“That’s it. You people have stood in my way long enough. I’m going to clown college.”

Homer J. Simpson, 1995

Osmoregulation of microalgae and release of climate relevant gases

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Abbreviations

BEH	-	ethylene bridged hybrid
CCN	-	cloud condensation nuclei
DMS	-	dimethylsulfide
DMS-Ac	-	dimethylsulfonioacetate
DMSHB	-	4-dimethylsulfonio-2-hydroxybutyrate
DMSO	-	dimethylsulfoxide
DMSP	-	dimethylsulfoniopropionate
FPD	-	flame photometric detector
GBT	-	glycine betaine
GC-MS	-	gas chromatography coupled mass spectrometry
HILIC	-	hydrophilic interaction liquid chromatography
HPLC	-	high performance liquid chromatography
IPCC	-	Intergovernmental Panel on Climate Change
LC-MS	-	liquid chromatography coupled mass spectrometry
SMM	-	S-methyl methionine
TMAB	-	trimethylammoniumbutyrate
TMAP	-	trimethylammoniumpropionate
UPLC	-	ultra performance liquid chromatography

Zusammenfassung

Marine Mikroalgen spielen aufgrund ihrer Eigenschaft als und Primärproduzenten von organischen Verbindungen eine entscheidende Rolle für das marine Nahrungsnetzwerk und sind beteiligt an verschiedenen globalen Stoffkreisläufen wie dem marinen Schwefelkreislauf. Hierbei spielen die Verbindungen Dimethylsulfoniumpropionat (DMSP), welches von zahlreichen Mikro- und Makroalgenarten synthetisiert wird, sowie dessen leicht flüchtiges Abbauprodukt, Dimethylsulfid (DMS), eine besondere Rolle. Letzteres trägt über Oxidationsprozesse in der Atmosphäre entscheidend zur Wolkenbildung über den Ozeanen bei und unterstreicht die Bedeutung des marinen Phytoplanktons für das globale Klima.

Durch die Spaltung von DMSP im basischen Milieu zu Acrylat und DMS bieten Gaschromatographiebasierte Analysemethoden die Möglichkeit einer indirekten Quantifizierung von DMSP als freigesetztes DMS. Aufgrund möglicher weiterer Verbindungen, welche unter basischen Bedingungen DMS freisetzen, liegt hier eine Quelle für systematische Fehler bei der indirekten Bestimmung von DMSP in biologischen Proben. Weiterhin liefern diese indirekten Methoden lediglich ein sehr vereinfachtes Bild über den komplexen marinen Schwefelkreislauf und die zugrundeliegenden Wechselwirkungen mit anderen zwitterionischen Verbindungen wie Glycinbetain (GBT), Dimethylsulfoniumacetat (DMS-Ac) oder Gonyol.

Ziel der vorliegenden Arbeit war es, mit Hilfe neuer LC–MS basierter Analysemethoden den Beitrag verschiedener zwitterionischer Verbindungen an wichtigen Osmoregulations- und Osmoadaptionsprozessen mariner Mikroalgen aufzuklären. Weiterhin sollte untersucht werden, inwieweit diese Verbindungen die Freisetzung von klimarelevantem DMS beeinflussen.

Beim Vergleich indirekter (GC-MS basierter) und direkter DMSP Quantifizierung mittels LC-MS wurde in Abhängigkeit von der untersuchten Algenart und deren Wachstumsphase eine um bis zu 14% niedrigere DMSP Konzentration in den direkten Messungen ermittelt. Diese Differenz konnte nicht auf biologische Variabilität oder Unterschiede in der Probenvorbereitung zurückgeführt werden. Somit müssen weitere basenlabile DMS-Quellen existieren, welche durch indirekte Messmethoden nicht separat erfasst werden können. Dieser systematische Fehler bei der indirekten Bestimmung von DMSP ist besonders bei quantitativen Untersuchungen, zum Beispiel physiologischer Prozesse, nicht zu vernachlässigen, da hier oft geringere DMSP-Konzentrationsschwankungen diskutiert werden. Weiterhin können diese neuen DMS-Quellen eine erhebliche ökologische und geochemische Bedeutung haben, da diese immerhin für ca. 120 Gmol biogener Schwefelemissionen pro Jahr verantwortlich gemacht werden können.

Durch simultane Quantifizierung verschiedener zwitterionischer Metaboliten in Phytoplankton-kulturen konnte gezeigt werden, dass neben DMSP auch Verbindungen wie Homarin, DMS-Ac und

GBT in erheblichem Maße an der Osmoadaption mariner Mikroalgen beteiligt sind. Diese Anpassung an verschiedene Salzgehalte spielt vor allem für kosmopolitische Arten eine wichtige Rolle, um in Habitaten mit unterschiedlichen Salinitäten überleben zu können. Experimente mit dem Dinoflagellaten *Prorocentrum minimum* zeigten, dass diese Spezies in einem großen Salinitätsbereich DMSP eher dazu nutzt ein bestimmtes Osmolytniveau aufrechtzuerhalten, wobei die Feinregulierung bei der Osmoadaption in diesem Bereich durch DMS-Ac und GBT erfolgt. Durch Übersichtsmessungen zahlreicher Phytoplanktonkulturen konnte weiterhin gezeigt werden, dass neben DMSP auch DMS-Ac in allen untersuchten Spezies vorkommt, wobei der relative DMS-Ac-Gehalt zwischen 0,1% und 1990%, bezogen auf DMSP, sehr variabel ist. Gonyol hingegen, welches zuvor lediglich in dem Dinoflagellaten *Gonyaulax polyedra* beschrieben wurde, konnte in allen untersuchten Dinoflagellaten und Haptophyten in relativen Anteilen von 6,2% bis 121%, bezogen auf DMSP, nachgewiesen werden. Dies zeigt die weite Verbreitung anderer Dimethylsulfoniumverbindungen, welche nicht über GC-basierte Messungen zugänglich sind.

Aufgrund der Tatsache, dass Phytoplankton assoziierte Bakterien als Hauptkonsumenten von DMSP und als größte Quelle von DMS gelten, wurde weiterhin untersucht, inwieweit die Dimethylsulfoniumverbindungen DMS-Ac und Gonyol die bakterielle Freisetzung der DMSP-Abbauprodukte DMS und Methanthiol (MeSH) beeinflussen. Zum einen zeigte sich, dass alle untersuchten Bakterienarten sowohl DMS-Ac als auch Gonyol in ähnlichem Ausmaß umsetzen, wobei dies jedoch ohne Freisetzung von DMS erfolgt. Hingegen deutet die Freisetzung von MeSH beim Abbau von Gonyol durch *Alcaligenes faecalis* beziehungsweise DMS-Ac durch *Ruegeria pomeroyi* auf einen entsprechenden Demethylierungs/Demethiolierungsweg hin. Da frühere Experimente von Reisch *et al*. keinen Umsatz von DMS-Ac durch das Enzym DmdA, welches den ersten Demethylierungsschritt dieses Abbauweges für DMSP katalysiert, zeigten, liefern die hier vorliegenden Ergebnisse erste Hinweise auf einen bislang unbekannten Abbaumechanismus von DMS-Ac sowie möglicherweise DMSP. Weiterhin wurde beobachtet, dass DMS-Ac und Gonyol die verschiedenen DMSP-Lyasen sowie den Abbau von DMSP durch Demethylierung/Demethiolierung beeinflussen. So konnte bei Anwesenheit von Gonyol in Kulturen von *R. pomeroyi* keinerlei Freisetzung DMS oder MeSH aus DMSP nachgewiesen werden. Bei den Spezies *Sulfitobacter* sp. und *A. faecalis* war dieser Effekt geringer ausgeprägt. Jedoch konnte auch hier eine signifikante Reduktion der DMSP-Lyaseaktivität beobachtet werden. Bei *Halomonas* sp., hingegen führte eine Kombination aus DMS-Ac und Gonyol zu einer signifikanten Reduktion an freigesetztem DMS aus DMSP, wobei eine Inkubation mit Gonyol allein keinen solchen Effekt hervorrief. Eine mögliche Ursache dieses von den anderen untersuchten Bakterienarten abweichenden Verhaltens, könnte in der Struktur der DMSP spaltenden Enzyme

zu suchen sein. Im Gegensatz zu *R. pomeroyi*, *A. faecalis* und *Sulfitobacter* sp., welche neben DMS als weiteres Reaktionsprodukt Acrylat liefern, führt die Spaltung von DMSP bei *Halomonas* sp. zu 3-Hydroxypropionat. Betrachtet man die weite Verbreitung von DMS-Ac und Gonyol in marinem Phytoplankton und die Salinitätsabhängigkeit der Osmolytzusammensetzung, ergibt sich ein sehr komplexes Bild an Wechselwirkungen verschiedener zwitterionischer Algenmetabolite mit bislang schwer abzuschätzenden Auswirkungen auf die Freisetzung von klimarelevantem DMS.

Durch Inkubationsversuche mit isotopenmarkiertem $^{13}C_2D_6$-DMSP konnte weiterhin ein direkter Aufnahmemechanismus von DMSP in marinem Phytoplankton nachgewiesen werden. Hierbei zeigte *Thalassiosira weissflogii*, welche kein eigenes DMSP produziert eine extrem schnelle DMSP-Aufnahme von 60% des zugegebenen $^{13}C_2D_6$-DMSP innerhalb von 2,5 Minuten. Die Tatsache, dass alle untersuchten Arten, inklusive *Emiliania huxleyi*, welche als einer der Hauptproduzenten von DMSP in den Ozeanen gilt, extern zugeführtes $^{13}C_2D_6$-DMSP in die Zellen schleusten, zeigt, dass marine Mikroalgen nicht nur als Hauptquelle, sondern auch als beträchtliche Senke von DMSP fungieren können.

In pelagischen Bakterien ist die Fähigkeit DMSP als leicht verfügbare Quelle reduzierten Schwefels sowie als Kohlenstoffquelle zu nutzen sehr weit verbreitet. Dieser Mechanismus wird auch im Zusammenhang mit der Ausbildung und Strukturierung von Biofilmen auf der Oberfläche verschiedener Makroalgen diskutiert. In der vorliegenden Arbeit konnte im Fall der marinen Braunalge *Fucus vesiculosus* gezeigt werden, dass die Beeinflussung der Biofilmausbildung und -strukturierung nicht nur über die Bereitstellung vorteilhafter Wachstumsbedingungen für Bakterien, sondern auch in Verbindung mit der Aminosäure Prolin über eine direkte Abschreckung bestimmter Bakterienarten erfolgen kann.

Durch die Anwendung direkter LC-MS basierter Analysemethoden bietet sich somit nun die Möglichkeit die ökologischen Wechselwirkungen und daraus folgenden klimatischen Einflüsse verschiedener zwitterionischer Phytoplanktonmetabolite zu untersuchen und einen genaueren Blick auf den sehr komplexen marinen Schwefelkreislauf zu werfen.

Summary

As primary producers of organic substances, marine microalgae are essential as basis for the marine food web and play an important role in many nutrient cycles such as the marine sulfur cycle. In this context, dimethylsulfoniopropionate (DMSP), which is synthesized by many marine micro and macroalgae, and its volatile cleavage product dimethylsulfide (DMS), which is involved in cloud formation over the oceans, are of particular importance.

Since DMSP is degraded under alkaline conditions to DMS and acrylate, gas chromatography (GC) based measurement are often used for an indirect determination of DMSP. However, there might be other DMS precursors present in biological samples. Therefore, direct back-calculations of measured DMS to DMSP have to be treated with caution. Thus, indirect GC-based methods provide just a very simplified view on the marine sulfur cycle and the interactions with other zwitterionic substances like glycine betaine (GBT), dimethylsulfonioacetate (DMS-Ac) or gonyol, which are not accessible via gas chromatography.

The aim of the present work was to determine, via novel LC-MS based analysis methods, the contribution of different zwitterionic compounds to important osmoregulation and osmoadaption processes in marine phytoplankton. Furthermore, the influence of these compounds on the release of climate relevant DMS by marine phytoplankton and bacteria was addressed.

A comparison of indirect, GC-MS based, and direct LC-MS based quantification of DMSP in marine phytoplankton cultures revealed partly substantial differences. Depending on the species and the corresponding growth stage, an overestimation of DMSP via indirect quantification of up to 14% could be determined. Due to the fact that these differences could not be ascribed to sample handling and preparation, other base labile DMS-precursors, which cannot be determined separately by indirect methods, must be present in phytoplankton cultures. This systematic error in indirect DMSP quantification is of particular interest when it comes to the investigation of physiological processes where minor differences in DMSP concentrations are discussed. Since non-DMSP DMS-precursors may account for approximately 120 Gmol of biogenic sulfur emissions per year, these substances might have a substantial ecological and geochemical relevance.

Through simultaneous quantification of different zwitterionic compounds in phytoplankton cultures, it could be shown that in addition to DMSP, the metabolites homarine, DMS-Ac and GBT are key players in osmoadaption processes. The adaption to different salinities of the medium is essential for vitality and survival of cosmopolitan living species. Experiments with the dinoflagellate *Prorocentrum minimum* showed that DMSP is used to provide a constant osmolyte level in this species over a wide range of salinities while DMS-Ac and GBT being highly regulated with regard to the osmoadaption in this range. Overview measurements of various phytoplankton

cultures revealed a universal distribution of DMS-Ac in all investigated species with relative abundances of 0.1% to 1990% compared to DMSP. However, gonyol, which was hitherto described only in the dinoflagellate *Gonyaulax polyedra*, was present in all investigated dinoflagellates and haptophytes with relative abundances of 6.2% to 121% compared to DMSP. This shows the wide distribution of other dimethylsulfonio-metabolites in marine phytoplankton that are not detectable via GC based measurements.

Phytoplankton associated bacteria are regarded as the main sink of DMSP and the major DMS source in the oceans. In this context, the potential effects of the microalgal metabolites DMS-Ac and gonyol on the release of the bacterial DMSP cleavage products DMS and methanethiol (MeSH) were addressed. In the present work, it could be shown that all investigated bacteria species were able to utilize DMS-Ac and gonyol in a similar extent as DMSP. However, there was no release of DMS detected during DMS-Ac as well as gonyol metabolism. The release of MeSH during incubation of *Ruegeria pomeroyi* with DMS-Ac and *Alcaligenes faecalis* with gonyol, respectively, suggested the involvement of a demethylation/demethiolation pathway in utilization of these metabolites. In fact, previous experiments of Reisch *et al*. showed no conversion of DMS-Ac by the enzyme DmdA, which catalyzes the first demethylation step of this pathway. Therefore, the present work delivers first evidence of a heretofore unknown DMS-Ac degradation mechanism, which could possibly recognize DMSP as well. Furthermore, it could be shown that the release of DMS and MeSH trough bacterial DMSP metabolism is modulated by the presence of DMS-Ac and gonyol. In fact, the presence of gonyol completely inhibited the release of these volatiles from DMSP in *R. pomeroyi*. Although this effect was less distinct in the species *A. faecalis* and *Sulfitobacter* sp., a significant reduction in DMSP-lyase activity was observed. However, while the combination of DMS-Ac and gonyol resulted in a significant reduction in DMS release from DMSP in *Halomonas* sp., the presence of gonyol alone had no such effect. A possible reason for the different behavior of *Halomonas* sp. lies in the structure of the involved DMSP-lyase. While the DMSP-lyases from *R. pomeroyi*, *A. faecalis* and *Sulfitobacter* sp. yield to DMS and acrylate, the by-product of the dddD DMSP-lyase from *Halomonas* sp. is 3-hydroxypropionate. Regarding the wide distribution of DMS-Ac and gonyol in marine phytoplankton species and their variable osmolyte composition in respect to different salinities, the relevance of different zwitterionic osmolytes to the marine sulfur cycle and their influence on the release of climate relevant DMS remain elusive.

Through incubation experiments with isotope labeled $^{13}C_2D_6$-DMSP, a direct DMSP uptake mechanism in marine phytoplankton could be proven. Here, *Thalassiosira weissflogii*, which does not produce DMSP, showed a very fast uptake of $^{13}C_2D_6$-DMSP within 2.5 minutes. The fact that all

investigated species, even the DMSP high-producer *Emiliania huxleyi*, assimilated the supplied $^{13}C_2D_6$-DMSP shows that marine phytoplankton is not only the main source of DMSP but also a substantial sink of this metabolite in the oceans.

Marine bacteria are known to use DMSP as readily available source for reduced sulfur species and as high energy nutrient. In this context, DMSP is widely discussed in formation and structuring of bacterial biofilms on marine macroalgae. Here, it was shown that the marine brown algae *Fucus vesiculosus* used a combination of DMSP and the amino acid proline to inhibit the bacterial attachment on the algal surface. Thus, DMSP can be not just regarded as a nutrient for marine bacteria bus also as effective deterrent to achieve a certain bacteria composition in the biofilm on marine macroalgae.

The application of novel direct LC-MS based methods for determination of different zwitterionic metabolites from marine phytoplankton provides a more detailed view on the marine sulfur cycle. This includes information about the various interactions mediated by these compounds as well as new information about their influence on the release of climate release DMS.

Introduction

Claw Hypothesis

In 1972, James E. Lovelock published a letter in *Atmospheric Environment* where he described the so called Gaia theory, named after the Greek personification of mother earth **[Lovelock 1972]**. The way living species are able to create and maintain favorable climatic condition for live led him to the attitude to regard the earth system and all living organisms as one single self-regulating entity. He corroborated his concept by the fact that the actual atmospheric composition with elemental nitrogen, a high oxygen concentration with traces of free methane and ca. 327 ppm of carbon dioxide (data from 1971, Manua-Loa Observatory, Hawaii **[Keeling 1976]**), is far away from the conceivable chemical equilibrium for a planet in this region of the solar system. The present state of a stable disequilibrium is more a consequence of constant activity by living organisms.
Fifteen years later, Charlson *et al*. released a review article **[Charlson 1987]**, which influenced many scientists in the following years working on the environmental impact of phytoplankton. Their CLAW hypothesis, named after the authors Charlson, Lovelock, Andreae and Warren, described the influence of marine phytoplankton on oceanic cloud formation via release of dimethylsulfide (DMS). This hypothesis describes that an increase in DMS synthesis by marine phytoplankton as result of a higher solar radiation and earth's surface temperature can lead, through DMS oxidation processes in the atmosphere, to an enhanced cloud formation and, in consequence, to a resulting lower solar radiation. This negative climate feedback mechanism has a potential stabilizing effect on the climate and could prevent greater atmospheric changes. Thus the works of Lovelock, Charlson, Andreae and Warren laid the foundation to a broad research field on the impact and importance of phytoplankton to the global climate.

Phytoplankton

The oceans, which cover about 70% of the global surface and contain about 50 times more soluble inorganic carbon than the atmosphere, are a huge sink for atmospheric carbon dioxide. About 30% of the anthropogenic CO_2 produced since the industrial revolution was sequestered by the oceans **[Raven and Falkowski 1999]**. These dissolved inorganic carbon species (carbon dioxide, bicarbonate and carbonate) are the basis for the enormous primary production by marine phytoplankton that accounts for about 50 Pg ($50 \cdot 10^{15}$ g) fixed carbon per year, which is roughly equal to the contribution of land plants **[Field 1998]**. It is impressive that this huge amount of primary production is achieved by organisms that represent only 0.2% of the primary producer biomass. As primary producers, the whole marine food web is dependent on these unicellular

organisms **[Link 2002]**, which are also the determining factor for production of fisheries industries **[Chassot 2010]**.

The term plankton is deduced from the Greek adjective *planktos* meaning wanderer or drifter, which describes the way these organisms move passively with currents in oceans and fresh water **[Hays 2005]**. However, plankton cells are able to change their depth, in a more or less limited extend, through active swimming or changing their buoyancy. The group of phytoplankton, which consists of at least eight different phyla of single celled photoautotrophic organisms, is numerically dominated by cyanobacteria **[Falkowski 2004]**. These organisms, formerly also called blue-green algae, are the only existing prokaryotic organisms that are able to release molecular oxygen through photosynthesis. The three principal eukaryotic phytoplankton clades, which dominate the today's oceans, are represented by diatoms, dinoflagellates and coccolithophores **[Falkowski 2004]**. In the following, the major properties and features of these organisms are described. The most prominent feature of diatoms is the ability to form very diverse silica containing cell walls with outstanding physical strength as mechanical protection against predators **[Hamm 2003]**. The enormous variety of sizes and forms of diatoms and also dinoflagellates often served as inspiration of scientists and artists as well (Figure 1). In contrast to dinoflagellates and coccolithophores, diatoms harbor a large cell vacuole for nutrient storage which occupies ca. 40% of the whole cell volume **[Falkowski 2004]**. Therefore diatoms are well adapted to habitats with frequent short pulse nutrient availability such as coastal upwelling zones or highly turbulent regions of the open ocean **[Smetacek 1999]**. Furthermore, benthic diatoms are also major participants in biofilm formation in coastal regions **[Middelburg 2000]**. Through extrusion of extracellular polymeric substances, diatoms provide important stabilization to the sediments surface against resuspension **[Holland 1974, de Brouwer 2005]**. In contrast to the silicate containing cell walls of diatoms, the cell walls of coccolithophores consist of small calcium carbonate plates with uncertain functions, which are assembled to a spherical shape **[Riebesell 2000]**. Therefore, by mineralization processes, these species are highly important as sink for dissolved CO_2 and carbonate in the oceans. The high reflectivity of the coccoliths is exploited in satellite observations and investigations of coccolithophore blooms in the oceans **[Reid 1990, Balch 1991]**. As well as diatoms, dinoflagellates show a huge diversity of different forms and sizes. Typical dinoflagellate cells possess two flagella that allow locomotion of the cells over shorter distances. Although less than half of the dinoflagellate species are photosynthetic active, they are key players in the marine environment synthesizing a variety of highly interesting metabolites which include compounds with partially very specific neurotoxic properties **[Taylor 1980, Shimizu 1993, Shimizu 1996, F. Delwiche 2007]**.

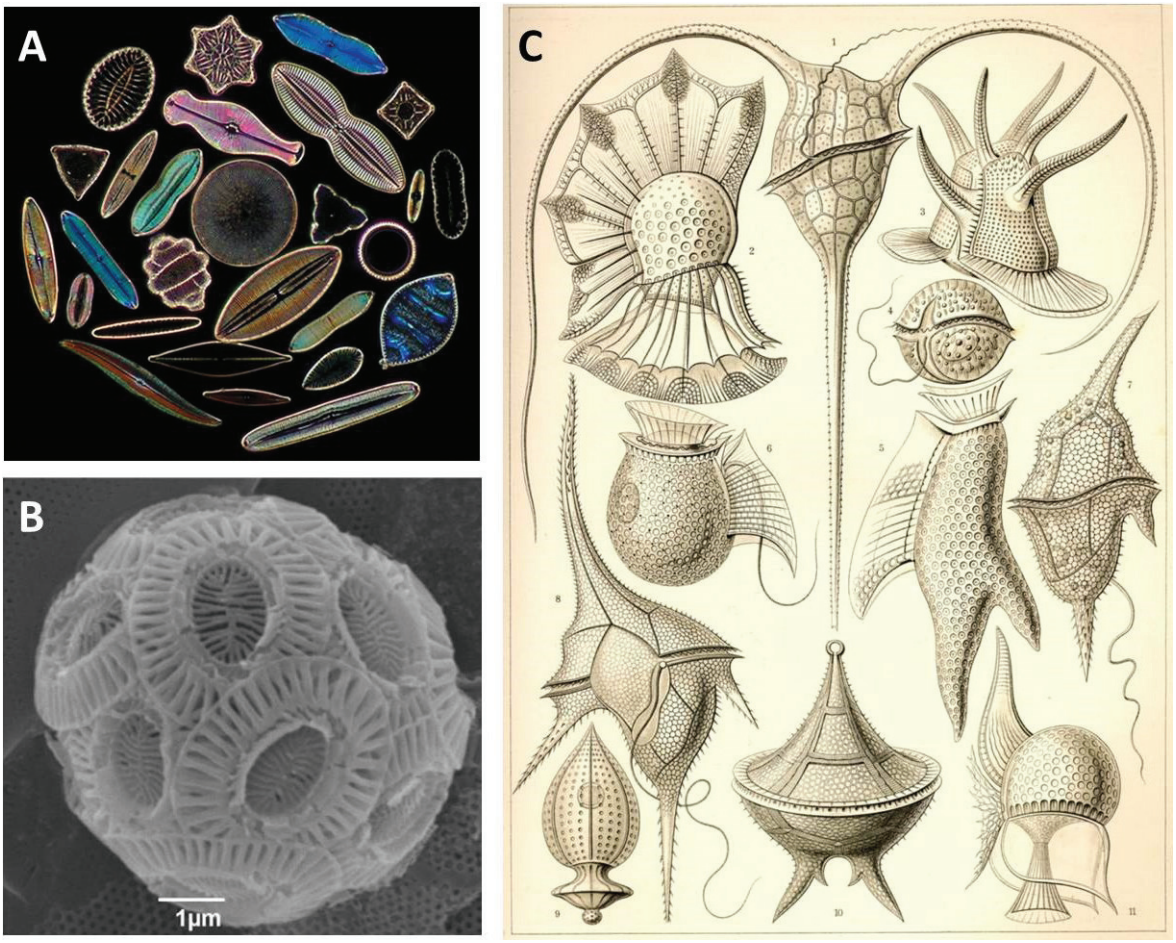

Figure 1: Images of different phytoplankton groups. A: Darkfield microphotographic picture of different arranged diatom cells by José R. Almodóvar, University of Puerto Rico, 100-fold magnification, taken from Nikonsmallworld 2008 Photomicrography Competition, Image of Distinction (http://www.nikonsmallworld.com). B: Scanning electron microscopic picture of a single cell of the coccolithophore *Emiliania huxleyi*, taken from [Triantaphyllou 2010]. C: Drawing of different dinoflagellate species by Ernst Haeckel, taken from [Haeckel 1904].

Blooms formed by marine phytoplankton can reach extents of hundreds of square kilometers. They are characterized by a very high growth rate of one certain species that ultimately dominates algal composition over a relatively short period in a specific area. When cell density reaches a maximum, depletion of nutrients causes cell death and a rapid collapse of the bloom occurs. High quality nutrients, which become available after bloom break-down, can be recycled by other plankton species or be transported to deeper water layers contributing crucial to several nutrient cycles such as phosphorus **[Paytan and McLaughlin 2007]**, nitrogen **[Arrigo 2005]** and sulfur **[Sievert 2007]**. In many coastal regions, there is a characteristic seasonal phytoplankton bloom cycle with a region typical succession of different algal blooms. Phytoplankton blooms are on one hand favorable to the marine food web and nutrient cycles, but on the other hand, they can be harmful to fisheries and human health. It is known that several microalgae species produce

toxic metabolites which can cause massive fish kills with negative effects on the fisheries industry **[Burkholder 1992, Bourdelais 2002]**. For example, the total economic loss on Korean aquaculture industry over the past three decades caused by harmful algal blooms (HABs) was estimated to 120 million USD **[Park 2013]**. The ecological impact of HABs can comprise a significant reduction in fish population due to release of ichthyotoxic compounds and largely increased coral mortality, which is mainly deduced to lower oxygen concentration and reduction in photosynthetic active radiation (PAR) during the blooms **[Gjosaeter 2000, Bauman 2010]**. While the fish population seems to recover very fast after such events **[Gjosaeter 2000]**, the effects on the benthic ecosystem are, due to the low growth rate of corals, rather prolonged. Furthermore, most poisoning syndromes of humans after shellfish consumption are caused by accumulation of toxic metabolites from phytoplankton in shellfish and fish **[Lefebvre and Robertson 2010, Mazzillo 2010]**. Formation and the potential toxicity of microalgal blooms are driven by several environmental factors like light intensity, nutrient availability and seawater salinity **[MacIntyre 1997, Hwang and Lu 2000, Errera and Campbell 2011, Graneli 2012]**. However, the effects of nutrient availability on toxin production of marine dinoflagellates are very diverse and vary according to the species and produced toxins **[Pistocchi 2011]**.

Osmoregulation and Osmoadaption

Due to the very limited ability of marine microalgae to move along longer distances, phytoplankton species need very efficient mechanisms to overcome environmental stress. In addition, cosmopolitan living species need to be very successful to adapt to different environmental conditions. A global view on the surface salinity of the oceans shows that the salt concentrations can vary from 6-8‰ in the Baltic Sea to 40‰ in the Red Sea. In general, regions with high freshwater influx, like estuarine areas of large rivers, lower temperatures and, therefore, lower evaporation rates show lower salinities than subtropical regions with high evaporation (Figure 2). Despite these relatively constant regional variations, the seawater salinity may also change over short timescales through glacier melt, seasonal changes in freshwater influx from rivers or heavy rainfalls **[McConnell 2009]**.

It is known that phytoplankton cells accumulate different highly polar substances to maintain their osmotic pressure in equilibrium with the surrounding seawater **[Kinne 1993]**. These compounds are often referred to as osmolytes or compatible solutes and comprise amino acids **[Fujii 1995, Liu 2000]**, complex alcohols **[Chen and Jiang 2009, Garza-Sanchez 2009]** and zwitterionic substances **[Dickson and Kirst 1986, Dickson and Kirst 1987b, Dickson and Kirst 1987a]**. In addition to osmoadaption, these compatible solutes can protect peptides from

denaturation and maintain enzyme activity under extreme conditions like very high and low temperatures, respectively, or high salt concentrations. It is assumed that the cryoprotective properties of compatible solutes rely on, to a certain extent, negative interaction of the solutes with the protein surface which is denoted as preferential exclusion. This means that the solute concentration in close vicinity of the protein is lower than in the surrounding solution, which prevents the protein hydrate shell from perturbation and, therefore, the protein itself from misfolding **[Arakawa and Timasheff 1985, Carpenter and Crowe 1988, Nishiguchi and Somero 1992]**.

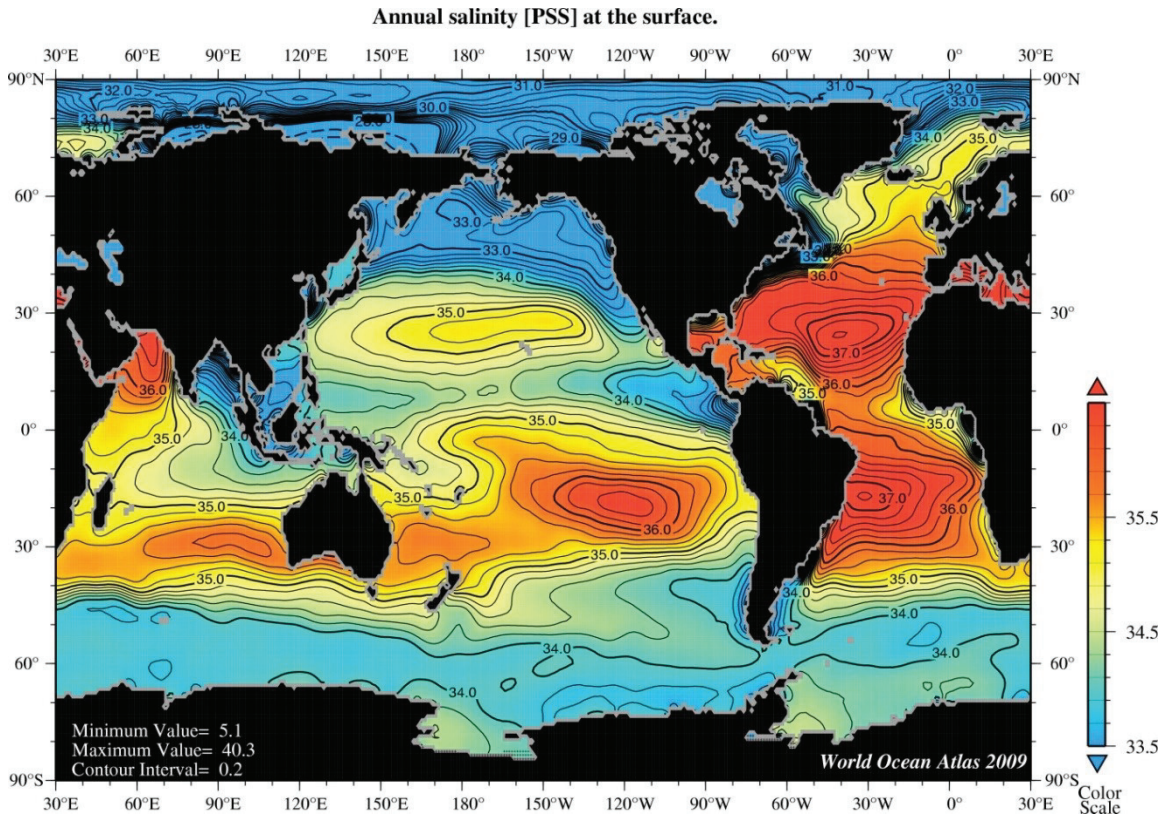

Figure 2: Global annual surface sea water salinity. Figure taken from world ocean atlas 2009 (https://www.nodc.noaa.gov/cgi-bin/OC5/WOA09F/woa09f.pl).

The structures of zwitterionic compounds, synthesized by marine phytoplankton, are very diverse and comprise aliphatic sulfonio- and ammonium salts as well as aromatic metabolites (Figure 3). Due to their high polarity as permanently charged organic compounds, they may all serve as possible osmolytes in marine microorganisms. The most discussed substances in this context are glycine betaine (GBT) and dimethylsulfoniopropionate (DMSP). These metabolites are nearly ubiquitous in the euphotic zone of the oceans and are well known in phytoplankton **[Dickson and Kirst 1986, Keller 1999]**, macroalgae **[Karsten 1991a, Karsten 1991b, van Alstyne and Puglisi**

2007] and higher plants **[Otte 2004, Ashraf and Foolad 2007]**. Besides their function to overcome osmotic stress in phytoplankton, organic osmolytes are involved in the regulation of cell buoyancy and, therefore, the ability of phytoplankton to migrate vertically through the water column **[Boyd and Gradmann 2002]**. Furthermore, these osmolytes are also present in marine animal species. GBT has been reported from all major invertebrate classes where it acts as a compatible solute to increase temperature- and salt stress resistance and may assist in transmethylation of phosphatidyl-ethanolamine **[Anthoni 1991]**. DMSP which was found in marine invertebrates is usually accounted to derive either from their diet or endosymbiotic algae **[Hill 1995, Hill 2000, Broadbent 2002, Van Alstyne 2006]**. Nevertheless, the functions of DMSP in marine invertebrates are not entirely understood. It is assumed to increase their resilience against osmotic, oxidative and thermal stress.

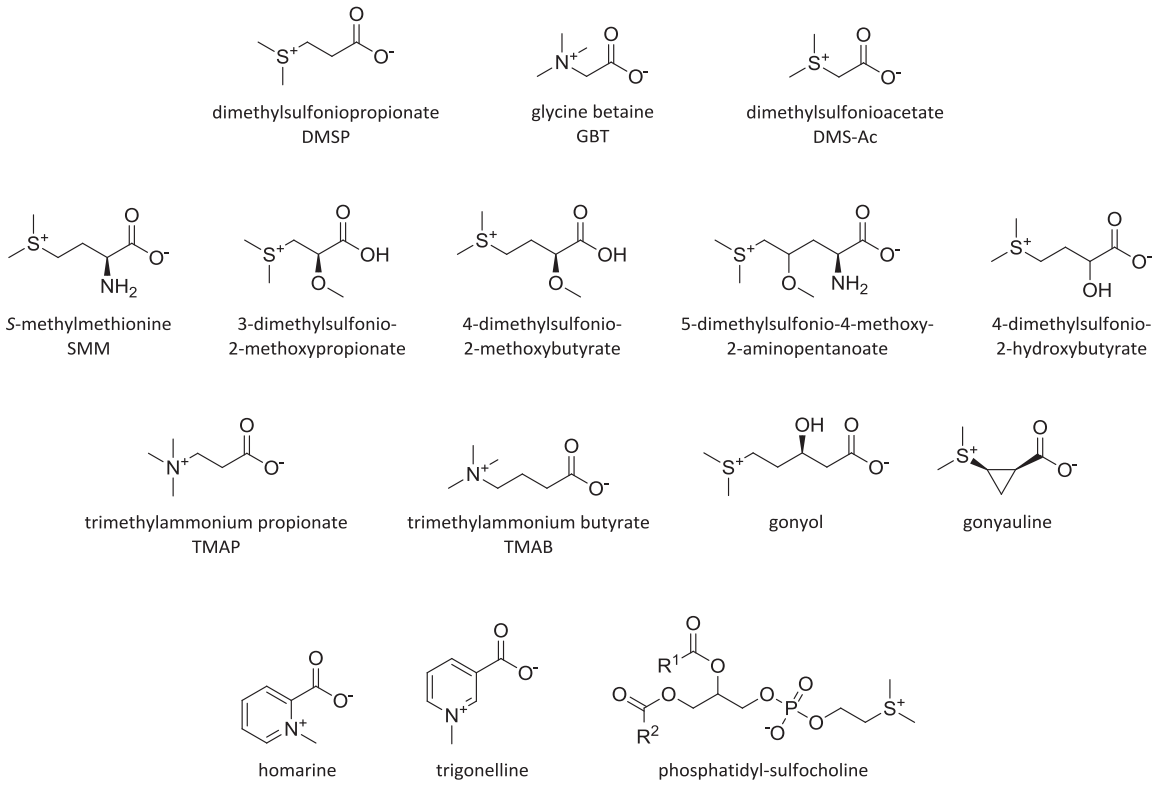

Figure 3: Diverse chemical structures of zwitterionic metabolites from marine phytoplankton. Structures comprise aliphatic dimethylsulfonio- and trimethylammonium-compounds as well as aromatic and cyclic metabolites. Dimethylsulfonio-moieties in polar phospholipids (phosphatidyl-sulfocholine) are known from the diatom *Nitzschia alba.*

It was also shown that major changes in microalgal metabolism can occur due to changes in seawater salinity. Röder *et al.* and Engström-Öst *et al.* have shown that the production of the toxic metabolites microcystin and yessotoxin from cyanobacteria and dinoflagellates, respectively,

are dependent on the salinity of the surrounding water **[Engström-Öst 2011, Röder 2012]**. Although these metabolites have no obvious osmoregulative function, they are highly affected by salinity changes. However, the mechanisms through which salinity triggers the biosynthesis of toxic metabolites remain unknown.

Dimethylsulfoniopropionate (DMSP)

As a key substance in the marine environment, the osmolyte DMSP is one of the most discussed zwitterionic metabolites. It was first isolated in 1947 by Challenger and Simpson from the red algae *Polysiphonia fastigiata* and identified as a precursor of highly volatile, smelly dimethylsulfide (DMS) **[Challenger and Simpson 1947]**. The biosynthesis of DMSP by algae and higher plants follows different mechanisms which all use the amino acid methionine as substrate. In micro- and macroalgae, DMSP synthesis is achieved through direct transamination, reduction, methylation and subsequent oxidative decarboxylation of the starting compound **[Gage and Rhodes 1997, Summers 1998, Stefels 2000]**. In contrast to algal DMSP biosynthesis, two different pathways were identified in higher plants, which start both with a methylation of methionine to *S*-methylmethionine (SMM) which can also act as methyl donor in the *S*-methylmethionine cycle for methionine synthesis **[Ranocha 2001]**. While the further reaction to dimethylsulfonio propionic aldehyde (DMSP-ald) in compositae follows a transamination/decarboxylation process, a pathway with DMSP-amine as intermediate in DMSP biosynthesis is proposed for gramineae (Figure 4) **[Stefels 2000]**.

DMSP is known to serve various functions in marine micro- and macroalgae. These comprise osmoprotection **[Van Bergeijk 2003, Lyon 2011]**, cryoprotection **[Karsten 1991a]** and antioxidative properties **[Sunda 2002, Husband 2012]**. It is also hypothesized that DMSP production in phytoplankton may be the result of an overflow mechanism of reduced substances and excessive energy **[Stefels 2000]**. In addition to the relevance of DMSP to marine algae as its producers, in was shown to mediate many interactions in the marine environment. For example, DeBose *et al*. determined that planktivorous reef fishes can use DMSP-cues as an infochemical to locate their prey **[DeBose 2008]**. Furthermore, it was shown that DMSP is involved as a precursor in an activated defense system of different marine macroalgae. Upon wounding the algae, through damage by grazers, DMSP is converted enzymatically to DMS and acrylate which both can act as feeding deterrents to sea urchins **[Van Alstyne 2001, Van Alstyne and Houser 2003]**. However, the actual composition of different active compounds can increase deterring effects of such activated defense systems. In this context, Wiesemeier *et al*. showed, that a mixture of DMS and trimethylamine (TMA), which is released by the brown alga *Dictyota dichotoma* upon

wounding, possesses significantly higher deterring activity against grazers than DMS or TMA alone **[Wiesemeier 2007]**.

