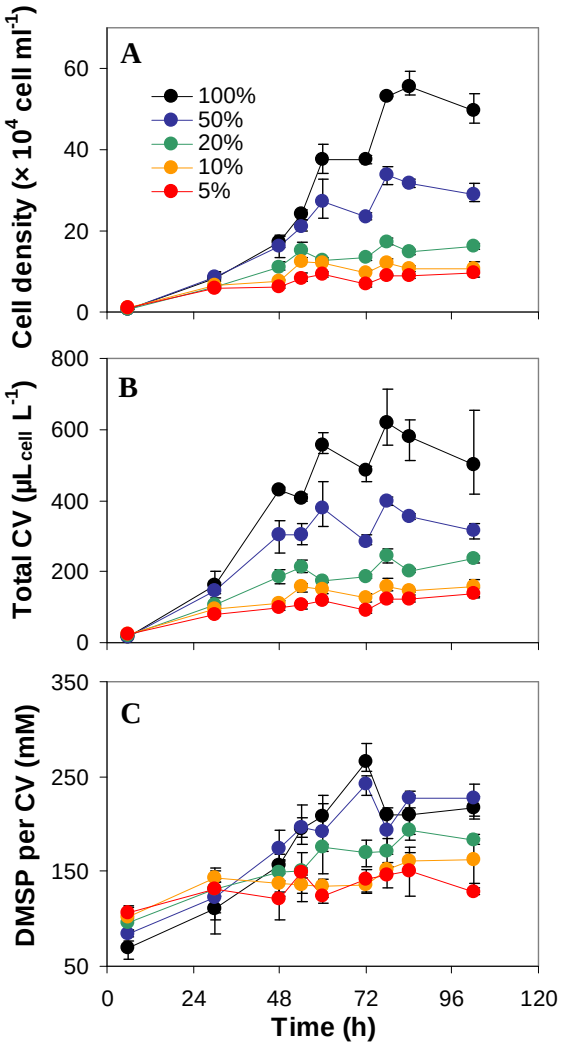

Figure 7.2 Effect of nitrogen availability on growth and DMSP parameters in batch cultures of *Crypthecodinium cohnii*. (A) Cell density. (B) Total cell volume (CV). (C) DMSP per CV. Each colour shows a different nitrogen treatment. Shown are average values with range of data (n=3). When no range bars are visible, the range of data was smaller than the symbol size.

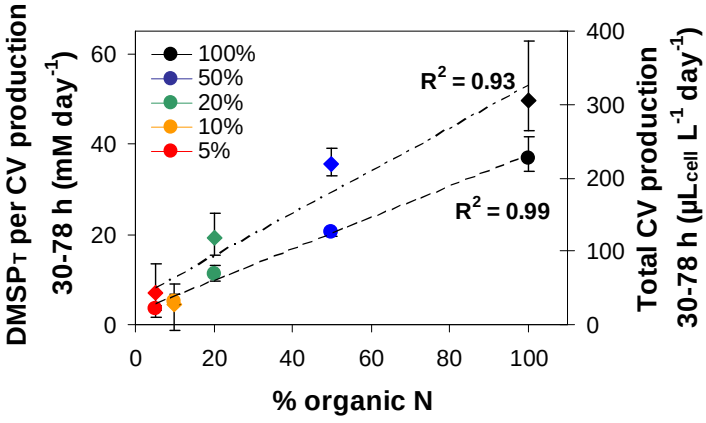

Figure 7.3 Regression of DMSP production and total cell volume (CV) production from 30 to 78 h of growth over nitrogen concentration (expressed as % of concentration in original recipe). Each colour visualises a different nitrogen treatment as shown in the legend. Each data point shows the average of 3 culture replicates. When no range bars are visible, the range of data was smaller than the symbol size.

To confirm the effect of nitrogen limitation on growth and DMSP content I tested the effect of nitrogen supplementation. The N addition was performed after 54 h of culture in nitrogen-deprived medium (10 %).

Figure 7.4 shows that growth parameters and DMSP concentration increased to reach similar maximum levels as the control culture with 100 % nitrogen. Nitrogen was thus, limiting the growth and DMSP production.

