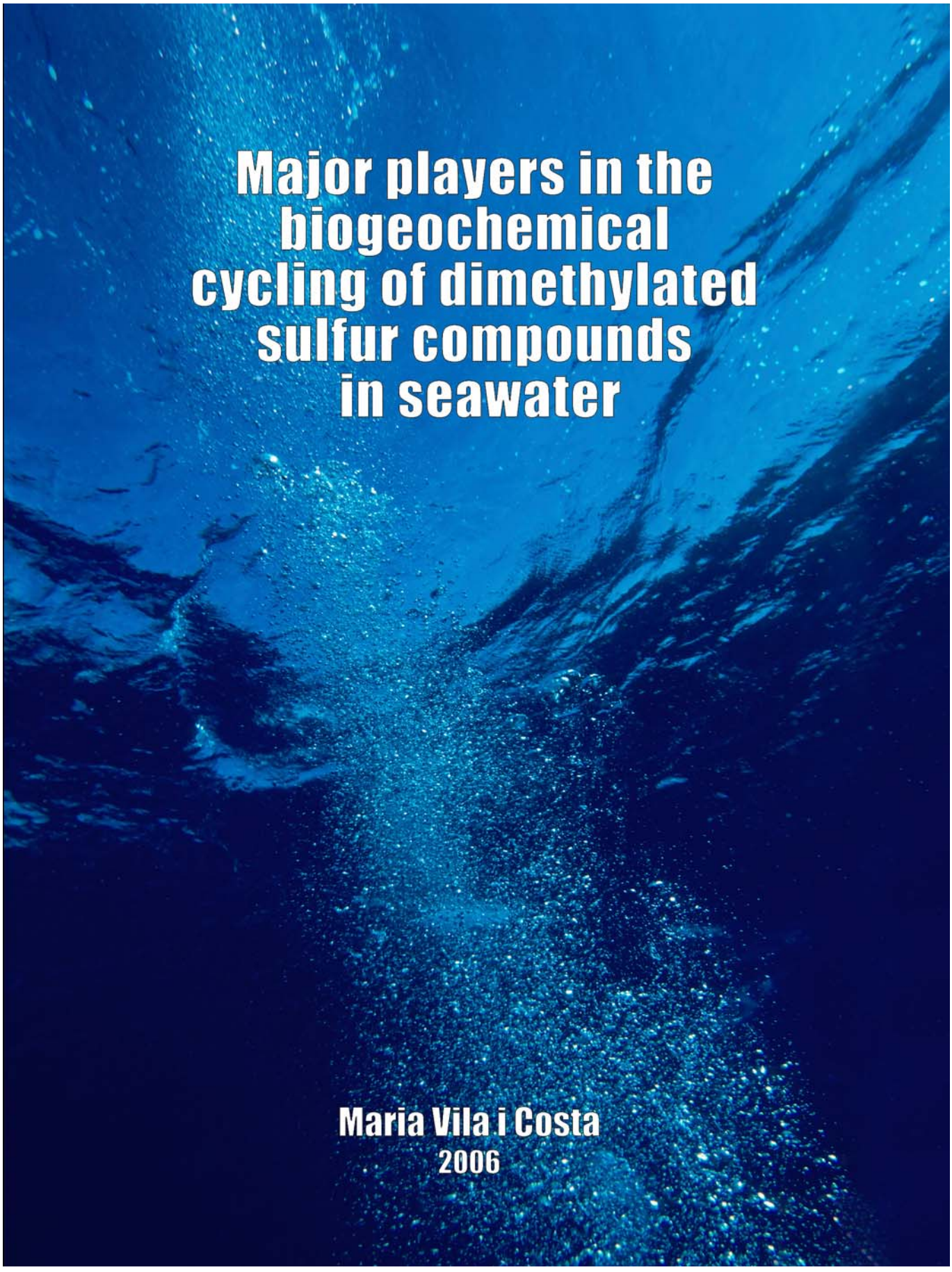
Major players in the biogeochemical cycling of dimethylated sulfur compounds in seawater
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Major players in the biogeochemical cycling of dimethylated sulfur compounds in seawater

Memòria presentada per optar al grau de Doctor en Ciències del Mar per la Universitat Politècnica de Catalunya per

Maria Vila i Costa

Vist-i-plau del director de la tesi

Dr. Rafel Simó
Investigador-CSIC

Scientific knowledge is a body of statements of varying degrees of certainty-some most unsure, some nearly sure, and none absolutely certain.

(Richard Feynman)

Aquesta tesi està dedicada a la meva gent,
que m'han acompanyat durant tot aquest temps.
I molt especialment, a l'Oriol, l'Andrea
i els meus pares, pilars imprescindibles.

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Resum

Des que Charlson et al. (1987) van publicar la seva controvertida hipòtesi que l'emissió de dimetilsulfur (DMS) per les algues podia contribuir a regular el clima, s'ha avançat notablement en el coneixement del cicle del sofre en els oceans. Malgrat això, queden encara aspectes importants sense resoldre; sobretot, saber quins factors controlen les concentracions d'aquest compost a l'aigua de mar. Les transformacions biogeoquímiques dels compostos dimetilats de sofre són el resultat de processos biològics (per exemple, la fisiologia i la diversitat dels organismes planctònics) i processos abiòtics (tals com la radiació solar, la velocitat del vent, la concentració de nutrients, entre d'altres). D'entre els processos biològics, hom creu que els bacteris tenen molta importància en el cicle, ja que la majoria de transformacions es donen a través de la fase dissolta. L'objectiu d'aquesta tesi és avaluar el paper que juguen els bacteris en el cicle del sofre a l'oceà superficial i identificar els factors principals que en determinen l'activitat. Mesures mensuals de la importància relativa dels principals processos d'aquest cicle durant 18 mesos en un lloc oligotròfic costaner (Badia de Blanes) han mostrat que la típica acumulació de DMS a l'estiu en les zones temperades i subtropicals (la que hom anomena "paradoxa d'estiu del DMS" perquè les màximes concentracions anuals de DMS es registren a l'estiu, 2-3 mesos després de les del seu precursor bioquímic, el DMSP) és causada per un increment de la producció biològica de DMS no compensada per les pèrdues de DMS (ventilació + fotòlisi + consum microbià). Els bacteris heteròtrofs explicaven només una part (52% de mitjana anual) de les transformacions totals de DMSP, fet que suggereix que les DMSP liases algals són una font de DMS més important del que es pensava. Per altra banda, la incorporació de DMSP pels bacteris va incrementar significativament durant l'estiu. Els principals responsables d'aquest consum eren membres dels grups Alphaproteobacteria (*Roseobacter* i SAR11) i Gammaproteobacteria, malgrat que la capacitat d'assimilar DMSP és un tret àmpliament distribuït entre els principals grups bacterians. Més que per la presència d'una comunitat bacteriana d'estiu especialment afina al DMSP, l'assimilació de DMSP semblava determinada per la contribució del DMSP a la disponibilitat total de sofre orgànic, expressada en la relació DMSP:Chla, que era màxima a l'estiu. A més, aquesta tesi ha permès descobrir una nova ruta metabòlica en el cicle marí del sofre: les algues no productores de DMSP (principalment les diatomees i els cianobacteris *Synechococcus* i *Prochlorococcus*) també assimilen una part del sofre del DMSP en la seva biomassa i, d'aquesta manera, eviten que sigui transformat a DMS. Finalment, per contrast amb l'alta labilitat i amplitud d'ús del DMSP, el DMS és usat com a font de carboni per un grup reduït de metilòtrofs especialitzats, que pertanyen principalment al grup *Methylophaga*. Quan hi ha disponibles altres fonts de carboni, el DMS s'utilitza principalment com a donador d'electrons i és convertit a DMSO.

Summary

Since Charlson et al. (1987) published the controversial hypothesis that dimethylsulfide (DMS) emission from phytoplankton could be involved in climate regulation, notable advances have improved our knowledge of the sulfur cycle in the oceans. Significant gaps still remain, however, mainly regarding the factors that control the concentrations of this compound in seawater. Biogeochemical transformations of dimethylated sulfur compounds result from biotic (e.g. physiology and diversity of plankton organisms) and abiotic (e.g. solar radiation, wind speed, nutrient concentrations) processes. Among biotic processes, it is believed that bacteria play a central role in the cycle since most transformations occur through the dissolved pool. This thesis has aimed to assess the role of bacterioplankton in the sulfur cycle of the ocean and to identify the main factors influencing their activity. Monthly measurements of the relative importance of the main processes of the cycle over a 18-month period in an oligotrophic coastal site (Blanes Bay) revealed that the typical summer DMS accumulation found in low temperate and subtropical regions (the called "DMS summer paradox" because maximum annual concentrations of DMS are recorded in summer, 2-3 months latter than their precursor DMSP) was due to an increase of biotic DMS production not matched by DMS losses (photolysis + ventilation + microbial consumption). Heterotrophic bacteria only accounted for a portion (annual average 52%) of total DMSP transformations, suggesting a major role of algal DMSP lyases as a source of DMS. Bacteria significantly increased DMSP incorporation in the summer period. The main contributors to DMSP assimilation were members of Alphaproteocbacteria (*Roseobacter* and SAR11) and Gammaproteobacteria, even though the capacity to assimilate DMSP is a widespread feature among the main broad groups of marine bacteria. Rather than having a bacterial population suited to use DMSP as a S source in summer, bacterial DMSP assimilation seemed to be determined by the contribution of DMSP to the total available organic S, expressed as DMSP:Chla ratio, that was highest in summer. A new player in the cycle has been discovered: low DMSP-producing phytoplankton (mainly diatoms and the cyanobacteria *Synechoccocus* and *Prochlorococcus*) can incorporate DMSP-sulfur into biomass and thus diverte a proportion of organic sulfur from being converted into volatile DMS. Finally, contrasting with the high lability of DMSP, DMS was consumed as a C source by a reduced group of specialist methylotrophs, mainly belonging to the *Methylophaga* group. When other carbon sources were available, DMS was mainly used as an electron donor and converted into DMSO.

General Introduction

Life on Earth modulates the flux of matter and energy that is exchanged among the land, atmosphere, ocean and sediments. Most of the exchanged materials flow on closed routes, called biogeochemical cycles. The study of these biogeochemical cycles involves the study of the interactions between living organisms and the physical processes of the planet, processes that convert the elements into many different chemical and physical states. Microorganisms have special relevance in biogeochemical cycling since they participate in most of the chemical transformations of the major elements constituents of living tissue (C, H, O, N, P and S).

The Global Sulfur Cycle

Sulfur is the 14th most abundant element in the Earth's crust where it is mainly found in igneous rocks (largely as pyrite (FeS_2) and gypsum ($CaSO_4.2H_2O$)). Sulfur naturally cycles through a lithosphere-ocean-biosphere-atmosphere system presenting different valence states ranging from +6 in SO_4^{2-} to -2 in sulfides. Figure 1 shows a simplified overview of the S cycle. Since the oxidation state +6 is the most stable under oxic conditions, SO_4^{2-} is the predominant form in oxic waters, soils and air. However, SO_4^{2-} is not easily exchangeable from one to other compartment (e.g. it is a very low volatile compound, difficult to be emitted to the atmosphere) and it is not metabolically active among all living organisms. In consequence, SO_4^{2-} has to be reduced to different S species in order to supply the sulfur requirements of all natural systems.

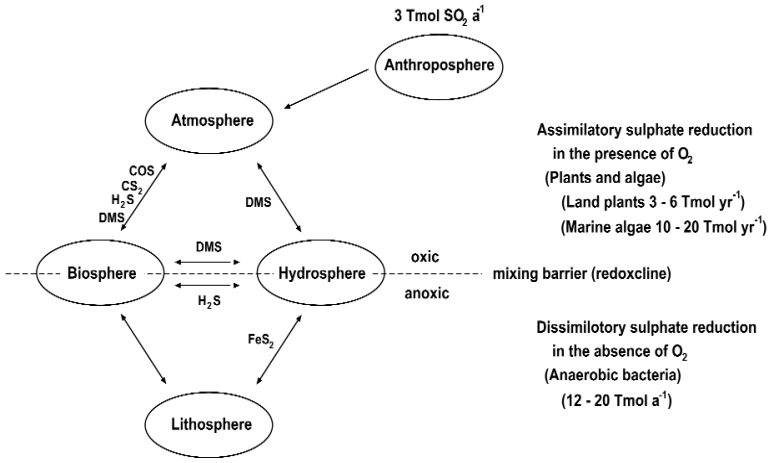


Figure 1. *Scheme of the main interactions of the global biogeochemical S cycle (from Andreae & Jaeschke, 1992).*

Sulfate is biologically reduced to sulfide (S^{2-}), which can chemically react to be incorporated into organic matter (e.g. methionine) or into minerals (generally in sediments, under anoxic conditions) or to be re-oxidized to sulfate. In the biosphere, S represents a minor but key nutrient for living organisms. The fact that animals and protozoa need to supply their sulfur demands with organosulfur compounds gives additional support to the idea that biochemical sulfate reduction processes are the driving forces of the S cycle. There are two main reduction pathways: dissimilatory and assimilatory sulfate reduction.

Dissimilatory sulfate reduction. The process associated with utilizing a substance as energy or electron source is called dissimilation. Dissimilatory sulfate reduction is a strictly anaerobic process carried out by sulfate-reducing bacteria that use sulfate as terminal electron acceptor instead of oxygen. It occurs largely in stratified anoxic water basins and in sediments of wetlands, lakes and coastal marine ecosystems. The main product of this reaction is H_2S that hardly escapes from the oxic/anoxic interface where it is mostly re-oxidised or precipitated in the form of FeS_2.

Assimilatory sulfate reduction. The process of using a substance for incorporation into biomolecules is called assimilation. Bacteria, cyanobacteria, fungi, eukaryotic algae and vascular plants can supply their sulfur requirements by reducing sulfate intracellularly to sulfur aminoacids (cysteine and methionine, which are incorporated into proteins) or other organosulfur compounds. This process is ubiquitous in both oxic and anoxic environments.

Biogenic sulfur compounds in the atmosphere

An important part of the S cycle occurs in the atmosphere due to the high volatility of a large number of S-containing compounds that facilitate their distribution among the different environmental compartments. The natural sources of S to the atmosphere are mainly derived from biological (reduced S volatiles) and volcanic activities (mostly SO_2). The biogenic sulfur gases have a short mean residence time in our oxidant atmosphere where they are converted to SO_2 and subsequently re-oxidized to SO_4^{2-} or H_2SO_4 by reaction with the radicals OH or H_2O. Thanks to the fact that no S gas is a long-lived or major constituent of the atmosphere, the analysis of the S emissions and depositions from the atmosphere permits to model the global S cycle. Figure 2 shows the estimated fluxes in a global S budget.

The values of these global estimations have to be seen with a high degree of caution. Factors such as the difficulty of accurately measuring some of the processes (e.g. the movement of S in soil dust, the sulfate associated with seaspray, or the emissions from soils and plants on the continents), the episodic nature of some events (e.g. volcanic eruptions or dust storms), or the latitudinal and seasonal variation of biogenic emissions, give uncertainties on the averaged

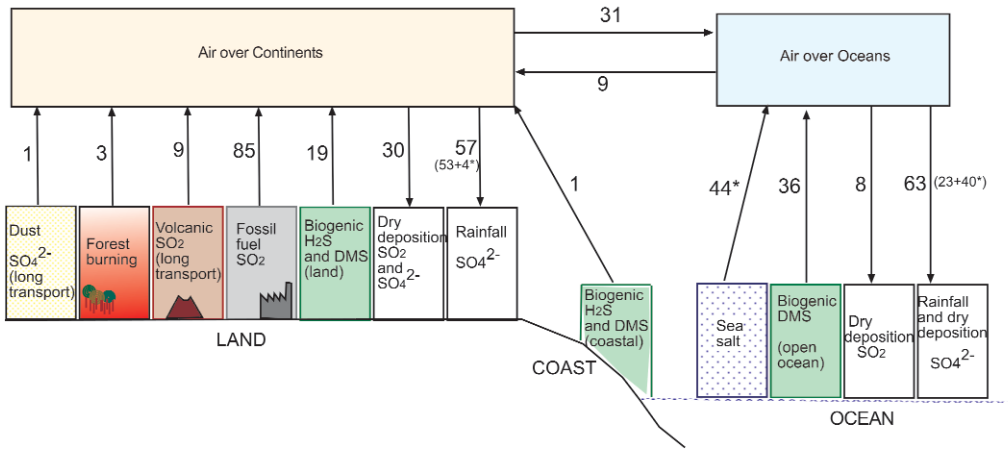


Figure 2. *Major input and output sulfur fluxes in the atmosphere (Tg (S) a^{-1}). Values averaged from Schlesigner 1991, Andreae & Jaeschke 1992 and Charlson et al. 1992. (*) indicates S in the seaspray. High uncertainty is associated with this process. The lowest estimate is given in the figure.*

fluxes that can differ by a factor of 10 among different studies (Schlesigner 1991, Andreae & Jaeschke 1992, Bates et al. 1992).

Overall, biogenic emissions account for ~60% of natural emissions with more than half of them accounted by marine biota. In land, the dominant gas emitted from freshwater wetlands and anaerobic soil is H_2S, with dimethylsulfide (DMS) and carbonyl sulfide (COS) playing a lower role. Fermentative decomposition of organic matter is probably the most important mechanism releasing S volatiles in soil, but high uncertainty is associated with this process due to the difficulty in obtaining representative data from land biomes. In contrast, many studies have well quantified the concentrations and distribution of DMS, the main biogenic source of S from sea-surface to the atmosphere (Lovelock et al. 1972, Kettle et al. 1999). Volcanic emissions (mainly SO_2) account for 20% of natural sources, with 90% of volcanic sulfur being emitted during non-erupting periods. Dust is transported throughout the troposphere due to wind blown erosion of soild, but these big particles (> 1μm) are rapidly deposited. Finally, seaspray aerosols have a high sulfate content but most of them are redeposited to the ocean through precipitation and dryfall.

The atmospheric sulfur cycle is highly perturbed by human activities. Anthropogenic emissions account for most of S fluxes to the atmosphere in the form of SO_2 and particulate sulfate. These are mainly deposited by rainwater; when the sulfur load is high, this wet deposition is called acid rain and may have hazardous effects (changes in rock weathering, damage on forest growth, deterioration of human-made architecture, etc). The main anthropogenic sources are coal burning and sulfide-ore smelting. Biomass burning (mainly as SO_2) can be also considered a

human sulfur emission since as much as 95% of global biomass burning is thought to be human initiated (Hileman 1990). Without human activities, net transport through the atmosphere would carry a small amount of S, mainly from oceans to land; nowadays, net transport occurs in the reverse direction.

Links to climate

Non-sea salt sulfate aerosols present in the low troposphere, either coming from oxidation of biogenic gases or anthropogenic SO_2, intercept incoming solar radiation and reduce the energy flux arriving at the Earth's surface. This cooling effect can be:

1. Direct: Sulfate aerosols directly scatter short-wavelength solar radiation.

2. Indirect: Sulfate aerosols cause cloud-mediated effects by acting as cloud condensation nuclei (CCN):

   2.1. Twomey effect on cloud albedo: an increase in CCN numbers produce more but smaller cloud droplets in a cloud whose liquid water content remains constant enhancing reflection of solar radiation (Twomey 1959)

   2.2. Albrecht effect on cloud lifetime: more but smaller cloud droplets reduce the precipitation efficiency and therefore enhance cloud lifetime and hence cloud reflectivity (Albrecht 1989).

Although in the past sulfate aerosols were considered to be of insignificant importance to global climate change, since the 90's their cooling effect has been included in climatic models as a forcing with opposite sign to that of the greenhouse gases (GHG). Some studies have suggested that the effect of sulfate aerosols is comparable in magnitude to current anthro pogenic GHG forcing, but their different geographical distributions prevent any compensation (Charlson et al. 1992). Paradoxically, the recent introduction of cleaner burning technologies is decreasing the anthropogenic sulfate aerosol burden in the atmosphere and reinforcing global warming. The possible reduction of aerosol cooling effect is being a matter of study with the regard on the introduction of industry in developing countries (Andreae et al. 2005).

Over the remote oceans, with low or null anthropogenic influences, biogenic emissions determine the amount of S processed in the atmosphere. Charlson et al. (1987) suggested a possible active feedback link between marine biota and climate regulation through the formation of sulfate aerosols from marine-exhaled DMS. The authors contextualized their hypothesis within a geophysiological thinking of the Earth: organisms influence the chemical composition of the atmosphere by producing oxygen, CO_2, DMS, etc., thus influencing climate, and setting the environmental conditions for an inhabitable planet (Lovelock & Margulis 1974).

The Marine Sulfur Cycle

The plankton-sulfur-cloud-climate system: the CLAW hypothesis

Oceans emit a variety of volatile sulfur species: DMS, CS_2, MeSH, dimethydisulfide (DMDS), H_2S and COS. DMS is by far the most abundant of these compounds in the surface ocean and, therefore, it is responsible for most of the S emissions, it is the main contributor to the natural acidity of the marine atmosphere, and it is the key compound to transfer S from the oceans to land (Lovelock et al. 1972).

In 1987, Charlson et al. published the provocative CLAW hypothesis (acronym of the names of the authors), which postulates a negative feedback through the plankton-sulfur-cloud-climate system (see Fig 3). Briefly, marine algae would produce DMS that would be emitted to the atmosphere where it would be oxidized by reaction with free radicals (particularly hydroxyl and nitrate) to form sulfate-aerosols. These, both by themselves and acting as CCN, would increase the albedo of the Earth (i.e., the reflection of solar radiation back to the space). The less solar radiation reaching the surface of the ocean would change sea-surface temperature and light fields, possibly decreasing the phytoplankton production of DMS. Less DMS would imply a negative feed-back loop, because fewer sulfate-aerosols would be formed and then more solar radiation would reach the surface of the sea, resulting in increased DMS production.

This algae-sulfur-climate loop was proposed as an example of how the planet self-regulates its temperature. Even though this hypothesis is still under test, defenders and detractors have generated a large number of scientific papers that have improved our knowledge of the sulfur cycle in the sea and in the atmosphere. Although we still have fundamental gaps in our understanding of this system, nowadays we know that DMS production results from complex interactions in the whole food web involving viruses, bacteria, grazers and algae, and only a minor percentage of the total DMS produced escapes to the atmosphere. In addition, DMS and its biogenic precursor, dimethylsulfoniopropionate (DMSP), are significant

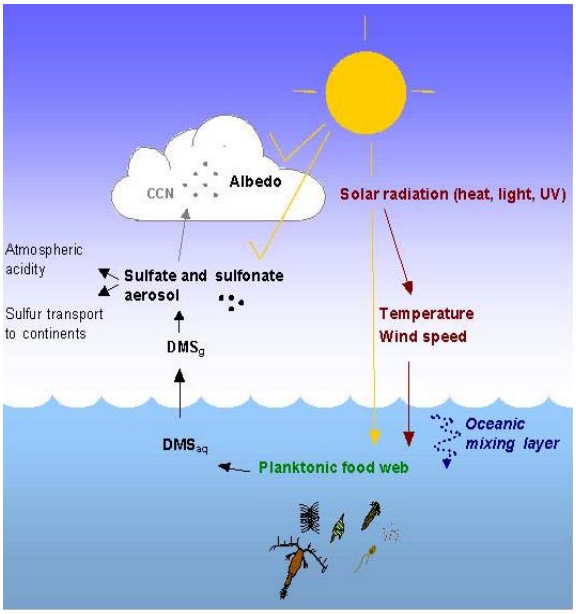


Figure 3. *Plankton-cloud-climate interactions through DMS prodution (from Simó et al. 2001).*

components of the fluxes of sulfur, carbon and energy that flow in many marine food webs (Kiene & Linn 2000a, Zubkov et al. 2001, Simó et al. 2002). The existence of convenient methods to measure trace aqueous concentrations of dimethylated sulfur compounds confers to these substances advantage over other components of the dissolved organic pool. The study of the transformations of dimethylated sulfur compounds can serve as a model to better understand biogeochemical transformations of labile organic compounds, marine ecosystem functioning and possible linkages between biogenic volatile compounds and climate.

The DMS(P) cycle. DMSP production

The biogenic precursor of DMS is dimethylsulfoniopropionate (DMSP), a reduced sulfur compound ubiquitous in the photic zone of the ocean. It is produced by different classes of phytoplankton, mainly Dinophyceae (dinoflagellates) and Prymnesiophyceae, through an assimilatory sulfate reduction process that involves methionine as precursor of DMSP. Intracellular concentrations of DMSP in high DMSP producers range from 100 to 500 mM. Other groups, such as Prasinophytes, Bacillariophyceae (diatoms except for those that grow in sea ice) and cyanobacteria generally produce low amounts of DMSP, intracellular concentrations ranging from 1 to 100 mM (Keller et al. 1989, Stefels 2000). In culture studies, DMSP accounts for most of the sulfur content of the cell in some species (Matrai & Keller 1994). In addition, field studies have shown that a significant proportion (from 0.2 to 9%) of the cellular carbon content of phytoplankton can be accounted for by DMSP (Kiene et al. 2000). Connecting with the C cycle, the percentage of primary production invested in DMSP synthesis has been quantified only in two studies ($\sim$ 7%, Simó et al. 2002, Burkill et al. 2002). Unfortunately, these two field studies were carried out during similar blooms of DMSP producers, which implies that for most oceanic sites the proportion of C fixation as DMSP still remains unknown.

Intracellular functions of DMSP in algal cells have not been fully elucidated. In terms of physiology, DMSP seems to act as compatible soluble to protect cells against external osmotic stress (Vairavamurthy et al. 1985, Kirst 1996) and as cryoprotectant in polar algae (Kirst et al. 1991, Karsten et al. 1996). Additionally, it has been suggested that DMSP can be produced to catch the excess of reducing power under unbalanced algal growth in N-limitation conditions (Stefels 2000). DMSP seems to be also a chemical signaling compound. DMSP breakdown products (DMS and acrylate, see next section) can act as deterrents of herbivore grazing due to the toxicity of acrylate (Wolfe et al. 1997) or they can act as attractors between algae and bacteria (Zimmer-Faust et al. 1996) and between zooplankton and birds (Nevitt et al. 1995). Recently, Sunda et al. (2002) proposed that DMSP can act as antioxidant in marine algae by effectively scavenging the reactive oxygen species (ROS) formed in oxidative stress situations (Fe limitation, high UV radiation, low CO_2 availability, etc). An intracellular antioxidant cascade would operate by sequentially converting DMSP to DMS and DMSO, all efficient ROS scavengers. This hypothesis has never been tested in field studies.

The structural homology between DMSP and another known osmolyte, glycine betaine, which contains N instead of S, and the existence of a transamination reaction in the DMSP synthesis in marine algae (Gage et al. 1997), lead to the hypothesis that DMSP production could be enhanced under N limitation conditions. Culture studies seem to support this idea (Keller & Korjeff-Bellows 1996) yet only for some species and not for others (Keller et al. 1999). A recent study with cultures of *Thalassiosira pseudonana* shows that other factors that also originate oxidative stress to cells also enhance DMSP production (Bucciarelli & Sunda 2003).

In the open ocean, factors that may promote DMSP production such as nutrient limitation or high UV radiation are typical of summer periods or oligotrophic regions, under conditions that also favor the presence of DMSP-producing taxa (such as small haptophytes and small dinoflagellates). Conversely, nutrient-replete waters favor taxa such as diatoms, which are generally low DMSP producers. Upon this observation, Simó (2001) suggested that possibly phytoplankton taxa succession rather than an acclimation switch between GBT and DMSP production by the same species could explain the high DMSP concentrations usually encountered in N-depleted waters. However, there is mounting evidence that intracellular DMSP concentrations not only change among taxa but also with the physiological state of the cells, which is influenced by the growth stage of a bloom or the dose of solar radiation (Matrai & Keller 1993, Slezak & Herndl 2003). In order to assess the factors that promote DMSP production in the ocean, field studies should be conducted over temporal or/and spatial scales that allow to capture both phytoplakton succession and physiological acclimation.

Food-web interactions. DMS production

The conversion of DMSP to DMS is catalyzed by the enzyme DMSP lyase following the reaction:

$$(CH_3)_2SCH_2CH_2COO^- \longrightarrow CH_3SCH_3 + CH_2{=}CHCOO^-$$

This enzyme is present with high activities in high-DMSP producers (Stefels & Dijkhuizen 1996, Steinke et al. 1998, Harada 2004) and in marine bacteria (Yoch 2002). Despite the algal origin of DMSP, DMS production can not be predicted from Chla concentrations (e.g. Kettle et al. 1999) neither primary production (Toole & Siegel 2004) due to the taxon-dependence of DMSP producers. In fact, not only algae but the whole food-web members are involved in DMS production (Fig. 4, Simó 2001). DMSP can break down inside or outside the algal cells. As commented above, intracellular lysis of DMSP can occur under oxidative stress conditions such as high doses of UV radiation or nutrient depletion, although this hypothesis has only be tested in culture studies (Sunda et al. 2002). DMSP is released into the dissolved pool through algal autolysis, viral attack and grazing, whereas DMSP exudation by healthy cells accounts for a minor amount of dissolved DMSP (Laroche et al. 1999).

Microzooplankton herbivores assimilate a fraction (~30%) of the DMSP contained in the ingested preys (Wolfe et al. 1994, Simó et al. 2002, Burkill et al. 2002, Tang & Simó 2003, Simó 2004). Another fraction of the ingested DMSP can be converted into DMS by DMSP lyases in the micrograzer's guts and vacuoles and released into the dissolved phase (Wolfe et al. 1994, Tang et al. 1999, Archer et al. 2001b), and a third fraction is released with fecal pellets and detrital material that make DMSP available for bacteria before sinking (Tang 2001). However, grazers mainly produce DMS during grazing by action of algal lyases after algal cell rupture (Wolfe & Steinke 1996), a process that also makes dissolved DMSP available to bacteria.

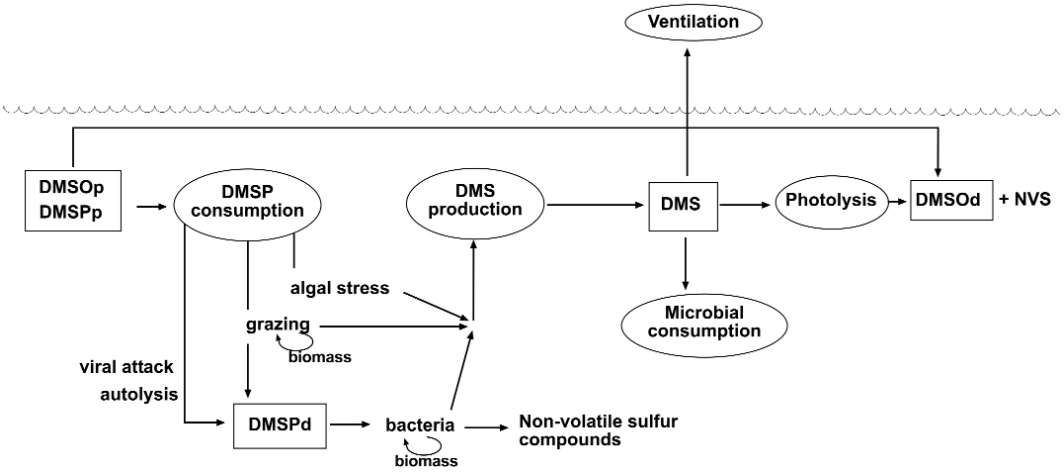


Figure 4. *Scheme of the DMSP/DMS cycle in the oceans. NVS: non-volatile sulfur compounds.*

Heterotrophic bacterioplankton is believed to play a major role in dissolved DMSP (DMSPd) transformations. Due to the zwitterionic nature of DMSP (it is a neutral molecule but carries (+) and (-) charges on different atoms), bacteria need an active transport system in the membrane to take up DMSP. Heterotrophic bacteria seem to use the same uptake system for DMSP and GBT (Kiene et al. 1998). Similar to what they do with GBT, bacteria can accumulate DMSP intracellularly without degradation for 6-30 h, which suggests for both molecules a possible role as compatible solutes under osmotic stress (Kiene & Linn 1999). Bacteria can also degrade DMSP through 2 major pathways (Fig. 5).

The "DMSP cleavage" pathway converts DMSP into DMS and acrylate that can be used as carbon and energy source (Ansede et al. 2001). The "DMSP double demethylation" pathway leads to non-volatile sulfur compounds that not seem to be used by bacteria (Visscher et al. 1992). Finally, "DMSP demethylation/demethiolation" gives rise to methanethiol (MeSH) production, which is used as a reduced S source and is assimilated primaryly into methionine and cysteine to build the proteins of the cell (Kiene et al. 1999). Despite that concentrations of

sulfate are 2-3 orders of magnitude higher than DMSP concentrations in seawater, marine bacteria may save energy by directly taking up sulfur in an already reduced form (Kiene et al. 1999). DMSP is a significant source of C and S for marine bacteria: it can supply from 1 to 15% of the C demand and 50 to 100% of the S demand (Kiene & Linn 2000a, Zubkov et al. 2001b, Simó et al. 2002).

DMSP demethylation dominates the fate of bacterial degradation of DMSP in most systems studied (Ledyard & Dacey 1996b, Kiene et al. 2000). However, factors that control whether DMSP is converted to DMS or MeSH still remain unknown. Kiene et al. (2000) proposed that, with enough availability of DMSPd, bacteria would switch from demethylation route to DMS

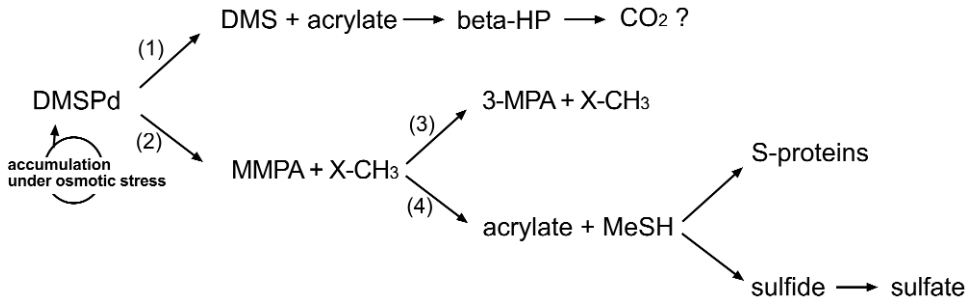


Figure 5. *Proposed pathways for bacterial DMSP degradation (from Yoch 2002 and Moran et al. 2003). (1) DMSP cleavage pathway (2) demethylation pathway (3) double demethylation pathway (4) demethylation/demethiolation pathway. beta-HP: beta hydroxypropionate; MMPA: 3-methylmercaptopropionate; MPA: 3-mercaptopropionate.*

production when bacteria saturated their S demands. An important difficulty arises when addressing this question: how to distinguish the contribution of bacteria to the bulk of DMSP transformations by the total community? In this thesis, we have combined the recently introduced radiotracer techniques (Kiene & Linn 2000b) with dark incubations of the total community (Simó & Pedrós-Alió 1999b, Simó et al. 2000) to assess the role of bacteria in DMSP transformations. For the first time, these two approaches have been combined in a field study.

Phylogentic identification of DMSP-consuming bacteria

If bacterial DMSP demethylation generally dominates over DMSP cleavage, and serves as an assimilative source of S for protein synthesis, factors influencing a major or minor DMSP assimilation are indirectly affecting the amount of DMS produced by bacteria. Recent studies have shown that heterotrophic bacterioplankton do not uniformly assimilate the components of the dissolved organic matter (DOM) pool. For example, members of the Bacteroidetes group

seem to have higher affinity to take up high molecular weight DOM compounds than low molecular weight DOM compounds (Cottrell & Kirchman 2000). Possibly, bacterial diversity is also affecting the total amount of DMSP assimilated by bacteria. But how widespread is the capacity to assimilate DMSP among marine bacteria still remains unknown. Previous works have provided evidence that *Roseobacter*, an abundant marine group belonging to Alphaprotoebacteria, could be major degraders of DMSP in seawater (González et al. 1999, González et al. 2000, Zubkov et al. 2001b). However, the good correlation found between DMSP consumption rates and bacterial production in the Gulf of Mexico (Kiene & Linn 2000a) suggests that DMSP may be consumed by more than a few phylotypes.

The study of the linkage between bacterial diversity and function *in situ* is not straightforward. Recent developed protocols of single-cell analysis (SCA) techniques have allowed studying metabolic capabilities of unculturable bacteria. In particular, two SCA techniques have revealed to be very useful with field samples: MARFISH and flow cytometry cell sorting. The MARFISH method results from the combination of microautoradiography (that allows visualizing the incorporation of a radiolabeled substrate by single cells under the microscope) and Fluorescence In Situ Hybridization (that allows to interrogate single cells about their phylogenetic identity by hybridizing their RNA with group-specific phylogenetic probes). Application of MAR-FISH in field studies have allowed determining some patterns of the DOM flux through different groups of bacteria (e.g. Alonso & Pernthaler 2006b) or the major contributors to total prokaryotic production (e.g. Cottrell & Kirchman 2003, Herndl et al. 2005). Flow cytometry cell sorting physically separates different populations within prokaryotic communities based upon the specific light scattering, fluorescence and size characteristics of each cell. Sorted cells can be further phylogentically identified (e.g. Zubkov et al. 2001b, Mou et al. 2005) or they can be quantified for their contribution to the total consumption of a given organic compound if the sample was previously incubated with the radiolabeled form of this compound. For example, Zubkov et al. (2003) discovered that *Prochlorococcus* consume methionine significantly competing with heterotrophic bacteria in the Arabian Sea.

DMS losses. DMS-degrading bacteria

There are three main pathways for DMS removal from the upper water column: ventilation to the atmosphere (e.g. Zemmelink et al. 2004), microbial consumption (e.g. Kiene & Bates 1990, González et al. 1999) and photolysis (e.g. Toole et al. 2003). Since, by definition, ventilation and photolysis are surface-enhanced, the relative quantitative importance of each sink depends on the depth interval considered, and varies over short-term scales by variation of their driving forces (wind-speed, solar radiation, etc) (Kieber et al. 1996, Simó & Pedrós-Alió 1999a, Toole et al. 2006).

Simó (2004) showed in a compilation work that, on average, 50-80% of DMS production was

lost as biological consumption. Although bacterial consumption notably controls DMS concentration in seawater, the very little information about the phylogenetic identity of DMS-consuming bacteria is restricted to culture studies (e.g. Visscher & Taylor 1994, de Zwart & Kuenen 1997, González et al. 1999). Based on the pioneering studies with isolates, Kiene et al. (1993) suggested that methylotrophic bacteria should be the main DMS consumers in seawater. However, this hypothesis has never been tested in studies in situ. Thus it still remains unknown whether DMS consumption is restricted to some phyla or is a widespread capacity among bacteria.

The remaining 20-50% of DMS production is lost through photolysis and ventilation. Ventilation generally is not such an important sink for oceanic DMS, except within the surface microlayer. Photolysis, conversely plays an important role in DMS loss. It is a function of the dose of solar radiation received and, furthermore, it is catalyzed by photosensitizers, mainly by components of the colored dissolved organic matter pool (cDOM) and by nitrate (Toole et al. 2003, Toole & Siegel 2004, Bouillon & Miller 2004).

The "DMS summer paradox"

Several seasonal studies in oligotrophic and coastal waters in temperate to subtropical zones have shown that maximum DMS concentrations occur in summer, around 2-months later than the maximum concentration of DMSP, when the concentration of Chla is at its annual minimum (e.g. Dacey et al. 1998, Uher et al. 2000). Known as the "DMS summer paradox" after (Simó & Pedrós-Alió 1999a), these authors suggested that the depth of the mixing layer could provide an explanation for this paradox by integrating all the environmental processes that affect DMS production, mainly phytoplankton succession, nutrient availability, and the different photoinhibitory effects of UV radiation on bacteria and phytoplankton. Toole & Siegel (2004) found an excellent seasonal correlation between UV doses and DMS concentrations in the Sargasso Sea, and suggested that, rather than being dictated by the classical biological factors (biomass, production), the seasonality of DMS concentrations is driven by physical factors acting on the ecosystem, particularly in non-bloom situations. In this context, DMS accumulation in the summer period may result from the combined effects of algal stress and inhibition of bacterial activity by UV radiation.

The "DMS summer paradox" shows the complexity associated with DMSP degradation and DMS production in marine food-webs. Despite the usefulness of integrative parameters to predict DMS concentrations in seawater, an understanding of the processes involved in the sulfur cycle will only be possible by simultaneous measurements of biotic and abiotic processes at multiple temporal and spatial scales. This will help to better identify the actors and mechanisms that play a role in the cycle and their relative importance. In this regard, bacteria seem to play a central

role, but fundamental gaps in our understanding of the factors that influence their DMS(P) related metabolisms still remain.

Aims and Outline of the thesis

The main objective of this thesis was to assess the role of bacterioplankton in the cycle of dimethylated sulfur compounds in the upper ocean. Since seasonality is a consistent and robust feature of the sulfur cycle, this thesis has been focused mainly in studies at annual scale. Specifically, the thesis addresses the following questions:

1. What is the seasonality of pools and transformations of dimethylated sulfur compounds and what are the physicochemical driving forces?

2. Are bacterioplankton the key component to explain the seasonality of DMS concentrations?

3. How widespread is the capacity to assimilate DMSP and DMS among microorganisms?

4. Which factors influence DMSP/DMS metabolism by bacteria?

The first question was assessed by simultaneous measurements of biotic and abiotic transformations of DMS and DMSP during a seasonal study in Blanes Bay, a coastal oligotrophic site, in order to determine the relative importance of each of these transformations in the sulfur cycle. The contribution of bacteria to total community DMSP transformations was elucidated by the use of two independent techniques never applied together in field studies (incubation with radiotracer and dark incubation of whole seawater, Chapter I).

The main DMSP consumers in seawater were identified by using the MARFISH method, with samples from the Gulf of Mexico and Blanes Bay (Chapter II and III). The seasonality of DMSP-consuming bacteria was analysed in order to find possible links between bacterial assemblage structure and DMSP assimilation and possible DMS production (Chapter III).

Chapter IV reports the contribution of DMSP to the fluxes of carbon and sulfur that flow through the first levels of the food-web and how this affects DMSP assimilation by bacteria. For the first time, this question has been addressed in an oligotrophic site, without the presence of blooms of DMSP-producing phytoplankton, and over a seasonal scale.

At the beginning of this thesis, when running some MARFISH samples, I saw under the microscope an unexpected ^{35}S-labeled diatom, suggesting DMSP assimilation. The work studying DMSP uptake by phototrophs is explained in Chapter V. Microautoradiography was used to follow DMSP assimilation by microphytoplankton and flow cytometry cell-sorting was used to analyse the contribution of picophototrophs.

Due to the low DMS-S assimilation by marine bacteria, all attempts to perform MARFISH technique with ^{35}S-DMS have failed. DMS-consumers were identified by enrichment experiments in contrasting coastal and open-ocean waters (**Chapter VI**).

The results of this research are presented in six chapters. Each chapter has the format of a scientific paper, and, even though some repetition can be found in the introductions, each chapter can be read independently.

CHAPTER I

Seasonal variability of the dynamics of dimethylated sulfur compounds in a coastal NW Mediterranean site

Vila-Costa, Maria; Kiene, Ronald P. and Simó, Rafel.

Abstract

Dimethylsulfide (DMS) is a biogenic oceanic gas with a potential large-scale influence on the climate of the Earth. Potential controls on the concentrations and emission fluxes of DMS are biological (e.g. physiology and diversity of plankton microorganisms), physical (e.g. solar radiation, wind speed) and chemical (e.g. nutrients, cDOM). Here we study for first time the seasonal variation of biotic and abiotic processes involved in the production and consumption of DMS and its precursor, dimethylsulfoniopropionate (DMSP), at a coastal sampling station in the NW Mediterranean. Monthly samplings over an 18-month period revealed that DMS concentrations in surface seawater were maximal in summer and minimal in winter, while algal-associated DMSP and dimethylsulfoxide (DMSO) concentrations did not follow total phytoplankton biomass (Chla) but phytoplankton succession and physiology, mirrored by the ratios DMSP:Chla and DMSO:Chla. Gross DMS production rates were higher in summer, coinciding with higher DMSP-to-DMS conversion yields, and exceeded microbial DMS consumption in this season. Heterotrophic bacteria only accounted for a portion (annual average 52%) of total DMSP transformations, suggesting that phytoplankton DMSP-lyases, either in stressed cells or upon grazing by herbivores, must play more important a role as DMS sources than generally believed. Photolysis and microbial consumption alternated their dominance among DMS sinks, with ventilation being a minor loss process. When dominant (from February to July), photolysis followed variations of cDOM, a known DMS photosensitizer.

Introduction

The biogenic marine gas dimethylsulfide (DMS) is the key compound that transfers sulfur from oceans to land through the atmosphere (Lovelock 1974). It is, by far, the most abundant form in which the oceans exhale volatile sulfur. In the atmosphere, DMS oxidizes to form sulfate aerosols that scatter solar radiation and act as cloud condensation nuclei, thereby potentially increasing cloud albedo and influencing the radiation balance of the Earth (Charlson et al. 1987, Charlson et al. 1992). Despite its global scale effects, the emission of DMS only represents a small percentage of total DMS produced in the surface ocean (Bates et al. 1994). DMS ventilation is controlled by the transfer velocity (temperature and wind-speed dependent) as well as by the sea-surface DMS concentration. Seasonal field studies and data compilations have shown that DMS tends to accumulate in summer months (e.g. Bates et al. 1987, Dacey et al. 1998, Simó & Pedrós-Alió 1999a, Uher et al. 2000) and at mid-to-low latitudes it even reaches its maximum concentrations two months later than its precursor, dimethylsulfoniopropionate, and coinciding with a minimum of surface chlorophyll concentrations. The causes of this "DMS summer paradox" (Simó & Pedrós-Alió 1999a) still remain unresolved, and they can only be explained with a better understanding of the couplings and decouplings of the biological, chemical and physical processes involved in DMS cycling and how they vary over an annual basis. However, there is a lack of seasonal studies that undertake simultaneous measurements of biological and abiotic transformations of dimetylated sulfur compounds.

DMS concentration in the upper ocean results from the balance between its production and loss processes. DMS results from enzymatic cleavage of DMSP, a compatible solute produced by a variety of species of phytoplankton (Keller et al. 1989). Intracellular DMSP is released into seawater through algal cell lysis caused by grazing, viral attack or autolysis, or by exudation (Simó 2001). Either in the algal cell or upon release, part of the DMSP is cleaved into DMS and acrylate by DMSP lyases present in some algae and bacteria (Stefels 2000, Yoch 2002).

Thus, the bulk of DMS production results from complex interactions in the food-web (Simó 2001). Having its sole known origin in algal DMSP, DMS can arise either from this compound in dissolved form in bulk seawater (DMSPd) or directly from the particulate pool (DMSPp). Transformations of DMSPd are carried out mainly by microorganisms (mainly heterotrophic bacteria but also non DMSP-producing phytoplankton (Chapter V)), as reflected in the high DMSPd turnover rates measured at different oceanic sites (Kiene & Linn 2000a, Zubkov et al. 2001a). The fate of DMSP degraded by bacteria is largely determined by a switch between two major degradation pathways. There is the non-assimilative route that converts DMSP into DMS, and an assimilative route by which DMSP largely fuels sulfur demands of heterotrophic bacteria (Kiene et al. 2000). However, there is considerable uncertainty regarding the factors that control this switch and the contribution of bacterial transformations to total DMSP transformations and how this affects the bulk of DMS production.

Second, DMS is produced from the particulate DMSP pool by its phytoplankton producers and by action of zooplankton. The factors that control these phyto- and zooplankton-mediated transformations are of different nature. Intracellular DMSP cleavage and DMS release by phytoplankton are suggested occur under oxidative stress conditions provoked by high UV radiation or nutrient limitation (Sunda et al. 2002). Grazing by meso- and microzooplankton gives rise to DMS either by action of algal DMSP lyases upon cell rupture (Dacey & Wakeham 1986, Wolfe & Steinke 1996), or by action of bacteria in the guts and vacuoles of the grazer while DMSP-containing algal cells are digested (Tang et al. 1999, Archer et al. 2001b). The relative importance of bacterioplankton, phytoplankton and zooplankton-mediated DMSP transformations and DMS production has hardly been quantified (Simó et al. 2002, Burkill et al. 2002).

There are three main processes for DMS removal: microbial consumption, photooxidation and ventilation to the atmosphere. Short-term studies in open-ocean waters have shown that the dominance of these removal pathways depends on physical forces (Simó & Pedrós-Alió 1999b). Microbial DMS consumption is inhibited by UV radiation (Slezak et al. 2001, Toole et al. 2006) and it is probably affected by other factors that regulate general bacterial activity, such as temperature or dissolved organic matter availability. DMS photooxidation occurs through secondary photosensitizes such as colored dissolved organic matter (cDOM) and nitrate, and it is dependent on incident solar radiation and sea-surface temperature (Toole et al. 2003, Toole et al. 2006). Ventilation depends mainly on wind speed and temperature (Nightingale et al. 2000).

In spite of the pytoplankton origin of DMSP, DMS concentrations generally do not correlate with chlorophyll *a* concentrations either at regional to global scales (Kettle et al. 1999) or in seasonal studies (Leck et al. 1990, Turner et al. 1996, Dacey et al. 1998). This is mainly due to the taxon-specificity and physiological functionality of DMSP production (Keller et al. 1989, Malin & Kirst 1997) and to the not straightforward conversion of DMSP into DMS. In non-bloom situations, DMS concentration seems to be rather modulated by physical forces inducing multiple biological responses (Simó 2004, Toole & Siegel 2004). Two physical parameters have been found to largely explain DMS varability in oceanic surface waters: the depth of the mixing layer (MLD, Simó & Pedrós-Alió 1999a) and the UV radiation dose (Toole & Siegel 2004). Both parameters are hypothesized to concur to have the same regulatory effect: in highly irradiated waters typical of summer conditions, stronger stratification of the water column favors high DMSP-producing phytoplankton, and high UV irradiances in shallow MLD generate high UVR doses that may inhibit the consumption of DMSP and DMS by non-pigmented bacteria and enhance the anti-oxidative responses of phytoplankton, all resulting in higher DMS production than consumption (Simó & Pedrós-Alió 1999a, Slezak et al. 2001, Sunda et al. 2002, Simó 2004, Toole & Siegel 2004). Unfortunately, no field studies of the seasonal variation of each of these biological and physical factors, and how they affect the DMSP and DMS dynamics have been conducted so far.

This is the first study where not only the major pools of dimethylated sulfur (DMS, DMSP, DMSO) but also the variation of biological processes (DMSP consumption by the total community and by bacteria specifically, DMS consumption, DMS production, DMSP-to-DMS conversion) and the variation of important DMS(P)-driving physicochemical parameters (solar radiation, cDOM concentrations, nitrate concentrations, wind speed) have been measured over an annual cycle. The aim was to obtain a better understanding of the biogeochemical sulfur cycle in the surface ocean and to identify the key parameters needed to model its dynamics.

Materials and Methods

Sampling. We sampled two consecutive days every month, from 13 January 2003 to 28 June 2004, in the Blanes Bay Microbial Observatory, NW Mediterranean, 41°40'N, 2° 48'E. Surface seawater was collected approximately 1 mile offshore by submerging acid-rinsed glass dark b ottles to a depth of 0.5 m avoiding bubbling. Bottles (2.5 L) were kept in the dark at in situ temperature until processing in Barcelona (usually 2 h after collection).

Basic data. Surface water temperature was measured in situ with a thermometer. Temperature profiles were determined using a Niskin bottle to collect water from different fixed depths (0, 5, 10, 15, 20 m) plus closer additional depths when an increment of 1°C was found. Mixed layer depths (MLD) were determined as the shallowest depth with a T= 0.2°C from surface temperature. Chlorophyll *a* (Chla) was measured by fluorometry (Turner Designs fluorometer) after filtering 150 ml through GF/F filters (Whatman) and extracting with 90% acetone for 24 h. Concentration of NO_3^- was determined spectrophotometrically using an Alliance Evolution II autoanalyzer following standard procedures (Grasshoff et al. 1983). Surface solar irradiances were obtained from the meteorological station of Malgrat, located 4.5 km south of Blanes Bay. The light extinction coefficient (k) was determined from PAR profiles using a Li-Cor radiometer. Daily ML-averaged solar radiation was calculated assuming an exponential decay of the daily-averaged surface solar irradiance ($EDAY_0$) with depth (z) following the equation $RADY_{UML} = RADY_0/(k \cdot MLD) \cdot (1-\exp(-k \cdot MLD))$ (see Vallina & Simó (*submitted*) for details).

Analysis of sulfur compounds. Surface seawater concentrations of DMS, DMSP and DMSO were determined following procedures described by Simó & Vila-Costa (2006) using purging, cryotrapping and sulfur-specific gas chromatography. Total DMSP and DMSO concentrations (DMSPt, DMSOt) were measured in non-filtered 20 to 50 ml sample aliquots. Compounds measured in 30 to 50 ml aliquots of seawater gently filtered through Whatman GF/F filters ($\sim$ 0.7 μm-porus-size) using a glass syringe were considered the "dissolved" fraction (DMSPd, DMSOd). The compounds retained on the GF/F filter were referred to as "particulate" (DMSPp, DMSOp). Those retained on polycarbonate filters of 5 μm-porus-size (Millipore) were considered the "particulate >5 μm" fraction. Acetate filters were discarded because they showed interferences with DMSO analysis. DMSO measurements were modified in the second part of

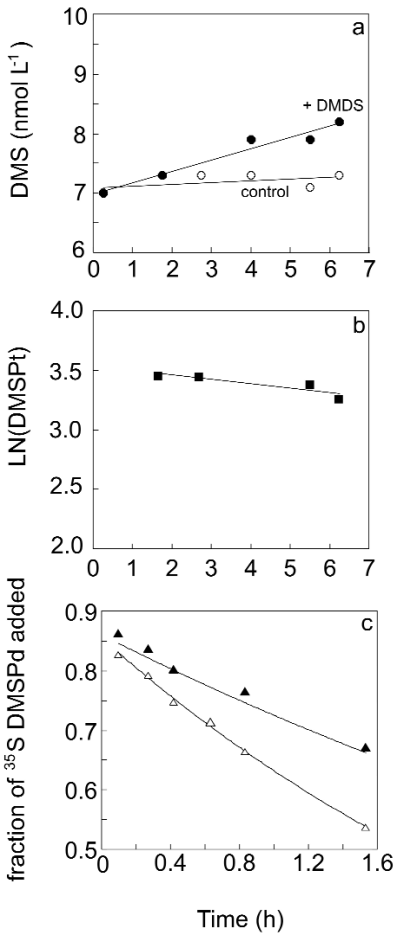


Figure 1. *Graphic results of the analytical methods employed to measure DMS production rates and DMS and DMSP consumption rates. These examples are taken from August 04, 2003. (a) Comparison between control (non-amended) and DMDS-amended bottles. The control bottle gives the net DMS production rate. The rate of increase of DMS concentration in the DMDS-amended bottle gives the gross DMS production rate. Microbial DMS consumption is calculated by subtraction of the two slopes. (b) DMSPt consumption by the total community using the "loss net curve" method. (c) Bacterial DMSPd consumption measured as the rate of disappearance of added ^{35}S-DMSPd (duplicate bottles are shown).*

this seasonal study (from September 2003 on). Briefly, after DMSP analysis, we added ¼ of a cobalt-doped $NaBH_4$ pellet (98%, Aldrich Chemical Co., Milwaukee, WI,USA) with no need for pH neutralization or acid addition. The same analytical precision was observed between both methods. The detection limit of the system was approximately 3 pmol S. The precision (%SE) of triplicate measurements was typically ~ 2% for DMS and DMSP and ~8% for DMSO measurements.