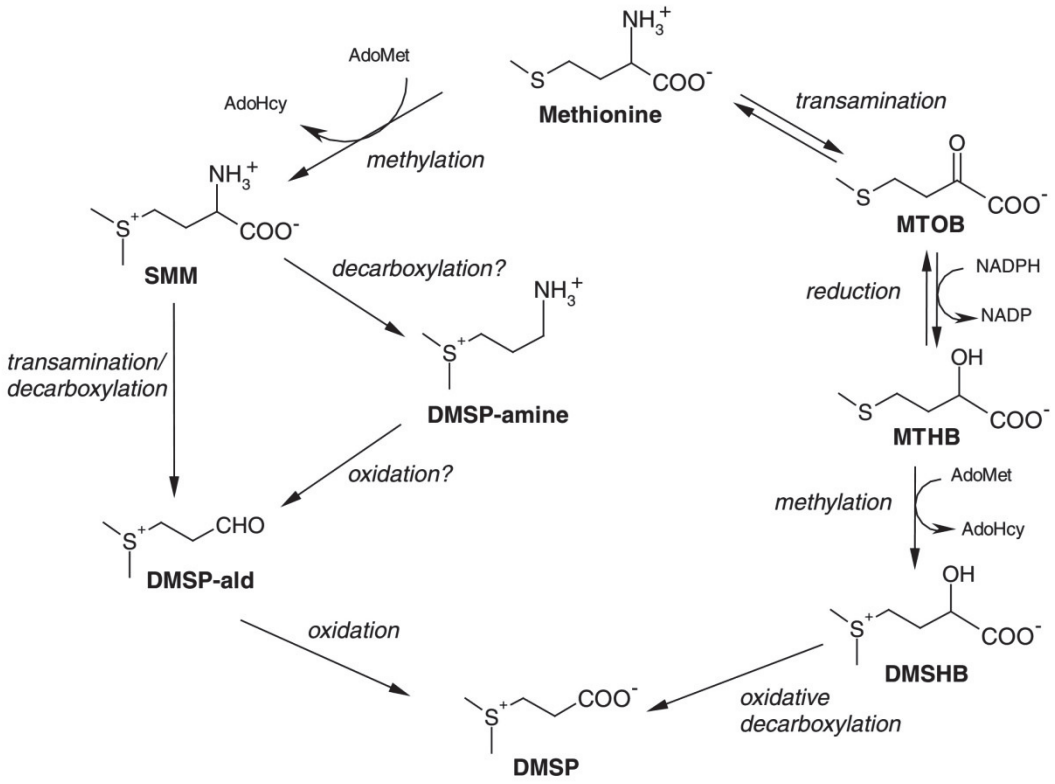

Figure 4: Schematic representation of DMSP biosynthesis pathways with methionine as starting compound, left: compositae; center: gramineae; right: marine algae. Figure taken from: [Stefels 2000].

Current research could show that, besides coral-endosymbiotic algae, also coral juveniles are able to synthesize DMSP which is the first description of DMSP biosynthesis by an animal **[Raina 2013]**. Corresponding genomic analysis revealed that over 65% of the coral-associated bacterial genera are capable to metabolize DMSP or DMS. Through attraction and provision of a favorable environment for DMSP and DMS degrading bacteria, DMSP is a crucial compound in structuring the coral-associated bacterial community and therefore the vitality of the coral itself **[Raina 2010, Raina 2013]**. Although the physiological functions of DMSP in animals remain elusive, the metabolite is assumed to be part of an activated defense mechanism in animals and, together with its cleavage products DMS and acrylate, a scavenger for reactive oxygen species **[Van Alstyne 2006, Raina 2013]**.

In fact, DMSP is a key substance in the marine ecosystem and food web. Besides its multiple physiological functions in marine microalgae, it is also highly important to other organisms like heterotrophic bacteria which are not able to synthesize DMSP themselves. When microalgal cells get injured or disrupted due to senescence, stress, viral lysis, microbial attack or zooplankton

grazing, intracellular DMSP leaks into the surrounding sea water **[Wolfe 1994, Bratbak 1995]**. Marine bacteria can take up the dissolved DMSP (DMSPd) as an energy rich nutrient and easily available source for reduced sulfur **[Vila-Costa 2006, Howard 2008, Dickschat 2010, Motard-Côté 2011]**. It is estimated that marine bacteria can satisfy approximately 95% of their needs for reduced sulfur and up to 15% of their carbon demands solely by assimilation and metabolization of DMSP **[Zubkov 2001]**. It was shown that the glycine betaine (GBT) uptake system, which is very common in marine bacterioplankton, also recognizes the structurally similar DMSP **[Kiene 1998]**. Such uptake systems are also present in *Escherichia coli*, which is able to take up DMSP and GBT via ProU and ProP transport systems **[Cosquer 1999]**. Through this direct uptake systems, DMSP can act as a compatible solute to overcome possible osmotic stress of the cells under unfavorable osmotic conditions **[Kiene 1998, Cosquer 1999]**.

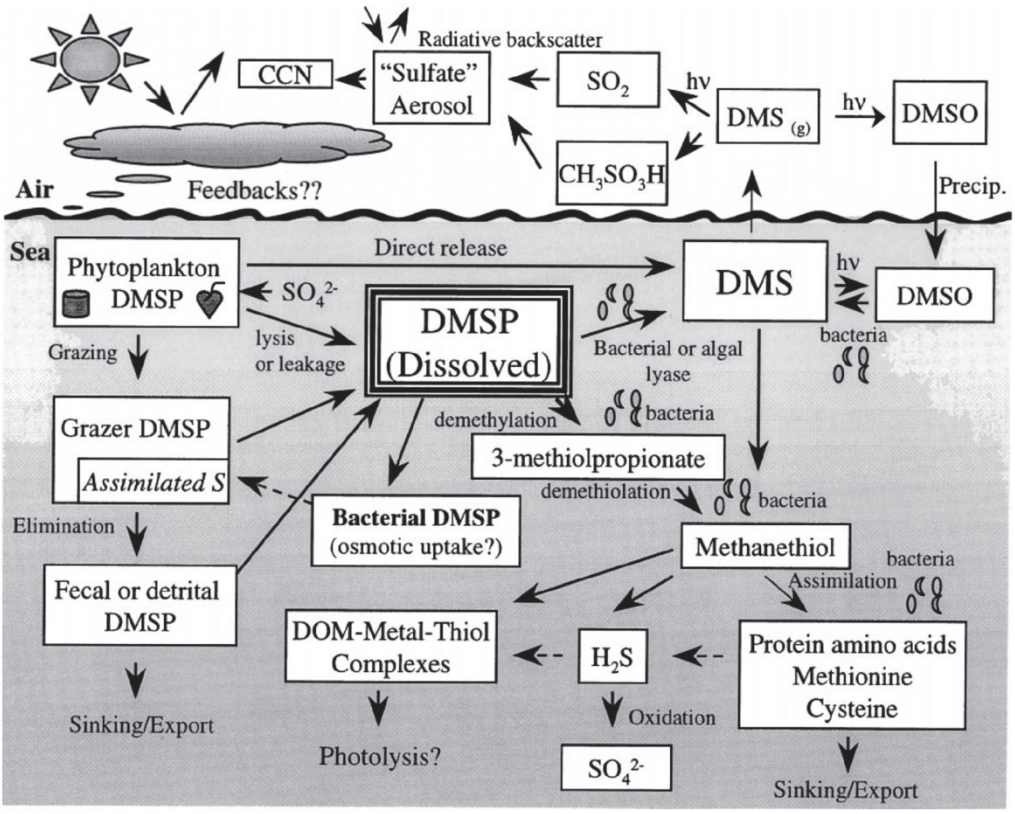

Figure 5: Schematic illustration of the current view of biogeochemical cycles of DMSP and DMS in the oceans and in the atmosphere. The possible feedback mechanisms of DMS derived cloud condensation nuclei (CCN) on DMSP biosynthesis in marine phytoplankton remain elusive. Figure taken from: [Kiene 2000].

The wide distribution of DMSP in the marine ecosystem, its presence in various different algae-, plant- and animal species and its highly diverse functions illustrate that DMSP plays a key role in the marine environment. Besides the highly polar DMSP, its volatile cleavage product

dimethylsulfide (DMS) is not lesser important to the marine ecosystem and the marine sulfur cycle (Figure 5).

Dimethylsulfide (DMS) and the marine sulfur cycle

The chemical half-life of DMSP in sterile seawater at 10°C was calculated as approximately 8 years **[Dacey and Blough 1987]**. In fact, the half-life of dissolved DMSP in the oceans is much lower. Due to microorganisms, which are present in high quantities in ocean water, dissolved DMSP is taken up and/or degraded very fast. The major group of DMSP-degrading organisms is, due to their quantity, marine bacteria. The two main degradation mechanisms of DMSP by bacterioplankton are the demethylation/demethiolation mechanism which yields methanethiol (MeSH) and the cleavage of dimethylsulfide (DMS) by DMSP-lyases **[Kiene and Linn 2000]**. Due to rapid incorporation of MeSH into macromolecules, the demethylation/demethiolation pathway is very important in respect to sulfur assimilation by marine bacteria **[Kiene 1999]** (confer Figure 5). The cleavage of highly volatile DMS from DMSP, however, is a rather less important pathway for sulfur assimilation by bacterioplankton **[Zubkov 2002]**. Which degradation pathway of DMSP is preferred by bacteria, is a very complex question and depends on the concentration of dissolved DMSP as well as temperature and the species composition of the bacterial community **[Taylor and Gilchrist 1991, Kiene and Linn 2000]**. Kiene *et al.* assume that around 75% of the DMSP, dissolved in seawater, undergo demethylation/demethiolation, 15% are recycled via direct uptake by bacteria and only 10% are degraded via DMS cleavage **[Kiene and Linn 2000]**. However, during a phytoplankton bloom collapse, where huge amounts of DMSP are released to the seawater, the proportions between DMS production and sulfur assimilation to macromolecules can alter significantly. As Kiene *et al*. showed, an increase of DMSP concentration from 0.5 nM to 20.5 nM resulted in a rise in DMSP to DMS conversion from 3% to 22% **[Kiene and Linn 2000]**. A large fraction of the DMS, released to the seawater, is oxidized photochemically or by bacterial oxygenase activity to dimethylsulfoxide (DMSO) or subsequently to sulfate **[Shooter and Brimblecombe 1989, Horinouchi 1999, del Valle 2009]**. The remaining dissolved DMS can be assimilated by bacteria **[Visscher 1995, Kiene and Linn 2000]** or, due to its high vapor pressure and hydrophobicity, exhaled from the seawater to the atmosphere (confer Figure 5).

In 1935, DMS was identified as the major component of the typical smell of decomposing macroalgae **[Haas 1935]**. As highly volatile substance with a characteristic smell it can serve as an infochemical for seabirds to find large groups of planktivorous fishes **[Nevitt and Bonadonna 2005]**. The enormous environmental importance of DMS can be observed on a rather global scale. DMS can be easily oxidized by air and sunlight, for example via reaction with hydroxyl radicals, to

DMSO, methanesulfinic acid, methanesulfonic acid, and sulfuric acid, which are usually subsumed as sulfurous aerosol **[Ayers and Gillett 2000, Sunda 2002]**. This sulfurous aerosol is known to act as highly efficient cloud condensation nuclei (CCN) and depicts the major source of clouds over the oceans, where concentrations of other CCN like dust, sooth or sulfur dioxide from anthropogenic activity or volcanic emissions are very low **[Fitzgerald 1991]**. An increased concentration of CCN in the atmosphere can lead to more clouds as well as brighter clouds with a higher reflectivity (albedo) to solar radiation **[Randall 1984]**. Changes in the cloud albedo strongly affect the amount of sunlight which is backscattered and reflected to space and, therefore, influences the earths' radiation budget and global surface temperatures. This climate feedback mechanism of DMS-derived CCN, which was the main proposition of the CLAW-hypothesis, is still discussed **[Charlson 1987]** and the quantity of this effect remains elusive, particularly with respect to global climate predictions **[Ayers and Cainey 2007]**.

By transportation with clouds, sulfur from the CCN can be deposited over land through rainfall. Via this sea to land transport a fraction of the sulfur washed as sulfate from landmass to the oceans is recycled .Current estimates of the DMS flux from the oceans to the atmosphere, based upon >47,000 measurements of DMS concentrations in surface seawater, is $28.1 \cdot 10^9$ kg S per year **[Carpenter 2012]**, which is consistent with previous calculations of 15-38.5 kg S per year. However, as shown by Kettle and Andreae, calculated DMS release to the atmosphere is highly dependent on the used flux model and the parameterization **[Kettle and Andreae 2000]**. Thus, DMS accounts for about 50% of the global biogenic sulfur emissions and contributes crucially to the global sulfur cycle **[Andreae 1990, Sievert 2007]**.

Non-DMSP dimethylsulfonio-metabolites

Through enzymatic activity or chemical degradation processes, other metabolites, which contain a dimethylsulfonio moiety, are potential additional sources of the climatically active DMS. Due to the osmoprotective functions of DMSP, *S*-methylmethionine (SMM), as an intermediate in DMSP biosynthesis, is strongly related to the salt stress response of higher plants. It was shown that *S*-methylmethionine accumulates in chloroplasts of *Wollastonia biflora* after salinization with artificial seawater **[Trossat 1998]**. The metabolite 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB) was identified as a precursor in DMSP biosynthesis in the green macroalgae *Enteromorpha intestinalis*and in the marine phytoplankton species *Emiliania huxleyi*, *Tetraselmis* sp. and *Melosira nummuloides*. A direct catabolism of DMSHB with release of DMS in *E. huxleyi* and *Tetraselmis* sp. is discussed **[Gage and Rhodes 1997]**. Several other dimethylsulfonio-metabolites with unknown physiological functions were isolated from different

red algae species. 4-dimethylsulfonio-2-methoxybutyrate, 3-dimethylsulfonio-2-methoxypropionate and 5-dimethylsulfonio-4-methoxy-2-aminopentanoate (for chemical structures confer Figure 3, page 19) were first investigated in *Rytiphloea tinctoria*, *Digenea simplex* and *Laphocladia lallemandi*, respectively **[Sciuto 1982, Sciuto 1988, Patti 1993]**. It was shown that 4-dimethylsulfonio-2-methoxybutyrate and 5-dimethylsulfonio-4-methoxy-2-aminopentanoate, respectively, release DMS through treatment with sodium hydroxide under elevated temperature. This suggests a possible contribution of these compounds to the oceanic DMS release **[Sciuto 1982, Sciuto 1988]**. The sulfonio-analogue of GBT, dimethylsulfonioacetate (DMS-Ac), which can be found among others in the macroalga *Ulva lactuca*, is easily assimilated by marine bacteria, where it can act as osmoprotectant **[Kiene 1998, Pichereau 1998, Cosquer 1999]**. The dimethylsulfonio-metabolites gonyol and gonyauline were first isolated from extracts of the dinoflagellate *Gonyaulax polyedra* **[Nakamura 1993, Nakamura 1997]**. While the latter influences the circadian rhythm of *G. polyedra*, there are no further studies on the physiological functions of gonyol **[Roenneberg 1991, Nakamura 1992]**. Besides the dimethylsulfonio-compounds mentioned above, which are all present in the cytoplasm, dimethyl sulfocholine can be found, as a polar phospholipid derivative, in the cell membrane of the non-photosynthetic diatom *Nitzschia alba* where it contributes to membrane polarity (confer Figure 3, page19) **[Anderson 1978]**.

Analysis of DMSP and other zwitterionic substances

First DMSP quantifications were based on the precipitation with ammonium reineckate from aqueous solutions. The formation of complexes with reineckate salt was formerly used for analysis of cations from heavy metals like mercury or copper in aqueous solution but was also applied for precipitation and further analysis of amino and ammonium compounds **[Tompsett 1964]**. Cantoni *et al.* used ammonium reineckate for isolation and subsequent spectrophotometric quantification of the corresponding DMSP complexes. Hereby they were able to determine the activity of DMSP-cleaving enzymes **[Cantoni and Anderson 1956]**. However, due to the possible interference of other zwitterionic compounds like the highly abundant GBT, this method was not suitable for the analysis of algal extracts containing a variety of different osmolytes. The by far mostly used method for DMSP quantification is based on an alkaline treatment of DMSP containing samples, which results in the cleavage of DMSP to DMS and acrylate. Subsequently, the released DMS can be analyzed via headspace gas chromatography (HS-GC) combined with different detector types. The measurement via flame photometric detector (FPD), which is very sensitive for the analysis of sulfur containing compounds, has the

advantage of a relatively simple instrumentation, high robustness and low costs. The combination of GC with mass spectrometry (MS), however, provides very low detection limits and the possibility to use isotope labeled internal standards for highly accurate and robust quantification. Depending on the type of sample application and used detector, the limits of detection are between 50 nM (headspace sampling with FPD) **[Vogt 1998, van Rijssel and Gieskes 2002]**, and 5 pM (solid phase microextraction with mass spectrometry) **[Yassaa 2006, Zhou 2009]**. The main drawback of an indirect quantification of DMSP via release of DMS is fact that other substances, which release DMS under alkaline conditions, result in erroneously elevated DMSP readings. Only few authors take account of this analytical issue and denote their findings as alkali-labile DMS-precursors **[Vogt 1998]**. At least this expression is more accurate than to equate measured DMS release with DMSP concentration in biological samples.

Due to the wide spectrum of different zwitterionic osmolytes with possibly similar functions and regulation mechanisms the simultaneous analysis of a large spectrum of different zwitterionic metabolites is an important task. First attempts for the simultaneous quantification of betaines and dimethylsulfonio-compounds were based on ion chromatography **[Gorham 1984]**. As the author stated, the separation and analysis of GBT, DMSP, DMS-Ac, trigonelline and homarine allow screenings of large numbers of plant samples and could provide useful information about of the relationship between betaine contents and drought stress of plants. However, the use of a UV detector made this approach more useful for the quantification of aromatic compounds like trigonelline and homarine which showed a 40 to 80-fold higher response than aliphatic analytes **[Gorham 1984]**. Due to the extensive sample preparation with drying of the organic extracts, dissolution of the residue in water and treatment of this solution with ion exchange material prior to the measurement, this method became no standard procedure for DMSP analysis of biological samples **[Gorham 1984, Colmer 2000]**. Another approach by Zhang *et al.* was the determination of DMSP via capillary electrophoresis, which was also applied on the quantification of several betaines like GBT and trigonelline in olive oils **[Zhang 2005, Sanchez-Hernandez 2011]**. By analysis of the corresponding *p*-bromophenacylester via UV-detector, the signal intensity of DMSP could be increased by factor 50 compared to direct measurement, with a detection limit of 5 µM **[Zhang 2005]**. Further improvements of this method were accomplished by Sánchez-Hernández *et al*. who combined the good separation of capillary electrophoresis with highly sensitive tandem mass spectrometry **[Sanchez-Hernandez 2011]**. Separation and simultaneous analysis of zwitterionic metabolites, including DMSP, GBT, proline betaine and trigonelline from coral tissues, was accomplished by Li *et al.* with a LC-MS system using a pentafluorophenyl column **[Li 2010]**. The very low detection limit of DMSP (8 nM) and the usage of standard HPLC systems, which are

available in many laboratories, underline the high potential of this method for analysis of zwitterionic metabolites. However, the main disadvantage of this method is the used ternary gradient which makes possible method development and optimization very complex.

A procedure which is based on liquid chromatography with a standard C-18 reversed phase column and a water-MeOH/THF binary gradient was introduced in 2007 by Wiesemeier and Pohnert **[Wiesemeier and Pohnert 2007]**. Retention of the highly polar DMSP on the column was increased through derivatization with pyrenyldiazomethane (PDAM), which allowed quantification both via UV-Vis detection and mass spectrometry. The authors suggested that the relatively high detection limit of the DMSP-PDAM ester of 590 nM with MS-detection could be reduced at least by factor 100 by application of a fluorescence detector. Due to the possibility to use MS as well as UV detection, this method provides linearity in a wide range of 2.93 µM to 11.72 mM DMSP **[Wiesemeier and Pohnert 2007]**.

Because of the high polarity of DMSP and other betaines, it immediately suggests itself to use LC-systems with a highly polar stationary phase for separation and quantification of these metabolites. The combination of ultra performance liquid chromatography (UPLC) with a polar BEH-HILIC (ethylene bridged hybrid – hydrophilic interaction liquid chromatography, Waters) column and mass spectrometric detection allowed DMSP quantification in microalgae extracts without time consuming sample preparation and derivatization and provided a detection limit of 60 nM **[Spielmeyer and Pohnert 2010]**. The BEH-HILIC stationary phase can be considered as a slightly modified normal silica phase whereby the ethylene bridges within the silica matrix provide only minor changes on the polarity of the particles. A similar approach using a polar Cogent Diamond Hydride silica column was published by Lenky *et al*. to quantify zwitterionic osmolytes in mammalian serum **[Lenky 2012]**. Despite the application of a triple quadrupole mass spectrometer and MRM-mode (multiple reaction monitoring), detection limits of DMSP of 100 nM were comparable to the method of Spielmeyer and Pohnert **[Spielmeyer and Pohnert 2010]**. However the MRM-mode ensured a proper quantification even of coeluting substances like, in this case, of DMSP and trimethylamine N-oxide (TMAO) **[Lenky 2012]**.

The numerous advances in the direct analysis of DMSP and other zwitterionic substances during the last decade provide very suitable methods with detection limits in the nanomolar range. These new techniques put us in the position to analyze a large variety of osmolytes within a single LC run and with high sensitivity. This allows a further and more detailed investigation of osmoregulation and adaption processes in marine algae and the involved metabolites.

Scope of the study

During the past years, the research focus regarding the environmental and geochemical relevance of marine phytoplankton laid mainly on the zwitterionic microalgae metabolite dimethylsulfonio-propionate (DMSP). Due to the commonly used GC-based indirect analysis of DMSP via DMS release, there is a lack of information about the relevance of other zwitterionic compounds like glycine betaine (GBT), dimethylsulfonioacetate (DMS-Ac) and gonyol, which are not detectable via such methods.

The aim of the present work was to apply novel direct liquid chromatography based method to provide a more detailed insight into the environmental and geochemical relevance of non-DMSP dimethylsulfonio-metabolites and other zwitterionic substances derived from marine microalgae. The possibility to couple liquid chromatography instrumentation with a highly sensitive mass spectrometric detector put us in the position use isotope labeled substances as internal standards for direct quantification of DMSP and other zwitterionic osmolytes as well as for investigation of physiological processes in marine phytoplankton.

One focus of the present work was to compare the commonly used GC-based DMSP quantification methods with direct (LC-based) methods to reveal the extent of possible systematic errors in indirect quantification of DMSP caused by other non-DMSP DMS-precursors. Possible implications on further research regarding environmental and geochemical relevance of DMSP and DMS were discussed. Furthermore, the DMSP uptake mechanism of different marine microalgae species were addressed via incubation experiments with synthetic isotope labeled $^{13}C_2D_6$-DMSP. This led to a better understanding about the capability of marine phytoplankton to act as major source as well as a sink for this multifunctional molecule in the oceans.

In order to obtain information about the contribution of different zwitterionic metabolites to osmoregulation and osmoadaption processes, the different strategies of two cosmopolitan microalgae species to adapt to salinities between 16‰ and 38‰ were investigated. Motivated by these results, the distribution of DMS-Ac and gonyol as non-DMSP dimethylsulfonio-metabolites in 13 different microalgae species from 4 different taxonomic groups was determined. Furthermore, the influence of DMS-Ac and gonyol on the DMSP metabolism of marine bacteria, which are the major sink of DMSP in the oceans, was addressed via quantification of the volatile DMSP degradation products DMS and MeSH. Since DMSP represents an important sulfur and energy source for marine bacteria, its contribution to the structuring of bacterial biofilms on the marine macroalgae *Fucus vesiculosus* was investigated.

Finally the present work should show the high potential of LC-MS methods for investigation of zwitterionic substances in marine algae and other environments. It could serve as encouragement

for other scientists to further investigate the ecological and geochemical relevance of these substances, which have mostly been overlooked in the past decades.

Publications

Manuscript A:

Astrid Spielmeyer, Björn Gebser and Georg Pohnert (2011). "Dimethylsulfide sources from microalgae: Improvement and application of a derivatization-based method for the determination of dimethylsulfoniopropionate and other zwitterionic osmolytes in phytoplankton." Marine Chemistry **124**: 48-56.

Summary: DMSP and other zwitterionic substances can be quantified via derivatization with pyrenyldiazomethane and analysis of the corresponding pyrenylmethylesters with UPLC-MS with a detection limit of 100 fmol (DMSP) on column. Improvement of the derivatization procedure led to a quantitative derivatization of DMSP from cell extracts and high reproducibility of the measurements. Comparison of the DMSP concentrations of different phytoplankton cultures with addition of D_6-DMSP as internal standard showed significant differences between direct (UPLC-MS) and indirect (GC-MS) methods. The differences in calculated DMSP concentrations of up to 14% (in *Emiliania huxleyi* RCC1217) indicate a possible overestimation of the DMSP concentration through indirect quantification via alkaline DMS release. Other zwitterionic dimethylsulfonio-metabolites (dimethylsulfonio acetate, *S*-methylmethionine, dimethylsulfonio-2-methyl-propionate) are able to release small amounts of DMS under alkaline conditions which can possibly contribute to the systematic error in indirect DMSP quantification.

Publication equivalents of contributing PhD students as coauthors according to the implementing provision of the doctoral regulations at the Faculty for Chemistry and Earth Sciences of the Friedrich Schiller University Jena:

	Author 1: **Spielmeyer**	Author 2: **Gebser**
Conception of the work	X	-
Planning of experiments	X	-
Data collection	X	X
Analysis and interpretation	X	X
Writing of manuscript	X	-
Proposed publication equivalents	1	0,5

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Dimethylsulfide sources from microalgae: Improvement and application of a derivatization-based method for the determination of dimethylsulfoniopropionate and other zwitterionic osmolytes in phytoplankton

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ABSTRACT

Dimethylsulfoniopropionate (DMSP) is a metabolite involved in central processes of phytoplankton physiology and ecology. Due to its high abundance, this molecule plays also a major role in global sulfur cycling. DMSP concentrations are usually indirectly determined via the base mediated release of dimethylsulfide (DMS) using gas chromatography (GC). However, because other potential precursors of DMS have been reported from macroalgae and phytoplankton, there is a substantial risk for an overestimation using an indirect method. We improve and validate a protocol for the derivatization and determination of DMSP in phytoplankton cultures. The improved method includes derivatization with 1-pyrenyldiazomethane (PDAM) and analysis via ultra performance liquid chromatography coupled with a mass selective detector (UPLC/MS). The protocol allows simultaneous qualitative and quantitative analysis of DMSP, other putative DMS precursors and osmolytes like glycine betaine (GBT). Detection limits are around 100 fmol on column allowing the quantification of DMSP from plankton field samples as well as from phytoplankton cultures. The DMSP content of several phytoplankton cultures was determined using the method involving derivatization of DMSP as well as an indirect headspace method determining DMS. For several algae strains, significant higher concentrations were obtained with the indirect determination. These results underscore the importance of other potential DMS precursors in phytoplankton samples that are not considered when using indirect methods for DMSP determination.

1. Introduction

Dimethylsulfoniopropionate (DMSP) is a metabolite produced by many marine microalgae and macroalgae. DMSP is involved in a wide variety of physiological and ecological processes. It can serve as an osmolyte, antioxidant and cryoprotectant (Karsten et al., 1991; Kirst et al., 1991; Sunda et al., 2002). Other zwitterionic compounds, such as glycine betaine, are discussed as alternative or additional metabolites involved in osmoregulation, and thus, a complex picture of algal osmoregulation arises (Dickson and Kirst, 1986; Keller et al., 1999a,b; Yoch, 2002).

DMSP is considered to be the most important precursor for the volatile sulfur compound dimethylsulfide (DMS) (Cantoni and Anderson, 1956; Turner et al., 1988). The enzyme DMSP lyase, which is located in different cellular compartments as DMSP, is capable to cleave DMSP into DMS and acrylic acid (de Souza et al., 1996; Stefels and Dijkhuizen, 1996; Wolfe and Steinke, 1996). Another pathway involved in the production of DMS from DMSP without concomitant formation of acrylic acid has been recently reported by Todd et al. (2007). DMS emissions contribute 13–37 Tg S per year to the atmosphere. This corresponds to 90% of the biogenic sulfur emissions from the ocean and almost 50% of the biogenic sulfur emissions worldwide (Kettle and Andreae, 2000). In the atmosphere, oxidation products of DMS serve as cloud condensation nuclei and are therefore thought to play a relevant role in climate regulation (Bates et al., 1987; Charlson et al., 1987). But DMS does not only play a role in the global sulfur cycle, it also mediates interactions on a small scale. Thus it serves as an infochemical in plankton interactions (Steinke et al., 2006) and along with acrylic acid it contributes to algal defense against grazers (van Alstyne et al., 2001; Steinke et al., 2002; Wiesemeier et al., 2007).

Base mediated cleavage of DMSP also leads to DMS release. This reaction is generally used for the indirect quantification of DMSP by determination of the volatile DMS via gas chromatography (GC). However, because other potential precursors of DMS have been reported in macroalgae and phytoplankton (Sciuto et al., 1982; Nakamura et al., 1992; Nakamura et al., 1993; Patti et al., 1993; Gage et al., 1997), methods relying on DMS quantification might lead to an overestimation of the DMSP content. Due to its instability and to chromatographic problems arising from its zwitterionic nature few methods for the direct determination of DMSP have been reported.

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Zhang et al. (2005) developed a protocol for the DMSP quantification using capillary electrophoresis. A HPLC method that requires preliminary purification of the sample and that uses unspecific UV detection at 194 nm has also been proposed for plant extracts (Colmer et al., 2000). Wiesemeier and Pohnert (2007) introduced a method using liquid chromatography/mass spectrometry for the determination of DMSP in macroalgae. DMSP is derivatized with 1-pyrenyldiazomethane (PDAM) to form an ester that can be analyzed on reversed phase LC columns (Fig. 1). Analysis of macroalgal samples is easily carried out with bulk material that can be flash frozen and directly extracted, but the use of this method for phytoplankton samples was problematic due to remaining lyase activity (Wiesemeier and Pohnert, 2007). This study demonstrated that the method is in principle transferable to phytoplankton samples; however, we had to realize that it requires substantial improvement to obtain validated results. Here we describe the transfer of the protocol to phytoplankton, its validation and its application for the investigation of 21 microalgal cultures. We compared our method for DMSP quantification with a previously established indirect method measuring base mediated DMS release. The indirect method for DMSP determination led for several algal strains to higher DMSP concentrations illustrating the importance of alternative DMS precursors. The scope of the method was also extended to other potential DMS precursors and glycine betaine (GBT) as representative zwitterionic osmolyte.

2. Materials and methods

2.1. Apparatus

LC separation was performed using an Acquity™ Ultraperformance LC (Waters, Milford, MA, USA) equipped with an Acquity UPLC™ BEH phenyl column (1.7 μm, 2.1 × 50 mm). The module was coupled to a Q-Tof micro-mass spectrometer (Waters Micromass, Manchester, England). GC separations were conducted with a PE Autosystem XL coupled to a quadrupole MS PE Turbomass detector (Perkin Elmer, Rodgau-Jügesheim, Germany). Both systems were calibrated before each sampling campaign. For NMR measurements Bruker Avance 200 MHz spectrometers were used.

2.2. Reagents

Anhydrous acrylic acid, betaine hydrochloride, dimethylsulfide, D_6-dimethylsulfide and 1-pyrenecarboxaldehyde were purchased from Sigma-Aldrich (Germany). Bromoacetic acid, methacrylic acid and S-methyl-methionine iodide were purchased from Alfa (Germany). All chemicals were of analytical grade. Methanol and water (ULC/MS grade) were purchased from Biosolve (Netherlands). Tetrahydrofuran (HPLC grade) was obtained from BDH Prolabo (France).

2.3. Synthesis of DMS precursors

The hydrochlorides of DMSP, D_6-DMSP and 3-dimethylsulfonio-2-methylpropionate (DMS-2-MP) were synthesized according to Chambers et al. (1987). Crude products were recrystallized from methanol/diethylether (1:2 v/v) with yields of 68%, 74% and 75%, respectively (NMR data for DMSP and D_6-DMSP in Wiesemeier and Pohnert, 2007, DMS-2-MP in Howard and Russell, 1997). Dimethylsulfonioacetate hydrobromide (DMS-Acetate) was synthesized according to Howard and Russell (1997) with a yield of 25% (^{1}H-NMR (200 MHz, D_4-MeOH): 4.47 (s, 2H), 3.00 (s, 6H)). All compounds were obtained with purities of 90–95%.

2.4. Pyrenyldiazomethane (PDAM)

PDAM is a commercially available reagent (Sigma-Aldrich, Molecular Probes). For economic reasons large batches were prepared by chemical synthesis. Therefore 1-pyrenecarboxaldehyde hydrazone was synthesized according to Nimura et al. (1988). The progress of the reaction was monitored by thin layer chromatography using a solvent mixture of petroleum ether and diethylether (1:1 v/v). The retention factors of the aldehyde and the hydrazone were 0.55 and 0.33, respectively. The product was used without purification for the further transformation (yield 94%, purity 95%). ^{1}H-NMR (200 MHz, D_6-DMSO): 8.76 (s, 1H), 8.70 (d, 1H), 8.40–8.01 (m, 8H), 7.16 (s, 2H).

For the oxidation of the hydrazone 170 mg dry silver oxide (Busch et al., 1936) and 1 g sodium sulfate were added to a solution of 150 mg hydrazone in 150 mL diethylether (Schroeder and Katz, 1954). The mixture was stirred for 5 h. The remaining solid was removed by filtering over glass wool and sodium sulfate. The solution was reduced to dryness via rotary evaporation. A red solid was obtained, which was stored at −20 °C. Purity was checked by ^{1}H-NMR. The purity was taken into account, when the PDAM solution for derivatization was prepared. ^{1}H-NMR (200 MHz, D_6-DMSO): 8.59–7.98 (m, 8H), 7.66 (d, 1H), 6.87 (s, 1H).

The purity of synthesized PDAM was 20–90%; best results were obtained with freshly prepared dry silver oxide. However, PDAM of low purity could also be used for the derivatization. Most of the impurities precipitate when samples are dissolved in the water/methanol/THF mixture and remaining impurities did not interfere with the detection of sulfonio-compounds.

2.5. DMS, DMSP and GBT determination from phytoplankton cultures

Unialgal but not axenic cultures were obtained from culture collections and propagated in autoclaved medium. Strains, temperature and culture media used for method comparison are listed in Table 1. Cultures for DMSP and GBT determination were cultivated at 13 °C (Table 3, except *Prorocentrum minimum* which was kept at 21 °C). Stock cultures were grown in a 14:10 light:dark cycle with light provided by Osram biolux lamps (PAR, 40 μmol m^{-2} s^{-1}). For method comparison cultures in exponential growth phase were diluted 1:5 and grown to mid-exponential phase. Samples were taken 5–7 h after the start of the light period. For analysis of DMSP and GBT cultures were grown to exponential phase and diluted 1:5. This process was repeated three times. The respective cultures were then split into three aliquots of equal volume, grown for additional 4 days and then used for further analysis.

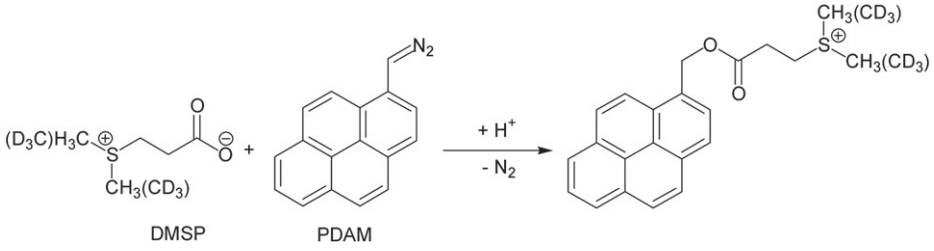

Fig. 1. Derivatization of DMSP with PDAM.

Table 1
Phytoplankton cultures, filtered volumes and cultivation conditions used for method development and comparison.

Species (strain)	Cells/mL (filtered volume)	Temperature	Culture media
Emiliania huxleyi (RCC[1] 1216)	245,000 (128 mL)	18 °C	HW sea salt professional[4] (33.33 g/L)
Emiliania huxleyi (RCC 1217)	600,000 (124 mL)	18 °C	was dissolved in double distilled
Emiliania huxleyi (CCMP[2] 1516)	425,000 (130 mL)	18 °C	water. For nutrients 400 µL of separately autoclaved Seramis®[5] for foliage plants were added.
Prorocentrum minimum	15,000 (60 mL)	21 °C	Artificial seawater
Skeletonema costatum (RCC 75)	640,000 (106 mL)	15 °C	Maier and Calenberg (1994)
Thalassiosira pseudonana (CCMP 1335)	1,200,000 (157 mL)	15 °C	
Thalassiosira weissflogii (RCC 76)	89,000 (515 mL)	18 °C	
Asterionella formosa (SAG[3] 8.95)	150,000 (480 mL)	18 °C	WC medium Maier and Calenberg (1994)
Microcystis aeruginosa (SAG 18.85)	545,000 (700 mL)	18 °C	Modified BB medium with vitamins
Scenedesmus obliquus (SAG 276-3a)	4,510,000 (700 mL)	18 °C	(3N-BBM + V) from CCAP/SAMS

[1] Roscoff culture collection (France).
[2] Center for culture of marine phytoplankton (Maine, USA).
[3] Culture collection of algae at Goettingen (Germany).
[4] Aquaristic.net, Babenhausen, Germany.
[5] MARS GmbH, Mogendorf, Germany.

Depending on cell density, cells were counted in Fuchs-Rosenthal or Neubauer haematocytometers using an upright microscope with phase contrast (DM2000, Leica, Heerbrugg, Switzerland).

2.6. Field and mesocosm sampling

Mesocosm experiments were conducted from week 16–18 in 2008 at the marine biological field station at Raunefjorden, Western Norway. A detailed description of the mesocosm setup and the cell counts is provided by Barofsky et al. (2010). Sampling was performed with 5 L plastic canisters that were transported immediately after sampling to the lab and stored in a cold room adapted to outside water temperatures until filtration. The filtration and derivatization protocol described below was also applied to field and mesocosm samples. Additional field samples were taken from the Baltic Sea in Strande (Lighthouse Bülk, Germany, 54.454°N, 10.199°E). Sampling was performed with 10 L plastic canisters that were transported in a cool box to Jena (Germany).

2.7. Sample preparation

Phytoplankton cultures were gravity or vacuum filtered on Whatman GF/C filters (60–700 mL, depending on cell density and expected DMSP content (Table 1) and 76–121 mL for simultaneous DMSP and GBT analysis (Table 3)). Filters were transferred into 15 mL Falcon® tubes filled with 2 mL methanol and 100 µL of a 200 µM D_6-DMSP aqueous solution as internal standard. Samples were stored for at least 6 days at −20 °C to ensure quantitative extraction before further preparation. Extraction could be accelerated by a 30 s treatment with an ultrasound finger (Bandelin Sonoplus HD 2070, 30% power setting). For method comparison four aliquots of 100 µL of each sample were derivatized and analyzed according to the protocol (see below). Four aliquots of 100 µL from the same solutions were used for the headspace determination of DMS (see below). For mesocosm experiments and investigations of natural plankton samples 1000–5000 mL were vacuum filtered on Whatman GF/C filters. These filters were transferred into 4 mL of methanol, containing 100 µL of a 100 µM D_6-DMSP aqueous solution. Aliquots of 3 mL were reduced to 100 µL under a stream of nitrogen and derivatized as described below. For Baltic Sea samples 30 and 100 mL were gravity filtered, and 1000, 3000 and 5000 mL samples were vacuum filtered. Filters were transferred into 1.5 mL of methanol, containing 100 µL of a 200 µM D_6-DMSP aqueous solution as internal standard. Storage was conducted at −20 °C. Aliquots of 100 µL were derivatized according to the protocol (see below).

2.8. Derivatization protocol

For derivatization 80 µL of a PDAM solution (2.5 mg mL^{-1} in THF) were added to the 100 µL sample which was then agitated on a vortexer for 5 min. Due to limited stability (Nimura et al., 1988) fresh PDAM solutions were prepared daily from a frozen solid stock of PDAM. The PDAM addition/5 min mixing sequence was repeated four times to obtain full conversion. Samples were reduced to dryness in a stream of nitrogen, taken up in 100–350 µL (depending on cell density and expected DMSP content) of water/methanol/THF (60:20:20 v/v/v) containing 0.1% formic acid and centrifuged (5 min, 16,000*g*). The supernatant was used directly for UPLC/MS measurements. Samples were measured directly or stored at −20 °C until analysis.