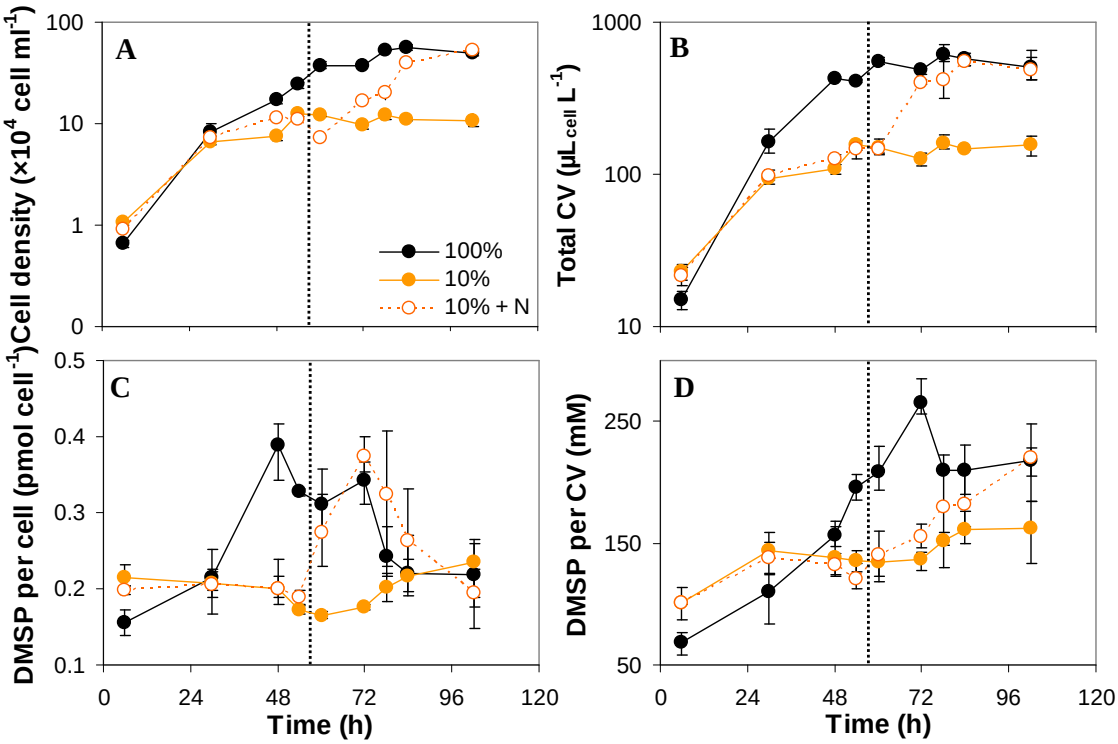

Figure 7.4 Effect of nitrogen addition (indicated by vertical dotted lines) in nitrogen limited medium on growth (A and B) and DMSP content (C and D). Comparison of cultures grown in normal recipe medium (100 % N, black line), N-limited medium (10 %, orange dashed line), N-limited medium (10 % N + addition of 100 % N, orange dotted line). Shown are average values with range of data (n=3). When no range bars are visible, the range of data was smaller than the symbol size.

7.4. Discussion

7.4.1. Effect of light on growth and DMSP content

Heterotrophic protists do not require light for growth, however, light interferes with several physiological functions such as growth, feeding and digestion. For instance, light cue determines the circadian cycle of cell divisions and feeding rates of heterotrophic protists (Jakobsen and Strom, 2004). Additionally, the growth rate has been observed to be higher in ciliates at 0.36 day^{-1} under 100 µmol photons m^{-2} s^{-1} of fluorescent light compared to almost null growth in the dark (Strom, 2001). On the other hand, the growth rate is 10-fold lower in the dinoflagellate *Crypthecodinium cohnii* under more than 3 W m^{-2} of fluorescent light compared to cells grown in the dark (Tuttle and Loeblich, 1975).

Crypthecodinium cohnii is a heterotrophic dinoflagellate that acquires organic matter by osmotrophy or, more rarely observed, by predation on unicellular red algae (Ucko et al., 1997). This species probably contains a remnant plastid (Sanchez-Puerta et al., 2007) but is incapable of photosynthesis. Nevertheless, this heterotroph contains 3 carotenoids (Tuttle and Loeblich, 1973) that protect cells against damage from sunlight (Withers and Tuttle, 1979). The abundance of these carotenoids is 4-fold lower in cells that are being kept in the dark compared to cells grown under 73 µmol photons m^{-2} s^{-1} of fluorescent light (calculated equivalent to 500 foot candles, reported by Tuttle and Loeblich 1973) and the higher growth rate observed in darkness could result from the resources saved by decreasing carotenoid synthesis (Tuttle and Loeblich, 1975). In our experiments, growth and DMSP content of *C. cohnii* were also significantly affected by light exposure. Similar to previous observations of lower growth rate under light exposure, I observed lower cell density and total cell volume in cultures exposed to light at 100 µmol photons m^{-2} s^{-1}.

Carotenoids are vital for *C. cohnii* cells exposed to light and carotenoid-deficient mutants show higher mortality rate under light exposure (Withers and Tuttle, 1979).

One function of carotenoids in photosynthetic organisms is the photo-protection of reaction centre complexes against photo-oxidative processes. With polyene chains of more than 9 double bonds, carotenoids quench the chlorophyll triplet state to avoid the generation of oxygen singlet or carotenoids may also directly quench oxygen singlet and other radical species. This quenching process occurs via formation of carotenoid triplet stage lower in energy than triplet chlorophyll or oxygen singlet and is followed by excessive energy dissipation as heat towards the return of carotenoid to its normal state (Vershinin, 1999; Frank and Brudvig, 2004). The photo-protective mechanism of carotenoids in a non-photosynthetic organism such as *C. cohnii* is unknown but carotenoids also have non-photosynthetic functions (Krinsky, 1978). They potentially act as antioxidant defence via scavenging and elimination of free radicals as described for some other non-photosynthetic organisms (Mathews, 1966; Krinsky, 1989; Vershinin and Lukyanova, 1993; Vershinin, 1999) and are likely to protect cells against reactive oxygen species (ROS) generation (H_2O_2, singlet O_2, radical $^{\cdot}OH$ and peroxides). Aerobic organisms are subject to ROS generation usually due to the intracellular metabolism in mitochondria, peroxisomes and enzyme systems and under particular exposure to UV, chemicals or toxins (Finkel and Holbrook, 2000).Given that DMSP and its by-products have shown capacities in scavenging ROS, hence similar function of cell protection against ROS (Sunda et al., 2002), the DMSP concentration may also be affected by light exposure analogous to carotenoid content. However, my experiments showed no difference in DMSP per CV in the presence or absence of light. On the other hand, *C. cohnii* possesses other active anti-oxidant mechanisms including iron-containing superoxide dismutases, enzymes that are able to degrade harmful superoxide radicals ($O_2^{\cdot -}$); (Dufernez et al., 2008). Therefore, carotenoids and superoxide dismutases appear to protect *C. cohnii* cells against photo-oxidation efficiently enough so that the additional antioxidant properties of DMSP may not be required. Nonetheless, this result does not exclude the potential use of DMSP in *C. cohnii* cells in other conditions of oxidative stress such as exposure to solar ultraviolet radiations or elevated concentrations of heavy metals (Okamoto et al., 2001) or under nutrient limitation (Bucciarelli and Sunda, 2003).