Biological DMS and DMSPt consumptions and DMS production. These were determined as described in Simó & Pedrós-Alió (1999b) and Simó et al. (2000). In brief, two amber bottles were incubated in the dark at *in situ* temperature for 8 h. Dimethyldisulfide (DMDS) was added to one of the bottles (260 nmol L^{-1} final conc.), to inhibit DMS consumption. Gross DMS production (nmol DMS $L^{-1}d^{-1}$) was measured as the rate of DMS accumulation in the +DMDS bottle. Microbial DMS consumption rate (nmol DMS $L^{-1}d^{-1}$) was calculated from the difference between the slope of the DMS time course in the +DMDS bottles and the slope in the control (where both production and consumption occur, Fig.1a).

DMSPt consumption by the whole community was measured using the method of the "net loss curve" in the dark (Simó et al. 2000). The rate constant of DMSPt consumption (d^{-1}) was obtained from the slope of the logarithmic disappearance of total DMSP concentrations in the control bottle (Fig. 1b). The DMSP consumption rate (nmol DMSPt $L^{-1}d^{-1}$) was calculated multiplying this rate constant by the initial DMSPt concentration.

DMS yield. The DMS yield is defined as the ratio between the rates of DMS production and DMSP consumption (Simó 2001). It is calculated as DMS production*100/ DMSP consumption.

Bacterial DMSP consumption. We incubated 30-ml seawater samples in the dark at in situ temperature with tracer levels of ^{35}S-DMSP (1000 dpm ml^{-1}, 5.79 Ci pmol^{-1}). Subsamples of 4 ml were taken over a short-term time course (< 2 h), acidified, and stored at 4°C for 24 h in the dark. Untransformed ^{35}S-DMSP was converted into volatile ^{35}S-DMS by injection of NaOH into sealed vials, and trapped in %3 H_2O_2-soaked wicks suspended in cups (Kiene & Linn 2000b). DMSP loss rate constant was calculated from the exponential loss of ^{35}S-DMSP with time (Fig. 1c). Bacterial DMSP consumption rate was calculated as the product of the DMSPd concentration and the loss rate constant.

DMS ventilation. Semi-hourly wind speeds were obtained from the meteorological station of Malgrat. Instantaneous DMS air-sea exchange transfer velocities (cm h^{-1}) were calculated from wind speed and sea-surface temperature using the parameterization of Nightingale et al. (2000). The DMS air-sea exchange fluxes (μmol m^{-2}d^{-1}) were calculated as the product of the DMS transfer velocity and DMS surface concentration. The ventilation rate (nmol DMS L^{-1}d^{-1}) was obtained by dividing the DMS exchange flux by the depth of the mixing layer. Semi-hourly DMS ventilation rates were averaged over the 24 h period between the two consecutive days of sampling.

Estimation of photolysis rates. Photolysis rates were estimated by budgeting the difference of surface DMS concentrations between the two consecutive days and measured DMS production and DMS losses (Simó & Pedrós-Alió 1999b). The assumption behind this budget was that we were sampling the same water mass during this 24 h period. This was checked by comparing field data such as temperature, Chla, inorganic nutrients concentrations, MLD and DMS. In most of the cases, the assumption was supported.

Calculation of loss rate constants. The rate constants (d^{-1}) of DMS loss processes (microbial, ventilation, photolysis) were obtained dividing the measured loss rates (nmol DMS L^{-1}d^{-1}) by the DMS concentration (nmol DMS L^{-1}) averaged for the 2 consecutive days.

Colored dissolved organic matter (cDOM). Subsamples of 50 ml were filtered through 0.2 μm pore-size, 47 mm diameter polycarbonate filters (Supor-200; gelman Sciences) using a HCl-rinsed polycarbonate filtration device (Millipore). Subsamples of 20 ml were stored at -20°C in ultraclean polypropylene vials until analysis. cDOM absorption values at 320 nm were measured using a UVIKON 923 UV/Vis spectrophotometer with 10 cm quartz cells. MilliQ water was used as a reference.

Results

Temperature, solar radiation, Chla and phytoplankton assemblages. Blanes Bay was characterized by a marked seasonality in water temperature 12.8 ± 1.6 °C in winter and 24.6

± 1.1 ºC in summer (Fig. 2). Based on the stratification of the water column, the summer period was considered from May to August, while winter period was from December to February. The solar radiation followed the same pattern as temperature. The mixing layer, daily mean solar radiation averaged 23.1 W m^{-2} in winter and increased by a factor of 10 (232.7 W m^{-2}) in summer (Fig. 2). The wind speeds did not vary significantly through the year (1.5 ± 0.3 m s^{-1}) and the salinity exhibited small variations in the range 36.0-38.7 psu.

The winter period was characterized by higher nutrient concentrations and moderate chlorophyll *a* concentrations (averaging 1.7 μg Chla L^{-1}). The typical winter bloom, mainly dominated by diatoms, was registered in the early March 2003 sampling. In December 2003, another winter bloom occurred, co-dominated by diatoms and haptophytes (Gutierrez et al. *in prep*). The summer periods exhibited low concentrations of inorganic nutrients and chlorophyll a (around 0.4 μg Chla L^{-1}) (Fig. 2). High abundances of *Synechococcus* were observed in the spring and summer months, whereas *Prochlorococcus* abounded in autumn.

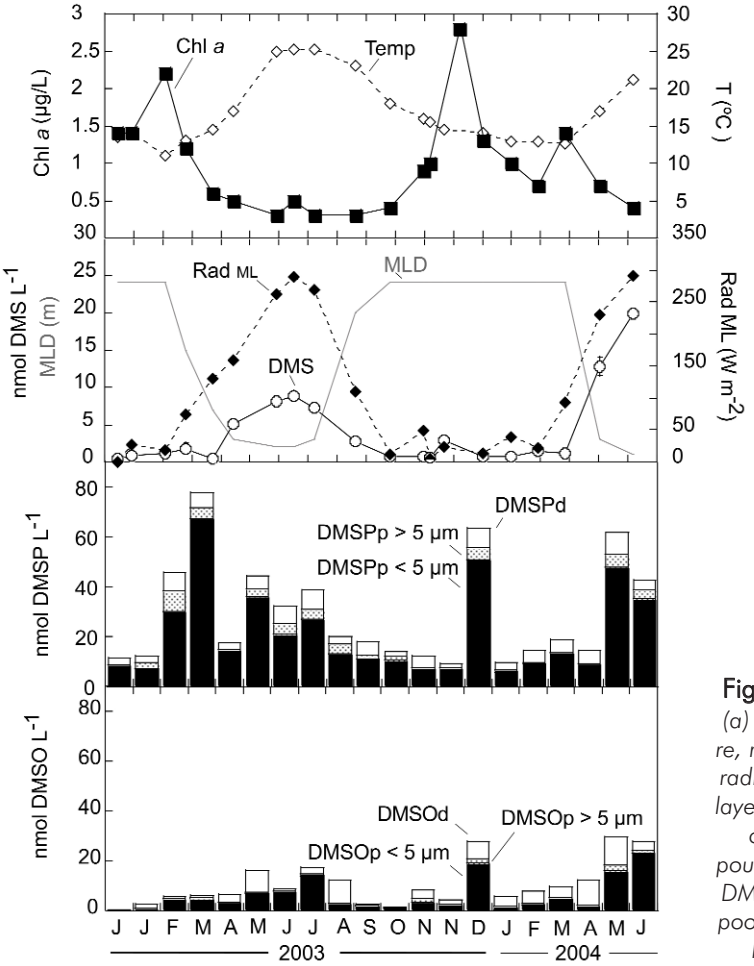


Figure 2. *Seasonal variation of: (a) Chla, sea-surface temperature, mixing layer depth (MLD), and radiation integrated in the mixing layer (Rad ML); (b) concentrations of dimethylated sulfur compounds; and (c) concentrations of DMSP and DMSO divided into 3 pools: particulate <5μm, particulate >5μm, and dissolved.*

Temporal variation in DMS, DMSP and DMSO concentrations. Surface DMS concentrations showed a strong seasonality, with lowest values in winter (0.9 ± 0.2 nmol DMS L^{-1}) and highest values in summer (11.3 ± 5.2 nmol DMS L^{-1}), hence a maximum summer-to-winter ratio of ca. 12 (Fig. 2). DMS concentrations were well correlated with the ML-averaged solar radiation (Vallina & Simó *submitted*) and it did not correlate significantly with total DMSP (DMSPt) or total DMSO (DMSOt) concentrations. The ratio DMS:Chla followed the same seasonal pattern as DMS concentrations (Fig. 3).

Fig. 2c shows the distribution of DMSPt into 3 pools: particulate DMSP < 5 μm (DMSPp<5), particulate DMSP >5 μm (DMSPp>5) and dissolved DMSP (DMSPd), and how they vary with time. The seasonal pattern of DMSPt was mostly contributed by that of DMSPp concentrations. The largest percentage of DMSPt was in the form of DMSPp<5. On annual average, DMSPp>5 only accounted for 10 ± 8 % of DMSPp indicating that the majority of DMSPp producers were in the size fraction smaller than 5 μm. DMSPp concentrations peaked (71.7 nmol L^{-1}) in March, one month after the peak of Chla, and in December 2003 (55.8 nmol L^{-1}), concomitant with a second annual peak of Chla. Values decreased during summer, reaching their annual lowest values in autumn. DMSPd never reached values higher than 8.8 nmol DMSP L^{-1} (annual average 5 ± 2 nmol DMSPd L^{-1}). The DMSPp:Chla ratio showed a similar pattern than DMS concentrations, reaching the maximum values in summer months (Fig. 3).

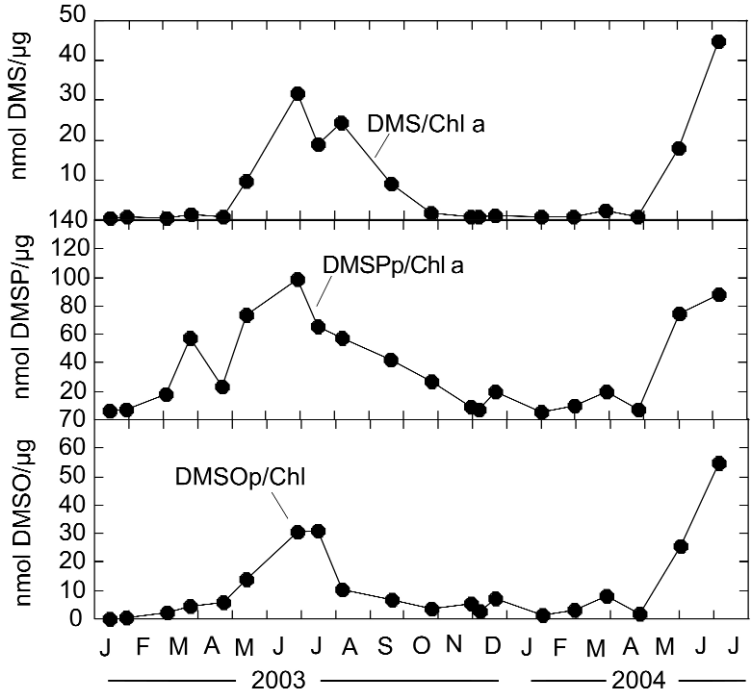
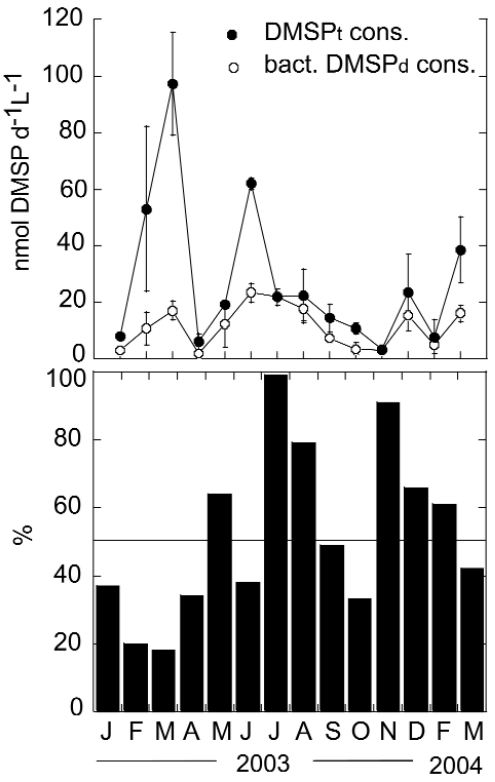


Figure 3. *Seasonality of Chla-normalized concentrations of dimethylated sulfur compounds (DMS, particulate DMSP, and particulate DMSO).*

Except for March 2003, DMSOt followed the pattern of DMSPt, but at lower concentrations. The seasonal variation of the 3 pools of DMSOt is shown in Fig. 2. Like DMSPp, most of the DMSOp (86 ± 21 %) occurred in the <5 μm fraction (DMSOp<5). In general, DMSOp concentrations increased from winter to summer. Maximum annual values were recorded in July 2003 (14.6 nmol DMSOp L^{-1}) and June 2004 (24.2 nmol DMSOp L^{-1}). Exceptionally, in December 2003, DMSOp reached 21 nmol L^{-1} in a peak concomitant with a peak of DMSPp. Annual DMSOd concentrations averaged 4 ± 4 nmol DMSOd L^{-1} and did not show a clear seasonal pattern. The ratio DMSOp:Chla showed the same pattern as DMS concentrations (Pearson's r= 0.95, N=20, p<0.01), with higher values in summer and lower in winter.

DMSP consumption by the plankton community and contribution of heterotrophic bacteria. The rates of DMSPt consumption by the total community ranged between 3.4 and 97.4 nmol DMSPt $L^{-1}d^{-1}$ (Fig. 4). The highest values were found in late winter, coinciding with the peak of DMSPt (March 2003) and in June 2003. The rate constant of DMSPt consumption did not change very significantly over the year; it averaged 0.79 ± 0.16 d^{-1}. This represents that, on annual average, 79% of the stock of DMSPt was renewed daily (Table 1).



Bacterial consumption of DMSPd (measured with ^{35}S-DMSPd) showed a variation pattern similar to that of total DMSP consumption but without the outstanding peaks in March and June (Fig. 4). It averaged 11.1 ± 7.2 nmol DMSPd $L^{-1}d^{-1}$. Bacteria accounted for 52.2 ± 25.2 % of total DMSP consumption (annual average). Higher contributions were observed in summer and early winter (Fig. 4).

Figure 4. *Monthly comparison of the rates of DMSP consumption by the total community (DMSPt cons) and bacterial DMSPd consumption (bact DMSPd cons). The bottom panel shows the contribution of bacteria to the total DMSP consumption. The line indicates the annual average (52%).*

Table 1. *Monthly variation in rate constants (d^{-1}) of biological processes (total DMSPt consumption, microbial DMSPd consumption and microbial DMS consumption) and abiotic processes (DMS ventilation and DMS photolysis). Seasonal averages are given at the end of the table. Winter:W, Spring:SP, Summer:SUM, Autumn:AU.*

Month	Date		MLD (m)	Total DMSPt consump. (d^{-1})	Microbial DMSPd consump. (d^{-1})	Microbial DMS consump. (d^{-1})	DMS ventilation (d^{-1})	DMS photolysis (d^{-1})
January	Jan 28, 03	W	24	0.8	1.2	2.3	0.0	0.0
February	Mar 04, 03	W	24	1.2	1.5	0.0	0.0	2.4
March	Mar 25, 03	SP	24	1.3	2.8	1.0	0.0	1.4
April	Apr 22, 03	SP	15	0.3	0.8	0.9	0.0	0.0
May	May 13, 03	SU	7	0.4	2.2	0.4	0.0	0.9
June	Jun 25, 03	SU	3	2.1	3.3	0.3	0.1	0.5
July	July 14, 03	SU	2	0.8	2.8	0.1	0.1	0.4
August	Aug 04, 03	SU	2	0.9	6.3	0.3	0.0	0.2
September	Sep 16, 03	AU	3	1.0	1.4	0.4	0.0	0.4
October	Oct 21, 03	AU	20	0.8	1.9	1.5	0.0	0.0
November	Nov 25, 03	AU	24	0.9	-	1.0	0.0	0.0
November	Dec 02, 03	AU	24	0.3	1.6	1.2	0.0	0.0
December	Dec 16, 03	W	24	0.4	2.0	0.0	0.0	0.7
January	Jan 26, 04	W	24	-	2.7	1.2	0.0	0.9
February	Feb 23, 04	W	24	0.6	1.0	1.5	0.0	0.4
March	Mar 22, 04	SP	24	1.4	3.1	0.7	0.0	0.0
April	Apr 19, 04	SP	24	0.2	-	0.5	0.0	0.1
May	May 25, 04	SU	3	0.0	-	0.5	0.1	0.1
June	Jun 28, 04	SU	1	1.9	-	0.4	0.2	0.1
	Mean	W	24	0.8	1.7	1.0	0.0	0.9
		SP	22	0.8	2.2	0.7	0.0	0.4
		SU	3	1.0	3.6	0.3	0.1	0.4
		AU	18	0.7	1.6	1.0	0.0	0.1
	Std error	*W*	*0.0*	*0.1*	*0.3*	*0.4*	*0.0*	*0.4*
		SP	*2.*	*0.3*	*0.6*	*0.1*	*0.0*	*0.3*
		SU	*0.9*	*0.3*	*0.7*	*0.0*	*0.0*	*0.1*
		AU	*5.0*	*0.2*	*0.1*	*0.2*	*0.0*	*0.1*

(-) no data

Table 2. *Pearson correlation coefficients (r) for biological rate constants of DMSP consumption by total community (DMSPt cons), bacterial DSPd consumption (DMSPd cons), DMS losses (microbial consumption (K bio), photolysis (K photo) and ventilation (K vent)), and gross DMS production rate, versus physical or easily measurable parameters. Significant correlations at the 95% confidence level are in bold.*

	units	MLD	SST	Rad(ML)	NO_3^-	cDOM	NH_4^+	DMSPp:Chla	DMSOp:Chla	DMS:Chla
K DMSPt cons	d^{-1}	-0.07	0.23	0.21	0.14	-0.03	0.32	0.47	0.40	0.48
K DMSPd cons	d^{-1}	-0.48	0.54	**0.60**	-0.26	-0.09	-0.11	0.50	0.36	**0.68**
DMS prod rate	nM d^{-1}	**-0.75**	**0.60**	**0.74**	-0.33	-0.11	0.03	**0.93**	**0.74**	**0.80**
DMS losses: K bio	d^{-1}	**0.52**	-0.42	**-0.60**	0.11	0.20	0.18	-0.53	-0.54	-0.56
K vent	d^{-1}	**-0.80**	**0.76**	**0.91**	-0.43	-0.37	0.06	**0.74**	**0.93**	**0.88**
K photo	d^{-1}	0.07	-0.35	-0.12	0.49	0.31	0.38	0.11	-0.09	-0.13

Gross DMS production. The DMSP-to-DMS conversion yield (DMS yield) represents the percentage of transformed DMSP that ends up as DMS formation. The annual variation of DMS yield (Fig. 5) showed roughly a seasonal pattern similar to that of DMS concentration, with maximum values during the summer period (20 ± 4.5 %). Two data points fell off this general pattern: January and May 2003, with higher yields but never beyond 37%.

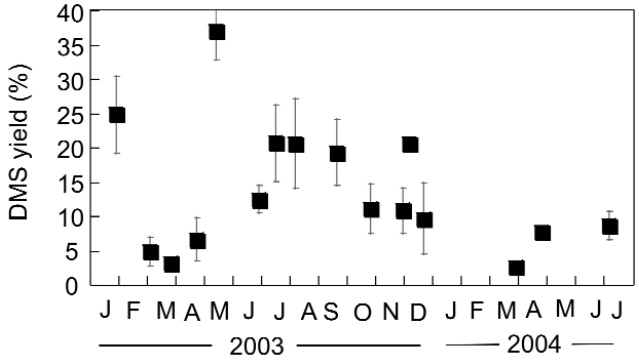
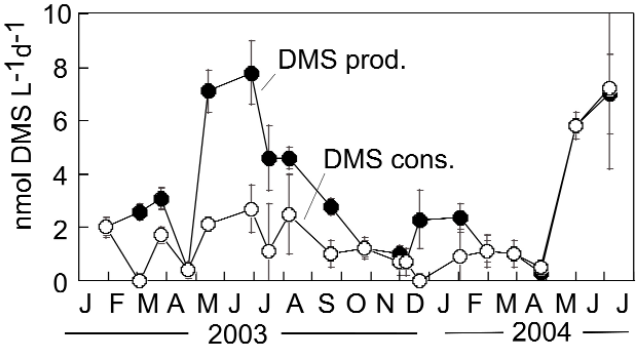


Figure 5. *Seasonal variation of the DMS yield (=DMSP consumption*100/DMS production).*

Figure 6 shows the annual variation of DMS production that, on average, was higher in summer months (6.0 ± 1.7 nmol DMS $L^{-1}d^{-1}$) than in the winter period (1.5 ± 0.7 nmol DMS L^{-1} d^{-1}). DMS production was significantly correlated with the ML-averaged solar radiation (Pearson's, r=0.74, n=20, p<0.001) and with the ratio DMSOp:Chla (Pearson's r=0.74, n=20, p<0.001), and it showed the highest correlation with the DMSPp:Chla ratio (Pearson's, r=0.93, n=20, p<0.001) (Table 2).

Figure 6. *Seasonal variation of DMS production and bacterial DMS consumption rates. .*



DMS loss processes: microbial consumption, ventilation and photolysis. Microbial consumption did not show a clear seasonal pattern over the year (Fig. 6). In general, DMS turnover rates were in the same range of magnitude than DMS production (1.7 ± 0.4 nmol DMS $L^{-1}d^{-1}$) with the exception of the summer months of 2003, when microbial DMS consumption (2.1 ± 0.7 nmol DMS $L^{-1}d^{-1}$) did not increase to reach the high levels of DMS production (6.0 ± 1.7 nmol DMS

$L^{-1}d^{-1}$). Microbial consumption rate constants averaged 0.7 ± 0.2 d^{-1} over the year, with significantly lower values in summer months (Table 1).

Ventilation never dominated amongst DMS sinks (Fig. 7). Highest ventilation rates were observed in summer (0.4 ± 0.1 nmol DMS $L^{-1}d^{-1}$, 3 nmol DMS $L^{-1}d^{-1}$ in June 2004), coinciding with shallower ML and higher DMS concentrations. Ventilation rate constants averaged annually 0.02 ± 0.01 d^{-1} (Table 1).

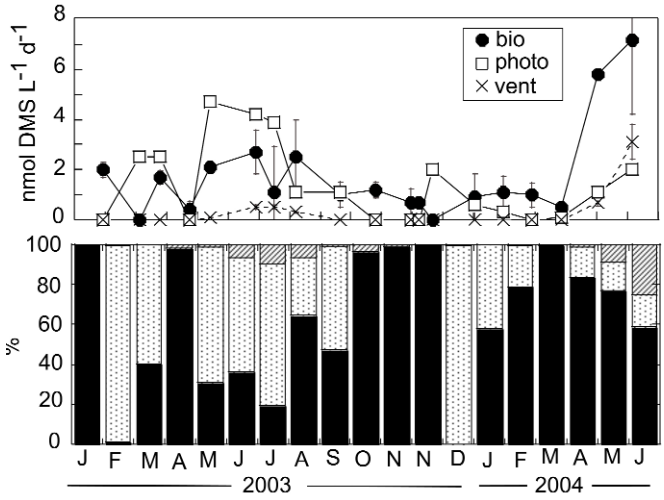


Figure 7. *Seasonal variation of DMS loss rates: microbial consumption (bio), photolysis (photo) and ventilation (vent). The vertical bars show the relative contribution of each DMS sink (bio=black, photo=doted, vent=crossed). It has to be noticed that in April 2003 we sampled after a stormy event.*

Photolysis was the dominant DMS sink from February to July 2003, but not in April 2003, when we sampled after a storm event (Fig. 8). Estimated photolysis rates averaged annually 1.4 ± 0.4 nmol DMS $L^{-1}d^{-1}$, while rate constants averaged 0.4 ± 0.2 d^{-1}. Photolysis rates showed a significant correlation with the ML-averaged solar radiation (Pearson's r= 0.54, n=19, $p<0.015$) but rate constants did not (Table 1). It is important to notice this distinction because rate constants are independent of DMS concentrations. Since photolysis rates were obtained by algebraically budgeting various measurements, they carry large standard errors (of the order of 60%) as calculated by error propagation.

Figure 8 shows the seasonal variation of the rate constants of DMS losses, along with the absorbance of chromophoric dissolved organic matter (cDOM) and the nitrate (NO_3^-) concentrations. The absorbance of cDOM did not exhibit a strong seasonality but decreasing trends from Chla peaks towards the late summer were visible probably due to the photobleaching effect (Toole et al. 2003). A strong increase in cDOM absorbance occurred during the stormy period between September and October 2003. Summer waters were characterized by very low NO_3^- concentrations (<0.09 μmol L^{-1}), which were higher, yet variable, during the rest of the year (0.21-7.11μmol L^{-1}). Both cDOM and NO_3^- have been suggested as photosensitizers for photochemical DMS destruction (Toole et al. 2003, Toole et al. 2004, Bouillon & Miller

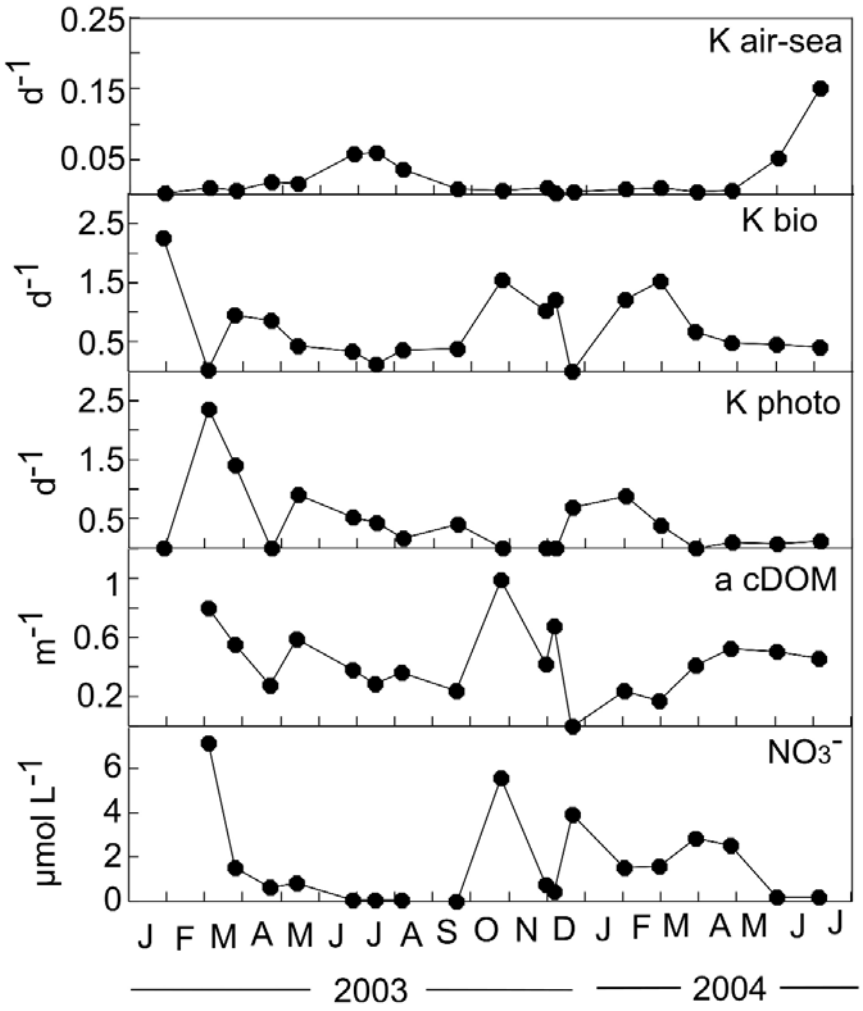


Figure 8. *Seasonal variation of the rate constants of DMS ventilation (K air-sea), DMS microbial consumption (K bio), and DMS photolysis (K photo). The absorbance of colored components of dissolved organic matter at 320 nm (a cDOM), and nitrate concentrations (NO_3^-) are also shown.*

2004). In our annual series, however, none of them showed a significant correlation with the photolysis rate constants except for the period February-July 2003, that is, when photolysis was the dominant DMS sink. No correlation was found either between the potential photosensitizers and dissolved DMSO, one of the major products of DMS photooxidation (Kieber et al. 1996).

Discussion

The concentrations of dimetylated sulfur compounds in surface seawater result from a tight web of biological and physicochemical processes that are expected to undergo seasonal variations. We found that not only the DMS concentration, also other variables like the DMSP:Chla and DMSO:Chla ratios showed clear seasonal patterns, with maximum values in summer and lower values in winter. This same seasonal pattern was followed by gross DMS production, which in turn was also well correlated with some physical factors such as the MLD and the ML-averaged solar radiation, suggesting that these physical forces might be driving the physiological responses of the organisms. Assessing the importance of the coupling between biological processes and physical factors and the sign of the potential forcings is crucial to understand the dynamics of dimethylated sulfur compounds in the ocean and their role in the global sulfur cycle.

DMS/DMSP/DMSO pools. DMS concentrations (0.5 - 8 nmol DMS L^{-1}) were in the same broad range of those found in the Mediterranean (0.3-7.3 nmol DMS L^{-1}, Simó et al. 1997) and in oligotrophic oceanic waters (0.5-4 nmol DMS L^{-1}, Dacey et al. 1998, 0.7-3.5 nmol DMS L^{-1}, Kettle et al. 1999). The pronounced seasonality of DMS concentrations observed in Blanes Bay is a common feature in the temperate and subtropical open ocean as well as in coastal regions where similar time series measurements have been performed (Leck et al. 1990, Berresheim et al. 1991, Turner et al. 1996, Dacey et al. 1998). In agreement with these studies, DMS concentrations peaked in mid summer, i.e., 2-3 months later than the DMS precursor DMSP, and in the season when phytoplankton biomass is at its minimum. This has been called the "DMS summer paradox" (Simó & Pedrós-Alió 1999a).

Most of the DMSP occurred in particulate form in the size-fraction smaller than 5 um. This size distribution did not change significantly over the year, suggesting that most DMSP producers were among the nanoplankton and below, despite the seasonal succession of the phytoplankton assemblages. Previous studies have reported similar results for other oceanic sites. Belviso et al. (2001) averaged values from highly contrasted trophic regions and found that DMSPp $<10\ \mu$m accounted for 65 ± 16 % of total DMSPp. Scarratt et al. (2002)observed that most of total DMSP in the northwest Atlantic was in the size-fraction 2-11 μm.

Attempts to correlate DMS or DMSP to Chla concentrations have failed in many studies (Leck et al. 1990, Turner et al. 1996, Dacey et al. 1998, Keller et al. 1999), due to the taxon-specificity of DMSP biosynthesis and a potential physiological regulation of the intracellular DMSP content. In our study, besides a lack of proportionality between dimethylated sulfur compounds and Chla we also observed a strong seasonality of the ratio DMS:Chla, similar to that found in other oceanic regions (see a compilation in Uher et al. 2000). A remarkable finding of our work is that the DMSP:Chla and DMSO:Chla ratios also exhibited this same seasonal pattern (Fig. 3). Identifying whether this pattern resulted mainly from

phytoplankton succession or physiological adaptations is not a straightforward task. The succession of phytoplankton in Blanes Bay has been studied (e.g. Mura et al. 1996). In brief, diatoms dominate in the winter period, followed by a succession towards assemblages with high abundances of *Synechococcus* (spring and late summer) and *Prochlorococcus* (autumn). In our annual study, from March to October microphytoplankton was dominated by haptophytes and, interestingly, in the size-fraction bigger than 3 μm, a peak of dinoflagellate numbers was observed from June to August (Gutierrez et al. *in prep*.). Thus, elevated abundances of two known groups of high DMSP producers (haptophytes and dinoflagellates, Stefels 2000) coincided in the summer period.

The fact that DMSO:Chla followed the same pattern as DMSP:Chla supports the suggestion that physiological adaptation also plays a role. An algal origin for DMSO has already been reported in previous studies (Simó et al. 1998, Simó & Vila-Costa 2006). With a culture manipulation study, Sunda et al. (2002) postulated that phytoplankton increase intracellular DMS and DMSP relative to Chla levels under exposure to high UV radiation doses. These authors proposed a cascade of reactions that starts with DMSP and evolves DMS and DMSO, which all scavenge hazardous reactive oxygen species and protect the cell against oxidative stress. It is very likely that this antioxidant adaptation contributed to the elevated DMS:Chla, DMSP:Chla and DMSO:Chla ratios obtained in summer and to the seasonal correlation between these ratios and the ML-averaged solar radiation.

DMSOd can result from DMS photolysis (Kieber et al. 1996), microbial DMS oxidation (del Valle & Kiene *submitted*) and algal DMSO release (Simó et al. 1998). The relative importance of each of these sources has never been quantified in field studies. The absence of any significant correlation between DMSOd and either DMS photolysis or DMS consumption rates could be explained by the fact that both biological DMS consumption and photooxidation give rise to various products. For example, Kieber et al. (1996) found that only 14% of DMS was converted to DMSO and del Valle & Kiene (*submitted*) demonstrated that DMS could be converted into DMSO or SO_4^{2-} and the switch between both pathways was probably dependent on the composition of the microbial assemblage. In contrast, DMSOp was positively correlated to DMSPp (Pearson's $r=0.66$, $p<0.01$, $n=20$). The ratio DMSP:DMSO has been found to be inversely correlated with seawater temperature (Simó & Vila-Costa 2006), a finding that tentatively supports the possible antioxidant role of DMSO in the algal cells.

DMSP loss processes: contribution of bacterioplankton. DMSPt consumption rates measured with the "dark net loss" method were always higher than DMSPd consumption rates measured with ^{35}S-DMSP. The range of DMSPt consumption (3-60 nmol DMSP $L^{-1}d^{-1}$) and DMSPd turnover (2-24 nmol DMSP $L^{-1}d^{-1}$) were similar to previous studies in coastal and open-ocean waters (1.4-16.8, Ledyard & Dacey 1996b; 4-28, Kiene & Gerard 1995; 7-76, Simó et al. 2000; 3.8 (open ocean)-39 (coastal), Kiene et al. 2000; 17-20, Zubkov et al. 2002; 1.1-20.5,

Malmstrom et al. 2005), never reaching the high values measured in dense blooms of strong DMSP producers (van Duyl et al. 1998, Simó et al. 2000).

Turnover times of DMSPt annually averaged 1.3 d, implying that 79% of the stock was renewed daily. This result is in agreement with the average of daily phytoplankton growth consumed by microzooplankton (67%) in the compilation by (Calbet & Landry 2004).

It is generally accepted that release of DMSPd and its subsequent use by heterotrophic bacterioplankton is the dominant mechanism for DMSP transformation (Kiene et al. 2000, Zubkov et al. 2002). DMSPd is indeed a very labile compound and a good substrate for the growth of a broad spectrum of marine bacteria (Malmstrom et al. 2004a, Chapter II). But algal DMSP is also transformed in the food web via a variety of other processes, including cleavage by the producer (Stefels 2000, Sunda et al. 2002) or loss upon zooplankton grazing on phytoplankton (Dacey & Wakeham 1986, Wolfe & Steinke 1996, Archer et al. 2001b, Archer et al. 2003, Tang & Simó 2003). The actual contribution of heterotrophic bacteria to community-mediated total DMSP loss remained unknown. By the simultaneous use of two different methods (dark DMSPt net loss and radiolabeled DMSPd loss), we have shown that, on average, 52% of total DMSP transformations occurred trough the DMSPd pool. This sets an upper end for the contribution of heterotrophic bacterioplankton to DMSP loss, since we have recently reported that non- or low-DMSP producing phytoplankton also act as a sink for DMSPd (Chapter V). Anyhow, this contribution of DMSPd consumers to DMSP loss changed over the year and, interestingly, it was minimal coinciding with the maxima of DMSPp production (February, March and June 2003, March 2004), leaving room for the dominance of other loss processes such as microzooplankton grazing. This finding suggests that the coupling between microzooplankton grazing and DMSP-producing phytoplankton growth was tighter during high biomass or production periods, as described previously for total phytoplankton assemblages (Strom et al. 2001). However, this likely higher grazing pressure in the winter period was not reflected in an increase of DMS concentrations as it could be expected after other studies (Belviso et al. 1990, Levasseur et al. 1996, Wolfe & Steinke 1996, Wolfe et al. 2000, Archer et al. 2003). This might have been masked by the low frequency of our sampling schedule. Overall, the role of phytoplankton and zooplankton in DMSP consumption is more relevant than previously believed and their impact as sources of DMS has to be explored in more detail.

DMS production versus microbial DMS consumption. Measurements of DMS production still represent a challenge. We chose to use the DMDS inhibition method (Wolfe & Kiene 1993, Simó & Pedrós-Alió 1999b). Although this method does not allow independent measurements of DMS consumption and production, and a possible stimulation of DMS production by addition of DMDS would give overestimates of DMS consumption, it has been successfully used in many studies to estimate DMS production (van Duyl et al. 1998, Simó & Pedrós-Alió 1999b, Wolfe et al. 1999, Simó et al. 2000).

Similar to the loss rates of DMSPt and DMSPd, DMS production and consumption rates were within the same range (0.1-7.7 nmol DMS $L^{-1}d^{-1}$) than most measured in other marine regions (0.5-3.6, Ledyard & Dacey 1996b, 0.3-5.1, Wolfe et al. 1999, 1.1-6.8, Yang et al. 2000, 1.1-8.1, Simó et al. 2000, see also a compilation in Simó 2004), and much lower than those measured in blooms of DMSP producers (Van Duyl et al. 1998, Simó et al. 2000).

In general, DMS consumption was of a magnitude similar to DMS production. The most salient exception was the late spring and summer months, and particularly May and June, when the increase of DMS production was not matched by DMS consumption (Fig. 6). Reasons for this decoupling might be either kinetic or environmental. Kinetic studies have consistently found that bacterial DMS consumption saturates at 10-30 nmol DMS L^{-1}, so that higher DMS concentrations caused by high production rates would lead to a decoupling between DMS consumption and production (Kiene & Service 1991, Kiene 1992, Wolfe & Kiene 1993, Ledyard & Dacey 1996a, Wolfe et al. 1999, Scaratt et al. 2000). Nevertheless, in studies conducted with blooms of DMSP producers high DMS consumption rates have been measured concomitantly with high DMS production rates (Kwint & Kramer 1996, Simó et al. 2000). Thus, inhibition of DMS consumption by environmental factors in the summer period seems a more plausible explanation. It is likely that UV radiation, enhanced by the shallow stratification of the water column, caused inhibition of DMS-consuming bacteria (Slezak et al. 2001, Simó 2004, Toole et al. 2006).

Interestingly, no inhibition of bacterial DMSPd consumption was apparent in the summer period. Rather, high rates were recorded in this period (Fig. 4) and, over he annual cycle, rate constants of DMSPd consumption were positively correlated with the ML-averaged solar radiation dose (Table 2). In contrast, microbial DMS consumption showed similar values over the year (Fig. 6), and, actually, the dramatic increase of DMS concentration in summer did not seem to stimulate DMS consumption as it has been observed in the aforementioned kinetic studies. Rather, rate constants of bacterial DMS consumption were negatively correlated with the ML-averaged solar radiation dose (Table 2). This different behavior through seasons may result from a different phylogenetic identity of DMS and DMSP consumers: DMSP consumption is carried out by a wide spectrum of marine bacteria (Chapter II, Malmstrom et al. 2004a) while DMS consumption seems to be restricted to some specific groups (Chapter V). It has been shown recently that some phylogenetic groups are more resistant to solar radiation than others (Alonso-Sáez et al. 2006). Additionally, other recent studies have shown the capacity of non-DMSP producing photoautotrophs to assimilate DMSPd from the medium (Malmstrom et al. 2005, Chapter V). Since pigmented-cells have higher resistance to photoinhibition, their contribution as a DMSPd sink could partially explain the higher DMSP consumption rates observed in summer. These findings contrasts with those of the laboratory studies reported by Slezak et al. (2001), who found that both DMSP and DMS consumption were inhibited by UV radiation.

DMS loss processes. In agreement with previous studies, ventilation was a minor DMS sink (Simó & Pedrós-Alió 1999a, Toole et al. 2006), whereas estimated photolysis and microbial consumption alternated their dominance throughout the year (Fig. 7). Ventilation was significantly higher in the summer period, due to the shallower stratification of the water column. DMS air-sea flux ranged from 0.032 to 3.1 μmol DMS $m^{-2}d^{-1}$, values slightly lower than those measured in oceanic sites (e.g. Berresheim et al. 1991, Turner et al. 1996).

Altogether, DMS losses averaged 0.4 d^{-1} ($\pm$ 0.2), thus giving turnover times of 2.5 days, slower than those measured in a bloom of coccolithophores (0.4-1.6 days, Simó & Pedrós-Alió 1999b) and within the range of those measured in the equatorial Pacific (1-4 days, Kieber et al. 1996) and in the North Atlantic drift (2.2-3.9 days, Wolfe et al. 1999).

In a short-term (15 days) study, Simó & Pedrós-Alió (1999b) observed that the relative importance of each DMS sink was strongly dependent on the variability of its physical driver. Over a seasonal scale in the Sargasso Sea, Toole et al. (2003) showed that photolysis was higher in the summer period mainly due to the higher doses of solar radiation. In Blanes Bay, photolysis dominated from February to July 2003, and microbial consumption dominated over the second half of that year and first half of 2004. DMS photolysis occurs through secondary photosensitizers (such as cDOM or NO_3^-) that absorb the photon flux at wavelengths $>$ 260 nm, leading to the photodestruction of DMS (Toole et al. 2003 and 2006). Variations of cDOM absorbance were well correlated with the DMS photolysis rate constant during the period of photolysis dominance (Fig. 8). During fall and winter, when microbial consumption was the dominant DMS sink, cDOM absorption was somewhat correlated with this biological loss. Although cDOM and DOC concentrations are independent of each other (Siegel et al. 2002), cDOM absorbance in fall and winter seems to reflect DOC bioavailability to bacteria. cDOM absorbances in Blanes Bay were similar to those measured in coastal stations (Toole et al. 2006), and slightly lower than values typical of the open ocean (Nelson & Siegel 2002). A period of stormy weather started late September 2003, possibly affecting the levels and composition of cDOM.

General picture of the seasonal cycle of dimethylated sulfur compounds in Blanes Bay. The methods we used to measure the fluxes of DMSP and DMS have one-day resolution time; they are not equal to monthly changes. Thus, we can not see the monthly trends of the fluxes, but we can track variations month by month, and compare contrasted periods as it is shown in Fig. 9, comparing winter versus summer.

In our year-round study, attempts to relate DMSP consumption to environmental factors only gave a significant correlation with the ML-averaged solar radiation dose (Table 2). Gross DMS production, microbial DMS loss, and DMS ventilation showed correlation with almost all physical factors: MLD, SST, and the ML-averaged solar radiation (Table 2). DMSP

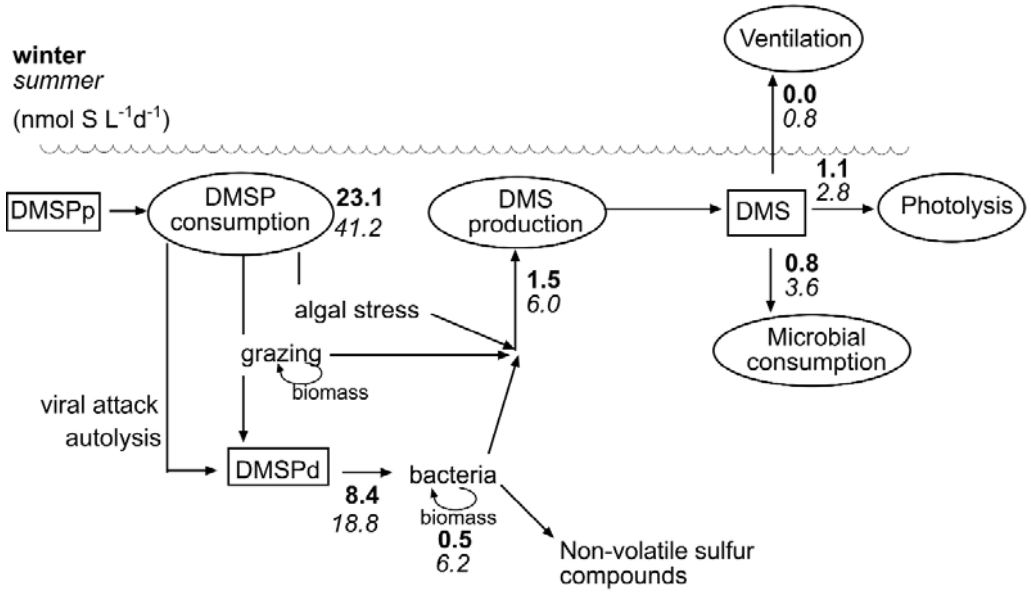


Figure 9. *Diagram of the DMS/P cycle comparing contrasted measured DMSP and DMS transformation rates (nmol S $L^{-1}d^{-1}$) in winter and summer. Data on bacterial DMSP assimilation is taken from Chapter III.*

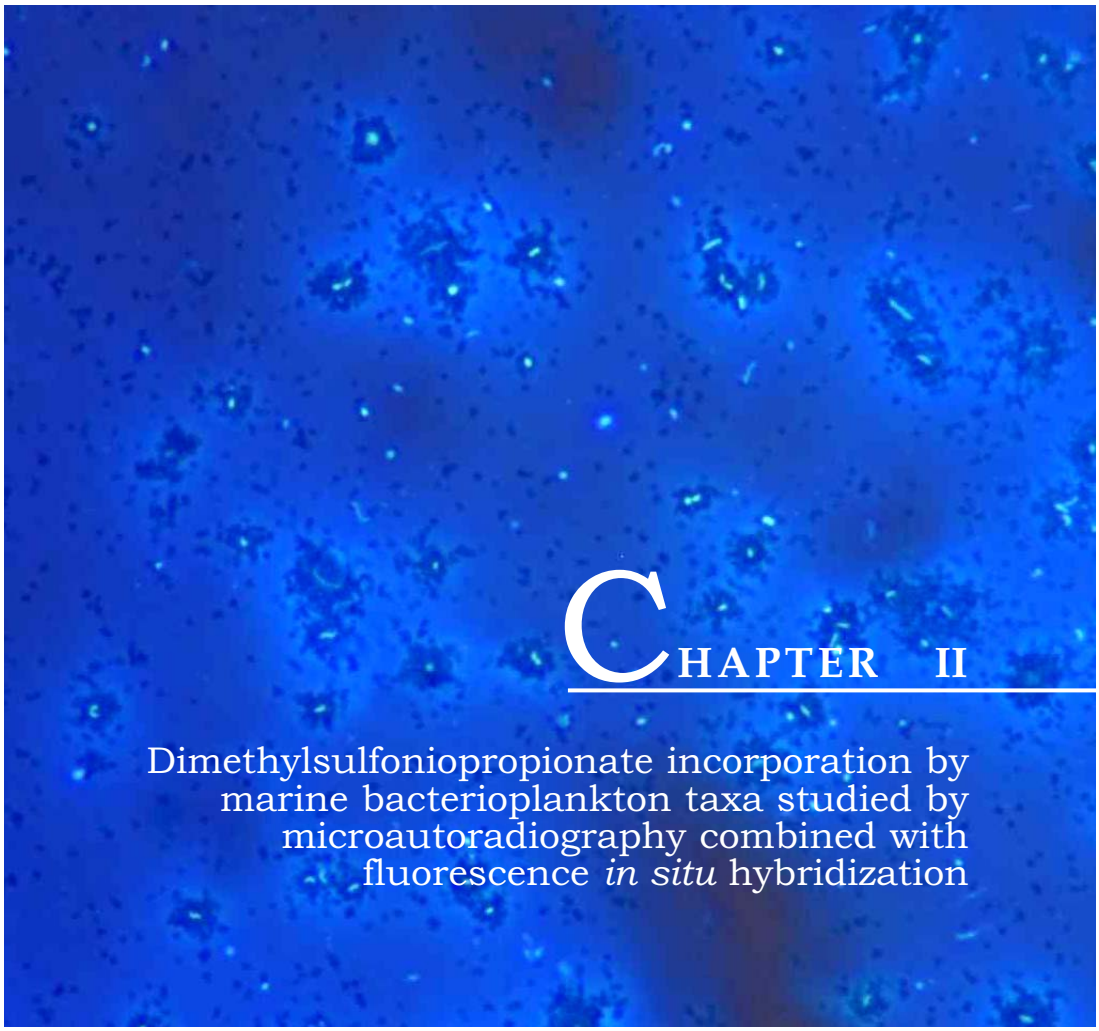
transformations seem to be mainly driven by the dynamics of the food-web, whereas DMS seems to ultimately result from physical forces as it had been postulated in previous studies (Simó & Pedrós-Alió 1999a, Toole & Siegel 2004).

DMSP consumption did not show the same year-round pattern as DMS concentration or production, which resulted in a seasonally changing yield of DMSP cleavage into DMS. Starting from 5% in February 2003, it increased to 20% in mid summer and decreased again as it went into autumn and winter. The annual average (12%) is at the lower end of DMS yields measured in other oceanic and coastal sites (mostly 5-80%, average 30%, Simó & Pedrós-Alió 1999a). We have shown that the DMS accumulation in the summer period (the "summer paradox") resulted from an increase of DMS production that was not matched by DMS losses. From our data, can we elucidate who were the main players in this decoupling?

As discussed before, the increase of DMS production in summer can not be attributed to bacterioplankton only. Although DMSPd consumption (mostly bacterial) accounted for most of all DMSP transformations (70 ±13%) in the summer period, bacterial DMS yield measured in August (8.0 ± 2.5%) did not fully explain DMS yield by total community (20.6 ± 4.0 %). In fact, bacterial DMSP assimilation was higher in this period (see Fig. 9, data from Chapter III) and diverted a larger fraction of DMSP from giving rise to DMS production. Upon grazing by

zooplankton, algal DMSP may follow 3 transformation pathways (Fig. 9): (1) release to the DMSPd pool, which becomes available to heterotrophic bacteria and non DMSP-producing phytoplankton, (2) assimilation by the grazer (Tang et al. 1999, Archer et al. 2001b, Tang & Simó 2003), and (3) cleavage into DMS by algal DMSP lyases, a process mediated by cell disruption during capture, ingestion or digestion (Dacey & Wakeham 1986, Wolfe & Steinke 1996). In a compilation work, grazing of microzooplankton has been found to be relatively constant at annual scale (Calbet & Landry 2004). Besides, mesozooplankton never accounted for more than 6% of the grazing impact of the whole community in a seasonal study taken 45Km south of our sampling site (Broglio et al. 2004). It seems thus plausible that a large proportion of the summer increase in DMS production comes from the DMSP-producers themselves. The anti-oxidant hypothesis proposes that DMS leaks out of the algal cell as a subproduct of a radical scavenging reaction chain in response to exposure to high levels of UV radiation (Sunda et al. 2002). Summer would then be the season when algal DMS release would be maximal. This conclusion is supported by the good correlation between DMS production rates, the DMSP:Chla and DMSO:Chla ratios, and the ML-averaged solar radiation doses.

Overall, our results provide insights into the complex mechanisms of the epipelagic ecosystem that configure the oceanic DMS cycle. The occurrence of the "DMS summer paradox" seems to support the hypothesized feed-back between climate and DMS production by marine biota at the seasonal scale. Climate forces typical of summer months, such the increase of solar radiation and the associated increase of the air-to-sea heat flux, result in shallower stratification of the water column that in turn magnifies the doses of solar radiation in the mixing layer. These physical forces drive abiotic transformation processes, and also the photobiological responses of organisms, as it has been observed in this study. Technical development is needed to quantitatively determine the relative contributions of the main players (phytoplankton, grazers and heterotrophic bacteria) to the production of DMS, and assess how these contributions are affected by solar radiation doses and other environmental forces.



CHAPTER II

Dimethylsulfoniopropionate incorporation by marine bacterioplankton taxa studied by microautoradiography combined with fluorescence *in situ* hybridization

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Abstract

The fraction of planktonic heterotrophic bacteria capable of incorporating dissolved dimethylsulfoniopropionate (DMSP) and leucine was determined at two coastal sites by microautoradioagraphy. In Gulf of Mexico seawater microcosm experiments, the proportion of prokaryotes that incorporated sulfur from ^{35}S-DMSP ranged between 27 and 51% of DAPI-positive cells; similar to or slightly lower than the proportion incorporating ^{3}H-leucine. In the NW Mediterranean coast, the proportion of cells incorporating sulfur from ^{35}S-DMSP increased from 5 to 42% from January to March, coinciding with the development of a phytoplankton bloom. At the same time, the proportion of cells incorporating ^{3}H-leucine increased from 21 to 40%. The combination of microautoradiography and fluorescence *in situ* hybridization (FISH) revealed that the *Roseobacter* clade (Alphaproteobacteria) accounted for 13 to 43% of the microorganisms incoporating ^{35}S-DMSP at both sampling sites. Significant uptake of sulfur from DMSP was also found among members of the Gammaproteobacteria and *Cytophaga-Flavobacterium* groups. *Roseobacter* and Gammaproteobacteria exhibited the highest percentage of DAPI-positive cells incorporating ^{35}S from DMSP (around 50%). Altogether, the application of microautoradiography with ^{35}S-DMSP combined with FISH indicated that utilization of S from DMSP is a widespread feature among active marine bacteria, comparable to leucine utilization. These results point toward DMSP as an important substrate for a broad and diverse fraction of marine bacterioplankton.

Introduction

The great majority of dimethylsulfoniopropionate (DMSP) produced by phytoplankton is rapidly cycled within the upper ocean and may be a major carrier for transferring sulfur and carbon among microorganisms in the marine food web (Bates et al. 1994, Kiene et al. 2000, Malin & Kirst 1997, Simó et al. 2002). Recent studies have shown that DMSP can account for 50% of the sulfur flux and approximately 10% of the carbon flux through various trophic levels in microbial food webs dominated by DMSP-producing phytoplankton (Burkill et al. 2002, Simó et al. 2002). Only a minor fraction of the gaseous by-products of DMSP degradation, mainly dimethylsulfide (DMS), escapes a tight cycling in the water column and vents to the atmosphere (Bates et al. 1994, Kiene & Linn 2000a, Ledyard & Dacey 1996b, Simó & Pedrós-Alió 1999b). Despite being a small leakage from a much larger flux, sea-to-air DMS emission is enough to constitute the largest natural source of tropospheric sulfur at a global scale (Andreae & Crutzen 1997).

DMSP is an intracellular reduced sulfur compound produced by a wide variety of unicellular algae to fulfill a number of physiological functions, mainly as a compatible solute and oxidant scavenger (Stefels 2000, Sunda et al. 2002). It is released into the dissolved organic matter (DOM) pool through algal autolysis, viral lysis and grazing by zooplankton (Simó 2001, Yoch 2002). Bacterioplankton are the main agents in the turnover of dissolved DMSP through two main pathways (Kiene et al. 2000, Yoch 2002): direct cleavage to DMS mediated by the enzyme DMSP-lyase, and demethylation/demethiolation to methanethiol (MeSH), a key intermediate for the assimilation of S into protein (Kiene et al. 1999, Kiene et al. 1996). As a substrate for heterotrophic bacterioplankton, DMSP has been shown to supply 1-15% of the carbon demand and 50-100% of the sulfur demand (Kiene & Linn 2000a, Zubkov et al. 2001b, Simó 2001). Kiene et al. (2000) hypothesized that bacteria exert a control on DMSP dynamics (including DMS production) by preferentially using the demethylation/demethiolation pathway over the lyase pathway when the dissolved DMSP concentration is low relative to the S demand. Crucial to this "bacterial switch" hypothesis (Simó 2001) is whether DMSP utilization is widespread among members of the bacterioplankton, allowing many baterial taxa to participate in DMSP dynamics, or whether it is characteristic of only a few phylotypes, potentially with more limited distribution and ecological impact.