2.9. UPLC/MS method

For UPLC separation 1–7 µL of the sample were injected using a loop injector. Water/methanol/THF (60:20:20 v/v/v) + 0.1% formic acid (solvent A) and water/methanol/THF (5:47.5:47.5 v/v/v) + 0.1% formic acid (solvent B) were used for gradient separation. Separation started at 100% A with the flow rate 0.45 mL min^{-1} for 1.50 min. The concentration of B was then increased to 100% over 0.50 min. This ratio was kept for 1.50 min and adjusted back to 100% A over 0.50 min. The column was reequilibrated with 100% A for at least 1 min. The void volume of the system was 0.3 min. Samples were cooled to 10 °C in the auto sampler and the column temperature was held at 35 °C during the separation. Mass measurements were performed in the ESI-positive mode. For DMSP analysis the mass range from 345 to 360 *m/z* was recorded. For the analysis of other DMS precursors and GBT the mass range was set from 330 to 385 *m/z* and 330 to 360 *m/z*, respectively. The MS parameters were as follows: capillary voltage 3000 V, sample cone 10 V, source temperature 150 °C, collision energy 5 V, ion energy 1.8 V.

2.10. GC sample preparation

Aliquots of 100 µL of the methanol extracts that were also used for derivatization (see above) were transferred into 4 mL glass vials. Directly after the addition of 400 µL NaOH (1 M) the vials were sealed and incubated at room temperature for 10 min. The headspace was extracted for 10 min using solid phase microextraction (SPME) (fiber:

divinylbenzene/carboxen/polydimethylsiloxan, Supelco, Germany) (Niki et al., 2004; Yassaa et al., 2006). DMSP calibration standards (5–200 μM, containing 20 μM D_6-DMSP) were treated in the same way. To test the potential release of DMS of other DMS precursors, standard solutions of S-methyl methionine, DMS-acetate and dimethylsulphonio-2-methyl propionate (DMS-2-MP) were treated in the same way.

2.11. GC/MS method

Separation was performed on a CP Volamine column (15 m × 0.32 mm, Varian, Darmstadt, Germany). Helium was used as carrier gas (30 kPa). The oven temperature was held for 2.5 min at 40 °C and subsequently increased to 180 °C (20 °C min^{-1}). Splitless injection was used with an injector temperature of 250 °C. Transfer line and detector were set to a temperature of 200 °C and 250 °C, respectively. Mass measurements were performed in the EI-positive mode. A mass range from 34 to 400 *m/z* was recorded. Ionization energy was 70 eV, and scan duration was 1 s with an interscan delay of 0.1 s. Deuterated and undeuterated DMS were detected at 1.44 and 1.47 min, respectively.

2.12. Statistics

Statistical analyses were performed with SigmaPlot (11.0).

3. Results

3.1. Derivatization protocol

Based on Wiesemeier and Pohnert (2007) we improved an existing LC/MS method for the determination of DMSP after derivatization with pyrenyldiazomethane (PDAM). Instead of cell concentration by centrifugation as introduced by Wiesemeier and Pohnert we reverted to filtration on GF/C filters. This resulted not in concentrated suspensions of the algae in seawater but rather in a pellet that could be easily extracted by methanol. Wiesemeier and Pohnert needed to extract the aqueous cell suspension using the Bligh and Dyer method and required an additional phase separation step. By monitoring the signals of the DMSP-PDAM ester (*m/z* 349) and DMSP (*m/z* 135) we found that the original procedure did not result in quantitative derivatization. We thus optimized the derivatization protocol by introducing repeated additions of small portions of PDAM and by adjusting reaction times. The derivatization was considered complete when no DMSP signal was observed. The repeated addition of PDAM to a methanolic solution containing DMSP was found to be more effective than a single addition of excess PDAM with extended reaction time. This was probably due to decomposition of PDAM. A five times repeated addition of 80 μL of a 2.5 mg mL^{-1} PDAM solution led to the best results. Between each addition step the samples were agitated on a vortexer for 5 min. This enabled a quantitative derivatization of up to 1 μmol DMSP in 50 μL of methanol. The derivatization protocol was transferable from methanolic standard solutions to samples with a complex matrix as found in phytoplankton samples. We also optimized LC conditions to obtain better peak shapes. A BEH phenyl column with an optimized gradient of mobile phases containing water, methanol, THF and formic acid gave better peak shapes and improved sensitivity. By introducing this protocol we were able to speed up the analytical process significantly and obtained six times lower limit of detection (LOD) for the injections of standard solutions compared to Wiesemeier an Pohnert (2007).

3.2. Method validation

Derivatized DMSP calibration standards were obtained from a stock solution by dilution in water/methanol/THF (60:20:20 v/v/v) + 0.1% formic acid. Internal standard was added to reach a final concentration of 20 μM D_6-DMSP. For the calibration curve the ratio of the peak areas of ion traces of undeuterated and deuterated DMSP was plotted relative to the DMSP concentration. Because of the mass increase from 349 *m/z* (M^+ DMSP) to 355 *m/z* (M^+ D_6-DMSP) due to deuteration both compounds can be quantified without interference despite nearly identical retention times (Fig. 2). Linearity was confirmed in the range of 0.5–300 μM.

The LOD and limit of quantification (LOQ) were defined as the amount of DMSP-PDAM required to reach a signal-to-noise ratio of ≥3 and ≥10, respectively, and were determined for standard solutions. The LOD and LOQ were 100 fmol or 35 pg on column and 500 fmol or 175 pg on column, respectively. These values refer only to the derivatization of a standard solution and the following analytical process and do not include sample preparation steps like filtration that leads to an enrichment of DMSP. Depending on filtration volume this would equal to a theoretical LOD of 2 nM (filtration volume of 100 mL) or 0.2 nM (1000 mL).

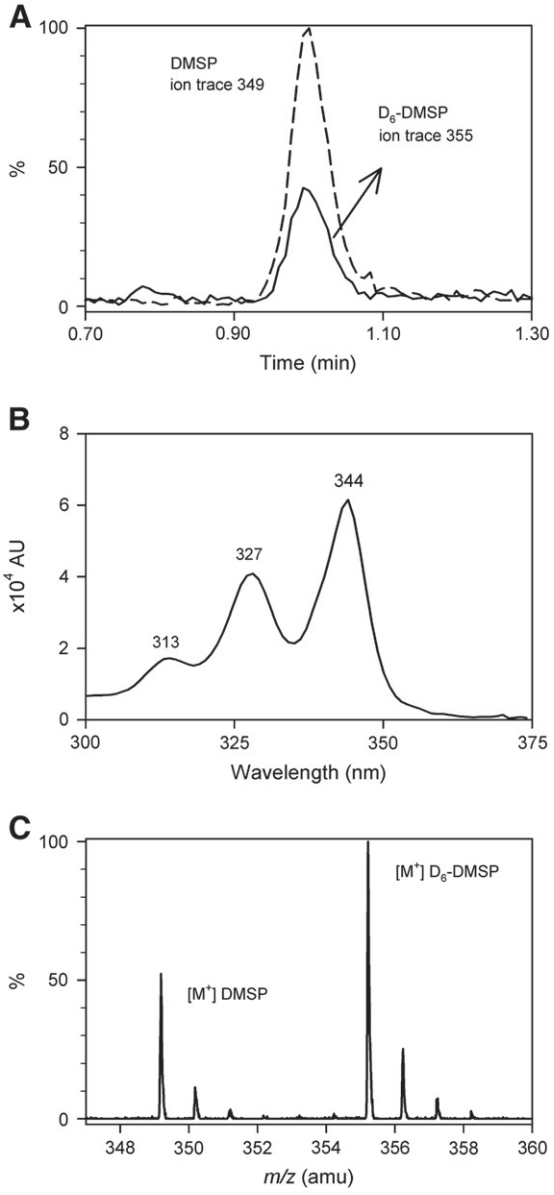

Fig. 2. (A) LC/MS chromatogram of a derivatized cell extract of *S. costatum* (410000 cells mL^{-1}, filtration volume 50 mL); UV-spectrum (B) and MS-spectrum (C) of derivatized calibration standard solution containing 10 μM DMSP and 20 μM D_6-DMSP (not background corrected).

A methanolic blank sample containing the derivatization reagent but no DMSP was injected 10 times and no signals were obtained at 349 and 355 *m/z*. For a culture blank 260 mL of medium were filtered and the filter was transferred into methanol. The sample was stored for 9 days at −20 °C and then an aliquot of 100 μL was derivatized and investigated according to the protocol. Measurement revealed no signals at 349 or 355 *m/z*.

Measurement precision was determined by repeated (6×) injection of calibration standards. The relative standard deviation (RSD) was 0.9–2.3% in the range of 5–300 μM. A maximum for the RSD was reached at the LOQ with 5%. If one culture is split up in three parts and derivatized, measurement precisions of 5% ($n=3$) are obtained (Table 2). If, however biological variability is involved and replicates of independent cultures are concerned coefficients of variance can be higher (Table 3). The precision of sample derivatization was determined using a methanolic cell extract of *Emiliania huxleyi* which was spiked with deuterated and undeuterated DMSP. Six aliquots of 800 μL (final concentration: DMSP 228 μM and D_6-DMSP 40 μM) were reduced to 50 μL, derivatized and analyzed according to the above described protocol. The precision of the method was 5.2%. To test the influence of the matrix on the derivatization yield, different volumes of one *Skeletonema costatum* culture were vacuum filtered (20–300 mL, 410,000 cells mL^{-1}) and worked up according to the protocol. DMSP concentrations determined from seven different culture volumes ranged from 0.29 to 0.32 pg DMSP per cell and no significant difference was found (ANOVA with Tukey post hoc test, $p>0.05$).

As deuterated compounds are more volatile than their non deuterated analogues, the effect of evaporation was tested. Therefore, 1.5 mL samples of six methanolic standard solutions were reduced to 50 μL in a stream of nitrogen and derivatized as described above. For the obtained recovery function neither constant nor proportional systematic errors were detected, indicating that evaporation has no influence on the accuracy of the method.

Recovery for algal samples was determined using a culture of *Thalassiosira weissflogii* (RCC76) that did not contain any natural DMSP. The culture was split into five aliquots (142 mL, 59200 cells mL^{-1}) and filtered. To each methanol extract DMSP (20, 50, 100 and 200 nmol) and D_6-DMSP (20 nmol) were added. The derivatized samples were stored 72 h at −20 °C and 24 h at 4 °C. The analysis gave a recovery of 95–105%.

To test for stability, derivatized culture samples and standard solutions were stored at −20 °C. After 6 months a recovery of >90% and >80% was found, respectively.

To test whether insufficiently suppressed lyase activitiy before addition of the deuterated standard has an influence, three different sample treatments were tested for six marine phytoplankton cultures. In each case one stock culture was divided into six aliquots that were filtered. Three treatments were conducted in duplicates. i) Two filters were transferred into 20 mL headspace vials that contained 4 mL of 1 M NaOH. Internal standard was added (100 μL of 200 μM D_6-DMSP) and the vials were sealed. Samples were incubated for 5 h in the dark at room temperature and analyzed via SPME GC/MS. Here, remaining lyase activity would not influence quantification as enzymatically produced DMS would be analyzed as well. ii) Two filters were transferred into methanol containing the internal standard (100 μL of 200 μM D_6-DMSP). The samples were sonicated (Bandelin Sonoplus HD 2070, 30% power setting, two continuous pulses for 15 s) and stored at −20 °C. Methanol extracts were analyzed after 5 h via SPME GC/MS as described above. By sonication intracellular DMSP and extracellular D_6-DMSP get mixed immediately, so both compounds would be cleaved by remaining enzymatic activity at the same time. iii) Two cell methanol extracts were stored without further treatment at −20 °C and analyzed after 6 days via SPME GC/MS. Here, cells are lysed slowly by methanol. If methanol treatment did not suppress lyase activity, extracellular D_6-DMSP would be cleaved before the enzyme gets into contact with the intracellular DMSP. In this case, higher DMSP cell contents should be observed for treatment iii. However, no significant differences were observed between any of the treatments (ANOVA, $p>0.05$). Thus, using deuterated DMSP as internal standard sample storage in methanol (*iii*) does not influence the quantification of DMSP compared to direct sample preparation (*i*).

Table 2
Comparison of DMSP contents in phytoplankton cells using GC/MS and LC/MS.

Species (strain)	pg DMSP/cell		Difference
	GC method	UPLC method	
Emiliania huxleyi (RCC 1216)	0.63 (0.02)	0.58 (0.01)	−9%***
Emiliania huxleyi (RCC 1217)	0.44 (0.01)	0.38 (0.01)	−14%***
Emiliania huxleyi (CCMP 1516)	0.43 (0.02)	0.40 (0.01)	−7%*
Prorocentrum minimum	10.84 (0.38)	9.84 (0.27)	−9%**
Skeletonema costatum (RCC 75)	0.34 (0.02)	0.33 (0.01)	−3%
Thalassiosira pseudonana (CCMP 1335)	0.021 (0.001)	0.020 (0.001)	−3%
Thalassiosira weissflogii (RCC 76)	<0.01	<0.01	
Asterionella formosa (SAG 8.95)	<0.01	<0.01	
Microcystis aeruginosa (SAG 18.85)	n.d.	n.d.	
Scenedesmus obliquus (SAG 276-3a)	n.d.	n.d.	

n.d., not detected.
*$p<0.05$; **$p<0.01$; ***$p<0.001$ (*t*-test, data sets fulfilled constraints of normal distribution and homogeneity of variance), mean $n=4$ (preparation and analysis of four independent samples of one culture), values in parentheses correspond to standard deviation.

Table 3
DMSP and GBT contents in phytoplankton cells.

Species[1] (strain)	Cells/mL (filtered volume)[2]	pg DMSP/cell	pg GBT/cell
Amphiphrora paludosa	46,000 (98)	n.d.	n.d.
Chaetoceros gracilis	706,000 (106)	n.d.	<0.01 (0.00)
Navicula sp.	1,140,000 (97)	<0.01 (0.00)	n.d.
Pavlova lutheri	986,000 (110)	n.d.	0.03 (0.00)
Rhodomonas sp.	119,000 (100)	n.d.	0.94 (0.15)
Thalassiosira weissflogii (RCC76)	65,000 (102)	n.d.	2.60 (0.53)
Chaetoceros didymus	136,000 (93)	0.07 (0.01)	n.d.
Prymnesium parvum	137,000 (93)	2.27 (0.13)	<0.01 (0.00)
Skeletonema costatum (RCC75)	1,276,000 (107)	0.27 (0.05)	<0.01 (0.00)
Skeletonema marinoi (G4)[3]	561,000 (114)	0.34 (0.03)	<0.01 (0.01)
Cryptochloris sp.	258,000 (103)	0.55 (0.01)	0.03 (0.00)
Isochrysis galbana	732,000 (100)	0.38 (0.02)	0.02 (0.00)
Phaeodactylum tricornutum (UTEX646)	1307,000 (102)	0.11 (0.03)	0.14 (0.03)
Prorocentrum minimum	2,000 (79)	34.86 (3.17)	6.96 (1.11)
Thalassiosira pseudonana (CCMP1335)	786,000 (103)	0.13 (0.00)	0.09 (0.01)

n.d. - not detected, mean $n=3$ (three independent cultures), values in parentheses correspond to standard deviation.
[1] All cultures were grown in artificial seawater (Maier and Calenberg, 1994).
[2] Filtration volume given in mL.
[3] Isolated in Raunefjord, Norway.

3.3. Detection of other potential DMS precursors

Other potential DMS precursors and DMSP can be determined and quantified simultaneously if the scan range of the mass spectrometer is increased (Fig. 3A). With a scan range from 330 to 385 *m/z* DMSP, dimethylsulfonio-2-methylpropionate (DMS-2-MP) and S-methylmethionine (SMM) can be detected at concentrations as low as 100 nM. The LOD of DMS-acetate was 1 μM. An unknown compound with *m/z* 393, the characteristic isotope pattern of a sulfur containing metabolite and the characteristic MS/MS and UV data from a pyrenylester (pyrenyl fragment at $m/z=215$ and λ_{max} 343.5 nm) was detected in all samples of *E. huxlexi* and *P. minimum* and in the mesocosm samples. Peak intensity of this signal reached up to 10% of

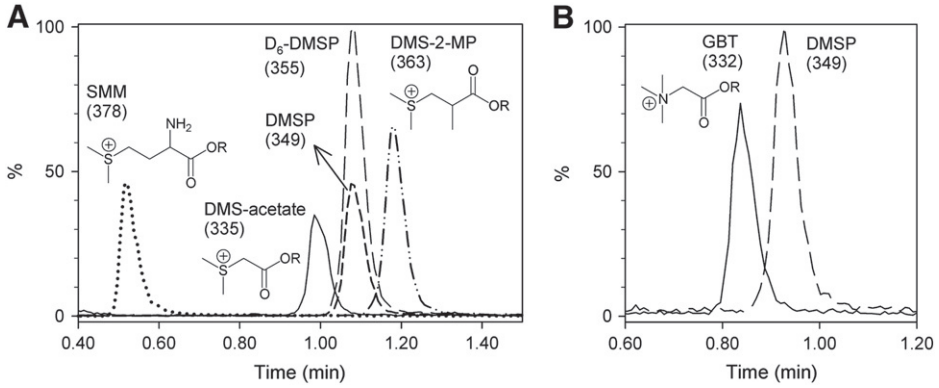

Fig. 3. Ion traces (monitored ions are given in brackets) of UPLC separations of (A) different putative derivatized DMS precursors (injection of 1 μL standard solution containing DMSP, DMS-acetate, DMS-2-MP and SMM, each 10 μM, with 20 μM D_6-DMSP as internal standard) and (B) derivatized methanol extract of *Phaeodactylum tricornutum* (injection volume 4 μL); ion traces were used to detect the respective derivatized compounds (*R* = pyrenylmethyl-group).

the DMSP signal. DMS release of SMM, DMS-acetate and DMS-2-MP after base treatment was quantified to verify the potential contribution of these compounds to the total DMS detected with the indirect method. Standard solutions were analyzed after 10 min, 1, 2 and 4 h, respectively. All compounds were capable to release DMS. However, in contrast to DMSP no complete conversion of the compounds to DMS and the respective acids took place. For SMM and DMS-acetate no time dependence was found. After 10 min as well as after 4 h incubation at room temperature 2–3.5% were converted to DMS. For DMS-2-MP 7% of the initial concentration were cleaved to DMS after 10 min, and 71% after 4 h at room temperature.

3.4. GBT and DMSP in phytoplankton cultures

Additionally, this method can be used for analysis of e.g. nitrogen-containing osmolytes like glycine betaine (GBT) without further adjustments to the protocol. Using a scan range of 330–360 *m/z* GBT possessed a LOD of 200 nM. Therefore, this method allows a simultaneous quantification of DMSP and GBT (Fig. 3B).

Fifteen marine phytoplankton cultures were analyzed concerning their GBT and DMSP content (Table 3). Cultures can be separated into four groups. There are species producing neither DMSP nor GBT; others produce only one of these substances. Both osmolytes are produced by one prymnesiophyte, the cryptophyte and the dinoflagellate investigated. Certain diatoms can also contain both compounds. The ratio is varying between 0.7 and >50 (calculated as ratio of the molar concentrations of DMSP/GBT per cell). *T. weissflogii* was the only tested diatom that contained no DMSP but elevated amounts of GBT (Table 3).

3.5. Comparison of the derivatization-based and the indirect DMSP quantification method

Several phytoplankton cultures were analyzed to determine their intracellular DMSP content both after derivatization via UPLC/MS and indirectly via base-released DMS using GC/MS (Table 2). In most samples from marine phytoplankton cultures the determined DMSP concentrations using the method based on derivatization and the indirect quantification differ significantly ($p<0.01$ or $p<0.001$, data sets fulfilled constraints of normal distribution and homogeneity of variance for *t*-test). In all cases the DMS concentrations from the NaOH-treated samples exceeded DMSP determined by UPLC/MS. This cannot be assigned to biological variability since in every comparison one methanol extract was split into eight aliquots of which four were used for the derivatization and four for the headspace measurements. As the UPLC/MS and the GC/MS method possess similar calibration functions ($y = 0.0517x$ and $y = 0.0483x$, respectively, $R^2 > 0.995$, x corresponds to DMSP and DMS in μM, y corresponds to area ratio of analyte and internal standard, relative standard error of the slope were 1.16% for the UPLC and 0.54% for the GC calibration function, respectively) and recovery rates, the different detected DMSP/DMS contents are also not the result of artificial bias due to different method sensitivities. UPLC and GC measurements were conducted within 5 days, so the influence of sample storage can be excluded as a reason for the obtained differences.

3.6. DMSP determination in field and mesocosm samples

We tested this method also during spring 2008 in Norwegian fjord waters. Filtration of 5 L of a field sample with a chlorophyll a (chl a) content of 1.56 ± 0.12 μg L^{-1} under non-bloom conditions was sufficient to produce a DMSP signal suitable for quantification (27.0 ± 1.0 nM DMSP). Nevertheless, independent studies in non-bloom situations in the Baltic Sea revealed that filtration volumes could be reduced to 1 L without loss of signal (data not shown). We also tested this method during a mesocosm experiment (for details on the mesocosms see Barofsky et al., 2010). During this experiment *S. marinoi* blooms of different intensities were induced by the addition of nutrients alone or by the addition of nutrients together with inoculation of a dense *S. marinoi* culture. The highest DMSP concentration could be detected in the mesocosm with the most intensive *S. marinoi* bloom (day 8 after inoculation, 2 L filtered, 228.2 ± 10.8 nM DMSP, chl a 17.58 ± 1.06 μg L^{-1}, *S. marinoi* 31600 cells mL^{-1}). Only addition of nutrients, which moderately increased cell counts compared to the surrounding plankton sample, gave intermediate results (day 5 after inoculation, 5 L filtered, 44.6 ± 1.7 nM DMSP, chl a 6.15 ± 0.11 μg L^{-1}).

Table 4
Comparison of method parameters of the direct quantification of DMSP using LC/MS introduced in this study (LC) and established indirect headspace methods (GC).

Parameter	LC	GC
LOD	100 fmol on column (2 nM with 100 mL filtration)	0.3 nM (10 mL filtration, Smith et al., 1999)
Indirect determination via DMS	No	Yes
Repeated injection possible?	Yes	No
Analysis of other potential DMS precursors?	Yes	No
Storage possible?	Yes	limited
Sample stability	recovery>90% after 6 months at −20 °C	
Required instrumentation	LC/MS	HS or SPME/GC with FID,[1] FPD[2] or MS

[1] Flame ionization detector.
[2] Flame photometric detector.

4. Discussion

We improved and evaluated a method that allows the detection of DMSP from phytoplankton cultures without alkaline cleavage. We developed the new protocol for UPLC/MS measurements, but the method can be generally transferred to widely available HPLC/MS equipment as shown previously (Wiesemeier and Pohnert, 2007). Compared to the original protocol by Wiesemeier and Pohnert (2007) several parameters have been changed to improve the extraction procedure and to improve the LOD. We could demonstrate that filtration is superior to concentration of the cells by centrifugation since the remaining water in the suspension after centrifugation leads to DMSP lysis during methanolic work-up. In contrast, methanol efficiently extracts DMSP from the cells on GF/C filters and suppresses lyase activity in the absence of excess water. Using this procedure we could avoid the Bligh and Dyer extraction described by Wiesemeier and Pohnert (2007) and save one extraction and concentration step. In contrast to the previous report we could test the stability of the methanol cell extracts and found that extracts can be stored for prolonged time (>4 weeks) which makes the method particularly suitable for field sampling campaigns where no immediate analysis can be performed. The derivatization protocol has also been optimized to achieve a quantitative transformation of DMSP. Furthermore, we switched to a phenyl column and developed a new eluent system to improve the peak shape and sensitivity. If only the detection of the DMSP standard is concerned we reached a six fold lower LOD compared to Wiesemeier and Pohnert (2007). Since the method was not validated for phytoplankton cultures in the previous report we cannot compare directly the overall improvement including the effects of the optimized extraction and derivatization procedure. Due to the low limit of detection (LOD) only small culture volumes are now required for DMSP quantification. The amount of detected matrix components is low.

Compared to the LOD of previously reported methods using indirect determination of DMSP after base mediated release of DMS (e.g. 0.3 nM for 10 mL filtration by Smith et al., 1999) the LOD for the method introduced here is higher (Table 4). Nevertheless, only 20 mL of algal cultures were required for a quantitative determination of DMSP. We evaluated if higher filtration volumes would interfere with the analysis and found for a *S. costatum* culture that a scale up of filtration volume to 300 mL can be easily performed without loss of sensitivity. The effects of filtration volume would, however, have to be verified independently if more fragile algal species are concerned. We also evaluated our method during non-bloom situations for plankton samples from the Baltic Sea. Low filtration volumes of 100 mL did not result in detectable signals, but filtration volumes of 1000 mL (or more) were suitable for DMSP detection. Filtration of large volumes can however cause artificial DMSP release by cell disruption that would not be compensated by the use of our internal standard, which is only added after filtration (Kiene and Slezak, 2006). We thus recommend the more sensitive indirect determination methods with lower LOD in cases where no quantitative information on the contribution of other DMS releasing metabolites is required. In cases where sensitivity is an issue, our method could be easily modified. For example, the sample volume of 100–350 μL that was selected due to the ease of handling can be reduced and the injection volume can be increased up to 20 μL. We estimate that this could lower the LOD by a factor of 10. Furthermore, filters can be transferred in less than 2 mL methanol. Our method is thus readily applicable to phytoplankton cultures and with some restrictions also to natural phytoplankton samples (Table 4).

The recovery rate for DMSP standard solutions using the indirect SPME GC method with an equilibration time of 10 min is higher than 90% (Niki et al., 2004). Therefore, the method introduced in this study can be considered at least comparable to the headspace approach. Validation shows that the derivatization/LC/MS protocol is very robust and that the risk of systematic errors is very limited. Cell density and therefore magnitude of matrix components has no influence on derivatization yield and solvent evaporation also does not affect the result. Storage of the samples is feasible over a prolonged period of time.

The use of a mass selective detector permits the use of a deuterated standard which bears major advantages for the determination of labile and hard to extract analytes, such as DMSP. Using D_6-DMSP as internal standard compensates for eventual losses during storage and sample preparation. This standard is transformed with identical kinetics as the undeuterated substance (Smith et al., 1999). Since the C-D- bond is not involved in the cleavage reaction of D_6-DMSP to D_6-DMS and acrylic acid it can be assumed that D_6-DMSP is enzymatically transformed with similar kinetics as DMSP. Losses due to remaining lyase activity or chemical degradation are thus compensated as well. Compared to other methods available for the detection of DMSP we add specificity since DMSP can be detected using its characteristic ion trace and robustness since the standard compensates for overlaying (a)biotic DMSP degradation reactions (Colmer et al., 2000; Zhang et al., 2005).

Using the new method we surveyed 21 phytoplankton cultures for their DMSP content (Tables 2 and 3). We found on a cellular basis a high DMSP value for the diatom *S. costatum* that was comparable with the coccolithophore *E. huxleyi*, but referred to cell volume intracellular DMSP concentrations of *S. costatum* are usually lower than in *E. huxleyi*. Diatoms are often considered to produce low levels of DMSP relative to dinoflagellates or prymnesiophytes, which was true for the other tested species like *T. weissflogii* (Keller et al., 1989). For the fresh water cultures, DMSP or DMS was not detected or were present in unquantifiable amounts. We applied our method also to samples from Norwegian Fjord waters and mesocosms with induced diatom blooms. This first comparison proves that in principle also DMSP from rather complex field samples can be detected and the observed quantitative values correspond well to the determined cell counts of *S. marinoi* in the field and the mesocosm (Barofsky et al., 2010).

Comparison of our analysis of a DMSP derivative and the indirect quantification via DMS reveals significant differences between DMSP content and DMS release of several phytoplankton cultures (Table 2). As the same culture extract was split and used for both analyses it can be excluded that the observed differences are the result of biological variability or different sample treatment during preparation. This is supported by the normal distribution of the determined concentrations. This procedure ensures that a direct comparison of DMSP content and the overall DMS release after base treatment is possible for all investigated samples. Standard deviations thus only include the method precision (RSD for UPLC 1.6–4.1% and for GC 2.1–6.7%). Discrepancies in results between the two methods are thus due to the contribution of other DMS precursors to the DMS release initiated by base treatment of the crude cellular preparations. It can be concluded that in most of the tested microalgae DMSP is not the only source for DMS. Throughout the detected samples the differences are not high, but often significant. If only an estimation of the DMSP content is concerned the established indirect method that requires less sophisticated equipment and no costly derivatization reagent is clearly sufficient. Errors of up to 15% arising due to additional DMS sources could be accepted in many cases, where the biological variability will often cause similar uncertainties. Nevertheless, if e.g. physiological investigations are concerned, where often minor DMSP variations or metabolic fluxes are discussed, it should be verified if also other DMS sources play important roles. This is also true for ecological investigations where other DMS precursors could have a significant impact as well. In these cases the use of the novel method would be clearly advised. If the role of DMSP in sulfur cycling is concerned it should be taken into account that even variations of 10% might correspond to several Tg sulfur on global scale and that currently no quantitative information is available for this non DMSP-derived sulfur. Also studies on the impact of environmental factors on the DMSP content should consider additional DMS precursors.

Our method offers the possibility to detect candidate molecules that could act as additional DMS precursors. A mix of synthetic

standards of several sulfonio-metabolites could be separated and detected without interference using the introduced method (Fig. 3A). Cell extracts were checked for peaks of other potential DMS precursors by increasing the scan range of the MS detector. All strains of *E. huxleyi* and *Prorocentrum minimum* showed a peak for *m/z* 393 that exhibited the characteristic isotope pattern of an organic metabolite containing one sulfur atom. MS/MS and UV measurements confirmed that the compound in question is a pyrenyl ester and is thus derived from esterification of a free acid (MS/MS: 393 (mol peak) and 215 amu (pyrenyl fragment), UV: λ_{max} 343.5 nm). The spectroscopic data and the retention time are in accordance with the reported substances 4-dimethylsulfonio-2-methoxybutyrate (Sciuto et al., 1982) and gonyol (Nakamura et al., 1993) but for a final verification which of the isomers corresponds to the detected natural product a comparison with synthetic standards would be required. Since this compound reached only peak intensities of ca. 10% of DMSP it is most likely not exclusively responsible for the increased DMS values.

All the above mentioned molecules released DMS upon base treatment. The DMS yields were variable from 2 to 71% depending on the structure of the metabolites. Nevertheless these small osmolytes might not be the only metabolites releasing DMS. It could be envisaged that also the higher molecular weight fraction could contain sulfonio-metabolites that could be converted to DMS.

DMSP concentrations might be connected to the concentrations of other osmolytes in algae. Of specific interest are other zwitterionic osmolytes, such as GBT that might act as partial replacements of DMSP if nitrogen is not limited (e.g. Keller et al., 1999a,b). Since the employed derivatization generally transforms carboxylic acids to pyrenylesters other osmolytes can be also quantified with our method. Indeed, the LOD of 200 fmol or 66 pg per μL injection for GBT is sufficient to simultaneously monitor DMSP and GBT in one run. As proof of principle we performed this simultaneous analysis for several phytoplankton cultures. Different culture protocols were used and the experiments were performed 1 year apart from each other. The observed differences of the absolute DMSP content for the cultures of the same strain (Tables 2 and 3) can therefore be attributed to biological variability. This variability can not only be dependent on strain and growth conditions, but also on growth rate. Keller et al. (1999a) reported e.g. for cultures of *P. minimum* highest DMSP cell contents during the early exponential phase that decreased by factor 4 with extended growth. Slight variability of the growth phases between the two campaigns could thus already explain the observed variability. Several algae belonging to prymnesiophytes, chryptophytes, dinoflagellates and diatoms contained DMSP and GBT. Some algae like the haptophyte *P. parvum* predominantly contained DMSP, while the diatom *T. weissflogii* exclusively contained GBT. While we determined ca. 7 pg GBT $cell^{-1}$ for *P. minimum* Keller et al. (1999a) reported for *P. minimum* (CCMP1329) that it does not produce GBT. Therefore, GBT production might be not only species but also strain specific. However, also GBT is a highly variable molecule over the growth cycle as pointed out by Keller et al. (1999a). Therefore, the observed differences might be due to different sampling points during the exponential growth phase. Several studies discuss an increased DMSP content per cell as a reaction to nitrate limited conditions as DMSP might replace N-containing osmolytes like GBT. However, in most studies only DMSP is determined and no information about GBT is available, so only assumptions could be made (Turner et al., 1988; Stefels and van Boekel, 1993; Bucciarelli and Sunda, 2003). Keller et al. (1999a,b) determined both compounds, but used two analysis techniques (HPLC and GC). The method presented here is able to quantify both metabolites simultaneously.

5. Conclusion

Here we introduce a validated method for the determination of particulate DMSP and related metabolites like other potential DMS precursors or the osmolyte GBT using derivatization with PDAM. It can be used for a broad range of different phytoplankton species including coccolithophores, diatoms or dinoflagellates. This method can thus be applied to answer a wide range of physiological questions.

Differences found by comparing direct and indirect methods for quantification of DMSP underline the importance of other DMS sources in phytoplankton. Therefore, care must be taken if previous results are discussed in a quantitative framework.

Acknowledgments

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Manuscript B:

Astrid Spielmeyer, Björn Gebser and Georg Pohnert (2011). "Investigations of the uptake of dimethylsulfoniopropionate by phytoplankton." ChemBioChem **12**(15): 2276 - 2279.

Summary: Marine microalgae possess an uptake mechanism for the zwitterionic osmolyte dimethylsulfoniopropionate (DMSP) which channels the molecule directly without previous transformation into the cells. DMSP uptake kinetics were highly species specific whereby the DMSP-non-producer *Thalassiosira weissflogii* showed a remarkably higher uptake than DMSP-producing species. The uptake of isotope labeled $^{13}C_2D_6$-DMSP by *T. weissflogii* and *Emiliania huxleyi* (RCC1242) had no effect on the natural intracellular concentrations of DMSP and glycine betaine which indicates that externally supplied DMSP is taken up in addition to the present osmolytes. It could be shown that marine phytoplankton can act as a considerable sink of DMSP in the oceans.

Publication equivalents of contributing PhD students as coauthors according to the implementing provision of the doctoral regulations at the Faculty for Chemistry and Earth Sciences of the Friedrich Schiller University Jena

	Author 1: **Spielmeyer**	Author 2: **Gebser**
Conception of the work	X	-
Planning of experiments	X	-
Data collection	X	X
Analysis and interpretation	X	X
Writing of manuscript	X	X
Proposed publication equivalents	1	0,75

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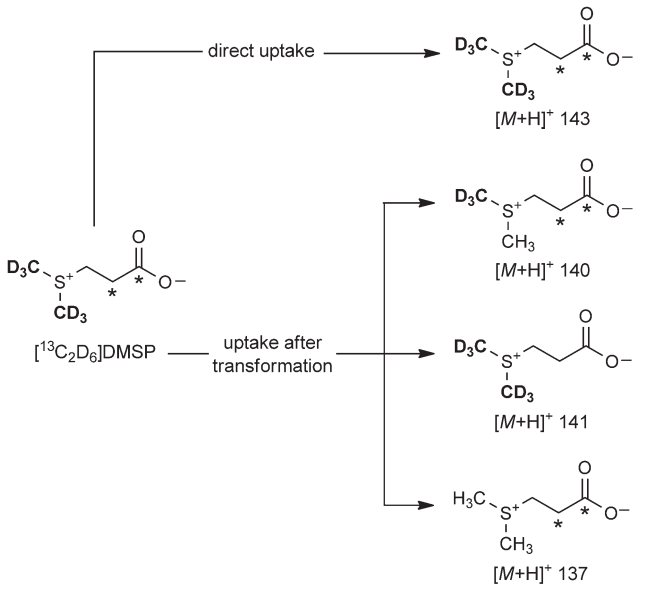
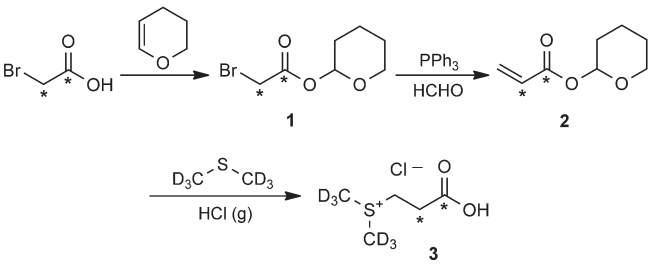
Investigations of the Uptake of Dimethylsulfoniopropionate by Phytoplankton

Astrid Spielmeyer, Björn Gebser, and Georg Pohnert*[a]

Dimethylsulfoniopropionate (DMSP), a zwitterionic metabolite, is produced by many marine micro- and macroalgae. Several physiological functions for this metabolite have been identified, including antioxidant,[1] cryoprotectant,[2] and osmolyte activity.[3] DMSP is also released by algae, and its concentration in seawater is usually a few nmol per liter (an overview is given in ref. [4]), but during mass occurrences of microalgae, the concentration can reach values above 100 nmol L^{-1}.[5] Given these relatively high concentrations, DMSP also serves as an important carbon and sulfur source for bacteria in the aquatic ecosystem.[6–8] Although sulfate is present in higher concentrations (ca. 10^7 times more), the uptake of reduced organic sulfur compounds such as DMSP is energetically favored.[9] DMSP and its degradation products can contribute up to 100% of the sulfur demand of certain bacteria.[10] Bacteria are known to produce volatile compounds from DMSP by demethylation/demethiolation to produce methanethiol or by lysis giving dimethylsulfide (DMS).[11] The ratio between these pathways has major implications for the global sulfur cycle. DMS emissions from the oceans into the atmosphere account for 17–34 Tg sulfur per year.[12] Sulfate aerosols formed from DMS serve as cloud condensation nuclei, thus making the role of DMSP and DMS relevant to the climate.[13,14] The central role of these metabolites in organismic interactions as well as in climate-relevant processes makes it important to gain information about their fate in seawater. Beside heterotrophic organisms, phytoplankton also have a relevant role in the uptake of dissolved DMSP, but only little quantitative information on this is available. Previous studies conducted with radiolabeled [^{35}S]DMSP showed radioactivity inside phytoplankton cells after incubation. Based on further indirect evidence, a direct uptake of DMSP by an uptake system that is also used for glycine betaine (GBT) was suggested.[15,16] However, the use of [^{35}S]DMSP did not allow a conclusion about the uptake mechanism and the intracellular metabolism to be drawn, as only the signal of the sulfur and not of DMSP itself is detected. Potential mechanisms for DMSP uptake and metabolism include transformation to intermediate demethylated species or lysis to DMS and acrylate. In this study, we present an approach to investigate the DMSP uptake mechanism and kinetics as well as its fate in phytoplankton by using an eightfold isotopically labeled DMSP ([$^{13}C_2D_6$]DMSP). LC/MS analysis allowed the pathways shown in Scheme 1 to be discriminated.



Scheme 1. Possible transformation of DMSP before uptake and the respective ion traces in cell extracts; * indicates ^{13}C.



Scheme 2. Synthesis of [$^{13}C_2D_6$]DMSP.

[$^{13}C_2D_6$]DMSP was synthesized according to Scheme 2 (see the Supporting Information for detailed procedures and spectroscopic data). For a first survey, this marker was applied at a concentration of 125 nM to non-axenic cultures of the three diatoms *Thalassiosira pseudonana*, *Thalassiosira weissflogii*, and *Skeletonema costatum*, the dinoflagellate *Prorocentrum minimum*, and two isolates of the coccolithophore *Emiliana huxleyi*, only one of which forms calcified coccoliths. We thereby covered the dominant oceanic algae that have been the subject of many studies relating to the production and function of DMSP. We included algae with high, low, and no DMSP cell content in order to survey whether a different uptake mechanism is active in these classes. Exponential cultures were grown over six days; on the first five days an aliquot equivalent to 125 nM [$^{13}C_2D_6$]DMSP was added. This procedure was

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selected so as to reach an equivalent of high environmental DMSP concentrations. After filtration and careful washing to remove surface-bound [$^{13}C_2D_6$]DMSP, the cells were extracted and investigated by ultra-performance liquid chromatography-MS (UPLC-MS) modified according to a reported procedure (Supporting Information).[17] In addition, the structure of labeled DMSP was confirmed by MS-MS analysis (see Figure 2B below). In all five investigated species, a [$^{13}C_2D_6$]DMSP signal could be unambiguously detected, but uptake occurred with different efficiency (Figure 1).

In the diatoms *S. costatum* and *T. pseudonana* as well as in the dinoflagellate *P. minimum*, the labeled DMSP accounted for about 10% of the total cellular DMSP (Figure 1). With reported DMSP cell contents of 0.27 and 0.13 pg per cell for *S. costatum* and *T. pseudonana* and 34.9 pg per cell for *P. minimum*, the absolute amount taken up is, however, strongly species dependent.[18] In contrast, the two isolates of *E. huxleyi* took up so little DMSP as to reach only about 1% of [$^{13}C_2D_6$]DMSP compared to the overall cellular concentration (Figure 1). For the strains of *E. huxleyi* investigated in this study, DMSP concentrations of 0.46 (RCC1242) and 0.58 (RCC1216[18]) pg per cell were determined. Apparently those algae that are able to maintain high DMPS levels by de novo biosynthesis do not rely substantially on external DMSP, even if it is administered at elevated concentrations for a long time. In a previous study that followed the uptake of radioactive DMSP, the dinoflagellate *K. brevis* and *E. huxleyi* did not take up any DMSP.[15] Our results indicate that dinoflagellates have the ability to take up DMSP and that minimal DMSP uptake can also be observed in *E. huxleyi* after prolonged incubation. These results have, however, to be interpreted with care because it cannot be fully excluded that small amounts of external labeled DMSP are carried over despite intense washing steps.