7.4.2. Effect of nitrogen availability on growth and DMSP content

The nutrient experiment of chapter 6 (section 6.3.3) suggested that N-limitation restrained the DMSP production in *C. cohnii* and I decided to investigate N-limitation by removing the N source of the medium in more detail. However, N-containing compounds (peptone, bacto-tryptone and yeast extract) are complex substrates that may also supply carbon, sulphur, phosphorus, trace metals, vitamins and other potential compounds in lesser proportions than nitrogen. Organic carbon is mainly supplied by glucose, sodium acetate and sodium succinate and is not considered to be limited by reducing the concentrations of N-containing compounds. Hence, it is likely that limitation of N-containing compounds will mostly affect N-dependant metabolic processes.

During the late exponential and stationary growth phases, the N-limitation resulted in lower DMSP per cell volume (CV) and this effect was confirmed by an increase of DMSP content in response to N supplementation after a period of limitation. In agreement with the conclusions of Chapter 6, the glucose depletion in late exponential and stationary growth phases triggered an increase in DMSP concentration that is dependent on the available organic source of nitrogen. This may be explained by the obligate requirement for the DMSP precursor, methionine (Uchida et al., 1996) which may be obtained directly or could be produced from the organic nitrogen source in the medium. Similarly, Gröne and Kirst (1992) proposed that the rate of DMSP synthesis is dependent on methionine availability in the autotroph *Tetraselmis subcordiformis* that accumulates more DMSP in methionine-enriched medium.

During the exponential growth phase, glucose was not limiting and the nitrogen limitation appeared to increase the DMSP per CV. Similar increase in DMSP concentrations during N-limitation have been reported. Turner et al. (1988) observed higher DMSP concentrations in field measurements of mixed phytoplankton population from areas of lower nitrate concentrations and in laboratory measurements of the coccolithophore *Emiliania huxleyi* cultured in low nitrate medium. Gröne and Kirst (1992) found higher DMSP content in *T. subcordiformis* cells cultured in N-deprived

medium. Bucciarelli and Sunda (2003) observed increased DMSP concentrations in N-limited cultures of *Thalassiosira pseudonana* and suggested that DMSP acts as a potential antioxidant in response to nutrient limitation. Similarly, the response of *C. cohnii* may also be a result of nutrient limitation that creates oxidative stress. On the other hand, the production of DMSP in conditions of N-deficiency may support the "overflow hypothesis" that describes the function of DMSP production and exudation to eliminate the excess sulphur (Stefels, 2000). Sulphur and nitrogen are both required for protein synthesis and the lack of nitrogen would result in sulphur excess. The production of DMSP, as observed, here, by the increase of DMSP concentration per CV during the exponential growth phase in conditions of N-limitation, may serve to catabolise excess sulphur and recycle amino groups to satisfy N-dependant metabolic processes.

Further experiments are necessary to confirm or eliminate these hypotheses: the "antioxidant hypothesis" could be tested with measurements of ROS production (for example H_2O_2, radical $^{\cdot}OH$, superoxide anion $O_2^{\cdot -}$ as described by Degli Esposti 2002) and checked if their concentration is increased by the N-limitation stress; the "overflow hypothesis" could be assessed by the measurement of the dissolved fraction of DMSP ($DMSP_d$) to check if the DMSP exudation is increased by the N-deprivation. However, if DMSP is re-metabolised instead of excreted no observation of $DMSP_d$ would be possible and in that case, the N:S ratio measurements of the cells could determine whether the elemental state of the cell is unbalanced.

As previously discussed, the N-limitation treatments may have resulted in decreased concentrations of other nutrients. However, in conditions of nitrogen limitation during the exponential growth phase, the increase of DMSP concentrations showed that sulphur was not limiting the DMSP production. Therefore, the decrease of nitrogen-containing compounds did not appear to generate a result in sulphur limitation. Moreover, the DMSP synthesis involves the anabolic machinery of enzymes that requires metals and vitamins. These elements did not appear to be limited by the lower levels of nitrogen-containing compounds. Finally, the use of defined organic media for the culture of *C. cohnii* such as media used by Tuttle and Loeblich (1975) would allow experimenting with more precision the limitation of nutrients by removing exactly one type of nutrient only.

7.5. Conclusion

The heterotrophic dinoflagellate *Crypthecodinium cohnii* is an obligate heterotroph that is able to grow in complete darkness, however, light interferes with growth. *C. cohnii* growth is reduced by light exposure but the DMSP per CV does not appear to be affected by the presence or absence of light. Therefore under similar conditions of light exposure where Tuttle and Loeblich (1973) observed that carotenoids were produced for photo-protection (fluorescent light of 100 and 73 µmol photons m^{-2} s^{-1} here and in Tuttle and Loeblich 1973 respectively), DMSP seemed ineffective as an antioxidant.

Growth and DMSP content of *C. cohnii* is, however, affected by the availability of organic nutrients in the medium. As previously observed, nitrogen was required for DMSP synthesis during late exponential and stationary growth phases where glucose depletion enhanced the increase of DMSP content. I confirmed here that DMSP concentrations were significantly dependent on external organic nitrogen concentrations.

Nitrogen limitation has a different effect during the exponential phase when glucose is not a limiting factor. In such conditions, nitrogen limitation enhanced the increase of DMSP content. The role of DMSP during the exponential growth phase of *C. cohnii* needs more investigation to determine whether it is a response to oxidative stress due to nutrient limitation or whether it is part of an overflow mechanism.

These results point out the multifunctional properties of DMSP within the same species. In the previous chapter, the role of DMSP as an osmolyte was proposed and demonstrated by some of the experiments. With the new results presented here, other potential roles of DMSP as an antioxidant or overflow metabolite were suggested in *C. cohnii* cells. Moreover, a strong relationship between the nitrogen availability and the DMSP content was observed and the novel data represent the first steps in elucidating the role of DMSP in heterotrophic organisms.