Few studies have tried to link the activity and phylogeny of DMSP-degrading bacteria in marine environments (González 1999, González et al. 2000, Ledyard & Dacey 1994, Yoch et al. 1997, Zubkov et al. 2001b). The bacterial assemblage associated with a bloom of the coccolithophore *Emiliania huxleyi* (an alga producing high amounts of DMSP) in the North Atlantic was dominated by *Roseobacter*, and a correlation was found between the abundance of this phylogroup and the concentration of DMSP (González et al. 2000). Similarly, in the northern North Sea, a single *Roseobacter* species dominated the bacterioplankton assemblage

associated with a bloom of DMSP-producing phytoplankton, and a close correlation between the abundance of this species and the loss rate of dissolved DMSP was found (Zubkov et al. 2001b). These field studies suggested a prominent role of the *Roseobacter* group among DMSP-degrading bacteria in the ocean, a capability that had been already demonstrated by experimental work with cultures of marine isolates (González et al. 1999, Kiene et al. 1999, Ledyard & Dacey 1994, Moran et al. 2003).

Despite the important body of evidence generated by this work, neither field studies nor culture work provide direct observations of the link between DMSP degradation and the *Roseobacter* group in the sea. First, spatial correlation between substrate concentrations or activity rates with the abundance of a particular phylotype does not definitely prove that the process is carried out by this phylotype. Second, culturable marine prokaryotes, even if members of major marine lineages, do not always represent the species and subspecies-level taxa that dominate *in situ* (Fuhrman et al. 1993, Giovannoni & Rappé 2000).

The extent of DMSP utilization among marine bacterioplankton, the phylogenetic composition of bacterial DMSP consumers, and the quantitative importance of DMSP consumers in the bacterial assemblage have not been determined. Single cell analysis methods which rely on interrogation of individual cells for information on identity and activity are the best experimental approach to answer these questions. Microautoradiography (Brock & Brock 1968, Pedrós-Alió & Newell 1989) provides information on the percentage of cells that take up a given compound by visualizing radioactivity incorporated into individual cells. The powerful combination of microautoradiography with fluorescence *in situ* hybridization (MicroFISH or STARFISH methods; Cottrell & Kirchman 2000, Lee et al. 1999, Ouverney & Furhman 1999) allows individual active cells to be assigned to phylogenetic groups.

We have developed protocols for microautoradiography and MicroFISH analyses of the extent of ^{35}S-DMSP-utilization activity in marine prokaryotes. Here we describe experiments carried out for optimization of methods and report on the application of these protocols to marine bacterioplankton communities from two sampling sites at different periods of the year. Throughout the study, ^{35}S-DMSP incorporation was compared to ^{3}H-leucine incorporation, since leucine is considered one of the most universal substrates for heterotrophic bacterioplankton (Kirchman et al. 1985).

Materials and Methods

Sampling. On 8 June 2001, surface seawater was collected in clear waters off Mobile Bay in the vicinity of Dauphin Island, in the northern Gulf of Mexico (30° 15' N, 88° 05' W) by submerging rinsed polycarbonate carboys to a depth of 0.5 m. Carboys were kept at ambient temperature in closed coolers until use in a microcosm experiment (see details below).

Table 1. Characteristics of the water samples used.

Sample	Temp (°C)	Salinity (psu)	Chl *a* (μg liter^{-1})	Prokaryotic cells (10^5) ml^{-1}	Bacterial production (pmol·liter^{-1}·h^{-1})	[$DMSP_d$] (nM)	[$DMSP_p$] (nM)	$DMSP_p$/chla (nmol μg^{-1})	AU-positive cells	
									[^{35}S]DMSP (%)	[^{3}H]leucine (%)
DIME, Gulf of Mexico[a] (8–16 June)										
Nutrient amended, day 2	27	33.3	1.00	13.6	64	5.2	24.4	24.4	50	70
Nutrient amended, day 5	27	33.3	1.67	13.6	378	5.0	46.1	27.6	51	45
Nutrient amended, day 8	27	33.3	1.39	20.0	468	4.4	73.8	53.1	51	63
Control, day 2	27	33.3	0.39	15.1	58	4.0	18.5	47.4	ND[b]	71
Control, day 5	27	33.3	0.27	10.0	41	2.5	13.0	48.1	38	47
Control, day 8	27	33.3	0.40	9.2	39	3.4	15.6	39.0	27	55
Blanes Bay, NW Mediterranean Sea										
13 January	13.5	37.4	1.35	6.1	3.8	2.7	8.8	6.5	5	21
28 January	14	37.5	1.51	8.2	2.6	2.2	7.8	5.2	10	25
4 March	11	36.1	2.21	8.9	42	11.6	42.4	19.2	42	40

[a] Each data point is the average of two replicate carboys. Amended, spiked with P and N at Redfield ratio.
[b] ND, not determined.

On 13 and 28 January and on 4 March 2003, surface seawater was collected in the same manner approximately 1 mile offshore in the Bay of Blanes (41° 40' N, 2° 48' E), 70 km north of Barcelona (NW Mediterranean). In this case, we sampled typical winter conditions and the onset of a natural phytoplankton bloom. Characteristics of the water samples are detailed in Table 1.

The Dauphin Island Microcosm Experiment (DIME). Gulf of Mexico water (salinity 33.3 ‰) was pre-filtered through a 130 μm mesh to exclude large zooplankton and was partitioned between four 25-liter polycarbonate carboys. Carboys were placed in a climate-controlled chamber set at 27°C (*in situ* temperature at the time of sampling) with an artificial light source (12 hours light and 12 hours dark, 200 μE cm^{-2}). Two carboys were amended with 10 μM NO_3^- and 0.6 μM PO_4^{3-} (labeled NUT1 and NUT2), and two carboys were maintained as controls (no amendments, labeled C1 and C2). The carboys were sampled daily at 10 am, immediately before the light was turned on. Two liters of water were collected into polycarbonate bottles by siphon with an acid rinsed pipette connected to silicon tubing. Gentle mixing of the carboys was provided once daily by turning them upside down twice before sampling. The microcosm experiment lasted for 8 days.

Chemical analyses. Chlorophyll *a* was measured by fluorometry in 90% acetone extracts (Parsons et al. 1984). Dissolved DMSP (DMSPd, GF/F filtrate) and particulate DMSP (DMSPp, retained by GF/F) were measured as DMS after alkaline hydrolysis. The evolved DMS was determined by gas chromatography, either by the headspace sweeping method (Kiene & Gerard 1994) for DIME or by water purge and trap for the NW Mediterranean samples (Simó et al. 1996).

Bacterial numbers and heterotrophic production. Samples for enumeration of bacteria were preserved with 0.2 μm pore-size filtered formaldehyde (final concentration, 2% [wt/vol]). Within 48 h, total cell counts of bacteria were determined by epifluorescence microscopy after staining cells with 4´, 6-diamidino-2-phenylindole (DAPI; 2 μg ml^{-1} for 10 min) and filtering them onto black 0.2 μm pore-size polycarbonate filters at a vacuum pressure of 100-200 mm Hg (Porter & Feig 1980).

Bacterial production was determined by incorporation of ^{3}H-leucine using the method of Kirchman et al. (1985) with the modifications of Smith & Azam 1992. For each seawater sample, live and killed (5% trichloroacetic acid) controls were incubated with ^{3}H-leucine (final concentration 20 nM for DIME; 40 nM for NW Mediterranean) for 1 to 2 h, at *in situ* temperature, in the dark.

Microautoradiography. Incubations for microautoradiography were carried out with samples from the DIME and from the NW Mediterranean (Table 1). Two radioactive substrates were used:

^{3}H-leucine (specific activity: 98.84 Ci mmol^{-1} in DIME, 161 Ci mmol^{-1} in the NW Mediterranean) and ^{35}S-DMSP (specific activity: 3.3 Ci mmol^{-1} in DIME, 203.1 Ci mmol^{-1} in the NW Mediterranean). Aliquots of 5 ml from each time point in DIME and 30 ml samples from the NW Mediterranean were incubated in the dark at in situ temperature with ^{3}H-leucine for ~ 5 hours or ^{35}S-DMSP for ~13 hours. A formaldehyde-killed control was prepared for each incubation. Incubations were stopped with formaldehyde (4% final concentration). Microautoradiograms were prepared by filtering 1 ml (DIME) or 5 ml (NW Mediterranean) aliquots through 0.2 μm pore-size polycarbonate filters (Nucleopore), thus providing 5 or 6 replicates per sample. The filters were rinsed twice with 0.2 μm pore-size filtered seawater to remove unincorporated radiolabel and were removed from the filtration apparatus without disconnecting the vacuum. Filters were air dried and stored at -20°C until processed.

Microautoradiography was carried out essentially as described by Pedrós-Alió & Newell (1989). In the darkroom, slides were dipped in melted NTB-2 nuclear track emulsion (Kodak, diluted 1:1 with filter-sterilized deionized water). The filter was carefully placed face-down on the emulsion. Slides were kept for 10 min on a metal tray in contact with ice. The slides were dried and exposed in the dark at 4°C for 20 days (^{35}S-DMSP) or 10 days (^{3}H-leucine).

The microautoradiograms were developed for 4 min in Kodak D19 developer (diluted 1:1 with distilled water), followed by a 30 s stop rinse in deionized water, and a 4 min soak in Kodak fixer. Slides were washed in tap water for 10 min, dipped in glycerol (1%) for 2 min, and stored inside a desiccator overnight, protected from light. The filter was gently peeled off and cells in the emulsion were stained with a drop of mounting solution (Vecta:Citifluor solution 4:1 v/v, plus DAPI at 1μg ml^{-1}) and covered with a coverslip. Total DAPI-positive and radiolabeled cells counts were counted with an Olympus BH microscope under simultaneous UV radiation epifluorescence and visible light transmitted illumination. Following this protocol, between 75 and 98% of the cells were transferred from the filter to the emulsion. Optimization of the incubation and exposure times is described in the Results section.

MicroFISH. The MicroFISH method consists of the combination of microautoradiography and fluorescence *in situ* hybridization (FISH) (Cottrell & Kirchman 2000, Lee et al. 1999, Ouverney & Furhman 1999). FISH was carried out on sections of the filter after incubation with radiolabel but before the microautoradiography step. Cells on filter sections were hybridized with the group specific oligonucleotide probes Eub338 (5'-GCTGCCTCCCGTAGGAGT-3') for Eubacteria, Ros536 (5'-CAACGCTAACCCCCTCCG-3') for *Roseobacter*, Alf968 (5'-GGTAAGGTTCCGCGCGTT-3') for Alphaproteobacteria, Gam42a (5'-GCCTTCCCA-CATCGTTT-3') for Gammaproteobacteria, CF319a (5'-TGGTCCGTGTCTCAGTAC-3') for *Cytophaga-Flavobacterium*, and Non338 (5'-ACTCCTACGGGAGGCAGC-3') as a negative control. Probes labeled with the cyanine dye CY3 at the 5'-end were purchased from Thermo Hybaid GmbH. Hybridization was carried out following the method of Pernthaler et al. (2001).

^{35}S labeling of DMSP. ^{35}S-DMSP for DIME (Gulf of Mexico) was synthesized biologically using the alga *Platymonas subcordiformis* and following the culture and extraction procedures of Kiene et al. (1998). For the NW Mediterranean, ^{35}S-DMSP was synthesized chemically from ^{35}S-L-methionine as described elsewhere (Malmstrom et al. 2004a). In both cases the ^{35}S-DMSP product was partially purified by solid phase, ion exchange extraction onto (Dowex-50, H^+). Further purification was achieved by evaporating the Dowex-50 eluate to dryness, reconstituting in 100 μl pure water, and injecting the sample into an HPLC equipped with a Whatman Partisil SCX cation-exchange column. The DMSP peak fraction was collected and subjected to a final Dowex-50 solid-phase extraction to remove phosphate from the HPLC eluent. Radiochemical purity was > 98% as judged by HPLC analysis and trapping of volatile ^{35}S after conversion of the ^{35}S-DMSP to ^{35}S-DMS by alkaline hydrolysis.

Results

Setting conditions for micrautoradiography (AU) with ^{35}S-DMSP. To optimize ^{35}S-DMSP incorporation by the water samples for subsequent microautoradiography, we carried out seawater incubations for different lengths of time and with different amounts of ^{35}S-DMSP. Results are shown in Fig. 1. The water sample used for these tests was from nutrient-enriched carboy NUT2 on day 2 of the DIME (Gulf of Mexico). Uptake and incorporation of ^{35}S into filterable material was monitored at several time points over 13 h by counting the filters in liquid scintillation cocktail (Ecolume, ICN Biomedicals). At selected time points, microautoradiography was performed on parallel filters. Since the full microautoradiography procedure took several weeks, optimization was based on the conditions which gave maximal radioactivity incorporated into filterable material. In Fig. 1a, total radioactivity on the filters is plotted as a function of time for varying concentrations of substrate. Maximal total uptake was reached after 10 - 14 hours. In Fig. 1b the same data set is plotted as a function of the added substrate concentration. Based on radioactivity incorporated into filterable material, we chose an incubation time of 13 hours and a DMSP addition of $5.6 \cdot 10^4$ dpm ml^{-1}, which was close to saturation of DMSP uptake. To optimize the exposure time of the emulsion, replicate slides from nutrient-amended carboy NUT2 on day 5 of DIME (with a ^{35}S-DMSP level of $5.6 \cdot 10^4$ dpm/filter) were exposed at 4 °C in the dark for varying times. The optimal exposure time for the microautoradiography was determined to be about 20 days (Fig. 2). In the case of ^{3}H-leucine, the exposure time was optimal at 10 days.

Using these experimental conditions, microautoradiography of marine bacterioplankton cells produced a well-defined crown of exposed silver grains with ^{3}H leucine labeling, making identification of positive cells easy (Fig. 3a). In the case of ^{35}S microautoradiography, the exposed grains were slightly more irregularly distributed around the cells, likely due to the higher energy of the beta particles emitted by ^{35}S (Fig. 3b). Nonetheless, identification of ^{35}S positive cells was also straightforward. The variability associated with microautoradiography

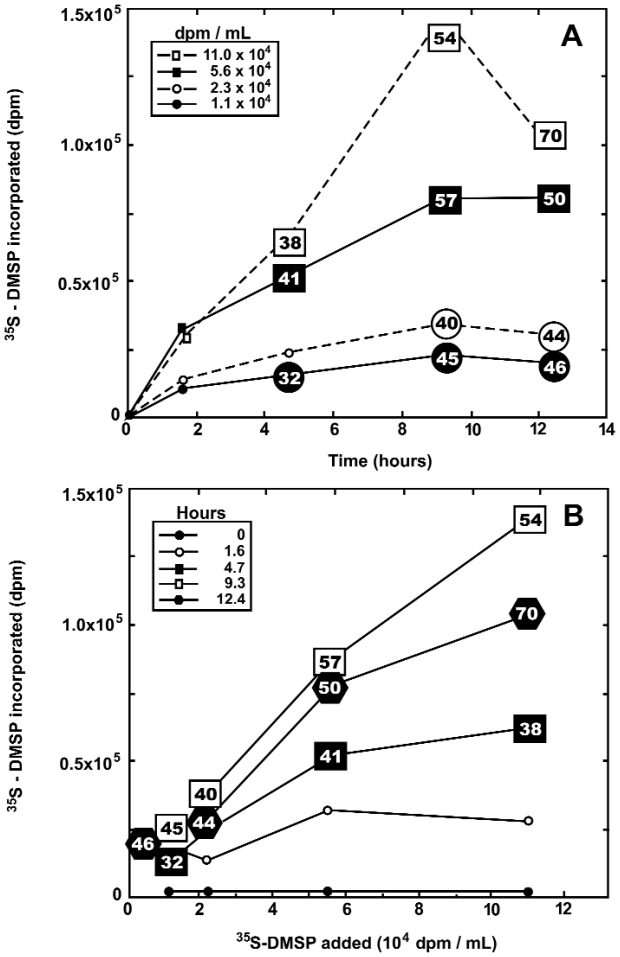


Figure 1. *Incorporation of ^{35}S-DMSP plotted as a function of incubation time (A) and substrate addition (B). The boxed numbers indicate the percent of cells incorporating DMSP at that time point and substrate concentration, if determined. The samples were from a nutrient-enriched carboy on day 2 of the DIME (Gulf of Mexico, June 2000).*

counts was assessed by comparing replicate sets of different samples. Four samples of ^{35}S-DMSP microautoradiography with 2-4 replicates of each sample gave coefficients of variation in AU positive counts of 7-20% (average 12%). For ^{3}H-leucine microautoradiography 11samples, with 2-4 replicates each gave coefficients of variation 12% (average 6%).

The Dauphin Island Microcosm Experiment (DIME). In response to the nutrient amendment, a phytoplankton bloom was induced that reached a peak chlorophyll *a* (Chla) concentration of ~3 μg L^{-1} after 4 days of incubation (Fig. 4a). In the control carboys, Chla remained constant around 0.5 μg L^{-1}. These dynamics were accompanied by increased DMSPp concentrations, up

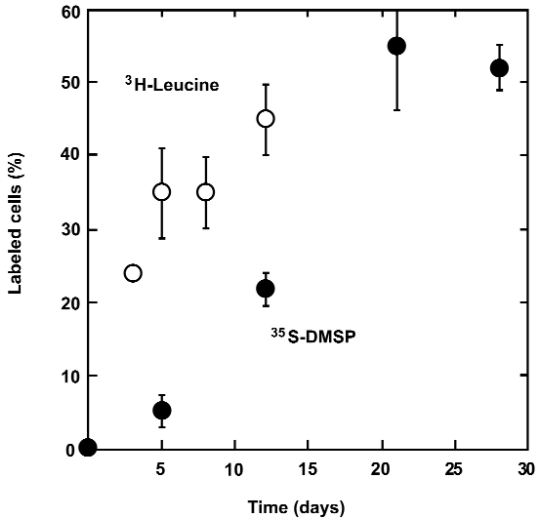


Figure 2. *Percent of labeled cells as a function of exposure time for microautoradiography with ^{35}S-DMSP (filled symbols) and ^{3}H-Leu (empty symbols). Error bars show the standard deviation of three replicates. The samples were from a nutrient-enriched carboy on day 5 of the DIME (Gulf of Mexico, June 2000).*

to 52 to 96 nM, in the nutrient-amended microcosms on day 8, while DMSPp concentrations remained constant at about 15 nM in the controls (Table 1). The DMSPp/chl ratio remained around 40-50 nmol μg^{-1} in the control microcosms throughout the experiment. In the amended microcosms, this ratio decreased to about 10 in the peak of the phytoplankton bloom (data not shown) and increased, as chlorophyll *a* decreased, to eventually reach values slightly higher than to those in the controls on day 8 (Table 1).

As a consequence of the phytoplankton bloom and its decay, bacterial leucine incorporation rates increased to around 600 pmol L^{-1} h^{-1} on day 6 and thereafter (Fig. 4b). In the control carboys, leucine incorporation remained below 100 pmol L^{-1} h^{-1}. Bacterial numbers increased very slowly throughout the experiment, averaging 1.40 x 10^6 cells ml^{-1} in the control carboys, and 2.0 x 10^6 cells ml^{-1} in the bloom carboys. A more detailed description of the microcosm bacterial communities is reported elsewhere (Pinhassi et al. 2005).

^{35}S-DMSP microautoradiography was carried out after the first 48 hours of the microcosm experiment (day 2), one day after the peak of chlorophyll in the enriched carboys (day 5) and when the DMSPp/chl ratio was highest (day 8) (Fig. 4). At the beginning of the experiment, the percent of cells assimilating ^{35}S-DMSP was 50%. A small decrease in positive cells occurred in the controls during the course of the experiment (down to 27% on day 8), but not in the nutrient-amended samples, where it remained approximately 50% (Fig. 5).

At the beginning of the experiment (day 2), similarly high percentages of ^{3}H-leucine labeled cells were recorded in the controls and the amended carboys, with an average of 71% positive cells. On day 5 the percentage of cells assimilating ^{3}H-leucine was similar to that of cells assimilating ^{35}S-DMSP in all carboys. On day 8, however, cells incorporating DMSP were significantly less abundant than those incorporating leucine (Fig. 5).

The composition of the heterotrophic bacterial assemblage was analyzed using a variety of

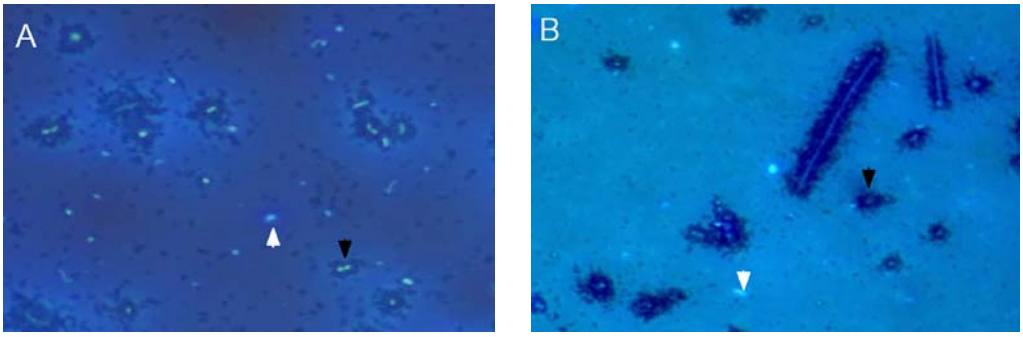


Figure 3. *Examples of microautoradiograms of ^{35}S-DMSP (A) and ^{3}H-Leu (B) labeled bacteria observed under transmitted light (for exposed silver grains) and epifluorescence microscopy (for DAPI stain). White arrows point to DAPI-stained non-radiolabeled cells, while black arrows point to DAPI-stained radiolabeled cells. Note morphological diversity of labeled cells and low background of exposed silver grains compared to labeled cells. The ^{35}S-DMSP and ^{3}H-Leu microautoradiograms are from a nutrient-enriched sample on days 2 and 8 of the DIME (Gulf of Mexico, June 2000).*

molecular techniques that focused on 16S rRNA genes: 16S rRNA clone libraries, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and FISH. The *Roseobacter* clade dominated the bacterioplankton community, and representatives of the *Cytophaga-Flavobacterium*, SAR11, and SAR86 groups were also important (Pinhassi et al. 2005). In order to test whether *Roseobacter* was the main bacterial group responsible for DMSP uptake, MicroFISH was performed on day 8 in the amended carboy C using fluorescent probes for eubacteria and for *Roseobacter* (Figs. 6 and 7). Hybridization with the Non338 probe did not give any positive cells, indicating that non-specific binding of the probes was not significant. The *Roseobacter* clade accounted for 23% of total (DAPI stained) prokaryotes (Fig. 6a). As many as 87% of *Roseobacter* cells identified by FISH were labeled with ^{35}S (Fig. 6b) whereas 50% of FISH-identified *Roseobacter* cells were labeled with ^{3}H-leucine (Fig. 6c). Among all the ^{35}S AU positive cells that occurred in this sample, 43% belonged to *Roseobacter* (Fig. 6d). In contrast, *Roseobacter* contributed only 21% of the ^{3}H-leucine labeled cells (Fig. 6e).

Coastal NW Mediterranean. Characteristics of the coastal NW Mediterranean Sea water at the time of sampling are given in Table 1. Chlorophyll *a* concentrations increased from 1.43 μg L^{-1} in January to 2.21 μg L^{-1} in March (Table 1), while DMSPp concentrations increased from 8 to 42 nM during this same period. As a consequence, the DMSPp:Chla ratio, which is a proxy for the relative abundance of DMSP-producers among the phytoplankton, was lower in January (6 nmol/μg) than in March (19 nmol/μg). Microautoradiography showed that the proportion of total DAPI-positive cells labeled with ^{35}S-DMSP and ^{3}H-leucine increased between January and March (Fig. 7). On the two sample dates in January, 5-10% of the DAPI-positive prokaryotes incorporated ^{35}S-DMSP, while 21-24% took up ^{3}H-leucine. In March, the percentage of cells labeled with ^{35}S-DMSP had increased to 42%, and was similar to the fraction of cells labeled with ^{3}H-leucine (40%).

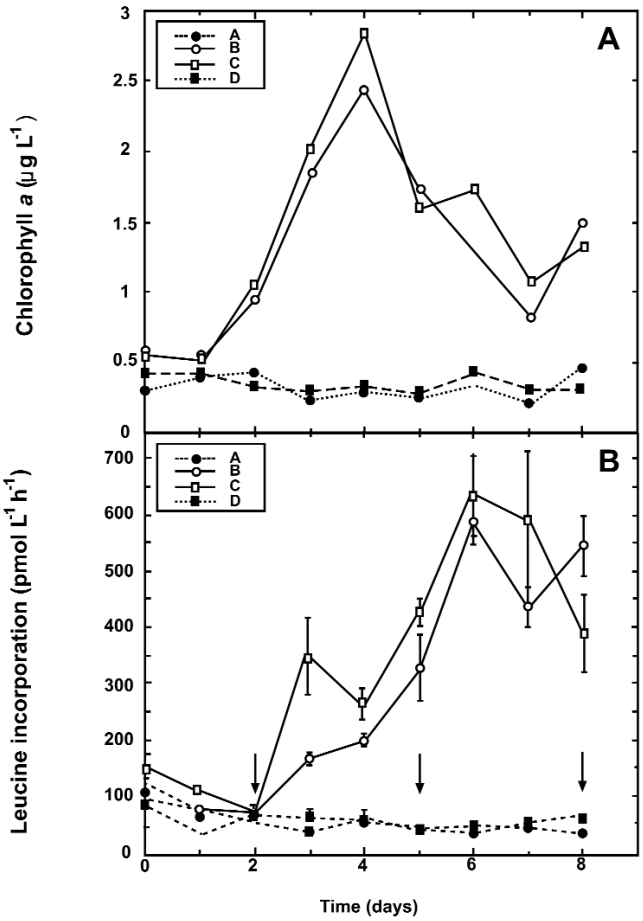


Figure 4. *Microbial dynamics in the DIME study (Gulf of Mexico, June 2000). A. Changes in chlorophyll a with time in control microcosms (filled symbols, carboys C1 and C2) and nutrient amended microcosms (empty symbols, carboys NUT1 and NUT2). Arrows indicate time points samples were incubated for microautoradiography. B. Changes in leucine incorporation with time. Error bars show standard error of three replicates.*

In both January and March samples, the *Roseobacter* clade accounted for an average of 16% of the DAPI counts according to FISH (Fig. 6a). Based on MicroFISH data, 13 - 19% of the ^{35}S-labeled cells could be identified by the *Roseobacter* probe (Fig. 6d). The percentage of *Roseobacter* cells that had assimilated ^{35}S-DMSP increased from 7 to 52% between January and March (Fig. 6b).

The fraction of *Roseobacter* cells that assimilated ^{3}H-leucine was higher than the fraction incorporating ^{35}S-DMSP in early January, but the two were very similar in March (Figs. 6b and c). The fraction of ^{3}H-leucine-labeled cells that could be identified by the *Roseobacter* probe

decreased from 29 to 17% from January to March as detected by MicroFISH (Fig. 6e). Nevertheless, the fraction of *Roseobacter* cells that assimilated ^{3}H-leucine remained constant (43 to 48%, Fig. 6c).

In the March sample we used FISH probes for other groups of bacteria in combination with AU. The different groups tested accounted for similar percentages of the total DAPI-positive prokaryote count (Fig. 6a). The Alphaproteobacteria and *Roseobacter* probes produced very similar numbers, suggesting that most of the Alphaproteobacteria that could be detected by FISH were indeed *Roseobacter*. As can be seen in Fig. 6b-c, all groups were active in both DMSP and leucine incorporation, suggesting that DMSP use is widespread among different phylogenetic groups of bacterioplankton.

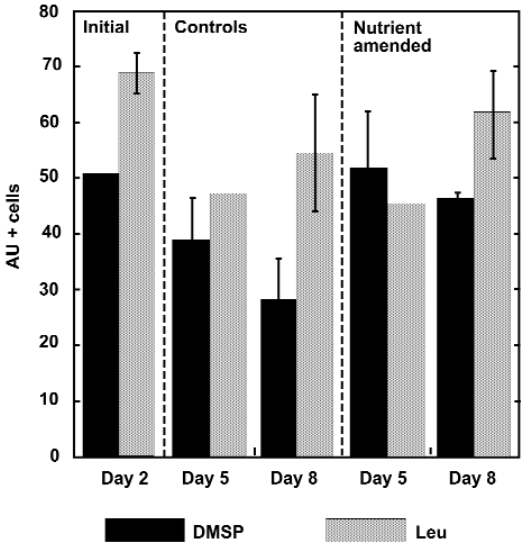


Figure 5. *Percent of cells taking up DMSP (black bars) and leucine (striped bars) in the DIME (Gulf of Mexico, June 2000). Error bars show standard deviation of two replicates.*

Figure 8 shows the percentage of total DAPI-positive prokaryotic cells that hybridized with each probe and incorporated each substrate for the March sampling. All groups had a significant fraction of cells that assimilated sulfur from ^{35}S-DMSP. *Roseobacter* and Gammaproteobacteria showed a similar percentage of DMSP and leucine labeled cells (i.e. they were close to the 1:1 line in the plot of Fig. 8). In the *Cytophaga-Flavobacterium* group, on the other hand, the percentage of cells that took up S from ^{35}S-DMSP was lower than that of cells incorporating ^{3}H-leucine.

Discussion

DMSP and the overall bacterial activity. Microautoradiography with ^{35}S-DMSP has provided a direct measure of the proportion of bacteria involved in utilization of this organic sulfur molecule. The percentage of bacteria that incorporate DMSP varies with sample characteristics but can be very high (Table 1, Figs. 5 and 7); under certain conditions, it can be similar to that of cells incorporating leucine. Leucine is widely accepted as a universal substrate for bacteria, to the extent that it is commonly used to provide estimates of bacterial heterotrophic production (Kirchman 2001). We thus take the number of cells assimilating ^{3}H-leucine as a measure of the

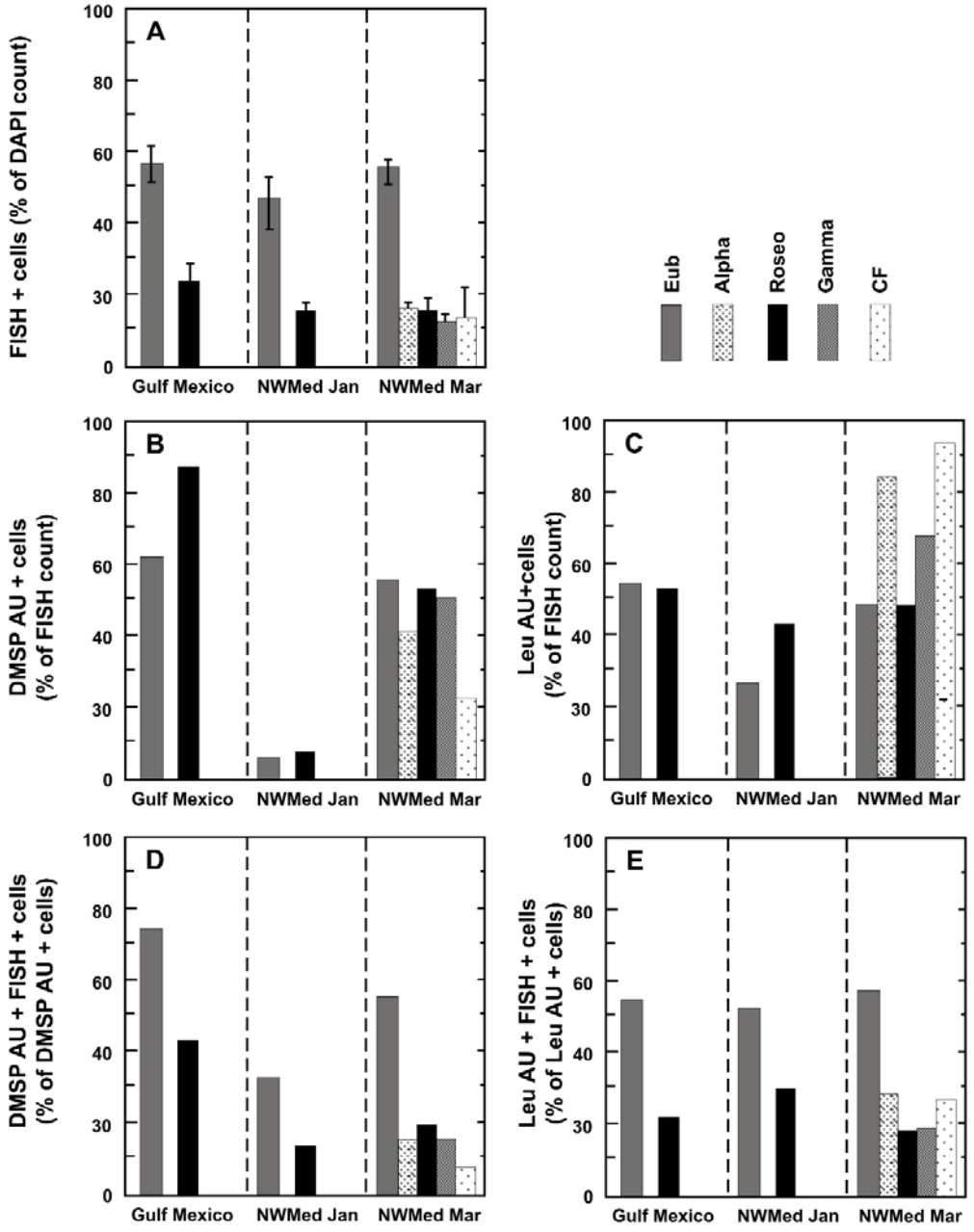


Figure 6. Results of MicroFISH in the DIME study (Gulf of Mexico, June 8-16, 2000) and in the Blanes Bay time series (NW Mediterranean, January 13 and March 4, 2003). A. FISH+: Cells hybridizing with each probe as percent of total DAPI count. B. DMSP AU+: Percent of cells hybridizing with each probe that was labeled with ^{35}S-DMSP. C. Leu AU+: Percent of cells hybridizing with each probe that was labeled with ^{3}H-leucine. D. DMSP AU+ FISH+: Percent of cells labeled with ^{35}S-DMSP that hybridized with each probe. E. Leu AU+ FISH+: Percent of cells labeled with ^{3}H-leucine that hybridized with each probe.

fraction of active heterotrophic cells. In our samples, when the percentage of active cells was higher (>40% of cells were ^{3}H-positive in either DIME or NW Mediterranean blooms), the incorporation of DMSP was widespread (≥ 40% of cells were ^{35}S-positive). When the bacterial assemblage was less active, such as in the January samples from Blanes Bay, the fraction of cells assimilating DMSP in that sample was also low (<10%, Table 1, Figs. 5 and 7).

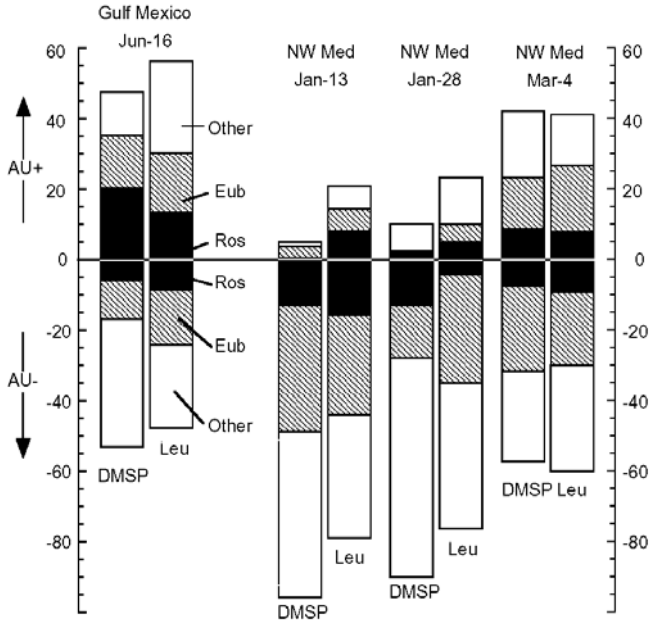


Figure 7. *Result of MicroFISH in the DIME study in the Gulf of Mexico (microcosm NUT2 enriched with nutrients, 3 days after the chlorophyll peak) and in the Blanes Bay time series (NW Mediterranean). Results are shown for ^{35}S-DMSP labeled and ^{3}H-leucine labeled cells separately. Values of labeled (AU+) cells appear above the x axis and those of non-labeled cells (AU-) below it. Two probes were used for FISH: Eubacteria (Eub) and Roseobacter (Ros). The percent of cells labeled with the Ros probe (black portion of bars) is shown as a subset of the percent of cells labeled with the Eub probe (black + dashed portion of bars). "Other" indicates DAPI stained cells that did not hybridize with the Eub probe.*

There are limitations of the microautoradiography method that call for caution in interpreting results. Exposed silver grains indicate the presence of a labeled atom or moiety, but no necessarily the original substrate molecule. In the case of ^{35}S-DMSP, which is a very labile substance that usually turns over in less than 1 day (Kiene 1996, Kiene et al. 2000, Pinhassi et al. 2005), degradation products (i.e., ^{35}S-DMS or ^{35}S-MeSH) may be released into seawater and assimilated by other bacteria during the incubation period of 10-14 hours. Hence, counts of ^{35}S AU positive cells assess direct and indirect incorporation of sulfur from DMSP and may therefore be an overestimate of cells actually incorporating DMSP. In any case, the conclusion that DMSP provided sulfur for a wide variety of marine bacteria is not altered. This indirect uptake of the isotope is unlikely to have occurred in the case of the ^{3}H-leucine, for which incubations were significantly shorter (5 hours), and because leucine is incorporated directly into proteins, with little degradation (Kirchman et al. 1985).

The low specific activity of the biologically-synthesized ^{35}S-DMSP and the fast decay rates of ^{35}S necessitated the use of a DMSP concentration in the DIME study (ca. 60 nM) that was well above

the endogenous DMSPd concentration. Therefore, AU-positive cells may represent the number of cells capable of incorporating DMSP, rather than those actively incorporating DMSP at *in situ* concentrations. In the NW Mediterranean, chemically synthesized ^{35}S-DMSP of higher specific activity was added at levels below the endogenous DMSPd concentrations (<0.3 nM). ^{3}H-leucine amendments in DIME (2.5 nM) were likewise above estimated endogenous concentrations (< 1 nM; Carlucci et al. 1984), while the added leucine levels in the NW Mediterranean (0.5 nM) were likely below endogenous levels. Therefore, since we applied the same criterion for the additions of DMSP and leucine, the comparisons of the relative extent of the incorporation of the two compounds by heterotrophic bacterioplankton are not affected. Comparison of microautoradiography results obtained with DMSP to those obtained with leucine show that, on some occasions, DMSP assimilation is as widespread as leucine assimilation, but on other occasions DMSP is assimilated by significantly fewer cells (Table 1, Figs. 5 and 8). Leucine is directly incorporated into bacterial proteins, which is the basis for its use as a measure of bacterial protein synthesis (Kirchman et al. 1985). Malmstrom et al. 2004a recently proposed that, since DMSP assimilation appears to satisfy most of the bacterial sulfur demand in different oceanic environments (e.g. Kiene & Linn 2000a, Simó et al. 2002), and all bacteria synthesizing protein need sulfur, then all bacteria synthesizing protein should assimilate DMSP. Our results indicate that natural assemblages of bacterioplankton may indeed rely heavily on DMSP as a sulfur source when it is available, but certainly not all bacteria synthesizing protein assimilate DMSP (Figs 7 and 8).

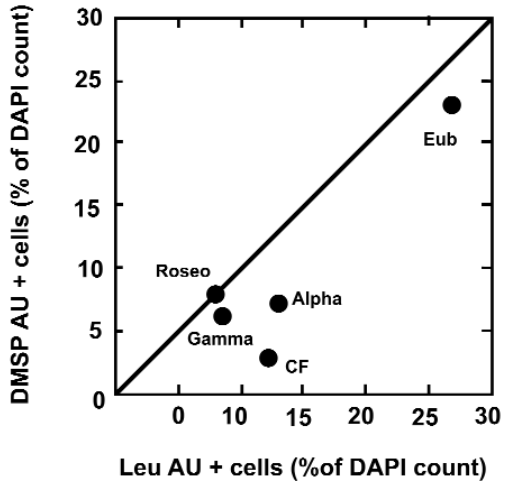


Figure 8. *Results of MicroFISH in Blanes Bay, NW Mediterranean (March 4). Comparison of ^{35}S-DMSP vs ^{3}H-leucine labeled cells (as percent of total DAPI counts) for each phylogenetic group tested. Diagonal indicates a 1:1 relationship, i.e. equivalent numbers of cells assimilating both substrates. Eub, Eubacteria; Ros, Roseobacter; Gamma, Gammaproteobacteria; Alpha, Alphaproteobacteria; CF, Cytophaga-Flavobacterium*

Exploring the links between Roseobacter and DMSP. In recent years, significant advances have been made in determining the identity of prokaryotic plankton in the ocean (e.g. see Giovannoni & Rappe 2000). A major remaining challenge is to connect microbial phylogeny with the role that microbes play in the biogeochemical cycles of key elements. The involvement of the *Roseobacter* clade in bacterial metabolism of dimethylated sulfur in marine environments represents a clear example of how such a connection can be approached (Moran et al. 2003).

By drawing jointly on the expertise of molecular microbial ecologists and biogeochemists, and by combining laboratory and field work, research on the linkage between DMSP cycling and the *Roseobacter* clade is as firmly established as for any other organic substrate and bacterioplankton taxon.

Previous studies have provided strong, yet circumstantial, evidence for a prominent role of *Roseobacter* in DMSP degradation. Every one of 15 cultured members of this group was able to cleave DMSP into DMS and acrylate, despite the fact that only half were isolated on DMSP-containing media (González et al. 1999). Five of the 15 isolates expressed the demethylation/demethiolation route, and all of these were also capable of incorporating DMSP sulfur into proteins (Kiene et al. 1999). In the open ocean, *Roseobacter* was found to be the most abundant group in the bacterial assemblage associated with blooms of high DMSP-producing phytoplankton (González et al. 2000, Zubkov et al. 2002). González et al. (2000) reported that *Roseobacter* DNA abundance was positively correlated with both chlorophyll and DMSP concentrations, while Zubkov et al. (2002) found that one species-level *Roseobacter* taxon was positively correlated with bacterial production and DMSP loss.

By means of MicroFISH with a *Roseobacter* probe, we observed that this group contributed to ^{35}S assimilation in all samples studied. In the sample for which the MicroFISH was done with the set of 5 phylogenetic probes (NW Mediterranean, March), *Roseobacter* and Gammaproteobacteria were the two largest contributors to ^{35}S-positive cells (Figs. 6 and 8). In agreement with the conclusion of Malmstrom et al. 2004a, these results indicate that *Roseobacter* is a prominent group among DMSP-utilizing bacterioplankton, yet by no means the only group involved.

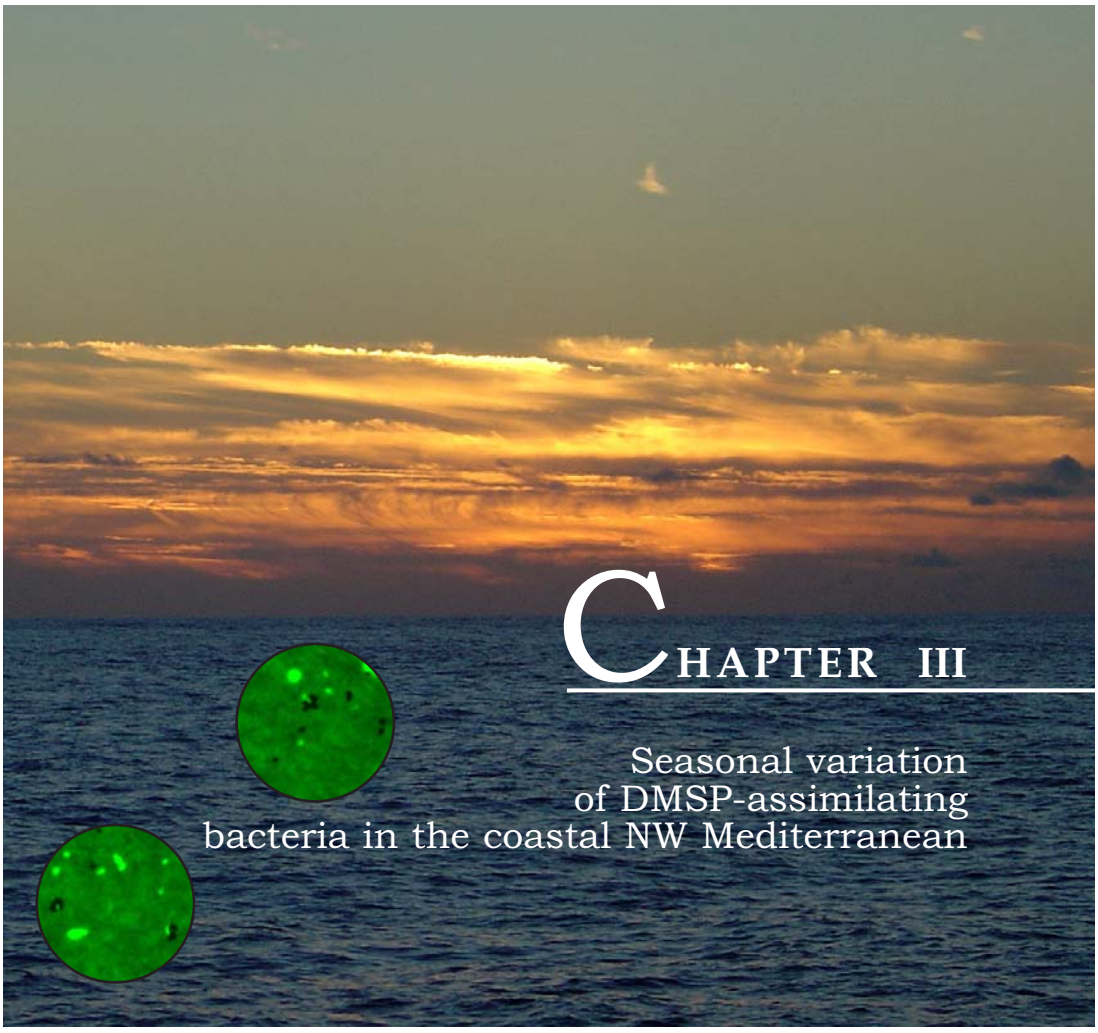
Implications for elucidating the DMS-DMSP cycle. The demethylation/demethiolation pathway, and the subsequent utilization of the methanethiol moiety as a S source, has been found in previous studies to dominate the microbial degradation of DMSP in seawater over the DMS-production pathway (Kiene et al. 2000, Kiene et al. 1996, Tang & Simó 2003). Single-cell resolution of DMSP uptake shows that the capability for incorporating S from DMSP is widespread among marine bacteria (Fig. 6b and d). The question of how widespread is the occurrence of the DMS-producing, DMSP lyase pathway among bacterioplankton remains yet to be answered.

Bacterial degradation by either of the two pathways is the fate of only a portion of algal DMSP in the ocean. Other fates include cleavage by algal DMSP-lyases during oxidative stress, grazing or autolysis, and assimilation by herbivores (Simó et al. 2002, Simó & Pedrós-Alió 1999a, Sunda et al. 2002, Tang & Simó 2003). Moreover, bacteria are not only involved in the degradation of DMSP but in the consumption of the evolved DMS and MeSH as well (Kiene & Bates 1990, Kiene et al. 1999). In particular, microbial consumption is one of the

major mechanisms for DMS loss from the surface ocean. As such, it is one of the major factors controlling DMS emission to the atmosphere (Simó 2001), and knowledge of the phylogeny and dynamics of the bacteria involved is crucial for understanding the marine DMS(P) cycle. Previous studies have suggested that DMS is a minor sulfur source, mainly used as a supplementary carbon source (Zubkov et al. 2001) by methylotrophic bacteria (Kiene 1993). The MicroFISH protocols developed in the present study, if applied with ^{35}S-DMS, could provide new insights into the microbial cycling of DMS.

Implications for the ecology and biogeochemistry of marine bacterioplankton. As stated by Giovannoni & Rappé (2000), the dominance of certain groups of heterotrophic prokaryotes in the surface ocean likely results from their competence in using labile dissolved organic matter derived from the primary producers. Several recent studies have shown that algal-derived DMSP, although occurring at nanomolar concentrations in seawater, turns over on the order of a few hours (Kiene et al. 2000). With techniques such as microautoradiography and MicroFISH, we have been able to examine coincidence of DMSP incorporation and identity in single cells. All the bacterioplankton groups examined had cells that exhibited the capability to assimilate sulfur from DMSP. In future studies, the comparison of MicroFISH with DMSP relative to other substrates (such as glucose and aminoacids) together with rate measurements of substrate utilization by bacteria should provide information on the importance of a rather small set of ubiquitous molecules released by phytoplankton for satisfying bacterioplankton C and energy demands.

The observation that *Roseobacter* and γ-Proteobacteria had a higher affinity for DMSP than other phylogroups (Fig. 8) is complementary to the findings of Malmstrom et al. (2004a) in a MicroFISH study of the NW Atlantic. These authors observed larger silver grain surface areas around *Roseobacter* cells relative to other phylogenetic groups, which led them to suggest that *Roseobacter* were more capable of utilizing DMSP on a per cell basis. If further studies confirm that affinity for DMSP varies among bacterial phylotypes, then we can speculate that conditions with a higher contribution of DMSP to the C and S pools and fluxes might favor bacterioplankton assemblages with a higher proportion of specialized DMSP-utilizers. In other words, we can speculate that succession of phytoplankton towards higher DMSP producers will be followed by succession of bacterioplankton towards better DMSP consumers. Obviously, addressing such a hypothesis will require a comprehensive application of the tools developed in the present study to natural communities, along with the determination of DMSP production, occurrence and transformation fluxes under changing environmental conditions, e.g. throughout time series or spatial gradients of trophic status.



CHAPTER III

Seasonal variation of DMSP-assimilating bacteria in the coastal NW Mediterranean

Vila-Costa, Maria; Pinhassi, Jarone; Alonso, Cecilia; Pernthaler, Jakob and Simó, Rafel.

Abstract

The contribution of major groups of heterotrophic bacteria to assimilation of sulfur from dissolved dimethylsulfoniopropionate (DMSPd) was analyzed in surface seawaters from Blanes Bay (NW Mediterranean) over an annual study. The percentage of bacteria assimilating DMSP-S showed a strong seasonal pattern, with a steady increase from winter (8 ± 5 %) to summer (23 ± 3 %). The same seasonal pattern was observed for the rate of DMSP-S assimilation. Members of the Alphaproteobacteria (*Roseobacter* and SAR11) and Gammaproteobacteria groups accounted for most of bacterial DMSP-S assimilation over the year and showed an increase of the percentage of cells incorporating DMSP during the summer period. Rather than a possible succession in the microdiversity of these groups towards higher-affinity DMSP consumers in summer, DMSP-S assimilation appeared to be determined by its role as S (and C) source. The annually-averaged percentage of DMSP-S-assimilating bacteria (15 ± 9 %) was lower than the percentage of leucine-incorporating cells (35 ± 16 %), suggesting that not all bacteria synthesizing protein incorporated DMSP-S. However, lower differences between both percentages were recorded in summer. Monthly experiments of substrate and nutrient addition revealed that, in summer, addition of DMSP plus phosphate provoked a stimulation of bacterial heterotrophic production comparable to that of glucose plus phosphate, while over the rest of the year glucose induced a higher response. Overall, our results suggest that the main contributors to DMSP-S assimilation are members of the Alphaproteobacteria and Gammaproteobacteria groups, and that bacterial DMSP-S assimilation increases during summer months when the contribution of DMSP a source of reduced S for bacteria is higher.

Introduction

Dimethylsulfoniopropionate (DMSP) is a ubiquitous compound in the upper ocean, where it represents a major pool of reduced sulfur (Bates et al. 1994). This compatible solute, produced by different phytoplankton taxa with suggested roles as osmoregulator, anti-oxidant and overflow of reducing power (Malin & Kirst 1997, Stefels 2000, Sunda et al. 2002) has been studied extensively since it was identified as the biogenic precursor of dimethylsulfide (DMS). DMS is a climatically active gas released by the oceans to the atmosphere where its oxidation products (sulfate and sulfonate aerosols) increase cloud albedo and thus they may partly quench the warming effect of increasing greenhouse gas concentrations (Charlson et al. 1987). Furthermore, DMSP is a significant component of the organic matter that flows through the food-web. During blooms of DMSP-producers, it can represent $\geq$ 50% of sulfur and $\sim$ 10% of carbon fluxes through various trophic levels (Burkill et al. 2002, Simó et al. 2002). The link between phytoplankton-associated DMSP and the pelagic food-web is the microbial loop. DMSP is released into the dissolved pool mainly through algal senescence, viral lysis, and grazing by micro- and mesozooplankton (Simó 2001). Dissolved DMSP (DMSPd) is mainly metabolized by heterotrophic bacteria and partly by non DMSP-producing phytoplankton (Chapter V). Bacteria degrade DMSP through two pathways: (1) cleavage to give rise to DMS production, or (2) demethylation. This latter route can be followed by a second demethylation, resulting in non-volatile sulfur compounds (Visscher et al. 1992), or by a demethiolation that leads to methanethiol (MeSH) formation, which goes primarily into bacterial macromolecules (protein synthesis) (Kiene et al. 2000). Thus, bacterioplankton is a key component that mediates the switch between DMS–producing and non-DMS-producing DMSP transformations.

Demethylation seems to be the dominant fate of bacterial DMSPd degradation in most studied systems (Kiene et al. 2000). Since a portion of total DMSPd demethylation ends up as assimilated sulfur, the identification of the factors that drive DMSP assimilation is of key importance to understand the biotic control of DMS production. Recent studies have suggested that not all bacteria uniformly assimilate DMSPd (Malmstrom et al. 2004a and 2004b). Probably, taxonomic composition is one of the factors influencing the extend of DMSPd assimilation by bacterioplankton assemblages, and indirectly, it may influence the preference of bacteria for either the cleavage or the demethylation route. This hypothesis needs to be verified with studies over long time scales, in view of the strong seasonal patterns of DMS concentrations observed at different sampling sites (e.g. Leck et al. 1990, Turner et al. 1996, Dacey et al. 1998, Chapter I).

The most successful method to identify the phylogeny of DMSP-assimilating bacteria *in situ* is MARFISH, a combination of microautoradiography (MAR) and fluorescence *in situ* hybridization (FISH) (Ouverney & Furhman 1999, Lee et al. 1999, Cottrell & Kirchman 2000). Using this technique, it has been observed that DMSP assimilation is a widespread feature among

different groups of bacterioplankton (Malmstrom et al. 2004a, Chapter II). In particular, members of the Alphaproteobacteria group, *Roseobacter* and SAR11, when abundant, seem to have a prominent yet not unique role in DMSP degradation in the sea (Malmstrom et al. 2004a and 2004b, Chapter II). During blooms of DMSP-producers, indeed, associated bacterial assemblages were dominated by *Roseobacter* taxa, and their abundance was positively correlated with DMSP concentration or DMSP turnover (González et al. 1999, Zubkov et al. 2001b). This high capability for DMSP metabolism was also observed in isolates of *Roseobacter* (González et al. 1999) and, very recently, the gene encoding for DMSP demethylation has been found in the genome of two representatives of *Roseobacter* and SAR11 taxa (Howard et al. 2006).

In Blanes Bay (NW Mediterranean) the seasonal succession of bacterial groups has been described in two independent studies (Schauer et al. 2003, Alonso-Sáez et al. *in press*). For example, in 2003, abundances of the SAR11 and *Roseobacter* groups showed opposite patterns: SAR11 abounded in summer, under high nutrient limitation conditions, while *Roseobacter* abundances followed Chla concentrations more closely (Alonso-Sáez et al. *in press*). That same year, the dynamics of dimethylated sulfur compounds were analysed. DMS concentration showed a strong seasonal pattern, with maximum concentrations in summer, whereas DMSP concentration peaked in March. Bacterial DMSP consumption rates were higher in summer, suggesting that the summer bacterial assemblage had a higher capability for DMSP utilization (Chapter I).