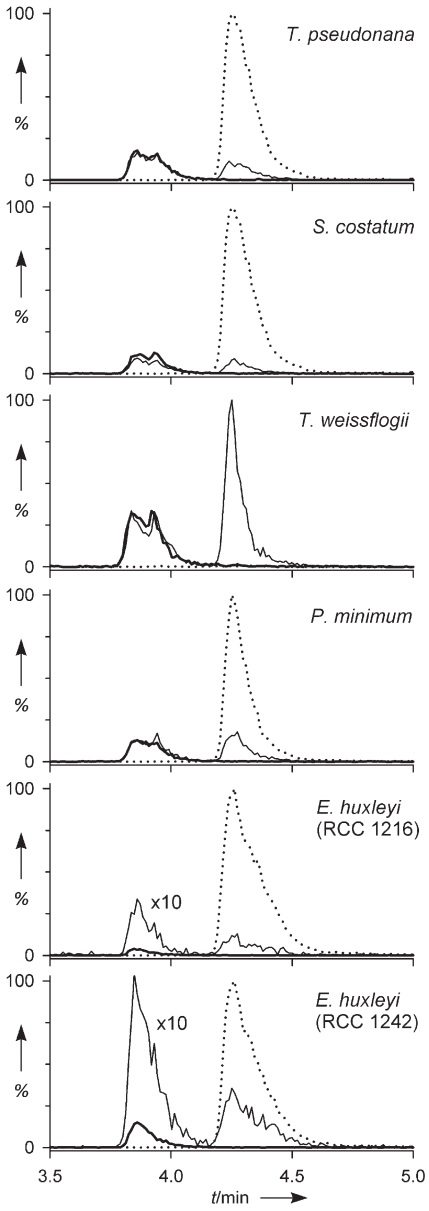

Figure 1. [$^{13}C_2D_6$]DMSP uptake in different phytoplankton cultures; depicted are the ion traces of [$^{13}C_2D_6$]DMSP (thin solid line, *m/z* 143) and of unlabeled DMSP produced by the cell (dotted line, *m/z* 135, both signals occur at the retention time 4.3 min). In the control cultures, no signal at *m/z* 143 was observed at the retention time of DMSP (bold solid line).

The most intense [$^{13}C_2D_6$]DMSP signal was observed for the diatom *T. weissflogii*, although this strain does not produce quantifiable amounts of DMSP (Figure 1).[18] This is in accordance with previous findings that non- or low producers of DMSP exhibit a pronounced uptake.[15] It might be speculated that *T. weissflogii* relies entirely on the uptake of DMSP, which is omnipresent in seawater, to fulfill cellular functions. The UPLC-MS data obtained with all cell extracts were also surveyed for potential DMSP transformation products, but no signals of labeled DMSP products other than [$^{13}C_2D_6$]DMSP were detected (data not shown). Thus, the uptake of DMSP occurs directly without previous transformation as indicated in the upper lane of Scheme 1. This supports the concept of an uptake through a zwitterion-selective transporter.[16] Interestingly, no metabolic activity leading to reversible transformation of DMSP in the cells takes place within the assay period; this indicates a slow or no turnover of cellular [$^{13}C_2D_6$]DMSP.

The uptake kinetics for cultures of *E. huxleyi* (RCC1242, 793000 cells per mL) and *T. weissflogii* (RCC76, 81000 cells per mL) were analyzed to obtain a better picture of transport processes. Therefore, the cells were treated with 200 nM [$^{13}C_2D_6$]DMSP, and uptake was determined within the first 60 min after this addition. Only [$^{13}C_2D_6$]DMSP was detected, and no isotope scrambling due to rapid metabolic processes occurred in either strain (Figure 2C and D). In the coccolithophore, the [$^{13}C_2D_6$]DMSP content per cell increased for 20 min and then reached a plateau (Figure 3A). Overall, 30% of the initially added [$^{13}C_2D_6$]DMSP was taken up by the cells. Both the unlabeled DMSP and the GBT content per cell were constant over the investigated time range of 60 min (Figure 3B and C). Thus, the [$^{13}C_2D_6$]DMSP does not replace the cellular DMSP, but is taken up in addition. The low proportion of [$^{13}C_2D_6$]DMSP taken up by *E. huxleyi* compared to the cellular concentration will not cause major differences in the osmomolarity of the cells, and thus a regulation to balance cellular processes might not be required.

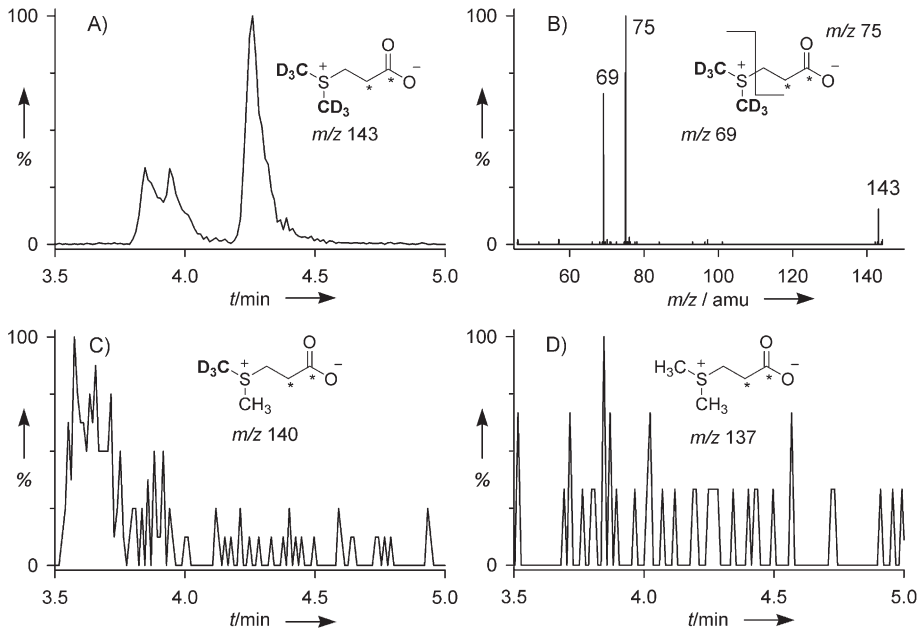

Figure 2. Uptake mechanism of DMSP. A) [$^{13}C_2D_6$]DMSP in a cell extract of *T. weissflogii*; B) MS-MS of the [$^{13}C_2D_6$]DMSP signal; C), D) ion traces of two possible transformation products show that DMSP is taken up directly.

A different kinetic was observed for DMSP uptake in the diatom *T. weissflogii*. Here, 60% of the supplied [$^{13}C_2D_6$]DMSP was already taken up after 2.5 min (Figure 4 A). Only a slight increase was observed for the other sampling points, and after 20 min approximately 70% of the externally administered [$^{13}C_2D_6$]DMSP was detected in the cells. In the steady state, *T. weissflogii* had a DMSP content of 1.73 fmol [$^{13}C_2D_6$]DMSP per cell; this is comparable to that of *E. huxleyi* (2.14 fmol DMSP per cell). However, *T. weissflogii* has a higher cell volume than *E. huxleyi*, so lower intracellular DMSP concentrations result in the diatoms. As observed in *E. huxleyi*, the GBT content per cell of *T. weissflogii* remained constant (Figure 4 B). It remains an open question how changes in osmolarity during this process are compensated for by the diatom.

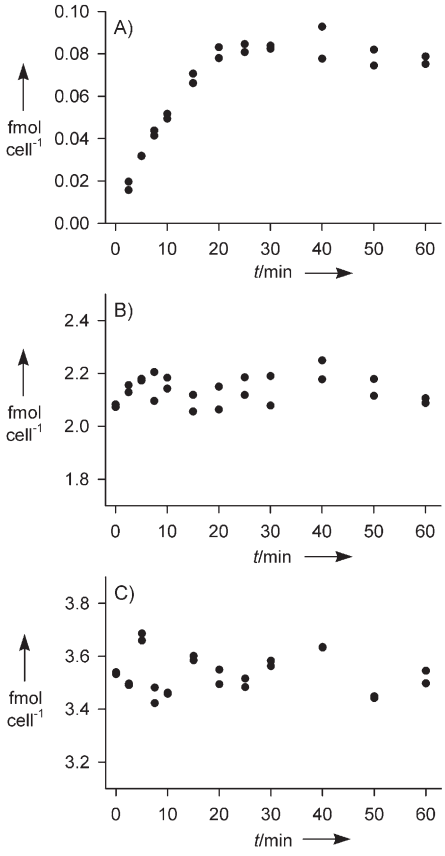

Figure 3. Kinetics of [$^{13}C_2D_6$]DMSP uptake in *E. huxleyi*. Monitoring of the A) [$^{13}C_2D_6$]DMSP, B) DMSP, and C) GBT content of the cells over the indicated time span after incubation with [$^{13}C_2D_6$]DMSP. Measurements were performed in duplicate.

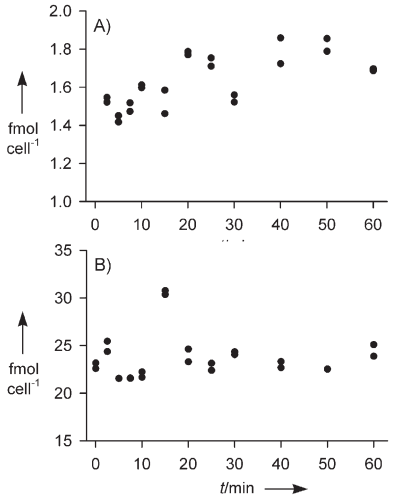

Figure 4. Kinetics of [$^{13}C_2D_6$]DMSP uptake in *T. weissflogii*. Monitoring of the A) [$^{13}C_2D_6$]DMSP and B) GBT content of the cells over the indicated time span after incubation with [$^{13}C_2D_6$]DMSP. Measurements were performed in duplicate.

No DMSP uptake was observed after 20 min for either culture. This has major implications for the determination of DMSP uptake rates. In previous studies, incubations of, for example, 5 min with the radioactive substrate were used to calculate DMSP uptake rates per hour.[16] According to our results, the uptake rate might be overestimated by this approach as a steady state is reached surprisingly quickly. This suggests a

highly active DMSP-uptake system that acts almost immediately after changes in the external DMSP concentrations occur.

Our results shed new light on the impact of phytoplankton on the fate of oceanic DMSP. It is interesting to note that no short- or long-term metabolism of the DMSP taken up by the cells occurred. In particular, the fact that the diatom *T. weissflogii*, which does not produce this metabolite, showed a fast uptake of considerable amounts of $[^{13}C_2D_6]$DMSP proves that algae can act as substantial sinks of this metabolite in the oceans.

Experimental Section

Details of the procedures for the synthesis of labeled DMSP and spectroscopic data for the intermediates and the end product as well as incubation and extraction procedures may be found in the Supporting Information.

Acknowledgements

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Keywords: dimethylsulfoniopropionate · liquid chromatography · mass spectrometry · phytoplankton · uptake mechanisms

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Supporting Information

Investigations of the Uptake of Dimethylsulfoniopropionate by Phytoplankton

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cbic_201100416_sm_miscellaneous_information.pdf

Equipment: For analytical separation an Acquity Ultraperformance LC (Waters, Milford, MA, USA) equipped with a Merck ZIC-HILIC column (3.5 µm, 2.1 x 100 mm, purchased from di2chrom, Marl, Germany) was used. The module was coupled to a Q-ToF micro mass spectrometer (Waters Micromass, Manchester, England).

Reagents: 3,4-Dihydro-2*H*-pyrane, 37% aq. formaldehyde and 3-(methylthio)propanal were purchased from Alfa Aesar (Germany). Triphenylphosphine, D_6-dimethylsulfide, iodomethane and ethyl bromoacetate were purchased from Sigma Aldrich (Germany).

Synthesis

Tetrahydro-*2H*-pyran-2-yl 1,2-$^{13}C_2$-2-bromoacetate (1): $^{13}C_2$-bromoacetic acid (0.285 g, 2 mmol) was dissolved in 3,4-dihydro-2*H*-pyrane (10 mL, previously purified by distillation over sodium). After 4 h stirring at room temperature, the solution was washed with $NaHCO_3$ (10% aq., 10 mL). The aqueous phase was extracted three times with diethyl ether (10 mL), dried over sodiumsufate, and the combined organic phases were evaporated under reduced pressure to give **1** (445 mg, 98%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.40-1.92 (m, 6H, CH_2), 3.85 (dd, ^1J(H,C)=153.32 Hz, ^2J(H,C)=4.57 Hz, 2H, CH_2), 3.61-3.80 (m, 1H, CH_2), 3.82-4.01 (m, 1H, CH_2), 6.04 (q, ^3J(H,H)=2.38 Hz, 1H, CH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 18.05, 24.67, 25.96 (d, ^1J(C,C)=65.18 Hz), 28.83, 63.07, 94.42, 165.99 (d, ^1J(C,C)=65.18 Hz); MS (EI, 70 eV): *m/z* 142 (41), 140 (41), 97 (85), 96 (54), 95 (100), 94 (64), 93 (13), 81 (52), 79 (54).

Tetrahydro-*2H*-pyran-2-yl 1,2-$^{13}C_2$-acrylate (2): To a vigorously stirred solution of triphenylphosphine (840 mg, 3.2 mmol) in dry dichloromethane (30 mL) **1** (430 mg, 1.9 mmol) was added quickly in dry dichloromethane (10 mL). After 50 s stirring at room temperature, a solution of formaldehyde (37% aq., 0.8 mL) and $NaHCO_3$ (10% aq., 12 mL) was added rapidly. The resulting mixture was stirred for another 2 h at room temperature. After extraction with ethyl acetate the solvent was removed under reduced pressure and the crude product was purified by column chromatography over silica gel 60 to give **2** (95 mg, 20%). R_f of **2** (petroleum ether/ethyl acetate 3:1) = 0.61. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.45-1.92 (m, 6H, CH_2), 3.63-3.76 (m, 1H, CH_2), 3.81-3.98 (m, 1H, CH_2), 5.85 (ddd, ^3J(H,H)=14.09, ^2J(H,C)=9.70, ^2J(H,H)=2.20 Hz, 1H, CH*H*), 6.13 (dddd, ^1J(H,C)=163.20 Hz, ^3J(H,H)=16.74 Hz, ^2J(H,C)= 10.34 Hz, ^2J(H,H)=4.39 Hz, 1 H, CH=C), 6.00 - 6.07 (m, 1H, CH), 6.40-6.50 (m, 1H, C*H*H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 18.59, 24.92, 29.17, 63.24, 92.85, 128.56 (d,^1J(C,C)=73.62

Hz), 130.85, 164.73 (d, ^{1}J(C,C)=73.61 Hz); MS (EI, 70 eV): *m/z* 85 (29), 84 (21), 74 (11), 57 (100), 56 (22), 55 (59).

3-([D_6]dimethylsulfonio)-1,2-$^{13}C_2$-propionate (3): Excess HCl(g) was bubbled through a well stirred solution of **2** (75 mg, 0.47 mmol) and [D_6]dimethylsulfide (0.05 mL, 0.68 mmol) in CH_2Cl_2 (0.5 mL) for 10 min at room temperature. Gaseous HCl was obtained by adding concentrated sulfuric acid to sodium chloride. The emerged white precipitate was filtered, dissolved in a very small volume of methanol and recrystallized by addition of diethyl ether to give **3** (39 mg, 39%) in a purity of >95% (NMR). ^{1}H NMR (400 MHz, CD_3OD): δ 2.96 (dtd, ^{1}J(H,C)=131.00 Hz, ^{3}J(H,H)=7.00 Hz, ^{2}J(H,C)=6.60 Hz, 2H, CH_2), 3.45-3.57 (m, 2H, CH_2); ^{13}C NMR (101 MHz, CD_3OD): δ 27.81 (d, ^{1}J(C,C)=56.74 Hz), 29.75 (d, ^{1}J(C,C)=56.74 Hz), 173.58 (d, ^{1}J(C,C)=55.21 Hz); MS (EI, 70 eV): *m/z* 125 (27), 74 (64), 68 (100), 57 (38), 50 (50), 46 (48), 27 (71).

DMSP analysis: For UPLC separation, an eluent system of water + 2% acetonitrile and 0.1% formic acid (solvent A) and 90% acetonitrile +10% water with 5 mmol L^{-1} ammonium acetate (solvent B) was used. Separation started with 100% B and a flow rate of 0.45 mL min^{-1} for 1.00 min. Within 5.50 min the proportion of A was increased to 80%. Then the gradient was set back to 100% B within 0.60 min. The column was equilibrated for 2.90 min, resulting in a total analysis time of 10 min. Injection of the sample solution (1-10 µL) was performed using a loop injector. The auto sampler temperature was held at 4 °C, the column temperature was set to 35 °C. Mass measurements were performed in the ESI-positive mode, recording the mass range from 105 to 200 *m/z* using a scan rate of 0.6 s and an inter-scan delay of 0.1 s. The following MS parameters were applied: capillary voltage 3000 V, sample cone 10.0 V, source temperature 120 °C, desolvation gas temperature 300 °C, collision energy 3.0 V, ion energy 1.8 V. For qualitative MSMS analysis collision energy was set to 10 V with a scan rate of 0.5 s.

Cultivation: Unialgal cultures of the diatoms *Thalassiosira weissflogii* (RCC76), *Thalassiosira pseudonana* (CCMP1335), *Skeletonema costatum* (RCC75), the dinoflagellate *Prorocentrum minimum* and the coccolithophore *Emiliania huxleyi* (RCC1242 and RCC1216) were propagated in autoclaved medium at 14 °C (diatoms) or 18 °C with a 14:10 light:dark cycle. Light was provided by Osram biolux lamps with an intensity of 40 µmol photons m^{-2} s^{-1}. The dinoflagellate and diatom cultures were cultivated in artificial seawater.[19] Medium for cul-

tures of *E. huxleyi* was prepared by dissolving 33.33 g L^{-1} HW sea salt professional (aquaristic.net, Babenhausen, Germany) in MicroPure water (MicroPure, TKA, Germany). 400 µL of separately autoclaved Seramis for foliage plants (MARS GmbH, Mogendorf, Germany) were added to this seawater preparation.

Sample preparation: For determination of DMSP uptake, 100 µL of 50 µmol L^{-1} aqueous [$^{13}C_2D_6$]DMSP solution was added to 40 mL of the respective phytoplankton culture. The addition was conducted five times on five consecutive days (day 1 to 5). On day 6, the cultures were gravity filtered on Whatman GF/C and the cells on the filter were washed with 20 mL of the respective medium. The filter was transferred into methanol. For each strain a control culture was treated in the same way without addition of [$^{13}C_2D_6$]DMSP.

For determination of DMSP uptake kinetics, 100 µL of a 200 µmol L^{-1} aqueous [$^{13}C_2D_6$]-DMSP solution was added to 100 mL of cultures of *E. huxleyi* and *T. weissflogii*, respectively. After 2.5, 5, 7.5, 10, 15, 20, 25, 30, 40, 50 or 60 min, 5 mL of the culture was vacuum filtered on Whatman GF/C (700 mbar). The filter was rinsed with medium (3 x 2 mL) and transferred into 0.7 mL methanol containing 100 µL of a 100 µmol L^{-1} aqueous gonyol solution as internal standard (synthesized, unpublished data). Extracts were stored at -80 °C. 100 µL of these extracts were diluted with 100 µL water/acetonitrile (10:90 *v/v*) and centrifuged (5 min, 16000 *g*). The supernatant was directly used for UPLC analysis. Standard solutions of $^{13}C_2D_6$-DMSP and gonyol were prepared in water/acetonitrile (10:90 *v/v*).

Manuscript C:

Mahasweta Saha, Martin Rempt, Björn Gebser, Jan Grueneberg, Georg Pohnert and Florian Weinberger (2012). "Dimethylsulphopropionate (DMSP) and proline from the surface of the brown alga Fucus vesiculosus inhibit bacterial attachment." Biofouling **28**(6): 593-604.

Summary: Bioassay guided fractionation of surface extracts of the brown algae *Fucus vesiculosus* revealed anti-attachment activity of the polar fraction. Structure elucidation via NMR and GC-MS led to the identification of the zwitterionic metabolite DMSP and the amino acids proline and alanine. Attachment experiments with different bacterial species, which were isolated from *F. vesiculosus* and other marine macroalgae, showed anti-attachment properties of DMSP. While natural concentrations of DMSP and proline inhibited attachment of bacteria that were not related to *F. vesiculosus*, the species *Rheinheimera baltica* and *Shewanella baltica*, which were isolated from *F. vesiculosus* showed no such effect. In contrast to the common belief that surface active compounds in aquatic environment need to be rather non-polar to increase persistence and its biological activity, it could be shown that also highly polar substances like DMSP and proline are effective fouling inhibitors. Furthermore, the low sensitivity of *R. baltica* and *S. baltica* to these metabolites suggests that they play an important role in formation and composition of species specific biofilms on the surface of macroalgae.

Publication equivalents of contributing PhD students as coauthors according to the implementing provision of the doctoral regulations at the Faculty for Chemistry and Earth Sciences of the Friedrich Schiller University Jena:

	Author 1: **Saha**	Author 2: **Rempt**	Author 3: **Gebser**	Author 4: **Grüneberg**
Conception of the work	X	-	-	-
Planning of experiments	X	-	-	-
Data collection	X	X	X	-
Analysis and interpretation	X	X	X	X
Writing of manuscript	X	-	-	-
Proposed publication equivalents	1	0,5	0,5	0

Biofouling: The Journal of Bioadhesion and Biofilm Research

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Dimethylsulphopropionate (DMSP) and proline from the surface of the brown alga Fucus vesiculosus inhibit bacterial attachment

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Dimethylsulphopropionate (DMSP) and proline from the surface of the brown alga *Fucus vesiculosus* inhibit bacterial attachment

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It was demonstrated previously that polar and non-polar surface extracts of the brown alga *Fucus vesiculosus* collected during winter from the Kiel Bight (Germany) inhibited bacterial attachment at natural concentrations. The present study describes the bioassay-guided identification of the active metabolites from the polar fraction. Chromatographic separation on a size-exclusion liquid chromatography column and bioassays identified an active fraction that was further investigated using nuclear magnetic resonance spectroscopy and mass spectrometry. This fraction contained the metabolites dimethylsulphopropionate (DMSP), proline and alanine. DMSP and proline caused the anti-attachment activity. The metabolites were further quantified on the algal surface together with its associated boundary layer. DMSP and proline were detected in the range 0.12–1.08 ng cm^{-2} and 0.09–0.59 ng cm^{-2}, respectively. These metabolites were tested in the concentration range from 0.1 to 1000 ng cm^{-2} against the attachment of five bacterial strains isolated from algae and sediment co-occurring with *F. vesiculosus*. The surface concentrations for 50% inhibition of attachment of these strains were always <0.38 ng cm^{-2} for DMSP and in four cases <0.1 ng cm^{-2} for proline, while one strain required 1.66 ng cm^{-2} of proline for 50% inhibition. Two further bacterial strains that had been directly isolated from *F. vesiculosus* were also tested, but proved to be the least sensitive. This study shows that DMSP and proline have an ecologically relevant role as surface inhibitors against bacterial attachment on *F. vesiculosus*.

Keywords: chemical defence; *Fucus*; DMSP; proline; anti-bacterial; antifouling

Introduction

Marine macroalgae produce a diverse range of chemical compounds that play a significant ecological role in the marine environment including protection against natural enemies (eg microbes and herbivores), settlement cues and competitive interactions (reviewed in Paul et al. 2011). Chemical compounds from seaweeds are also known to inhibit fouling organisms, including bacteria that are primary colonisers (reviewed in Qian et al. 2010).

Seaweeds provide a microniche rich in nutrients that promotes bacterial colonisation, which in turn can have detrimental effects on the host (Littler and Littler 1995; Sunairi et al. 1995; Sawabe et al. 1998; Vairappan et al. 2001). Thus, there should be a demand for defence against bacteria in seaweeds and chemical antifouling defence has been reported in a number of studies (eg de Nys et al. 1991; Schmitt et al. 1995; Brock et al. 2007). However, the ecological roles of specific algal inhibitors against microbes have only been demonstrated for a few species (eg Kubanek et al. 2003; Paul et al. 2006; Lane et al. 2009; Persson et al. 2011; Saha et al. 2011). Furanones from *Delisea pulchra* have been quantified and investigated as antifoulants (de Nys et al. 1998; Dworjanyn et al. 1999), while less is known about the role of other algal metabolites in terms of inhibiting colonisation by microbes (but see Paul et al. 2006; Nylund et al. 2008; Saha et al. 2011).

In the Western Baltic, the perennial rockweed *F. vesiculosus* occurs mainly between mean sea surface level and 3 m depth. A variety of abiotic stress factors are common in this habitat and some of these have been predicted to increase in the course of ongoing climate change. During the past decades, *F. vesiculosus* has retreated from the deeper parts of its former distribution range in the Baltic Sea (Vogt and Schramm 1991), which is presumably due to the combined action of eutrophication and epibiosis (Rohde et al. 2008). The jeopardised existence of this alga in the course of climate change makes it important to understand its interaction with potential fouling organisms in detail.

In spite of high bacterial densities in the Kiel fjord [$0.7–2.24 \times 10^6$ ml^{-1} seawater (mean of monthly samplings between 2005 and 2008), H.J. Hoppe and R. Koppe, personal communication], *F. vesiculosus* still

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manages to remain largely free from fouling by bacteria as well as macrofouling during most times of the year (Wahl et al. 2010). This observation suggests the deployment of chemical defence metabolites (Brock et al. 2007; Saha et al. 2011), which may be produced either by the alga itself or by its surface associated biofilm.

Non-polar compounds have mainly been reported as algal antifoulants (Schmitt et al. 1995; de Nys et al. 1998; Nylund et al. 2008; but see Harder et al. 2004 for a report on polar compounds). This may be due to the fact that non-polar compounds have a higher chance of being accumulated at algal surfaces than polar compounds (Jennings and Steinberg 1997), which in turn may have a higher probability of diffusion into the surrounding water column in absence of strong interionic interactions or hydrogen bonds.

A previous study on the chemical defence of *F. vesiculosus* revealed the presence of surface associated non-polar and polar active metabolites against bacterial attachment (Saha et al. 2011). Both the hexane fraction (containing mostly dissolved non-polar metabolites) and the methanol fraction (containing relatively polar dissolved metabolites) of the surface extract of *F. vesiculosus* inhibited bacterial attachment strongly and to a similar extent, while the water fraction was less efficient (Saha et al. 2011). Investigation of the non-polar extract has led to the identification of the compound fucoxanthin as inhibitor of bacterial attachment in *F. vesiculosus* (Saha et al. 2011).

The present study focuses on the identification of the metabolites responsible for the anti-attachment activity in the MeOH fraction of the initial surface extract of *F. vesiculosus* and the assessment of their possible ecological role as natural anti-bacterial deterrents. Bioassay-guided fractionations of the MeOH fraction were used, in order to identify extractable polar metabolites that inhibited bacterial attachment. Isolated bacteria from *F. vesiculosus* and also from seaweeds co-occurring with *F. vesiculosus* were used as test organisms, in order to detect ecologically relevant antimicrobial effects. The concentrations of identified deterrent metabolites on the surfaces along with surface boundary layers, and in total tissue extracts of field collected *F. vesiculosus* were also determined. In addition, the contribution of bacteria and microalgae associated with the algal surface to deterrence was investigated.

Materials and methods

Algal material

For the purpose of fractionation and chromatography, 7.7 kg of *F. vesiculosus* (*ca* 100 individuals) were collected from the littoral zone of Kiel Fjord, Germany (54°26'N/10°11'E) in December 2009 and transported to the laboratory in a cool box. Prior to extraction, the plants were held in saturated seawater to avoid desiccation and damage. For surface quantification, young algal individuals (n = 4) with a maximum length of 10 cm were collected from Laboe, Germany (54°40′N/10°21′E) in March 2011. For the study of surface concentrations after elimination of associated bacteria, the material was collected from the littoral zone of Kiel fjord (54°33′N/10°16′E) in November 2011. The latter two samplings were processed immediately. For the purpose of quantifying active intracellular compounds, *F. vesiculosus* (n = 3) plants were collected from the littoral zone of Kiel Fjord (54°27′N/10°11′E), Germany in May 2012.

Bacteria

Cytophaga sp. KT0804 (isolated from *Halidrys siliquosa* and also detected on *Saccharina latissima*) and *Bacillus aquimaris* (isolated from *Halidrys siliquosa* and also detected on *Desmarestia aculeata* and *Ahnfeltia plicata*) were used in all the bioassays in order to screen the activity of extracts and compounds against these two representatives of Gram-negative and Gram-positive microorganisms, respectively. Five additional strains isolated from *F. vesiculosus*, neighbouring algal species and marine sediment were used to test pure DMSP and proline, viz. *Rheinheimera baltica*, *Shewanella baltica* (both isolated from *F. vesiculosus*), *Ulvibacter littoralis* (isolated from the brown alga *Fucus serratus*), Alteromonadaceae E1 (isolated from the red alga *Polysiphonia stricta*), and marine sediment bacterium ISA 7311 (isolated from marine sediment). (Strains were isolated and identified by F. Symanowski, unpublished data.) The bacterial strains were grown in nutrient enriched medium (5 g peptone + 3 g yeast in 1 l of filtered seawater) at 20°C and 16 psu. All strains were maintained as cryostocks at −80°C (Saha et al. 2011).

Extraction of metabolites

Prior to extraction, the algal fronds (except for the 7.7 kg algal material) were scanned for surface area quantification using the image analysis software Image J (National Institute of Health, Bethesda, Maryland, USA; for details see Saha et al. 2011). Since quantifying the surface area of 7.7 kg of algal material (used for the purpose of bioassay-guided fractionation) was impractical, the total surface area was calculated by multiplying the wet weight of 7.7 kg by 25.57 $cm^2\ g^{-1}$ (1 g of algal wet weight corresponded to *ca* 25.57 cm^2 (SD ± 1.88) of algal surface area, for details see Saha et al. 2011). For surface extraction, algal fronds were

dipped for 10 s into a stirred mixture of methanol (MeOH): hexane (1:1 v/v). This method has been previously identified as non-destructive (see Supplementary material in Saha et al. 2011). Larger thalli were cut prior to extraction and care was taken that the cut ends had no contact with the solvent in order to prevent leaching of intracellular compounds (Saha et al. 2011). The resulting extract was immediately filtered through GF/A filters (Whatman, $\varnothing = 15$ mm) to remove particles and the solvent was evaporated under reduced pressure at $< 20^{\circ}$C using a rotary evaporator. The resulting residue was redissolved in hexane to remove the non-polar metabolites that inhibit bacterial attachment, such as fucoxanthin. The step was repeated until the solvent appeared colourless, indicating redissolution of fucoxanthin. The remaining extract after this hexane treatment was taken up in MeOH to dissolve the more polar metabolites. The final residue after this MeOH treatment contained highly polar compounds and was dissolved in water (HPLC grade, Roth GmbH).

Bioassays

Newly inoculated liquid cultures of bacteria were incubated for 18–20 h until their optical density (OD) was in the range 0.5–0.8 ($\lambda = 600$ nm; Beckman Du® 650 spectrophotometer). The bacteria in suspension were transferred to 96 well plates (flat bottom, polystyrene, Greiner®) with the compounds, size-exclusion chromatography (SEC) fractions or solvent controls. The 96 well plates were incubated for 1 h on a shaking table (100 rpm) at 20°C. The bacterial suspension was then removed from the wells and unattached cells were eliminated by gently rinsing twice with sterile sea water (SSW) (16 psu, collected from the Kiel fjord). The attached cells were quantified by staining (10 min) with the fluorescent DNA–binding dye Syto 9 (0.005 mM) (Invitrogen, GmbH). The fluorescence was subsequently measured (excitation 477–491 nm, emission 540 nm) in a plate reader (Hidex Chamaeleon, Turku, Fi) as a proxy for bacterial cell attachment density.

Bioassay-guided fractionation with a surface volume based assay

The polar methanolic fraction was fractionated by size exclusion chromatography (SEC) on Sephadex LH20 (65 × 2 cm, Sigma-Aldrich, Germany) with isocratic MeOH elution (UV detector 254 nm, Biorad biologic chromatography system). The fractions were collected at time intervals of 5 min (flow rate 2.5 ml min^{-1}) and pooled on the basis of the observed UV peaks (Figure 1). The SEC fractions were tested at the

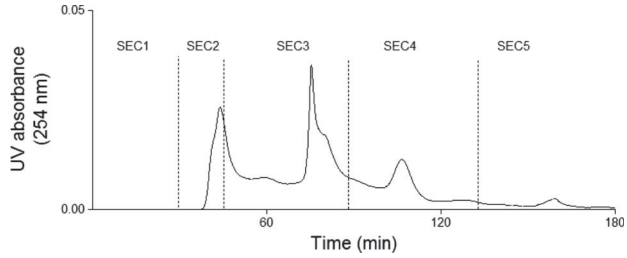

Figure 1. Size-exclusion fractionation of the MeOH fraction of surface extract of *F. vesiculosus*. Chromatographic parameters: Sephadex LH20 (2 × 65 cm); 1 ml injection; isocratic elution with MeOH at 2.5 ml min^{-1}. Vertical dotted lines indicate the fraction combination.

natural concentration that was present in the algal surface volume. This natural concentration was calculated as algal surface area × 30 μm (estimated thickness of the surface boundary layer). Ninety-seven μl of the bacterial suspension were added to the wells. Three μl of extract/SEC fractions (dissolved in DMSO) present at 33.3 times the natural concentration were added to the suspension so that the tested fractions in the final mixture were diluted to their natural concentration. Bacteria were exposed to DMSO concentrations of 3% and never > 5%, in order to prevent toxic effects (Saha et al. 2011). DMSO containing the fractionated solvent residue was taken as a control when the SEC fractions were tested. This was done to ensure that the presence of the solvent did not confound the results. To prepare the SEC solvent residue fractions, an equivalent amount of solvent containing no extract was fractionated in a similar manner to the MeOH fraction.

Structural elucidation of attachment inhibiting compounds

For nuclear magnetic resonance spectroscopy (NMR) the active SEC fraction was evaporated to dryness and dissolved in CD_3OD. Structural elucidation of DMSP was based on 1D- and 2D-NMR spectroscopy and comparison with synthetic DMSP hydrochloride. DMSP hydrochloride was synthesised by applying general procedures (see Chambers et al. 1987). This metabolite could, however, not explain all observed signals in the NMR (see Supplementary information, Figure S1). [Supporting material is available *via* a multimedia link on the online article webpage.]

The active LH20 fraction was further analysed by gas chromatography with mass spectrometric detection (GC-MS) after silylation in pyridine. The sample was evaporated to dryness and redissolved in 1 ml of pyridine. To this solution 10 μl of N-methyl-N-

trimethylsilyl-trifluoroacetamide (MSTFA) were added and the sample was heated for 1 h to 40°C and injected directly into the GC-MS system.

A GCT-Premier (Waters Micromass, UK) time-of-flight mass spectrometer coupled to an Agilent 6890N gas chromatograph was used for analysing the derivatised samples. Injection to the GC was done in split mode (split ratio 1). A Agilent DB5-MS column (29.5 m × 0.25 mm and 0.25 μm film) was used for separation at constant flow of 1 ml min^{-1} of helium 5.0, and 1 μl of the sample was injected into the system. The injector and transfer line were kept at 280°C. The oven temperature was initially held at 40°C for 5 min and then increased by 30°C min^{-1} to 305°C, and this temperature was held for 5 min. Metabolites were tentatively identified using the NIST library and the identity of the amino acids proline and alanine was verified by co-injection with derivatised commercially available amino acids.

Quantification of DMSP, proline and alanine

Quantification of DMSP in the surface extracts was done by liquid chromatography with mass spectrometric detection (LC-MS) according to Spielmeyer and Pohnert (2010). Briefly, the samples were diluted with 900 μl of water:acetonitrile (1:9, v/v) and centrifuged (5 min, 16,000 *g*). The supernatants were directly used for UPLC analysis by injection of 20 μl using a loop injector. For UPLC separation an Aquity UPLC™ BEH HILIC column (1.7 μm, 2.1 mm × 50 mm) and an eluent system of water +2% acetonitrile (solvent A) and acetonitrile (solvent B) was used. The solvent gradient (flow 0.25 ml min^{-1}) started with 10% A held for 0.40 min, set to 60% A at 0.41 min and held at this solvent composition for 1.70 min. At 1.71 min the flow rate was increased to 0.60 ml min^{-1} within 0.20 min to accelerate washing for 0.75 min. A Q-ToF Micro time-of-flight mass spectrometer (Waters Micromass, UK) was used for detection and quantification in ESI-positive mode.

Surface and intracellular quantification of DMSP for samples collected in November 2011 and May 2012, respectively, was done according to Spielmeyer et al. (2011). Quantification of proline (both surface and intracellular) and alanine (surface only) was done using the same LC-MS method after external calibration with three concentrations of the commercially available amino acids. The concentrations of the calibration standards were 0.074 μM, 0.74 μM, and 7.4 μM for alanine, and 0.049 μM, 0.49 μM and 4.9 μM for proline. Alanine was not quantified for samples treated with antibiotics. Total tissue quantification of DMSP and proline was also determined. The purpose of this was to investigate whether the surface and total tissue concentrations were correlated, and the total tissue concentrations were therefore expressed as ng cm^{-2} of surface of extracted algal thallus.

Surface area based test of attachment inhibiting components

After the identification of the attachment inhibiting components these were tested as a surface coating against bacteria. Proline, alanine, and the two control compounds valine and isoleucine were from Sigma Aldrich, Germany, while DMSP was prepared following Chambers et al. (1987). DMSP or proline (dissolved in MeOH), as well as alanine, valine or isoleucine (all dissolved in water) were pipetted into 96 well plates and the solvent was evaporated *in vacuo*, in order to coat the bottom and side walls of the wells with different surface area concentrations. A bacterial suspension of 108 μl (to the wells coated with DMSP and proline) or 100 μl (to the wells coated with alanine, valine and isoleucine) was added. MeOH or SSW were used as the solvent control, respectively. Replication was 4-fold for each tested concentration.

Estimating the microbial contribution of surface associated deterrents

Since epibiotic bacteria may contribute to surface extracted proline or DMSP, the concentrations of these compounds on the surface of *F. vesiculosus* were compared for individuals with different densities of associated microorganisms. In order to reduce the bacterial density, *F. vesiculosus* was treated with antibiotics. Three individuals were split into two parts of comparable wet weight and each part was maintained separately in a 3 l conical flask containing 2.5 l of sterilised filtered seawater. One part was supplemented with 100 ppm each of the antibiotics Vancomycin (Ratiopharm) and Cefotaxim (Hexal), while the second part was used as a control individual without antibiotics. All individuals were maintained for 4 days under continuous aeration at 16°C, with a 16:8 h (light/dark) regime at 20 μmol m^{-2} s^{-1}. The health status of the treated thalli was monitored daily. At the end of the experiment the algae were photographed for image analysis, surface extracted and DMSP and proline were subsequently quantified in the MeOH fraction as described above.

Prior to extraction, 1 cm^2 of the algal tips was swabbed with cotton tips, in order to count the associated bacteria and diatoms. The cotton tip was vortexed for 30 s in an Eppendorf vial containing 1 ml of SSW. The relative abundance of diatoms and any other possible photoautotrophs in a 100 μl subsample

was determined by measuring the fluorescence of chlorophyll *a* at 485 nm (excitation) and 677 nm (emission) in 96 well plates (Greiner®), using a plate reader. Subsequently, the relative density of all microfoulers (including bacteria and diatoms) was determined by staining all the particles in the same 100 μl subsample with the fluorescent DNA-binding dye Syto 9 (Invitrogen GmbH). After addition of Syto 9 at a final concentration of 0.005 mM and incubation for 10 min in darkness the fluorescence was measured (excitation 477–491 nm, emission 540 nm), using the same plate reader. The relative abundance of diatoms (and other possible photoautotrophs) was subtracted from this value, in order to determine the relative bacterial abundance amongst treated and non-treated individuals.

Statistical analysis

The activity strength of the extracts, SEC fractions and the individual compounds was expressed as the log effect ratio. A log effect ratio value of 0 (ie equal number of bacteria in wells with extract and in wells without extract) indicates that the tested extract had no effect on attachment, whereas a negative log effect ratio value indicates an inhibitory and a positive log effect ratio value indicates an attractive effect, respectively. Thus, a log effect ratio of -1 represents a 10 fold reduction whereas a value of $+1$ represents a 10 fold enhancement of bacterial settlement due to the extract.