Chapter 8 Synthesis: progress and perspectives

In this section, I describe the major issues investigated throughout this thesis. The main results are summarised and discussed in relation with the progress achieved and from the perspective of further research that could be developed.

8.1. DMSP and DMS measurement in dinoflagellate cultures

Regarding performing DMSP and DMS measurements on dinoflagellate culture samples, time was invested in tests to optimise the sampling and handling strategy before setting up experiments. The analytical uncertainty of the methods was assessed and the potential sources of variation in DMSP and DMS concentrations were investigated.

For particulate DMSP ($DMSP_p$) measurement, I examined the variability in $DMSP_p$ concentration over the day and over the growth. Diel cycle in DMSP concentrations have been observed in other phytoplankton species such as in batch cultures of the coccolithophore *Emiliania huxleyi* and suggested for the dinoflagellates *Alexandrium tamarense* and *Scrippsiella trochoidea* in their natural environment (Merzouk et al., 2004; Bucciarelli et al., 2007). Here, I showed for the first time that DMSP concentration per cell volume increased over the photoperiod in batch cultures of the photosynthetic dinoflagellate *Heterocapsa triquetra* (Chapter 3). During growth in batch cultures, the physiological conditions of the phytoplankton cells generally change over time in response to nutrient depletion and cell ageing. I found that DMSP concentrations increased at the end of the exponential growth phase (Chapter 3). This variability is critical for deciding upon culture sampling time during an experiment. To exclude diel variability in DMSP concentration, cultures were sampled at the same time

(mid-time) within the photoperiod each day. In addition sampling was done at the same point of the growth phase, usually mid-exponential phase to avoid the effect of nutrient limitation.

A comparison of values obtained from measurement of total DMSP ($DMSP_T$) with the sum of all its measured individual fractions, i.e. $DMSP_p$, $DMSP_d$ and DMS, suggested a missing fraction of 25 % in the additive value. No study has ever reported the measurements of all fractions in culture or field samples that would fully validate the different methods used for these 4 parameters. The investigation of the major fraction $DMSP_p$ (72 %) using 3 different filtration methods (hand vacuum pump, automated syringe pump and filtration by gravity) only showed an increase in $DMSP_p$ concentration in one experiment where it was higher using the automated syringe pump rather than using the hand vacuum pump (Chapter 3). When using the syringe pump, I observed that increasing the delivery speed resulted in increasing DMSP concentration and reducing the analytical uncertainty. Nevertheless, the analytical uncertainty was in a similar range when using the syringe pump and the hand vacuum pump (Chapter 3).

The filtration step is critical for $DMSP_p$ measurement and as Kiene and Slezak (2006) pointed out it can move DMSP from the particulate to the dissolved fraction. In dinoflagellate cultures especially, filtration may create even more damage as many species are known to be very fragile to turbulence. The filtration of a dinoflagellate sample increases the analytical uncertainty (relative standard deviation or RSD) for $DMSP_p$ measurement: within an experimentt detailed in chapter 3, I obtained 1.1 - 16.4 % RSD for $DMSP_p$ and 0.6 - 5.2 % RSD for $DMSP_T$ measurement without filtration. The measurement of $DMSP_T$ avoids filtering culture samples and it gives data with less analytical uncertainty so it was favoured in this study (chapter 3). A similar technique was also applied by Keller et al (1989a) to determine DMSP concentrations of 123 phytoplankton species and by Yost and Mitchelmore (2009) who measured the DMSP content of the dinoflagellate *Symbiodinium spp*. Therefore, $DMSP_T$ is a rather common representation of DMSP content in phytoplankton. Nonetheless, using the method I described in detail in Chapter 3 and 4, I obtained the best analytical precision (6 - 32 % of, RSD, for culture samples n=9 on average in 9 species) given that Yost and Mitchelmore obtained RSD between 34 and 76 % (n=3 - 4). By eliminating the filtration step, this method also allowed fast measurements (including sampling and sample

preparation) in quick succession that would not have been possible with $DMSP_p$ measurement (time response of *C. cohnii* DMSP adjustment to glucose limitation, Chapter 6 section 6.4.3).

DMS measurements were performed on culture samples filtered with the syringe pump at the highest delivery speed available (14 ml min^{-1}) in agreement with findings on $DMSP_p$ measurements. The filtrate was introduced into the purge tube with a sample loop device to ensure injection of constant volume. This entire system described in Chapter 3 was aimed at ensuring satisfactory replication of DMS measurements in dinoflagellate cultures (Chapter 6, section 6.3.1). This system led to a low analytical uncertainty for standards (2 %) but larger for culture samples (25-33 %). I observed that agitation of culture can affect the DMS measurement by either increasing or decreasing its value. I also found that DMS concentrations were increased by increased time of filtration and large sample volume. According to these results I performed filtration of small volume samples (5 ml) using the syringe pump set at the maximum delivery speed of 14 ml min^{-1}.

$DMSP_d$ measurement includes the same steps for sample preparation as DMS measurement plus the addition of NaOH to the filtrate sample. The variations in DMS concentration that may result from the handling and the filtration of the culture are likely to be similar for $DMSP_d$ measurement. Some investigations should be done to optimise $DMSP_d$ measurements in dinoflagellate cultures. I have observed large variation on preliminary $DMSP_d$ measurements (Chapter 3) and the analytical uncertainty could be tested and improved using the syringe pump at high delivery speed according to the results obtained for DMS and $DMSP_p$. Some tests could also be done on agitated and non-agitated flasks to determine if the handling of the flask would have any effect on $DMSP_d$ measurement.