In the time series study from early 2003 to early 2004, we used an improved protocol of the MARFISH method (see Alonso & Pernthaler 2005 and Annex II) to address the link between the succession of bacterial groups and DMSP dynamics. The objectives of this work were: (1) to determine the seasonality of bacterial DMSP-sulfur assimilation; (2) to identify the main DMSP-S-assimilating bacteria using phylogenetic probes for the main broad groups, namely Alphaproteobacteria, Gammaproteobacteria, CFB (Cytophaga-Flavobacterium-Bacteroidetes) and specifically for *Roseobacter* and SAR11 phyla; (3) to analyze the seasonal variation of the contribution of each of these groups to DMSP-S-assimilation. We compared DMSP-incorporation results with incorporation of leucine, which is used as a universal substrate by heterotrophic bacteria.

Materials and Methods

Sampling. Surface seawater samples were collected 1 mile offshore in Blanes Bay (the Blanes Bay Microbial Observatory, 41°40'N, 2°48'E) by submerging a rinsed amber glass bottle (2.5 L) an arm length into the water. Samples were kept in the dark at *in situ* temperature until being processed in the laboratory (usually ~2 h after sampling). Samples were taken monthly over an annual cycle from 25 March 2003 to 22 March 2004.

Chemical analyses. Chlorophyll *a* (Chla) was measured by fluorometry in 90% acetone extracts(extraction overnight at 4°C) from 150 ml samples filtered through GF/F. DMS in 40-50 ml of GF/F filtered water was measured using a purge and cryotrapping system and sulfur-specific gas chromatography as described by Simó et al. 1996. Total DMSPt concentrations were determined in 20-40 ml of whole seawater by alkaline hydrolysis overnight and analysis of the evolved DMS.

DMSP incorporation rate. A 15 ml sub-sample of whole seawater was incubated in the dark at *in situ* temperature without headspace for 18 hours with a trace addition of ^{35}S-DMSP (1000 dpm ml^{-1}; 5.79 Ci pmol^{-1}). Triplicate aliquots (5ml) were filtered through nylon filters (GN, Millipore, 0.2-μm-pore-size) using a gentle vacuum (<5 cm Hg) and rinsed with 0.2 μm-filtered seawater (FSW). Macromolecules were precipitated by treating filters with cold aliquots (5 ml) of trichloroacetic acid (TCA 5%) for 5 min. Filters were then rinsed twice with MilliQ water. Radioactivity in the TCA-rinsed filters was determined in 5 ml of scintillation cocktail (Optimal HiSafe) using a Beckman scintillation counter. The precision (%SE) of triplicate measurements averaged $\sim$ 1%. Incorporation of ^{35}S-DMSP in formalin-killed controls was $\leq$ 1.5 % that in live samples.

The percentage of incorporated ^{35}S into macromolecules from initially added ^{35}S-DMSP was multiplied by the DMSPd consumption rate (nM DMSPd/d) (see Chapter I) to calculate the DMSP incorporation rate. It is assumed that all initial ^{35}S-DMSP added to the sample (1000 dpm/ml=0.0004 nM) was consumed during the 18 h of incubation.

Quantification of populations by CARD-FISH. The abundance of bacterial populations was determined by FISH with horseradish peroxidase-label nucleotide probes (HRP probes) and catalyzed reporter deposition (CARD-FISH) following the protocol described by Pernthaler & Pernthaler (2002) with the only modification that samples were fixed with formaldehyde (4% final conc., overnight at 4°C) instead of paraformaldehyde. To permeabilize the cells, filters were treated with lysozyme (37°C, 1h) and then with Achromopeptidase (37°C, 30 min). HRP probes used were the following (in parenthesis the % of formamide of the hybridization): EUBI-III, to target most Bacteria (55%, Daims et al. 1999), ALF968, to target most Alphaproteobacteria (45%, Neef 1997), GAM42am to target most Gammaproteobacteria (55%, Manz W et al. 1992) and CF319 to target most Cytophaga-Flavobacterium-Bacteroidetes (CFB, 55%, Manz W et al. 1996). Two subgroups of Alphaproteobacteria were also selected: ROS537 (*Roseobacter* clade, 55%, Eilers et al. 2001) and SAR11-441R (SAR11 cluster, 45%, Morris et al. 2002). Hybridizations were carried out for 2 h at 35°C, except SAR11-441R, ALF968 and EUBI-III that were incubated overnight to improve the signal. Nonspecific binding was examined by hybridizing with NON338 probe (Amann et al. 1995). Counterstaining of CARD-FISH preparations was done with 4,6-diamidino-2-phenylindole (DAPI, 1 μg ml^{-1}). An image analysis system (described by Cottrell & Kirchman 2003) were used to count a minimum of 1000 DAPI-positive cells. These counts were verified with manual counts for a subset of samples for all probes used.

MARFISH. We followed the recent developed MARFISH method that combines microautoradiography and CARD-FISH to follow radioactive substrate incorporation by specific phylogenetic groups (Alonso & Pernthaler 2005). Briefly, 30 ml samples were incubated in the dark at *in situ* temperature for 18 hours with trace additions of ^{35}S-DMSP ($\leq$ 0.4 nM, specific activity: 5.79 Ci/pmol) and ^{3}H-leucine (0.5 nM, specific activity: 161 Ci/mmol). Incubations were stopped with formaldehyde (final conc. 4%) and fixed overnight at 4°C. A formaldehyde-killed control was prepared for each incubation. Aliquots (5ml) were filtered using a gentle vacuum (<5 cm Hg) through 0.2-μm-pore-size polycarbonate filters (Millipore, GTTP, 25 mm diameter) and rinsed twice with 0.2μm-FSW. No differences were observed if filters were rinsed with 0.2 μm-miliQ water instead. Filters were air dried and stored at -20°C until processed. Filter sections were hybridized following the CARDFISH procedure and glued onto microscopic slides using a two-component epoxy adhesive (UHU plus sofortfest; UHU GmbH, Bühl, Germany). CARDFISH counts were performed cutting a small piece of these filters. For the MAR procedure, in the darkroom, slides were dipped in melted (46°C) NTB-2 nuclear track emulsion (diluted 1:1 with agarose 1%, Kodak). Slides were kept for ~10 min on a metal ice cold bar until solidification of the emulsion and then exposed in the dark at 4 °C. The exposure times were tested individually in the most contrasted samples regarding DMSP incorporation uptake and bacterial production (6 out of 12). The optimal exposure time was 20 days for ^{35}S-DMSP filters and 14 days for ^{3}H-leucine. The July month presented exceptionally high activity and exposures were stopped after 2 days for both radioisotopes. The microautoradiograms were developed for 3 min in Kodak D19 developer, followed by 30 s of rinsing with distilled water and 3 min in Kodak Tmax fixer. Slides were washed in tap water for 10 min and dried overnight in a desiccator. Cells were stained with DAPI (1 μg/ml) for 3 min at 4 °C, washed with MilliQ water and ethanol (80%), and then stored at -20°C until counting. Total radiolabeled cell counts were determined manually with a Olympus BX61 epifluorescence microscope. Killed-controls were evaluated with probe EUBI-III.

Short-term nutrient limitation bioassays. The effect of nutrient additions on the growth of heterotrophic bacteria was examined monthly, from January 2003 to July 2004. For details on bacterioplankton C, N and P limitation in the Bay of Blanes see Pinhassi et al. (2006). Seawater was transferred to acid-rinsed 250 ml polycarbonate bottles (Nalgene), thoroughly rinsed with MilliQ-water and sample water. Phosphorus was added as Na_2HPO_4 (0.6 μM P) singly, or in combination with 24 μM C as DMSP or glucose, in duplicates. After incubation for 24 h at *in situ* temperature in the dark, bacterial production was measured as ^{3}H-leucine incorporation following the protocol of Smith and Azam (1992).

Results

The strong seasonality of the air-to-sea heat flux in Blanes Bay originated a marked seasonality of the sea-surface temperature and a shoal stratification (2-3 m of mixing depth) in the summer

period (from May to August). Blanes Bay can be considered an oligo-to-mesotrophic sampling site. Concentrations of Chla in winter (December to February) averaged 1.7 ± 0.7 μg L^{-1}, coincident with higher concentrations of nutrients, and decreased to 0.4 ± 0.1 μg L^{-1} in summer (Fig. 1).

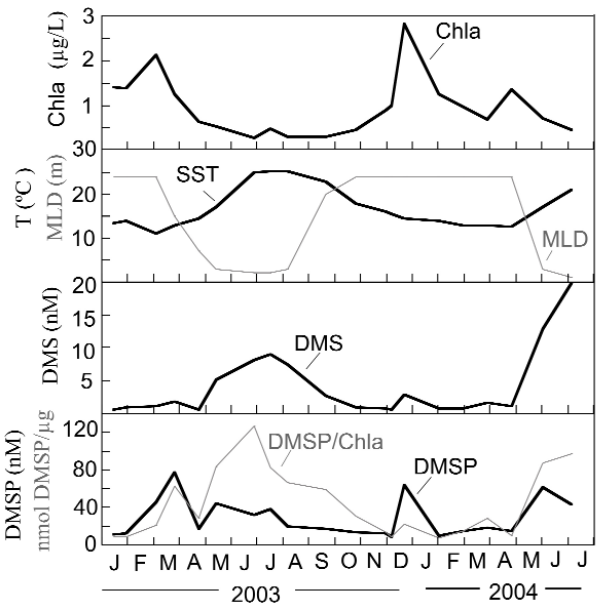


Figure 1. *Seasonality of chlorophyll a (Chla), sea-surface temperature (SST), mixing layer depth (MLD), and concentrations of DMS and total DMSP. Chla-normalized DMSPt concentrations are shown in grey in the bottom panel.*

The concentration of DMS also showed a strong seasonality, reaching the maximum values in summer, coinciding with the strongest and shallowest stratification of the water column (Fig. 1). DMSPp concentrations did not match Chla concentrations but probably followed the succession and physiology of phytoplankton. As a result, the DMSP:Chla ratio varied notably among seasons, with a very similar pattern to that of DMS concentrations (Chapter I).

The seasonality of the bacterial assemblage composition has been described by Alonso-Sáez et al. (*in press*). The CARDFISH numbers obtained from our MARFISH filters after 18 hours of incubation were not statistically different (t-test, $p>0.05$) than the in situ CARDFISH numbers determined by these authors. Briefly, Alphaproteobacteria dominated the percent of DAPI counts annually. This group was mostly contributed by SAR11 over the year, whereas *Roseobacter* abundance matched Chla concentrations and never reached values higher than 8% of DAPI-stained cells. The second most abundant group was CFB, with constant numbers throughout the year (~11%). Finally, the third most abundant group was Gammaproteobacteria, which showed a similar pattern to that of sea-surface temperature, with abundances ranging from 1 to 8% (Fig. 2).

July 2003 has to be regarded as an unusual sampling event that seems to fall off any seasonal pattern, since bacterial production was exceptionally high and the bacterial community was clearly dominated (30%) by one only phylotype: *Graciecola* sp., Alteromonadaceae, Gammaproteobacteria (Alonso-Sáez et al. *in press*).

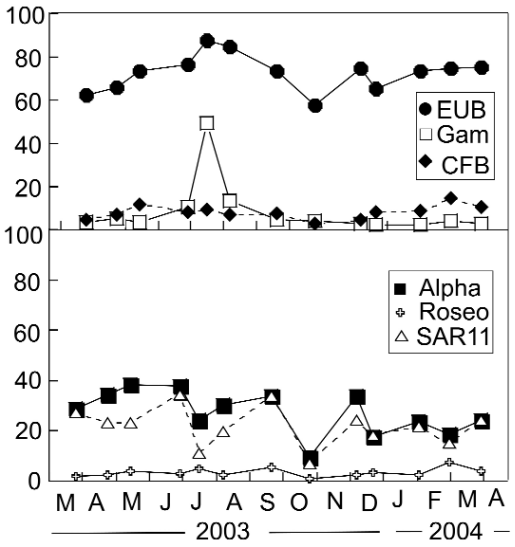


Figure 2. *Percentages (with respect to total DAPI counts) of Eubacteria (EUB) and the main broad groups of marine bacteria, namely Alphaproteobacteria (Alpha), Gammaproteobacteria (Gam), and Cytophaga-Flavobacterium-Bacteroidetes (CFB), as detected by CARD-FISH. In the Alpha group, percentages of Roseobacter and SAR11 phyla are also shown.*

Seasonality of DMSP-S assimilation rate and proportion of bacteria assimilating DMSP. The rate of DMSP-S assimilation showed a strong seasonal pattern with significant higher values ($p<0.05$) in summer compared with the rest of the year. From January to April, assimilation rates averaged 0.6 ± 0.1 nM DMSP/d, representing 8% of total DMSPd bacterial consumption. Coincident with the onset of water column stratification in May, assimilation increased and kept high (6.2 ± 2 nM/d) over the summer, accounting for 33% of total DMSPd bacterial transformations. In September, when the stratification broke, values decreased again and settled to ~ 0.5 nM/d until March 2004 (Fig. 3).

In general, the percentage of EUB+ cells (cells that hybridized with the universal EUB probe) that assimilated DMSP (cells labeled as EUB+DMSP+) showed a similar seasonal pattern to that of the DMSP-S assimilation rate (Fig. 3). From March to June, the percentage of EUB+DMSP+ increased from 6 to 26% and then decreased until December 2003 when they increased again. This latter increase was comparatively higher than that observed for the DMSP-S assimilation rate. On annual average, the percentage of EUB+DMSP+ cells was 15 ± 9 %. The percentage of EUB+ cells that incorporated leucine (EUB+LEU+) was equal to or

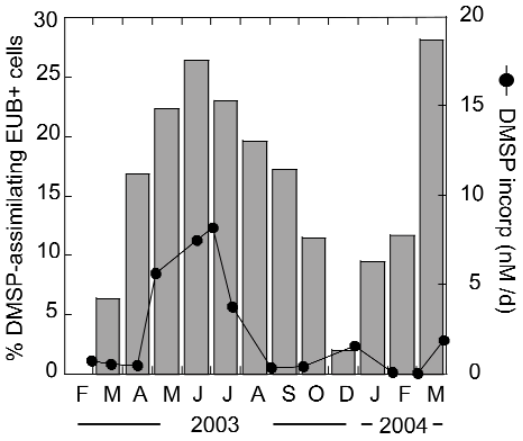


Figure 3. *Seasonality of the percentage of DMSP-assimilating Eubacteria. DMSP incorporation rates are overlaid as the solid black line.*

higher than EUB+ cells incorporating DMSP (Fig. 4). Differences were highest in June and July 2003, and during the winter (November 2003 to March 2004). On annual average, the percentage of EUB+LEU+ cells was 35 ± 16 %.

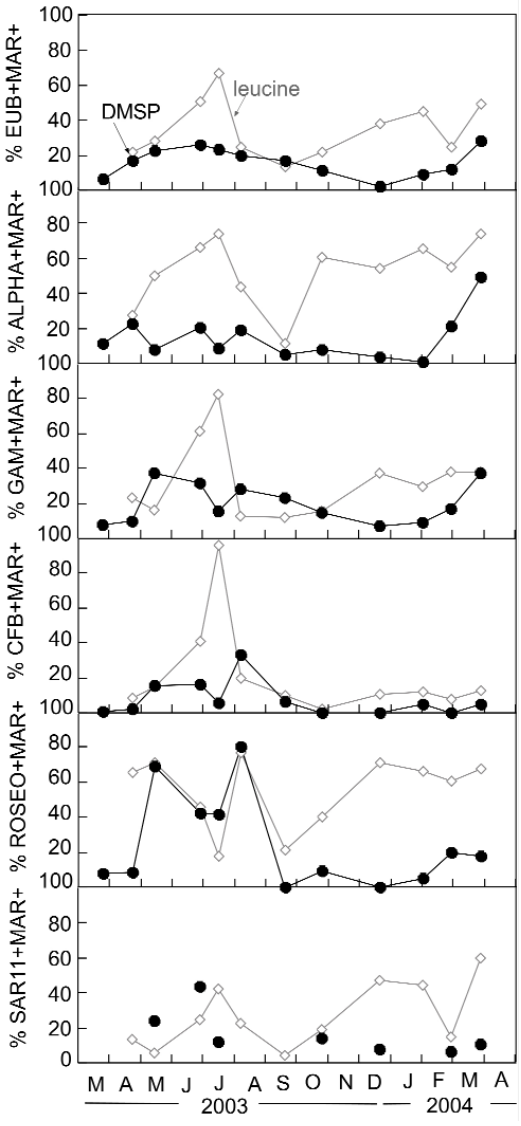


Figure 4. *Seasonal variation of the percentage of DMSP-assimilating (black) and leucine-assimilating cells (grey) within each bacterial group: Eubacteria (EUB), Alphaproteobacteria (ALPHA), Gammaproteobacteria (GAM), Cytophaga-Flavobacterium-Bacteroidetes (CFB), Roseobacter (ROSEO), and SAR11.*

Seasonality of DMSP- and leucine-assimilating bacteria within the phylogenetic groups. The proportion of Alphaproteobacteria that were active at DMSP-S assimilation (ALFA+DMSP+) did not show a clear seasonal pattern but similar values over the year except in March 2004 (11 ± 8 %, Fig. 4). Much higher percentages were recorded for ALFA+LEU+ cells (53 ± 18 %). These showed a dramatic decrease in September 2003, a month characterized by the highest phosphorus limitation of bacterial production (Pinhassi et al. 2006). Among subgroups of Alphaproteobacteria, the percentage of DMSP-S-assimilating *Roseobacter* cells (ROSEO+DMSP+) increased in May 2003 and showed similar or even higher values than those assimilating leucine (ROSEO+LEU+) during the summer (Fig. 4). Over the rest of the year, values of ROSEO+DMSP+ were low (7 ± 6 %). In contrast, the fraction of ROSEO+LEU+ was consistently high over the year (56 ± 20 %). Due to methodological problems, the percentage of SAR11 cells active at DMSP-S assimilation could only be measured in 7 months (Fig. 4). In general, values of SAR11+DMSP+ (15 ± 13 %) were lower than SAR11+LEU+ percentages (29 ± 18 %) except for May and June 2003, when SAR11+DMSP+ cells went up to over 20 and 40%, respectively, reaching values higher than those of SAR11+LEU+ cells.

The fraction of Gammaproteobacteria active at DMSP-S assimilation (GAMMA+DMSP+) showed a marked seasonality with uniform higher numbers (28 ± 9 %) during summer and September, a decrease towards December, and a further increase into March 2004 (Fig. 4). The proportion of GAMMA+LEU+ showed a peak in July 2003, coincident with the dominance of a single phylum of Gammaproteobacteria (Alonso-Sáez et al. *in press*). A similar trend was observed for CFB (Fig. 4). The summer percentages of CFB+DMSP+ cells (18 ± 11%) and CFB+LEU+ cells (43 ± 36 %) contrasted with the low values observed in the winter period (2 ± 2 % and 7 ± 4 %, respectively).

Contribution of each group to substrate assimilation relative to their abundances. The contribution of each group to the total number of cells assimilating a substrate was calculated from the fraction of active cells in the group and its abundance (with respect total EUB+ cells) relative to the percentage of total bacteria (EUB+ cells) active at substrate assimilation. For example, the contribution of Alphaproteobacteria to the total number of cells assimilating DMSP would be:

((ALFA+DMSP+/ALFA+)*(ALFA+/EUB+)) /(EUB+DMSP+/EUB+)

where ALFA+DMSP+/ALFA+ is the percentage of DMSP-assimilating Alphaproteobacteria cells in the Alphaproteobacteria group; ALFA+/EUB+ is the abundance of Alphaproteobacteria with respect to EUB-hybridized cells; and EUB+DMSP+/EUB+ is the percentage of DMSP-assimilating EUB-hybridized cells among total EUB-hybridized cells.

The comparison of these group contributions to total DMSP-S- and leucine-assimilating cells with their contribution to the composition of the bacterial assemblage is shown in Fig. 5. The group Gammaproteobacteria showed most of the data points on or near the 1:1 line, i.e., their contribution to the assimilation of both substrates was that expected from their relative abundance. The group Gammaproteobacteria, and particularly the *Roseobacter* and SAR11 clades, were overrepresented among DMSP-assimilating cells in the summer samples, whereas over the rest of the year they were underrepresented. This contrasts with the consistent overrepresentation of Alphaproteobacteria in the assimilation of leucine, mainly due to Roseobacter, while fewer SAR11 than their relative abundance assimilated leucine. CFB were consistently underrepresented in the assimilation of both compounds over most of the year.

Seasonal variation of the phylogeny of DMSP- and leucine-assimilating cells. The dominance of Alphaproteobacteria in DMSP and leucine assimilation was a common feature all over the year, except in July 2003 when both assimilations were dominated by Gammaproteobacteria. In the summer months, *Roseobacter* and SAR11 explained all the Alphaproteobacterial DMSP assimilation (when SAR11 hybridization was performed), whereas in winter an unknown Alphaproteobacteria fraction also accounted for a portion of DMSP incorporation (Fig. 6). Annually, members of the SAR11 clade were responsible for most of Alphaproteobacterial DMSP

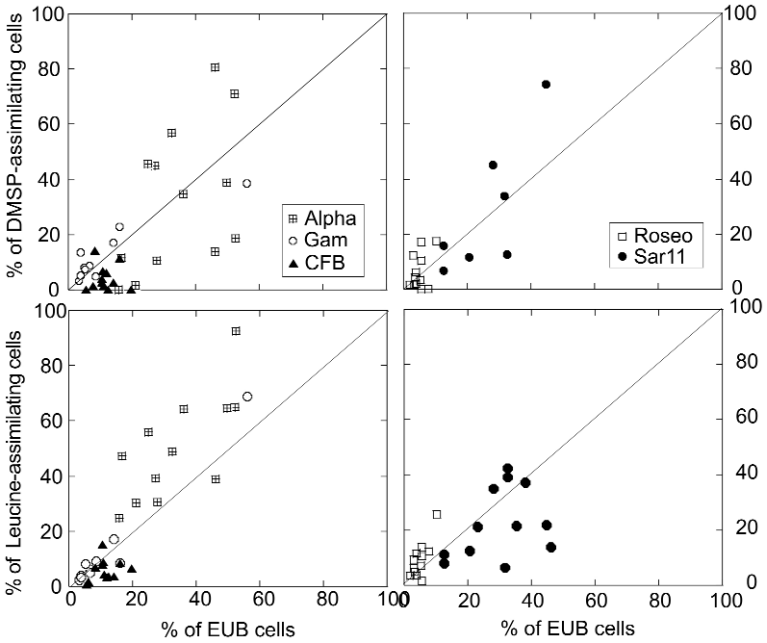


Figure 5. *Contribution of each bacterial phylogenetic group (Alphaproteobacteria (Alpha), Gammaproteobacteria (Gam), Cytophaga-Flavobacterium-Bacteroidetes (CFB), Roseobacter (ROSEO), and SAR11) to total substrate uptake versus the relative abundance of the group (calculated with respect to EUB cells). Data points on the 1:1 line indicate equal contribution to substrate uptake and to community composition.*

assimilation (62 ± 33 %) with *Roseobacter* also accounting for a significant proportion (25 ± 25 %). However, a clear seasonal pattern could not be determined. Similarly, SAR11 accounted for the largest fraction of Alphaproteobacterial leucine uptake over the year, with higher contributions in the winter period (63 ± 35 %) than in the summer period (25 ± 12 %). The contributions of the *Roseobacter* group were lower in summer (10 ± 5 %) than in winter (27 ± 18 %).

A clearer seasonal pattern was observed in the contribution of Gammaproteobacteria to DMSP assimilation, with higher contributions in summer (17 ± 15% on average, and 10 ± 6 % excluding July) contrasting with lower values over the rest of the year (7 ± 3 %). The highest Gammaproteobacterial contributions to leucine uptake were observed in June (17%) and July (68%), with values averaging ~5 ± 2 % during the rest of the year.

Despite the fact that CFB represented the second most abundant group, generally it accounted for the smallest annual proportion of the cells assimilating both substrates (4 ± 1% and 6 ± 1 % of DMSP and leucine incorporation, respectively). However, CFB showed a slightly higher

contribution to leucine uptake in summer (10 ± 4%) that was only observed in March and August in DMSP assimilation.

On annual average, ~40% (± 24) of the cells that had assimilated DMSP or leucine could not be attributed a phylogenetic identity with the broad probes used. In general, this fraction was higher in winter and lower in summer for both compounds.

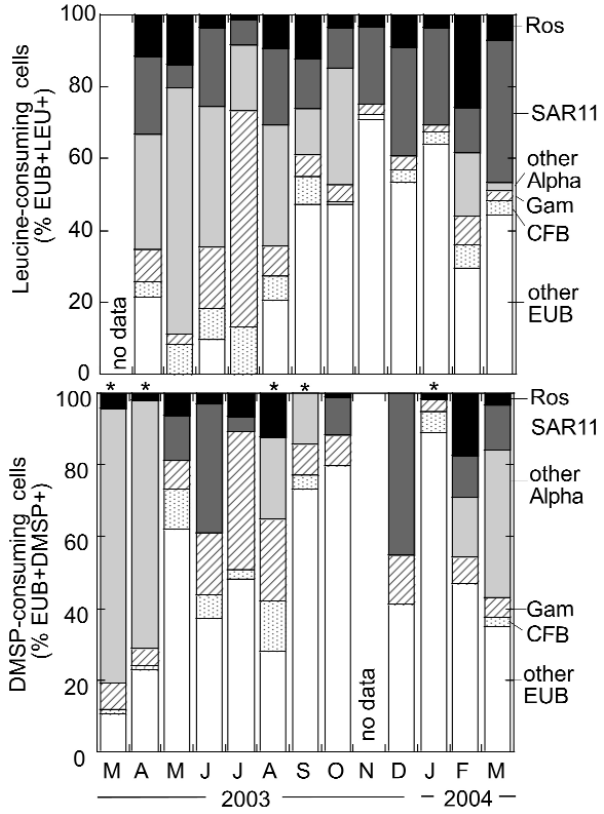


Figure 6. *Seasonal variation of the contribution of each phylogenetic group (Alphaproteobacteria (Alpha), Gammaproteobacteria (Gam), Cytophaga-Flavobacterium-Bacteroidetes (CFB)) to total substrate assimilation (leucine, top panel; DMSP, bottom panel). The Alphaproteobacteria group is divided among Roseobacter (Ros), SAR11, and unidentified Alpha (other Alpha). (*) months when microautoradiograms of SAR11 could not be performed.*

Short term nutrient limitation bioassays. Addition of glucose in combination with P caused increases in bacterial heterotrophic production (leucine incorporation) that, in 14 out of 19 monthly experiments, were significantly higher than those after addition of P only (Fig. 7). This suggests that over most of the year a secondary C limitation occurred associated with a strong and consistent P limitation of bacterial growth. In 10 experiments, addition of DMSP + P stimulated bacterial production over that with P only, particularly from April to October 2003 and from March to July 2004. That is, DMSP was an efficient C source during summer periods. Interestingly, in three experiments (June and July 2003, and June 2004) combined additions of DMSP + P caused significantly higher bacterial heterotrophic production than combined additions of glucose + P.

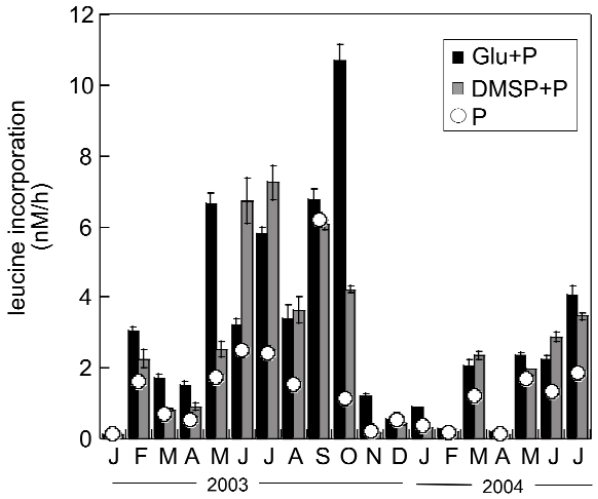


Figure 7. *Seasonal variation of bacterial production responses to phosphate and substrate additions. Leucine incorporation rates in samples incubated for 24 h after enrichment with P (0.6 μM Na_2HPO_4), P plus glucose, or P plus DMSP (24 μM C)*

Discussion

The motivation of this work was to describe the seasonality of the number and phylogenetic affiliation of DMSP-assimilating bacteria in order to assess the importance of bacterial assemblage composition in controlling the fate of DMSP in the pelagic marine environment. Heterotrophic bacteria play a major role in the DMS cycle since most DMSPd is demethylated by them to eventually supply their sulfur demands and only a small percentage is cleaved into DMS (Kiene et al. 2000). However, factors influencing bacterial DMSP assimilation remain mostly unknown and, contrasting with the well described seasonality of DMS and DMSP concentrations in different sampling sites (e.g. Dacey et al. 1998, Uher et al. 2000, see compilation in Simó & Pedrós-Alió 1999a), little is known about the seasonality of their transformation processes, including DMSP assimilation.

In our study, DMSP assimilation rates showed a strong seasonality, with significant maximum values in summer. Slezak et al. (2001) reported on significant inhibition of bacterial DMSP degradation by surface levels of solar radiation (UV + PAR) in samples from the Adriatic and coastal North Sea. In fact, several studies agree to show the negative effects on bulk metabolic activities of bacterioplankton caused by UV radiation (Herndl 1997, Jeffery et al. 2000). Summer months are characterized by higher solar radiation doses in shallower mixing layers (1-3 m in our sampling site, from May to August, Fig. 1) thus enhancing the photo-damage on non-pigment-protected cells. During the summer period in our sampling site, however, bacteria not only significantly increased their rate of DMSP incorporation by a factor of 15 with respect to winter values but also increased the percentage of DMSP-assimilating cells (Fig. 3). The similar seasonal pattern observed for the ratio DMSP:Chla (Fig. 1) suggests that the succession

of phytoplankton assemblages towards higher-DMSP producers in summer (Chapter IV) could have increased the contribution of DMSP into the fluxes of reduced sulfur compounds, thus increasing the role of DMSP as S source for bacteria. In order to know if, additionally, the summer bacterial assemblage was more suited to use DMSP as a S source, we used the MAR-CARD-FISH technique.

The percentage of bacteria assimilating DMSP-S was well correlated with the rate of DMSP-S assimilation suggesting the widespread capacity among bacteria to incorporate DMSP. Indeed, DMSP was consumed by members of the 3 main groups of marine bacterioplankton: Alphaproteobacteria, Gammaproteobacteria and CFB, in agreement with the results of previous studies (Malmstrom et al. 2004a, Chapter II). Differences in the contribution of each group also agree with the previous studies: Alphaproteobacteria dominated DMSP-S assimilation over the year, as was observed in the Gulf of Maine and the Sargasso Sea (Malmstrom 2004a and 2004b) and at the same sampling site (Blanes Bay) in January and February 2003 (Chapter II). This group also dominated leucine incorporation over the year, in support of the observation that Alphaproteobacteria, along with Gammaproteobacteria, prefer the uptake of low-molecular-weight compounds (LMW) whereas CFB are more suited to use high-molecular-weight substrates (Cottrell & Kirchman 2000). Accordingly, Gammaproteobacteria was the second main contributor to DMSP assimilation, while CFB had a minor contribution.

The use of the SAR11 and *Roseobacter* probes is justified because these two abundant subgroups of marine Alphaproteobacteria have shown a high capability for DMSP transformations (Moran et al. 2003, Malsmtrom et al. 2005). Interestingly, the gene encoding for DMSP demethylation has been found in cultured representatives of both clades (Howard et al. 2006). SAR11 was found to be the most abundant group in our seasonal study, with higher abundances in the summer months, coinciding with higher nutrient limitation of bacterial growth (see Alonso-Sáez et al. *in press*, Pinhassi et al. 2006). In Gulf of Mexico and Sargasso Sea waters, Malmstrom et al. (2004b) observed that SAR11 accounted from 31 to 47% of total DMSP-S assimilation. We obtained similarly high values only in the summer samples, whereas over the rest of the year SAR11 contributed ~12% of total DMSP assimilation. A seasonal succession of bacterial phyla within the SAR11 clade might explain this observation. However, despite the fact that clone libraries of the same samples revealed a high microdiversity among SAR11 clones all the year round, they did not show any clear succession pattern (Alonso-Sáez et al. *in press*).

Roseobacter phyla are usually found associated with algal blooms (Buchan et al. 2005). In blooms of DMSP producers, such as the coccolithophore *Emiliania huxleyi*, *Roseobacter* abundances were well correlated with DMSP concentrations (González et al. 2000) or DMSP consumption rates (Zubkov et al. 2002), thus reinforcing their prominent role as DMSP

consumers that had been observed with isolates (González et al. 1999). In Blanes Bay, the phytoplankton succession to high DMSP producers in summer was not followed by an increase in *Roseobacter* abundance, which rather matched total Chla concentrations (Alonso-Sáez et al. in press). Despite the lack of a marked seasonality among *Roseobacter* microdiversity, it is interesting to note that the increase in the percentage of DMSP-assimilating *Roseobacter* cells in summer coincided with the presence of a DGGE band that had the closest match to another DGGE band found in previous microcosm nutrient-enrichment study where 87% of *Roseobacter* cells assimilated DMSP-S (Pinhassi et al. 2005, Chapter II). However, due to the very low abundances of *Roseobacter* in summer, their contribution during this period was not significantly increased.

In addition, the seasonality of the DMSP assimilation rates was not correlated with that of the abundances of *Roseobacter*. Our results agree with the conclusion of previous MARFISH studies that *Roseobacter* alone could not explain all DMSP assimilation (Malmstrom et al. 2004a, Chapter II). Further recent studies have observed the competitive advantageous capacity of this group to take up leucine at much lower concentrations than other groups (Alonso & Pernthaler 2006a). The consistent high percentages (56%) of *Roseobacter* cells active at leucine uptake over the year compared to the average 23% of DMSP-assimilating *Roseobacter* cells reflect the high capacity of the group to take up LMW-DOM components in general rather than showing a preference for DMSP assimilation.

The increased proportion of *Roseobacter* and SAR11 cells assimilating DMSP-S during summer was not reflected in an increase of DMSP-assimilating Alphaproteobacteria cells. This could be explained because the general probe of Alphaproteobacteria (Alf968) has a mismatch against all the SAR11 clones from Blanes Bay samples, as discussed by Alonso-Sáez et al (*in press*). The Alf968 probe can, thus, underestimate the abundances of Alphaproteobacteria because some SAR11 cells do not get hybridized. This effect could be especially important during the summer period when the abundances of SAR11 cells were highest.

Remarkably, Gammaproteobacteria showed a contribution to DMSP-S assimilation that was consistent with their abundance over the year. In general, this group contributed equally to DMSP-S assimilation than to leucine assimilation. This finding confirms the prominent role of Gammaproteobacteria in DMSP-S assimilation suggested by Vila et al. (Chapter II). As a matter of fact, in blooms of DMSP-producing phytoplankton where the bacterial communities were dominated by Alphaproteobacteria, members of Gammaproteobacteria were present also at significant numbers (González et al. 2000, Zubkov et al. 2002). Moreover, Mou et al. (2005) found that 16S rRNA sequences belonging to the Gammaproteobacteria group contributed 14-21% of the abundance of DMSP-enriched communities in 2 of 4 samples collected from salt marsh waters. Similarly, enrichment of estuarine and salt marsh waters with DMSP resulted in the predominance (80%) of Gammaproteobacteria isolates (Ansede et al. 2001). Thus, our results

provide evidence for more important a role of Gammaproteobacteria in DMSP consumption in sea-surface waters than previously considered.

CFB did not contribute significantly to DMSP-S assimilation in 8 of the 12 monthly samples, and they were consistently underrepresented in the assimilation of DMSP-S relative to their abundance *in situ*. This result is in agreement with previous observations (Malmstrom et al. 2004a, Chapter II) and supports the suggestion by Cottrell & Kirchamn (2000) of a preference of this group for degrading high-molecular-weight compounds.

Close values of DMSP- and leucine-assimilating cells were observed also in summer (except for the unusual high percentage of active-leucine cells in July 2003). The assimilation of leucine can be taken as an indicator of active cells, since leucine is widely used for bacterial protein synthesis (Kirchman 2001). The annual average of leucine-active cells was $\sim$35 $\pm$ 16 %, in the range of typical values obtained in other marine sites (Smith & del Giorgio 2003). The annual percentage of DMSP-assimilating cells was lower (15 $\pm$ 9 %), and significantly lower than values (48 $\pm$ 10 %) observed in the Gulf of Maine and Sargasso Sea (Malmstrom et al. 2004a). Reduced percentages of DMSP- versus leucine-assimilating cells in all the major bacterial groups during much of the year suggest that not all bacteria synthesizing protein assimilated DMSP. This finding contrasts with the good correlation found between DMSPd consumption rates and leucine incorporation rates found in temperate open-ocean and subtropical coastal waters (Malmstrom et al. 2004b, Kiene & Linn 2000a). Although sulfate is very abundant in seawater and bacteria have the capability to reduce it, so that they never get limited by this element, the use of available S in a reduced form would be energetically advantageous. Kiene et al. (1999) demonstrated the incorporation of the CH_3SH moiety from DMSP into S-aminoacids, so that it is very plausible that bacterioplankton are well suited to use DMSP, when it is available, to save energy for protein synthesis. The excellent correlation that we found between bacterial heterotrophic production (^{3}H-leucine incorporation) and ^{35}S-DMSP assimilation rates (Chapter IV) suggests that our determinations of the percentage of DMSP-assimilating cells with microautoradiography might be underestimates if cells were not accumulating enough ^{35}S to be visually detected once their S demands were satisfied (see also Annex II).

The results of the enrichment experiments highlight the role of DMSP as S and C source. The elevated bacterial production caused by combined additions of DMSP+P, relative to addictions of P-only, in all cases took place during spring and summer, coinciding with an increase in the proportion of bacterioplankton that assimilated DMSP-S. This coincided with the period of increasing DMSP:Chla ratios, which can thus be proposed as a proxy for the quantitative role of DMSP as a supply of reduced S and C to bacterial S and C demands (Chapter IV). On several occasions in summer the stimulation of DMSP+P additions was as large as, or even larger than the stimulation caused by additions of glucose+P, indicating that DMSP is a highly labile organic compound for marine bacteria to use. This corroborates recent findings from the

C-limited coastal Gulf of Mexico, where the addition of DMSP alone triggered as large an increase in bacterial leucine incorporation as glucose addition (Pinhassi et al. 2005).

Conclusions

In Blanes Bay, DMSP-S assimilation showed a strong seasonal pattern with maximum values in summer. Also in summer we observed concomitant higher percentages of leucine-incorporating cells, and higher stimulation of bacterial production by additions of DMSP plus phosphate. All this indicated that either DMSP-S was being assimilated by a very broad and diverse fraction of the bacterial assemblage or DMSP-specialists, if occurring, were very abundant in summer. Our results confirm the widespread capacity of the main groups of heterotrophic bacteria to assimilate DMSP-S, although it was more efficiently incorporated by members of the Alphaproteobacteria and Gammaproteobacteria groups. The abundances of major bacterial groups over the year were not related to the seasonality observed in DMSP-S assimilation. Although our phylogenetic characterization of bacterioplankton only resolves broad taxonomic groups, finer parallel analyses with clone libraries did not reveal a clear intra-group succession that could explain the higher assimilation in summer. Rather, DMSP-S was more assimilated when it contributed more to S sources for bacteria, independently of any detectable shift in the composition of the bacterial assemblage. This study highlights the importance of DMSP as a S source for marine bacteria under oligo- to mesotrophic conditions, particularly in summer highly irradiated waters.

CHAPTER IV

Seasonality of DMSP contribution to S and C fluxes through phytoplankton and bacterioplankton in a NW Mediterranean coastal site

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Guadayol, Òscar; Vázquez-Domínguez, Evaristo and Simó, Rafel.

Abstract

The contribution of dimethylsulfoniopropionate (DMSP), the precursor of the climatic active gas dimethylsulfide (DMS), to the fluxes of sulfur and carbon through the first levels of the food-web was investigated throught a seasonal study in Blanes Bay (NW Mediterranean coast). In general, the contribution of DMSP to the algal sulfur content increased from winter (47 $\pm$ 22 %) to the annual maximum values in summer (104 $\pm$ 8 %), possibly due to a succession of the phytoplankton assemblages towards higher-DMSP producers. Estimated DMSP production showed roughly a similar seasonal pattern to that of primary production, which showed maximum values during the winter period. Considering DMSP as a product of photosynthesis, the percentage of primary production invested into DMSP synthesis was, for most of the year, 1-7%. DMSP supplied most of the sulfur demand and $\sim$5% of total carbon demand of heterotrophic bacteria during summer, whereas over the rest of the year these supplies were 55% and 2%, respectively. Even though the percentage of primary production invested into DMSP did not significantly increase in summer, the decrease of Chla concentrations led to an increase of the ratio DMSP:Chla, concomitant with a significant increase of DMSP assimilation by bacteria. In conclusion, our results suggest that in more oligotrophic and irradiated waters (summer), DMSP accounts for a larger share of the fluxes of C and S. We propose the ratio DMSP: Chla as a good indicator of the contribution of DMSP to the C and S demands of bacteria.

Introduction

Dimethylsulfoniopropionate (DMSP) is a ubiquitous organic sulfur compound produced in the euphotic zone by many species of marine phytoplankton, especially those belonging to the *Dinophyceae* and *Prymnesiophyceae* classes (Keller et al. 1989). Research on DMSP was greatly stimulated when its role as the precursor of dimethylsulfide (DMS) was discovered. DMS is the most abundant volatile organic sulfur compound in the upper ocean. It is emitted from the oceans to the atmosphere where its oxidation products (sulfur aerosols) can influence the Earth's climate system through direct scatter of solar radiation and by promoting cloud formation and enhancing cloud albedo (Charlson et al. 1987). Previous research has revealed that DMSP is a major pool of organic sulfur in the epipelagic ocean, and the study of the complex cycle of DMSP transformations has shown that it is a source of reduced S (and a potential source of C) for marine heterotrophic bacteria (Kiene & Linn 2000a, Simó et al. 2002, Zubkov et al. 2002), herbivore protozoans (Burkill et al. 2002, Simó et al. 2002, Tang & Simó 2003, Simó 2004), and even non-DMSP-producing phytoplankton (Chapter V). Thus, DMSP is cycled and transformed through the different trophic levels of the food web (Tang et al. 1999, Archer et al. 2001a, Simó et al. 2002). Nevertheless, the very few field studies that have quantified the contribution of DMSP to the fluxes of S and C through the food-web were conducted with blooms of DMSP-producing phytoplankton (Simó et al. 2002, Burkill et al. 2002). Since these blooms are not a dominant feature of the ocean, the role of DMSP as carrier of S and C through the food-web in the different possible environmental and ecological settings still remains unknown.

DMSP is believed to act in the algal cells as osmoregulator, cryoprotector and antioxidant (Vairavamurthy et al. 1985, Kirst et al. 1991, Sunda et al. 2002). Other functions such as methyl donor in metabolic reactions and overflow of reducing power under unbalanced growth conditions have been also suggested (Stefels 2000). The contribution to algal C and S in the form of the DMSP molecule has been found to be significant in samples from cultures and in several field studies. In general, the fraction of phytoplankton carbon that occurs as DMSP ranges from 0.2 to 9% (Kiene et al. 2000). Interestingly, blooms of DMSP-producers such as *Emiliana huxleyi* had yielded similar values (7%) for the fraction of total primary production invested in DMSP synthesis (Simó et al. 2002, Archer et al. 2002).

The reduced sulfur carried by algal DMSP goes up in the food web through direct grazing on algae or through the microbial loop that recycles the dissolved DMSP (DMSPd) again into particulate material. In fact, only a minor fraction (yet significant) of algal-DMSP-sulfur is assimilated (incorporated into biomass) by the herbivore, or retained as DMSPp and transferred up the food chain (Tang & Simó 2003). Grazers mainly act to catalyze the release of DMSPd and DMS (Tang et al. 1999, Archer et al. 2001b, Simó et al. 2002). Thus, the amount of DMSPd eventually assimilated by heterotrophic bacteria has a relevant role in regulating the quantity of DMSP-sulfur that flows to the higher trophic levels.

DMSPd can supply 1-15% of the carbon demands and almost all of the sulfur demands of heterotrophic bacterioplankton (Kiene & Linn 2000a, Simó et al. 2002, Zubkov et al. 2002). DMSP provides the sulfur requirements of bacteria by supplying the incorporation of the MeSH moiety, which goes primarily into the synthesis of sulfur aminoacids and proteins (Kiene et al. 1999). This demethylation/demethiolation route seems to dominate DMSP degradation generally in the surface ocean. However, bacteria can also cleave DMSP to produce DMS or to double-demethylate DMSP to produce non volatile sulfur compounds. Kiene et al. (2000) suggested that, with enough available DMSPd, the saturation of the sulfur demands of bacteria would induce a switch from the demethylation to the cleavage route, increasing the production of DMS by bacteria.

Unfortunately, the few studies that have quantified the sulfur demand of bacteria have not addressed the seasonality of this demand. The temporal variation is important since several studies in different oceanic sites have pointed to a strong seasonality of sea-surface DMS concentrations (e.g. Dacey et al. 1998, see compilation in Simó & Pedrós-Alió 1999a). A very recent study has shown that this seasonality is also observed in DMSP cycling dynamics (Chapter I). Particularly, in Blanes Bay and during this study, DMSP assimilation by heterotrophic bacteria was significantly higher during the summer period, coinciding with a higher DMSP:Chla ratio. The seasonal coupling found between bacterial DMSP assimilation and the DMSP:Chla ratio should reflect the relative role of DMSP in C and S fluxes through the food web and we would thus expect a higher participation of DMSP in the fluxes in summer when DMSP assimilation is higher.

We report a seasonal study of the contribution of DMSP to the fluxes of C and S through phytoplankton and heterotrophic bacterioplankton in a coastal oligo- to mesotrophic site. For the first time, the variability of DMSP production by phytoplankton, and the variability of bacterial sulfur and carbon demands supplied by DMSP assimilation are described along with the evolution of the environmental and trophic conditions over an annual cycle.

Materials and Methods

Environmental factors. We monthly sampled two consecutive days in Blanes Bay (Blanes Bay Microbial Observatory, NW Mediterranean, 41°40'N, 2° 48'E) from January 2003 to June 2004. This site is located approximately 1 mile offshore, over a 24-m deep water column. Surface seawater was collected by carefully submerging two acid-cleaned amber glass bottles (2.5 L) to a depth of 0.5 m, avoiding bubbling. Bottles were kept in the dark at *in situ* temperature until they were processed, approximately 1-2 h after sampling. Vertical temperature profiles were determined by collecting water from different depths (0, 5, 10, 15 and 20 m) using a Niskin bottle and measuring temperature with a mercury thermometer. A more accurate vertical profile was performed when a difference of temperature >1 °C was recorded

in the first 5 m of depth. Chlorophyll a (Chla) concentration was measured by fluorometry (Turner Designs fluorometer) in extracts (90% acetone, 4°C, overnight) of 150 ml of seawater filtered through GF/F filters (Whatman). Concentration of NO_3^- was determined spectrophotometrically using an Alliance Evolution II autoanalyzer following standard procedures (Grasshoff et al. 1983).

DMSPp and DMSPt analyses. Total DMSP (DMSPt) and particulate DMSP (DMSPp) were measured as DMS after alkaline hydrolysis of either 40 ml of whole seawater (DMSPt) or GF/F-retained particles (DMSPp) from 40 ml seawater subsamples. The evolved DMS was determined with the purge, cryotrapping and sulfur-specific gas chromatography system described by Simó et al. 1996.

DMSP production rate. DMSP production rate was estimated by budging the concentrations of DMSPt from the two consecutive days corrected by the measured DMSPt consumption as follows:

$$\text{DMSP prod} = ([\text{DMSP}]_2-([\text{DMSP}]_1-((\text{DMSP consumption})*(t_2-t_1)))/(t_2-t_1)$$

Based on the similarity of physical parameters (T, salinity), Chla, and dimethyalted sulfur concentrations (DMS, DMSP and DMSO) between the two consecutive sampling days, we assumed we sampled the same water mass. DMSPt consumption was measured using the method of "net loss curve" in the dark (Simó et al. 2000, see also Chapter I).

Particulate primary production. Incorporation of carbon into the particulate fraction was measured by the ^{14}C-technique (Steeman-Nielsen 1952) generating P-E curves. Water for incubations was collected and dispensed in aliquots (70 ml) that were introduced in tissue culture bottles, which were then spiked with 10 μCi of ^{14}C-bicarbonate. There were 13 clear bottles and 1 dark (covered with aluminium foil) under constant light conditions in a controlled-temperature bath maintained at *in situ* temperature and in a gradient of light irradiance (ca. 10-1000 μmol fotons m^{-2} h^{-1}). Circulating water connected to a water bath maintained the temperature. Light was measured with a small size spherical light meter (Illuminova AB, Sweden).

After 2 hours of incubation, water was filtered through 0.2 μm pore size cellulose ester Millipore filters. These filters were put into scintillation vials and left for 24 h in a HCl saturating atmosphere. Finally, 4 ml of scintillation cocktail (Optiphase Hisafe 2) were added in each vial and radioactivity was measured in a Beckman liquid scintillation counter. Total in situ pPP was determined from the P-E curve and the *in situ* irradiance obtained with a Li-Cor sensor (Li-193S).

Bacterial DMSP consumption and incorporation. Rate constants for bacterial DMSPd consumption were measured following the exponential disappearance of ^{35}S-DMSP added at

trace concentrations (< 0.01 nM) to 30 ml of sample and incubated in the dark at the *in situ* temperature. Bacterial DMSPd consumption rates were calculated as the product of DMSPd concentrations and the loss rate constants (Chapter I).

The percentage of DMSP incorporated into macromolecules was calculated as the proportion of ^{35}S-DMSP retained on the filter with TCA-precipitated particulate material versus initial radioisotope added. Briefly, 15 ml sub-sample of whole seawater was incubated in the dark at *in situ* temperature without headspace for 18 hours with a trace addition of ^{35}S-DMSP (1000 dpm ml^{-1}; 5.79 Ci pmol^{-1}). Triplicate aliquots (5ml) were filtered through nylon filters (GN, Millipore, 0.2-μm-pore-size) using a gentle vacuum (<5 cm Hg) and rinsed with 0.2 μm-filtered seawater (FSW). Macromolecules were precipitated by treating filters with cold aliquots (5 ml) of trichloroacetic acid (TCA 5%) for 5 min. Filters were then rinsed twice with MilliQ water and counted using a Beckman scintillation counter. The precision (%SE) of triplicate measurements averaged $\sim$ 1%. Incorporation of ^{35}S-DMSP in formalin-killed controls was $\leq$ 1.5 % that in live samples. DMSP incorporation rate was obtained by multiplying the percentage of initial ^{35}S-DMSP added retained in the filters by DMSPd consumption rate (see Chapter III).

DMS consumption. Surface waters were incubated for 8-12 h in the dark at the *in situ* temperature in acid-rinsed, amber glass bottles (2.5 L) without any amendment (control treatment) and, parallely, with dimethyldisulfide addition (260 nM final conc., DMDS treatment). Assuming that DMDS selectively inhibits DMS consumption (Wolfe & Kiene 1993), the DMS consumption rate was obtained by the difference between the slope of DMS accumulation in the DMDS treatment (gross DMS production) and the slope of the time course of DMS concentration in the control treatment (net DMS production). For further details see Simó et al. (2000) and Chapter I.

Bacterial Production (BP). Bacterial heterotrophic production was determined from the incorporation of ^{3}H-leucine into protein using the method of Kirchman et al. (1985) with the modifications of Smith & Azam (1992) Briefly, 1.2-ml triplicate live and duplicate killed (5% trichloroacetic acid, TCA) subsamples were incubated with ^{3}H-leucine (40 nM final conc.) for 2 h, at the *in situ* temperature, in the dark. Incubations were stopped by addition of 120 μl of cold TCA 50% and frozen (-20°C) until further processing by centrifugation and TCA rinsing. Leucine incorporation rates were converted to bacterial production rates with empirical conversion factors that ranged from 1.0 kgC mol^{-1} on average in summer to 1.9 kgC mol^{-1} on average in winter (details in Alonso-Sáez et al., *submitted*).

Bacterial respiration rates (BR). Respiration rates were obtained by linear regression of oxygen concentration vs. time in incubations (0-24 hs) of ca. 130 ml of filtered seawater in boro-silicate glass bottles. Samples were filtered through 0.8 μm mixed cellulose esters filters to

include exclusively the prokaryote fraction. The bottles were siphoned twice with sample seawater before they were closed with their stoppers. Five replicates of the initial-time samples were immediately fixed with Winkler reagents. The remaining five replicates were submerged inside dark coolers filled with tap water, which were maintained in an isothermal chamber. Previously to each determination, the water inside the coolers was stabilized to the correct temperature during at least 12 hours. After 24 hours, samples were fixed with Winkler reagents, and dissolved oxygen was determined with an automatic titrator based on potentiometric endpoint detection (Oudot et al. 1988). The average standard error between replicate bottles was 0.53 μM O_2. Oxygen consumption rates were transformed to carbon units assuming a respiratory quotient of 1. All oxygen decrease rates were significant with a $p < 0.05$, except in one occasion when $p = 0.07$, indicating that the assumption of linearity in the oxygen consumption with time held in most of our determinations.

Bacterial Carbon Demand (BCD). BCD was calculated as the sum of bacterial production (BP) and bacterial respiration (BR) as follows: BCD= BP + BR.

Results

Seasonal changes in the ecosystem. The marked seasonality of air temperature and heat flux in Blanes Bay originated a strong stratification of the water column in summer (from May to August) with a typically 3-m deep mixed layer (Fig. 1). This period showed a rather high water temperature (22°C), low NO_3^- concentrations (0.24 μM) and low Chla concentrations (0.4 μg L^{-1}) at the surface. The phytoplankton assemblage was dominated (60%) by phototrophs smaller than 3 μm and *Synechococcus* abounded during this period (39790 $\pm$ 8413 cells/ml). Contrastingly, the winter period (from December to February) was characterized by a well-mixed water column, lower water temperature (13°C, i.e., similar to deeper Mediterranean waters), high NO_3^- concentrations (2.8 μM), and higher Chla concentrations (1.7 μg L^{-1}) mostly contributed by phototrophs larger than 3 μm (70%). The typical winter diatom bloom occurred in February 2003, and another peak of Chla was recorded in December 2003, when the phytoplankton assemblage was co-dominated by diatoms and haptophytes (Gutierrez et al. *in prep.*). Salinity ranged 36.0-38.7 psu over the year, indicating that the sampling station does not have a major influence of freshwater coastal discharges.

The concentrations of DMSPp ranged between 7 and 72 nM. Higher values were recorded in March 2003 (72 nM) and December 2003 (56 nM). DMSPp concentrations decreased towards summer to reach the lowest values in autumn (September-November, 10 $\pm$ 1 nM). In general, DMSPp peaked one month after the peaks of Chla except in December 2003 when both peaks were concomitant (Fig. 1). A detailed description of the time series of DMSP and other dimethylated sulfur compounds is given elsewhere (Chapter I).