1-way-ANOVA was conducted to compare the log effect ratio distributions. Homogeneity of variances was tested using Levene's test ($p < 0.05$). Shapiro-Wilk's test was used to test for normal distribution ($p < 0.05$). *Post hoc* comparisons were made using Tukey's honest significant difference test (HSD, $p < 0.05$). The t-test was used to compare bacterial abundances amongst anti-attachment treated and non-treated *F. vesiculosus* individuals. The computer program Statistica, (StatSoft, Tulsa, OK, USA) was used to conduct all statistical tests, including correlation analyses of proline and DMSP surface concentrations and the abundances of bacteria and microalgae. For the analysis of the effects of surface coated compounds, functions that described best fits of the data were computed by iterative adaptation, using the software package Prism 4 (GraphPad, La Jolla, CA) and the logistic function $Y = \text{Min} + (\text{Max-Min})/(1 + 10\hat{}(\log(EC_{50})\text{-X}))$. In this function X and Y represent compound concentration and attachment response, respectively. Min, Max and EC_{50} are constants describing minimal and maximal responses and the necessary concentration required for 50% inhibition of attachment, respectively.

Results

Bioassay-guided fractionation of the polar fraction

The SEC of the *F. vesiculosus* MeOH fraction, yielded five fractions, SEC1 to SEC5 (Figure 1), out of which SEC3 produced a significant inhibitory effect on bacterial attachment (*Cytophaga* sp. KT0804, $n = 3$, $F = 12$, $p < 0.001$; *B. aquimaris*, $n = 3$, $F = 5$, $p < 0.02$, one-way ANOVA, Tukey's HSD, Figure 2).

Structure elucidation of attachment inhibiting compounds

The ^{1}H-NMR spectrum of fraction SEC3 (Supplementary Figure S1) [Supplementary material is available *via* multimedia link on the online article webpage] exhibited signals characteristic for DMSP. HSQC, COSY and HMBC measurements confirmed this structure. The structure was verified by comparison with an authentic standard. The structural elements of two amino acids were also detectable in the ^{1}H-NMR-spectrum of the active fraction. A comparison of the chemical shifts of two spin systems suggested the presence of alanine and proline. The comparison of the proton and carbon shifts showed good accordance with literature values. No indication for a peptide bond of DMSP with an amino acid could be detected in the HMBC data, suggesting a mixture of these three dominant metabolites. The mass spectra and retention times were in perfect consonance with amino acid standards derivatised according to the same protocol (Supplementary Figures S2 and S3). [Supplementary material is available *via* multimedia link on the online article webpage.]

Surface quantification and activity of DMSP and free amino acids

The natural concentrations of DMSP, proline and alanine were found to be in the range of 0.12–1.08 ng

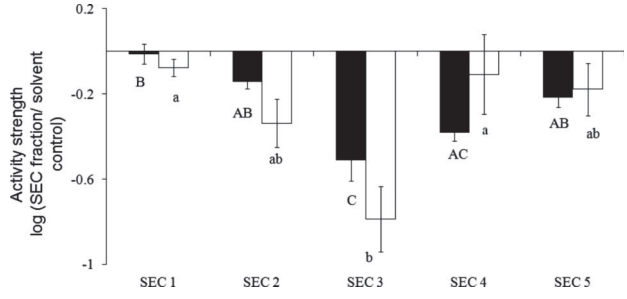

Figure 2. Anti-attachment activity of the SEC fractions (mean $\pm$ SE, $n = 3$). Different capitals and small letters indicate significantly different treatment responses of *Cytophaga* sp. KT0804 (■) and *B. aquimaris* (□) (Tukey's test, $\alpha = 0.05$).

cm^{-2} (mean 0.40 ng ± SD 0.45), 0.09–0.59 ng cm^{-2} (mean 0.45 ng ± SD 0.43) and 0.09–1.25 ng cm^{-2} (mean 0.47 ng ± SD 0.54), respectively. The surface coated DMSP inhibited the attachment of six out of seven of the tested strains in the concentration range 0.1–1000 ng cm^{-2} (Figure 3i and ii). Based on best-fitting logistic functions the maximal attachment inhibition of five isolates from co-occurring algae and sediment was in the range −0.08 to −0.59. The necessary dose of DMSP for 50% attachment inhibition of three of these isolates was <0.38 ng cm^{-2}, while two isolates responded maximally at the lowest concentration that was tested (0.1 ng cm^{-2}), so that an EC_{50} could not be computed (Table 1). The three most sensitive strains were *U. littoralis* and Alteromonadaceae E1, which were maximally inhibited with respect to attachment at all DMSP concentrations tested, and *B. aquimaris*, which showed the strongest maximal inhibition. The two least sensitive strains were those that had been isolated from *F. vesiculosus*, viz. *Rheinheimera baltica* and *Shewanella baltica*. While *S. baltica* was only slightly inhibited at concentrations <1000 ng cm^{-2}, DMSP had a weak pro-attachment effect on *R. baltica* (Figure 3ii). A relatively weak inhibition was also observed with strain ISA 7311.

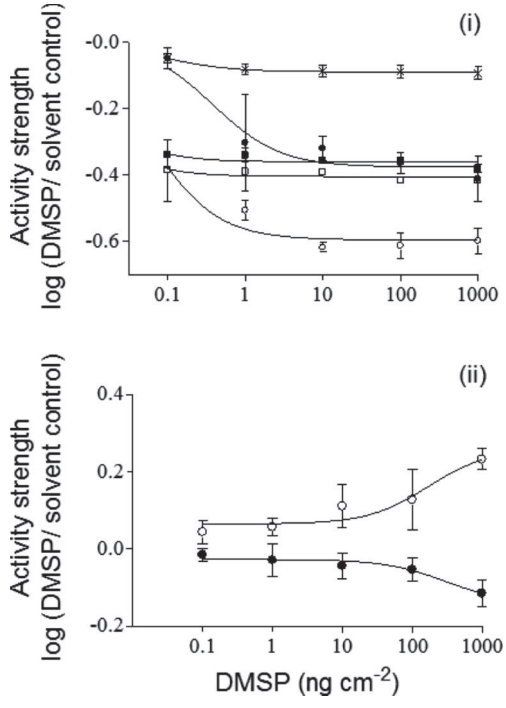

Figure 3. Anti-attachment activity of surface coated with DMSP against (i) *Cytophaga* sp. KT0804 (●), *B. aquimaris* (○), *U. littoralis* (□), Alteromonadaceae E1 (■), and marine sediment bacterium ISA 7311 (X) and (ii) *R. baltica* (○) and *S. baltica* (●). Mean ± SE, n = 4, lines represent best fitting logistic functions. See Table 1 for details of best fitting functions shown in (i).

Surface coated proline also inhibited the attachment of most of the tested strains in the concentration range 0.1–1000 ng cm^{-2} (Figure 4i). The maximal attachment inhibition of the five isolates from co-occurring algae and sediment was in the range −0.12 to −0.55. The required dose of proline for 50% inhibition of attachment of the same isolates was computed to be always <1.66 ng cm^{-2} (Table 2). *U. littoralis*, Alteromonadaceae E1 and *B. aquimaris* were the three most sensitive strains and responded nearly maximally over the whole concentration range (Figure 4i). Also, as with DMSP, *R. baltica* was not deterred, but attracted by proline, and the second bacterial isolate that originated from *F. vesiculosus* was only weakly inhibited (Figure 4ii), similar to strain ISA 7311 (Figure 4i). Alanine inhibited the attachment of *Cytophaga* sp. KT0804 and *Bacillus aquimaris* in a similar manner when tested in the concentration range 0.984–984 ng cm^{-2} (Figure 5). Based on best-fitting logistic functions the maximal attachment inhibition of *Cytophaga* sp. KT0804 and *B. aquimaris* by alanine was −0.28 and −0.25, respectively and the required dose for 50% inhibition of attachment was 18.11 and 9.72 ng cm^{-2}, respectively (Table 3).

Valine and isoleucine did not show any significant anti-attachment activity. Rather, valine had a tendency to promote bacterial attachment at lower concentrations, particularly in *Cytophaga* sp. KT0804. Isoleucine had a strong attachment promoting effect on *Cytophaga* at higher concentrations and on *B. aquimaris* at lower concentrations (Supplementary Figures S4 and S5). [Supplementary material is available *via* multimedia link on the online article webpage.]

Intracellular quantification of DMSP and proline

The total tissue concentrations of DMSP and proline were found to be in the range 506.2–876.2 ng cm^{-2} (mean 683.53 ng cm^{-2} ± SD 185.49) and 25.03–52.06 ng cm^{-2} (mean 39.47 ng cm^{-2} ± SD 13.6), respectively.

Effect of microorganisms on the surface concentrations of proline and DMSP

Algal fronds that had been treated with antibiotics did not look unhealthy or morphologically different when compared to the untreated controls. Although there was a reduction in the relative bacterial cell abundance in individuals of *F. vesiculosus* treated with antibiotics when compared to the controls (data not shown), there was no statistically significant difference in the relative bacterial abundance (t-test, $p > 0.05$). The abundance of microorganisms was negatively correlated with the surface concentration of proline (Figure 6i, $r^2 = -0.97$,

Table 1. Maximal attachment inhibition by DMSP and necessary concentration of DMSP for halfmaximal inhibition (EC_{50}) as computed from best fitting logistic functions for five different bacterial isolates.

Strain tested	*B. aquimaris*	*Cytophaga* sp. KT0804	*U. littoralis*	M.s.b. ISA7311	Alteromonadaceae *E1*
Maximal attachment inhibition [log (DMSP/ solvent control)]	−0.59 (−0.64 to −0.54)	−0.37 (−0.46 to −0.28)	−0.40 (−0.41 to −0.39)	−0.08 (−0.10 to −0.07)	−0.36 (−0.37 to −0.34)
EC_{50} [ng cm^{-2}]	0.05 (0.02 to 0.12)	0.38 (0.06 to 2.15)	<0.1	0.08 (0.01 to 0.41)	<0.1
r^2 of fit	0.45	0.44	0.32	0.21	0.12

Note: 95% CI in brackets; no EC_{50} could be computed when approximately full inhibition was observed at the lowest tested concentration (0.1 ng cm^{-1}); M.s.b. = Marine sediment bacterium.

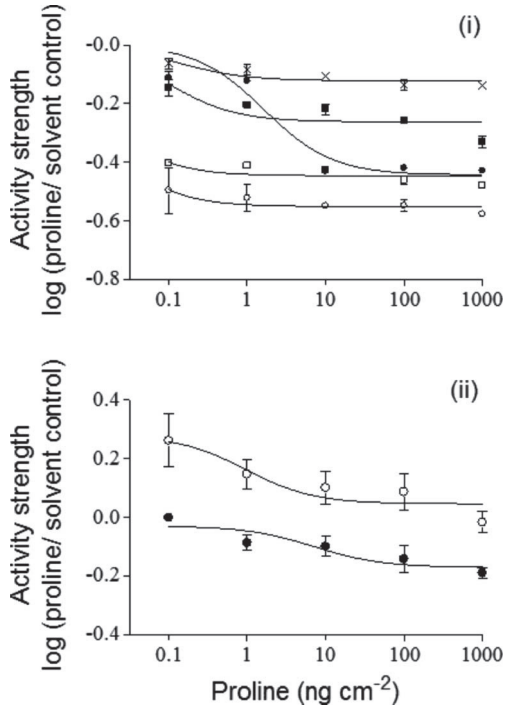

Figure 4. Anti-attachment activity of surface coated with proline against (i) *Cytophaga* sp. KT0804 (●), *B. aquimaris* (○), *U. littoralis* (□), Alteromonadaceae E1 (■) and marine sediment bacterium ISA 7311 (X) and (ii) *R. baltica* (○) and *S. baltica* (●). Mean ± SE, n = 4, lines represent best fitting logistic functions. See Table 2 for details of best fitting functions shown in (i).

$p < 0.05$) and not correlated with the surface concentration of DMSP (Figure 6ii, $r^2 = 0.11$, $p > 0.05$). Additionally, the abundance of microalgae was not correlated with the surface concentration of proline (Figure 6iii, $r^2 = 0.26$, $p > 0.05$) and also not correlated with the surface concentration of DMSP (Figure 6iv, $r^2 = 0.25$, $p > 0.05$).

Discussion

Bioassay-guided fractionation of the methanolic fraction of *F. vesiculosus* resulted in the identification of three relatively polar metabolites, viz. DMSP, proline and alanine. DMSP and proline reduced bacterial attachment when they were tested at their natural concentrations. Thus, there was no complete inhibition of bacterial attachment, but the active metabolites were efficient enough to reduce bacterial attachment in a selective manner. This corresponds with the fact that *F. vesiculosus* is never completely free of associated epibionts. Even in the least epiphytised specimens, ~5% of the surface is covered by a sparse biofilm (Wahl et al. 2010).

Despite the fact that the NMR indicated that proline, alanine and DMSP were present in surface extracts at nearly identical concentrations, no indication of a peptide linkage between the metabolites was detected in the NMR and MS data. That the amino acids result from the cleavage of a peptide initially produced by the alga or by epibiotic microorganisms cannot be completely excluded. Such cleavage would, however, not be due to the sample handling, since NMR analysis of the reference peptide captopril that underwent a similar treatment as the extract, showed no hydrolysis during the procedure (data not shown). Proline and several other free amino acids have previously been detected at the surface of *F. vesiculosus* (Lachnit et al. 2010) and the presence of DMSP in tissue of *F. vesiculosus* has also been reported (Howard and Russell 1995; Lyons et al. 2007).

The methionine derived secondary metabolite DMSP has several different physiological and ecological functions in marine algae. DMSP along with its associated compounds has so far been shown to function as a cryo-protectant and as an anti-oxidant (Van Alstyne 1988; Karsten et al. 1996; Sunda et al. 2002). Enzymatic cleavage of DMSP leads to the production of dimethlysulphide (DMS) and acrylic acid (Cantoni and Anderson 1956) and may be catalysed by numerous marine bacterial taxa (Howard et al. 2008). Furthermore, DMS and acrylic acid play a role in the anti-grazing defence of marine algae (Van Alstyne et al. 2001; Lyons et al. 2007). The present

Table 2. Maximal attachment inhibition by proline and necessary concentration of proline for halfmaximal inhibition (EC_{50}) as computed from best fitting logistic functions for five different bacterial isolates.

Strain tested	*B. aquimaris*	*Cytophaga* sp. KT0804	*U. littoralis*	M. s. b. ISA7311	Alteromonadaceae *E1*
Maximal attachment inhibition [log (proline/ solvent control)]	−0.55 (−0.59 to −0.50)	−0.44 (−0.48 to −0.40)	−0.44 (−0.46 to −0.43)	−0.12 (−0.14 to −0.10)	−0.26 (−0.29 to −0.23)
EC_{50} [ng cm^{-2}]	<0.1	1.661 (0.94 to 2.9)	<0.1	0.13 (0.04 to 0.40)	<0.1
r^2 of fit	0.08	0.87	0.31	0.38	0.47

Note: 95% CI in brackets; no EC_{50} could be computed when approximately full inhibition was observed at the lowest tested concentration (0.1 ng cm^{-1}); M.s.b. = Marine sediment bacterium.

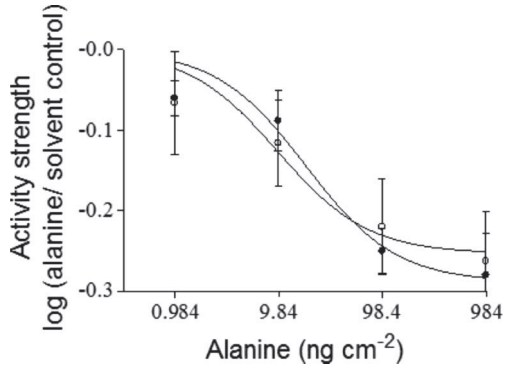

Figure 5. Anti-attachment activity of surface coated with alanine against *Cytophaga* sp. KT0804 (●) and *B. aquimaris* (○). Mean ± SE, n = 3, lines represent best fitting logistic functions. See Table 3 for details of best fitting functions.

Table 3. Maximal attachment inhibition by alanine and necessary concentration of alanine for halfmaximal inhibition (EC_{50}) as computed from best fitting logistic functions for five different bacterial isolates.

Strain tested	*B. aquimaris*	*Cytophaga* sp. KT0804
Maximal attachment inhibition [log (alanine/ solvent control)]	− 0.25 (−0.35 to −0.15)	−0.28 (−0.36 to −0.21)
EC_{50} [ng cm^{-2}]	9.72 (1.18 to 79.93)	18.11 (5.06 to 64.8)
r^2 of fit	0.42	0.73

Note: 95% CI in brackets.

study reports for the first time the surface based role of DMSP as an inhibitor of bacterial attachment. An earlier study by Jackson and Stukey (2007) on the cord grass *Spartina alterniflora* ruled out an effect of DMSP as anti-foulant on epiphytic algae. A spatial, temporal and taxonomic variation in the presence of this sulphonium compound amongst macroalgae has been reported (Van Alstyne and Puglisi 2007; Lyons et al. 2010). DMSP has been quantified in studies of macroalgae and coral reef invertebrates (Howard and Russell 1995; Van Alstyne et al. 2006; Lyons et al. 2010) and has repeatedly been reported to be present in green algae. The quantification studies with macroalgae were based on the total dry weight and to the authors' knowledge no concentration determination in the surface boundary layer has so far been demonstrated. DMSP concentrations ranging from 0.12 to 1.08 ng cm^{-2} were found for *F. vesiculosus* in the present study. The attachment of five tested bacterial strains not originating from *F. vesiculosus* was inhibited by 50% when DMSP was present at surface concentrations within or below this range. In contrast, the compound proved to have at this concentration range no effect on two bacterial isolates originating from *F. vesiculosus*. Rather, DMSP promoted the attachment of one of these isolates, *R. baltica*, at concentrations of 10 ng cm^{-2} or more, which corresponds with the fact that certain bacteria are known to be attracted to microscale pulses of DMSP (Seymour et al. 2010), while some use DMSP as a dominant nutrient and metabolise it very quickly (Dickschat et al. 2010). Many microalgae and green algae are known to be DMSP producers (eg Kasamatsu et al. 2004). However, no positive correlation amongst relative microalgal or bacterial abundance and DMSP was detected in the present study, suggesting that associated diatoms or other microorganisms were not responsible for the presence of DMSP on the surfaces of *F. vesiculosus*.

Several studies have demonstrated the anti-microbial activity of proline rich peptides (eg Yang et al. 2009). In the present study an anti-bacterial role of the free amino acid, surface associated in *F. vesiculosus* at concentrations of up to 0.59 ng cm^{-2}, has been found. Four out of the five tested bacterial strains (not *Cytophaga* sp.) not originating from *F. vesiculosus* were inhibited by at least 50% when proline was present at 0.59 ng cm^{-2}, which shows that this amino acid is

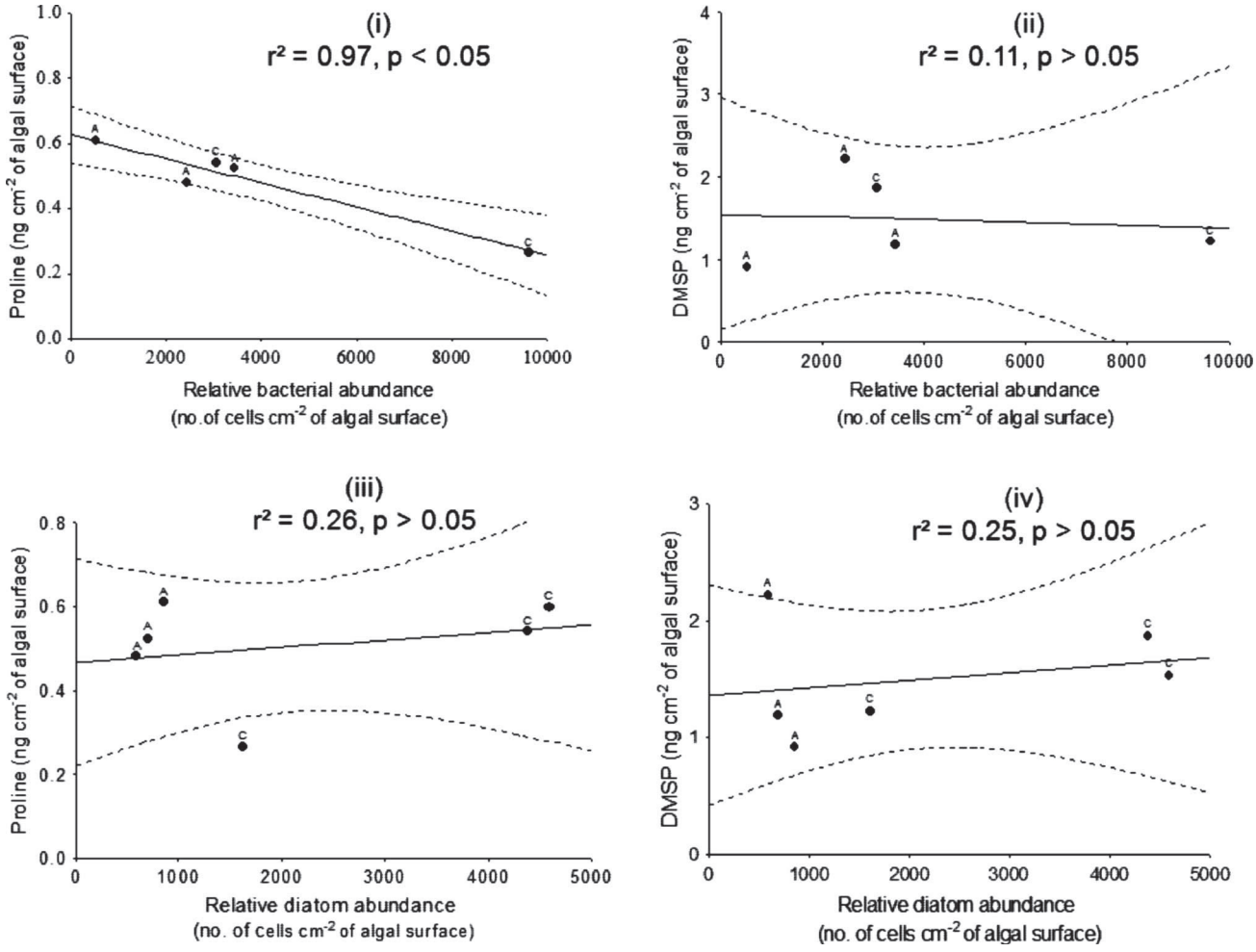

Figure 6. Relationships between surface proline (i, iii) and DMSP (ii, iv) concentrations and the relative abundance of bacteria (i, ii) and microalgae (iii, iv) on *F. vesiculosus*. Individuals treated (A) and non-treated (C) with antibiotics were compared. Straight lines indicate best fitting linear functions; dotted lines indicate 95% CI.

clearly present at a sufficient concentration to deter bacterial attachment on the algal surface. On the contrary, the attachment of *R. baltica* and *S. baltica* (isolated from the surfaces of the alga itself) was promoted and weakly inhibited, respectively, when proline was present at natural concentration, corresponding to the fact that several bacteria are known to use free amino acids as a suitable source of carbon (eg Kim et al. 2009). A significant negative correlation was observed amongst bacterial abundance and proline concentration, which indicates that surface associated bacteria are probably not major contributors of proline. Likewise, the densities of microalgae were also not positively correlated with proline concentrations on the *F. vesiculosus* surfaces.

Lane et al. (2009) investigated the localisation of anti-fungal compounds, (bromophycolides) from the red alga *Callophycus serratus*. Probing of the algal surface revealed that these compounds were present only on distinct light coloured surface patches (and not on other areas of the algal surface) at sufficiently high concentrations to inhibit fungal growth (Paul et al. 2011). The authors hypothesized that the alga maintains these compounds internally and releases them at distinct surface sites. Both DMSP and proline are known to function as algal osmolytes (Edwards et al. 1987; Yoshiba et al. 1997). Thus, it might be expected that these compounds are more concentrated in the whole algal tissue in comparison with the surface. Secretion of DMSP and proline by *F. vesiculosus* or any other alga has not been reported previously. In the present study, DMSP and proline were about 1,700 and 90 times more concentrated in the total tissue in comparison with the surface. The surface presence of DMSP and proline might result from their secretion by *F. vesiculosus* or because of their leakage from cells that are damaged by natural causes (eg grazing or microbial attack). In any case, the different ratios of

tissue and surface concentrations of DMSP and proline indicate that both compounds are released to the surface by different mechanisms.

Alanine inhibited the attachment of *Cytophaga* sp. and *B. aquimaris* by 50% at concentrations of 18.11 and 9.72 ng cm^{-2}, respectively. This amino acid only has a feeble anti-attachment effect when compared to DMSP and proline, given that a maximal concentration of 1.25 ng cm^{-2} has been detected on *F. vesiculosus*. In contrast to proline and alanine, valine and isoleucine had an attachment promoting effect, which seems to suggest that the anti-bacterial activity of the former two amino acids is specific and not a general feature of amino acids. However, these attachment promoting effects were detected at lower rather than higher concentrations, which suggests interacting attachment reducing effects at higher concentrations.

The effects of DMSP and proline apparently vary when different bacterial species or strains are tested. Bacteria isolated from *F. vesiculosus* were generally the least sensitive towards both compounds. Interestingly, the same strains were also relatively insensitive towards fucoxanthin, a third attachment inhibiting compound from *F. vesiculosus* (Saha et al. 2011). Thus, the variable sensitivity of bacteria towards DMSP, proline and fucoxanthin apparently results in a specific bacterial community on the algal surface, which corresponds with the observation that bacterial communities associated with *F. vesiculosus* are indeed specific (Lachnit et al. 2009). An additional selective effect on the composition of bacterial communities associated with *F. vesiculosus* certainly results from growth inhibiting components that are also present in surface extracts of the alga (Wahl et al. 2010), but have so far not been identified.

It is commonly believed that surface active compounds should be non-polar in order to be effective as fouling inhibitors in the aquatic environment as such metabolites are more likely to persist over prolonged time periods on surfaces (Jennings and Steinberg 1997). However, the effectiveness of DMSP and proline as microbial inhibitors shows that this view is too simplistic. The compounds were active not only when tested together (SEC3 fraction) at surface boundary layer concentrations, but also when tested singly in terms of surface area concentration, thus proving their efficacy. The retention time even of polar compounds may be prolonged by the presence of interionic interactions and hydrogen bonds within the surface boundary layer or by mucus at the algal surface (Jennings and Steinberg 1997), which could increase the efficiency of polar inhibitors. A mechanism of controlled release (eg Salgado et al. 2008) may even be effective with more polar antifoulants. Indeed, compared to less polar algal surface associated attachment inhibitors DMSP, proline and alanine all reach relatively low concentrations in the range between 0.09 and 1.25 ng cm^{-2}. In contrast, fucoxanthin was detected at concentrations between 700 and 9,000 ng cm^{-2} (Saha et al. 2011), furanones from *Delisea pulchra* at 100 to 500 ng cm^{-2} (de Nys et al 1998; Dworjanyn et al. 1999) and 1,1,3,3-tetrabromo-2-heptanone in *B. hamifera* at 3600 ng cm^{-2} (Nylund et al. 2008). This difference between polar and non-polar compounds may perhaps be due to differential solubilities in the surrounding water. Nonetheless, the polar compounds identified here are obviously sufficiently concentrated to affect associated microorganisms. The mean concentration of fucoxanthin on *F. vesiculosus* surfaces is $\sim$8,000 times higher than that of DMSP, but at the same time the necessary dose of fucoxanthin for inhibition of bacterial settlement is 19,000 times higher (Saha et al. 2011). Despite its higher polarity DMSP thus seems to be a relatively more efficient anti-bacterial compound on *F. vesiculosus* surfaces than fucoxanthin. Obviously, a high bioactivity may compensate for a low concentration of a compound.

In conclusion, this study shows that DMSP and proline, along with the non-polar metabolite fucoxanthin (Saha et al. 2011), have an ecologically relevant role as natural antibacterial compounds in *F. vesiculosus*. This alga thus uses a multiple defence strategy against microfoulers. Interestingly, all the deterrents detected so far in this alga are relatively widespread or even universally present among macroalgae and it may therefore be expected that they could be relevant for the antimicrobial defence of other species. This is particularly the case for DMSP, tissue concentrations of which reach more than 10 times higher in green seaweeds than in *F. vesiculosus* (Lyons et al. 2010) and could thus potentially be more concentrated on green algal surfaces.

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Supplementary Information

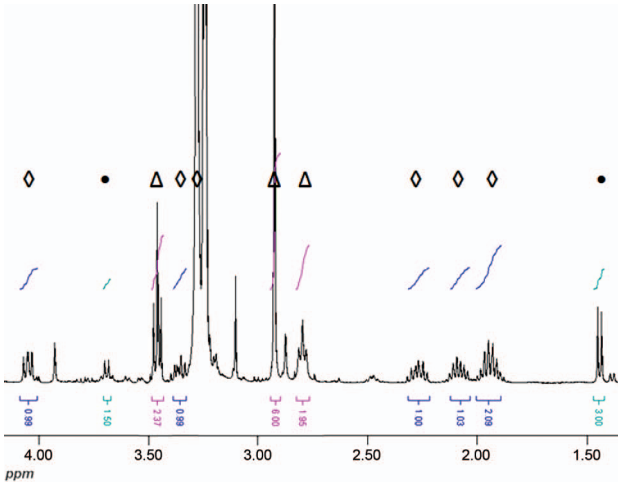

Figure S1. ^{1}H-NMR-spectrum (600MHz, CD_3OD) of the active LH20 fraction: alanine (●), proline (◇), DMSP (Δ). One proton of proline falls together with the solvent signal. Given values of integrals represent intensities within the compounds, not between different compounds.

DMSP
^{1}H-NMR (600 MHz, CD_3OD) δ ppm 3.49 (t, J = 6.77 Hz [2H]), 2.93 (s, [6H]), 2.74 (t, J = 6.79 Hz [2H]).
^{13}C-NMR (150 MHz, CD_3OD) δ ppm 175.08, 41.88, 30.21, 26.33

Proline
^{1}H-NMR (600 MHz, CD_3OD) δ ppm 4.05 (dd, J = 8.48, 6.61 Hz [1H]), 3.42–3.34 (m [1H]), 3.22–3.17 (based on COSY and HSQC), 2.28 (m [1H]), 2.09 (m [1H]), 2.01–1.88 (m [2H]).
^{13}C-NMR (150 MHz, CD_3OD) δ ppm 176.75, 62.46, 47.38, 30.24, 24.95.

Alanine
^{1}H-NMR (600 MHz, CD_3OD) δ ppm 3.67 (m [1H]), 1.45 (d, J = 7.20 Hz [3H]).
^{13}C-NMR (150 MHz, CD_3OD) δ ppm 176.28, 51.59, 17.00.

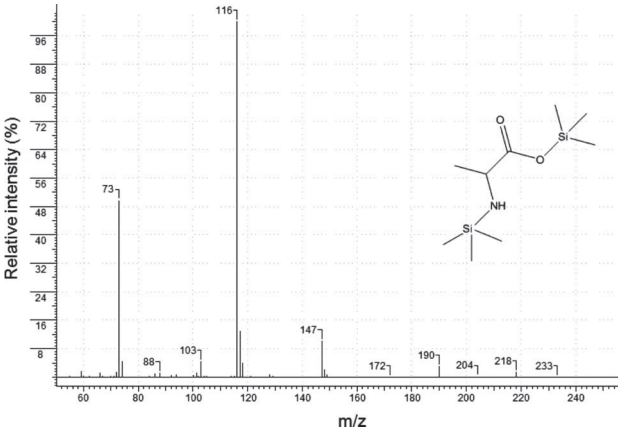

Figure S2. Mass spectrum of alanine derivatised to (*S*)-trimethylsilyl 2-(trimethylsilylamino) propanoate.

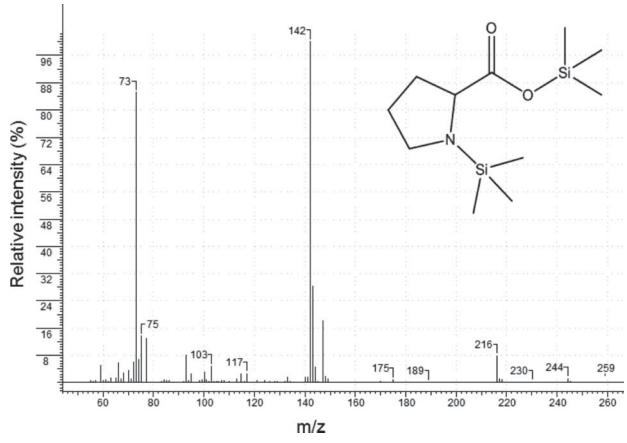

Figure S3. Mass spectrum of proline derivatised to (*S*)-trimethylsilyl 1-(trimethylsilyl) pyrrolidine–2-carboxylate.

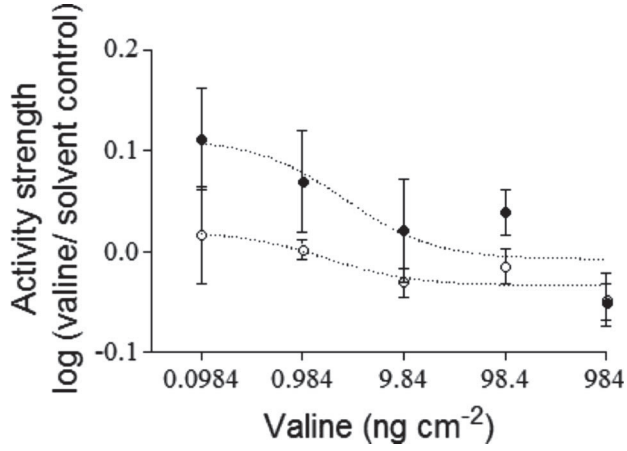

Figure S4. Pro-attachment activity of surface coated valine against *Cytophaga* sp. KT0804 (●) and *B. aquimaris* (○). Mean ± SE, n = 4, lines represent best fitting logistic functions.

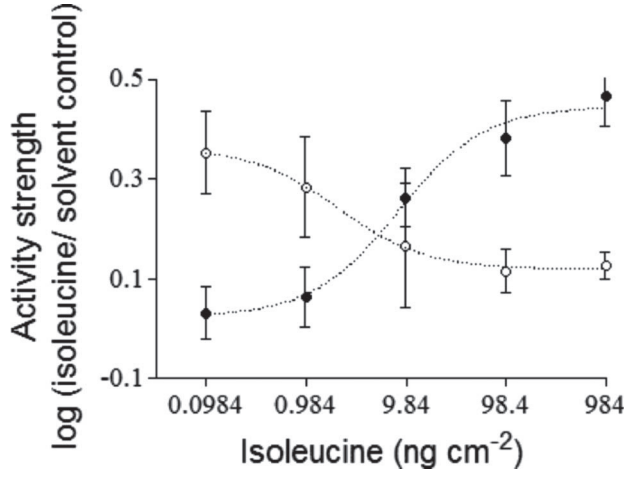

Figure S5. Pro-attachment activity of surface coated isoleucine against *Cytophaga* sp. KT0804 (●) and *B. aquimaris* (○). Mean ± SE, n = 4, lines represent best fitting logistic functions.

Manuscript D:

Björn Gebser and Georg Pohnert (2013). "Synchronized regulation of different zwitterionic metabolites in the osmoadaption of phytoplankton." Marine Drugs **11**(6): 2168-2182.

Summary: Marine phytoplankton harbors a wide spectrum of zwitterionic osmolytes to regulate turgor according to the surrounding seawater. Evaluation of average cell sizes and intracellular concentration of zwitterionic osmolytes of the cosmopolitan marine microalgae *Prorocentrum minimum* and *Emiliania huxleyi* revealed two different adaption mechanisms to distinct seawater salinities. Over a wide salinity range, the coccolithophore *E. huxleyi* showed a significant decrease of cell volumes under higher salinities with nearly constant intracellular concentration of zwitterionic osmolytes. However, the dinoflagellate *P. minimum* responded with increasing concentration of glycine betaine (GBT) and dimethylsulfonio acetate (DMS-Ac) when cultivated in medium with higher salinity. The highly increased concentration of DMSP in *E. huxleyi* at a salinity of 38‰ compared to 34‰ with constant cell volumes suggest a lower critical cell size at which investment in synthesis of new osmolytes is more favorable than further cell size reduction. Furthermore, the significant increase of the DMS-Ac and GBT concentrations in *P. minimum* with a constant DMSP content between salinities of 20‰ and 32‰ indicates highly osmoregulative properties of these metabolites. It could be shown that the intracellular composition of zwitterionic osmolytes is not just species specific, but also highly dependent on the salinity of the surrounding seawater.

Publication equivalents of contributing PhD students as coauthors according to the implementing provision of the doctoral regulations at the Faculty for Chemistry and Earth Sciences of the Friedrich Schiller University Jena:

	Author 1: **Gebser**
Conception of the work	X
Planning of experiments	X
Data collection	X
Analysis and interpretation	X
Writing of manuscript	X
Proposed publication equivalents	1

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Article

Synchronized Regulation of Different Zwitterionic Metabolites in the Osmoadaption of Phytoplankton

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Abstract: The ability to adapt to different seawater salinities is essential for cosmopolitan marine phytoplankton living in very diverse habitats. In this study, we examined the role of small zwitterionic metabolites in the osmoadaption of two common microalgae species *Emiliania huxleyi* and *Prorocentrum minimum*. By cultivation of the algae under salinities between 16‰ and 38‰ and subsequent analysis of dimethylsulfoniopropionate (DMSP), glycine betaine (GBT), gonyol, homarine, trigonelline, dimethylsulfonioacetate, trimethylammonium propionate, and trimethylammonium butyrate using HPLC-MS, we could reveal two fundamentally different osmoadaption mechanisms. While *E. huxleyi* responded with cell size reduction and a nearly constant ratio between the major metabolites DMSP, GBT and homarine to increasing salinity, osmolyte composition of *P. minimum* changed dramatically. In this alga DMSP concentration remained nearly constant at 18.6 mM between 20‰ and 32‰ but the amount of GBT and dimethylsulfonioacetate increased from 4% to 30% of total investigated osmolytes. Direct quantification of zwitterionic metabolites via LC-MS is a powerful tool to unravel the complex osmoadaption and regulation mechanisms of marine phytoplankton.

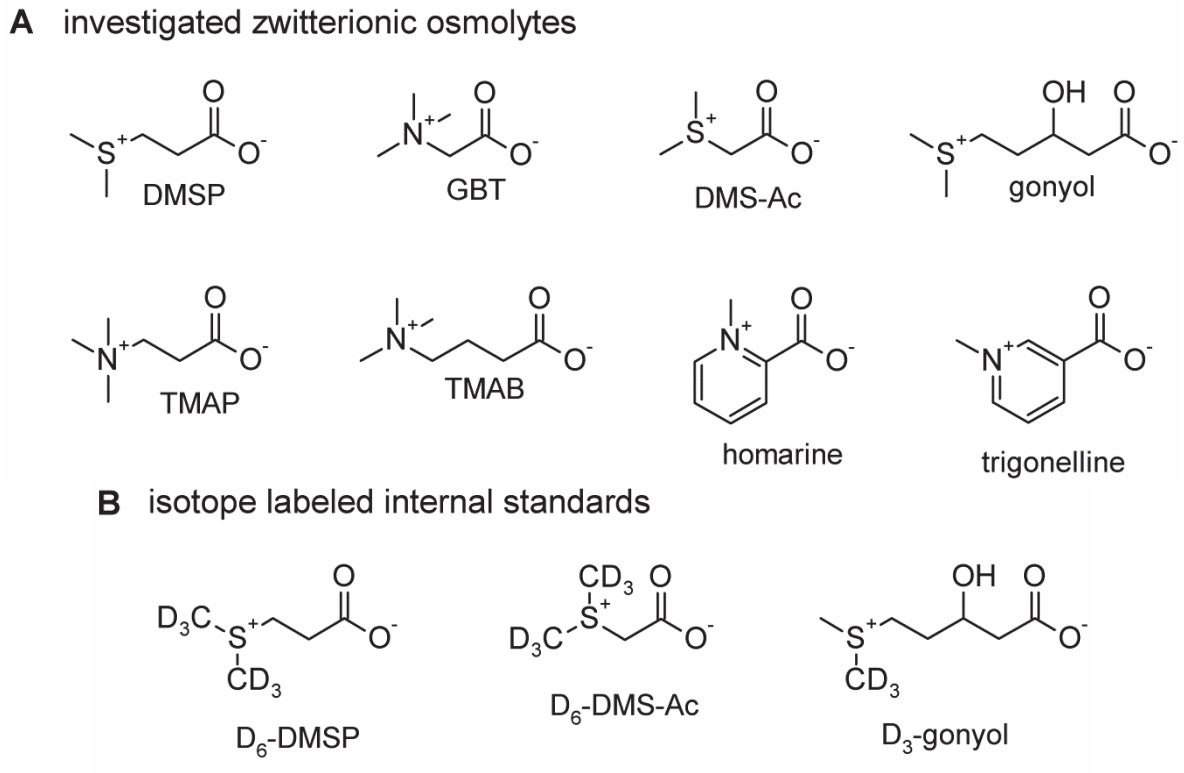
Keywords: *Emiliania huxleyi*; *Prorocentrum minimum*; dinoflagellates; haptophytes dimethylsulfoniopropionate (DMSP); gonyol; dimethylsulfide (DMS); osmoadaption

1. Introduction

Marine algae harbor different highly polar organic substances to maintain osmotic pressure balance with the surrounding sea water [1]. Organic osmolytes include polyols [2,3], amino acids [4,5] and zwitterionic substances [6–8]. Certain zwitterionic metabolites may fulfill multiple physiological roles in algae e.g., as osmolytes, antioxidants and cryoprotectants [9–11]. The zwitterionic dimethylsulfoniopropionate (DMSP) is considered to be a key player in this context and since it also serves as precursor for the volatile dimethylsulfide (DMS), it has attracted much attention [12–14]. DMS is among the most important sources of biogenic sulphur to the atmosphere contributing 13–37 Tg S emissions per year [15]. Another dominant zwitterionic osmolyte produced by marine phytoplankton is the nitrogen containing glycine betaine (GBT) [16]. In addition to these well studied osmolytes there are many other zwitterionic substances found in marine phytoplankton like gonyol [17], homarine [6], trigonelline [16], dimethylsulfonioacetate (DMS-Ac, dimethylthetin), trimethylammonium propionate (TMAP), and trimethylammonium butyrate (TMAB) with poorly understood physiological functions [6,18,19]. The interplay of all these potential osmoregulators under different salinities has hitherto not been addressed, which might be explained with methodological problems in the quantification of such small and highly polar metabolites. Recently we established a direct LC-MS method for simultaneous quantification of DMSP and glycine betaine, which is extended here for the comprehensive monitoring of all above mentioned zwitterionic metabolites [20,21].