8.2. The contribution of the dinoflagellate group to the DMSP and DMS oceanic pool

Several laboratory and field studies have pointed out the occurrence of DMSP in dinoflagellate cells. The commonly referred studies done by Keller et al., (Keller, 1988/1989; 1989a; 1989b) reported the DMSP content of 25 dinoflagellate strains amongst 123 phytoplankton species and strains. They concluded that dinoflagellates are a significant DMSP-producing group.

Twenty years later, Stefels et al. (2007) recommended that the dinoflagellate group should be inserted as DMS producers in ocean climate models. However, so far only 1 model has included this group, and in this model dinoflagellates are mixed in with other nanophytoplankton (Vogt et al., 2010). Le Quéré et al (2005), did incorporate the dinoflagellate group when describing a 10 Plankton Functional Types (PFT) representative ecosystem for the use of modellers. The photosynthetic dinoflagellates were placed in the "mixed phytoplankton group" and, even though described as non efficient DMS producers, their DMSP production parameterisation was equivalent to that of the PFT "DMS producers" (12 mmol mol^{-1} C). By directly measuring DMSP and carbon content in 8 photosynthetic dinoflagellates, I obtained a DMSP content value very close to this (10.4 mmol mol^{-1} C, Chapter 4). The heterotrophic dinoflagellates were placed in the "proto-zooplankton" PFT with no parameterised DMSP production. It is now clear that several heterotrophic species synthesise DMSP (listed in Chapter 5) but currently the extent of this ability amongst the large diversity of the heterotrophic dinoflagellates is unknown. The limited amount of data available so far and the large diversity of the dinoflagellates including both photosynthetic and heterotrophic species makes it difficult for scientists to accurately describe, incorporate or parameterise the DMSP and DMS contribution of this group.

In this thesis, I provided more data on DMSP content and DMSP lyase activities (DLA) for dinoflagellates and gathered the existing data to summarise the available knowledge with the aim of giving a better understanding and description of the DMSP and DMS contribution of dinoflagellates. I produced DMSP measurements of 9 species from my laboratory work and gathered together a total of 61 concentration values for

DMSP per cell volume from the literature (Chapters 4 and 5). By analysing this dataset I observed that dinoflagellates contain DMSP in highly variable concentrations that spread over 6 orders of magnitude difference (per cell volume) between species. Furthermore, the dataset includes the broadest span of values and the highest values reported to date in any phytoplankton group. Moreover, I concluded that several factors appeared to affect the DMSP content of dinoflagellates: species with a haptophyte-like plastid and bioluminescent species (Pyrocystales order from the Longhurst oceanic province CAMR in the East Pacific) contained significantly lower concentration of DMSP whereas species from the Mediterranean province had higher concentration of DMSP. Additional measurements of species belonging to these subgroups would help to reinforce or refute these conclusions which are based on the 61 values available per cell volume. From this database I also noticed that species from the southern hemisphere and the poles are underrepresented and need more investigation.

The causes underlying the variability in DMSP content of dinoflagellates are still unclear and might be related to the multifunctional role of this sulphur compound. I also noted that the osmolyte role of DMSP is unlikely in species that produce very low DMSP concentrations (Chapter 5).

The DMSP content of dinoflagellates makes them a potential source of DMS. Some dinoflagellates are able to synthesise DMSP lyase that may cleave DMSP to DMS and acrylate. I detected *in vitro* DMSP lyase activity in 5 dinoflagellate species from the 9 species tested in this study (Chapter 4). Given that very few DLA data have been published for dinoflagellates, this increases the DLA dataset to data for 23 strains and 17 species (Chapter 5). More effort in increasing the number of species assessed for DLA would be worthwhile as it is now clear that some dinoflagellates can produce substantial amounts of DMSP and DMSP lyase. The release of DMS from DMSP occurs in seawater through the action of algal and bacterial enzymes (Steinke et al., 1996; Yoch, 2002; Todd et al., 2009). The fraction due to algal enzyme activities and the overall contribution from dinoflagellates may be substantial in areas where dinoflagellates form blooms (Steinke et al., 2002a). Sampling and measuring DLA in several dinoflagellate blooms from diverse areas and environments, as for instance in blooms of Mediterranean species which have high DMSP concentrations or in recurring dense blooms of *Karenia brevis* in the Gulf of Mexico, would further assess the

potential impact of dinoflagellate DLA on DMS release in the ecosystem. Also, the bloom period of maximum DLA per cell or per cell volume could be investigated by performing successive DLA measurements over the initiation, development and termination of a dinoflagellate bloom. The contribution of the dinoflagellate group in comparison with other phytoplankton groups able to produce DMSP lyases could be assessed by performing DLA measurements over phytoplankton successions.

The classification of DMSP-rich species I listed for some dinoflagellate species in Chapter 5 and especially bloom-forming species would help to identify potential areas of elevated DMSP concentrations and to estimate the amount of associated DMS production. Additional measurements of DMSP:Chl *a* ratio in the lab (Chapter 4) and in the field open doors towards future estimation of oceanic DMSP concentrations from Chl *a* satellite data as it is now possible to describe the dominant assemblages of phytoplankton taxa by remote sensing using a range of accessory pigments (Alvain et al., 2008). This tool could be applied to estimate global oceanic DMSP concentrations given that DMSP measurements are limited to the tracks of scientific cruises. Nevertheless, an estimation of DMSP:Chl *a* concentration for the dinoflagellate group could hardly be realistic due to the broad variation in DMSP content across dinoflagellate species.

Furthermore, dinoflagellate populations seem to be affected by the changing climate. Changes in abundance due to increased sea surface temperature have been observed (Edwards et al., 2006) but consequences of ocean acidification for this group are not known yet. Future research on the effect of ocean warming and acidification on dinoflagellate populations and their DMSP content could be critical for predicting the future DMS emissions and climate interactions and scenarios. Dinoflagellate blooms are often monitored for toxicity by many countries and some of this monitoring could be associated with sampling and analysis for DMS and DMSP to substantially increase the available field database and prospect for DMSP-rich species and oceanic areas.