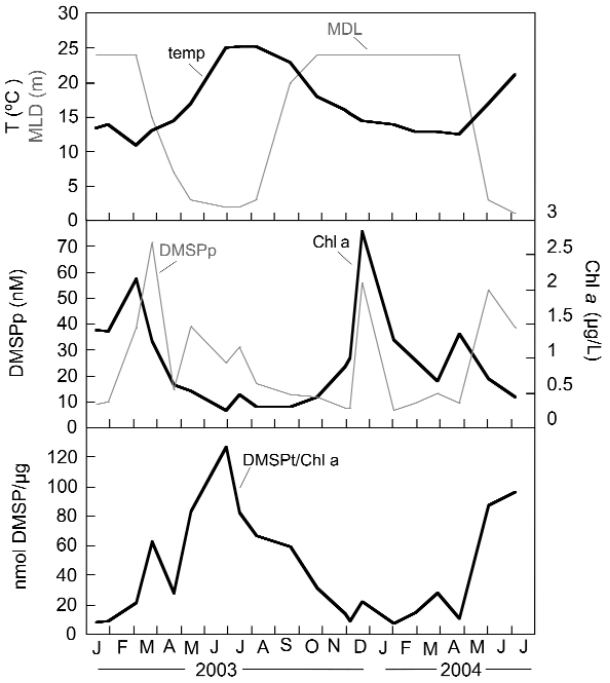

Figure 1. *Variation of sea-surface temperature (temp), mixing layer depth (MLD), chlorophyll a (Chl a) and particulate DMSP (DMSPp) concentrations during the course of the time-series study in Blanes Bay. The seasonality of the ratio DMSPp:Chla is also shown.*

Seasonality of DMSP contribution to carbon and sulfur fluxes through phytoplankton. Phytoplankton particulate organic carbon (POC) was calculated using experimental ratios of C:Chla obtained from the concentrations of pico-, nano- and microphytoplankton in the same samples (Guadayol et al. *in prep.*) except for the period from January to June 2003 and May-June 2004, when these measurements were not available and had to be estimated. Considering photoacclimation as one of the main driving factors of the C:Chla ratio (Geider et al. 1997, Taylor et al. 1997), we assumed a sigmoidal increase of C:Chla in this period (equivalent to the increase of the dose of solar radiation integrated in the mixing layer) from 20 (typical winter value) to 57 (August measured value). To convert POC to Particulate Organic Sulfur (POS), we used a C:S ratio of 57, which is the mean of values obtained in cultures by Matrai & Keller (1994) and Ho et al. (2003) for a number of marine phytoplankton species belonging to the groups of dinoflagellates, haptophyte-coccolithophores, and diatoms. DMSP-carbon content in cells was calculated considering that 1 mole of DMSP contains 5 moles of DMSP-carbon. DMSP accounted for most of organic S (104 ± 8%) and a substantial fraction of organic C (9 ± 1%) of algae in summer, contrasting with significant lower values the rest of the year (44 ± 11% for S and 4 ± 1% for C). In fact, the seasonality of both contributions was strongly marked, inversely correlated to the depth of the mixing layer (Pearson's correlation coefficient $r=0.75$, $p<0.01$, $n= 18$, taking out December 2003). On annual average, DMSP contributed 6 ± 1% of algal carbon and 70 ± 10% of algal sulfur content (Table 1).

Table 1. *DMSP and C and S pools and fluxes through phytoplankton. POC: particulate organic carbon, POS: particulate organic sulfur. Standard desviations (SD) of empirical measurements are given in parenthesis. Averages (± SE) are shown for different seasonas (W: winter, SP: spring, SU:summer, AU:autumn).*

Month	Date		DMSPp	Chla	C:Chla[a]	DMSP-C/ algal POC	DMSP-S/ algal POS[b]	DMSP prod	Prim.Prod. (PP)	DMSP-C prod/PP
			nM	*µg L^{-1}*	*µg µg^{-1}*	*%*	*%*	*nM S d^{-1}*	*nM C d^{-1}*	
January	Jan 28, 03	W	9.8 *(1.4)*	1.4 *(0.1)*	*20.0*	2.1	23.9	12.5 *(0.9)*		
February	Mar 04, 03	W	38.4 *(11.7)*	2.2 *(0.1)*	*22.0*	4.9	55.4	59.4 *(29.3)*	8710	3.4
March	Mar 25, 03	SP	71.7 *(0.0)*	1.2 *(0.5)*	*27.3*	12.6	143.9	31.0 *(18.1)*	3587	4.3
April	Apr 22, 03	SP	14.8 *(0.0)*	0.6 *(0.2)*	*35.7*	4.0	45.5	6.0 *(3.0)*	2163	1.4
May	May 13, 03	SU	39.1 *(2.8)*	0.5 *(0.0)*	*40.1*	11.1	126.2	18.2 *(8.8)*	1855	4.9
June	Jun 25, 03	SU	25.1 *(1.1)*	0.3 *(0.1)*	*56.0*	10.5	120.2	57.5 *(6.1)*	614	46.8
July	July 14, 03	SU	30.9 *(3.9)*	0.5 *(0.0)*	41.3	9.6	109.2	42.1 *(3.9)*	5826	3.6
August	Aug 04, 03	SU	17.1 *(4.1)*	0.3 *(0.1)*	57.0	6.1	69.0	9.3 *(9.6)*	2078	2.2
September	Sep 16, 03	AU	12.5 *(1.7)*	0.3 *(0.1)*	50.9	4.9	56.4	21.7 *(4.8)*	1621	6.7
October	Oct 21, 03	AU	11.9 *(0.0)*	0.4 *(0.1)*	39.5	4.1	46.3	5.1 *(3.3)*	959	2.7
November	Nov 25, 03	AU	7.7 *(3.0)*	0.9 *(0.1)*	32.2	1.6	18.5	11.3 *(0.0)*	4252	1.3
November	Dec 02, 03	AU	7.4 *(0.0)*	1.0 *(0.0)*	32.2	1.4	15.7	3.4		
December	Dec 16, 03	W	55.8 *(1.3)*	2.8 *(0.3)*	10.3	11.4	129.8	29.0 *(5.8)*	14684	1.0
January	Jan 26, 04	W	6.5 *(1.8)*	1.3 *(0.5)*	41.0	0.7	8.5		4088	
February	Feb 23, 04	W	9.6 *(1.6)*	1.0 *(0.0)*	33.7	1.7	19.9	9.6 *(1.3)*	5234	0.9
March	Mar 22, 04	SP	13.4 *(0.9)*	0.7 *(0.1)*	18.3	6.6	74.9	33.5 *(9.8)*	6759	2.5
April	Apr 19, 04	SP	9.3 *(0.7)*	1.4	20.6	2.0	22.8	0.3 *(8.6)*	3469	0.0
May	May 25, 04	SU	53.0 *(2.1)*	0.7	*50.9*	8.8	100.2		924	
June	Jun 28, 04	SU	38.7 *(0.0)*	0.4	*60.2*	8.8	99.8	89.3 *(14.2)*	1115	40
	Mean (*SE*)	W	24.0 (*9.8*)	1.7 (*0.3*)	25.4 (*5.4*)	4.2 (*1.9*) 2.4 (*0.8*)[c]	47.5 (*22.0*) 26.9 (*9.0*)[c]	27.6 (*10.2*)	8179 (*2129*)	1.8 (*0.6*)
		SP	27.3 (*14.8*)	1.0 (*0.2*)	25.5 (*3.9*)	6.3 (*2.3*)	71.8 (*26.3*)	17.7 (*8.5*)	3995 (*785*)	2.1 (*0.9*)
		SU	34.0 (*5.1*)	0.4 (*0.1*)	50.9 (*3.5*)	9.1 (*0.7*)	104.1 (*8.2*)	43.3 (*13.1*)	2069 (*785*)	19.5 (*9.0*) 3.6 (*0.5*)[d]
		AU	9.9 (*1.4*)	0.7 (*0.2*)	38.7 (*4.4*)	3.0 (*0.9*)	34.2 (*10.1*)	10.4 (*4.1*)	2277 (*871*)	3.6 (*1.4*)

[a]Estimated C:Chla ratios labeled in *italic*.
[b]Ratio C:S=57 averaged from Matrai and Keller (1994) and Ho et al. (2003).
[c]Winter average excluding Dec 16, 2003.
[d]Summer average excluding June 2003 and June 2004.

DMSP production ranged from 0.3 to 89 nM S d^{-1}. A peak of DMSP production was observed in February (59 nM S d^{-1}) concomitant with a peak of primary production (8710 nM C d^{-1}). In general, the seasonal variation of DMSP production followed that of particulate primary production (pPP) except in June 2003 and June 2004 when peaks of DMSP production were not accompanied with peaks of pPP (Fig. 2). Particulate PP was higher during the winter period (8179 ± 2129 nM C d^{-1}) and lower in summer (2069 ± 785 nM C d^{-1}, Table 1). An unusual peak of pPP was observed in July 2003 (see also Alonso-Sáez et al. *submitted*), concurrent with high values of DMSP production.

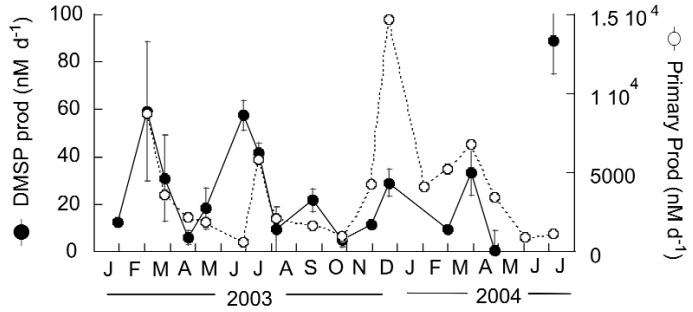

Figure 2. *Estimated rates of DMSP production and particulate primary production during the course of the time-series study in Blanes Bay.*

The percentage of pPP invested into DMSP production was calculated by applying the same conversion factor used for DMSP-C to POC ratios, so that 5 x DMSP production (nM S d^{-1}) was DMSP-C production (nM C d^{-1}). The contribution of DMSP-C to pPP ((DMSP-C production*100)/PP) did not show a clear seasonality over the year (Table 1). On annual average, 3 ± 1% of carbon fixed by phytoplankton was used to synthesize DMSP, excluding the values from both Junes (2003 and 2004). In these two months, due to the increase of DMSP production not matched by pPP, the contribution of DMSP to pPP reached outlying values as high as 40-47% (Table 1).

Seasonality of DMSP and DMS consumption versus bacterial heterotrophic production. Bacterial DMSP and DMS consumptions, as well as bacterial heterotrophic production (BP) were measured from January 2003 to February 2004. DMSP consumption was not significantly correlated with bacterial production (BP). Peaks of DMSP consumption were observed in March 2003 and December 2003 and during the summer period. BP also showed the highest values during the summer, except for June 2003, and in February and December 2003, concurrent with the maximum annual values of Chla (Fig. 3).

Contrastingly, a significant ($p < 0.01$) linear correlation was found between DMSP-S incorporation (DMSP-S assimilated into macromolecules) and BP (Pearson's $r = 0.7$, $n = 13$,

$p<0.01$), as shown in Fig. 3. An even stronger correlation was found when the data point of June 2003 was excluded (Pearson's $r=0.9$, $p< 0.001$, $n=12$). DMSP-S was more efficiently assimilated during the summer (34 ± 5%), while the rest of the year assimilation ranged from 1 to 22%.

Bacterial DMS consumption did not show the same seasonal pattern as BP. DMS consumption rates did not show a clear seasonality, but similar values were recorded over the year (Fig. 3).

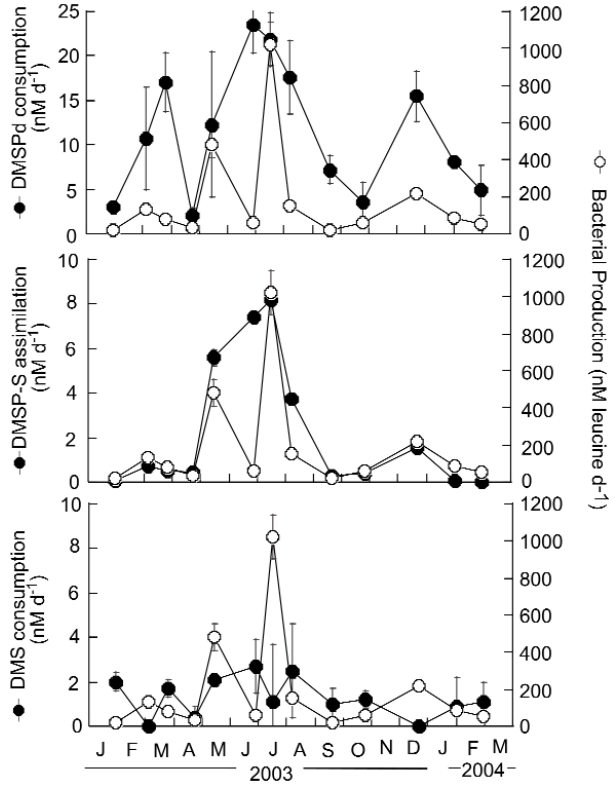

Figure 3. *Variation of bacterial DMSPd consumption, bacterial DMSP-S assimilation, bacterial DMS consumption, and bacterial heterotrophic production (^{3}H-leucine incorporation) during the course of the time-series study in Blanes Bay. Note the different scales of the turnovers of DMSP and DMS.*

Seasonality of DMSP contribution to carbon and sulfur fluxes through bacterioplankton. Heterotrophic bacterial carbon demand (BCD) was calculated from empirical bacterial production (BP) and bacterial respiration (BR). Since most of the incorporated organic carbon was respired, the seasonality of BR drove the seasonality of BCD (Alonso-Sáez et al. *submitted*). Over the year, DMSP contributed from 0.5 to 6% of BCD (Table 2). Beginning with low spring values (1.3 ± 0.8%), this contribution increased as conditions became more oligotrophic in summer (4.9 ± 0.4%) and declined again in autumn. DMSP supported significant proportions of BCD in December 2003 (6%), concomitant with the peak of Chla. The percentage of BCD supplied by DMSP showed a seasonal pattern similar to that of DMSP consumption to the extent that both parameters were positively correlated (Pearson's $r=0.75$, $p<0.01$, $n=11$).

Table 2. *DMSP fluxes and C and S fluxes through bacterioplankton.BP: bacterial production (^{3}H-leucine); BR<0.8: bacterial respiration in the fraction <0.8 µm; BCD: bacterial carbon demand; BSD: bacterial sulfur demand. Standard desviations (SD) of empirical measurements are given in parenthesis. Averages (± SE) are shown for different seasonas (W: winter, SP: spring, SU:summer, AU:autumn).*

Month	Date		DMSPd turn rate *nM DMSP d^{-1}*	DMSPd ass rate *nM DMSP d^{-1}*	DMSPd assimilated *%*	BP *nM C d^{-1}*	BR<0.8 *nM C d^{-1}*	BCD *nM C d^{-1}*	DMSP-C cons/BCD *(%)*	DMSP-S assim/ BSD *(%)*
January	Jan 28, 03	W	3.0 (*0.1*)	0.05 (*0.00*)	1.8 (*1.1*)	19.5 (*2.9*)				23.7
February	Mar 04, 03	W	10.7 (*5.8*)	0.71 (*0.12*)	6.6 (*2.1*)	129.6 (*6.4*)				47.1
March	Mar 25, 03	SP	17.1 (*3.3*)	0.52 (*0.02*)	3.0 (*0.7*)	78.2 (*2.2*)	3995.8	4074.0	2.1	56.9
April	Apr 22, 03	SP	2.1 (*0.8*)	0.44 (*0.01*)	21.5 (*0.7*)	31.7 (*5.4*)	2105.0	2123.9	0.5	120.2
May	May 13, 03	SU	12.3 (*8.2*)	5.58 (*0.38*)	45.5 (*4.7*)	483.3 (*73.8*)	621.7	1105.0	5.6	99.3
June	Jun 25, 03	SU	23.5 (*3.2*)	7.44 (*0.04*)	31.7 (*1.3*)	61.8 (*3.5*)	2000.0	2061.8	5.7	(1035.2)
July	July 14, 03	SU	21.9 (*3.0*)	8.18 (*0.05*)	37.3 (*1.6*)	1021.4 (*121.0*)	1885.8	2907.2	3.8	68.9
August	Aug 04, 03	SU	17.6 (*4.1*)	3.72 (*0.01*)	21.2 (*0.3*)	152.5 (*9.0*)	1726.7	1879.1	4.7	210.0
September	Sep 16, 03	AU	7.2 (*1.6*)	0.30 (*0.02*)	4.1 (*1.0*)	20.2 (*3.6*)	2385.8	2406.0	1.5	125.7
October	Oct 21, 03	AU	3.6 (*2.2*)	0.38 (*0.01*)	10.7 (*0.5*)	62.1 (*7.2*)	2402.4	2464.5	0.7	53.2
December	Dec 16, 03	W	15.5 (*2.8*)	1.53 (*0.01*)	9.8 (*0.5*)	217.9 (*11.3*)	1113.3	1331.3	5.8	60.4
January	Jan 26, 04	W	8.1	0.07	0.9 (*0.3*)	86.7 (*16.8*)	3056.7	3143.3	1.3	7.3
February	Feb 23, 04	W	4.9 (*2.8*)	0.02 (*0.01*)	0.5 (*0.2*)	54.9	1027.5	1082.4	2.2	3.8
	Mean (*SE*)	W	8.4 (*2.2*)	0.5 (*0.3*)	3.9 (*1.8*)	101.7 (*34.2*)	1732.5 (*513.2*)	1852.3 (*503.1*)	3.1 (*1.1*)	28.4 (*11.1*)
		SP	9.6 (*6.1*)	0.5 (*0.0*)	12.3 (*9.2*)	54.9 (*23.3*)	3050.4 (*771.9*)	3105.3 (*790.9*)	1.3 (*0.8*)	88.5 (*31.6*)
		SU	18.8 (*2.5*)	6.2 (*1.0*)	33.9 (*5.1*)	429.7 (*217.0*)	1558.5 (*317.3*)	1988.3 (*369.9*)	4.9 (*0.4*)	126.1 (*37.1*)[a]
		AU	5.4 (*1.8*)	0.3 (*0.0*)	7.4 (*3.3*)	41.1 (*20.9*)	2394.1 (*8.3*)	2435.3 (*29.2*)	1.1 (*0.4*)	89.5 (*36.3*)

[a] Summer average excluding June 2003.

A molar C:S ratio of 86 was used to convert BP to heterotrophic bacterial sulfur demand (BSD). This is a conservative value taken from the elemental composition of marine bacteria analyzed by Fagerbakke et al. (1996). During the stratified period (from April to September), DMSP supplied all the sulfur requirements of bacteria and some values even exceeded 100% (Table 2). During the rest of the year, DMSP contributed from 4 to 60% (average 36%) of bacterial sulfur demands.

Discussion

In this study, we mainly have shown that the contribution of DMSP to the C and S pools and fluxes through phytoplankton and bacterioplankton in a coastal oligo- to mesotrophic site varies seasonally with higher values in summer than over the rest of the year. Since in this site summer conditions lead to far stronger oligotrophy than winter conditions, our results suggest that DMSP contributes more to the fluxes of C and S in communities suited to live under oligotrophic conditions.

The gradual increase in the DMSP-S (or DMSP-C) contribution to algal POS (or POC) from winter to the general maximum annual values in summer might have resulted either from a succession in the phytoplankton assemblages or from changes in the physiological state of the algal cells (mirrored in the C:Chla ratio). On one hand, several seasonal studies of the succession of phytoplankton communities in NW Mediterranean coastal waters have concurred that cyanobacteria (low DMSP producers) and dinoflagellates (high DMSP producers) generally dominate along with nano-flagellates and pico-eukaryots in stratified, oligotrophic and highly irradiated waters (i.e., in summer conditions) while diatoms dominate in the mixed and N-replete waters typical of winter and autumn (e.g. Mura et al. 1996). Common phototrophic nano-flagellates are high DMSP producers (Liss et al. 1994). Much less is known of the DMSP content of phototrophic picoeukaryots; usual size distribution of DMSPp concentrations, however, indicate that smaller phytoplankton must have higher intracellular DMSP concentrations (e.g. Belviso et al. 1993, see also Chapter I). Diatoms are generally low DMSP producers (Keller et al. 1989) and their typical winter bloom in February 2003 coincided with a lower (55%) contribution of DMSP to the algal S content. On the other hand, high irradiances and N- or P-limitation can decrease the per cell pigment content so that summer phytoplankton are characterized by higher C:Chla ratios (Geider et al. 1997). In the latitude of Blanes Bay, however, the amplitude of the seasonal variation of modeled C:Chla ratios is typically of a factor of 4 (from ca. 40 gC $gChla^{-1}$ in winter to ca. 160 gC $gChla^{-1}$ in summer; Taylor et al. 1997). The range of our experimental C:Chla ratio varied by a factor of 3 (18.3-60.2 gC $gChla^{-1}$). Conversely, the estimated percentage of algal POS in the form of DMSP varied by a factor of 10 (from 9-16 % in winter to 109-120 % in summer; Table 1). Hence, it seems that the DMSP:POS ratio in our time series is driven more by succession (low-DMSP producing (winter) towards high-DMSP producing (summer) phytoplankton) than by physiology.

The calculated average 104% contribution of DMSP to phytoplankton S content in summer exceeds the upper limits of the range (50-100%) of cultured species with high intracellular DMSP levels (Matrai & Keller 1994). Uncertainties associated with these calculations explain this over-estimation (>100%). First, uncertainty is associated with the estimation of cell volume from microscopic analyses, since most times only two dimensional measurements of cell size can be done. When possible, the volume of each cell was calculated applying the formulae proposed by Hillebrand et al. (1999), but in radially asymmetric cells the closest 2-parameter geometrical shape was assumed. This may cause a bias in the estimation of biovolumes, especially when radially asymmetric cells are dominant (Guadayol et al. *in prep*.). Furthermore, the molar C:S ratio differs among algal species and varies over the growth cycle (Matrai & Keller 1994, Ho et al. 2003). In consequence, due to the succession of phytoplankton and their physiological acclimation to variable light conditions, both the C:Chla and the C:S ratios are expected to change over the year. However, despite this body of uncertainties, our results clearly indicate that DMSP was the dominant constituent of algal S during the summer period when the most oligotrophic conditions occurred. In winter and autumn, lowest percentages were recorded (48% and 34% respectively), indicating that ~50% of algal S was in other forms of organic S (e.g. S-containing aminoacids).

On an annual average, DMSP-C accounted for 6% of algal POC. This value is within the range of those obtained from different sampling sites (0.2-9%, Kiene et al. 2000) but in the lower range of the values estimated in a very oligotrophic site such as the Sargasso Sea (7.2-39 %, Andreae 1990). Interestingly close to this value, an annual average of 8% of pPP was invested into DMSP synthesis. If we exclude the two June months, when data were inexplicably high, an average of 3% of pPP occurred through DMSP synthesis, a slightly lower proportion than those found in waters dominated by high-DMSP producers (7%, Simó et al. 2002, Archer et al. 2002).

A remarkable finding of our work is the similar pattern between DMSP production and pPP (if we take out the two outlying Junes of 2003 and 2004). It is remarkable because these are two totally independent determinations: DMSP production was estimated by balancing DMSP concentrations and DMSP consumption between 2 consecutive days, and pPP was measured with the ^{14}C fixation method and PE curves. DMSP synthesis is a light-stimulated process that requires reducing power but it is still unknown whether DMSP production is a process strictly coupled to photosynthesis (Stefels 2000). In a study with North Atlantic waters dominated by high-DMSP producers, Simó et al. (2002) found excellent correlations between DMSP production and PP both over diel and multi-day scales, but such a comparison has never been attempted at a seasonal scale. In our study, both parameters, yet showing obvious co-variation (Fig. 2), were not significantly correlated. Sunda et al. (2002) suggested that DMSP production could be stimulated under oxidative stress situations (N-limitation, high UV radiation, low CO_2 availability, etc) because of the role of DMSP and derivatives in scavenging stress-induced OH radicals. If so, the rapid conversion of DMSP-to-DMS in the antioxidant reaction cascade in the

cell might be obscuring any relationship. However, DMSP production did not follow the same seasonality than DMS production (see Chapter I). Other functions of this compound such as osmoregulation or cryoprotection are hardly thought to drive the monthly variability of DMSP production in Blanes Bay, in view of the low variation range of salinity (36-38 psu) and the relatively warm waters all year round (11-25 °C). The similar patterns of DMSP production and pPP, although no strongly correlated, suggest that DMSP biosynthesis is related to photosynthesis, yet through seasons this relationship is modulated by the succession of phytoplankton composition and, probably, by different physiological responses to environmental changes.

The fate of the DMSP metabolized by heterotrophic bacteria can be diverse. DMSP can be cleaved to DMS or can be demethylated into non-volatile sulfur compounds (double demethylation) or MeSH (demethylation+demethiolation). The latter has been suggested to be quantitatively dominant and serves to supply most of the reduced sulfur demands of bacteria (Kiene et al. 2000). This diversity of metabolic pathways explains the weak correlation between DMSP consumption rates and bacterial production as measured by ^{3}H-leucine incorporation, since the latter is strictly a measure of protein synthesis rather than a measure of general activity (Kirchman et al. 1985). Contrastingly, the strong correlation between DMSP-S incorporation and bacterial production confirms the role and efficiency of DMSP as a supplier of the MeSH moiety to be incorporated into proteins (Kiene et al. 2000). In this sense, both ^{3}H-leucine and ^{35}S-DMSP incorporation rates would be proxies for protein synthesis. The implication of this similarity, therefore, is that DMSP would be as universal a bacterial substrate as leucine, at least in our site of Blanes Bay. Since a succession of bacterial assemblages over the year has been observed at the sampling site (Alonso-Sáez et al. *in press*), the good match between DMSP assimilation and BP indicates that DMSP incorporation is a widespread capability among different taxonomic groups of bacterioplankton (Malsmtrom et al. 2004a, Chapter II and III). On the other hand, the lack of a relationship between DMS consumption and BP suggests that DMS assimilation as a S source for protein synthesis is a minor process (Zubkov et al. 2002, del Valle & Kiene *submitted*) and reflects the higher degree of specialization of the bacteria able to metabolize DMS (Chapter VI).

The contribution of DMSP-C to bacterial carbon demand averaged 3 ± 1%. This average is almost identical to those found in shelf (3.4%) and oceanic waters (3.1%) of the Gulf of Mexico (Kiene & Linn 2000a). In summer, the supply of bacterial C demand in the form of DMSP increased to 5%, coinciding with increasing oligotrophy and a higher DMSP-C to POC contribution. These values represent a significant contribution to total C demands by an individual substrate alone. Very few studies have focused on individual components of the dissolved organic pool; this issue has generally been assessed by adding together groups of molecules in pools such as dissolved free aminoacids (DFAA) or proteins, which have been seen to account for as much as 30% of C demands (Keil & Kirchman 1991, Rich et al. 1996). In Long Island Sound , a single DFAA (Alanine) showed C supply values similar to those we found for

DMSP (8%, Fuhrman 1987). This lends support to the suggestion that DMSP is a very labile C source for bacteria, especially significant in oligotrophic conditions.

The contribution of DMSP-S to bacterial sulfur demand was always higher than that of DMSP-C. Despite the fact that sulfate largely dominates the pool of sulfur in the oceans (ca. 28 mM in dissolved salts), bacteria may be energetically favored by directly taking up S in a reduced form (such as DMSP) for satisfying their cellular S requirements (Kiene et al. 1999). During the summer period, estimated DMSP-S incorporation exceeded 100% of bacterial S demands, concurrent with higher efficiencies of DMSPd-S assimilation (34 ± 5%). Similar results were obtained by Zubkov et al. (2002) in waters of the North Sea and by Simó et al. (2002) in the NW Atlantic waters, both dominated by blooms of the coccolithophore *Emiliania huxleyi* (a high-DMSP producer), and by Kiene & Linn (2000a) in temperate waters from the Gulf of Mexico. Contrastingly, during the winter period, DMSP accounted for 28 ± 11% of S demands, so that other sources of S were used. When DMSP-S incorporation was compared with bacterial S demands all over the time series, most of the values fell close to, or slightly below, the 1:1 line (Fig. 4) indicating that DMSP was probably the largest, but not the sole, S source for heterotrophic bacteria.

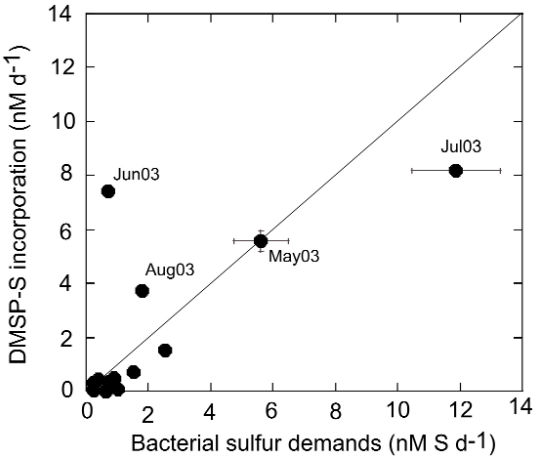

Figure 4. *DMSP-S incorporation vs. bacterial sulfur demands (BSD). BSD were calculated from bacterial production using a ratio C:S=86 (see text). The 1:1 line is included as a reference.*

These calculations are subject to a rather large uncertainty. The molar C:S ratio determined for bacterioplankton in seawater from Norway (Fagerbakke et al. 1996) varied between June (C:S=54) and October (C:S=140), suggesting they might exhibit seasonal variation. Other published ratios are higher by a factor of 2 to 5 (C:S=248; Cuhel et al. 1982). The fact that we used the average ratio (C:S=86) reported by Fagerbakke (1996) as a fixed value to calculate the bacterial sulfur demand all over the year may explain why in some months DMSP apparently accounted for more than 100% of the BSD. This overestimation is a common feature in previous studies where different C:S ratios were used and, therefore, it calls for the need to generate more data to better constrain the elemental composition of bacteria.

On an annual average, 57% of particulate DMSP circulated through DMSPd utilization, pointing to the importance of free-living heterotrophic bacteria (and potentially non

DMSP-producing phytoplankton) as end users of algal DMSP. In the summer months, when the algal assemblage was dominated by high-DMSP producers characterized by higher DMSP:POS ratios, a larger percentage of the DMSP-S was assimilated by bacteria. Since the DMSP:POS ratios were derived primarily from measured DMSP:Chla ratios, we propose the latter as a good indicator of the role of DMSP in the S fluxes (and, according to the results discussed above, also the C fluxes) through the lower trophic levels of the food web.

CHAPTER V

DMSP uptake by marine phytoplankton

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Abstract

Dimethylsulfoniopropionate (DMSP) accounts for most of the organic sulfur fluxes from primary to secondary producers in marine microbial food-webs. Incubations of natural communities and axenic cultures with radio-labeled DMSP showed that dominant phytoplankton groups of the ocean: the unicellular cyanobacteria *Prochlorococcus* and *Synechococcus*, and diatoms, as well as heterotrophic bacteria take up and assimilate DMSP-sulfur, thus diverting a proportion of plankton-produced organic sulfur from emission into the atmosphere.

Introduction

Dimethylsulfoniopropionate (DMSP) is synthesized by ubiquitous phytoplankton taxa as a solute probably for osmoregulatory and antioxidant purposes (Malin & Kirst 1997, Stefels 2000, Yoch 2002, Sunda et al. 2002). DMSP is the precursor of the climate-active gas dimethylsulfide (DMS), the main natural source of sulfur to the global atmosphere and a major aerosol and cloud droplet precursor over the ocean (Charlson et al. 1987, Simó 2001, Andreae & Crutzen 1997). Enzymatic cleavage of DMSP into volatile DMS is the fate of only a fraction (generally <50%) of all DMSP produced (Simó & Pedrós-Alió 1999a). Recent research has revealed that algal DMSP plays an important role in food-web processes supplying sulfur and carbon to heterotrophic bacteria and, to a lesser extent, to microzooplankton herbivores (Kiene et al. 1999, Kiene et al. 2000, Simó et al. 2002, Simó 2004). Thus, the biogeochemical fate and function of DMSP is largely determined by a switch between conversion into DMS and sulfur assimilation by microorganisms, which in turn depends on the composition, structure and dynamics of the planktonic food-web. The ability to assimilate DMSP-sulfur seems to be widespread among taxa of heterotrophic bacterioplankton (Malmstrom et al. 2004a, Chapter II) and has also been observed in the cyanobacterium *Synechococcus* (Malmstrom et al. 2005). Our work is aimed to find out if major non-DMSP producing phytoplankton also assimilate DMSP-sulfur.

Materials and Methods

Flow cytometry cell sorting. Whole water samples (50-100 ml) were incubated with additions of ^{35}S DMSP (0.1 nM, specific activity 130-350 Ci mmol^{-1}) for 4-24 hours in the light (mimicking *in situ* light intensities, no UV) and in the dark in acid-cleaned glass serum vials (see Table 1). Samples were fixed with 1% paraformaldehyde + 0.05% glutaraldehyde (final conc.), frozen in liquid nitrogen, and stored at -80°C. Different populations were identified and sorted using a FACSCalibur flow cytometer-cell sorter (Becton Dickinson). Sorted cells were collected onto 0.2 μm nylon filters and assayed by liquid scintillation counting. Number of sorted cells: Heterotrophic bacteria: 100,000-400,000, *Synechococcus*: 30,000-130,000, *Prochlorococcus*: 30,000-90,000, autofluorescent picoeukaryotes: 5,000-25,000. Killed controls were performed by addition of the fixative (in the dark) 30 min before addition of the radioisotope, and parallel incubation with live samples. Assimilation of ^{35}S DMSP-sulfur in killed controls was 2.3% that in live samples.

Microautoradiography. Incubations with ^{35}S DMSP (203.1 Ci mmol^{-1}) and preparation of the microautoradiograms were conducted exactly as previously described (Chapter II) but filtering aliquots of 30 ml of sample through 5 μm polycarbonate filters. Cells were transferred from the filter onto the surface of a photographic emulsion and exposed in the dark for 20 days at 4 °C.

Table 1. Basic parameters of the different samples analyzed by flow cytometry cell sorting. Sampling dates: Blanes Bay (Blanes Bay Microbial Observatory), March 22, 2004; Gran Canaria, October 3, 2005; Sargasso Sea, July 17, 2004; Pensacola Pier, June 19, 2004; Gulf of Mexico, May 25, 2004.

Location		T (°C)	Chl-*a* (mg m^{-3})	$DMSP_t$ (nM)	$DMSP_d$ turn (d^{-1})	BP (nM leu d^{-1})	Incubation time (h)
Blanes Bay	41°40N 2°48E	13	0.74	29.5	3.1	0.5	4.0
Gran Canaria	28°00N 15°40W	22	0.61	59.5	28.0	11.6	8.4
Sargasso Sea	30°23N 64°56W	27	0.04	9.8	1.2	0.3	9.0
Pensacola Pier	30°19N 87°9W	30	1.60	17.0	59.0	30.0	4.3
Gulf of Mexico	29°50N 87°5W	29	0.44	6.0	n.d.	6.3	24.0

$DMSP_t$: total (particulate + dissolved) DMSP

$DMSP_d$ turn: dissolved DMSP turnover

BP: bacterial heterotrophic production as determined by ^{3}H-leucine incorporation

n.d. not determined

Once developed, cells were stained with DAPI (1 μg L^{-1}, 4',6-diamino-2-phenylindole dihydrochloride) and counted manually under an epifluorescence microscope. A halo of black silver grains around a cell indicates ^{35}S occurrence in the cell.

DMSP-sulfur assimilation. Microautoradiography and flow cytometry cell sorting samples were preserved with fixatives that cause cells to lose cytoplasm ^{35}S-DMSP (Kiene & Linn 1999). In these samples, ^{35}S-radiolabels in cells indicate assimilation of ^{35}S from DMSP into cell structure.

Cultures. Axenic cultures of T. *pseudonana* (CCMP1335) and *T. oceanica* (CCMP 1005) were obtained from the CCMP (Center for Culture of Marine Phytoplankton, Maine, USA). Cultures were grown in f/4 medium at 22 °C under fluorescent light (105 μmol quanta $m^{-2}s^{-1}$; 12:12 h light:dark cycle). Aliquots of 20 ml of exponentially growing cultures were incubated with additions of either tracer ^{35}S DMSP (<0.1 nM, specific activity 130 Ci mmol-1) or ^{14}C GBT (7 nM, specific activity 57 mCi $mmol^{-1}$) for 12 h. Substrate uptake was determined in triplicate by gravity-filtering 1 ml of sample through 1.2 μm nylon filters. Formalin-killed controls showed <1.3% of added radioisotopes in the cells.

DMSP concentrations and turnover. Total DMSP concentrations were measured by gas chromatography by using analysis protocols described in Kiene 1996 and Simó et al. 1996.

Dissolved DMSP turnover (DMSPd turn) and bacterial production (^{3}H-leucine incorporation) were determined in the dark following standard protocols described in Kiene & Linn (2000a) and Smith & Azam (1992).

Results and Discussion

To investigate the distribution of DMSP-sulfur uptake and assimilation among picoplankton, we used flow cytometry cell sorting and measured assimilation by pico-phototrophs and heterotrophic bacteria using radio-labeled DMSP. Surface seawater samples were collected from the coasts of the Gulf of Mexico, the NW Mediterranean and Gran Canaria Island, and from the Sargasso Sea (Table 1). After light and dark incubations with ^{35}S-DMSP, sample aliquots were passed through the flow cytometer and sorted into four major groups: heterotrophic bacteria, *Prochlorococcus*, *Synechococcus*, and autofluorescent pico-eukaryotes. All groups showed some capability of assimilating DMSP-sulfur (Fig. 1). The most significant DMSP-sulfur assimilators were heterotrophic bacteria followed by *Prochlorococcus*, *Synechococcus* and pico-eukaryotes. Incubation of samples in the light stimulated DMSP-sulfur assimilation by pico-phototrophs by as much as a factor of 2.2. The phototrophs accounted for 10-34% of pico-planktonic DMSP consumption in the light, with the remaining 66-90% being carried out by heterotrophic bacteria.

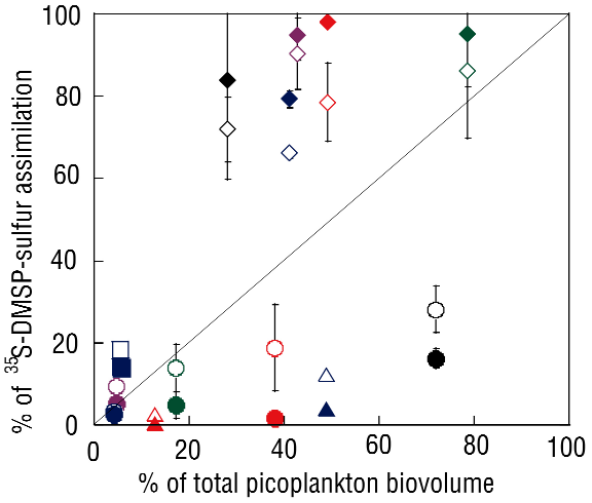


Figure 1. *Contribution of different groups of picoplankton to total picoplankton ^{35}S-DMSP-sulfur assimilation versus their contribution to the total picoplankton biovolume. Filled and open symbols orrespond to dark and light incubations, respectively. (diamonds: heterotrophic bacteria; circles: Synechococcus; squares: Prochlorococcus; triangles: picoeukaryotes). Green: Blanes Bay (NW Mediterranean); black: off Dauphin Island (Gulf of Mexico); purple: Sargasso Sea; red: Pensacola beach (Gulf of Mexico); blue: Gran Canaria Island. Error bars represent standard deviation of the mean (n=2-6). The 1:1 line is included as a reference.*

Until now the only phototrophs for which DMSP use had been observed were *Synechococcus*. It seems that, in a similar way to heterotrophic bacteria (Kiene et al. 1999) and *Synechococcus* (Malmstrom et al. 2005), *Prochlorococcus* may also benefit from using a reduced sulfur source such as DMSP, probably because it saves the energy required to reduce sulfate. Studies with cultured and natural assemblages of heterotrophic bacteria have provided evidence for a common membrane transporter for DMSP and glycine betaine (GBT) (Kempf & Bremer 1998, Kiene et al. 1998) and, interestingly, putative GBT transporter genes have been found in the genome of *Prochlorococcus marinus* MIT9313 (Rocap et al. 2003).

It is remarkable that, in one of the samples (Gran Canaria Island), eukaryotic pico-phytoplankton also showed significant incorporation of ^{35}S from ^{35}S-DMSP (Fig. 1). It is possible, however, that some of these eukaryotes were mixotrophic bacterivores that had fed on ^{35}S-radio-labeled bacteria.

We used microautoradiography with ^{35}S-DMSP to follow DMSP-sulfur assimilation by organisms larger than 5 μm collected through during an annual time series in the coastal Mediterranean. Consistent with our flow-cytometric observations on pico-eukaryotes, many phytoplankton cells, including dinoflagellates, cryptophytes, and diatoms became radio-labeled (Fig. 2). Mixotrophy by bacterivory has been described for dinophytes, cryptophytes and haptophytes (Raven 1997), but not for diatoms, which consequently must have directly taken up ^{35}S from dissolved radio-labeled DMSP.

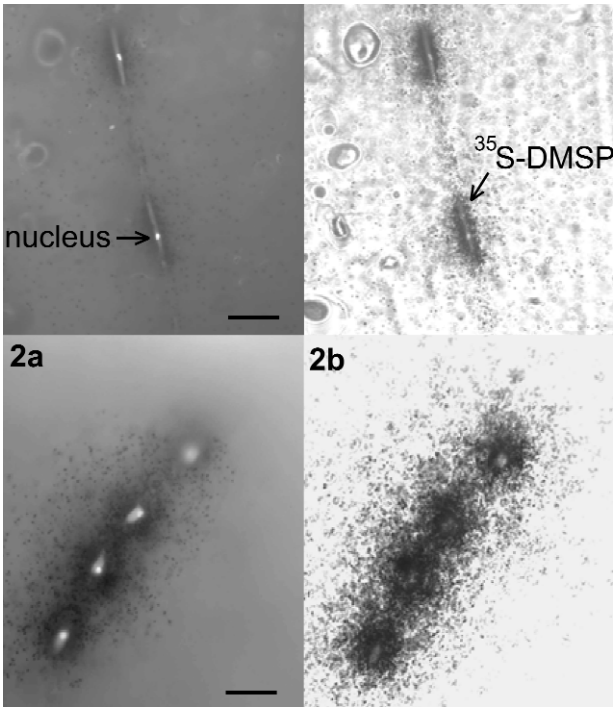


Figure 2. *Photomicrographs of two species of diatoms: (1) Pseudo-nitzschia sp. and (2) Chaetoceros sp. occurring in a natural phytoplankton assemblage from Blanes Bay (NW Mediterranean), after being processed by microautoradiography. (a) Epifluorescence micrographs under UV light, showing DAPI-stained nuclei. (b) Same cells observed under transmitted light. Black dots surrounding cells indicate assimilation of ^{35}S-DMSP-sulfur by diatoms. Scale bar is 10 μm.*

The DMSP-to-chlorophyll (DMSP:Chla) ratio is a good indicator of how strong a DMSP producer a phytoplankton assemblage is, and how much of the available carbon and sulfur are accounted for by DMSP (Kiene et al. 2000, Simó et al. 2002). We found that the proportion of diatoms that had assimilated ^{35}S-DMSP-sulfur, compared with independently measured DMSP:Chla ratios from parallel samples (Fig. 3), followed a very similar pattern through the annual course of sampling, with highest values observed in June and August. In other words, higher numbers of DMSP-sulfur assimilating diatoms did not occur when these phytoplankters were more abundant (late winter) but when DMSP was more abundant with respect to total sulfur and carbon fluxes (summer).

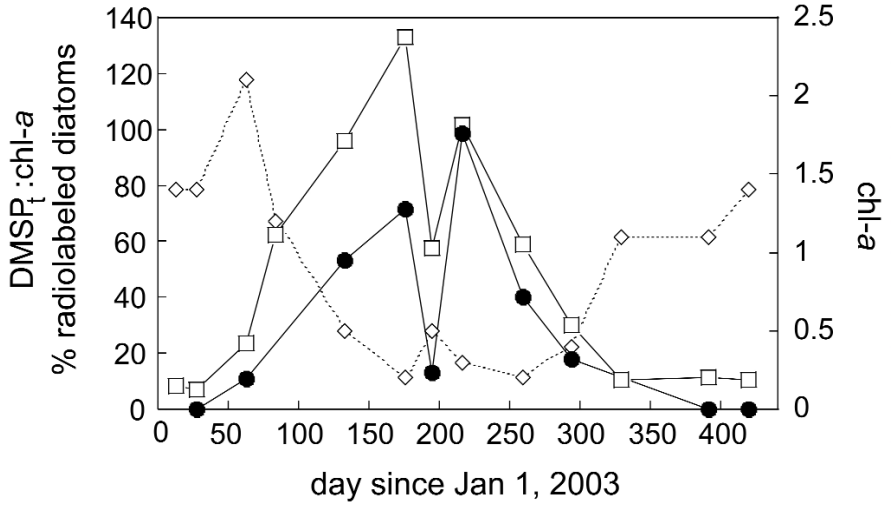


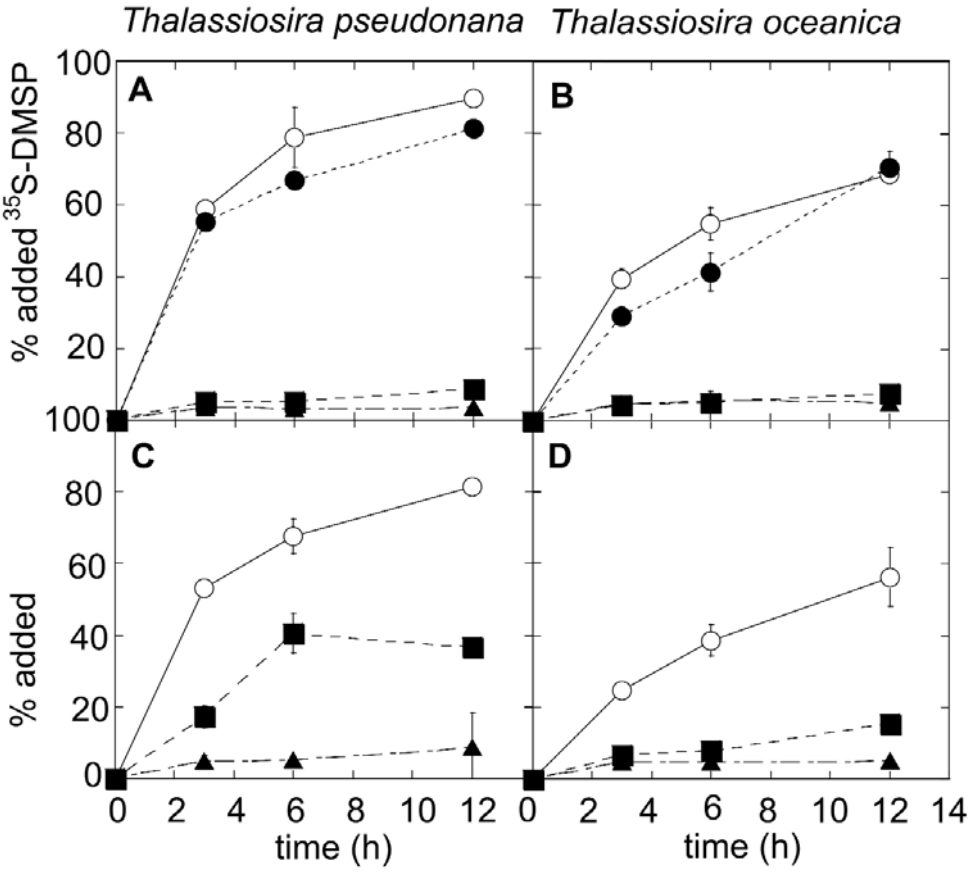
Figure 3. *Annual variation of the percentage of DMSP-sulfur assimilating diatoms (circles) and the in situ DMSP:chl-a ratio, nmol·μg^{-1} (squares) in surface waters of Blanes Bay (NW Mediterranean). Chla concentration (μg·l^{-1}) is also shown (diamonds).*

DMSP is a zwitterion that cannot cross cell membranes without a specific transporter (Kiene et al. 1998). DMSP-sulfur assimilation by diatoms implies, therefore, that either these algae have a DMSP transport system or they were taking up by-products of ^{35}S-DMSP degradation by bacteria, such as ^{35}S-methanethiol. To check for the capability of diatoms to take up DMSP, we grew two axenic strains of the centric diatoms *Thalassiosira pseudonana* (CCMP1335) and *Thalassiosira oceanica* (CCMP1005). We chose these two species for their low cellular DMSP content (1.3 and 0.9 mM, respectively) and their small size (ca. 5 and 8 μm diameter, respectively).

After 12 hours of incubation with trace concentrations of ^{35}S-DMSP, both species had taken up most of it. Contrasting with what was observed with picophototrophs, light only stimulated

uptake by ~10% in diatoms (Fig. 4). When non-labeled DMSP was added at a concentration of 10 μM, the uptake of ^{35}S-DMSP was almost completely suppressed. Addition of 10 μM of non-labeled glycine betaine (GBT) produced the same effect (Fig. 4, top panels). Trace amounts of ^{14}C-GBT were taken up by both species and, likewise, when 10 μM of non-labeled GBT was added, ^{14}C-GBT uptake was suppressed. Addition of 10 μM of DMSP inhibited ^{14}C-GBT uptake by half in *T. pseudonana* and by a third in *T. oceanica* (Fig. 4, bottom panels). The two species of *Thalassiosira* seemed to use the same transport system for both compounds, in a similar way to heterotrophic bacteria (Kempf & Bremer 1998, Kiene et al. 1998). Genes encoding for a putative GBT membrane transporter have been found in the genome of this same *T. pseudonana* CCMP1335 strain (Armbrust et al. 2004, www.membranetransport.org).

Figure 4. *Uptake of ^{35}S-DMSP (top panels, circles) and ^{14}C-GBT (bottom panels, circles) by axenic ultures of Thalassiosira pseudonana (left panels) and T. oceanica (right panels). Filled and open symbols correspond to dark and light incubations, respectively. Time series of isotope uptake in the presence of potential competitive inhibitors, 10 μM of non-radio-labeled DMSP (squares) and 10 μM GBT (triangles), are also shown. Error bars correspond to standard deviation from triplicate measurements.*



Our results provide evidence that diatoms and the two major groups of pelagic non-filamentous cyanobacteria can take up and use DMSP. Production of DMSP, although ubiquitous in the ocean, is taxon dependent and, to some extent, size-dependent too: small haptophytes and dinoflagellates are generally high producers, whereas diatoms (except for those that grow in sea-ice) and cyanobacteria are low or non-producers (Keller et al. 1989, Corn et al. 1996, Belviso et al. 2001). Tests with two axenic strains of the haptophyte *Emiliana huxleyi* (CCMP373, CCMP374) and the dinoflagellate *Karenia brevis* (CCMP 2281), strong and moderate DMSP producers respectively, revealed no uptake of ^{35}S-DMSP (Table 2). Our results, thus suggest that low- or non-DMSP-producing diatoms and cyanobacteria consume DMSP released by high producing phytoplankton partners.

Table 2. *Intracellular DMSP concentrations and percentage of ^{35}S DMSP uptake by different strains in axenic culture. The percentage of ^{35}S DMSP uptake is the proportion of added ^{35}S DMSP (<0.01 nM) that remained in the cells after 6 h. of incubation. Values are the means of triplicate measurements (standard deviations in parentheses). Formalin-killed controls of all species averaged 0.7± 0.6 % ^{35}S DMSP uptake.*

Species	Strain	Group	Intracellular DMSP (mM)	DMSP uptake (%) light	dark
Emiliania huxleyi	CCMP373	Haptophyte	150-400	0.2 (0.0)	0.3 (0.1)
Emiliania huxleyi	CCMP374	Haptophyte	200-400	0.2 (0.0)	0.3 (0.1)
Karenia brevis	CCMP2281	Dinoflagellate	15-30	2.7 (0.6)	n.d.
T. pseudonana	CCMP1335	Diatom	1.3-1.8	78.9 (8.4)	66.9 (1.8)
T. oceanica	CCMP1005	Diatom	0.9-1.3	55.1 (4.5)	41.6 (5.4)

n.d.=not determined. *T. pseudonana* = *Thalassiosira pseudonana*. *T. oceanica* = *Thalassiosira oceanica.*

But what is the quantitative relevance of this process in nature? *Prochlorococcus* are numerically the dominant phytoplankters in the oligotrophic central oceanic gyres, and tend to be replaced by *Synechococcus* in productive tropical waters and in the transitional zones to temperate waters (Partensky et al. 1999). Both co-occur in the euphotic zone with small high-DMSP producing eukaryotic algae. In upwelling regions and in waters that receive pulses of nutrients from continental discharges or by episodic or seasonal mixing, diatoms grow among a background of small algae, and tend to dominate primary production (Smetacek et al. 1990). We have shown that pico-phototrophs contributed 10-34% of DMSP-sulfur assimilation in the light-exposed waters studied. The contribution of total phytoplankton (including diatoms) is harder to quantify. Size fractionated DMSP-sulfur assimilation experiments conducted in the

surface Sargasso Sea in April 2002 and July 2004 revealed that, in the dark, 100% of the DMSP-sulfur assimilation was carried out by microorganisms smaller than 0.6 μm (i.e., mostly heterotrophic bacteria) whereas, in the light, assimilation was stimulated by two-to-three fold, and 50-70% of the total assimilation was by organisms larger than 0.6 μm (i.e., mostly phototrophic prokaryotes and all eukaryotes).

All of our incubations were conducted in the absence of UV radiation, hence, it is likely that we are underestimating the relative contribution of phytoplankton (UV-protected by pigments) versus heterotrophic bacterioplankton as DMSP-sulfur sinks. In any case, phytoplankton DMSP utilization confirms a major role of DMSP as a carrier for sulfur and carbon through multiple levels of marine microbial food webs (Kiene et al. 2000, Simó et al. 2002). Ours results show that, in the illuminated conditions of the surface ocean, phytoplankton assimilate DMSP-S in similar proportions to heterotrophic bacterioplankton, both making up the assimilation of ca. 20% of total DMSP consumption. If we include also the assimilation by microzooplankton (ca. 20%), then the total assimilative consumption by microbial food web components is of similar magnitude to DMS production (ca. 10-50% of total DMSP turnover) and much higher than eventual DMS ventilation to the atmosphere (ca. 3%) (Fig. 5). Assimilation of DMSP, therefore acts to regulate sulfur emissions into the atmosphere, with potential important consequences to the global biogeochemical sulfur cycle and climate (Charlson et al. 1987, Andreae & Crutzen 1997).

Another broad implication of our results refers to the use of organic substrates by phytoplankton. Our data add to previous observations (Malmstrom et al. 2005, Paerl 1991, Moore et al. 2002, Zubkov et al. 2003) to demonstrate that widespread and numerically dominant phytoplankton groups are capable of taking up essential elements in reduced organic forms. This, together with the phagotrophic bacterivory described in many algal taxa (Raven 1997, Jones 1994) further reveals how metabolically versatile phytoplankton are as a fundamental ecological player in the ocean, and how challenging it becomes to implement their dynamics in oceanic biogeochemical models.