We investigated the composition of zwitterionic metabolites in two cosmopolitan algae after adaptation to different salinities. Due to their occurrence in diverse regions of the ocean these algae need efficient strategies to adapt to the variable seawater salinities in different habitats. We selected dominant algae that have become model organisms for the study of phytoplankton. The coccolithophore *Emiliania huxleyi* is present in all oceans except Arctic and Antarctic regions [22]. Also the dinoflagellate *Prorocentrum minimum* can be found in many regions like the North Atlantic [23,24], in Eastern Pacific [25] and the Baltic Sea [26]. Such a broad distribution in marine waters of different salinity requires efficient adaptation. In the present study we perform a comprehensive, simultaneous analysis of the zwitterionic metabolites DMSP, GBT, dimethylsulfonioacetate, gonyol, homarine, trigonelline, trimethylammonium propionate (TMAP) and trimethylammonium butyrate (TMAB) (Scheme 1). Adaptation to different salinities is achieved by a concerted adjustment of several different zwitterionic metabolites. In the two investigated species, osmoadaptation is reached by fundamentally different regulative processes. The osmolyte composition of phytoplankton is thus, not only species specific, but also variable under different conditions.

Scheme 1. Chemical structures of zwitterionic osmolytes. (**A**) Investigated zwitterionic osmolytes: dimethylsulfoniopropionate (DMSP), glycine betaine (GBT), dimethylsulfonioacetate (DMS-Ac), gonyol, trimethylammonium propionate (TMAP), trimethylammonium butyrate (TMAB), homarine and trigonelline. (**B**) Isotope labeled internal standards: D_6-dimethylsulfoniopropionate (D_6-DMSP), D_6-dimethylsulfonioacetate (D_6-DMS-Ac) and D_3-gonyol.

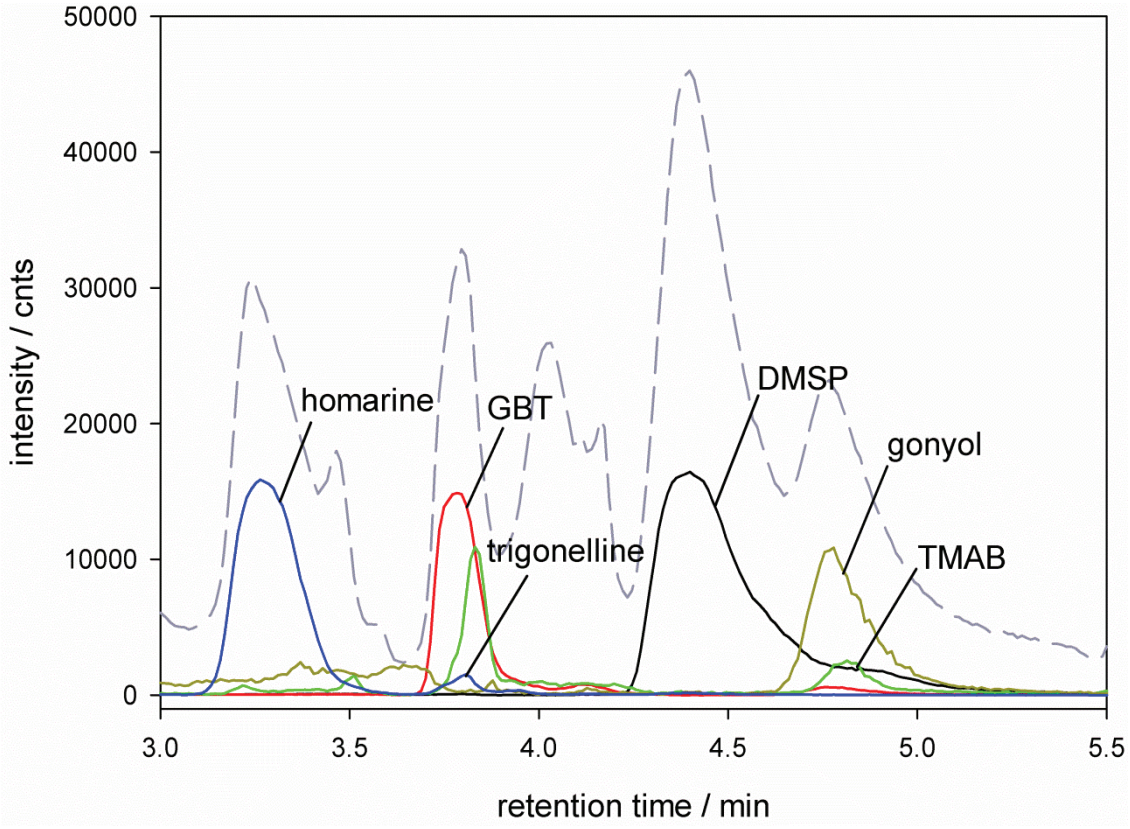


2. Results and Discussion

Several studies showed that the concentration of intracellular osmolytes in different algae species are highly dependent on their growth phase [16,27]. In order to avoid overlaying effects of adaptation to different salinities and growth phase, we ensured that every replicate sampling was performed at the beginning of the stationary phase. At this point all replicates are comparable and observed variations of the intracellular osmolyte concentrations can be attributed to the adaption to different salinities. Using established filtration and extraction protocols we could reliably generate samples for profiling of zwitterionic osmolytes [20,21]. Our previously introduced method of HPLC separation on a ZIC-HILIC column and mass spectrometric detection using an ESI q-Tof-mass spectrometer allowed direct monitoring of DMSP and glycine betaine [20,21]. Since this method is highly sensitive for the detection of zwitterionic metabolites we can now extend its scope for the additional simultaneous monitoring of gonyol, DMS-acetate, homarine, trigonelline, trimethylammonium propionate (TMAP) and trimethylammonium butyrate (TMAB). HPLC did not allow a baseline separation of all mentioned analytes. The molecular ion traces of DMSP ([M + 1] *m/z* = 135), GBT ([M + 1] *m/z* = 118), DMS-Ac ([M + 1] *m/z* = 121), gonyol ([M + 1] *m/z* = 179), TMAP ([M + 1] *m/z* = 132), TMAB ([M + 1] *m/z* = 146), homarine ([M + 1] *m/z* = 138) and trigonelline ([M + 1] *m/z* = 138) could however be reliably

integrated and quantified relative to the internal isotope labeled standard D_3-gonyol. Figures 1 and 2 show example chromatograms and ion traces of *E. huxleyi* and *P. minimum* extracts grown in 26‰ salinity medium (ion traces of GBT, DMS-Ac, gonyol, TMAP, TMAB and trigonelline are 10-times amplified). The identity of all analytes was verified by co-injection with commercially available glycine betaine (Sigma Aldrich, Germany), trimethylammonium butyrate (Sigma Aldrich, Germany) and trigonelline (Sigma Aldrich, Germany). All other standard compounds used for quantification (DMSP, D_6-DMSP, DMS-Ac, D_6-DMS-Ac, gonyol, D_3-gonyol, homarine and TMAP) were synthesized in our lab. The co-injection data and synthesis of standard compounds are documented in the supplementary material.

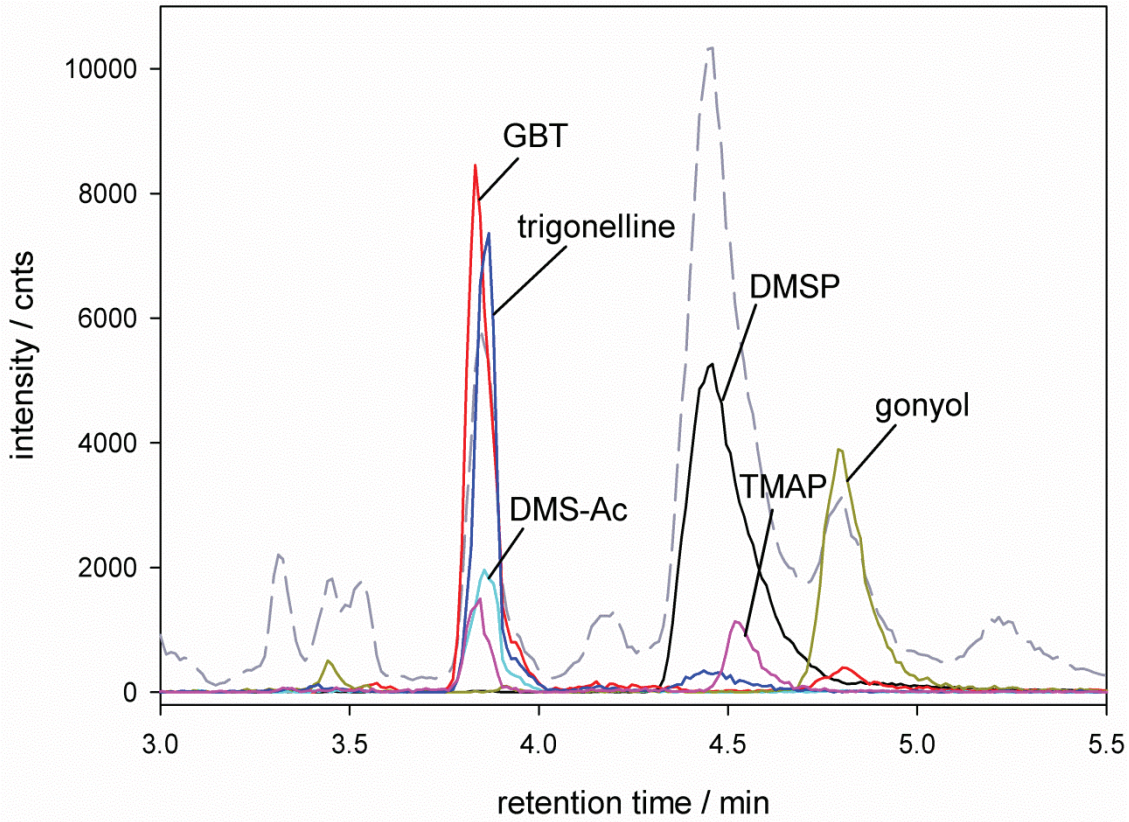
Figure 1. HPLC-MS separation of zwitterionic metabolites from *E. huxleyi* RCC1216. Total Ion Count (TIC --) and ion traces of dimethylsulfoniopropionate (DMSP), [M + 1] *m/z* = 135 ─), glycine betaine (GBT, [M + 1] *m/z* = 118 ─), trimethylammonium butyrate (TMAB, [M + 1] *m/z* = 146 ─), gonyol ([M + 1] *m/z* = 179 ─), homarine and trigonelline ([M + 1] *m/z* = 138 ─). Ion traces of GBT, gonyol, TMAB and trigonelline are 10-times amplified.



In order to allow adaptation of the algae to the respective salinities we inoculated a starting culture grown at 28‰ salinity for *P. minimum* (artificial seawater according to Maier and Calenberg [28]) and 30‰ salinity for *E. huxleyi* (HW sea salt medium according to Spielmeyer [29]) into three hyposaline (16‰, 20‰, 26‰) and two hypersaline (32‰ and 36‰ for *P. minimum* and 34‰ and 38‰ for *E. huxleyi*) media. Medium salinities between 30‰ and 34‰ mentioned above correspond to open ocean seawater conditions. Higher salinities of over 36‰ can be found in regions with high

evaporation like the Red Sea or Mediterranean Sea. Hyposaline conditions correspond to regions with high freshwater influx like the Black Sea, Baltic Sea or estuarine areas of large rivers. Thus, chosen salinities in this study represent a selection of common conditions encountered in nature. The cultures were maintained with regular re-inoculation into the salinity adjusted media for four weeks. The fully adapted cultures were then used as starting stock for the experiments.

Figure 2. HPLC-MS separation of zwitterionic metabolites from *P. minimum*. Total Ion Count (TIC --) and ion traces of dimethylsulfoniopropionate (DMSP) ([M + 1] *m/z* = 135 —), glycine betaine (GBT) ([M + 1] *m/z* = 118 —), dimethylsulfonioacetate (DMS-Ac, [M + 1] *m/z* = 121 —), gonyol ([M + 1] *m/z* = 179 —), trigonelline ([M + 1] *m/z* = 138 —) and trimethylammonium propionate (TMAP, [M + 1] *m/z* = 132 —). Ion traces of GBT, DMS-Ac, gonyol, TMAP, and trigonelline are 10-times amplified.



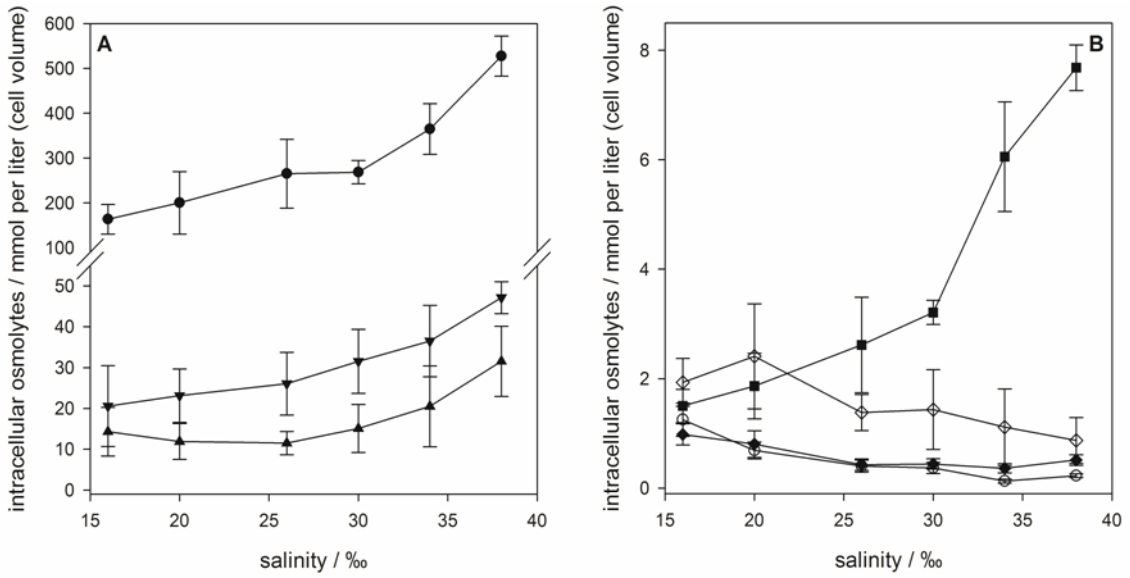
In order to monitor the growth of all replicates and to determine the beginning of the stationary phase for collecting samples, chlorophyll A fluorescence was measured at least every two days with daily measurements in later growth phases. Interestingly medium salinity had no obvious effect on the length of the exponential growth phase but affected maximum cell densities. All replicates reached the stationary phase within 17 days (±1 day) after inoculation (data shown in the supplementary material).

2.1. Emiliania huxleyi

In the survey of zwitterionic metabolites we detected the well-known osmolytes DMSP, gonyol and glycine betaine. In addition, we found homarine, trigonelline, TMAP and TMAB in lower amounts. The nitrogen containing zwitterionic homarine is described in several marine invertebrates [30,31] and

in few microalgae like *Amphidinium carterae* and has previously also been detected in *E. huxleyi* (CCMP378) in low concentrations (0.2 mM to 0.5 mM) [16]. Trigonelline is not known as common metabolite from phytoplankton but has also been found in the microalgae *Chrysochromulina* sp., *Skeletonema costatum* and *Tetraselmis* sp. [16]. It was recently also detected in Laminariales brown algae [32,33]. TMAP and TMAB were to the best of our knowledge not described as natural products before. The sulfur containing DMS-acetate, known from other phytoplankton sources, was not detected.

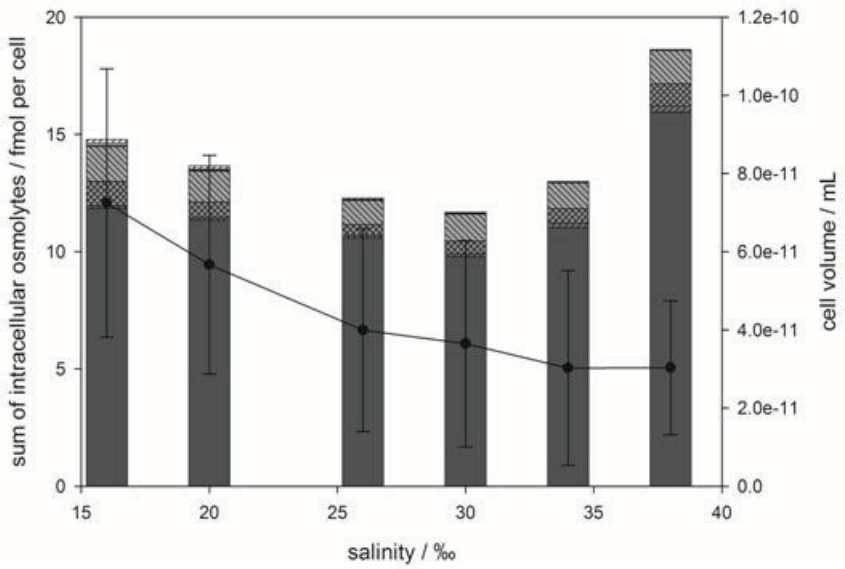
Figure 3. Intracellular concentrations of zwitterionic osmolytes of *E. huxleyi* RCC1216 as a function of medium salinity. (**A**) Major osmolytes: dimethylsulfoniopropionate (DMSP) (●), glycine betaine (GBT) (▲) and homarine (▼). (**B**) Minor osmolytes: gonyol (■), trimethylammonium propionate (TMAP) (♦), trimethylammonium butyrate (TMAB) (○) and trigonelline (◊). Concentrations are normalized to cell volume, error bars represent standard deviation (biological replicates, $N = 5$).



Quantification of the sulfur containing osmolytes DMSP and gonyol and the nitrogen containing osmolytes GBT, homarine, TMAP, TMAB and trigonelline revealed that DMSP is the major zwitterionic osmolyte at all tested salinities. Intracellular concentrations ranged from 164 ± 33 mM (with respect to cell volume) at 16‰ medium salinity to 527 ± 44 mM at 38‰ (Figure 3A). The increase in concentration with rising salinity was more pronounced at higher values. The second most abundant zwitterionic osmolytes were GBT and homarine that were present in concentrations between 15.3 ± 6.0 mM at 16‰ and 31.5 ± 8.6 mM at 38‰ for GBT and 20.6 ± 9.9 mM at 16‰ and 47.2 ± 3.9 mM at 38‰ for homarine, respectively. The ratio between the major osmolytes, DMSP, GBT and homarine, remains at approximately 100:6:10 over the whole range of tested salinities suggesting that all pathways involved were regulated in similar ways. The less abundant gonyol also increased with increasing salinity (1.5 ± 0.3 mM at 16‰ to 7.7 ± 0.4 mM at 38‰), but a more pronounced increase was observed between the two highest salinities where the concentration at 38‰ was double that at 30‰ (Figure 3B). While the intracellular concentrations of DMSP, GBT, homarine and gonyol increased with increasing salinity in agreement with the expected behavior of osmolytes, the nitrogen containing compounds, TMAP, TMAB and trigonelline, all decreased in their

concentration (Figure 3B). These minor abundant zwitterionic metabolites have not yet been described or quantified in algal cultures, but were possibly included as fractions of a complex mixture of quaternary ammonium compounds in previous surveys [7,8]. The decrease of intracellular TMAP, TMAB and trigonelline concentrations with increasing salinity, however, suggests that these zwitterionic metabolites play no role in osmoadaptation. Measurement of cell diameters and calculation of average cell volume revealed a considerable reduction of the cell volume with rising salinity (from 7.2×10^{-11} mL at 16‰ to 3.0×10^{-11} mL at 38‰) (Figure 4). In agreement with the literature this indicates that adjustment of the volume is an additional response to salinity changes [6,34]. If the concentration of zwitterionic compounds per cell is considered (Figure 4), it becomes clear that investment into the production of sulfur containing osmolytes is predominantly relevant at elevated salinity, while cell volume compensation seems to be more important at lower salinities.

Figure 4. Total concentration of intracellular zwitterionic osmolytes (bar graph) and cell volume (line graph) of *E. huxleyi* as a function of medium salinity. Bars represent concentrations of dimethylsulfoniopropionate (DMSP) (▬), gonyol (▨), glycine betaine GBT (▩), homarine (▩), trimethylammonium propionate (TMAP) (▭), trimethylammonium butyrate (TMAB) (▭) and trigonelline (▨). Osmolyte concentrations are normalized per cell, error bars represent standard deviation (biological replicates, $N = 5$).



Previous works regarding intracellular DMSP concentrations of *E. huxleyi* by Spielmeyer *et al.* (52 mM, HW sea salt medium, 30‰ salinity) [29], van Rijssel *et al.* (ca. 195 mM, artificial seawater according to Veldhuis) [35,36] and Keller (145 mM, K-medium, ca. 33‰ salinity, based on natural seawater) [16] show that intracellular DMSP concentrations can be highly dependent on the selected strain, cultivation conditions or sample processing procedures. Keller *et al.* also revealed differences in DMSP concentration between the growth stages of *E. huxleyi* (145 mM in exponential and 32.3 mM in stationary growth phase) [16]. Furthermore Bucciarelli *et al.* observed DMSP variation between 223 mM and 318 mM at the beginning and end of light period, respectively [37]. At an intermediate salinity of 30‰ a cellular DMSP concentration of 268 mM was found in this study confirming that our

findings are within the range of literature data. Maximum DMSP concentrations of 527 mM measured in this study at the highest salinity of 38‰, which is about twice the concentration we found at a salinity of 30‰ illustrate the high influence of medium salinity on the concentration of this osmolyte. The large variations of detected concentrations between the different literature studies show that osmoadaption and regulation are very complex mechanisms and quantitative comparisons between different studies have to be treated with caution. It is therefore a particular strength of the current investigation that all osmolytes were recorded within a single experiment and one single extract, thereby minimizing inter-experiment variability and allowing the direct analysis of the response of multiple metabolites. GBT concentrations between 15.3 mM and 31.5 mM are in the same range as determined by Spielmeyer (20–35 mM) [21] and Keller (8.1–32.6 mM) [16]. In our study, homarine contributes significantly, with up to 10% to the total osmolyte content. While this metabolite was also detected in traces in *E. huxleyi* before an osmoregulatory function in this organism has not been described up to now [16]. Dickson and Kirst, however, showed a contribution to osmoregulation of homarine in the prasinophyte *Platymonas subordiformis* [6].

2.2. Prorocentrum minimum

In contrast to *E. huxleyi*, higher salt content of the medium had no effect on average cell size of *P. minimum* (Figure 5). Thus, adaptation to different salinities must rely on changes in osmolyte concentrations. However, the osmoadaptation of *P. minimum* has been poorly explored to date.

As in *E. huxleyi* DMSP was the dominant zwitterionic metabolite in *P. minimum* contributing 29.3%–40.0% of the investigated osmolytes at any given salinity. The composition of the other zwitterions was fundamentally different compared to *E. huxleyi*. At low salinities trigonelline was the second most abundant metabolite, while GBT and DMS-Ac increased with increasing salinity (Figure 6). The presence of GBT and trigonelline in *P. minimum* is in contrast to results by Keller *et al.* who did not detect these metabolites in this organism [16]. Gonyol, TMAP and TMAB were only detected in minute amounts (Figure 6B). The osmoadaptation of this dinoflagellate followed a fundamentally different pattern of zwitterion concentration changes compared to *E. huxleyi*. At the lowest salinity of 16‰, DMSP was by far the most abundant osmolyte with 12.6 ± 0.7 mM referred to cell volume (Figure 6A). Between 20‰ and 32‰ DMSP concentration remained almost constant at a level around 18.6 mM (concentration at 20‰). Only at the highest and lowest salinity tested was a change in concentration of DMSP observed (Figure 6A).

In contrast GBT and DMS-Ac concentration increased dramatically from 0.25 ± 0.04 mM to 4.96 ± 0.27 mM and from not detectable concentrations to 4.29 ± 0.23 mM, respectively. This suggests that DMSP is maintained at a high but constant level under average salinities and only plays a role in osmoadaption under extreme salinity conditions. The significant increase of GBT and DMS-Ac with increased salinity contributes most to the changes of the osmolyte composition of *P. minimum* (Figure 5). This suggests that GBT and DMS-Ac are highly regulated if adaptation to intermediate salinity changes is concerned. Regarding the minor abundant zwitterionic metabolites it could be shown that gonyol concentration remains constant at 0.49 ± 0.05 mM over the whole salinity range (Figure 6B). Concentrations of TMAP, and trigonelline decreased from 0.731 ± 0.081 mM at 16‰ to 0.159 ± 0.019 mM at 36‰ and from 1.62 ± 0.31 mM at 16‰ to 0.654 ± 0.055 mM at 36‰,

respectively. Also, the concentration of TMAB dropped from 0.357 ± 0.026 mM at 16‰ to 0.046 ± 0.007 mM at 20‰, corresponding to a decrease of 87%. TMAP was not detectable at higher salinities. These metabolites did not, therefore, contribute significantly to the overall changes of the osmolyte pool (Figure 5).

Figure 5. Total concentration of intracellular, zwitterionic osmolytes (bar graph) and cell volume (line graph) of *P. minimum* as a function of medium salinity. Bars represent concentrations of DMSP (▬), GBT (▨), DMS-Ac (▩), gonyol (▧), TMAP (▤), TMAB (▭) and trigonelline (▨). Osmolyte concentrations are normalized per cell, error bars represent standard deviation (biological replicates, $N = 5$).

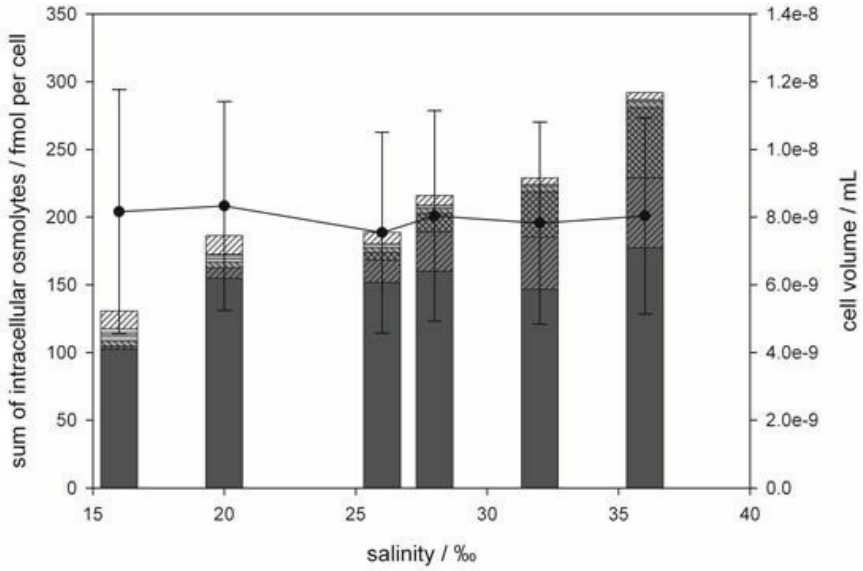
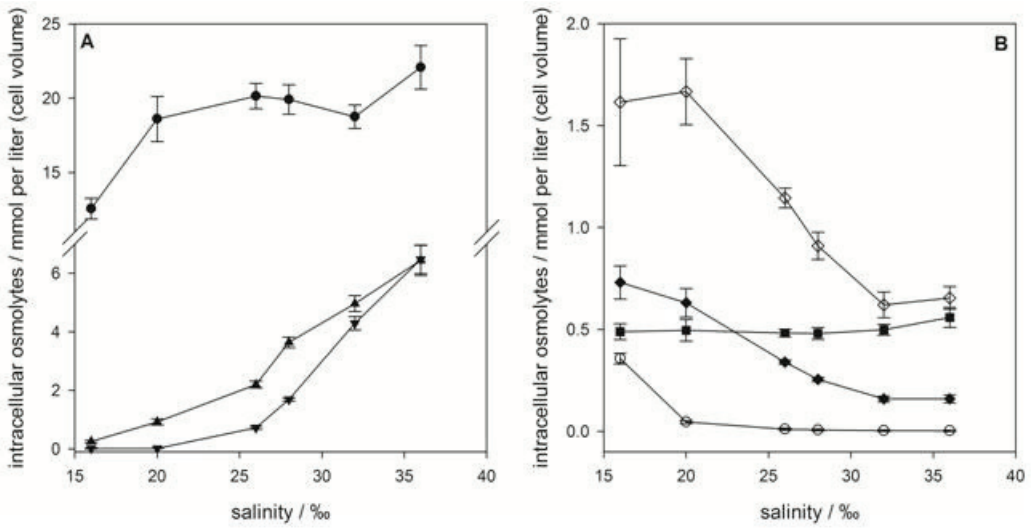


Figure 6. Intracellular concentrations of zwitterionic osmolytes of *P. minimum* as a function of medium salinity. (**A**) Major osmolytes: dimethylsulfoniopropionate (DMSP) (●), glycine betaine (GBT) (▲) and dimethylsulfonioacetate DMS-Ac (▼); (**B**) minor osmolytes: gonyol (■), trimethylammonium propionateTMAP (♦), trimethylammonium butyrateTMAB (○) and trigonelline (◊); concentrations are normalized to cell volume, error bars represent standard deviation (biological replicates, $N = 5$).



The pronounced changes in osmolyte composition of *P. minimum* are in accordance with a lack of adjustment of cell size under different salinities. Compensation thus solely depends on osmolytes, which are orchestrated in an unexpected way. Apparently, fundamentally different metabolic pathways are involved in the adaptation of the two investigated model algae; how these are addressed remains open and will be subject to further studies. Besides the investigated osmolytes, numerous other metabolites can be affected by salinity changes. Some metabolites might have obvious function in osmoregulation, while others, such as the toxins of different harmful algae like cyanobacterium *Anabaena* sp. [38] and the dinoflagellate *Protoceratium reticulatum* [39], are also affected by salinity changes. These compounds might be rather affected via indirect influences of other metabolic pathways.

3. Experimental Section

3.1. Cultivation of Microalgae

Medium for cultures of *Emiliania huxleyi* (obtained from the Roscoff Culture Collection, Roscoff, France, strain number RCC1216) was prepared according to Spielmeyer *et al.* [20]. Concentration of the sea salt (HW sea salt professional, aquaristik.net, Babenhausen, Germany) was modified to obtain salinity of 16‰, 20‰, 26‰, 30‰, 34‰ and 38‰, respectively. The amount of added nutrients was not changed.

Prorocentrum minimum was cultivated in artificial seawater medium according to Maier and Calenberg [28]. Salinity of the medium was adjusted by modifying the content of the main salts mix, resulting in salinities of 16‰, 20‰, 26‰, 28‰, 32‰ and 36‰. At 28‰ salinity the main salts were NaCl (24 g/L), $MgSO_4 \cdot 7H_2O$ (8 g/L), KCl (0.75 g/L), $CaCl_2 \cdot 2H_2O$ (1.5 g/L), HEPES (1.2 g/L), $NaHCO_3$ (0.2 g/L). Concentration of trace metals and vitamins were not changed. Final salinities of the media were measured using a portable refractometer with automatic temperature compensation (VWR, Germany). All media were autoclaved before use.

Microalgae were cultivated as standing cultures in 50 mL polystyrene cell culture bottles (Carl Roth GmbH, Germany) with membrane filter screw caps for gas exchange. Stock cultures of *E. huxleyi* and *P. minimum* were adapted to the different salinities by cultivation in the respective media over a period of four weeks. During this time, cultures were diluted 1:1 every fourth day to maintain an exponential growth phase. After adaption to the different salinities, these cultures were used for inoculation of all biological replicates used for osmolyte quantification. Initial cell densities for the experiment were 4 kcells/mL for *P. minimum* and 20 kcells/mL for *E. huxleyi* respectively. For each salinity, five biological replicates were prepared. All cultures were grown at a temperature of 14 °C ± 2 °C with a 14:10 light:dark cycle. Light was provided by Osram biolux lamps with an intensity of 40 µmol photons $m^{-2}s^{-1}$.

The growth stages of every replicate were determined by daily measurement of *in vivo* fluorescence of a 200 µL sample using a microplate reader (Mithras LB 940, Berthold Technologies, Bad Wildbad, Germany). The excitation and emission wavelengths were set to 430 nm and 655 nm, respectively.

3.2. Cell Counting and Size Measurement

For determination of the final cell densities, *E. huxleyi* samples were counted after fixation with glutaraldehyde using a Cytomics FC 500 flow cytometer (Beckman Coulter, Krefeld, Germany) with CXP-software, air-cooled argon-ion laser (20 mW, 488 nm) and standard filters. The discriminator was set to side scatter and samples were analyzed for 1 min at a flow rate of 30 µL/min. Data were normalized to 3.6 µm polystyrene beads (Beckman Coulter, Krefeld, Germany) measured at 620 nm using CXP analysis software. *P. minimum* samples were fixed with Lugol and counted in Fuchs-Rosenthal chamber using a Leica DM2000 (Heerbrugg, Switzerland) upright microscope with phase contrast.

Pictures for cell size measurements were taken with a Leica DFC280 system. Average cell diameters were calculated using 200 randomly taken pictures of one replicate for every salinity to obtain representative data. Calculations of the average cell volumes based on a spherical shape, including the coccosphere for *E. huxleyi* and an oblate spheroid for *P. minimum*, whose minor axis was calculated from average diameter *vs.* height ratios of 50 different cells.

3.3. Sample Preparation

Samples for measurement were taken at the beginning of the stationary phase (6 h after beginning of the light period) by vacuum filtration of 25 mL of the cell culture on Whatman GF/C filters (700 mbar). The filters were immediately transferred into 15 mL Falcon® tubes, and 2 mL of methanol for extraction of osmolytes was added. After 30 min at room temperature, extracts were stored at −80 °C. After thawing, samples were centrifuged for 2 min at 6.000 rcf and 100 µL of the supernatant were diluted with 900 µL acetonitrile and 100 µL of an aqueous solution of an internal standard mixture (D_3-gonyol, D_6-DMSP, D_6-DMSAc). After centrifugation (5 min, 16000 rcf), the supernatant was directly used for ultra performance liquid chromatography (UPLC) analysis.

3.4. Equipment

For analytical separation an Aquitiy UPLC (Waters, Milford, MA, USA) equipped with a SeQuant ZIC®-HILIC column (5 µm, 2.1 × 150 mm, SeQuant, Umeå, Sweden) and a SeQuant ZIC®-HILIC guard column (5 µm, 2.1 × 20 mm, SeQuant, Umeå, Sweden) was used. A Q-ToF micro mass spectrometer (Waters Micromass, Manchester, England) with electrospray ionization was used as a mass analyzer.

3.5. Osmolyte Analysis

For separation of osmolytes via UPLC we used the method of Spielmeyer [20] with water + 2% acetonitrile and 0.1% formic acid (solvent A) and 90% acetonitrile + 10% water with 5 mmol/L ammonium acetate (solvent B) as eluent system. The flow rate was set to 0.60 mL/min. As internal standards for quantification of DMSP and DMS-Ac we used D_6-DMSP and D_6-DMS-Ac, respectively. For all other osmolytes, we used D_3-gonyol as internal standard. For proper quantification of all examined zwitterionic substances, relative response factors were determined by measurement of an equimolar mixture of all used labeled and unlabeled compounds. Response factors were calculated by comparison of the peak area of the analytes with the peak area of the corresponding internal standard.

Syntheses of standard compounds that are not commercially available are described in the supplementary materials.

4. Conclusions

LC-MS analysis of zwitterionic metabolites provides a powerful tool to unravel osmoregulation and osmoadaption mechanisms for a better understanding of the ability and success of cosmopolitan living algae to deal with a wide spectrum of salinities in different habitats. With simultaneous analysis of a large set of zwitterionic metabolites in the coccolithophore *E. huxleyi* and the dinoflagellate *P. minimum,* we were able to distinguish between two completely different strategies of osmoadaption of these two marine algae. While *E. huxleyi* reacts with a lower cell size to higher salinities to overcome osmotic stress, cell volumes of *P. minimum* remained constant over the whole range of salinity (16‰–38‰). The ratio of the main osmolytes in *E. huxleyi* remained more or less constant, which underlines the strategy of osmoadaption by cell size adjustment, whereas osmolyte composition and cellular concentrations in *P. minimum* changed in a pronounced way with increasing salinity. Therefore, intracellular concentrations of zwitterionic osmolytes and the whole osmolyte composition are not just species specific, but also highly dependent on the salinity of the culture medium and consequently, on the marine habitat.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Materials

S1. Synthesis of Standard Compounds

S1.1. DMSP and D_6-DMSP

DMSP and D_6-DMSP as hydrochloride were synthesized according to Chambers [1] by bubbling gaseous hydrogen chloride through a solution of anhydrous acrylic acid (Fluka, Germany) and dimethyl sulfide (Sigma Aldrich, Germany) or D_6-dimethyl sulfide (Sigma Aldrich, Germany) in dichloromethane and subsequent recrystallization of the resulting white solid in MeOH/Et_2O.

S1.2. DMS-Ac and D_6-DMS-Ac

DMS-Ac and D_6-DMS-Ac as hydrobromide were synthesized according to Howard [2] by addition of commercially available dimethyl sulfide (Sigma Aldrich, Germany) and D_6-dimethyl sulfide (Sigma Aldrich, Germany), respectively, to a stirred solution of bromoacetic acid (Fluka, Germany) in dichloromethane. The resulting white solid was recrystallized in MeOH/Et_2O.

S1.3. Trimethylammonium Propionate

To a stirred solution of 100 mg of dimethylaminopropionic acid in 2 mL methanol were added 50 mg K_2CO_3 and 200 mg iodomethane (Sigma Aldrich, Germany) at room temperature. After two days, the resulting mixture was acidified by addition of aqueous hydroiodic acid (Alfa Aesar, Germany). Trimethylammonium propionate as hydroiodide was precipitated by addition of Et_2O as white solid, which was recrystallized in MeOH/Et_2O.

^{1}H-NMR (400 MHz, D_2O) δ ppm: 2.94 (2H, t, J = 7.68 Hz), 3.12 (9H, s), 3.64 (2H, t, J = 7.50 Hz); ^{13}C-NMR (50 MHz, D_2O) δ ppm: 27.75, 52.75, 61.27, 172.98; ESI-MS *m/z* 132.12 $[M + H]^+$; ESI-MS-MS (parent ion: *m/z* 132, collision energy: 15 eV) *m/z* 132.12 $[M]^+$, 73.08 $[M - C_3H_9N]^+$, 60.13 $[C_3H_{10}N]^+$, 59.12, 58.11.