8.3. The biological role of DMSP and DLA in heterotrophic dinoflagellates

Some heterotrophic dinoflagellates are able to synthesise DMSP and species such as *Crypthecodinium cohnii* contain DMSP in rather high concentrations (61 – 255 mM). Heterotrophic dinoflagellates are unique amongst free-living heterotrophs in this respect. This ability might originate from a common photosynthetic ancestor of the dinoflagellates (McFadden, 2001) and may provide some support to the evolutionary theory of a photosynthetic ancestor for the dinoflagellate group. Some heterotrophic dinoflagellates such as *C. cohnii* and *Oxyrrhis marina* are thought to harbour a vestigial plastid because they synthesise plastid-targeted genes and peptides (Sanchez-Puerta et al., 2007; Slamovits and Keeling, 2008). However, whilst *C. cohnii* contains DMSP, none has been found in *O. marina*. Indeed this species has been proved useful for studies on the incorporation of DMSP by grazing DMSP-producing prey and by osmotrophy because it does not contain DMSP (Salo et al., 2009).

The open question is: why have some heterotrophic dinoflagellates conserved the ability to produce DMSP if, as far as I am aware, no other heterotrophs synthesise it? I investigated the role of DMSP in *C. cohnii* and found in common with some photosynthetic organisms that DMSP acts as an osmolyte (Chapter 6). Heterotrophic marine organisms are subject to essentially the same osmotic variations experienced by photosynthetic marine organisms and require the same sort of adaptive mechanisms including osmolyte synthesis. In other microzooplankton including ciliates, osmoregulation may be performed by variation of amino acid composition and abundance (Kaneshiro et al., 1969).

The DMSP concentration in *C. cohnii* cells is also dependent on nutrient availability. Nutrient limitation appeared to enhance DMSP accumulation (Chapter 6 and 7). In the case of glucose limitation, DMSP is probably accumulated in replacement of glucose-derived osmolytes. The determination of the polysaccharide composition in *C. cohnii* cells and its variation in glucose-deprived medium could confirm this hypothesis. Additionally, the analysis of other potential osmolytes such as amino acids and their derivatives generally including proline and glycine betaine (Welsh, 2000) could be conducted in *C. cohnii* cells. In case of nitrogen limitation, DMSP is accumulated potentially as an overflow mechanism or possibly as an antioxidant in

response to nutrient-limitation stress. However, in conditions of D-glucose limitation compensated with L-glucose, the absence of DMSP increase suggested that if L-glucose was incorporated but non-assimilated by the cells, the nutrient-limitation stress would not cause any DMSP increase. To clearly eliminate the hypothesis of nutrient-limitation stress triggering a DMSP increase, the incorporation of L-glucose by *C. cohnii* cells has to be tested by performing experiments with *C. cohnii* cells grown in medium composed of radiolabelled L-Glucose as the unique source of glucose. The antioxidant hypothesis could be tested by measuring the reactive oxygen species in *C. cohnii* cultures in N-limited and N-replete conditions which could indicate whether there is an oxidative stress due to nutrient-limitation. The overflow mechanism hypothesis could be assessed by determining if there is any exudation of DMSP in N-limited conditions and measuring the potential fraction of extracellular DMSP concentration in comparison with N replete conditions.

The production of DMSP lyase has been observed and quantified in *C. cohnii* and it is not yet known whether other heterotrophic dinoflagellates are also able to produce DMSP lyase and, thereby represent a potential source of DMS. DLA measurements in other heterotrophic dinoflagellates should be done in future research. The presence of a DMS-producing enzyme in heterotrophic dinoflagellates might suggest that these organisms could use DMS as a grazer deterrent as has been suggested for phytoplankton (Wolfe and Steinke, 1996; Wolfe et al., 1997). Grazing experiments with *C. cohnii* and other heterotrophic dinoflagellates or other protists could be done to determine whether this function exists for DMS in these organisms.

C. cohnii is also used in the biotechnology research area for the production of ω-3-fatty acids (Mendes et al., 2009). I have observed exceptionally fast responses to external stimuli such as the DMSP synthesis in response to transfer of culture in glucose-deprived medium and which occurred within a few seconds (Chapter 6). Such response could be dependent on gene expressions and regulations as it is reported for yeasts which can change the expression of 40 % of their genes within minutes in response to nutrient changing conditions. If a similar molecular mechanism could happen in less than a minute it could lead towards several applications: it could be utilised by genetic modification toward the production of any key products in the same way that yeasts are used as laboratory factories for drug production (Szczebara et al.,

2003) or the responsible genes could be introduced in plasmids of bacteria to accelerate any metabolite production. The identification of such genes and mechanisms would also shed light on the DMSP metabolism in heterotrophic dinoflagellates.

8.4. Further issue- DMSP synthesis and its subcellular localisation

A major issue that remains for the DMSP production by phytoplankton research field is the DMSP pathway and its subcellular location. *Wollastonia biflora* seems to produce DMSP in the chloroplast (Trossat et al., 1996) and the same might happen for phytoplankton but no evidence has been reported yet. Before starting this study, I had hoped that investigating the DMSP content of dinoflagellate species that harbour different plastid types would give an indication on the role of the plastid in DMSP synthesis. However, more variation in DMSP content was observed between species holding the same plastid type than between species with different plastid types. This result was strongly influenced by the fact that species holding peridinin plastids and heterotrophic dinoflagellates are by far the most common types of species in the ocean today. Rather few non-peridinin plastid species are currently available in culture and the species harbouring haptophyte-like plastids showed lower DMSP content than species with a peridinin plastid. This in itself was surprising given that haptophytes in general have substantial DMSP content. It seems that the conservation or loss of genes may be more likely to affect the DMSP content rather than the plastid type.