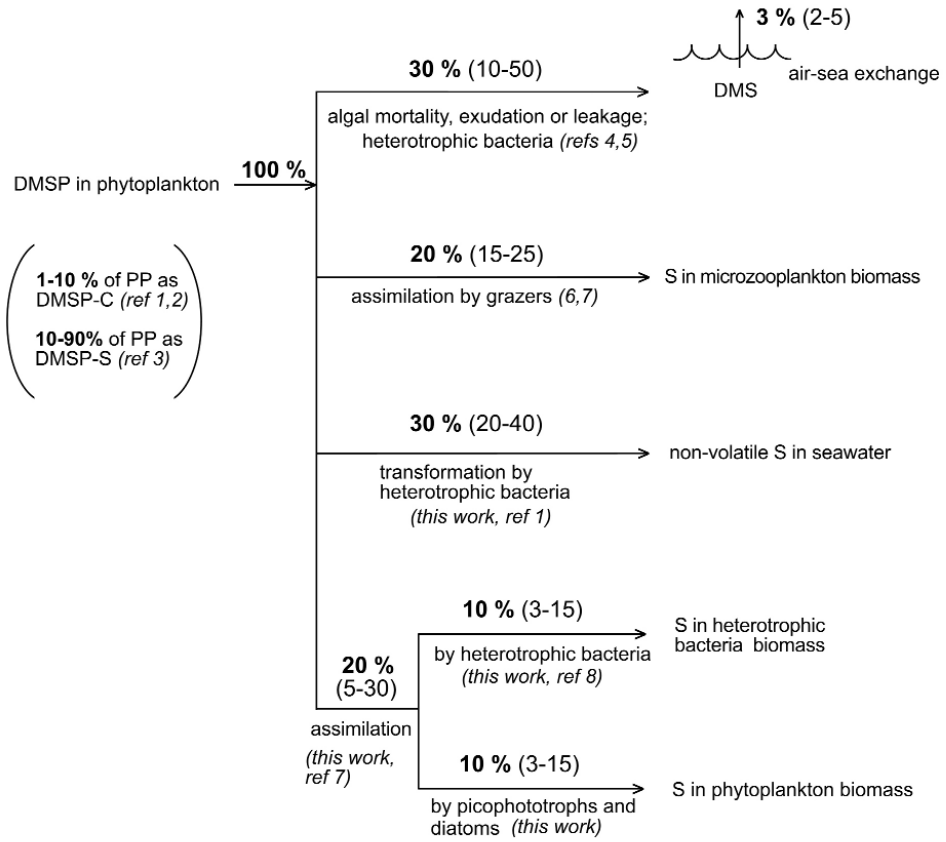
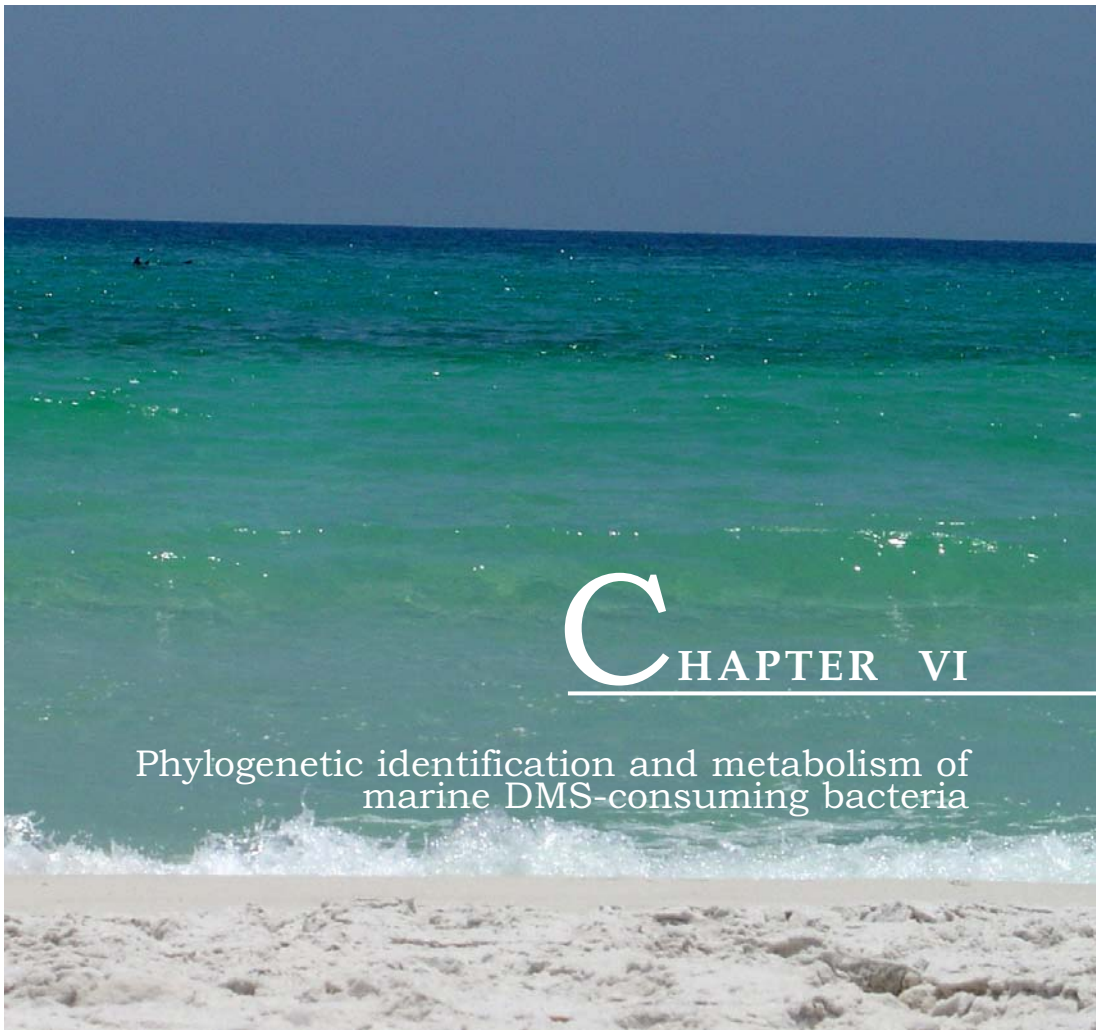


Figure 5. *Estimates of the relative importance of DMSP transformations in the microbial food web. All percentages are referred to 100% of DMSP consumption (= DMSP production). Estimated ranges are given in parentheses. Estimates of the percentage of primary production (PP) as DMSP-S were obtained assuming an average phytoplankton C:S (mol/mol) ratio of 70 (ref 3) and considering that the C:Chla (g/g) and the DMSP:Chla (nmol/g) ratios vary with irradiance and trophic status, resulting in variable DMSP:C ratios. The endpoints of the range would be 20 (C:Chla) and 2 (DMSP:Chla) for productive, mixed waters dominated by diatoms, and 200 (C:Chla) and 200 (DMSP:Chla) for oligotrophic, stratified and highly irradiated waters (refs. 1,2, and unpublished results). Among DMS sinks, only climate relevant air-sea exchange is shown in the diagram. The other DMS sinks (microbial consumption and photolysis) account for the remaining 27% losses. Microzooplankton assimilation has been calculated assuming a 30% assimilation of total DMSP ingested (ref. 6) and 70% daily ingestion of the phytoplankton stock (ref 7).*

Specific references of Figure 5:
ref 1: Kiene et al. 2000, ref 2: Taylor et al. 1997, ref 3: Ho et al. 2003, ref 4: Simó & Pedrós-Alió 1999a, ref 5: Chapter I, ref 6: Simó 2004, ref 7: Calbet & Landry 2004, ref 8: Kiene et al. 1999.



CHAPTER VI

Phylogenetic identification and metabolism of marine DMS-consuming bacteria

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Abstract

Microbial consumption is one of the main processes, along with photolysis and ventilation, that remove the biogenic trace gas dimethylsulfide (DMS) from the surface ocean. Although a few isolates of marine bacteria have been studied for their ability to utilize DMS, little is known about the characteristics or phylogenetic affiliation of DMS consumers in seawater. We enriched coastal and open-ocean waters with different carbon sources to stimulate different bacterial communities (glucose-consuming bacteria, methyl-group consuming bacteria, and DMS consumers) in order to test how this affected DMS consumption and to examine which organisms might be involved.

DMS consumption was greatly stimulated in the DMS addition treatments whereas there was no stimulation in the other treatments. Analysis of microbial DNA by two different techniques (sequenced bands from DGGE gels and clone libraries) showed that bacteria grown specifically with the presence of DMS were closely related to the genus *Methylophaga*. We also followed the fate of consumed DMS in some of the enrichments. DMS was converted mostly to DMSO in glucose or methanol enrichments, whereas it was converted mostly to sulfate in DMS enrichments, the latter suggesting use of DMS as a carbon and energy source. Our results indicate that unlike the biochemical precursor of DMS, dimethylsulfoniopropionate (DMSP), which is consumed by a broad spectrum of marine microorganisms, DMS seems to be utilized as a carbon and electron source by specialists. This is consistent with the usual observation that DMSP turns over at much higher rates than DMS.

Introduction

Dimethylsulfide (DMS) is an organic sulfur gas that represents the main natural source of sulfur from the oceans to the atmosphere, where it has been suggested to play a major role in aerosol formation, cloud albedo and the radiation balance of the Earth (Andreae 1990). DMS is a degradation product of dimethylsulfoniopropionate (DMSP), a compound synthesized by a wide spectrum of phytoplankton species in the euphotic zone of the ocean. Phytoplankton DMSP is transformed intracellularly or released to seawater by exudation or algal mortality, mainly through grazing, viral attack and autolysis. Once it is in dissolved form, a fraction of this DMSP is converted to DMS through a process mediated mainly by bacterioplankton (Kiene et al. 2000). The concentration of DMS in surface seawater depends on this production but it is also controlled by three removal processes: ventilation to the atmosphere, photolysis and microbial consumption. The relative importance of these processes is variable and depends on physical forcing factors. Dominance of ventilation has only been observed under high wind speed conditions, while microbial consumption and photolysis generally are the main DMS sinks (Kiene & Bates 1990, Kieber et al. 1996, Simó & Pedrós-Alió 1999b).

Despite the importance of biological DMS consumption as a mechanism that controls the DMS flux to the atmosphere, in situ studies of the phylogenetic identification of the bacteria involved have never been attempted. Based on the first isolates from aerobic and anaerobic environments, it was hypothesized that pelagic DMS consumers would be methylotrophic bacteria (Kiene 1993). However, the very few strains isolated from seawater have shown that DMS consumers can potentially utilize a variety of metabolisms. They can be obligate methylotrophs (Hoeft et al. 2000, de Zwart & Kuenen 1997), bacteria that grow on a variety of carbon sources, not just DMS (Visscher & Taylor 1994, González et al. 1999), and those able to obtain energy from the oxidation of the resulting inorganic sulfur compounds once DMS is degraded (Fuse et al. 2000, González et al. 1999). Whether DMS consumption in the ocean is a process carried out by specialists or by a broad diversity of bacteria still remains unresolved.

In order to identify which bacteria consume DMS in seawater, we carried out enrichment experiments with coastal and open-ocean waters with 3 different C sources: glucose, methanol (to stimulate methylotrophs), and DMS (to specifically stimulate DMS consumers). We compared different enriched communities by DGGE, sequenced specific bands from the DGGE gels and performed clone libraries from DMS treatment. By incubation with trace additions of ^{35}S-DMS, we followed the metabolic fate of consumed DMS into 3 major product pools: the fraction assimilated into cell structure, the fraction converted to DMSO and the fraction converted to sulfate. Application of substrate enrichments, molecular tools and radiotracer methods to coastal and open-ocean waters have allowed us to identify the marine bacteria that grow on DMS as the carbon and energy source and to elucidate the metabolic fate of DMS consumption in different bacterial communities.

Materials and Methods

Enrichment with different C_1 compounds. Coastal seawater collected at Dauphin Island, Alabama, located in the Northern Gulf of Mexico (30°159N, 88°059W), was amended, in duplicate, with different methylated substrates (10 μM of C) in order to stimulate different methyl-consuming bacterial populations and test their ability to consume DMS. This concentration of substrates did not inhibit DMS consumption (Wolfe & Kiene 1993). The methylated substrates tested were the following: MMA (monomethylamine), DMA (dimethylamine), TMA (trimethylamine), MeOH (methanol), MDTMA (equimolar mixture of MMA+DMA+TMA+ MeOH), DMSO (dimethylsulfoxide), MSA (methanesulfonic acid), GBT (glycine betaine) and DMS. Duplicate seawater samples without any organic amendments served as controls. All the samples (30 ml in 40 ml gas-tight acid-washed glass vials) were incubated in the dark at in situ temperature (30°C). When the DMS concentration in the DMS enrichment had decreased to <1 μM of DMS (2 μM of carbon) (after about 50 hrs), 500 nM of DMS was added to all the treatments and subsequent changes in DMS concentration were monitored by analysis of the headspace (Fig. 1). In the DMSO enrichment, where microbial DMSO reduction had already yielded 400 nM of DMS, the concentration after the DMS addition was 900 nM.

Enrichment with glucose, methanol, and DMS. Surface coastal (Pensacola Pier, 30°19.6N, 87°9.6W, 1.2 μg/L Chla) and open-ocean (Sargasso Sea, 30°49.6N, 64°58.9 W, 0.041 μg/L Chla) seawaters were amended with either 5 μM C of glucose (to stimulate the general heterotrophic community), methanol (to stimulate methylotrophs), DMS (to stimulate specific DMS consumers) and DMSO (to stimulate DMSO consumers; Sargasso Sea only). Concentrations of glucose and methanol were low enough to avoid inhibition of DMS consumption (Wolfe & Kiene 1993). Nitrate (1 μM) and phosphate (0.0625 μM) were added to all treatments, including controls with no growth substrate addition. Coastal seawater from Pensacola was diluted 90% with 0.2 μm-filtered seawater from the same location in order to minimize contributions by phytoplankton carbon and grazing. Non-perturbing DMS concentrations were added to all treatments (except for the DMS enrichment) in order to follow the variation of DMS consumption in these treatments (see Fig. 2). When the DMS was completely consumed in the DMS enrichment, all the carbon sources and nutrients were re-added to all treatments at the same initial concentration. Duplicate samples (2.5 L) of each treatment were incubated in the dark at in situ temperature (27°C for both waters). Subsamples were taken at different time points in order to measure bacterial DMS consumption activity parameters. At the end of the experiments, DNA from each enrichment was extracted for phylogenetic characterization of bacterial communities by DGGE and clone libraries.

DMS concentrations. DMS concentration from each enrichment at different time points was quantified by gas chromatography (Shimadzu 14A GC), either by headspace injection or by using the purge and cryotrapping system described by Kiene (1996).

DMS turnover and fate of DMS. DMS turnover rates and the fate of DMS were determined using ^{35}S-DMS as described by del Valle et al. (*submitted*). By using this approach DMS-sulfur was traced into sulfate, macromolecules (Kiene & Linn 2000b), and DMSO. Briefly, turnover rates constants were taken as the absolute value of the slope of a linear regression of the natural log of the fraction of ^{35}S-DMS converted to non-volatile (NV) products versus incubation time, assuming first-order uptake kinetics. The DMSO yield was calculated as the percentage of ^{35}S lost from the NV product pool when this fraction was treated with $TiCl_3$ in order to reduce ^{35}S-DMSO to ^{35}S-DMS. The sulfate yield was calculated based on the activity left on solution once the ^{35}S-sulfate present in the NV fraction was precipitated with Ba^{2+}. No abiotic ^{35}S-DMS losses were found in controls consisting of 0.2-μm filtered seawater from the enrichments which were amended with ^{35}S-DMS in parallel to live samples.

Bacterial Production. Bacterial activity was measured by the incorporation of ^{3}H-leucine into trichloroacetic acid (TCA)-insoluble material (Kirchman 1993) using the centrifugation method (Smith & Azam 1992). 20 nM Leucine were added to triplicate 1.5 ml samples plus 1 killed blank and incubated for 1 h at in situ temperature in the dark.

DNA extraction and DGGE. At the end of the experiment, we collected microbial biomass in the 3- to 0.2 μm size range by filtering ca 2 L of enriched water through a 3-μm-pore size Nucleopore filter and a 0.2 μm-pore-size Durapore filter (Millipore, 25mm). After filtration, the Durapore filters were stored in cryovials filled with 1.8 ml lysis buffer (40 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.3, 0.75 M sucrose) at -80°C. Extraction of DNA was performed following Schauer et al. (2000) using, sequentially, incubations with lysosyme solution (1mg ml^{-1}, 37°C, 45 min), Proteinase K (0.2 mg ml^{-1}) and sodium dodecyl sulfate (1%), at 55°C for 1 hr. The nucleic acids were extracted by phenolization and concentrated in a Centricon-100 (Millipore). The quality of recovered DNA was checked by agarose gel electrophoresis. Nucleic acid extracts were stored at -80°C. One microliter of the extracted DNA was used to amplify the bacterial 16S rDNA by PCR (Polymerase Chain Reaction). It was performed exactly as described by Schauer et al. 2000, except that the reverse primer we used (907rM) had an extra ambiguity. We used the bacterial specific primer 358f (5'-CCT AGC GGA GGC AGC AG-3') with a 40 bp GC-clamp, and the universal primer 907rM (5'-CCG TCA ATT CMT TTR AGT TT-3'), which amplifies a 550 bp DNA fragment of bacterial 16rDNA.

About 800 μg of each PCR product was loaded for each sample on a 6% polyacrylamide gel with a DNA-denaturant gradient ranging from 40 to 80%. The gel was run at 100 V for 16 h at 60°C in 1 X TAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). It was stained with the nucleic acid stain SybrGold (Molecular Probes) and visualized with UV in a Fluor-S MultiImager (Bio-Rad ChemiDocTM XRS) with the Multi-Analyst software (Bio-Rad). High resolution images were analyzed with the software Quantity One (Bio-Rad) to detect DGGE bands, quantify their intensity (using a Gaussian model), and identify the same band position

across the different lanes (samples) of the gel. A matrix was constructed with the presence and relative intensity of individual bands in each lane. This matrix was used to calculate a distance matrix with City-block distances and a dendrogram with Ward's method using the software Statistica 6.0 (StatSoft, Inc.).

Sequencing and phylogenetic analysis. Numbered DGGE bands in Fig 3 were excised from the gel and kept in 20 μl of MilliQ water overnight at 4°C. 5 μl of supernatant were re-amplified with the original primer set. Part of the PCR product was checked by DGGE together with the original sample to verify the correct position of the band. The PCR product was purified by QIAquick PCR Purification Kit (QUIAGEN) and quantified in agarose gel. The sequences obtained were compared with public database DNA sequences using BLAST to determine their phylogenetic affiliation. Chimeric sequences were detected with the RDP CHECK_CHIMERA program (Cole et al. 2005) and by comparison of trees derived from different parts of the alignments.

Clone libraries and RFLP analysis. For cloning, bacterial 16S rRNA gene was amplified between positions 27 and 1492 (Escherichia coli 16S rRNA gene sequence numbering), using the primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). PCR mixtures contained 10 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.5 μM, 1.25 U Taq DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer. Reactions were carried out in an automated thermocycler (Biometra) with the following cycle: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, and a final extension step of 10 min at 72°C. The PCR product was cloned with the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Putative positive colonies (169 and 234, Sargasso and Pensacola respectively) were picked, transferred to a multi-well plate containing Luria-Bertani medium and 7% glycerol, and stored at -80°C. PCR product from each clone was digested at 37°C overnight with HaeIII (Invitrogen) and run in 2.5% low melting point agarose gel. Different band patterns (43 and 30) were chosen for partial sequencing from Sargasso and Pensacola samples.

Nucleotide sequence accession numbers. Eighty-nine 16S rRNA gene sequences were sent to EMBL database (http: //www.ebi.ac.uk/embl) and received the following accession numbers: from AM238543 to AM238615 for the clone libraries (Table 3 and 4) and from AM238633 to AM238648 for the DGGE bands (Table 2).

Results

Consumption of added DMS after enrichments with different C_1 compounds. Different DMS consumptions rates were observed after addition of 500 nM of DMS to seawaters previously enriched with different C_1 compounds. No significant changes in DMS concentration relative to

the non-enriched control were observed in MA, DMS, TMA, MeOH, MDTMA, MSA or GBT enrichments (10 μM C, Fig. 1). Significant consumption of the 500 nM added DMS were only observed in the DMS and DMSO treatments. In the DMS treatment, the initial 500 nM of DMS was consumed in 7.5 hours, yielding an apparent consumption rate as high as 1600 nM d^{-1}. In the DMSO treatment, the initial DMS concentration was 900 due to some DMS production from DMSO, but DMS was consumed within 25 h. Slow net DMS consumption during the first 10 h. was probably due to concurrent production of DMS.

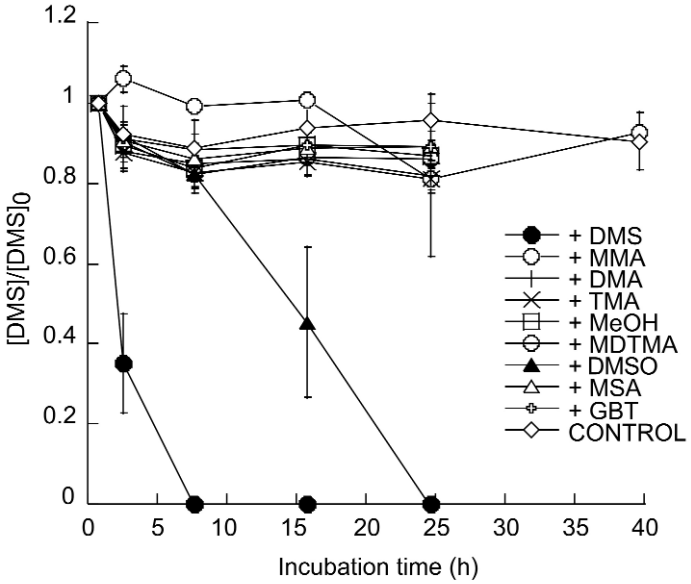


Figure 1. *Time course of the fraction of initial DMS remaining after 50 h of preincubation with methylated substrates and the subsequent addition of 500 nM DMS (MMA: monomethylamine, DMA: dimethylamine, TMA: trimethylamine, MeOH: methanol, MDTMA: mixture of MMA+DMA+TMA+MeOH, DMSO: dimethylsulfoxide, MSA: methanesulfonic acid, GBT: glycine betaine).*

DMS dynamics in enrichments with glucose, methanol, DMS and DMSO. Coastal and open-ocean waters were enriched with glucose, methanol, DMS and DMSO in order to follow DMS dynamics in different bacterial communities. Glucose addition increased bacterial production (leucine incorporation) relative to the control by a factor of 5-9. The increase factors were 2-6 after methanol addition, 3 after DMS addition, and 2 after DMSO addition (data not shown). In the samples enriched with glucose or methanol, the non-perturbing DMS concentration added at the initial time to follow DMS dynamics remained nearly constant during the entire experiment (Fig. 2). In contrast, after a lag of 3 days (coastal waters) and 8 days (open-ocean waters), the samples enriched with DMS consumed all initial DMS (2.5 μM) over 0.5-2 days. Once DMS was consumed, a new DMS addition was made at the same initial concentration, with concomitant re-additions of the other substrates to those treatments. Each

time a new DMS addition was made to the DMS depleted treatment, the added DMS was consumed with faster apparent rates. Yet little DMS consumption occurred in the glucose or MeOH treatments, even after the readditions.

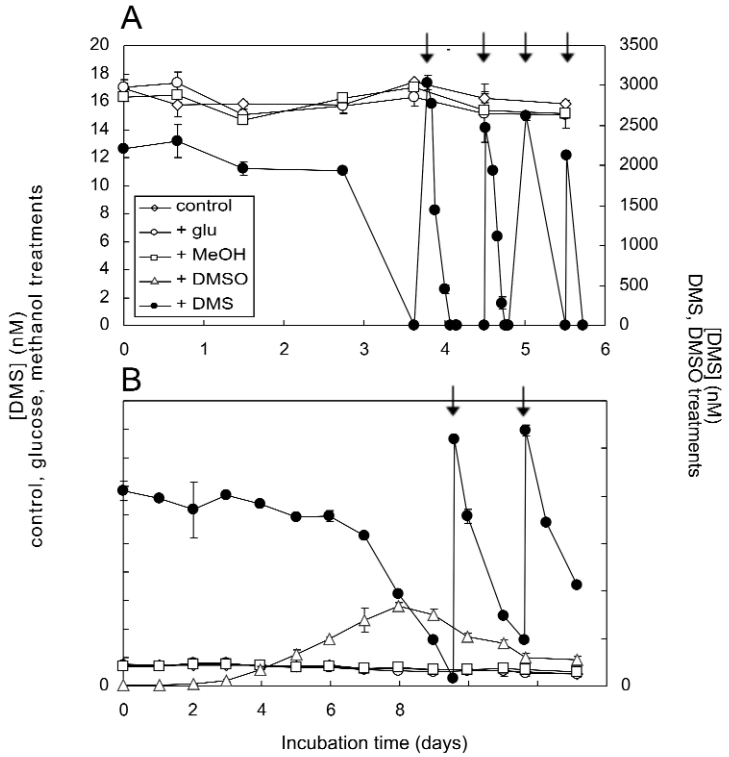


Figure 2. *Changes in DMS concentrations during the enrichment experiments done with coastal waters from Pensacola (A) and open-ocean waters from Sargasso Sea (B). Arrows indicate each re-addition of carbon sources to all treatments at initial concentrations (5 μM of carbon). Only one replicate is shown for the +DMS treatment because of the time elapsed between replicates (replicates showed the same behavior). Note that a DMSO-addition treatment was done only in Sargasso Sea waters.*
Error bars represent standard error.

In the 2.5 μM DMSO treatment (Sargasso Sea), the DMS concentration increased from 8 nM to 840 nM in 8 days. Assuming that all DMS evolved from DMSO reduction, this represents a conversion of DMSO to DMS of 33%.

The DMS consumption normalized to the bacterial production was calculated for all treatments in the beginning and at the end of the experiment (Table 1). Different stimulation of DMS consumption was observed between treatments. After enrichment with glucose and methanol, normalized DMS consumption decreased by a factor of 100 with respect to the initial water in Pensacola and by a factor of 10 in the Sargasso Sea. This resulted from increased bacterial

production with no increase of DMS consumption. In the DMS treatments, the normalized DMS consumption rate was 3760 nM DMS. h^{-1}/nM leucine. h^{-1} in Pensacola waters and 219.9 in Sargasso waters at the end of the experiment. These represent ~1400 times (Pensacola) and ~800 times (Sargasso Sea) the values obtained in the controls. In the DMSO treatment, the normalized DMS consumption was around 70 times that of the control and 1/10 that of the DMS treatment.

Fate of sulfur from consumed DMS. Incubations with ^{35}S-DMS were used to track the fate of the DMS sulfur utilized by bacteria into three pools: macromolecules, DMSO and sulfate (Table 1). The fate of DMS in the initial waters was very similar at both locations. On average, 3% of consumed ^{35}S-DMS was incorporated into macromolecules, 70% of DMS was converted to DMSO and ca 20% was converted to SO_4^{2-}. Similar percentages were found in the glucose-treated Sargasso Sea water near the end of the experiment. These percentages changed dramatically in the DMS and DMSO treatments at the end of the experiment: Incorporation into macromolecules occurred at a similar percentage (2.6% on average) but only 10% of DMS was converted to DMSO, while most of the ^{35}S-DMS was transformed to SO_4^{2-} (88%). Incubations with ^{14}C-DMS (substrate concentration of 60 nM) could be only performed in the DMS enrichments with Pensacola waters. After 17 hours, bacteria assimilated 24.8% (std. desv. 3.5%) of added ^{14}C-DMS into particle-associated macromolecules, indicating that carbon was retained much more than sulfur.

Table 1. *DMS consumption normalized to bacterial production (nM.h^{-1} DMS/nM.h^{-1} leucine) and fate of assimilated ^{35}S-DMS.*

				Fate of ^{35}S-DMS in cells (%)		
Location	time (day)	treatment	DMS turnover/BP	Macro-molecules	DMSO	SO_4^{2-}
Penascola	0	initial water	2.57	3.7	68.6	27.7*
	4.7	control	0.30	6.0	-	-
		MeOH	0.03	7.1	-	-
		Glucose	0.03	6.9	-	-
		DMS	3760	1.3	0.9	95.0
Sargasso	0	initial water	0.28	3.3	70.1	13.5
	12.2	control	0.07	-	-	-
		MeOH	0.03	-	-	-
		Glucose	0.02	4.6	88.3	12.3
		DMS	219.9	1.2	12.3	87.9
		DMSO	20.62	5.2	9.9	85.5

* estimated value
- no data

Phylogenetic identification of microbial populations after enrichment with glucose, methanol, DMS and DMSO. We ran 2 DGGE gels with the extracted and PCR-amplified microbial DNA at the end of the enrichments done with Pensacola and Sargasso Sea waters (Fig. 3). A total of 16 bands were found in the DMS treatments in both waters. Initial waters had on average 22 bands.

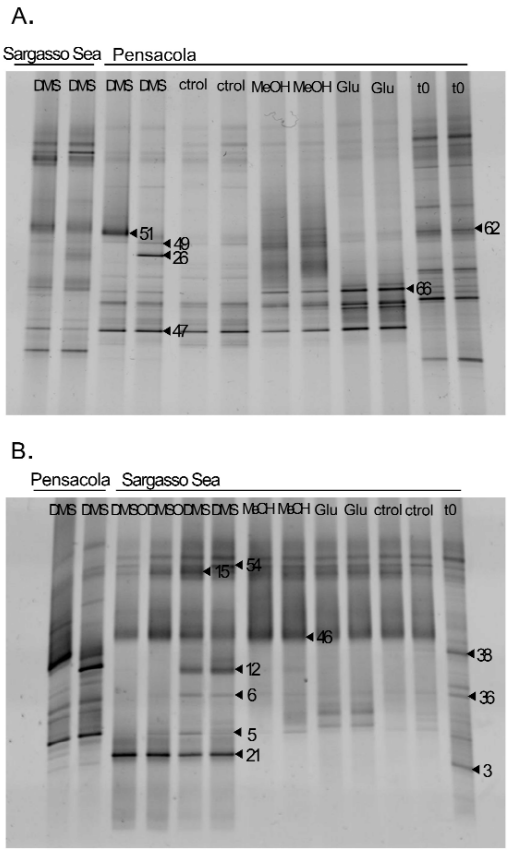


Figure 3. *DGGE gels containing 16S rRNA fragments (PCR amplified with primers 358f and 907rM) of final bacterial community for enrichment experiments from Pensacola (A) and Sargasso Sea waters (B). Each treatment had two replicates. Numbers in the gels mark sequenced bands. Note that each gel contains also the DMS treatment from the other location as a reference.*

The most intense bands in the DMS-treated waters were found almost exclusively in this treatment: bands 12 and 21 (Sargasso); bands 26 and 51 (Pensacola). Bands 12 and 26 were also found in the MeOH treatment but with much lower intensity. Only one intense band was found in the DMSO treatment (Sargasso), and it was band 21, i.e. one of the intense bands found in the DMS treatment.

The dendograms that resulted from statistical analysis of the intensity matrix (presence or absence and intensity of bands) of all gels (using Ward's method, City-block distances) separated the treatments into 3 clusters (Fig. 4). Initial waters were clearly separated from the treated-and-incubated waters of the same experiment. Control, glucose and MeOH were grouped in one cluster in the Sargasso Sea, and the DMS and DMSO treatments were grouped in another one. In Pensacola waters, the DMS treatment was grouped with the control, while glucose and MeOH made up another cluster. The DMS treatments from the two locations were not grouped together in any of the dendograms.

We excised and sequenced 16 bands. These bands accounted for a mean of 66% of the total band intensity of the DMS treatments. The specific bands of the DMS treatments were found to be closely related to the *Methylophaga* group (Table2- bands 51, 26, 12). Band 21 was not closely related to any relevant cultured marine bacteria. It showed a 97% similarity to clone SIMO-2190, an uncultured Gammaproteobacteria isolated from salt marsh sediments of

Table 2. *Phylogenetic affiliations of bacterial 16S rRNA gene sequences from bands excized from the DGGE gel Sargasso and Pensacola, as indicated by BLAST analyses in GenBank. Also shown is in which enrichments the phylotypes were mostly found.*

Phylotype	GenBank accession no.	Closest relative in GenBank; accession no.	Similarity (%)	Group	Closest cultured match, accession number	Similarity %
Sargasso 21	AM238639	Uncult. Clone SIMO-2190; AY711556	97.3	Gamma	*C. orbicularis symbiont (X84979)*	94
Sargasso 12	AM238640	Uncult. Isolate SA-37; AJ495636	98.9	Gamma	*Methylophaga* sp. *V4.MO. 19 (AJ244762)*	93
Sargasso 5	AM238641	*Oceanicola batsensis* strain HTCC2597; AY424898	98.8	*Roseobacter* (Alpha)		
Sargasso 6	AM238642	Uncult. Bacterium SB-23-CS; AJ319860	99.2	Alpha	*Roseobacter* sp. *NT N37 (AB166982)*	98
Sargasso 15	AM238643	Marine bacterium AY-56; AJ298372	99.6	Bacteroidetes	*Flavobacteriaceae* str. *SW072 (AF493679)*	91
Sargasso 54	AM238644	Bacteroidetes bacterium clone PI_RT302; AY580649	95.8	Bacteroidetes	*Flavobacteriaceae bacterium U43 (AJ623288)*	93
Sargasso 46	AM238645	*Thiomicrospira* sp. JB-A2; AF013974	96.6	Gamma		
Sargasso 3	AM238646	*Synechococcus* sp. WH 8109; AY172836	100	Cyanobacteria		
Sargasso 36	AM238647	*Roseobacter* sp. clone EF100–65C12; AY627371	99.4	Alpha	*Roseobacter* sp. *LA7 (AF513438)*	98
Sargasso 38	AM238648	Clone JL-SCS-M57; AY664063	99.6	SAR116 (Alpha)	*Mesorhizobium* sp. *rops-3 (AY490125)*	87
Pensacola 26	AM238633	Clone HMMVCen-13; AJ704666	96.5	Gamma	*Methylophaga marina (X95459)*	94
Pensacola 51	AM238634	Clone HMMVCen-13; AJ704666	95.9	Gamma	*Methylophaga thalassica (AY536563)*	95
Pensacola 49	AM238635	Clone DI-33; AY573535	99.2	Bacteroidetes	*Marine Eubacterial* sp. *(L10944)*	96
Pensacola 47	AM238636	Clone AS-19; AJ391181	99.8	Alpha	*Methylarcula* sp. *WED1.2 (AY536563)*	97
Pensacola 66	AM238637	*Roseobacter* sp. HYL-SA-18; DQ008594	99	Alpha		
Pensacola 62	AM238638	Clone PI_4d12f; AY580667	99.2	Bacteroidetes		

Sapelo Island (see http://simo.marsci.uga.edu/). Members of the *Roseobacter* group were present in all treatments (bands 5, 6, 36, 66), including that with DMS and one of the replicates of the DMSO treatment. Typical Sargasso Sea groups, such as SAR116 (Alphaproteobacteria) and *Synechococcus* were only found in the initial water (bands 38 and 3).

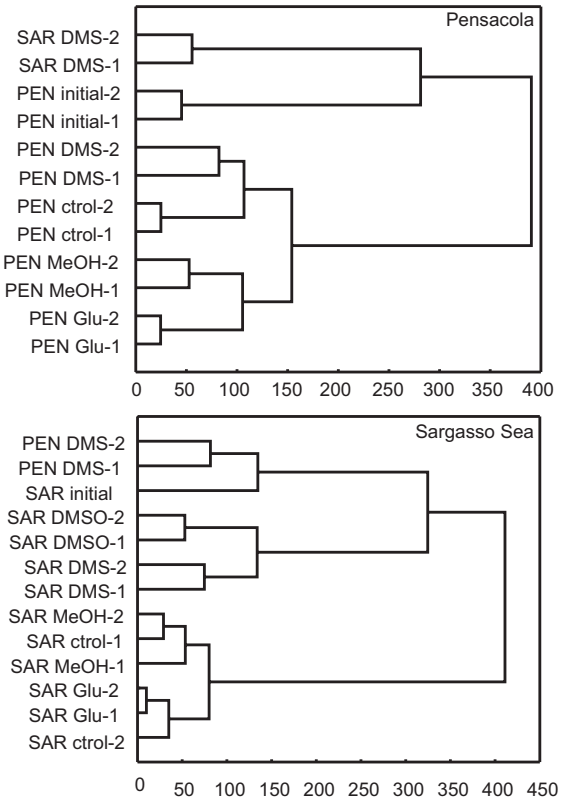


Figure 4. *Dendogram (Ward's method, City-block distances) according to the band pattern from both DGGE (Pensacola and Sargasso Sea waters)*

Clone libraries from DMS treatments. Two clone libraries were generated from DNA collected at the final time point of DMS enrichments of Pensacola and Sargasso waters. Figure 5 represents the percentage of the main clone groups in both libraries. The phylogenetic affiliation of every clone sequence retrieved can be found in Table 3 and 4 at the end of the chapter. In both waters, the highest percentage of clones corresponded to Gammaproteobacteria (55%). Within this group, *Methylophaga* were present in both waters, with a percentage of 41.2% of the total sequenced clones from Pensacola, and 18.4% of the clones from the Sargasso Sea. *Thiomicrospira*, chemolithoautotrophic bacteria, were present in DMS-enriched Sargasso Sea waters, where it accounted for 10.2% of the clones. Alphaproteobacteria accounted for 23% of total clones in both libraries, with significant presence of *Roseobacter* in Pensacola and of SAR11 in Sargasso Sea waters. The third most abundant group was Bacteroidetes, which on average accounted for 17% of the total clones in both libraries. Other groups represented less than 4% of total clones.

Attempts of the authors to use further complementary approaches such as microautoradiography with ^{35}S-DMS and fluorescent in situ hybridization of RNA (MAR-CARD-FISH, see Alonso & Pernthaler 2005) failed due to very low ^{35}S assimilation by microorganisms.

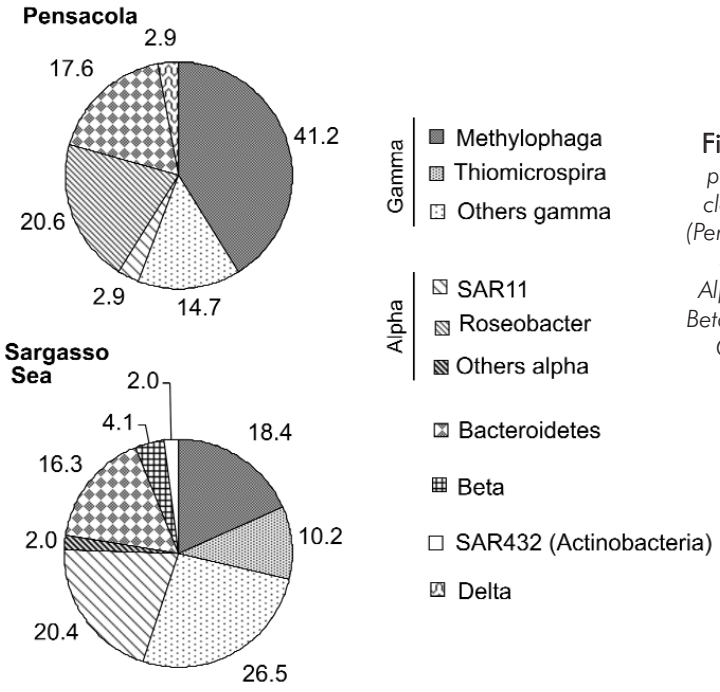


Figure 5. *Percentage and phylogenetic affiliation of clone sequences retrieved (Pensacola and Sargasso Sea clone libraries). Alpha: Alphaproteobacteria, Beta: Betaproteobacteria, Gamma: Gammaproteobacteria, Delta: Deltaproteobacteria.*

Discussion

Studies of DMS-consuming bacteria isolated from soil and marine and freshwater sediments have shown that they were also capable of consuming other C_1 compounds such as methylamine (Suylen et al. 1986), trimethylamine (Oremland et al. 1989, Lomans et al. 2001), methanethiol (Lomans et al. 2001) or dimethyl disulfide (Smith & Kelly 1988). On the basis of the early studies and the observation that ^{14}C-DMS consumption in seawater was inhibited by additions of methylated compounds (Wolfe & Kiene 1993), it was hypothesized that methylotrophs should be the main DMS consumers in seawater (Kiene 1993). However, this hypothesis had never been tested with natural marine communities. Our experiments with coastal seawater enriched with several different C_1 compounds, other than DMS or DMSO, showed no stimulation of DMS consumption. We used C_1 compounds like methylated amines or methanol that are known to be growth substrates for a variety of methylotrophs (Colby & Zatman 1973, Michalik & Raczynskabojanowska 1976, Sieburth et al. 1993). No higher DMS consumption rates were detected either when heterotrophic bacteria were supplied with nutrients

or stimulated with glucose. In contrast, DMS consumption was highly stimulated when DMS or DMSO was supplied as growth substrate. Most probably, DMSO stimulated DMS consumption indirectly via conversion into DMS in the first few hours.

Our results suggest that DMS utilization as a growth substrate was carried out by specialized methylotrophs rather than all bacteria or all methylotrophs. This specialization in microbial DMS metabolism contrasts with that of dissolved DMSP, the biochemical precursor of DMS, which is consumed by a wide spectrum of marine bacteria (Malmstrom et al. 2004a, chapter II). This difference is reflected in the turnover times of both compounds in the surface ocean. While dissolved DMSP turns over typically in 0.1 to 1 day (Kiene & Linn 2000a, Zubkov et al. 2002, Malmstrom et al. 2005), DMS generally turns over in 0.5 to 4 days (Simó 2004).

In samples from Pensacola and Sargasso Sea enriched with DMS or DMSO, where DMS consumption was greatly stimulated, 88% of DMS was converted to sulfate (Table 1). A metabolic route from DMS to sulfate has been described in *Hypomicrobium* sp. and isolates belonging to the *Roseobacter* group: DMS was metabolized via conversion to methanethiol and then to HS^-, with a heterotrophic use of the methyl groups, and HS^- in turn was converted into SO_3^{2-} and subsequently oxidized to SO_4^{2-} through lithotrophic growth (de Bont et al. 1981, González et al. 1999). Our finding that DMS-consuming bacteria assimilated 25% of added ^{14}C-DMS confirms the first heterotrophic steps of this route, where methyl groups of DMS were used as a carbon source. This significant carbon assimilation from DMS agrees with previous results of Wolfe & Kiene (1993). Use of DMS degradation products (such as HS^-) as energy source could have stimulated the complementary lithotrophic metabolism too, as it has been observed in cultures of DMS consumers such as *Hypomicrobium* sp., *Thiobacillus* sp., *Methylophaga sulfidovorans* sp. and unidentified isolates from seawater (Suylen et al. 1986, Smith & Kelly 1988, Visscher & Taylor 1993, de Zwart & Kuenen 1997, Hoeft et al. 2000). As many as 10% of the clones in the clone library from Sargasso Sea DMS-enriched waters were closely related to *Thiomicrospira*, chemolithoautotrophic bacteria that use reduced sulfur compounds as energy source (Kuenen & Veldkamp 1972). In addition, band 21 was the most intense band in the DMS and DMSO treatments from the Sargasso Sea-DGGE gel. This band however could not be assigned to a taxonomic group since its closest matches are uncultured organisms. Similarities of band 21 with clones from marine sediments from Sapelo Island (SIMO project) and autotrophic sulfur-oxidizing bacteria (Fig. 6), suggest that this bacterium belongs to a group able to obtain energy from the oxidation of inorganic sulfur compounds. It still remains unknown if these lithotrophic bacteria were using exclusively the inorganic products of DMS degradation or if they could directly metabolize DMS.

A few very intense bands were found exclusively in the DGGE gels of the DMS and DMSO treatments from both Pensacola and the Sargasso Sea. The molecular biology tools (DGGE and clone libraries) used to identify the stimulated bacteria agreed to point to the *Methylophaga*

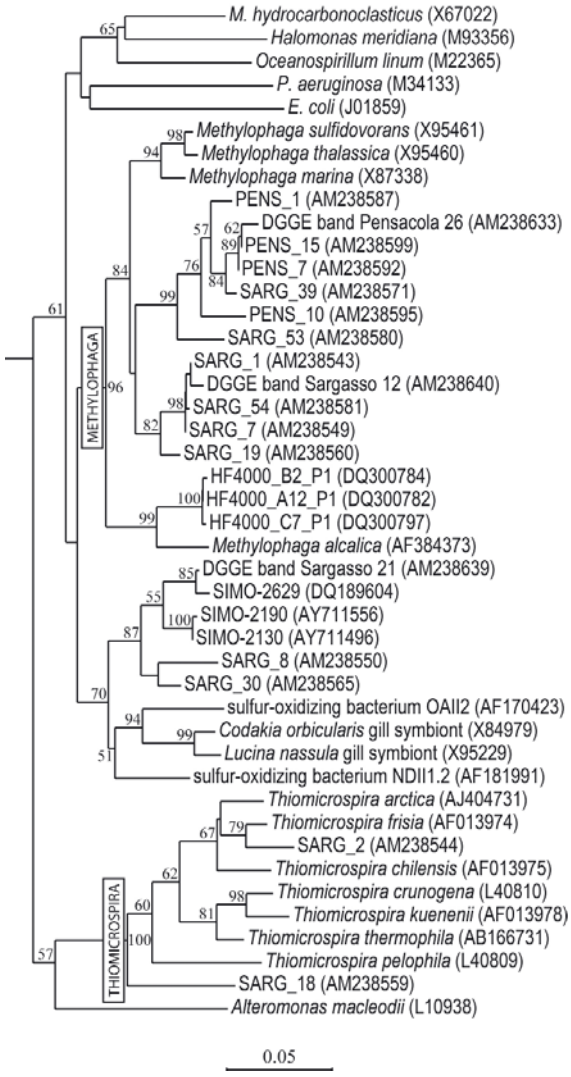


Figure 6. *Unrooted distance matrix tree showing the phylogenetic relationships of sequences from the clone libraries and DGGE bands closely related to the genera Methylophaga and Thiomicrospira. Multiple sequence alignment was performed with CLUSTAL W (Chenna et al. (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31:3497-500 PubMedID: 12824352). The tree was generated based on a Jukes-Cantor distance matrix and the Neighbour-Joining method using the PHYLIP package (Felsenstein, J. (1989) PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics 5: 164-166). The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 100 replicates. The numbers at the nodes are bootstrap values higher than 50%. Scale bar represents evolutionary distance. Only positions 180-673 (E. coli numbering system) were considered. For this part of the alignment, PENS_1 had the same sequence as PENS_2, PENS_9, PENS_12, PENS_18, PENS_20, PENS_23, PENS_30, PENS_37, PENS_43, and DGGE band Pensacola 51; SARG_1 had the same sequence as SARG_11; SARG_2 had the same sequence as SARG_10, SARG_28 and SARG_51; and SARG_30 had the same sequence as SARG_34.*

group as the most efficient marine DMS consumers. Evidence for the capability of *Methylophaga* to consume DMS was reported by de Zwart & Kuenen (1996). Those authors isolated the aerobe, obligate methylotroph *Methylophaga sulfidovorans* from microbial mat sediments in an estuarine intertidal region, and observed that it was able to oxidize DMS to thiosulfate. The first description of the genus *Methylophaga* was done by Janvier et al. in 1985. Bacteria belonging to this genus had a marine origin. They consumed methanol and other C_1 compounds but were not methanotrophs. Statistical analysis of the distribution and intensities of DGGE bands in our experiments showed that DMS-enriched microbial assemblages were not related to methanol-enriched assemblages, pointing out that these *Methylophaga*-like bacteria used preferentially DMS as a carbon source (Fig. 4).

High conversion of DMSO to DMS was observed in the DMSO treatment. The capability to reduce DMSO is a widespread feature among bacterioplankton in freshwater environments (Griebler & Slezak 2001). DMSO is used as electron acceptor and its reduction rate has been used as a measure of respiratory activity of bacteria. Some studies on the characterization of DMS consumers have been done with DMSO additions because this compound is easier to handle than DMS, and DMS eventually builds up in DMSO-rich media (de Bont et al. 1981, Suylen et al. 1986). However, microbial reduction of endogenous DMSO in seawater has never been reported. Preliminary attempts to quantify DMSO consumption showed that it was a slow process (Kiene & Gerard 1994, Simó et al. 2000, Kiene, *unpublished results*). In fact, the pathways for DMSO loss from seawater still remain unclear. Our observations suggest that bacterial reduction could be an important sink for dissolved DMSO in the ocean.

When only endogenous substrates or high levels of added glucose or MeOH were present, the DMS-consuming specialists did not dominate the bacterial community and DMS was mainly transformed to DMSO (Table 1). This DMS transformation could be a result of 2 processes: either DMS could be used as energy source (electron donor) or DMSO could be a product of DMS cometabolism, that is, enzymatic conversion of DMS without any energetic benefit for the organism (Alexander 1967). Previous studies have shown that both pathways are possible. Zhang et al. (1991) observed that a strain of *Pseudomonas acidovorans* isolated from a peat biofilter oxidized DMS to DMSO only when carbon sources other than DMS were present. Conversion of DMS to DMSO was also observed in certain anoxygenic phototrophic bacteria that used DMS as electron donor (Zeyer et al. 1987, Visscher & Van Gemerden 1991). Recently, Hirano et al. (2003) reported that *Marinobacterium* sp. strain DMS-S1 converted DMS to DMSO in the light by excretion of photosensitizing factors such as FAD and riboflavin. These two latter light-mediated metabolisms can be discarded in our enrichments since they were conducted in the dark. On the other hand, Juliette et al. (1993) and Fuse et al. (1998) found that ammonia monooxygenase (AMO) and methane monooxygenase (MMO) of marine nitrifying and methanotrophic bacteria, respectively, oxidized DMS to DMSO. Although the occurrence of a specific enzyme for the oxidation of DMS to DMSO still remains unknown, the presence of

monooxygenases in our enrichments could have driven the conversion of DMS to DMSO without any energetic benefit for bacteria. This latter explanation seems more likely since DMS consumption did not increase with glucose or nutrient additions.

Conclusions

With the aim of identifying the phylogeny and metabolic patterns of pelagic DMS-consuming bacterioplankton, we carried out enrichments with a wide array of substrates, determined the metabolic fates of DMS using radiotracers, and used molecular fingerprinting analysis and clone libraries. Altogether, our results suggest that DMS utilization as a carbon source is a specialized process where DMS is mainly transformed into sulfate. Inorganic products of DMS degradation can be utilized as an energy source by the same or other bacteria through a lithotrophic metabolism. Bacteria closely related to the *Methylophaga* group seem to be the most efficient users of DMS carbon in DMS-enriched waters. The relevance of *Methylophaga* for DMS consumption *in situ* is still uncertain and further studies on their distribution and metabolism in the water column will be required. When other carbon sources (e.g. glucose or MeOH) were available, DMS was transformed mainly into DMSO. Whether or not such transformation is carried out by specialist bacteria remains unknown.