*S1.4. Homarine (*N-*Methyl Picolinic Acid Hydroiodide)*

N-methylpicolinic acid hydroiodide was synthezised by addition of 150 mg iodomethane (Sigma Aldrich, Germany) to a well stirred suspension of 100 mg picolinic acid (Alfa Aesar, Germany) in 2 mL propylene carbonate. After two days, 10 mL Et_2O were added, and the resulting yellow solid was recrystallized in MeOH/Et_2O (for synthesis see also [3]).

^{1}H-NMR (400 MHz, MeOD) δ ppm: 4.95 (3H, s), 8.06 (1H, t, J = 6.26 Hz), 8.44 (1H, d, J = 7.63 Hz), 8.51 (1H, t, J = 7.78 Hz), 8.85 (1H, d, J = 5.19 Hz); ^{13}C-NMR (101 MHz, MeOD) δ ppm: 127.97, 130.51, 145.44, 146.71, 149.69, 163.90 (the signal of the methyl group was overlapped by the signals of the solvent and therefore not visible, cf. [4]); ESI-MS (positive) *m/z* 138.08$[M + H]^+$; ESI-MS-MS (positive, parent ion: *m/z* 138, collision energy: 15 eV) *m/z* 138.08 $[M + H]^+$, 124.09 $[M - CH_3 + H]^+$, 106.08, 96.09, 94.11 $[M - COOH + H]^+$, 78.08.

S1.5. Ethyl-3-hydroxy-5-methylthiopentanoate

In a three-necked flask (100 mL) with 5 g zinc powder 40 mL dry diethylether and 0.3 mL chlorotrimethylsilane were added under argon atmosphere. The resulting suspension was refluxed for 20 min. After dropwise addition of 3.3 g (19.8 mmol) ethyl-bromoacetate and 2.02 g (19.4 mmol) 3-(methylthio)propionaldehyde reaction mixture was refluxed over an additional 2.5 h. After cooling to room temperature 50 mL 3 M HCl were added and the resulting mixture was stirred for 20 min. The solution was then extracted with Et_2O and combined organic phases were washed with $NaHCO_3$ solution and water. Removal of the solvent gave a yellowish residue which was purified by silica gel column chromatography (petroleum ether/AcOEt = 4:1) to give 937 mg (25%) of the desired product as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 1.26 (3H, t, J = 7.14 Hz), 1.64–1.87 (2H, m), 2.10 (3H, s), 2.36–2.54 (2H, m), 2.55–2.71 (2H, m), 4.06–4.22 (3H, m); ^{13}C-NMR (50 MHz, $CDCl_3$) δ ppm: 14.10, 15.46, 30.33, 35.53, 41.22, 60.69, 66.88, 172.62; EIMS *m/z* (relative intensity, 70 eV) 192.08 $[M]^+$ (18), 174.07 $[M - H_2O]^+$ (23) , 144.08 (29), 129.03 $[M - H_2O - C_2H_5O]^+$ (46), 107.07 (32), 100.04 (22), 99.04 (16), 98.03 (39), 87.02 (21), 85.02 (20), 75.03 $[M - C_5H_9O_3]^+$ (20), 71.02 (28), 70.04 (22), 61.01 $[M - C_6H_{11}O_3]^+$ (100).

S1.6. 3-Hydroxy-5-methylthiopentanoic Acid

To 475 mg Ethyl-3-hydroxy-5-methylthiopentanoate in a 4 mL screw cap vial, 2 mL 10% NaOH solution was added and shaken for 30 min at room temperature until the mixture became a homogeneous solution. After washing with Et_2O the yellowish solution was acidified with conc. HCl to pH 1. The aqueous phase was extracted five times with 2 mL Et_2O. Removal of the solvent gave 275 mg (63%) of an orange, viscous liquid.

^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 1.68–1.91 (2H, m), 2.11 (3H, s), 2.47–2.58 (2H, m), 2.58–2.67 (2H, m), 4.15–4.25 (1H, m), 5.90 (1H, br. s); ^{13}C-NMR (50 MHz, $CDCl_3$) δ ppm: 15.44, 30.32, 35.29, 41.07, 66.99, 176.91; ESI-MS (negative) *m/z* 163.09 $[M - H]^-$.

S1.7. D_3-Gonyol (as Hydroiodide)

100 mg of 3-hydroxy-5-methylthiopentanoic acid were dissolved in 1 mL acetone. After addition of 0.05 mL Iodomethane-d_3 the reaction mixture was stirred for 12 h at room temperature while an orange oily liquid precipitated out of the solution. After removal of the solvent under reduced pressure and reprecipitation of the resulting residue in MeOH/Et_2O 145 mg (77%) of D_3-gonyol hydroiodide were obtained.

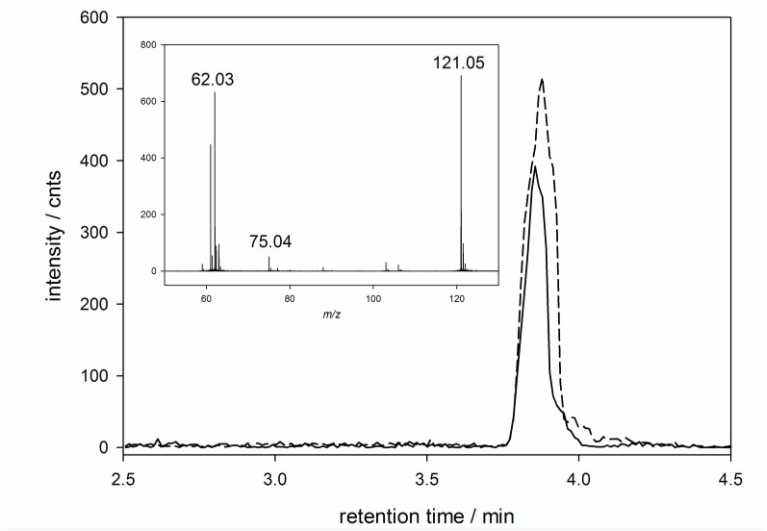
^{1}H-NMR (400 MHz, MeOD) δ ppm: 1.89–2.01 (1H, m), 2.07–2.17 (1H, m), 2.47–2.60 (2H, m) 2.94, 2.95 (3H, ss), 3.37–3.53 (2H, m), 4.10–4.19 (1H, m); ^{13}C-NMR (101 MHz, MeOD) δ ppm: 25.84, 26.19, 31.98, 42.40, 42.77, 67.83, 174.78; ESI-MS (positive) *m/z* 182.02 $[M + H]^+$; ESI-MS-MS (positive, parent ion: *m/z* 182, collision energy: 15 eV) *m/z* 182.02 $[M + H]^+$, 117.11 $[M + H - C_2H_3D_3S]^+$, 99.10 $[M + H - H_2O - C_2H_3D_3S]^+$, 89.07, 87.10, 75.09, 71.10, 66.09 $[C_2H_4D_3S]^+$, 57.08, 55.10.

S2. Identification of Different Osmolytes

Chemical structures of all previously unknown osmolytes in cell extracts were verified by ESI-MS-MS experiments and co-injection with standard compounds. Collision energy for all ESI-MS-MS experiments was set to 15 eV. In the following ion traces of investigated osmolytes of cell extracts (solid lines), standard compounds (dashed lines) and corresponding mass spectra are shown.

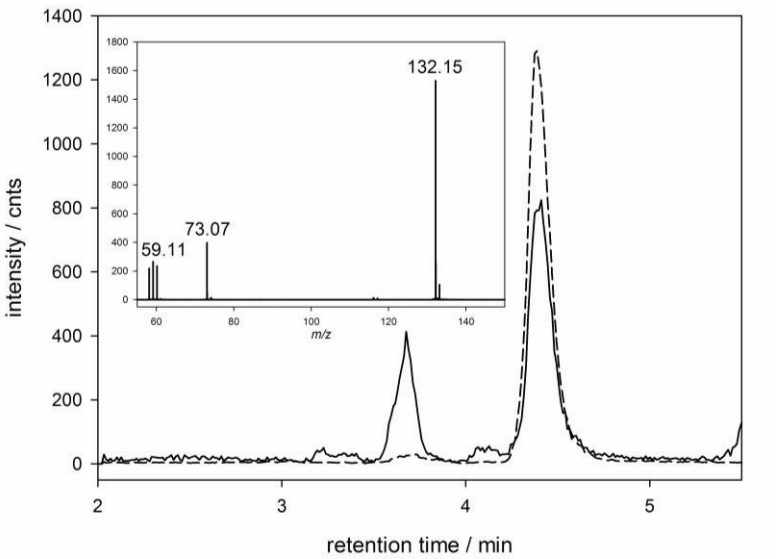
S2.1. DMS-Acetate

Figure S1. Ion traces (*m/z* = 121) of dimethylsulphonioacetate (DMS-Ac) in cell extracts (—), corresponding standard compound (--) and ESI-MS-MS spectrum of DMS-Ac (parent ion: *m/z* = 121).



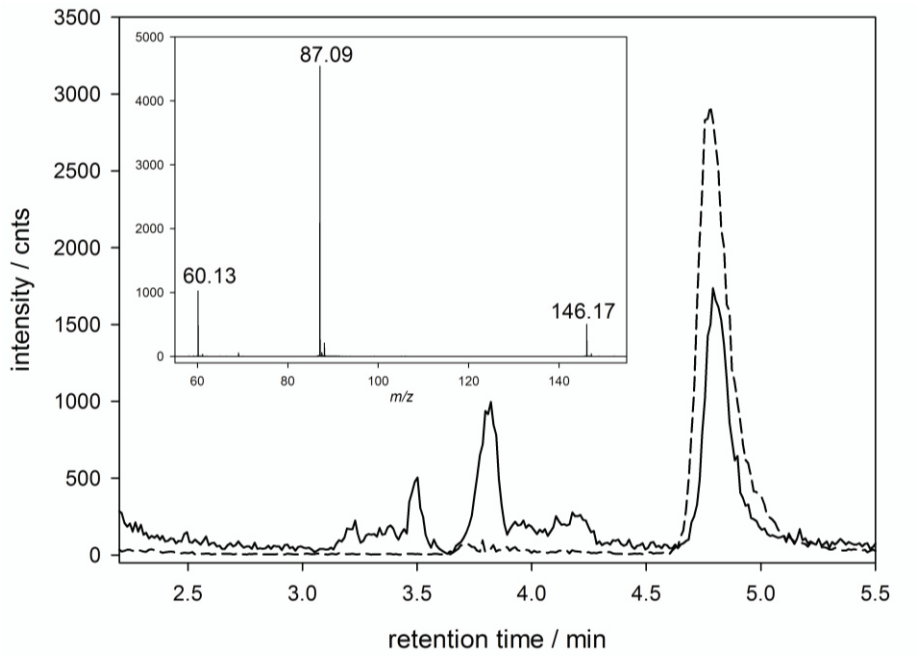
S2.2. TMAP

Figure S2. Ion traces (*m/z* = 132) of trimethylammoniumpropionate (TMAP) in cell extracts (—), corresponding standard compound (--) and ESI-MS-MS spectrum of TMAP (parent ion: *m/z* = 132).



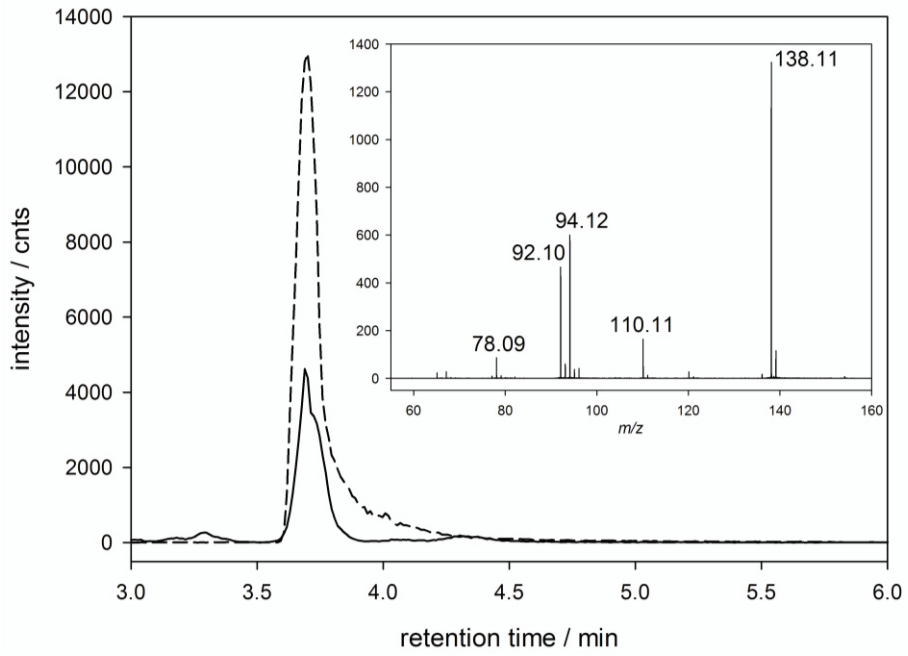
S2.3. TMAB

Figure S3. Ion traces (*m/z* = 146) of trimethylammoinimbutyrate (TMAB) in cell extracts (—), corresponding standard compound (--) and ESI-MS-MS spectrum of TMAB (parent ion: *m/z* = 146).



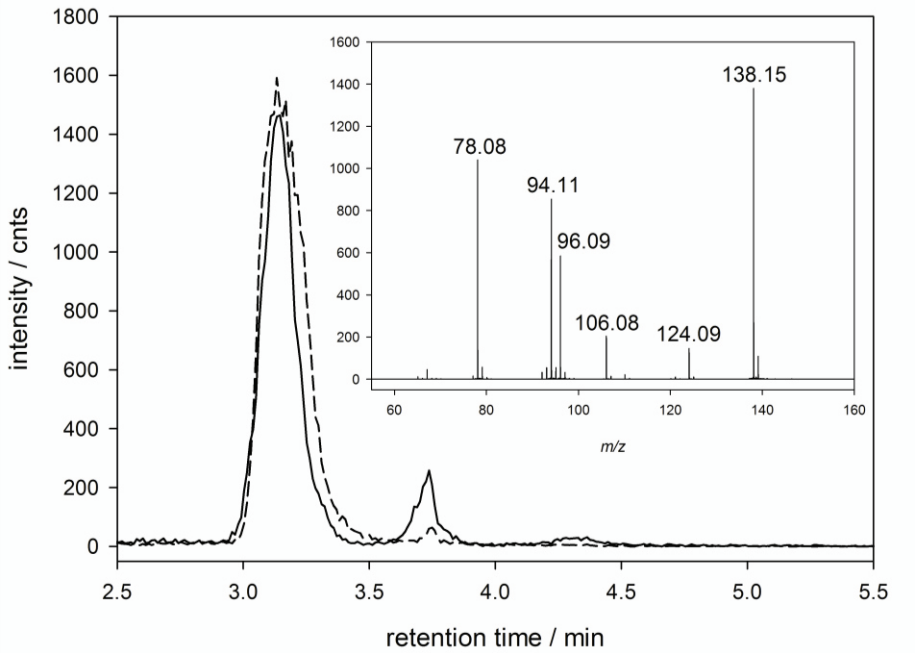
S2.4. Trigonelline

Figure S4. Ion traces (*m/z* = 138) of trigonelline in cell extracts (—), corresponding standard compound (--) and ESI-MS-MS spectrum of trigonelline (parent ion: *m/z* = 138).



S2.5. Homarine

Figure S5. Ion traces (*m/z* = 138) of homarine in cell extracts (—), corresponding standard compound (--) and ESI-MS-MS spectrum of homarine (parent ion: *m/z* = 138).



S3. Growth Curves (*in Vivo* Chlorophyll-A Fluorescence Data)

Figures S6 and S7 show measured *in vivo* chlorophyll-A fluorescence of *E. huxleyi* and *P. minimum* over the growth curve. Last data point represents the day of sample collection and extraction of the algae.

Figure S6. *In vivo* chlorophyll-A fluorescence during growth curve of *E. huxleyi* RCC1216 cultures grown in HW sea salt medium with salinities of 16‰ (—), 20‰ (—), 26‰ (—), 30‰ (—), 34‰ (—) and 38‰ (—); error bars represent standard deviation between biological replicates, last data point is day of sample collection and cell extraction.

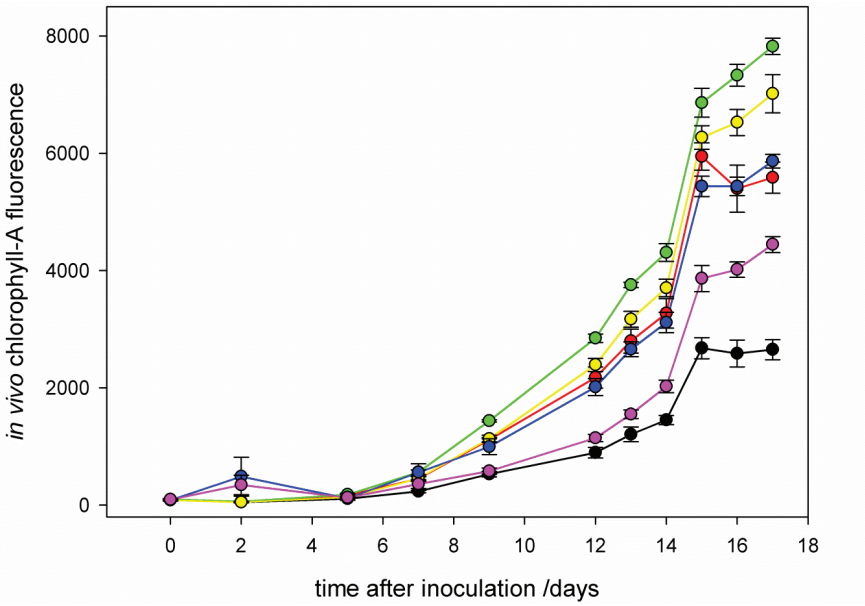
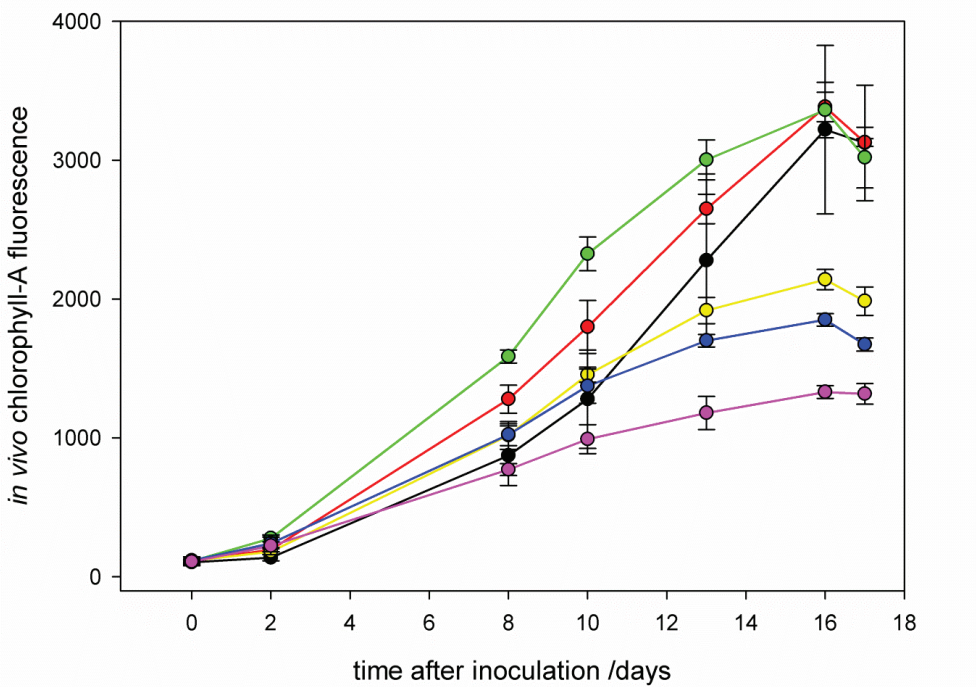


Figure S7. *In vivo* chlorophyll-A fluorescence during growth curve of *P.minimum* cultures grown in artificial seawater with salinities of 16‰ (—), 20‰ (—), 26‰ (—), 28‰ (—), 32‰ (—) and 36‰ (—); error bars represent standard deviation between biological replicates, last data point is day of sample collection and cell extraction.



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Manuscript E:

Björn Gebser, Michael Steinke and Georg Pohnert (unpublished). "Phytoplankton-derived small zwitterionic dimethylsulfonio-compounds are widespread and interfere with microbial DMSP/DMS sulfur cycling"

Submitted to: Applied and Environmental Microbiology

Date of submission: April 29, 2015

Summary: Besides dimethylsulfoniopropionate (DMSP), other dimethylsulfonio-compounds like dimethylsulfonio acetate (DMS-Ac) and gonyol can be found in phytoplankton cultures with highly diverse relative abundances. Marine bacteria which are, due to their metabolic activity, a major sink for DMSP in the oceans are able to metabolize DMS-Ac and gonyol as well and with partly comparable efficiencies. Release of MeSH in *Ruegeria pomeroyi* and *Alcaligenes faecalis* cultures after incubation with DMS-Ac and gonyol, respectively, indicate possible yet unknown demethylation/demethiolation mechanisms. Additionally, the composition of dimethylsulfonio-osmolytes in the surrounding medium affects the different degradation pathways for these compounds and thus the release of methanethiol (MeSH) and the climate active gas dimethylsulfide (DMS).

Publication equivalents of contributing PhD students as coauthors according to the implementing provision of the doctoral regulations at the Faculty for Chemistry and Earth Sciences of the Friedrich Schiller University Jena:

	Author 1: **Gebser**
Conception of the work	X
Planning of experiments	X
Data collection	X
Analysis and interpretation	X
Writing of manuscript	X
Proposed publication equivalents	1

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Jena,29.04.2015

Manuscript Submission

Dear Editor,

Please find enclosed our manuscript entitled " Phytoplankton-derived small zwitterionic dimethylsulfonio-compounds are widespread and interfere with microbial DMSP / DMS sulfur cycling". It reveals that most microalgae from the plankton produce a complex mixture of small sulfur containing osmolytes. The influence of these osmolytes on bacterial metabolism is then established in detail. We conclude that aspects of the marine sulfur cycle have to be re-visited and therefore think that this contribution matches perfect the scope of Applied and Environmental Microbiology.

Sincerely,

On behalf of all co-authors

Phytoplankton-derived small zwitterionic dimethylsulfonio-compounds are widespread and interfere with microbial DMSP / DMS sulfur cycling

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Running Title: Dimethylsulfonio compounds and bacterial sulfur cycle

Abstract

The marine planktonic sulfur cycle is substantially fuelled by the phytoplankton osmolyte dimethylsulfoniopropionate (DMSP). This metabolite can be transformed by bacteria, which results in the emission of the volatile sulfur species dimethylsulfide (DMS) and methanethiol (MeSH). It is generally accepted that bacteria thereby contribute significantly to DMSP turnover. In this context we show that the other low molecular weight zwitterionic dimethylsulfonio-compounds dimethylsulfonioacetate (DMSA) and gonyol are widely distributed in phytoplankton. DMSA was found in all thirteen surveyed phytoplankton species and gonyol was detected universally in haptophytes and dinoflagellates. We show that these prevalent metabolites can be degraded by all tested bacteria. But upon addition of DMSA and gonyol only *Ruegeria pomeroyi* and *Alcaligenes faecalis* released elevated amounts of MeSH. In addition gonyol and DMSA modulate bacterial DMS release. In *Ruegeria pomeroyi* gonyol even quantitatively inhibited DMS production if offered in mixtures with DMSP. We argue that the prevalence of DMSA and gonyol as well as their potential to interfere with DMSP-metabolism might result in a modulation of the planktonic sulfur cycle.

Introduction

The ubiquitous marine algal metabolite dimethylsulfoniopropionate (DMSP) and its cleavage product dimethylsulfide (DMS) play key roles in the marine sulfur cycle (1, 2). The zwitterionic DMSP is predominantly produced by marine phytoplankton and fulfills various physiological functions as an osmoprotectant (3), a cryoprotectant (4, 5) or an antioxidant (6). An estimated annual production of DMSP of around 10^9 tons fuels the marine sulfur cycle and it is thus not surprising that marine bacteria and algae have evolved multiple pathways to utilize this resource (7, 8). Marine bacteria can sustain up to 95% of their sulfur and 15% of their carbon requirements through metabolization of

DMSP (9). Two major metabolic pathways for the degradation of DMSP have been reported from bacteria (Fig. 1A). The demethylation/demethiolation pathway initially leads to the formation of 3-(methylthio)propionate that is the substrate for the release of methanethiol (MeSH) (10). The first step of this pathway is encoded in the *dmdA* gene which is widely distributed in marine bacteria (11-13). The DMSP cleavage pathways to DMS are catalyzed by several different enzymes forming either acrylate (14) or 3-hydroxypropionate as further reaction products (15).

Other metabolites that contain the dimethylsulfonio structural element found in DMSP have been described for marine phytoplankton. This includes dimethylsulfonioacetate (dimethylthetin, DMSA) and gonyol (Fig. 1B) (16-18). In fact it is estimated that, depending on the species, up to 10% of the marine DMS may derive from non-DMSP sources (19). It was further shown that DMSA is recognized by the glycine betaine uptake system in marine bacteria (20) and can be used for osmoregulative functions (21).

Until recently dimethylsulfonio-compounds besides DMSP were considered to be rather exotic and were reported from only a few algal species. This view might however change, with a recently introduced method for the direct monitoring of low molecular weight zwitterionic metabolites including the dimethylsulfonio-derivatives described above (19, 22). Using this methodology we surveyed the two globally important microalgae *Emiliania huxleyi* and *Prorocentrum minimum* for the regulation of such zwitterionic metabolites during osmoacclimation (16). Gonyol, a metabolite described in only one dinoflagellate until then, was found in both of the tested species and DMSA was detected in *P. minimum*. This prompted us to undertake a survey of the distribution of these metabolites in a broader selection of phytoplankton species that is presented here. Indeed we found high abundance and broad distribution of DMSA and gonyol. We therefore also addressed their potential function as sulfur sources or mediators of sulfur metabolization in the four model bacteria *Ruegeria pomeroyi* DSS-3, *Halomonas* sp. HTNK1, *Alcaligenes faecalis* M3A and *Sulfitobacter* sp. EE-36. These marine bacteria are well known DMSP catabolizers that use a set of different DMSP-dependent DMS-production pathways involving the central enzymes listed in Table 1.

Experimental section

Cultivation of bacteria Stock cultures of *Ruegeria pomeroyi* DSS-3 and *Sulfitobacter* sp. EE-36 were grown in marine basal medium MBM (23). *Alcaligenes faecalis* M3A and *Halomonas* sp. HTNK1 were cultivated in M9 minimal medium (Sigma-Aldrich, Deisenhofen, Germany). All cultures were grown under gentle shaking at 28°C with addition of 10 mM sodium succinate as carbon source. Out of these stock cultures samples, taken from exponential growth phase, were used for further incubation experiments with addition of different substrates.

Incubation experiments Prior to incubation experiments aliquots of the bacteria cultures were washed three times by centrifugation and subsequent resuspension in succinate free medium to remove the high excess of organic carbon. For incubation experiments all bacteria cultures were diluted with succinate-free medium to the same optical density of OD = 0.1. For incubation experiments 450 µl of these cultures were transferred into autoclaved 4.92 ml screw cap vials with sterile PTFE/silicone septa. After addition of ca. 10 µl of an aqueous solution of the substrates (DMSP, DMSA and racemic gonyol) to a final concentration of 3.33 µM the vials were sealed and placed on a heated shaker at 28°C for 24 h. The large headspace of the vials was needed to enrich the volatile sulfur compounds DMS and MeSH for further quantification. For all treatments including a control without any addition of substrates four biological replicates were prepared.

After 24 h of incubation methanethiol (MeSH) and dimethylsulfide (DMS) were quantified using headspace sampling and direct injection into a GC-FPD system (see below).

Control measurements of the synthetic substrates revealed that the synthetic gonyol standard released just minor amounts of DMS upon base treatment. In fact only 1.9 ± 0.1% DMS was released under alkaline conditions. This low amount of DMS release can be ascribed to remaining impurities from the chemical synthesis. The composition of these base labile impurities remains unknown. This DMS background caused by gonyol was subtracted from DMS concentrations in all gonyol containing treatments.

GC-FPD measurement of MeSH and DMS For quantification of MeSH and DMS in the headspace of the samples, the sealed vials were flushed with oxygen-free nitrogen for 1 min at a flow rate of 60 ml/min. After cryogenic enrichment of the samples which was carried out according to Steinke *et al.* (24), the sample was introduced to the gas chromatograph (GC-2010, Shimadzu) equipped with a 30 m x 0.53 mm 5 µm HP-1 capillary column, (Agilent, Wokingham, UK) and a flame photometric detector, by rapid heating of the sample loop using freshly boiled water. The CG-oven was set isothermally at 40°C with helium as carrier gas at a flow rate of 10.56 ml/min. The flame gases for the FPD, compressed air and hydrogen, were set to 70 ml/min and 60 ml/min, respectively. Calibration for DMS was done by pipetting <10 µl of aqueous DMSP standard solutions to 450 µl 1M NaOH in a 4.92 ml screw cap vial with PTFE/silicone septa. The vials were sealed immediately after addition of the DMSP standard. After incubation for 24 h at 30°C the samples were analyzed as outlined above. Possible matrix effects of the different cultivation media (MBM and M9) on the DMS calibration were not investigated. For MeSH calibration 10.9 mg sodium methanethiolate (Sigma Aldrich) was dissolved in 1 ml 10M NaOH as stock solution. Higher dilutions of the stock solution were prepared in 1M NaOH. After pipetting <10 µl of the standard solution to 450 µl 2M sulfuric acid in 4.92 ml screw cap vials, the samples were incubated and analyzed as mentioned above. To quantify the amount of possible impurities in the synthetic DMSA and gonyol, standard solutions were added to 450 µl 1M NaOH in 4.92 ml screw cap vials leading to a concentration of 3.33 µM and incubated for 1 h at 30°C before analysis with GC-FPD as mentioned above. Samples were vigorously shaken several times during incubation and prior to the measurements to achieve equilibrium between liquid and gas phase.

Quantification of unmetabolized substrates After quantification of DMS and MeSH, 100 µl methanol was added to 100 µl of bacteria culture. These suspensions were stored at -20°C until further measurement. Unmetabolized substrates were quantified via a direct UPLC-MS method for zwitterionic osmolytes according to Gebser and Pohnert (16). Samples for UPLC-MS analysis were prepared from 50 µl of the suspensions that were added to 200 µl acetonitrile/water 9:1 in micro

centrifuge cups. After addition of the respective isotope-labeled internal standards (D_6-DMSP, D_6-DMSA and D_3-gonyol, respectively) samples were centrifuged (2 min at 16000 g) to remove remaining cell fragments and the supernatant was measured via UPLC-MS. Relative response factors of the substrate and the corresponding internal standard were determined by addition of equimolar concentrations of the analyte and the internal standard to the extracts of the untreated bacteria cultures. After dilution with acetonitrile/water, the samples were analyzed via UPLC-MS.

Substrate-mix treatments For determination of possible synergistic or antagonistic effects of the different substrates on the MeSH and DMS release, treatments with all substrate combinations of DMSP, DMSA and gonyol were made. For these experiments bacteria cultures were cultivated and washed as outlined for incubation experiments. After addition of the different substrate combinations (final concentration of each substrate 3.33 µM), MeSH and DMS were quantified as described above. The test for possible impurities of the pure DMSA and gonyol causing DMS background were done as described above with a DMSA/gonyol standard solution (3.33 µM each).

Quantification of bacterial growth To determine the effect of the substrates on bacterial growth, stock cultures were cultivated and washed as outlined above. Bacteria cultures were transferred to autoclaved 20 ml headspace vials with cotton stopper for further cultivation (28°C, shaking). After addition of 3.33 µM DMSP, DMSA and gonyol, respectively, bacterial growth was monitored for 72 h by measuring the optical density at 600 nm using a two beam UV-Vis spectrophotometer (Specord M42, Carl Zeiss Jena, Jena, Germany) and standard single use polystyrene cuvettes (Sarstedt AG & Co., Nümbrecht, Germany). For each strain, a control without any substrate addition was prepared. All treatments were carried out as triplicates.

Bacterial consumption of MeSH and DMS In order to determine the consumption of highly volatile DMS and MeSH by the different investigated bacteria, cultures were prepared as mentioned above (4.92 ml screw cap vial, 450 µl culture, OD = 0.1). To each culture, less than 10 µl of a freshly prepared aqueous solutions of DMS (Sigma-Aldrich, Germany) or MeSH (Fisher Scientific, Germany),

were added as substrate (final concentration 3.33 µM). Vials were sealed immediately after addition of the substrate solution and samples were incubated for 24 h at 28°C under continuous shaking. Control treatments included non-inoculated MBM and M9 media. After incubation, 25 µl of gaseous phase were taken from the headspace of the samples with a gastight syringe (Hamilton, Switzerland) and injected into a GC-MS system (Thermo Finigan, ISQ) equipped with a Zebron ZB-1ms column (60 m x 0.25 mm x 1 µm, Phenomenex, Germany). Oven temperature was set at 50°C. Remaining DMS and MeSH in the gas phase were determined by calculation of the peak areas of the corresponding mass traces, m/z = 62 and m/z = 48, respectively. Measurements for bacterial consumption of DMS and MeSH and unspecific reactivity of MeSH were performed with five individual replicates.

Unspecific interaction/reaction of MeSH Due to the high reactivity of MeSH, we determined possible unspecific particle interaction with the bacteria samples. After measurements for bacterial consumption of MeSH, the corresponding samples were boiled for 2 min to stop bacterial metabolism and to evaporate possible remaining MeSH. After cooling to room temperature, an aqueous solution of MeSH was added as mentioned above and the samples were incubated again for 24 h at 28°C. Remaining MeSH in the gas phase was determined via GC-MS as outlined for bacterial consumption of MeSH and DMS.

Results and discussion:

Distribution of dimethylsulfonio-metabolites

We selected 13 phytoplankton species (7 diatoms, 3 dinoflagellates, a cryptophyte, and 2 haptophytes) to screen for intracellular DMSA and gonyol concentrations. As reported previously, gonyol was abundant in *Lingulodinium polyedrum*, the dinoflagellate from which it was initially isolated (16-18). In addition, our screening revealed that all dinoflagellates tested contained this metabolite in significant quantities of up to 23.6% compared to the relative abundance of DMSP

(Table 2). Also the haptophytes *E. huxleyi* and *Isocrysis galbana* contained around 5% gonyol relative to DMSP. In contrast, gonyol was not detected in any of the diatoms investigated.

We detected DMSA in all species tested (Table 2). In the diatom *Stephanopyxis turris* and the cryptophyte *Rhodomonas sp*. DMSA exceeded the amount of DMSP more than 10-fold. In other species the amount varied between 23% and 0.1% relative to DMSP. Given the few reports of this osmolyte, its universal distribution is rather surprising and suggests an underestimation of the variety of dimethylsulfonio metabolic pathways in the literature. The analytical procedures predominantly used for quantification of DMSP rely on its conversion to DMS during chemical hydrolysis with strong base and detection of the released DMS (25-28). The circumstance that DMSA does not cleave DMS under alkaline conditions (data not shown) may contribute to the fact that it has been overlooked in many previous studies. Furthermore, the analytical procedure for detection of DMSP does not distinguish between DMSP and other dimethylsulfonio-metabolites as possible sources for DMS from alkaline hydrolysis. Due to these methodological limitations, most previous studies do not provide the entire profile of dimethylsulfonio metabolites. In contrast, the method used in this study allows the direct quantification of a multitude of low molecular weight zwitterionic metabolites including DMSP, DMSA and gonyol (22, 29). It can be concluded that the few reports of non-DMSP dimethylsulfonio-metabolites in the literature are not representative for the actual diversity of this compound class in nature.

As a consequence of this broad distribution of non-DMSP dimethylsulfonio-metabolites the question arises how these compounds influence and contribute to the marine microbial sulfur cycle. Laboratory experiments that challenged bacteria with pure DMSP showed a significant turnover of this compound (9, 30-32) but the results in Table 2 suggest that they will rather be frequently exposed to a complex mixture of dimethylsulfonio-metabolites including DMSP, DMSA and gonyol. To characterize the bacterial utilization and degradation of these zwitterionic osmolytes, we chose the four well-studied model species *Ruegeria pomeroyi* DSS-3, *Halomonas* sp. HTNK1, *Alcaligenes faecalis* M3A and *Sulfitobacter* sp. EE-36 for which information about DMSP cleavage

activities, corresponding genes and the demethylation/demethiolation pathway are available (33-39).

Substrate utilization

The metabolism of the individually added substrates DMSP, DMSA and gonyol by the studied bacteria (OD = 0.1) was calculated as the percentage of the initial substrate concentration (3.33 µM) remaining after 24 h incubation. The applied substrate concentration is high compared to the batch availability of these compounds in natural seawater which is typically in the low nanomolar range (40). However, during senescence and collapse of phytoplankton blooms, microenvironments high in organics release nutrients to the surrounding seawater. Phytoplankton associated bacteria are therefore commonly exposed to pulses of high nutrient concentration which includes metabolites such as DMSP, DMSA and gonyol. In this context, our findings describe processes associated with such microenvironments rather than the often low nutrient conditions of the surrounding seawater.

Our values for utilization of DMSP, DMSA and gonyol, respectively, represent the metabolic activity regardless of the used degradation or conjugation pathway. It is remarkable that *R. pomeroyi* quantitatively utilized all of the three added substrates (Fig. 2A), although no volatile products were detected from gonyol (Fig. 2E). Due to the total conversion of DMSP and gonyol by *R. pomeroyi* a possible preference in utilization between these compounds could not be detected. Since the added, synthetic, gonyol was a racemic mixture, the pathways in gonyol metabolism are obviously not enantioselective. In the other bacterial cultures transformation of the administered substrates was obvious but not quantitative despite similar initial optical densities at the start of the incubations. DMSP, DMSA and gonyol were utilized with similar efficiency in *Sulfitobacter* sp. and *Halomonas* sp. (Fig. 2B, D). Both bacteria metabolized ca. 45% of the initially supplied substrates within 24 h of incubation. Furthermore, no discrimination between the three substrates could be observed. In contrast, *A. faecalis* showed a significantly higher transformation of DMSP (77.8 ± 16.2%) compared to DMSA (55.2 ± 1.4%, t-test: $P = 0.029$, $n = 4$) and gonyol (43.9 ± 1.2%, $P = 0.029$), respectively (Fig.

213 2C).These results demonstrate that the widely distributed zwitterionic metabolites DMSA and gonyol
214 can be readily metabolized by marine bacterioplankton involved in DMSP degradation. Due to the
215 consumption of ca. 45% of gonyol in *Sulfitobacter* sp., *Alcaligenes faecalis* and *Halomonas* sp., it
216 remains unclear whether the involved enzymes show a preference for one of the enantiomers or
217 recognize both stereoisomers as substrate.

218 **Release of MeSH and DMS from DMSP, gonyol and DMSA**

219 Since we showed that DMSP, DMSA and gonyol were metabolized by all investigated bacteria we
220 aimed to categorize the pathways involved by quantifying the volatile sulfur-metabolites MeSH and
221 DMS. The release of these volatiles from synthetic DMSP, DMSA and gonyol was determined by
222 headspace extraction and GC/FPD measurements in bacterial cultures which were not pre-
223 acclimated to the utilization of these substrates. Control measurements in non-inoculated medium
224 with added substrates revealed that DMSP, DMSA and gonyol did not release any of these volatiles in
225 the absence of bacteria (data not shown). Therefore DMS and MeSH release results from the intrinsic
226 enzymatic activity in the examined bacteria (Fig. 2).

227 Bacteria have been shown to consume the volatiles DMS and MeSH (31, 41) so their final
228 concentrations may be affected by gross production and consumption processes in our experiments.
229 To assess DMS and MeSH consumption we conducted short-term (24h) incubations and quantified
230 their concentrations in the absence (medium control) and presence of bacteria using GC-MS. There
231 was no significant difference in DMS concentration between *R. pomeroyi*, *Sulfitobacter* sp., *A. faecalis*
232 and the corresponding medium controls (see Figures 2 and 3 in supplementary material). This
233 indicates that DMS was not metabolized in significant amounts so that gross consumption was
234 negligible. *Halomonas* sp. showed 8% consumption of DMS compared to non-inoculated M9
235 medium. As a consequence, measured DMS concentrations should be regarded as good
236 approximation for net accumulations over the incubation period in this species. Analogous
237 experiments with MeSH as substrate showed that all cultures quantitatively metabolized MeSH

238 (Figures 2 and 3 in supplementary material). Inactivated bacteria (boiled controls) showed the same
239 concentrations as the medium controls so that non-specific loss of the reactive MeSH in the presence
240 of organic material can be ruled out (see Figures 2 and 3 in supplementary material). This suggests
241 that the net production of MeSH in our experiments is an underestimate of the gross production rate
242 resulting from close coupling of production and consumption processes.