Several questions remain: Is DMSP produced in the plastids of dinoflagellates and other microalgae? Is it possible that heterotrophic dinoflagellates synthesise DMSP in a remnant plastid or is DMSP produced in a different subcellular location? The purification of plastids in dinoflagellates appears to be a very delicate and difficult process due to the fact that plastids are fragile and are never isolated completely intact as shown by the loss of proteins (Wang et al., 2005). However, the separation of cytoplasmic and plastidic fractions could be a satisfactory preliminary approach towards determining the major subcellular distribution of DMSP.

The DMSP pathway in microalgae is assumed to be similar to the one described for the green macroalgae *Enteromorpha intestinalis* (Gage et al., 1997) after detection of the same key intermediates in a range of microalgal cells. However, this pathway has not been fully verified and there is scope for further investigation. The identification of enzymes involved in the DMSP pathway (such as the enzyme that oxidizes the DMSP precursor, DMSP-aldehyde found by Trossat et al., 1996) could be used for identifying the subcellular localisation of DMSP biosynthesis according to the presence of an N-terminal targeting signal or by using an immunolabelling technique such as that used to locate proteins and enzymes in Snapdragon flowers (Kolosova et al., 2001).

The future identification of the genes involved in DMSP and DMSP lyase synthesis would represent a landmark for the DMSP research area. If they were conserved, such genes could be used to rapidly screen a high number of phytoplankton species and strains. This would lead to the identification of potential DMSP and DMS producers in cultures and in the field similar to the detection of toxin production genes that are used for monitoring harmful algal blooms. Molecular methods could allow detection of the presence of such genes with just a few cells using the PCR-amplification. The detection of DMSP-producing genes would thus be possible in species that are difficult to grow in culture and in field samples of low cell density. Moreover, the generation of mutants deficient in DMSP-production genes could be used in comparative eco-physiological experiments with normal clones in order to determine the role and benefit of DMSP. However, recent work has shown that the bacterial genes involved in DMSP lyase synthesis appear to be rather diverse with 3 genes encoding for 3 different enzymes that catalyse diverse reactions of DMS production from DMSP (Todd et al., 2007; Johnston et al., 2008; Todd et al., 2009). If the same proves true for DMSP production and DMSP lyase synthesis genes in phytoplankton this would limit their application as suggested above. Nevertheless, the investigation on genes involved in DMSP and DMSP lyase synthesis in dinoflagellate and other phytoplankton cells would be of great interest.

Appendix I List of species

All the species mentioned in the thesis are listed here with their author names and date when available. Species are divided into Class and listed by alphabetic order.

Class Bacillariophyceae

Cylindrotheca closterium (Ehrenberg) Reimann & Lewin 1964

Class of Dinophyceae

Akashiwo sanguinea (Hirasaka) Hansen & Moestrup 2000
Alexandrium affine (Inoue & Fukuyo) Balech 1985
Alexandrium catenella (Whedon & Kofoid) Balech 1985
Alexandrium minutum Halim 1960
Alexandrium monilatum (Howell) Balech 1985
Alexandrium ostenfeldii (Paulsen) Balech & Tangen 1985
Alexandrium tamarense (Lebour) Balech 1995
Amphidinium carterae Hulburt 1957
Amphidinium operculatum Claparède & Lachmann 1859
Azadinium spinosum Elbrächter & Tillmann 2009
Ceratium arcticum (Ehrenberg) Cleve 1901
Ceratium fusus (Ehrenberg) Dujardin 1841
Ceratium longipes (Bailey) Gran 1902
Ceratium tripos (Müller) Nitzsch 1817
Ceratocorys horrida Stein 1883
Crypthecodinium cohnii Seligo 1952
Dinoclonium sp Pascher 1927
Dinophysis acuminata Claparède & Lachmann 1859
Dinophysis acuta Ehrenberg 1839
Dinophysis caudata Saville-Kent 1881