Table 3. *Phylogenetic affiliations of clone sequences retrieved (Pensacola clone library)*

Group	Clone (Acc.Number)	Closest Match (Acc.Number)	Similarity % (n° bases)[a]	Cultured Closest Match (Acc.Number)	Similarity % (n° bses)	% clones
α Proteobacteria	PENS_6	Uncultured Rhodobacteraceae	99.9 (888)	*Methylarcula* sp. BIO-24 (AJ534207)	95.7 (851)	2.6
Rhodobacterales	AM238591	bacterium (DQ234240)				
	PENS_41	Uncultured Rhodobacteraceae	99.9 (868)	*Methylarcula* sp. (AJ534207)	95.7 (832)	0.4
	(AM238614)	bacterium (DQ234240)				
	PENS_25	Roseobacter sp.	98.9 (871)			0.9
	(AM238604)	(AY745856)				
	PENS_33	Rhodobacteraceae bacterium	95.1 (828)	*Roseobacter* sp. DSS-8 (AF098493)	92.8 (808)	0.4
	(AM238609)	N° 63 (AB180391)				
	PENS_40	Rhodobacteraceae bacterium	97.9 (847)	*Roseobacter gallaeciensis*	95.7 (828)	0.4
	(AM238613)	N° 63 (AB180391)		(AY881240)		
	PENS_39	*Thalassobius mediterraneus*	99.4 (866)			0.4
	(AM238612)	(AJ878874)				
Rickettsiales	PENS_26	Uncultured marine	99.4 (866)	Pelagibacter ubique	99.0 (866)	0.4
	(AM238605)	bacterium (DQ009191)		(AF510192)		
γ-Proteobacteria	PENS_31	*Alteromonas* sp.	92.4 (802)			0.4
Alteromonadales	(AM238608)	(AB015135)				
Thiotrichales	PENS_5	Uncultured bacterium	99.1 (870)	*Marinobacter* sp. Trimyema-1	91.7 (827)	1.3
	(AM238590)	(DQ300843)		(AJ292527)		
	PENS_1	Uncultured bacterium	95,1 (755)	*Methylophaga marina*	94.7 (751)	**54.3**
	(AM238586)	(AF468294)		(X95459)		
	PENS_2	Uncultured bacterium	95.0 (766)	*Methylophaga marina*	94.7 (764)	**15.8**
	(AM238587)	(AF468294)		(X95459)		

	PENS_9 (AM238594)	Uncultured bacterium (AF468294)	94.2 (842)	*Methylophaga marina* (X95459)	94.9 (804)	0.9
	PENS_12 (AM238597)	Uncultured bacterium (AF468294)	94.1 (790)	*Methylophaga marina* (X95459)	94.8 (810)	0.9
	PENS_18 (AM238601)	Uncultured bacterium (AF468294)	93.1 (716)	*Methylophaga marina* (X95459)	94.7 (750)	0.9
	PENS_20 (AM238602)	Uncultured bacterium (AF468294)	93.1 (716)	*Methylophaga marina* (X95459)	93.2 (672)	0.4
	PENS_23 (AM238603)	Uncultured bacterium (AF468294)	95.2 (794)	*Methylophaga marina* (X95459)	94.6 (788)	0.9
	PENS_30 (AM238607)	Uncultured bacterium (AF468294)	94.1 (788)	*Methylophaga marina* (X95459)	94.7 (748)	0.4
	PENS_37 (AM238610)	Uncultured bacterium (AF468294)	93.9 (814)	*Methylophaga marina* (X95459)	94.1 (772)	0.9
	PENS_43 (AM238615)	Uncultured bacterium (AF468294)	93.9 (812)	*Methylophaga marina* (X95459)	94.1 (770)	0.4
	PENS_10 (AM238595)	Uncultured marine bacterium (DQ009130)	94.3 (848)	*Methylophaga marina* (X95459)	93.7 (799)	2.6
	PENS_3 (AM238588)	Uncultured γ-proteobacterium (AY580765)	96.5 (738)	*Methylophaga thalassica* (X95460)	91.5 (670)	0.9
	PENS_7 (AM238592)	*Methylophaga marina* (X95459)	94.9 (797)			2.6
	PENS_7 (AM238599)	*Methylophaga marina* (X95459)	94.7 (793)			4.3
Pseudomonadales	PENS_28 (AM238606)	Uncultured γ-proteobacterium CHAB-III-7 (AJ240921)	99.7 (879)	*Pseudomonas stutzeri* ATCC 17591 (U26261)	91.0 (653)	0.9
Oceanospirillales	PENS_8 (AM238593)	*Neptunomonas naphtovorans* (AF053734)	93.5 (839)			0.9
δ-Proteobcateria	PENS_4 (AM238589)	Uncultured δ –proteobacterium (AY580409)	94.6 (722)	*Bacteriovorax* sp. (AY294217)	88.9 (770)	1.3
Bdellovibrionales						

CFB	PENS_11 (AM238596)	Sphingobacteria bacterium SKA50 (AY317117)	99.6 (840)	*Winogradskyella poriferorum* (AY848823)	96.9 (717)	1.3	
	PENS_14 (AM238598)	Unidentified marine eubacterium (L10944)	97.0 (822)	*Levinella nigricans* (AF039294)	89.7 (533)	0.9	
	PENS_16 (AM238600)	Uncultured bacteroidetes bacterium (AY580666)	99.6 (826)	*Flexibacter canadensis* (AB078046)	89.1 (589)	1.3	
	PENS_38 (AM238611)	Uncultured Flavobacteria bacterium (DQ189977)	92.7 (683)	*Pibocella ponti* (AY771744)	90.5 (535)	0.4	

[a] The numbers in parantheses are the number of bases used to calculate the levels of sequence similarity

Table 4. *Phylogenetic affiliations of clone sequences retrieved (Sargasso clone library)*

Group	Clone (Acc.Number)	Closest Match (Acc.Number)	Similarity % (n° bases)[a]	Cultured Closest Match (Acc.Number)	Similarity % (n° bses)[a]	% clones
α Proteobacteria	SARG_4	Uncultured marine bacterium (DQ009191)	99.8 (905)	Pelagibacter ubique (AF510192)	99.0 (898)	13.0
Rickettsiales	(AM238546)					
	SARG_15	Uncultured bacterium (AY093481)	99.2 (894)	Pelagibacter ubique (AF510192)	91.8 (826)	3.6
	(AM238556)					
	SARG_25	Uncultured bacterium (AY093481)	99.3 (899)	Pelagibacter ubique (AF510192)	91.8 (833)	2.4
	(AM238562)					
	SARG_56	Uncultured bacterium (AY093481)	99.2 (896)	Pelagibacter ubique (AF510192)	92.1 (830)	0.6
	(AM238583)					
	SARG_17	Uncultured marine bacterium (DQ071171)	98.0 (820)	Pelagibacter ubique (AF510192)	97.1 (830)	0.6
	(AM238558)					
	SARG_35	Uncultured marine bacterium (DQ009205)	99.7 (899)	Pelagibacter ubique (AF510192)	98.8 (830)	0.6
	(AM238568)					
	SARG_37	Uncultured marine bacterium (DQ009250)	98.9 (901)	Pelagibacter ubique (AF510192)	97.2 (884)	0.6
	(AM238569)					
	SARG_40	Uncult. α-proteobacterium (U75252)	98.7 (865)	Pelagibacter ubique (AF510192)	97.8 (857)	0.6
	(AM238572)					
	SARG_44	Uncult. α-proteobacterium (U75252)	98.9 (843)	Pelagibacter ubique (AF510192)	98.0 (835)	0.6
	(AM238575)					
	SARG_58	Uncult. α-proteobacterium (AY828383)	99.4 (712)	Pelagibacter ubique (AF510192)	99.0 (709)	0.6
	(AM238584)					
Rhizobiales	SARG_55	Uncult. marine eubacterium Hstp131	97.3 (863)	*Hyphomicrobium* sp. (AF408954)	94.6 (839)	0.6
	(AM238582)	(AF159659)				

β Proteobacteria	SARG_43	*Burkholderia multivorans* (AY486372)	91.5 (671)			0.6
Burkholderiales	(AM238574)					
	SARG_59	*Burkholderia multivorans* (AY486372)	91.1 (767)			0.6
	(AM238585)					
γ-Proteobacteria	SARG_1	*Methylophaga thalassica* (X95460)	95.7 (798)			26.6
Thiotrichales	(AM238543)					
	SARG_7	*Methylophaga thalassica* (X95460)	95.2 (810)			1.2
	(AM238549)					
	SARG_11	*Methylophaga thalassica* (X95460)	95.1 (821)			0.6
	(AM238553)					
	SARG_19	*Methylophaga thalassica* (X95460)	95.7 (826)			1.2
	(AM238560)					
	SARG_54	*Methylophaga thalassica* (X95460)	96.0 (820)			0.6
	(AM238581)					
	SARG_6	Uncult. γ-proteobacterium (AY701420)	96.9 (563)	*Methylophaga thalassica* (X95460)	99.3 (557)	0.6
	(AM238548)					
	SARG_33	Uncult. γ-proteobacterium (AY711496)	93.6 (682)	*Methylophaga marina* (X95459)	95.6 (502)	0.6
	(AM238566)					
	SARG_53	*Methylophaga marina* (X95459)	95.0 (809)			0.6
	(AM238580)					
	SARG_39	Uncultured bacterium (AF4682294)	94.2 (845)	*Methylophaga marina* (X95459)	94.5 (803)	1.2
	(AM238571)					
	SARG_2	Uncult. γ-proteobacterium (AJ879931)	98.1 (891)	*Thiomicrospira* sp. JB-A2 (AF013974)	96.3 (829)	7.1
	(AM238544)					
	SARG_10	Uncult. γ-proteobacterium (AJ879931)	97.5 (854)	*Thiomicrospira* sp. JB-A2 (AF013974)	95.5 (784)	0.6
	(AM238552)					
	SARG_18	Uncult. γ-proteobacterium (AJ879931)	92.9 (737)	*Thiomicrospira* sp. JB-A2 (AF013974)	93.4 (689)	0.6
	(AM238559)					
	SARG_28	Uncult. γ-proteobacterium (AJ879931)	98.1 (856)	*Thiomicrospira* sp. JB-A2 (AF013974)	96.1 (786)	1.2

	(AM238563)					
	SARG_51 (AM238578)	Uncult. γ-proteobacterium (AJ879931)	98.0 (872)	*Thiomicrospira* sp. JB-A2 (AF013974)	96.0 (802)	0.6
	SARG_30 (AM238565)	Uncult. γ-proteobacterium (DQ189604)	96.8 (521)	*Thiomicrospira chilenzis* (AF013975)	93.2 (411)	1.8
	SARG_34 (AM238567)	Uncult. γ-proteobacterium (DQ189604)	96.8 (521)	*Thiothrix* sp. EJ2M-B (AB042545)	89.3 (503)	0.6
	SARG_3 (AM238545)	Uncult. proteobacterium (AY711496)	97.4 (788)	*Thiothrix* sp. CT3 (AF148516)	89.3 (560)	14.2
	SARG_24 (AM238561)	Uncult. proteobacterium (AY711496)	97.3 (787)	*Thiothrix* sp. CT3 (AF148516)	89.2 (559)	0.6
Alteromonadales	SARG_8 (AM238550)	Uncultured *Alteromonas* sp. (AY726981)	92.2 (635)	*Pseudomonas fluorescens* (AY771747)	88.2 (732)	0.6
	SARG_52 (AM238579)	Uncultured *Alteromonas* sp. (AY726981)	92.3 (828)	*Alteromonas* sp. JL-S9 (AY745861)	92.9 (718)	0.6
Pseudomonadales	SARG_9 (AM238551)	Uncultured bacterium (AY700629)	99.5 (882)	*Moraxella cuniculi* (AF005189)	90.1 (299)	1.2
Legionellales	SARG_13 (AM238554)	*Legionella landfill* (AB233212)	89.9 (537)			1.2
CFB	SARG_5 (AM2385447)	Uncultured bacterium (AY701420)	97.9 (750)	*Formosa algae* (AY228461)	89.1 (687)	1.2
	SARG_16 (AM238557)	Uncultured bacterium (AY907780)	98.5 (849)	*Formosa algae* (AY228461)	90.0 (700)	0.6
	SARG_29 (AM238564)	Uncult. marine bacterium AY-56 (AJ298372)	99.4 (534)	*Muricauda* sp. DOKDO 023 (DQ191182)	89.3 (818)	1.2
	SARG_46 (AM238576)	Uncult. marine bacterium AY-56 (AJ298372)	99.6 (535)	*Muricauda* sp. DOKDO 023 (DQ191182)	89.1 (743)	0.6
	SARG_49 (AM238577)	Uncult. marine bacterium AY-56 (AJ298372)	99.6 (535)	*Muricauda* sp. DOKDO 023 (DQ191182)	89.2 (809)	0.6

	SARG_41 (AM238573)	Uncultured Flavobacteria bacterium (DQ189977)	92.8 (684)	*Muricauda* sp. DOKDO 023 (DQ191182)	88.3 (674)	0.6
	SARG_38 (AM238570)	Uncult. bacterium (DQ270634)	91.5 (689)	Saprospiraceae bacterium MS-Wolf1-H (AJ786322)	87.9 (642)	1.2
High G+C Firmicutes	SARG_14 (AM238555)	Uncult. bacterium (AB193930)	99.1 (854)	Acidimicrobidae bacterium (AY673309)	85.3 (474)	5.3

SYNTHESIS

Synthesis and Main Conclusions

The main objective of this thesis was to assess the role of heterotrophic bacterioplankton in the cycle of dimethylated sulfur compounds in seawater. Since most transformations of DMSP and DMS occur through the dissolved pool, bacteria (primary consumers of dissolved organic matter (DOM) in seawater) are a key component to better understand the dynamics of the cycle.

This research has been conducted from 2 perspectives:

1. From an ecosystem perspective, by assessing the relative role of bacteria within the plankton community and under the influence of environmental variables. The cycle of dimethylated sulfur compounds in seawater involves a complex web of interactions among members of the plankton community that are influenced by both biotic and physico-chemical factors. DMSP and DMS transformations in seawater, the relative contribution of bacteria, and their potential couplings with abiotic forcing factors have been investigated during a seasonal study in an oligotrophic coastal site (Chapter I). Also, the contribution of DMSP to the fluxes of S and C through the first levels of the food web has been estimated over an annual cycle, and its role in driving bacterial sulfur transformations has been discussed (Chapter IV).

2. From an organism/population perspective, by identifying the bacterial taxa involved in DMSP and DMS consumption. Since different phylogenetic groups of bacteria contribute differently to the transformation fluxes of DOM, it was likely that the consumption rate and fate of DMSP and DMS transformations were influenced by the taxonomic composition of the bacterial community. Chapter II addresses this question by the use of the single-cell analysis (SCA) technique MARFISH. This technique has also been used to find possible linkages between phylogeny of DMSP consumers and the seasonality of DMSP transformations (Chapter III). By using two other SCA techniques, microautoradiography and flow cytometry cell-sorting (called cell-sorting hereafter), we have discovered a new route in the cycle, the competitive uptake of DMSP by phototrophic microorganisms (Chapter V). Finally, identification of DMS-consuming bacteria in seawater has been achieved by combining enrichment experiments with molecular fingerprinting tools (Chapter VI).

The thesis has addressed the following questions:

What is the seasonality of the pools and transformations of dimethylated sulfur compounds in an oligotrophic site, and what are the physicochemical driving forces? Are bacterioplankton the key component to explain the seasonality of DMS concentrations?

A consistent and common feature of the DMS cycle, as observed in different study sites, is the strong seasonality of DMS concentrations in the upper ocean. In low temperate and subtropical

regions, DMS reaches its annual maximum concentrations in summer, 2-3 months later than its precursor, DMSP, and at a time when surface phytoplankton biomass and production are at their annual minima. This has been called the "DMS summer paradox". DMS concentrations in the upper ocean result from the balance between production and loss processes. Recent hypotheses suggest that summer DMS accumulation results from the inhibition of bacterial DMS consumption by increased doses of UV radiation as well as from the leakage of DMS by UV-stressed phytoplankton cells. Photolysis, although expected to increase in summer, would not be large enough a sink to compensate the increase in the production term. The exhaustive seasonal study of the relative importance of biotic and abiotic processes of the cycle conducted in Blanes Bay (Chapter I) has revealed that increased DMS production during the summer period was not matched by DMS losses.

In agreement with previous studies, photolysis and biological consumption alternated their dominance among DMS losses, with ventilation being a minor sink. Contrasting with kinetic studies, where higher DMS concentrations stimulated bacterial DMS consumption, the rate constants for DMS consumption decreased in summer, yielding similar consumption rates all year round (Chapter I). Photolysis did not correlate with solar radiation doses; rather, only when dominant, it correlated with the absorbance of cDOM, a known DMS photosensitizer.

If UV radiation was causing inhibition of bacterial DMS consumption in summer, it should also be inhibiting bacterial DMSPd consumption. A remarkable finding of this thesis is that maximum DMSPd consumption rates concurred with maximum DMSP concentrations (after the typical winter bloom) and, unexpectedly, in summer. The former is explained by the observation, through field and kinetic experiments, that higher DMSP concentrations induce faster DMSP consumption by bacteria. Conversely, the summer peak in DMSP consumption contrasts with the expected inhibitory effect of UV on bacteria. The main 2 routes of DMSPd metabolism are DMS production and DMSPd demethylation (that eventually fuels sulfur incorporation into proteins). DMS accumulation in summer could occur if higher DMSPd consumption rates gave rise to increased bacterial DMS production. However, our observations showed a proportionally larger fraction of DMSPd being assimilated by heterotrophic bacteria in summer. Furthermore, in August 2003 the bacterial DMS yield (that is, the proportion of DMSPd consumption that gave rise to DMS production) accounted for only a half of the DMS yield by the total plankton community. Clearly, bacteria were not the only players that explained DMS seasonality. On annual average, bacteria accounted for 52% of total DMSP transformations.

All over the year, most of the particulate DMSP (DMSPp) occurred in phytoplankton cells smaller than 5 μm. Therefore, microzooplankton were expected to be the main grazers of DMSP-containing cells. A compilation of an extensive data base by Calbet & Landry (2004) showed that grazing by microzooplankton is relatively constant through productive and oligotrophic seas, and also over seasonal scales. Interestingly, their global average of daily

phytoplankton growth consumed by microzooplankton (67%) is in good agreement with our 79% (annual average) of the DMSPp stock that is renewed daily. Hence, increased grazing on DMSP-producing algae was not a likely cause for higher DMS production in summer.

Our results suggest that direct DMS release by stressed algal cells (Sunda et al. 2002) was an important source of DMS in summer. The similarities in the seasonal patterns of the DMS:Chla and DMSOp:Chla ratios with those of the DMS concentrations and production rates suggests that these two easily measurable ratios can be regarded as proxies of the physiological state of the DMSP-producing cells and their role as DMS sources.

How widespread is the capacity to assimilate DMSP among heterotrophic bacteria?

In **Chapter II** and **III** we have used the MARFISH technique to identify the phylogenetic affiliation of marine DMSP-consumers. In each study, however, we added different concentration of ^{35}S-DMSP and used different protocols to process MARFISH samples. In the mesocosms experiment with waters from the Gulf of Mexico (**Chapter II**), due to the low specific activity of the ^{35}S-DMSP stock, addition of measurable amounts of the isotope were accompanied by DMSPd levels higher than the endogenous concentrations (ca. 60 nM). This provided information about the number of cells capable of incorporating DMSP. Contrastingly, in Blanes Bay (**Chapter II** and **III**), we incubated the samples with trace additions of ^{35}S-DMSP ($\leq$ 0.5 nM), which provided information about the *in situ* active DMSP consuming bacteria. In the Gulf of Mexico, percentages of ^{35}S-labeled cells were similar to ^{3}H-leucine assimilating cells, whereas in the seasonal study in Blanes Bay, percentages of ^{35}S-labeled cells never exceeded those of ^{3}H-labeled cells. This result suggests that capability to assimilate DMSP is a widespread feature among bacterioplankton, but not all bacteria synthesising proteins are using DMSP as a S source. However, a technical limitation of the MARFISH protocol used in Blanes Bay study calls for caution when interpreting these results. As explained in Annex 2, for a proper use of this technique it is crucial to determine the optimal exposure time to avoid silver grains over-coating the active cells. Thus, a shortening of the exposure time to avoid masking the most active cells can compromise the detection of the least active cells. This might lead to underestimation of the total number of DMSP-assimilating bacteria and to the impression that they are many fewer than leucine-assimilating cells. However, the good positive correlation between bacterial production (^{3}H-leucine incorporation rates) and ^{35}S-DMSP assimilation rates throughout the annual study in Blanes Bay (**Chapter IV**) suggests that te bulk of bacteria synthesizing protein were also assimilating DMSP.

Both the Gulf of Mexico and the Blanes Bay studies (**Chapter II** and **III**) have shown that the capacity to assimilate DMSP is a widespread feature among the main broad phylogenetic groups of marine heterotrophic bacteria. However, two groups of bacteria generally dominated DMSP incorporation in Blanes Bay all the year round: Alphaproteobacteria and Gammaproteobacteria, whereas CFB (Cytophaga-Flavobacterium-Bacteroidetes) played a

minor role. This result is in good agreement with the higher affinity showed by Alphaproteobacteria and Gammaproteobacteria groups for taking up low-molecular-weight compounds (Cottrell & Kirchman 2000). Among Alphaproteobateria, the SAR11 and *Roseobacter* clades accounted for most of DMSP assimilation. Abundances of both groups showed opposite patterns: *Roseobacter* followed Chla concentrations (peaking in winter) whereas SAR11 abounded in the N-depleted summer. Since bacterial communities associated with blooms of DMSP-producers were dominated by *Roseobacter* (González et al. 2000, Zubkov et al. 2001), we expected to find good correlations between *Roseobacter* abundances and DMSP consumption rates. However, as a general seasonal pattern, the contribution of *Roseobacter* to DMSP consumption was less than that expected from their abundance over most of the time, and so were those of SAR11 and Gammaproteobacteria. They all contributed to DMSP consumption equally or higher than their abundance during summer, when they increased the percentage of active cells. This is a surprising result, especially for the *Roseobacter* group, wich showed a consistent high activity at consuming ^{3}H-leucine all the year round. Why was there such a higher affinity for DMSP in summer? A seasonal succession of bacterial groups towards better suited DMSP consumers in summer is unlikely, since an exhaustive description of bacterial assemblages using different molecular techniques (Alonso-Sáez et al. *in press*) did not reveal any detectable succession pattern. Rather, DMSP assimilation could be determined by the relative importance of DMSP as a S source for bacteria relative to other organic S sources (see next).

What factors control DMSP assimilation by bacteria?

A strong seasonality was found for DMSP assimilation rates in Blanes Bay, with significantly higher values during the summer period. DMSP assimilation was well correlated to the percentage of ^{35}S-DMSP-assimilating cells and, interestingly, inversely correlated to the depth of the mixing layer (**Chapter III**). The shoaling of the mixing layer also originated an increase of the DMSP:Chla ratio. This ratio can be seen as a proxy of DMSP contribution to the C and S contents of phytoplankton (**Chapter IV**), which increased towards summer with phytoplankton succession. Accordingly, DMSP-sulfur satisfied most of bacterial sulfur demands in the summer period contrasting with a smaller contribution (28 $\pm$11%) in winter. All these results lead us to suggest that bacterial incorporation of DMSP-S is determined by the contribution of DMSP to the available fluxes of organic S, indicated by the DMSP:Chla ratio. If so, DMSP will have more relevant a role as a S source for bacteria under oligotrophic conditions than in nutrient-replete, productive waters.

Does DMSP uptake occur in phototrophs? If so, what are the implications for the oceanic sulfur cycle?

A new route in the cycle has been discovered: low DMSP-producing phytoplankton (mainly diatoms and the cyanobacteria *Synechoccocus* and *Prochlorococcus*) compete with

heterotrophic bacteria as sinks for DMSPd by means of light stimulated uptake (**Chapter V**). This unexpected result has broad implications:

1. Phytoplankton may take advantage to take up DMSP under damaging situations for heterotrophic bacteria, such as strong UV radiation in the shallow upper layer in summer. It is likely that, in our seasonal study, phytoplankton contributed significantly to the higher DMSP incorporation observed during the summer period, even though heterotrophic bacteria apparently were not inhibited. First, *Synechoccocus* were more abundant in summer. If their contribution to DMSP assimilation is proportional to their abundance, as observed in different temperate sampling sites (see **Chapter V**), they would have been contributing increasingly to the measured DMSPd incorporation. Secondly, higher percentages of DMSP-S-assimilating diatoms were observed in summer when their abundance was not significantly contributing to algal biomass but DMSP was contributing the most to algal S and C. Thus, similar to what was concluded in **Chapter IV** for heterotrophic bacteria, the DMSP:Chla ratio can be used to assess the availability of DMSP to supply the fluxes of organic S to non-DMSP-producing phytoplankton.

2. It provides evidence for the uptake of essential elements (in this case, S) in reduced organic forms by non-phagotrophic phytoplankton.

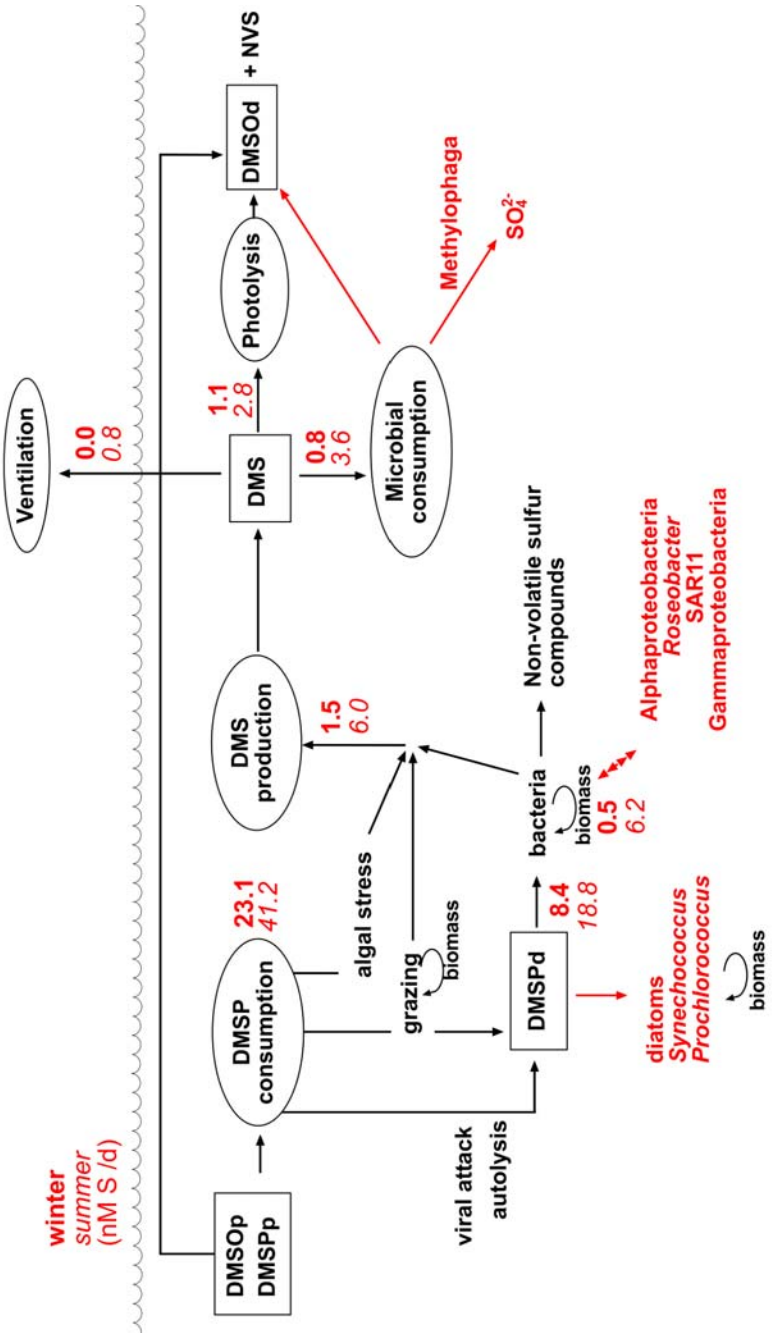
3. By incorporating DMSP-sulfur, phytoplankton divert a proportion of organic sulfur from being converted into volatile DMS.

How widespread is the capacity to assimilate DMS among microorganisms?

Due to the low assimilation of ^{35}S-DMS by bacteria, all attempts to perform MARFISH with ^{35}S-DMS incubations have failed. Alternatively, we enriched coastal and open-ocean waters with different C sources and analyzed the enriched bacterial community by fingerprinting molecular techniques in order to determine if DMS consumption was a widespread or specialized (e.g. restricted to methylotrophs) feature among marine heterotrophic bacteria. Since bacterial DMS consumption can be the dominant DMS sink in many marine systems, it was surprising to find that its use as a S source was a minor route. DMS was mainly converted to DMSO and probably used as a source of electrons when other C sources were available. Their use as a C source was restricted to some specialized methylotrophs, mainly belonging to the *Methylophaga* group. Interestingly, DMS consumption also stimulated lithotrophic metabolism but bacteria using inorganic sulfur compounds as energy source could not be identified (**Chapter VI**). Success at identifying DMS consumers in studies *in situ* might be achieved with ^{14}C-DMS incubations and MARFISH.

Summary

The main findings of this thesis are summarized in the following Figure, where the results of our work are indicated in red. (NVS: non-volatile sulfur compounds; winter: December-February; summer: May-August).



Future Research

Understanding the complexity of the sulfur cycle in the oceans requires studies of simultaneous biotic and abiotic transformations of dimethylated sulfur compounds at different temporal and spatial scales. The seasonal study in a coastal oligotrophic site (Blanes Bay) carried out in this thesis have provided insights into the seasonality of bacterial DMS and DMSP activities and the phylogenetic affiliation of DMSP consumers. Also, it has allowed finding possible couplings between biotic and abiotic processes and physical or biological parameters. However, some questions have remained unanswered and new questions have been opened.

Possibly, the major challenge that this thesis opens for future research arises from the discovery of a novel route in the cycle: DMSPd uptake by phytoplankton. The role that DMSP plays in the algal or cyanobacterial cell remains unknown. Is it used solely as a S source or may phytoplankers be taking it also as an osmoprotectant? Which other function can it play in cells? Does phytoplankton DMSP uptake increase with oxidative stress? This subject could be elucidated by the use of molecular methods for the analysis of ecophysiological processes in phytoplankton that are currently under development. Also, the successful utilization of single-cell analysis techniques such as cell sorting in studies *in situ* can be used further to assess the quantitative contribution of non-producing DMSP phytoplankton to DMSP assimilation under different regimes. Finally, genome sequencing of representative algal strains may allow to identify the gene responsible for organic S assimilation and determine how widespread this capacity is.

A remarkable finding of this thesis is that the role of algal DMSP lyases as a DMS source is more important than it had been recognized hitherto. Combination of radiotracer methods with dark incubations of whole seawater has allowed the quantification of the contribution of bacteria to DMSP consumption by the total community. Similar approaches might yield better determinations of the relative importance of the algal, zooplankton and bacterial sources of DMS.

The key factors that determine the "bacterial switch" between DMS production and DMSP demethylation have been successfully investigated. Today, unfortunately, no single cell analysis technique is available to link bacterial phylogeny to enzymatic activities that do not lead to substrate incorporation into the cell structure. Genomic and metagenomic data will soon be available to allow constraining bacterioplankton sulfur cyling arrays. The combination of DMSP microarrays and quantitiave PCR to look for patterns of presence and expression of genes responsible for DMSP metabolism, along with measurements of bulk metabolic activities, are a promising avenue to decipher the factors that regulate DMSP cycling and fate.

The dose of solar radiation appears to have a very important role as a driving force of the DMS/DMSP cycle in non-bloom situations. Certainly, further studies assessing the influence of this abiotic factor will help to better understand the dynamics of the cycle.

The seasonality of the concentrations of dimethylated sulfur coumponds is a robust feature in temperate waters. In this thesis, it has been demonstrated that this seasonality also occurrs in the major processes of the cycle. More research focused on observing the variability of the main processes of the cycle, and how they vary with environmental factors at different temporal and spatial scales, will allow developing and/or constraining more accurate and realistic models of DMS dynamics.

ANNEXES

(or how to survive Single *Hell* Techniques…)

Annex I.

Flow cytometry cell sorting using a FACSCalibur System

Some flow cytometers are able to sort cells after they have been identified according to their fluorescence or size. Cell sorting involves physically separating desired specific cell population from the remaining heterogeneous community, and depositing them into tubes for further study. This technique has been successfully applied in field studies. It has been used, for example, for determining the contribution of subpopulations to the total uptake of a radiolabeled substrate (see e.g. Zubkov et al. 2003), the distribution of primary production among different picophytoplankton groups (Li 1994), or the determination of the phylogenetic affiliation of specific sorted groups of bacteria (see e.g. Zubkov et al. 2001b, Mou et al. 2005).

Only two groups of flow cytometers are able to do cell sorting: the relatively sofisticated high-speed sorters (such as the FACSVAntage, FACSAria, Cytopeia Influx Dako MoFlo flow cytometers) which use mechanical electrostatic sorting, or the much simpler FACSCalibur which does it mechanically. Advantages and disadvantages are summarized in the following table for the two machines that are more common:

Mechanical (FACSCalibur)	Electrostatic (FACSVantage
Low speed sorter (max 300 cells sec^{-1})	High speed sorter (10000 cells sec^{-1})
Flow cell	Stream in air (nozzle tip)
No need to align laser	Alignment of the lasers
Mechanical sort	Electrostatic sort
Sheath can be varied	Saline sheath
Sort in one way	Sort in two ways
3 levels of purity	3 levels of purity
No aerosol *(safer to sort samples with toxic substances)*	Creates aerosols
Capable of shipboard work	Complicated shipboard work
High dilution of sample with sheath fluid	Low dilution

Of the different flow cytometry cell sorting applications possible in microbial ecology, we were interested in separating populations that had incorporated a radiolabeled tracer, a technique for which the main needs are that:

- the whole procedure can be made safe by isolating the pathways of circulation of the sheath fluid and the sample.
- there are no aerosols being generated.
- the lower the amount of work needed to prepare the setup, the better.

Thus, and even if the mechanical sorters are very slow and they dilute a lot the sample, they were still the best option. If we had wanted to do molecular work with the sorted material, then both the high dilution and the low sort rate would have generated many problems. High dilution with sheath fluid, even if sterile, would create many PCR artifacts.

In this thesis, we have used a FACSCalibur system with the mechanical sorting option. A catcher tube, located in the upper portion of the flow cell, moves in and out of the sample stream to collect the desired cells at a rate of up to 300 per second. We have used this technique in order to elucidate the specific contribution of bacteria and picophytoplankton subpopulations to the total assimilation of ^{35}S-DMSP. Since incubations with this radiolabeled substrate were performed before cell sorting, we were able to avoid the two major problems associated to mechanical cell sorting: the damaging effects on the physiology of the sorted cells, and the low sorting efficiencies combined with a high dilution of the sorted cells. The optional use of a postsorting Cell Concentrator was rejected because the recover efficiency of cells with this system is very low (Jochem 2005). In consequence, the major problem we had was the tedious filtration of large volumes of sorted specific populations (e.g. to sort 30000 *Synechococcus* generated 70 ml to be filtered if their abundance was 109165 cells/ml and 1000 ml if the concentration was 2207 cell/ml) .

Different protocols were tested to create good controls. No differences were observed by fixing controls with 1% paraformaldehyde + 0.05% glutaraldehyde (final conc.) or 2% formaldehyde (final conc.) by (**a**) adding the radiolabeled compound after 30 min. of fixation and freezing immediately the sample (-80°C) or (**b**) adding the radiolabeled compound after 30 min. of fixation and freezing after the incubation time of alive samples. However, it was very important to very methodically clean the tubing of the cytometer (see the protocol) and the filtration setup (with miliQ and EtOH) before running control samples. We recommend running controls always before running live samples.

A critical step was the type of filter that had to be used to collect the cells. Kiene & Linn (1999) showed that the retention of radiolabeled cells in policarbonate (PC) and polyester (PE) filters was lower than in Nylon, polyethersulfone (PES) or mixed cellulose esters (MCE) filters. Accordingly, in our samples, PVC housing filters (GS Millipore) or Nylon filters (GN Millipore) showed the highest retention of radioactivity and good reproducibility. It was very important to never let the filters dry to avoid the explosion of cells and, if possible, not to use more than 1 filter to collect all sorted cells. In the same work Kiene & Linn (1999) reported that fixation and

filtration cause loss of any intracellular compound that is not assimilated into the cell structure. Thus, our sorting results are equivalent to assimilation of the radiolabeled substrate into biomass, while we lose any information on cytoplasmatic retention of free compounds. We also did tests comparing the effect cleaning the filters after filtration of sorted cells with 0.2 μm-filtered seawater or with trichloroacetate (TCA) and we did not find any significant differences.

To assess the feasibility and efficiency of the flow sorting, increasing numbers of cells (30000 to 350000) were sorted (Fig. 1). This procedure is always better than simply sorting several accounts of the same number of cells (and using the variability associated to this replication). The standard error of the slope of the regression equations is a measure of the combined variability of the per-cell tracer incorporation and the variability of the sorted procedure itself.

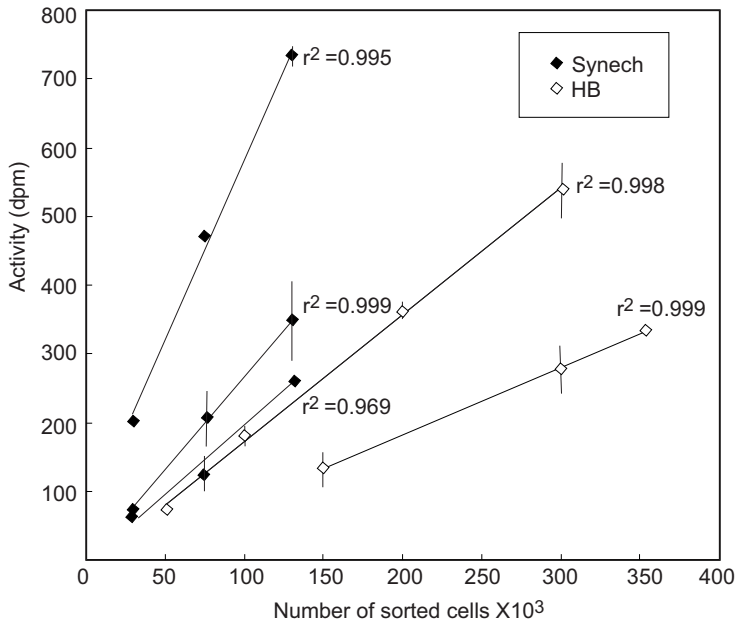


Figure 1. *Radioactivity retained in cells (dpm) as a function of the number of sorted cells.*

Detailed protocol for flow cytometry cell sorting designed to studying the assimilation of radiolabeled substrates by different subpopulations.

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1. Incubation of the sample with the radiolabeled compound.
2. Fixation and storage.
3. Setup of the cytometer.
4. Sorting cells.
5. Filtration.
6. Calculations.

1. Incubation of the sample with radiolabeled compounds

- Collect your sample (20-30 ml) in adequate vials depending on the experiment you want to run (e.g. quartz bottles for experiments considering the effect of UV) and take an extra vial for control (e.g. 20 ml, Falcon tube).

- Fix 1.8 ml of the initial sample with P+G (1% paraformaldehyde + 0.05% glutaraldehyde, final conc.), leave it in the dark for 10 min and freeze it with liquid N_2. (*)

- Add P+G to the control vial and wait 15-30 min in the dark before addition of the radiolabeled substrate. Other fixatives are possible.

- Add to live samples a trace concentration (<0.5 nM) of a given radiolabeled sub strate (at the highest specific activity possible). Count an aliquot (100 μl or less) in the scintillation counter to know exactly the initial concentration of added isotope.

- Incubate your samples in the dark or in the light at in situ temperature for a period of time, depending on the substrate and the activity of your community (typically for 4 hours).

(*) this sample will give the initial cell concentration of your sample
(**) keep this vial until you count all your sorting samples if the half-life of your isotope is of the order of days (e.g. ^{35}S = 87.1 days).

2. Fixation and storage

- Stop your incubations by adding freshly unfrozen P+G to sample.
- Leave on bench in the dark for 10 min to allow complete cell fixation.

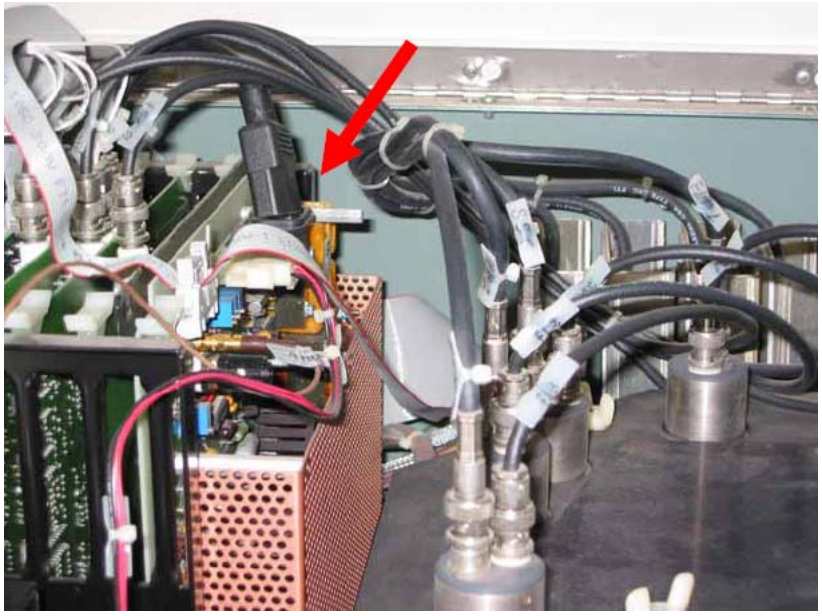
- Aliquot your samples (+ control) into 5 ml cryovials correctly labeled
- Deep freeze in liquid N_2
- Store the samples at -80ºC.

3. Setup of the cytometer

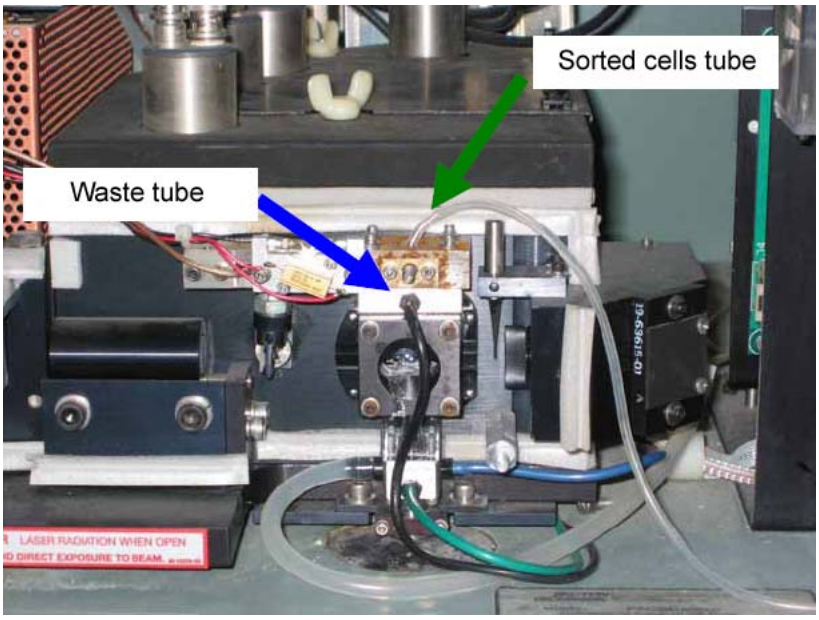
Important: The protocol of how to count algae and bacteria with the FACScalibur flow cytometer can be found in http://www.cyto.purdue.edu/cdroms/flow/vol5/index.htm or ftp.icm.csic.es/pub/gasol/Manuals/FACS/Citometry.html (Gasol 2000). Here, we directly describe how to sort radioactive cells and assume that you know everything else.

If the flow cytometer is usually used for non-radioactive samples, when sorting radiolabeled cells, some connections (see below) and the needle should be changed. All this material is labeled as HOT.

- Connect the sorting board by pressing the button in the board (we keep this one usually disconnected, because if not needed it unnecessarily heats the flow cytometric chamber)



- Connect the wasting collection tube and the sample stream tube (the one that collects the sorted cells). Use a big bottle to collect the very diluted HOT waste ("Waste" reservoir) and falcon tubes (50 ml) to collect the sorted cells. However, during the cleaning of the cytometer (see below), a plastic bottle (1 L) can be used to collect the sample stream or one can derive it to the "waste" reservoir.



- Switch on the flow cytometer (not yet the computer).

Cleaning of the cytometer (this should be done every day we do sorting)

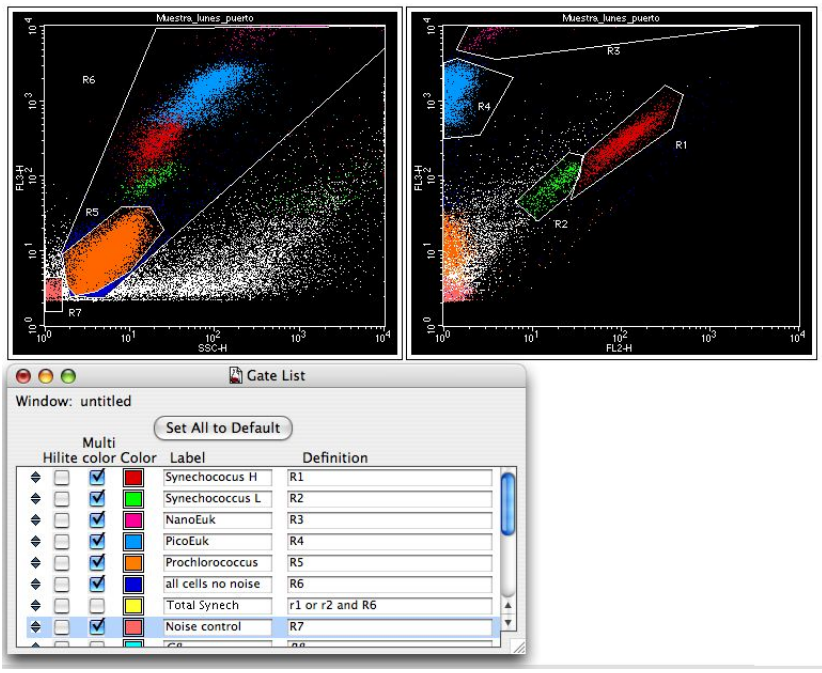
- Put a tube with Diluted bleach (approx. ~20%).
- Change the Sheath liquid tank (MilliQ tank) for a tank filled with diluted bleach (approx. ~20%).
- Bypass the saline filter of the fluidics system.

- Start with the pressure.
- (A) PRIME 5 times: push the PRIME, change the tube by a new one filled with MilliQ, push RUN, let the tube placed at the end point of the sorting gets filled, and bubbles disappear. Push PRIME again.
- Put a new tube with Diluted bleach and let it circulate at HIGH during 30 min.

- Empty the "waste" reservoir.
- Take the pressure out and rinse the detectors in the entrance tank with MilliQ
- Change the tank with bleach by one with FACSFlow or 0.2 μm-filtered seawater (or miliQ with NaCl 35% prefiltered through 0.2 μm PC filters).
- Repeat procedure (A)
- Put a new tube with FACSFlow and let it circulate in HIGH during 30 min.
- Defreeze your samples in the dark.
- Empty the "waste" reservoir.

4. Sorting cells

- Turn on the computer and open the CellQuest program.
- Open the correct CellQuest document for your analysis.
- Run an aliquot (1 ml) of your sample as if you were counting a non-radioactive marine sample. Do this is following the protocol as in Gasol 2000. Obtain the concentration of your sample by the weight-speed method.
- Under the menu Windows, open "show palette". Draw the gates of interest with the tool to mark areas.
- Under the menu Gates, open "Gate list". In order to strictly sort the desired subpopulations, combination of different gates can be performed using the terms AND, OR, NOT (see picture below). Check the CellQuest manual for details on the semantics rules.



- Under the menu Cytometer, open "Sort counters". Place the windows wherever you like on the desktop.
- Under the menu Acquire, open "Sort Setup". Define: (1) the sort gate: based on the gate list, (2) sort count: number of cells to sort (3) sort mode: single cell
- Under the menu Acquire, open "Acquisition and Storage". Tell again the sort gate and a number of cells higher than the number of cells to sort. Since the efficiency the sorter is ca. 60%, we recommend to write at least 2x the value to sort in order to make sure you sort all the cells you want.
- Place the Falcon tubes (50 mL) correctly labeled to collect the sorted cells.
- Put a large volume of sample in a cytometer tube (3-4 ml).
- Vortex the tube.
- Put the machine in STANDBY.
- Put the tube with the sample in the cytometer.
- Protect the tube from light with an aluminium foil.
- Put the machine in RUN.
- Prepare the machine for saving data.
- In the Acquisition Control window, push "Pause", then "Abort", then make sure "Setup" is off, and then push Acquire. The sorting will start to run. Collect the sorted cells in corrected labeled Falcon tubes (50 mL).

Notes

- The stream sample does not stop after sorting. Remember not collecting noise in the Falcons.
- Run your samples in increasing order of concentration. We recommend starting with control, phytoplankton and bacterioplankton (if you do both groups).
- Between samples, to clean the cytometer tubings, put a tube with FACSFlow or 0.2 μm-filtered seawater, PRIME 3 times, and let it circulate in HIGH during 10 min.
- When running phytoplankton samples, turn off the lights of the lab. *Prochlorococcus* are extremly sensitive to light. If their concentration in the sample is low, sorting takes longer and they can lose fluorescence.
- Sometimes, if sorting is done for a long time (and specially when sorting dilute subpopuplations), the populations appear to slowly. We have empirically found that just by vortexing the tube and putting it again in the cytometer the populations appear again in their place.

5. Filtration

- Store the Falcons at 4°C until filtration. We recommend filtering the same day of sorting.
- Clean the filtration kit (columns and filtration setup) with milliQ water, EtOH and milliQ water again.

- Check that there is no radioactivity in your filtration setup by filtering milliQ water and counting the filter in the scintillation counter.
- Filter the sorted cells using, if possible, one filter per subpopulation. Always use Nylon and cellulose acetate 0.2 μm-pore size filters (GN or GS 0.2 μm-size-porus filters, 25 mm, Millipore).
- Place the filter in a vial with Optiphase Highphase II scintillation cocktail.
- Vortex
- Sample is now ready for scintillation counting. However, better counts are obtained if at least 24 hours in the dark pass before it is counted.
- To know assimilation by the total community, filter an aliquot of sample (per triplicate) through 0.2 μm-pore size filters and count. Correct the number of dpm per ml by the dilution generated by the fixative.

6. Calculations

1. Convert cpm to dpm. It depends in the scintillation counter, the type of cocktail used and the type of vials. Most scintillation counters compute it automatically (by comparison to a known source of radioactivity). If that were not the case, quenching curves have to be prepared and counted.

2. Divide the dpm obtained from sorted cells by the number of cells sorted (dpm/cell of the desired subpopulation).

3. To compare the assimilation between different subpopulations, normalize the cell volume of your sorted cells. We used the following ratios: (HB: heterotrophic bacteria, Synech: *Synechococcus*, Prochl: *Prochlorococcus*, PICOeuk: Picoeukaryotes)

	diameter (μm)[a]	volume[b]	v/v(HB)[c]	standard
HB	0.4	0.034	1	1
Syench	1	0.524	15.625	10
Prochl	0.6	0.113	3.375	5
PICOeuk	2	4.189	125	100

[a]: Aproximate averaged diameter of cells obtained from Sieracki et al. (1995), Zubkov et al. (2000), Gasol et al. (*in prep.*)
[b]: volume calculate assuming spherical volume of cells ($4/3*\Pi*r^3$)
[c]: Standardized by volume of HB.

4. To know the contribution of a desired subpopulation to total isotope assimilation:

a= dpm/sorted cells
b= concentration of desired subpopulation (cells/ml)
c= assimilation by total community (total dpm/ml)

Contribution of the desired subpopulation to total isotope assimilation = a*b/c

Annex II.

Microautoradiography and its combination with fluorescence *in situ* hybridization techniques

Microautoradiography (MAR) is a useful method for determining the *in situ* assimilation of radiolabeled substrates by microorganisms. Samples are incubated with the radiolabeled form of the substrate of interest. Cells are collected in a filter that is put in contact with a photographic emulsion. When the crystals of silver bromide (AgBr) from the emulsion are impacted by the radioactivity coming from the active cells, the AgBr precipitates into Ag nuclei that, after photographic development, become visual black grains surrounding the active cells that can be easily identified under the microscope. This is one of the earliest single-cell methods; the first reports of its use in aquatic microbial ecology were published in 1959 (Saunders 1959), and Thomas Brock used it to quantify the *in situ* growth rate of a conspicuous freshwater bacterium (Brock 1967). The technique was also successfully applied to study uptake of radiolabeled substrates by cells of different size, from micro- (e.g. Paerl 1991) to picoplankton (e.g. Ouverney & Furhman 1999).

We used MAR in Chapter V of this thesis to study the incorporation of ^{35}S-DMSP by diatoms and other microplankters.

MAR can also be combined with the FISH technique (*Fluorescence In Situ Hybridization*) and it then allows the phylogenetic identification of active marine heterotrophic bacteria. Initially, MAR was combined to classical FISH (DeLong et al. 1989, Amann et al. 1990). This combination is what we used in Chapter II following exactly the protocol developed by Cottrell & Kirchamn (2000), which improved the first protocols published (Lee et al. 1999, Ouverney & Furhman 1999). Since CARD-FISH (CAtalysed Reporter Deposition-FISH) was suggested to be much better for detection of the bacteria that thrive in the ocean, with relatively low rRNA content (Pernthaler & Pernthaler 2002), Alonso & Pernthaler (2005) and Herndl et al. (2005) produced different flavors of a modified protocol that combined microautoradiography with CARDFISH. In the Herndl et al. (2005) version, the cells are transferred from the filter onto the emulsion, whereas in the Alonso & Pernthaler (2005), the emulsion is placed on top of the cells. We used the Alonso & Pernthaler protocol in Chapter III.

A summary of the different protocols available can be seen in Fig. 1.

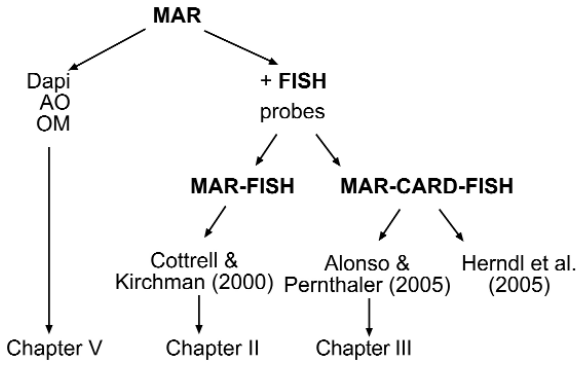


Figure 1. Summary of the different protocols of MAR. Protocols used in each Chapter are specified. DAPI: 4',6'-diamidino-2-phenylindole hydrochloride; AO: acridine orange; OM: optical microscopy.

The following table gives an overview of the advantages and disadvantages of the protocols used in Chapters II and III.

Advantages and disadvantages of MAR+FISH vs MAR+CARDFISH (Alonso's flavor)

MAR + FISH	MAR + CARDFISH
(+) Good quality of DAPI staining	(-) Bad quality of DAPI staining
(+) No masking of cells by the silver grains coating on the top of active cells.	(-) Optimization of exposure times is needed for each experiment. (but see Sintes & Herndl 2006)
(+) Optimization use of all the (expensive) emulsion	(-) Not all the open emulsion can be used
(-) Low transference of cells to the emulsion	(+) No transfer step
(-) Complicated handling in the dark.	(+) Easy handling
(-) Necessary use of a red light bulb in the dark, increasing the risk of background exposition in the microautoradiograms.	(+) No need for a red light
(-) Fluorescence of hybridized cells is often too dim	(+) Excellent visualization of hybridized cells
(-) More time consuming protocol. You usually do fewer replicates	(+) More replicates are possible because of easier protocol.
(-) MAR has to be performed after the FISH procedure	(+) CARDFISH can be performed after MAR (good for short half-life isotopes)

The two main problems we had in using the MAR+FISH protocol (**Chapter II**) were the low efficiency in the transfer of cells from the filter to the emulsion, and the low fluorescence of the hybridized cells.

When using 0.2 μm-porus-size filters (Polycarbonate, Millipore), the efficiency of transfer was, on average, 57% if microautoradiograms were stored for 5 min in the desiccator after development and 69% if they were stored overnight (see Fig. 2). This efficiency was very variable and it was almost impossible to obtain good microautoradiograms (that is, transfer efficiencies >80%) without damaging the emulsion (even if microautoradiograms were embedded in glycerol 1%). Thus, a lot of replicates had to be done in order to obtain confident results.

Interestingly, this limitation of the transfer step did not occur when using 5 μm-porus-size filters (also PC, Millipore), maybe because the filters are thinner and less sticky.

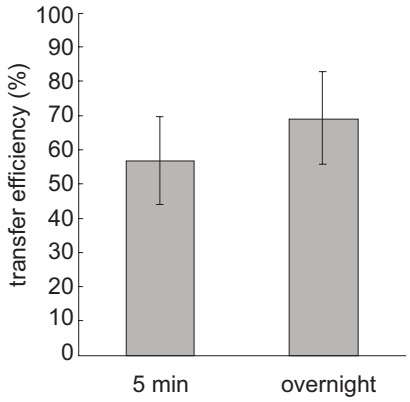


Figure 2. *Comparison of the transfer efficiency of microautoradiograms dried for 5 minutes or overnight in the desiccator.*

The low sensitivity of FISH hybridizations is mainly due to the low ribosomal content of bacterial cells in marine samples, since FISH probes are linked directly to a fluorochrom. The improved CARD-FISH protocol solves this problem by using probes linked with horseradish peroxidase (HRP) that amplifies the signal of the cells by catalyzing reactions of structural transformations of fluorescent molecules that finally bind to the proteins of the cell. All attempts we performed to combine MAR and CARD-FISH modifying the Cottrell & Kirchman (2000) protocol resulted unsuccessful. Others, however, seem to have been successful (e.g. Herndl et al. 2005).

Alonso & Pernthaler (2005) eliminated the transfer step in their protocol by covering the filters already attached to the slides with the photographic emulsion. CARDFISH hybridized cells are bright enough to be perfectly visualized under the microscope, but this is not the case of DAPI fluorescence that can not be detected. Thus, the percentage contribution of FISH-positive cells to the total community has to be counted separately (see the protocol below). Also, it is crucial

to optimize the exposure times for each experiment and isotope used (Fig. 3). Long exposure times originate too high densities of silver grains coating the most active cells. Short exposure times are desired, even though the detection of the least active cells can be lost.

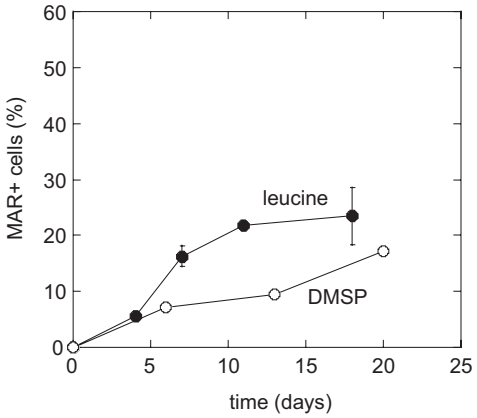


Figure 3. *Percentage of MAR+ cells as a function of different exposure times of MAR preparations with ^{35}S-DMSP and ^{3}H-leucine incubations from samples taken in Blanes Bay in September (^{35}S-DMSP) and October (^{3}H-leucine).*

From a set of filters assayed (polycarbonate track-etched, cellulose nitrate and anodisc filters), polycarbonate filters resulted to be the best support for both MAR and CARDFISH procedures (see the PhD thesis of Cecilia Alonso, 2006). However, surprisingly, when we used 0.2-μm-pore-size 25-mm polycarbonate track-etched filters from two different companies (Millipore and Whatman Nucleopore), we obtained different results (Fig. 4).

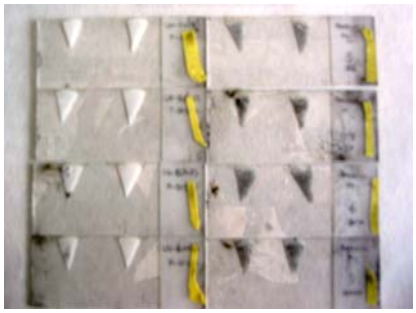


Figure 4. *Filters from the same dipping and exposed in the same box. The only difference was that filters on the left (ready to be counted) were from Millipore and filters on the right (with background) were from Whatman Nuclepore.*

Our experience during this thesis is that all methods and procedures should be carefully followed, because there are several steps in which modifications might bring lower efficiency; even though we don't know the exact reason for each detail, we do know that modifications may easily not work. We have written all these tricky empirical details on the protocol below.

MAR-CARD-FISH PROTOCOL

Maria Vila-Costa & Laura Alonso-Sáez

1. Incubation of the sample with radiolabeled compounds

• Fill up the incubation vials using adequate vials depending on the experiment you want to run (e.g. quartz bottles for experiments considering the effect of UV. If no UV transmittance is required, use e.g. ultra-clean falcon tubes) with sample water (20-30 ml are typical), and the killed controls with a smaller volume (10 ml).

• Add 2% (final concentration) formaldehyde (or paraformaldahyde) to the killed controls and wait 3-5 minutes.

• Add the radiolabeled compound(*).