Dinophysis norvegica Claparède & Lachmann 1859

Dinothrix sp Pascher

Dissodinium lunula (Schütt) Pascher

Durinskia baltica (Levander) Carty & Cox

Durinskia capensis Pienaar, Sapiai & Horiguchi 2007

Galeidinium rugatum Tamura & Horiguchi 2005

Gambierdiscus toxicus Adachi & Fukuyo 1979

Gonyaulax spinifera (Claparède & Lachmann) Diesing 1866

Gymnodinium acidotum Nygaard 1949

Gymnodinium chlorophorum Elbrächter & Schnepf 1996

Gymodinium fuscum (Ehrenberg) Stein 1878

Gymodinium nelsoni Martin 1929

Gyrodinium aureolum Hulburt 1957

Gyrodinium impudicum Fraga & Bravo 1995

Heterocapsa pygmaea Lobelich, Schmidt & Sherley 1981

Heterocapsa triquetra (Ehrenberg) Stein 1883

Karenia brevis (Davis) Hansen & Moestrup 2000

Karenia mikimotoi (Miyake & Kominami ex Oda) Hansen & Moestrup 2000

Karenia umbella de Salas, Bolch & Hallegraeff 2004

Karlodinium armiger Bergholtz, Daugbjerg & Moestrup 2006

Karlodinium australe de Salas, Bolch & Hallegraeff 2005

Karlodinium veneficum (Ballantine) Larsen 2000

Lepidodinium viride Watanabe, Suda, Inouye, Sawaguchi & Chihara 1990

Lingulodinium polyedrum (Stein) Dodge 1989

Noctiluca sp Suriray 1836

Ostreopsis sp Schmidt 1901

Oxyrrhis marina Dujardin 1841

Peridiniopsis berolinense (Lemmermann) Bourelly

Peridinium bipes Stein 1883

Peridinium gatunense Nygaard 1925

Peridinium quinquecorne Abé 1927

Peridiniella catenata (Levander) Balech 1977

Perkinsus atlanticus Azevedo 1989

Pfiesteria piscicida Steidinger & Burkholder 1996

Pfiesteria shumwayae Glasgow & Burkholder 2001

Prorocentrum arcuatum Issel 1928

Prorocentrum belizeanum Faust 1993

Prorocentrum lima (Ehrenberg) Dodge 1975

Prorocentrum micans Ehrenberg 1833

Prorocentrum sp. Ehrenberg 1834

Protoceratium reticulatum (Claparède & Lachmann) Butschli 1885

Pyrocystis noctiluca Murray ex Haeckel 1890

Pyrodinium sp Plate 1906

Scrippsiella trochoidea (Stein) Balech ex Loeblich 1965

Symbiodinium microadriaticum Freudenthal 1962

Symbiodinium pilosum Trench & Blank 2000

Symbiodinium sp Freudenthal 1962

Takayama helix de Salas, Bolch, Botes & Hallegraeff 2003

Takayama tasmanica Bolch & Hallegraeff 2003

Tovellia sanguinea Moestrup, Hansen, Daugbjerg, Flaim & D'andrea 2006

Class Prymnesiophyceae

Emiliania huxleyi (Lohmann) Hay & Mohler 1967

Phaeocystis sp Lagerheim 1893

Phaeocystis pouchetii (Hariot) Lagerheim 1896

Appendix II Bioluminescent and non-bioluminescent species

The dinoflagellate species presented in chapter 5 are listed in the table below with description of bioluminescence property and the source of this data. CCMP means that information about bioluminescence was available on the CCMP website (Wilson, 2009).

Species	Bioluminescence	Data source
Alexandrium minutum	No	No record as bioluminescent
Prorocentrum sp. IIB2B1	No	Unknown
Gyrodinium impudicum	No	No record as bioluminescent
Scrippsiella trochoidea NIES-369	No	No record as bioluminescent
Dinophysis acuminata	No	No record as bioluminescent
Heterocapsa pygmaea CCMP1322	No	CCMP, (Wilson, 2009)
Prorocentrum arcuatum	No	No record as bioluminescent
Amphidinium carterae CCMP1314	No	CCMP
Crypthecodinium cohnii CCMP316	No	CCMP
Heterocapsa triquetra CCMP449	No	CCMP
Scrippsiella trochoidea CCMP1331	No	CCMP
Symbiodinium microadriaticum CCMP827	No	CCMP
Amphidinium carterae CCMP1314	No	CCMP
Scrippsiella trochoidea CCMP1599	No	CCMP
Amphidinium operculatum CCAP1102/6	No	No record as bioluminescent
Heterocapsa triquetra NIES-7	No	No record as bioluminescent
Amphidinium carterae CCMP1314	No	CCMP
Alexandrium minutum CCMP113	No	CCMP
Amphidinium carterae	No	CCMP
Gymnodinium nelsoni	No	No record as bioluminescent
Alexandrium tamarense CCM P115	Yes	CCMP
Alexandrium tamarense CCMP116	No	CCMP
Alexandrium tamarense CCMP1771	Yes	CCMP
Thoracosphaera heimii L603	No	CCMP
Cachonina niei CACH	No	No record as bioluminescent
Prorocentrum micans CCMP 691	No	CCMP
Heterocapsa sp. CCMP450	No	CCMP
Alexandrium fundyense CCMP1719	Yes	CCMP

Scrippsiella trochoidea CCAP1134/1	No	No record as bioluminescent
Prorocentrum minimum CCMP1329	No	CCMP
Alexandrium fundyense CCMP1846	Yes	CCMP
Protoperidinium pellucidum	Potentially yes	Listed by Poupin et al., 1999
Gymnodinium sp. 94GYR	No	Unknown
Prorocentrum minimum CCMP1329	No	CCMP
Amphidinium carterae CCMP1314	No	CCMP
Crypthecodinium cohnii CCMP316	No	CCMP
Polarella glacialis CCMP1138	No	CCMP
Prorocentrum micans SB1	Potentially yes	Listed by Poupin et al., 1999
Amphidinum carterae CCAP1102/1	No	Never reported to be
Kryptoperidinium foliaceum CCMP1326	No	CCMP
Gonyaulax spinifera LY11363	No	Personal communication (Dr David Green, SAMS, UK)
Gymnodinium simplex CCMP419	No	CCMP
Ceratium furca	Potentially yes	Listed in Tett, 1971 and Poupin et al., 1999
Gymnodinium simplex CCAP1117/3	No	CCMP
Gymnodinium nelsoni GSBL	No	No record as bioluminescent
Lingulodinium polyedra LP2810	Yes	Personal communication (Joanne Field, CCAP, UK)
Alexandrium tamarense CCMP 115	Yes	CCMP
Karenia brevis CCMP2281	No	CCMP
Gonyaulax spinifera CCMP409	No	CCMP
Lingulodinium polyedra CCMP1738	Yes	CCMP
Karlodinium veneficum CCMP415	No	CCMP
Gambierdiscus toxicus GT2	No	No record as bioluminescent
Lingulodinium polyedrum CCAP1121/2	No	Personal communication (Maria Salta, NOC, UK)
Lingulodinium polyedrum CCMP405	Yes	CCMP
Pyrocystis lunula CCMP731	Yes	CCMP
Karenia mikimotoi CCMP430	No	CCMP
Karenia mikimotoi CCMP429	No	CCMP
Ceratium longipes 090201	No	No record as bioluminescent
Pyrocystis noctiluca CCMP732	Yes	CCMP
Pfiesteria shumwayae CCMP2089	No	CCMP
Pfiesteria piscida CCMP1830	No	CCMP
Symbiodinium spp	No	No record as bioluminescent
Peridinium gatunense	No	No record as bioluminescent

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