• Incubate the vials at *in situ* temperature (out of light) typically for 4 hours, but longer (up to 12-24 h) depending on the substrate and concentration.

(*) The specific activity should be as high as possible. The different compounds can be added in low concentration as tracers (<0.5 nM) or high concentrations typical of bacterial production measurements (20-40 nM Leu) depending on the process one wants to study.

2. Fixation

• Add formaldehyde (or paraformaldehyde) to 2% (final concentration) and let the sample fix overnight at 4°C in the dark.

3. Filtration

• Set up the filter column with a 0.45 nitrocellulose filter supporting a white 0.22 μm polycarbonate track-etched filter (Millipore). Suck some milli Q water through the nitrocellulose filter before laying the polycarbonate on the top of it.

• Filter (typically) 5 ml of fixed sample (depending on the concentration of cells in the sample). It is recommended to have 3-4 replicate filters for each sample. (*)

• Rinse each filter three times with milli Q water filtered through 0.2 μm.

• Dry the filters on drying paper, label them with a pencil and store them in the freezer (-20°C).

(*) If you want to collect the microplankton fraction for MAR, pre-filter the sample using a 5 μm polycarbonate filter (Millipore).

4. CARD-FISH

A detailed protocol of our CARDFISH procedure is described in the PhD thesis of Laura Alonso-Sáez (2006). In brief, the outline of this technique is:

4.1. Cell immobilization via embedding the filters in 0.1 % low-gelling point agarose.
4.2. Permeabilization of cells with 10 mg/ml Lysonzyme solution (1 h, 37°C) and Achromopeptidase solution (60U/ml, 30 min, 37 °C).
4.3. Cutting of filters in sections (label them with a lead pencil).
4.4. Hybridization of cells with HRP-oligonucleotide probes overnight at 35°C.
4.5. Washing the filters in a washing buffer solution (5 min, 37 °C)
4.6. Amplification of the signal by incubating filters with fluorescent tyramide (15 min PBS at room temperature, 15 min tyramide solution at 46°C, 10 min PBS at room temperature in the dark).
4.7. Washing the filters with tap water, milliQ water, and ethanol.

Important: Cut a little piece of the filter and stain the cells with a mounting mixture of 4 Citifluor: 1 Vecta containing DAPI (1 μg/ml). Count this sample under the microscope to know the contribution of CARDFISH+ cells to the total community.

5. Dipping (Autoradiography)

Prior to the dipping, photographic emulsion (KODAK NTB2) has to be aliquoted in Falcon tubes (50ml) containing 10 ml of emulsion each (in the dark). Each falcon tube has to be covered with black tape and several folders of aluminium foil in order to protect them from the light. Store Falcon tubes with emulsion at 4°C until the dipping. Use one tube (10ml emulsion) for around 25 slides.

- Glue the filters (with the cells face up!) onto slides using epoxy-glue (UHU plus). Up to 3-4 pieces of filters can be glued onto one slide.

Before putting the filters, cut one little peace of the corner of each slide in order to be able to detect (in the dark) in which side of the slides the filters are. Alternatively, you can stick a label tape on the top of the slide at the same face where filters are.

Slightly wet the back of the filters with milliQ water (in a Petri dish) before putting them onto the slide with the glue. This helps maintaining the filters attached to the slides once they dry.

• In the dark room, melt the Falcon with emulsion in a 43°C water bath for 1hour.

• Cover a metal bar with ice for at least 15 min before the dipping, and dry it well before starting.

• Set the labeled slides with the filters (in order) in a rack.

• In the dark, add 10 ml of warm agarose solution (0.2% w/v) to the Falcon tube containing the photographic emulsion (1:1 dilution). Mix the tube very slowly.

• Put the tube back in the water bath to melt and wait for 5 minutes.

• Dip the first slide in the diluted emulsion, by placing it inside the Falcon tube and turning it upside-down smoothly 2 or 3 times.

• Open the Falcon tube and place the slide on the metal bar with the face with the filters up.

• Repeat it for all the slides and filters.

• Allow the emulsion on the slides to gel on the metal bar for about 7 minutes (no longer since cold causes background on the microautoradiograms)

• Once solidified, transfer slides to dark boxes, seal them tightly and wrap them in aluminum foil. (Use different black boxes for different exposure times. If possible, add drierite desiccants in the box to assure dryness during the exposure).

• Place the dark boxes in the fridge and expose them at 4°C. (Exposure times need to be optimized for every experiment).

6. Development of samples

• Fill up four containers with developer (Kodak D19, diluted 1:1 with milli Q water), milliQ water, fixative (Kodak Tmax, diluted 1:4 with milli Q water) and tap water.

• Turn off the lights.

• Take the slides out of the box and place them in the developer for 3 min.

• Rinse with milliQ water for 30 sec.

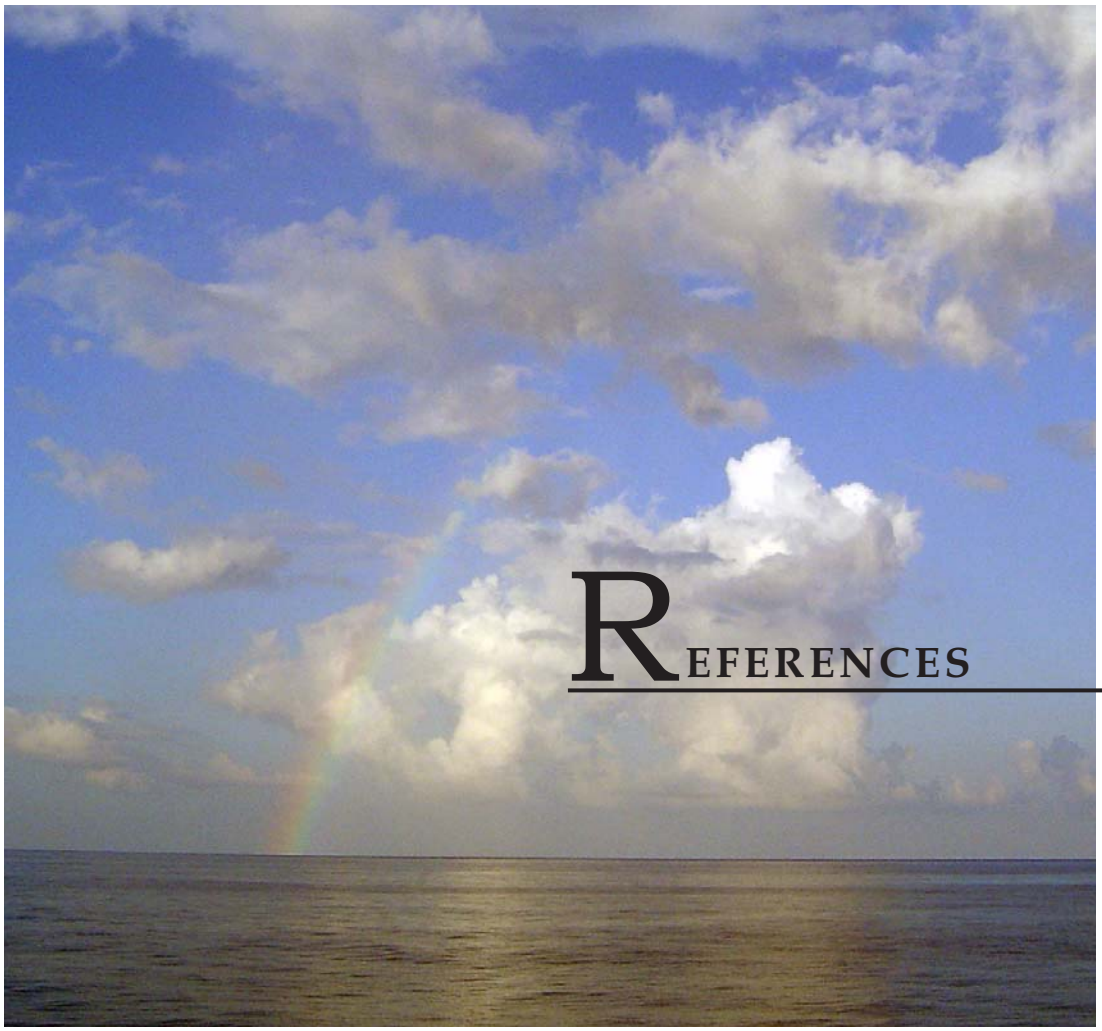
- Fix with Kodak fixer for 3 min.
- Rinse with tap water for 10 min.
- Place the slides inside a desiccator overnight, and protected from light.

7. DAPI Staining

- Cover the filters with DAPI (1 μg/ml), and stain for 3 min at 4°C.
- Clean the slides with abundant milliQ water and ethanol.
- Prepare the mounting solution: 4 Vecta Shield: 1 Citifluor (*).
- Pour one drop of the mounting solution over each filter and place a coverslip.
- Count under the microscope (UV-light for DAPI, Blue-light for Alexa488 and transmitted light for silver grains).

(*) Alternatively, the mounting solution can be prepared following this protocol:

10 ml glycerol
2 ml Vecta Shield
1 ml PBSx20
Add DAPI to a final conc. 1μg/ml
Adjust pH at 9.5 (with pH-paper).



REFERENCES

Albrecht BA (1989) Aerosols, cloud microphysics, and fractional cloudiness. Science 245:1227-1230.

Alexander (1967) Agriculture and the Quality of Our Environment, Vol. N.C. Brady, Washington D.C.

Alonso C, Pernthaler J (2005) Incorporation of Glucose under Anoxic Conditions by Bacterioplankton from Coastal North Sea Surface Waters. Appl. Environ. Microbiol. 71:1709-1716

Alonso C, Pernthaler J (2006a) Concentration-Dependent Patterns of Leucine Incorporation by Coastal Picoplankton. Appl. Environ. Microbiol. 72:2141-2147

Alonso C, Pernthaler J (2006b) Roseobacter and SAR11 dominate microbial glucose uptake in coastal North Sea waters. Environ Microbiol 8:2022-2030

Alonso C, (2006) Identify and activity of marine microbial populations as revealed by single cell techniques. PhD thesis. Max-Planck-Institut für Marine Mikrobiologie.

Alonso-Sáez L, Gasol JM, Lefort T, Hofer J, Sommaruga R (2006) Effect of Natural Sunlight on Bacterial Activity and Differential Sensitivity of Natural bacterioplankton Groups in Northwestern Mediterranean Coastal Waters. Appl. Environ. Microbiol. 72:5806-5813.

Alonso-Sáez L, Balagué V, Sà EL, Sánchez O, Gonzalez JM, Pinhassi J, Massana R, Pernthaler J, Pedros-Alio C, Gasol JM (*in press*) Seasonality in bacterial diversity in NW Mediterranean coastal waters: assessment through clone libraries, fingerprinting, and fluorescence in situ hybridization. FEMS Microbiol Ecol.

Alonso-Sáez L, Vázquez-Domínguez E, Pinhassi J, Cardelús C, Sala MM, Lekunberri I, Vila-Costa M, Balagué V, Unrein F, Massana R, Simó R, Gasol JM (*submitted*) Seasonality of bacterial carbon use in NW Mediterranean coastal waters and controlling factors.

Alonso-Sáez L (2006) Links between bacterial diversity and carbon cycling in the sea. PhD thesis.Institut de Ciències del Mar-CSIC. ftp://cucafera.icm.csic.es/pub/gasol/tesi_laura

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56:1919-1925.

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev. 59:143-169.

Andreae MO (1990) Ocean-atmosphere interactions in the global biogeochemical sulfur cycle. Mar. Chem. 30:1-29.

Andreae MO, Jaeschke W (1992) Sulphur Cycling on the Continents (scope 48), Vol. Scientific Committee On Problems of the Environment.

Andreae MO, Crutzen PJ (1997) Atmospheric aerosols: biogeochemical sources and role in atmospheric chemistry. Science 276:1052-1058.

Andreae MO, Jones CD, Cox PM (2005) Strong present-day aerosol cooling implies a hot future. Nature 435:1187-1190.

Ansede JH, Friedman R, Yoch DC (2001) Phylogenetic Analysis of Culturable Dimethyl Sulfide-Producing Bacteria from a Spartina-Dominated Salt Marsh and Estuarine Water. Appl.Environ. Microbiol. 67:1210-1217.

Archer S, Widdicombe C, Tarran G, Rees A, Burkill P (2001a) Production and turnover of particulate dimethylsulphoniopropionate during a coccolithophore bloom in the northern North Sea. Aq. Microb. Ecol. 24:225-241.

Archer SD, Stelfox-Widdicombe CE, Burkill PH, Malin G (2001b) A dilution approach to quantify the production of dissolved dimethylsulphoniopropionate and dimethylsulphi de due to microzooplankton herbivory. Aquat. Microb. Ecol. 23:131-145.

Archer SD, Gilbert FJ, Nightingale PD, Zubkov MV, Taylor AH, Smith GC, Burkill PH (2002) Transformation of dimethylsulfoniopropionate to dimethylsulfide during summer in the North Sea with an examination of key processes via a modelling approach,. Deep-Sea Res. II 49:3067-3101.

Archer SD, Stelfox-Widdicombe CE, Malin G, Burkill PH (2003) Is dimethyl sulphide production related to microzooplankton herbivory in the southern North Sea? Journal of Plankton Research 25:235-242.

Armbrust EV et al. (2004) The Genome of the Diatom Thalassiosira Pseudonana: Ecology, Evolution, and Metabolism. Science 306:79-86.

Bates T, Cline J, Gammon R, Kelly-Hansen S (1987) Regional and seasonal variations in the flux of oceanic dimethylsulfide to the atmosphere. J. Geophy. Res. 92:2930-2938.

Bates TS, Lamb BK, Guenther A, Dignon J, Stoiber RE (1992) Sulfur emissions to the atmosphere from natural sources. J. Atmos. Chem. 14:315-337.

Bates TS, Kiene RP, Wolfe GV, Matrai PA, Chavez FP, Buck KR, Blomquist BW, Cuhel RL (1994) The cycling of sulfur in surface sea water of the Northeast Pacific. J. Geophys. Res. 99:7835-7843.

Belviso S, Kim SK, Rassoulzadegan F, Krajka B, Nguyen BC, Mihalopoulos N, Buat-Menard P (1990) Production of dimethylsulfonium propionate (DMSP) and dimethylsulfide (DMS) by a microbial food web. Limnol. Oceanogr. 35:1810-1821.

Belviso S, Buat-Menard P, Putaud J-P, Nguyen B, Claustre H, Neveux J (1993) Size distribution of dimethylsulfoniopropionate (DMSP) in areas of the tropical northeastern Atlantic Ocean and the Mediterranean Sea. Mar. Chem. 44:55-71.

Belviso S, Claustre H, Marty JC (2001) Evaluation of the utility of chemotaxononmic pigments as a surrogate for particulate DMSP. Limnol. Oceanogr. 46:989-995.

Berresheim H, Andreae M, Iverson R, Li S (1991) Seasonal variations of dimethylsulfide emissions and atmospheric sulfur and nitrogen species over the Western North Atlantic Ocean. Tellus 43B:353-372.

Bouillon RC, Miller WL (2004) Determination of apparent quantum yield spectra of DMS photodegradation in an in situ iron-induced Northeast Pacific Ocean bloom. Geophys. Res. Lett. 31:L06310.

Brock TD (1967) Bacterial growth rate in the sea- direct analysis by thymidine autoradiography. Science 155:81-83.

Brock ML, Brock TD (1968) The application of microautoradiographic techniques to ecological studies. Mitt. int. Verein. theor. angew. Limnol. 15:1-29.

Broglio E, Saiz E, Calbet A, Trepat I, Alcaraz M (2004) Trophic impact and prey selection by crustacean zooplankton on the microbial communities of an oligotrophic coastal area (NW Mediterranean Sea). Aq. Microb. Ecol. 35:65-78.

Bucciarelli E, Sunda WG (2003) Influence of CO2, nitrate, phosphate, and silicate limitation on intracellular dimethylsulfoniopropionate in batch cultures of the coastal diatom Thalassiosira pseudonana. Limnology and Oceanography 48:2256-2265.

Buchan A, González JM, Moran MA (2005) An overview of the marine Roseobacter lineage. Appl. Environ. Microbiol. 71:5665-5677.

Burkill PH, Archer SD, Robinson C, Nightingale PD, Groom SB, Tarran GA, M.V. Z (2002) Dimethyl sulphide biogeochemistry within a coccolithophore bloom (DISCO): an over view. Deep-Sea Res. II 49:2863-2885.

Calbet A, Landry MR (2004) Phytoplankton growth, microzooplankton grazing, and carbon cycling in marine systems. Limnol. Oceanogr. 49:51-57.

Carlucci AF, Craven DB, Henrichs SM (1984) Diel production and microheterotrophic utilization of dissolved free amino acids in waters off Southern California. Appl. Environ. Microbiol. 48:165-170.

Charlson RJ, Lovelock JE, Andreae MO, Warren SG (1987) Oceanic phytoplankton, atmospheric sulfur, cloud albedo and climate. Nature 326:655-661

Charlson RJ, Anderson TL, McDuff RE (1992) The Sulfur Cycle. In: Butcher SS, Charlson RJ, Orians GH, Wolfe GV (eds) Global Biogeochemical Cycles, Vol 50. Harcourt Brace & Co., London, p 285-300

Colby J, Zatman LJ (1973) Trimethylamine metabolism in obligate and facultative methylo trophs. Biochemical journal 132:101-112.

Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM, Garrity GM, Tiedje JM (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 33:D294-D296.

Corn M, Belviso S, Partensky F, Simon N, Christaki U, Fukai E (1996) Origin and importance of picoplanktonic DMSP. In: Kiene RP, Visscher PT, Keller MD, Kirst GO (eds) Biological and environmental chemistry of DMSP and related sulfonium compounds. Plenum, New York.

Cottrell MT, Kirchman DL (2000) Natural assemblages of marine proteobacteria and members of the Cytophaga-Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. Appl. Environ. Microbiol. 66:1692-1697.

Cottrell MT, Kirchman DL (2003) Contribution of major bacterial groups to bacterial biomass production (Thymidine and Leucine Incorporation) in the Delaware Estuary. Limnol. Oceanogr. 48:168-178.

Cuhel RL, Taylor CD, Jannasch HW (1982) Assimilatory sulfur metabolism in marine microorganisms: considerations for the application of sulfate incorporation into protein as a measurement of natural population protein synthesis. Appl. Environ. Microbiol. 43:160-168.

Dacey JWH, Wakeham SG (1986) Oceanic dimethylsulfide: production during zooplankton

grazing on phytoplankton. Science 233:1314-1316.

Dacey JWH, Howse FA, Michaels AF, Wakeham SG (1998) Temporal variability of dimethylsulfide and dimethylsulfoniopropionate in the Sargasso Sea. Deep-Sea Res. 45:2085-2104.

Daims H, Brühl A, Amann R, Schleifer K, Wagner M (1999) The domain-specific probe eub338 is insufficient for the detectio of all Bacteria: development and evaluation of a more comprehensive probe set. System. Appl. Microbiol. 22:434-444.

de Bont JAM, van Dijken JP, Harder W (1981) Dimethyl sulphoxide and dimethyl sulfide as a carbon, sulphur and energy source for growth of Hyphomicrobium S. J. Gen. Microbiol. 127:315-323.

de Zwart JMM, Kuenen JG (1996) Aerobic DMS degradation in microbial mats: the use of a newly isolated species Methylopila sulfovorans in the mathematical description of sulfur fluxes in mats. In: Kiene RP, Visscher PT, Keller MD, Kirst GO (eds) Biological and environmental chemistry of DMSP and related sulfonium compounds. Plenum, New York.

de Zwart JMM, Kuenen JG (1997) Aerobic conversion of dimethyl sulfide and hydrogen sulfide by Methylophaga sulfidovorans: implications for modeling DMS conversion in a microbial mat. FEMS Microbiol. Ecol. 22:155-165.

del Valle D, Kiene RP (submitted) Depth-dependent fate of biologically-consumed dimethylsulfide in the Sargasso Sea.

DeLong EF, Wickham GS, Pace NR (1989) Phylogenetic strains: Ribosomal RNA-based probes for the identification of single cells. Science 243:1360-1363.

Eilers H, Pernthaler J, Peplies J, Glockner F, Gerdts G, Amman R (2001) Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. Appl. Environ. Microbiol. 67:5134-5142.

Fagerbakke KM, Heldal M, Norland S (1996) Content of carbon, nitrogen, oxygen, sulfur, and phosphorus in native aquatic and cultured bacteria. Aquat. Microb. Ecol. 10:15-27

Fuhrman JA (1987) Close coupling between release and uptake of dissolved free amino acids in seawater studied by an isotope dilution approach. Mar. Ecol. Prog. Ser. 37:45-52.

Fuhrman JA, McCallum K., A.A. D (1993) Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. Appl. Environ. Microbiol. 59:1294-1302.

Fuse H, Ohta M, Takimura O, Murakami K, Inoue H, Yamaoka Y, Oclarit JM, Omori T (1998) Oxidation of trichloroethylene and dimethyl sulfide by a marine Methylomicrobium strain containing a soluble methane monooxygenase. Biosci. Biotech. Biochem. 62:1925-1931.

Fuse H, Takimura O, Murakami K, Yamaoka Y, Omori T (2000) Utilization of dimethylsulfide as a sulfur source witht he aid of light by Marinobacterium sp. strain DMS-S1. Appl. Environ. Microbiol. 66:5527-5532

Gage D, Rhodes D, Nolte K, Hicks W, Leustek T, Cooper A, Hanson A (1997) A new route for the synthesis of dimethylsulfoniopropionate in marine algae. Nature 387:891-894.

Gasol JM (2000) How to count picoalgae and bacteria with the FACScalibur flow cytometer. Protocol.*http://www.cyto.purdue.edu/cdroms/flow/vol5/index.htm* or *ftp.icm.csic.es/pub/gasol/Manuals/FACS/Citometry.html*

Geider R, MacIntyre HL, and Kana TM (1997) Dynamic model of phytoplankton growth and accumulation: response of the balanced growth rate and the chlorophyll a:carbon ratio to light, nutirent limiation and temperature. Mar. Ecol. Prog. Ser. 148:187-200.

Giovannoni S, Rappe M (2000) Chapter 3: Evolution, diversity and molecular ecology of marine prokaryotes. In: Kirchman D (ed) Microbial Ecology of the Oceans, p 1-21.

González JM (1999) Bacterial populations in replicate marine enrichment cultures: assessing variability in abundance using 16S rRNA-based probes. Hydrobiol. 401:69-75.

González JM, Kiene RP, Moran MA (1999) Transformations of sulfur compounds by an abundant lineage of marine bacteria in the a-subclass of the class Proteobacteria. Appl. Environ. Microbiol. 65:3810-3819.

González JM, Simó R, Massana R, Covert JS, Casamayor EO, Pedrós-Alió C, Moran MA (2000) Bacterial community structure associated with a dimethylsulfoniopropionate-producing north Atlantic bloom. Appl. Environ. Microbiol. 66:4237-4246.

Grasshoff K, Ehrhardt M, Kremling K (1983) Methods of sea water analysis. Verl. Chem., Weinheim:63–97.

Griebler C, Slezak D (2001) Microbial activity in aquatic environments measured by dimethyl sulfoxiode reduction and intercomparison with connonly used methods. Appl. Environ. Microbiol. 67:100-109.

Harada H, M-A. Rouse, W. Sunda and R. P. Kiene. (2004) Latitudinal and vertical distributions of particle-associated DMSP lyase activity in the western North Atlantic Ocean. Can. J. Fish. Aquat. Sci.

Herndl G (1997) Role of ultraviolet radiation on bacterioplankton activity., Vol. Donat-P. Häder Herndl GJ, Reinthaler T, Teira E, van Aken H, Veth C, Pernthaler A, Pernthaler J (2005) Contribution of Archaea to total prokaryotic production in the deep Atlantic Ocean. Appl. Environ. Microbiol. 71:2303-2309.

Hileman (1990) Biomass Burning: Environment hurt more than thought. Chemical and Engineering News 26:4-5.

Hillebrand H, Dürselen CD, Kirschtel D, Pollingher D, Zohary T (1999) Biovolume calculation for pelagic and benthic microalgae. J. Phycol. 35:403–424.

Hirano H, Yoshida T, Fuse H, Endo T, Habe H, Nojiri H, Omori T (2003) Marinobacterium sp. strain DMS-S1 uses dimethyl sulphide as a sulphur source after light-dependent transformation by excreted flavins. Environ Microbiol 5:503-509.

Ho T-Y, Quigg A, Finkel ZV, Milligan AJ, Wyman K, Falkowski PG, Morel FMM (2003) The elemental composition of some marine phytoplankton. J. Phycol. 39:1145-1159.

Hoeft SE, Rogers DR, Visscher PT (2000) Metabolism of methyl bromide and dimethyl sulfide by marine bacteria isolated from coastal and open waters. Aq. Microb. Eco. 21:221-230.

Howard EC, Henriksen JR, Buchan A, Reisch CR, Bürgmann H, Welsh R, Ye W, González JM,

Mace K, Joye SB, Kiene RP, Whitman WB, Moran MA (2006) Bacterial Taxa That Limit Sulfur Flux from the Ocean. Science 314:649-652.

Janvier M, Frehel C, Grimont F, Gasser F (1985) Methylophaga marina gen. nov. and Methylophaga thalassica sp. nov. Marine Methylotrophs. International Journal of Systematic Bacteriology 35:131-139

Jeffery W, Kase J, Wilhelm S (2000) Ultraviolet radiation effects on bacteria and viruses in marine ecosystems. In: de Mora SJ, Demers S, Vernet M (eds) The effects of UV radiation on Marine Ecosystems. Cambridge University Press, p 206-236.

Jochem FJ (2005) Short-term physiologic effects of mechanical flow sorting and the Becton-Dickinson Cell Concentrator in cultures of the marine phytoflagellata Emiliania huxleyi and Mircomonas pusilla. Cytometry part A 65A:77-83.

Jones RI (1994) Mixotrophy in planktonic protists as a spectrum of nutritional strategies. Mar. Microb. Food Webs 8:87-96.

Juliette LY, Hyman MR, Arp DJ (1993) Inhibition of ammonia oxidation in Nitrosomas euopaea by sulfur compounds: thioethers are oxidized to sulfoxides by ammonia monooxygenase. Appl. Environ. Microbiol. 59:3718-3727.

Karsten UK, Kuck K, Vogt C, Kirst GO (1996) Dimethylsulfoniopropionate production in phototrophic organisms and its physiological function as cryoprotectant. In: Kiene RP, Visscher PT, Keller MD, Kirst GO (eds) Biological and environmental chemistry of DMSP and related sulfonium compounds. Plenum, New York.

Keil RG, Kirchman DL (1991) Contribution of dissolved free amino acids and ammonium to the nitrogen requirements of heterotrophic bacterioplankton. Mar. Ecol. Prog. Ser. 73:1-10

Keller AA (1989) Modeling the effects of temperature, light and nutrients on primary productivity: an empirical and a mechanistic approach compared. Limnol. Oceanogr. 34(1):82-95.

Keller MD, Bellows WK, Guillard RRL (1989) Dimethyl sulfide production in marine phytoplankton. In: Saltzman E, Cooper WJ (eds) Biogenic sulfur in the environment. Am. Chemical Soc., Washington, p 167-182.

Keller MD, Korjeff-Bellows W (1996) Physiological aspects of the production of dimethylsulfo niopropionate (DMSP) by marine phytoplankton. In: Kiene RP, Visscher PT, Keller MD, Kirst GO (eds) Biological and environmental chemistry of DMSP and related sulfonium compounds. Plenum, New York, p 131-142.

Keller MD, Matrai PA, Kiene RP (1999) Production of glycine betaine and dimethylsulfoniopro pionate in marine phytoplankton: I. Batch cultures. Mar. Biol. 135:237-248.

Kempf B, Bremer E (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high osmolality environments. Arch. Microbiol. 170:319-330.

Kettle AJ et al. (1999) A global database of sea surface dimethylsulfide (DMS) measurements and a procedure to predict sea surface DMS as a function of latitude, longitude, and month. Glob. Biogeochem. Cycles 13:399-444.

Kieber DJ, Jiao J, Kiene RP, Bates TS (1996) Impact of dimethylsulfide photochemistry on methyl

sulfur cycling in the Equatorial Pacific Ocean. J. Geophys. Res. 101:3715-3722.

Kiene RP, Bates TS (1990) Biological removal of dimethyl sulphide from seawater. Nature 345:702-705.

Kiene RP, Service SK (1991) Decomposition of dissolved DMSP and DMS in estuarine waters: dependence on temperature and substrate concentration. Mar. Ecol. Prog. Ser. 76:1-11.

Kiene RP (1992) Dynamics of dimethyl sulfide and dimethylsulfoniopropionate in oceanic seawater samples. Mar. Chem. 37:29-52.

Kiene RP (1993) Microbial sources and sinks for methylated sulfur compounds in the marine environment. In: Kelly DP, Murrell JC (eds) Microbial growth on C1 compounds, Vol 7. Intercept Ltd., London, p 15-33.

Kiene RP, Gerard G (1994) Determination of trace levels of dimethylsulfoxide (DMSO) in sea water and rainwater. Mar. Chem. 47:1-12.

Kiene RP, Gerard G (1995) Evaluation of glycine betaine as an inhibitor of dissolved dimethylsulphoniopropionate degradation in marine waters. Mar. Ecol. Prog. Ser. 128:121-131.

Kiene RP (1996) Production of methanethiol from dimethylsulfoniopropionate in marine surface waters. Mar. Chem. 54:69-83.

Kiene RP, Visscher PT, Keller MD, Kirst GO (eds) (1996) Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds, Vol. Plenum Press, New York.

Kiene RP, Hoffmann Williams LP, Walker JE (1998) Seawater microorganisms have a high affinity glycine betaine uptake system which also recognizes dimethylsulfoniopropionate. Aquat. Microb. Ecol. 15:39-51.

Kiene RP, Linn LJ (1999) Filter type and sampling handling affect determination of organic substrate uptake by bacterioplankton. Aquat. Microb. Ecol. 17:311-321.

Kiene RP, Linn LJ, González JM, Moran MA, Bruton JA (1999) Dimethylsulfoniopropionate and methanethiol are important precursors of methionine and protein-sulfur in marine bacterioplankton. Appl. Environ. Microbiol. 65:4549-4558.

Kiene RP, Linn LJ (2000a) Distribution and turnover of dissolved DMSP and its relationship with bacterial production in the Gulf of Mexico. Limnol. Oceanogr. 45:849-861.

Kiene RP, Linn LJ, Bruton JA (2000) New and important roles for DMSP in marine microbial communities. J. Sea Res. 43:209-224.

Kiene RP, Linn LJ (2000b) The fate of dissolved dimethylsulfoniopropionate (DMSP) in seawater: tracer studies using 35S-DMSP. Geochim. Cosmochim. Acta 64:2797-2810.

Kirchman DL, K'nees E, Hodson R (1985) Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Appl. Environ. Microbiol. 49: 599-607.

Kirchman DL (1993) Leucine incorporation as a measure of biomass production by heterotrophic bacteria., Vol Handbook of methods in aquatic microbial ecology. Boca Raton: Lewis Publishers.

Kirchman DL (2001) Measuring bacterial biomass production and growth rates from leucine

incorporation in natural aquatic environments. Methods Microbiology 30:227-237

Kirst GO, Wolff TH, Nothnagel J, Wanzek M, Ulmlke R (1991) Dimethylsulphoniopropionate (DMSP) in ice-algae and its possible biological role. Mar. Chem. 35:381-388.

Kirst GO (1996) Osmotic adjustment in phytoplankton and macroalgae: the use of dimethylsulfoniopropionate (DMSP). In: Kiene RP, Visscher PT, Keller MD, Kirst GO (eds) Biological and environmental chemistry of DMSP and related sulfonium compounds. Plenum, New York, p 121-129.

Kuenen JG, Veldkamp H (1972) Thiomicrospira pelophila, gen. n., sp. n., a new obligately chemolithotrophic colourless sulfur bacterium. Antonie van Leeuwenhoek Journal of Microbiology and Serology 38:241-256.

Kwint RLJ, Kramer KJM (1996) Annual cycle of the production and fate of DMS and DMSP in a marine coastal system. Mar. Ecol. Prog. Ser. 134:217-224.

Laroche D, Vezina AF, Levasseur M, Gosselin M, Stefels J, Keller MD, Matrai PA, Kwint RLJ (1999) DMSP synthesis and exudation in phytoplankton: a modeling approach. Mar. Ecol. Prog. Ser. 180:37-49.

Leck C, Larsson U, Bagender LE, Johansson S, Hajdu S (1990) Dimethyl sulfide in the Baltic Sea: annual variability in relation to biological activity. J. Geophys. Res. 95:3353-3364.

Ledyard KM, Dacey JWH (1994) Dimethylsulfide production from dimethylsulfoniopropionate by a marine bacterium. Mar. Ecol. Prog. Ser. 110:95-103.

Ledyard KM, Dacey JWH (1996a) Kinetics of DMSP-lyase activity in coastal seawater. In: Kiene RP, Visscher PT, Keller MD, Kirst GO (eds) Biological and environmental chemistry of DMSP and related sulfonium compounds. Plenum, New York, p 325-335.

Ledyard KM, Dacey JWH (1996b) Microbial cycling of DMSP and DMS in coastal and oligotrophic seawater. Limnol. Oceanogr. 41:33-40.

Lee N, Nielsen PH, Andreasen H, Juretschko S, Nielsen JL, Schleifer K-H, Wagner M (1999) Combination of fluorescent in situ hybridization and microautoradiography - A new tool for structure-function analyses in microbial ecology. Appl. Environ. Microbiol. 65:1289-1297.

Levasseur M, Michaud S, Egge J, Cantin G, Nejstgaard J, Sanders R, Fernandez E, Solberg P, Heimdal B, Gosselin M (1996) Production of DMSP and DMS during a mesocosm study of an Emiliania huxleyi bloom: Influence of bacteria and Calanus finmarchicus grazing. Mar. Biol 126:609-618

Li WKW (1994) Primary production of prochlorophytes, cyanobacteria, and eucaryotic ultraphytoplankton: measurements from flow cytometeric sorting. Limnol. Oceanogr. 39:169-175.

Liss P, Malin G, Turner S, Holligan PM (1994) Dimethyl sulphide and phaeocystis: a review. Journal of Marine Systems 5:41-53.

Lomans BP, Luderer R, Steenbakkers P, Pol A, van Der Drift C, Vogels GD, Op den Camp HJ (2001) Microbial populations involved in cycling of dimethyl sulfide and methanethiol in freshwater sediments. Appl. Environ. Microbiol. 67:1044-1051.

Lovelock JE, Maggs RJ, Rasmussen RA (1972) Atmospheric dimethyl sulfide and the natural sulfur cycle. Nature 237:452-453

Lovelock JE (1974) CS2 and the natural sulphur cycle. Nature 248:625-626.

Lovelock JE, Margulis L (1974) Atmospheric homeostasis by and for the biosphere: the gaia hypothesis. Tellus 26:2-9.

Malin G, Kirst GO (1997) Algal production of dimethyl sulfide and its atmospheric role. J. Phycol. 33:889-896.

Malmstrom RR, Kiene RP, Kirchman DL (2004a) Identification and enumeration of bacteria assimilating dimethylsulfoniopropionate (DMSP) in the North Atlantic and Gulf of Mexico. Limnol. Oceanogr. 49:597-606.

Malmstrom RR, R. P. Kiene, M. Cottrell and D. L. Kirchman. (2004b) Contribution of SAR11 bacteria to C, N, and S cycling in the North Atlantic Ocean. Appl. Environ. Mircobiol. 70:4129-4135.

Malmstrom RR, Kiene RP, Vila M, Kirchman DL (2005) Dimethylsulfoniopropionate (DMSP) assimilation by Synechococcus in the Gulf of Mexico and Northwest Atlantic Ocean. Limnol. Oceanogr. 50:1924-1931.

Manz W, Amann R, Ludwig W, Wagner M, KH S (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol. 15:593-600.

Manz W, Amann R, Ludwig W, Vancanneyt M, H S (1996) Application of a suite of16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga-Flavobacter-Bacteroides in the natural environment. Microbiology 142:1097-1106.

Matrai PA, Keller MD (1993) Dimethylsulfide in a large-scale coccolithophore bloom in the Gulf of Maine. Contin. Shelf Res. 13:831-843.

Matrai PA, Keller MD (1994) Total organic sulfur and dimethylsulfoniopropionate (DMSP) in marine phytoplankton: Intracellular variations. Mar. Biol. 119:61-68.

Michalik J, Raczynskabojanowska K (1976) Oxidation of methanol by facultative and obligate methylotrophs. Acta biochimica polonica 23:375-386.

Moore LR, Post AF, Rocap G, Chisholm SW (2002) Utilization of different nitrogen sources by the marine cyanobacteria Prochlorococcus and Synechococcus. Limnol. Oceanogr. 47:989-996.

Moran MA, Gonzalez JM, Kiene RP (2003) Linking a bacterial taxon to sulfur cycling in the sea: studies of the marine Roseobacter group. Geomicrobiol. J. 20:375-388.

Morris RM, Rappé MS, Connon SA, Vergin KL, Slebold WA, Carlson CA, Giovanonni SJ (2002) SAR11 clade dominates ocean surface bacterioplankton communities. Nature 20:806-810.

Mou X, Moran MA, Stepanauskas R, González JM, Hodson RE (2005) Flow-Cytometric Cell Sorting and Subsequent Molecular Analyses for Culture-Independent Identification of Bacterioplankton Involved in Dimethylsulfoniopropionate Transformations. Appl. Environ.

Microbiol. 71:1405-1416.

Mura MP, Agustí S, Cebrian J, Satta M (1996) Seasonal variability of phytoplankton biomass and community composition in Blanes Bay (1992-1994). Publ. Espec. Inst. Esp. Oceanogr. 22:23-29.

Neef (1997) Anwendung der in situ-Einzelzell-Identifizierung von bakterin zur populationsanalyse in komplexen mikrobiellen Biozönosen. Ph. D. Thesis. Technische Universität Nünchen Munich Germany.

Nelson NB, Siegel DA (2002) Chromophoric DOM in the open ocean. In: Hansell DA, Carlson CA (eds) Biogeochemistry of marine dissolved organic matter. Academic, p 547-578.

Nevitt GA, Veit RR, Kareiva P (1995) Dimethyl sulphide as a foraging cue for Antarctic Procellariiform seabirds. Nature 376:680-682.

Nightingale P, Malin G, Law C, Watson A, Liss P, Liddicoat M, Boutin J, Upstill-Goddard R (2000) In situ evaluation of air-sea exchange parameterizations using novel conservative and volatile tracers. Glob. Biogeochem. Cycles 14:373-387.

Oremland RS, Kiene RP, Mathrani I, Whiticar MJ, Boone DR (1989) Description of an estuarine methylotrophic methanogen which grows on dimethyl sulfide. Appl. Environ. Microbiol. 55:994-1002.

Oudot C, Gerard R, Morin P, Gningue I (1988) Precise shipboard determination of dissolved oxygen (Winkler procedure) for productivity studies using a commercial system. Limnol.Oceanogr. 33:146-150

Ouverney CC, Furhman JA (1999) Combined microautoradiography--16S rRNA probe technique for determiation of radioisotope uptake by specific microbial cell types in situ. Appl. Environ. Microbiol. 65:1746-1752.

Paerl HW (1991) Ecophysiological and trophic implications of light-stimulated amino acid utilization in marine picoplankton. Appl. Environ. Microbiol. 57:473-479.

Parsons TR, Maita Y, Lalli CM (1984) A manual of chemical and biological methods for seawater analysis. In. Pergamon Press, Oxford, p 173

Partensky F, Blanchot J, Vaulot D (1999) Differential distribution and ecology of Prochlorococus and Synechococcus in oceanic waters: a review. In: Charpy L, Larkum AWD (eds) Marine Cyanobacteria, Vol 19. Bull. Inst. Oceanogr., Monaco, p 457-475.

Pedrós-Alió C, Newell. SY (1989) Microautoradiography study of thymidine uptake in brackish waters around Sapelo island, Georgia, USA. Mar. Ecol. Prog. Ser. 55: 83-94

Pernthaler A, Pernthaler J, R A (2002) Fluorescence in situ identification and catalyzed reporter deposition for the identification of marine bacteria. Appl. Environ. Microbiol. 68:3094-3101.

Pernthaler J, Glöckner FO, Schönhuber W, Amann R (2001) Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes, Vol. J.H. Paul Academic Press, London, United Kingdom.

Pinhassi J, Simó R, González J, Vila M, Alonso-Sáez L, Kiene RP, Moran M, Pedrós-Alió C (2005) Dimethylsulfoniopropionate Turnover Is Linked to the Composition and Dynamics

of the Bacterioplankton Assemblage during a Microcosm Phytoplankton Bloom. Appl. Environ. Microbiol. 71:7650–7660.

Pinhassi J, Gomez-Consarnau L, Alonso-Saez L, Montserrat Sala M, Vidal M, Pedros-Alio C, Gasol J (2006) Seasonal changes in bacterioplankton nutrient limitation and their effects on bacterial community composition in the NW Mediterranean Sea. Aquatic Microbial Ecology 44:241-252.

Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25:943-948.

Raven JA (1997) Phagotrophy in phototrophs. Limnol. Oceanogr. 42:198-205.

Rich JH, Ducklow HW, Kirchman DL (1996) Concentrations and uptake of neutral monosaccharides along 140oW in the equatorial Pacific: contribution of glucose to heterotrophic bacterial activity and DOM flux. Limnol. Oceanogr. 41:595-604.

Rocap G et al. (2003) Genome divergence in two Prochlorococcus ecotypes reflects oceanic niche differentiation. Nature 424:1042-1047.

Saunders, GW (1959) The application of radioactive tracers to the study of lake metabolism. PhD Thesis, Univ. of Michigan, 103 pp.

Scarratt, MG, Levasseur M, Schultes S, Michaud S, Cantin G, Vézina A, Gosselin M, deMora SJ. (2000) Production and consumption of dimethylsulfide (DMS) in North Atlantic Waters. Mar Ecol Prog Ser 204:13-26.

Scarratt MG, Levasseur M, Michaud S, Cantin G, Gosselin M, De Mora SJ (2002) Influence of phytoplankton taxonomic profile on the distribution of dimethylsulfide and dimethylsul foniopropionate in the northwest Atlantic. Mar. Ecol. Prog. Ser. 244:49-61

Schauer M, Massana R, Pedrós-Alió C (2000) Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. FEMS Microbiol Ecol 33:51-59.

Schauer M, Balagué V, Pedrós-Alió C, Massana R (2003) Seasonal changes in the taxonomic composition of bacterioplankton in a coastal oligotrophic system. Aquat. Microb. Ecol. 31:163-174.

Schlessinger WH (1991) The Global Sulfur Cycle. In: Academic Press (ed) Biogeochemistry: an analysis of global change. Harcourt Brace Jovanovich, p 336-348.

Sieburth JM, Johnson PW, Church VM, Laux DC (1993) C1 bacteria in the water column of hesapeake Bay, USA .3. Immunological relationships of the type species of marine monomethylamine-oxidizing and methane-oxidizing bacteria to wild estuarine and oceanic cultures. Marine ecology-progress series 95:91-102.

Siegel DA, Maritorena NB, Nelson NB, Hansell DA, Lorenzi-Kayser M (2002) Global distribution and dynamics of colored dissolved and detritial organic materials. J. Geophs. Res. 107:3228.

Sieracki ME, Haugen EM, Cucci TL (1995) Overestimation of heterotrophic bacteria in the Sargasso Sea: direct evidence by flow and imaging cytometry. Deep-Sea res. 42:1399-1410.

Sintes E, Herndl GJ (2006) Quantifying Substrate Uptake by Individual Cells of Marine Bacterioplankton by Catalyzed Reporter Deposition Fluorescence In Situ Hybridization Combined with Microautoradiography. Appl. Environ. Microbiol. 72:7022-7028.

Simó R, Grimalt JO, Albaiges J (1996) Sequential method for the feild determination of nanomolar concentrations of dimethyl sulfoxide in natural waters. Anal. Chem. 68: 1493-1498.

Simó R, Grimalt JO, Albaigés J (1997) Dissolved dimethylsulphide, dimethylsulphoniopropionate and dimethylsulphoxide in western Mediterranean waters. Deep-Sea res. II 44:929-950.

Simó R, Hatton AD, Malin G, Liss PS (1998) Particulate dimethylsulfoxide in seawater: production by microplankton. Mar. Ecol. Prog. Ser. 167:291-296.

Simó R, Pedrós-Alió C (1999a) Role of vertical mixing in controlling the oceanic production of dimethyl sulphide. Nature 402:396-399.

Simó R, Pedrós-Alió C (1999b) Short-term variability in the open ocean cycle of dimethylsulfide. Glob. Biogeochem. Cycles 13:1173-1181.

Simó R, Pedrós-Alió C, Malin G, Grimalt JO (2000) Biological turnover of DMS, DMSP, and DMSO in contrasting open-sea waters. Mar. Ecol. Prog. Ser. 203:1-11.

Simó R (2001) Production of atmospheric sulfur by oceanic plankton: biogeochemical, ecological and evolutionary links. Trends Ecol. Evol. 16:287-294.

Simó R, Archer SD, Pedrós-Alió C, Gilpin L, Stelfox-Widdicombe CE (2002) Coupled dynamics of dimethylsulfoniopropionate and dimethylsulfide cycling and the microbial food web in surface waters of the North Atlantic. Limnol. Oceanogr. 47:53-61.

Simó R (2004) From cells to globe: approaching the dynamics of DMS(P) in the ocean at multiple scales. Can. J. Fish. Aquat. Sci. 5:673-684.

Simó R, Vila-Costa M (2006) Ubiquity of algal dimethylsulfoxide in the surface ocean:Geographic and temporal distribution patterns. Marine Chemistry 100:136-146

Slezak D, Brugger A, Herndl GJ (2001) Impact of solar radiation on the biological removal of dimethylsulfoniopropionate and dimethylsulfide in marine surface waters. Aq. Microb. Ecol. 25:87-97.

Slezak D, Herndl GJ (2003) Effects of ultraviolet and visible radiation on the cellular concentrations of dimethylsulfoniopropionate in Emiliania huxleyi (strain L). Mar. Ecol. Prog. Ser. 246:61-71.

Smetacek V, Scharek R, Nöthig EM (1990) Seasonal and regional variation in the pelagial and its relationship to the life history cycle of krill. In: Kerry KR, Hempel G (eds) Antarctic Ecosystems Ecological Change and Conservation. Springer, Berlin, p 103-114.

Smith DC, Azam F (1992) A simple, economical method for measuring bacterial protein synthesis rates in seawater using 3H-leucine. Mar. Microb. Food Webs 6:107-114.

Smith E, del Giorgio PA (2003) Low fractions of active bacteria in natural aquatic communities. Aquat. Microb. Ecol. 31:203-208.

Smith NA, Kelly DP (1988) Isolation and physiological characterization of autotrophic sulphur

bacteria oxidizing dimethyl disulphide as sole source of energy. J. Gen. Microbiol. 134:1407-1417.

Steeman-Nielsen E (1952) The use of radio-active carbon for measuring organic production in the Sea. J. Cons. Expl. Mer. 18:117-140.

Stefels J, Dijkhuizen L (1996) Characteristics of DMSP-lyase in Phaeocystis sp. (Prymnesiophyceae). Mar. Ecol. Prog. Ser. 131:307-313.

Stefels J (2000) Physiological aspects of the production and conversion of DMSP in marine algae and higher plants. J. Sea Res. 43:183-197.

Steinke M, Wolfe GV, Kirst GO (1998) Partial characterization of dimethylsulfoniopropionate (DMSP) lyase isozymes in 6 strains of Emiliania huxleyi. Mar. Ecol. Prog. Ser. 175:215-225.

Strom SL, Brainard JL, Holmes JL, Olson MB (2001) Phytoplankton blooms are strongly impacted by microzooplankton grazing in coastal North Pacific Waters. Mar. Biol 138:355-368.

Sunda W, Kieber DJ, Kiene RP, Huntsman S (2002) An antioxidant function for DMSP and DMS in marine algae. Nature 418:317-320.

Suylen GMH, Stefess GC, Kuenen JG (1986) Chemolithotrophic potential of a Hyphomicrobium species, capable of growth on methylated sulphur compounds. Arch. Microbiol. 146:192-198.

Tang K, Damm H, Visscher PT (1999) Dimethylsulfoniopropionate (DMSP) in marine copepods and its relation with diets and salinity. Mar. Ecol. Prog. Ser. 179:71-79.

Tang K (2001) Defecation of dimethylsulfoniopropionate (DMSP) by the copepod Acartia tonsa as functions of ambient food concentration and body DMSP count. J. Plank. Res 23:549-553.

Tang KW, Simó R (2003) Trophic uptake and transfer of dimethylsulfoniopropionate in simple planktonic food chains. Aq. Microb. Ecol. 31:193-202.

Taylor AH, Geider RJ, Gilbert FJH (1997) Seasonal and latitudinal dependencies of phytoplankton carbon-to-chlorophyll a ratios: results of a modelling study. Marine Ecology-Progress Series 152:51-66.

Toole DA, Kieber DJ, Kiene RP, Siegel DA, Nelson NB (2003) Photolysis and the dimethylsulfide (DMS) summer paradox in the Sargasso Sea. Limnol. Oceanogr. 48:1088-1100.

Toole DA, Siegel DA (2004) Light-driven cycling of dimethylsulfide (DMS) in the Sargasso Sea: Closing the loop. Geophys. Res. Lett. 31:L09308.

Toole, D. A., Kieber D. J., Kiene R. P., White E. M., Bisgrove J., del Valle D. A., Slezak D. (2004) High dimethylsulfide photolysis rates in nitrate-rich Antarctic waters. Geophys. Res. Lett., 31: L11307.

Toole DA, Slezak D, Kiene RP, Kieber DJ, Siegel DA (2006) Effects of solar radiation on dimethylsulfide cycling in the western Atlantic Ocean. Deep-Sea res. I 53:136-153.

Turner SM, Malin G, Nightingale PD, Liss PS (1996) Seasonal variation of dimethyl sulphide in the North Sea and an assessment of fluxes to the atmosphere. Mar. Chem. 54:245-262.

Twomey S (1959) The nuclei of natural cloud formation. Part II: The supersaturation in natural clouds and the variation of cloud droplet concentration. Geophys. Pure Appl. 43:243–249.

Uher G, Schebeske G, Barlow RG, Cummings DG, Mantoura RFC, Rapsomankiks SR, Andreae MO (2000) Distribution and air-sea exchange of dimethyl suphide at the European western continental margin. Mar. Chem. 69:277-300.

Vairavamurthy A, Andreae MO, Iverson RL (1985) Biosynthesis of dimethylsulfide and dimethylpropiothetin by Hymenomonas carterae in relation to sulfur source and salinity variations. Limnol. Oceanogr. 30:59-70.

Vallina S, Simó R (submitted) Tight coupling of DMS to the solar radiation dose in the Global Ocean surface.

van Duyl FC, Gieskes WWC, Kop AJ, Lewis WE (1998) Biological control of short-term variation in the concentration of DMSP and DMS during a Phaeocystis spring bloom. J. Sea Res. 40:221-231.

Visscher P, Van Gemerden H (1991) Production and consumption of dimethylsulfoniopropionate in marine microbial mats. Appl. Environ. Microbiol. 57:3237-3242.

Visscher PT, Diaz MR, Taylor BF (1992) Enumeration of bacteria which cleave or demethylate dimethylsulfoniopropionate in the Caribbean Sea. Mar. Ecol. Prog. Ser. 89:293-296.

Visscher PT, Taylor BF (1993) A new mechanism for the aerobic catabolism of dimethyl sulfide. Appl. Environ. Microbiol. 59:3784-3789.

Visscher PT, Taylor BF (1994) Demethylation of dimethylsulfoniopropionate to 3-mercaptopropionate by an aerobic marine bacterium. Appl. Environ. Microbiol. 60:4617-4619.

Wolfe GV, Kiene RP (1993) Radioisotope and chemical inhibitor measurements of dimethyl sulfide consumption rates and kinetics in estuarine waters. Mar. Ecol. Prog. Ser. 99:261-269.

Wolfe GV, Sherr EB, Sherr BF (1994) Release and consumption of DMSP from Emiliania huxleyi during grazing by Oxyrrhis marina. Mar. Ecol. Prog. Ser. 111:111-119.

Wolfe GV, Steinke M (1996) Grazing-activated production of dimethyl sulfide (DMS) by two clones of Emiliania huxleyi. Limnol. Oceanogr. 41:1151-1160.

Wolfe GV, Steinke M, Kirst GO (1997) Grazing-activated chemical defense in a unicellular marine alga. Nature 387:894-897.

Wolfe GV, Levasseur M, Cantin G, Michaud S (1999) Microbial consumption and production of dimethyl sulfide (DMS) in the Labrador Sea. Aquat. Microb. Ecol. 18:197-205 Wolfe GV, Levasseur M, Cantin G, Michaud S (2000) DMSP and DMS dynamics and microzooplankton grazing in the Labrador Sea: application of the dilution technique. Deep-Sea res. I 47:2243-2264.

Yang G, Zhang J, Li L, Qi J (2000) Dimethylsulfide in the surface water of the East China Sea. Continent. Shelf Res. 20:69-82.

Yoch DC (2002) Dimethylsulfoniopropionate: Its Sources, Role in the Marine Food Web, and Biological Degradation to Dimethylsulfide. Appl. Environ. Microbiol. 68:5804-5815.

Yoch DC, Ansede JH, Rabinowitz KS (1997) Evidence for intracellular and extracellular dimethylsulfoniopropionate (DMSP) lyases and DMSP uptake sites in two species of marine bacteria. Appl. Environ. Microbiol. 63:3182-3188.

Zemmelink HJ, Dacey JWH, Hintsa EJ (2004) Direct measurements of biogenic DMS fluxes from the oceans. Can. J. Fish. Aq. Sc. 61:836-844.

Zeyer J, Eicher P, Wakeham SG, Schwartzenbach RP (1987) Oxidation of dimethyl sulfide to dimethyl sulfoxide by phototrophic purple bacteria. Appl. Environ. Microbiol. 53:2026-2032.

Zhang L, Kuniyoshi I, Hirai M, Shoda M (1991) Oxidation of dimethylsulfide by Pseudomonas acidovorans DMR-11 isolated from peat biofilter. Biotechnol. Lett. 13:223-228

Zimmer-Faust R, de Souza MP, Yoch DC (1996) Bacterial chemotaxis and its potential in marine dimethylsulfide production and biogeochemical sulfur cycling. Limnol. Oceanogr. 41:1330-1334

Zubkov MV, Sleigh MA, Burkill PH (2000) Assaying picoplankton distribution by flow cytometry of underway samples collected along a merdional transect across the Atlantic Ocean. Aq. Microb. Ecol. 21:13-20

Zubkov M, Fuchs B, Burkill P, Amann R (2001a) Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. Appl. Environ. Microbiol. 67:5210-5218

Zubkov MV, Fuchs BM, Archer SD, Kiene RP, Amann R, Burkill PH (2001b) Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopro pionate in an algal bloom in the North Sea. Environ. Microbiol 3:304-311

Zubkov MV, Fuchs BM, Archer SD, Kiene RP, Amann R, Burkill PH (2002) Rapid turnover of dissolved DMS and DMSP by defined bacterioplankton communities in the stratified euphotic zone of the North Sea. Deep-Sea Res. Part II 49:3017-3038

Zubkov MV, Fuchs BM, Tarran GA, Burkill PH, Amann R (2003) High Rate of Uptake of Organic Nitrogen Compounds by Prochlorococcus Cyanobacteria as a Key to Their Dominance in Oligotrophic Oceanic Waters. Appl. Environ. Microbiol. 69:1299-1304.

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Gaston Rébuffat, *Horitzons conquerits*

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Apaga l'espelma
Apaga la foguerada dels ulls
Apaga el cap, que divaga
Ho apagues tot, i reculls.

Enric Casassas


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