243 The different bacteria converted DMSP to DMS with various efficiencies. While DMS concentration
244 after 24 h of incubation of *R. pomeroyi* with 3.33 µM DMSP was 47 ± 36 nM (Fig. 2E), *A. faecalis*
245 showed a 32-fold higher DMS release of 1510 ± 730 nM (Fig. 2G).These findings are consistent with
246 experiments by Todd *et al.* (42) and Curson *et al.* (34) who calculated the DMSP-lyase activities of
247 *R. pomeroyi* and *A. faecalis* as ca. 2 pmol (DMS) µg (Protein)$^{-1}$ min^{-1} and 60 pmol (DMS) µg (Protein)$^{-1}$ min^{-1}, respectively. Our data regarding DMSP-dependent DMS production are likely underestimates
249 since bacteria were not pre-exposed to DMSP and, therefore, were not acclimated to utilize this
250 substrate. Thus direct comparison of our data with results from other studies on DMSP consumption
251 in bacteria that were grown on high concentrations (5 mM) of DMSP to maximize expression of
252 enzymes required for DMSP catabolism (34, 39, 42) have to be treated with caution. We could not
253 demonstrate DMS release from substrates other than DMSP (Fig. 2E-H). This indicates that enzymes
254 involved in DMS production in these model organisms are highly substrate-specific. This high
255 specificity might be explained by the enzyme mechanism recently identified for the DMSP lyase
256 DddQ, from *Ruegeria lacuscaerulensis* (43). This lyase relies on abstraction of an acidic alpha proton
257 from DMSP resulting in concomitant beta-elimination of DMS and the release of acrylate. This
258 elimination mechanism is excluded for the shorter chain length homolog DMSA and the longer chain
259 length homolog gonyol.

260 In contrast to the lyase-pathway, substrate utilization via the demethylation/demethiolation
261 pathway is apparently not limited to DMSP. In contrast to the control, we detected substantial MeSH
262 release after incubation of *R. pomeroyi* with DMSA (790 ± 350 nM MeSH; Fig. 2E). While MeSH
263 release from DMSP is described in bacteria (10, 38), Reisch *et al.* (2008) show that the purified DMSP-

dependent demethylation enzyme DmdA which catalyzes the first reaction step of the demethylation/demethiolation pathway does not recognize DMSA. This lack of activity would explain that MeSH release compared to the control was not increased in *Sulfitobacter* sp. and *Halomonas* sp. In *R. pomeroyi*, however, a hitherto unidentified enzymatic activity is apparently responsible for MeSH release from DMSA. These results thus indicate the existence of a different demethylation/demethiolation pathway in bacteria that accepts DMSA as substrate. Interestingly, this alternative pathway is quite efficient, since MeSH release from DMSA in *R. pomeroyi* (790 ± 350 nM) was higher than the demethylation/demethiolation activity for DMSP that accounted for 390 ± 31 nM MeSH release (Fig. 2E). Due to a possible lower outlier in the DMSA measurements (Dean-Dixon test, $N = 4$, $\alpha = 0.1$) the difference is not significant ($P = 0.343$) but can be regarded as a major trend (without outlier: $P \leq 0.001$). The importance of this newly identified source for MeSH production lies in the high relevance of MeSH for sulfur assimilation by marine bacteria (31, 44). In *A. faecalis* MeSH release in the presence of DMSA followed a different pattern. Here DMSA resulted in a decrease of MeSH in comparison to the untreated cultures. Whether this effect is a consequence of decreased production or increased consumption of MeSH remains unclear. In contrast to *R. pomeroyi*, *A. faecalis* responded to gonyol with a significantly higher MeSH release (140 ± 3 nM) compared to the untreated control ($P = 0.029$). It remains unclear whether this can be ascribed to a higher demethylation/demethiolation activity or a decrease in MeSH metabolism of *R. pomeroyi*.

Interestingly, gonyol interfered with natural MeSH release observed in the control culture of *Sulfitobacter* sp. (715.7 ± 50.6 nM). In contrast, MeSH release was not affected by the addition of DMSP (564.0 ± 271.4 nM; $P = 0.314$) or DMSA (613.0 ± 169.8 nM; $P = 0.290$) (Fig. 2F). A possible antibacterial function of gonyol which could explain this result can be excluded since a disc diffusion test with *Sulfitobacter* sp. and different gonyol concentrations was negative (data not shown) and growth curves did not differ with respect to the presence and absence of this substrate (Fig. 2 in supplementary material).

In all investigated cases besides activity of DMSP catabolism in *A. faecalis*, the release of volatile sulfur compounds explained only a minor fraction of the overall transformed substrates. It is thus obvious that the bacteria tested utilize sulfur and presumably carbon of all administered substrates and that volatile emission represents only a minor pathway. In certain combinations (gonyol with *R .pomeroyi* and *Sulfitobacter* sp. or DMSA with *A. faecalis*) no volatile emission was observed despite substantial transformation of the administered substrates.

Inhibition of DMSP metabolization by gonyol and DMSA

The experiments described above indicate that DMSA and gonyol might interfere with the release of volatile sulfur metabolites (Fig. 2E-G). To explore the inhibitory action in a systematic manner, addition of combinations of DMSP, DMSA and gonyol to the bacteria cultures were tested. Compared to the controls (Fig. 2) we observed species-specific inhibitory effects of DMSA and gonyol on the enzymes involved in DMSP metabolism. These effects manifested in a modulation of the release of volatile sulfur compounds. An addition of an equimolar DMSP/DMSA mixture (both at 3.33 µM) to *R. pomeroyi* indicated an antagonistic effect of DMSA on the net release of MeSH (272 ± 215 nM; Fig. 3A) compared to 1180 ± 350 nM as the sum of the single treatments (Fig. 2E). Gonyol addition in any of the administered combinations resulted in no net MeSH or DMS production in *R. pomeroyi* (Fig. 3A), which is in agreement with the findings on the effect of gonyol in isolation (Fig. 2E).

The inhibitory action of gonyol on the MeSH production in *Sulfitobacter* sp. which was observed in the single substrate treatments (Fig. 2F) could also be observed in the mixed substrate experiments (Fig. 3B). The fact that there are significant concentrations of MeSH in treatments where gonyol is present in combination with at least one other osmolyte (DMSP, DMSA) might indicate a protective effect of DMSP and DMSA on the inhibitory influence of gonyol (compare Figures 2F and 3B). In *A. faecalis* and *Halomonas* sp. no inhibitory effect of gonyol on MeSH net release was observed (Fig. 3C, D). The release of MeSH in *A. faecalis* (Fig. 3C) likely resulted from the addition of gonyol which is consistent with the results from single substrate treatments (Fig. 2G).

Notably the presence of DMSA and gonyol, respectively, seems to decrease net DMS release. This inhibitory activity is not connected to the properties of the metabolites as substrates for volatile sulfur emission since DMS production was universally inhibited by gonyol in *R. pomeroyi* (Fig. 3A). Together with its wide distribution, this implies a substantial impact of the microalgal osmolyte gonyol on the DMSP catabolism in marine bacterioplankton. A similar but less dramatic effect was also observed in *Sulfitobacter* sp. and *Halomonas* sp. where DMS concentrations were 44% ($P \leq 0.001$) and 23% ($P \leq 0.001$) lower, respectively, in the treatment containing DMSP and DMSA plus gonyol compared to the DMSP/DMSA treatment.

A. faecalis with all three osmolytes DMSP, DMSA plus gonyol showed significantly higher net DMS-production with 3026.0 ± 169.6 nM than in the DMSP plus gonyol treatment (1974.5 ± 533.5 ; $P = 0.009$) and nearly the same as in the treatment containing only DMSP and DMSA ($P = 0.134$, Fig. 3C). Together with the result that DMSA is not a source for DMS (Fig. 2G) this suggests a protective effect of DMSA on the DMS-production activity in *A. faecalis*.

Halomonas sp. showed a slightly different pattern than the other bacteria (Fig. 3D). The addition of gonyol did significantly affect DMS-release in the DMSP/DMSA treatment (399.5 ± 15.2 nM; $P = 0.144$) compared to DMSP/DMSA/gonyol (308.8 ± 6.3 nM). Treatments of DMSP/gonyol (377.2 ±21.8 nM) and DMSP/DMSA (399.5 ± 15.2 nM) were not significantly different ($P = 0.144$). This pattern could be caused by different types of DMSP-cleaving enzymes in this model organism. The DddD enzyme from *Halomonas* sp. leads to 3-hydroxypropionate as by-product of the DMSP cleavage reaction (15, 35) whereas all other DMS-producing enzymes of the bacteria tested here co-produce acrylate (Fig. 1) (34).

Taken together these results show that the phytoplankton-derived zwitterionic dimethylsulfonio compounds DMSA and gonyol affect the release of climatically active DMS and MeSH by marine bacteria. Given their wide distribution (Table 2) this has potentially significant implications for previous considerations on the marine sulfur cycle. Additional sources for sulfur containing volatiles

339 have to be taken into account as well as possible inhibitory effects that might serve as indirect
340 regulators of plankton dynamics.

341 **Conclusion**

342 We show that the zwitterionic algal osmolytes DMSA and gonyol are widely distributed in
343 phytoplankton. As a consequence, bacterial communities will often be exposed to mixtures of these
344 structurally-related dimethylsulfonio-metabolites. The compounds and their inhibitory effect on the
345 bacterial sulfur metabolism were highly species-specific. All bacteria tested were capable of
346 transforming these substrates. However the transformation pathways apparently differed.
347 *R. pomeroyi* and *A. faecalis* can enzymatically metabolize DMSA and gonyol, respectively, under
348 MeSH release, while *Sulfitobacter* sp and *Halomonas* sp. do not produce any volatiles from these
349 sources. The enzymatic release of MeSH from DMSA suggests a so far unrecognized
350 demethylation/demethiolation pathway. Furthermore, gonyol strongly interfered with DMSP
351 metabolization in *R. pomeroyi* and resulted in a complete suppression of volatile release. This
352 suggests that gonyol affects the marine sulfur cycle by modulating the transformation of other
353 potential substrates including DMSP. Future studies should consider the differential effects of these
354 molecules on purified enzymes as well as in complex plankton samples to further our understanding
355 of the mechanisms in bacterial degradation of DMSP and related substances.

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Table and figure captions

Table 1: Enzymes for DMSP-dependent DMS production (ddd) identified in model organisms used in this work

Table 2: Relative abundance of DMSA and gonyol in different phytoplankton cultures, values are normalized to DMSP concentration, (n.d.) not detected

FIG 1 A) Major catabolic pathways of DMSP. B) Alternative metabolites containing the dimethylsulfonio structure element found in phytoplankton.

FIG 2 Mean utilization (in %) of the different substrates and concentrations of volatile sulfur compounds methanethiol (MeSH) and dimethylsulfide (DMS) released by *Ruegeria pomeroyi* DSS-3 (A, E), *Sulfitobacter* sp. EE-36 (B, F), *Alcaligenes faecalis* M3A (C, G) and *Halomonas* sp. HTNK1 (D, H) after 24 h incubation. Error bars represent standard deviation between biological replicates, N=4. Control measurements of bacteria cultures without substrate addition are referred to as pure culture.

FIG 3 Mean concentrations of volatile sulfur compounds methanethiol (MeSH) and dimethylsulfide (DMS) in combined substrate treatments with *Ruegeria pomeroyi* DSS-3 (A), *Sulfitobacter* sp. EE-36 (B), *Alcaligenes faecalis* M3A (C) and *Halomonas* sp. HTNK1 (D) after 24 h incubation. Error bars represent standard deviation between biological replicates, N=4.

509 Table 1: Enzymes for DMSP-dependent DMS production (ddd) identified in model organisms used in
510 this work

Species	**ddd enzymes**	**References**
Ruegeria pomeroyi DSS-3	dddP dddQ dddW	(45) (42) (46)
Sulfitobacter sp. M3A	dddL	(37)
Alcaligenes faecalis EE-36	dddY	(34)
Halomonas sp. HTNK1	dddD	(33)

511

512

513 Table 2: Relative abundance of DMSA and gonyol in different phytoplankton cultures, values are
514 normalized to DMSP concentration, (n.d.) not detected

Species	**Taxonomic group**	**Strain no.**	**DMSA (%)**	**gonyol (%)**
Chaetoceros compressum	diatom	CCMP168	6.0	n.d.
Navicula sp.	diatom	I15	4.8	n.d.
Nitzschia cf pellucida	diatom		0.9	n.d.
Phaeodactylum tricornutum	diatom	CCMP2561	0.6	n.d.
Stephanopyxis turris	diatom		1040	n.d.
Thalassiosira weissflogii	diatom	RCC76	24	n.d.
Thalassiosira rotula	diatom	RCC776	4.4	n.d.
Rhodomonas sp.	cryptophyte		1990	n.d.
Isochrysis galbana	haptophyte		0.4	6.2
Emiliania huxleyi	haptophyte	RCC1217	0.4	4.5
Prorocentrum minimum	dinoflagellate		2.3	7.9
Amphidinium carterae	dinoflagellate	CCMP1314	0.1	23.6
Lingulodinium polyedrum	dinoflagellate	SCCAP K-0982	3.8	121

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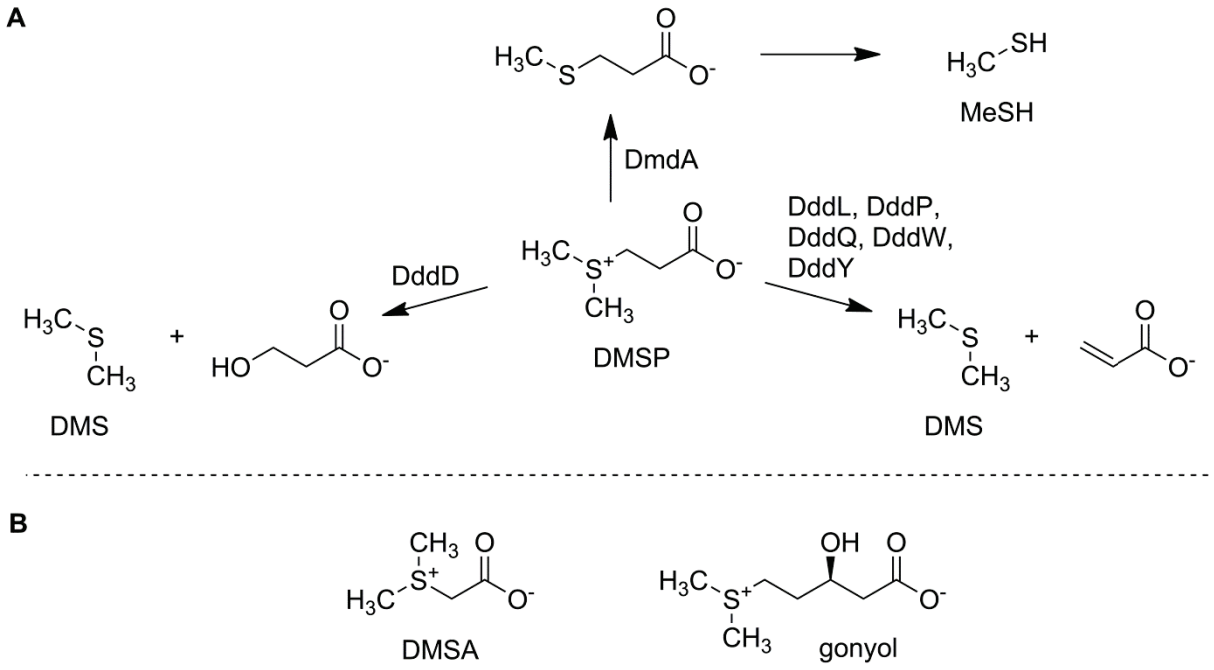
517 FIG 1 A) Major catabolic pathways of DMSP. B) Alternative metabolites containing the
518 dimethylsulfonio structure element found in phytoplankton.



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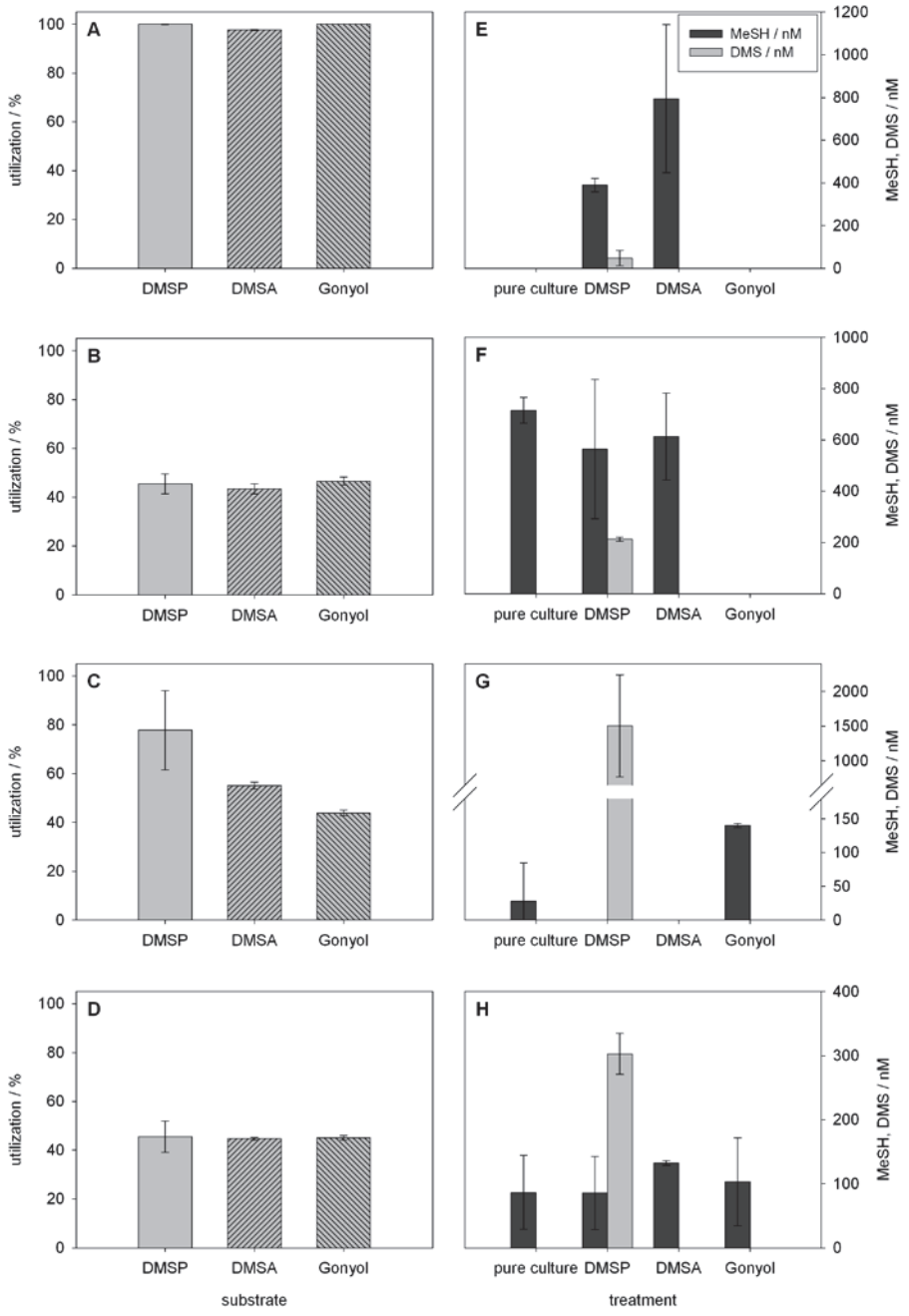
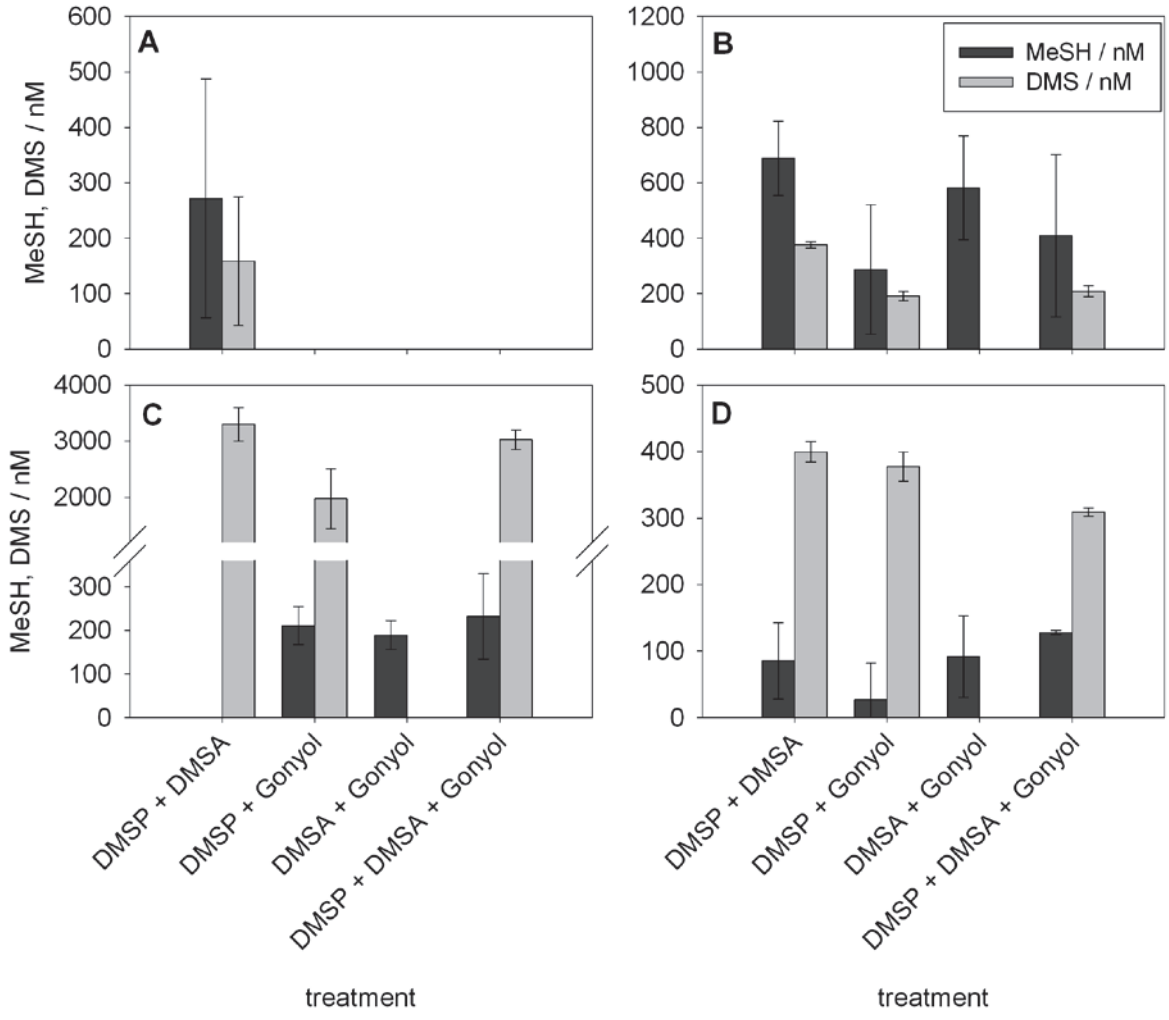
521 FIG 2 Mean utilization (in %) of the different substrates and concentrations of volatile sulfur
522 compounds methanethiol (MeSH) and dimethylsulfide (DMS) released by *Ruegeria pomeroyi* DSS-3
523 (A, E), *Sulfitobacter* sp. EE-36 (B, F), *Alcaligenes faecalis* M3A (C, G) and *Halomonas* sp. HTNK1 (D, H)
524 after 24 h incubation. Error bars represent standard deviation between biological replicates, N=4.
525 Control measurements of bacteria cultures without substrate addition are referred to as pure
526 culture.



527

528 FIG 3 Mean concentrations of volatile sulfur compounds methanethiol (MeSH) and dimethylsulfide
529 (DMS) in combined substrate treatments with *Ruegeria pomeroyi* DSS-3 (A), *Sulfitobacter* sp. EE-36
530 (B), *Alcaligenes faecalis* M3A (C) and *Halomonas* sp. HTNK1 (D) after 24 h incubation. Error bars
531 represent standard deviation between biological replicates, N=4.



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Phytoplankton-derived small zwitterionic dimethylsulfonio-compounds are widespread and interfere with microbial DMSP / DMS sulfur cycling

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Supplementary materials:

Synthesis of zwitterions:

DMSP and D_6-DMSP (as hydrochloride):

DMSP and D_6-DMSP were synthesized according to Chambers [1] by passing gaseous hydrogen chloride through a solution of anhydrous acrylic acid (Fluka, Germany) and dimethyl sulfide (Sigma Aldrich, Germany) or D_6-dimethyl sulfide (Sigma Aldrich, Germany), respectively. Dichloromethane was used as solvent. Recrystallization of the resulting white solid from methanol/diethyl ether (MeOH/Et_2O) gave DMSP and D_6-DMSP, respectively, as white needles.

DMSA and D_6-DMSA (as hydrobromide):

DMS-Ac and D_6-DMS-Ac were synthesized according to Howard [2] by addition of dimethyl sulfide (Sigma Aldrich, Germany) and D_6-dimethyl sulfide (Sigma Aldrich, Gemany), respectively, to a well-stirred solution of bromoacetic acid (Fluka, Germany) in dichlorormethane. Recrystallization was carried out from MeOH/Et_2O.

Rac-gonyol and rac-D3-gonyol (as hydroiodide):

Synthesis of racemic gonyol and D_3-gonyol was carried out according to Gebser [3]. Methylation of the thioether group of 3-hydroxy-5-methylthiopentanoic acid in the final step was carried out in acetone using iodomethane (Sigma Aldrich, Germany) and D_3-iodomethane (Sigma Aldrich, Germany), respectively, as alcylating agent. Reprecipitation of the raw product from MeOH/Et_2O gave gonyol and D_3-gonyol, respectively, as yellow, partially crystalline oil.

Growth curve of bacteria:

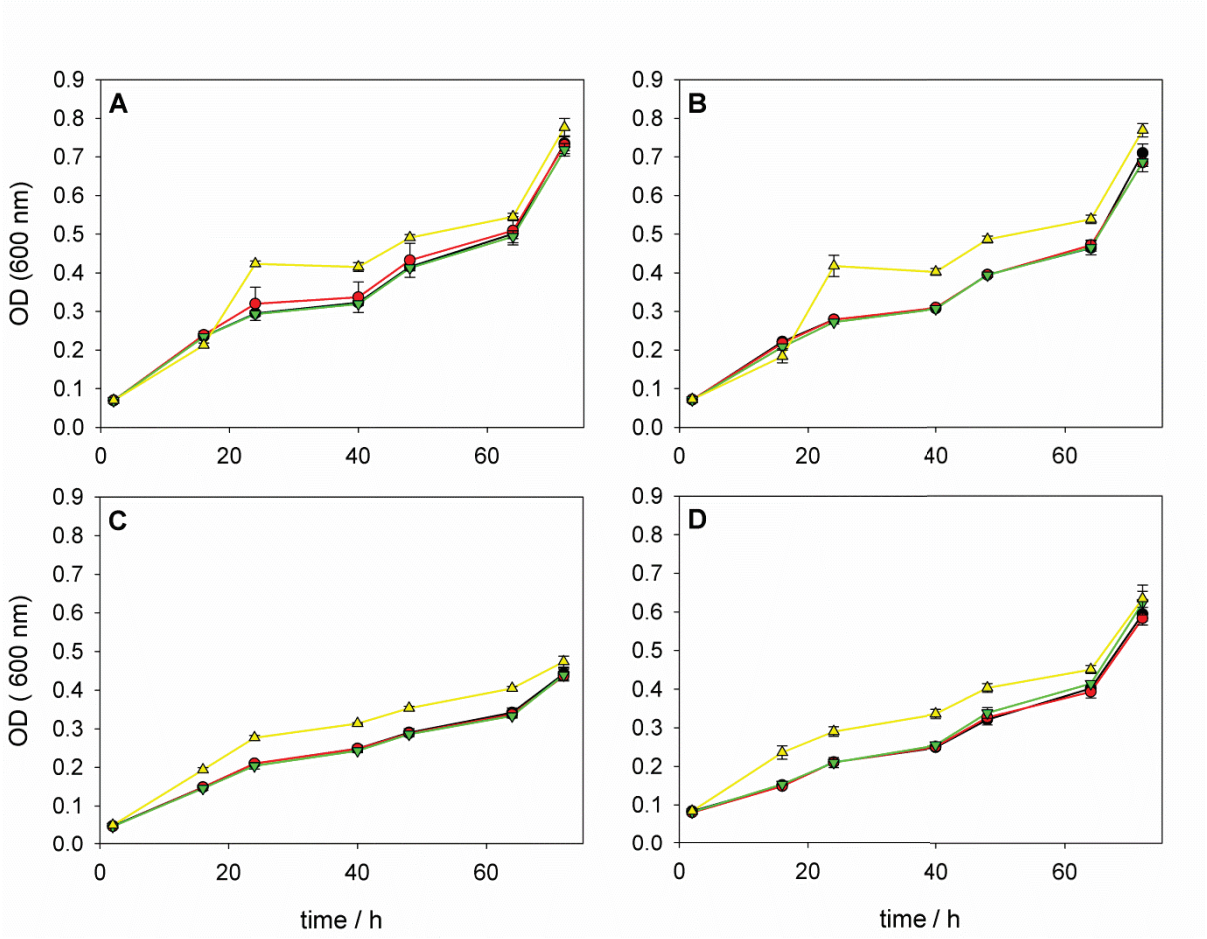

Figure 1: Growth curve of *Ruegeria pomeroyi* DSS-3 (A), *Sulfitobacter* sp. (B), *Alcaligenes faecalis* (C) and *Halomonas* sp. (D) over 72h at 28°C under constant agitation; bacteria were cultivated in MBM (A, B) and M9 minimal medium (C, D) without organic substrate addition (●), with addition of DMSP (●), DMSA (▼), and gonyol (△), respectively; concentration of substrates were 3.33 µM each; error bars represent standard deviation of biological replicates (N=3)

Bacterial consumption of MeSH and DMs

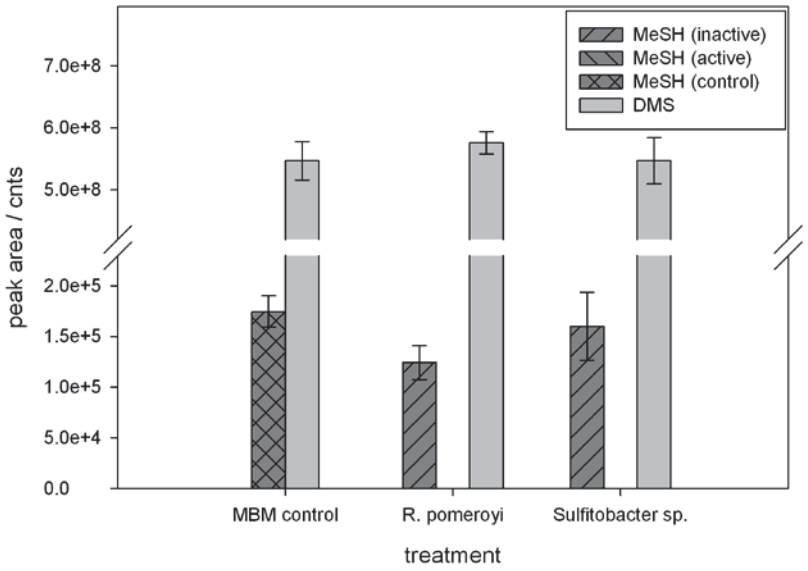

Figure 2: Bacterial consumption of DMS and MeSH, respectively over 24 h at 28°C under constant agitation. Columns represent the peak area of the corresponding mass traces of MeSH (*m/z* = 48) and DMS (*m/z* = 62), respectively. MBM control corresponds to non-inoculated marine basal medium. Active and inactive treatments correspond to live and inactivated (by boiling) cultures, respectively. Error bars represent standard deviation of individual replicates (N=5).

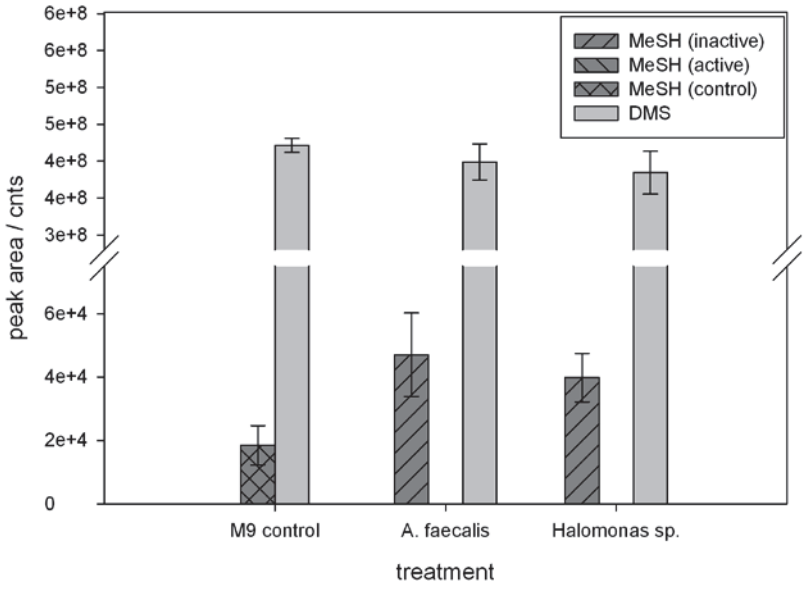

Figure 3: Bacterial consumption of DMS and MeSH, respectively over 24 h at 28°C under constant agitation. Columns represent the peak area of the corresponding mass traces of MeSH (*m/z* = 48) and DMS (*m/z* = 62), respectively. M9 control corresponds to non-inoculated M9 minimal medium. Active and inactive treatments correspond to live and

inactivated (by boiling) cultures, respectively. Error bars represent standard deviation of individual replicates (N=5)

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Manuscript F:

Mohamed B. M. Ali, Gregor C. Welna, Ahmed Sallam, Regina Martsch, Christiane Balko, Björn Gebser, Olaf Sass and Wolfgang Link (unpublished) "Association analyses of drought and frost tolerance in German winter faba beans (*Vicia faba* L.)."

Submitted to: Crop Science

Date of submission: March 30, 2015

Summary: The global climate change with increasing temperatures in central Europe will lead to larger potential cultivation area for drought and frost tolerant crops like winter types of faba bean (*Vicia faba* L.). By determination of different physiological properties like compatible solute concentration (proline, glycine betaine, soluble sugars), membrane stability and water content, monitoring of frost tolerance features and comparison with available genomic information of *Vicia faba* L., 21 putative quantitative trait loci (QTL) regarding frost tolerance were identified. Furthermore, the identification of promising inbred lines would allow breeders a more selective cultivation towards improving the dehydration and frost tolerance of *V. faba.*

Publication equivalents of contributing PhD students as coauthors according to the implementing provision of the doctoral regulations at the Faculty for Chemistry and Earth Sciences of the Friedrich Schiller University Jena:

	Author 1: **Welna**	Author 2: **Sallam**	Author 3: **Gebser**
Conception of the work	-	-	-
Planning of experiments	-	-	-
Data collection	X	X	X
Analysis and interpretation	X	X	X
Writing of manuscript	X	X	-
Proposed publication equivalents	0,5	0,5	0

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Association analyses of drought and frost tolerance in German winter faba beans (Vicia faba L.)

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Keywords:	Other legumes, Temperature stress, Water stress, Other grain crops, Crop genetics

Association analyses of drought and frost tolerance in German winter faba beans (*Vicia faba* L.)

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ABSTRACT

In parts of Central Europe, such as Germany, climate change will lead to increasing acreage utilization for winter types of faba bean (*Vicia faba* L.) with improved tolerance to drought and frost. Here, we present the first genome-wide association analysis focusing on drought and frost stress in a set of 189 German winter bean lines. We assessed proline, glycine betaine, soluble sugars, water content, membrane stability, and SPAD in leaves of juvenile plants, with and without wilting. To describe frost tolerance under growth chamber conditions, we monitored the frost symptoms of juvenile plants, such as loss of color and turgidity, frost survival, and regrowth after frost—achieving medium to high repeatabilities ($0.43 < h^2 < 0.93$). With 175 SNP and 1147 AFLP, a total of 1322 (mostly mapped) DNA markers were utilized. We detected a total of 21 putative QTL for seven of the traits, each explaining 6–15% of the phenotypic variance. Several phenotypically promising inbred lines were identified. The SNP-based macro-synteny to *Medicago truncatula* will assist with the search for the corresponding candidate genes. The present results will greatly improve the prospects for including winter faba beans into German rotations in the near future.

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The faba bean (*Vicia faba* L.) is a partially allogamous diploid annual grain legume (pulse crop) with 2n = 2x = 12 chromosomes and a genome size of about 13,000 Mb (Satovic et al., 2013). Its mature seed comprises about 45% starch and about 30% protein, and is used for human consumption and animal nutrition. According to FAOSTAT (2014), the global faba bean acreage was about 2 Mio ha in 2013, ranking between the global acreages of lentils and lupines. China is the country with the highest faba bean production, and home to about 45% of the global acreage. Another 14% of faba bean acreage is in the Maghreb, and a similar proportion lies near the river Nile in Egypt, Sudan, and Ethiopia. Australia has about 5% of the global acreage, and the largest American grower is Peru with 3% (nearly 57,000 ha). In 2013, about 2/3 of the total EU acreage (~230,000 ha) existed north of the Alps and Pyrenees. Germany's faba bean acreage was 16,500 ha in 2013, and increased to 20,500 ha in 2014, with organic field comprising about half of this small acreage (FAOSTAT, 2015; EUROSTAT, 2015).

The current IPCC report (IPPC, 2014) considers it a virtual certainty that we will experience more frequent high temperatures and corresponding dry spells, with the continued occurrence of occasional cold winter extremes. Thus, the genetic improvement of abiotic stress tolerance is an important objective. In the face of a greater threat of drought, many temperate crops benefit from autumn sowing and the correspondingly faster development in spring and summer. Given the state of climate change, such winter crops promise higher and more reliable yield. The predicted milder winters will further allow autumn sowing even for crops with limited winter hardiness—such as winter faba bean. Genetic improvements of their winter hardiness will open even more opportunities for autumn sowing.

Over the past decade, a number of studies have reviewed the abiotic stress tolerance of the faba bean. Stoddard et al. (2006) describe field-based and controlled-condition experiments assessing faba bean tolerance to drought and frost, with consideration of physiological issues such as ^{13}C discrimination and proline accumulation. They further

mentioned tolerant genotypes, such as ILB938 (drought tolerant) and Côte d'Or (frost tolerant). Khan et al. (2007) presented experimental evidence pointing to stomatal conductance, leaf temperature, and ^{13}C discrimination as promising tools for breeding drought tolerant faba beans. In their 2010 report focusing on honest drought avoidance, Khan et al. proposed ^{13}C discrimination as the "gold standard" for assessing transpiration efficiency. They cite Amede and Schuster (1999) with regard to the lack of osmotic drought adjustment of known faba bean types, and describe a need for screening of a genetically more diverse set of germplasm. They underscored that the realization of genetic progress related to drought tolerance will require not a single trait approach but the combination of different aspects of abiotic and biotic stress tolerance.

Duc et al. (2011) reported details of the abiotic stress responses of the faba bean, mentioning the relatively large amount of water that a faba bean seed must imbibe to germinate. They discuss the potential osmotic drought adjustment of the faba bean, as well as the promise of interspecific crosses for frost tolerance. The latter cannot yet be realized as interspecific crosses with the faba bean have, to date, been unsuccessful.

Across large parts of the UK and many coastal regions of France, the winter conditions are mild enough to grow current winter bean cultivars, such as 'Wizard' (Smith, 2015). However, winters may occasionally be too harsh for these crops to be grown in Germany and the more continental parts of Europe, north of the Pyrenees and Alps. Link et al. (2010) reported the current knowledge of breeding and the research status regarding winter hardiness and frost tolerance, concluding that a genetically wider set of accessions must be studied to uncover genotypes with higher tolerance to both drought and frost. Such work has been performed by Olszewski (1996) who found new frost-tolerant "genitors" (ILB3187, ILB2999, ILB3245, and ILB14), and by Khazaei et al. (2013) who identified new drought-tolerant types along with their typical physiological profile.

The faba bean was long considered a "genomic orphan" crop but, since 2008, increasing genetic information has become available (Ellwood et al., 2008; Alghamdi et al., 2012). Recently, an appreciable number of SNP markers have become available for the faba bean (Webb et al., 2015; Kaur et al., 2014). Substantial additional progress is expected based on the high level of synteny between *Vicia faba* (genome not yet sequenced) and *Medicago truncatula* (genome sequenced; Webb et al., 2015; Smýkal et al. 2015; Khazaei et al., 2014). Findings from the sequenced genomes of *Arabidopsis thaliana* and *Medicago truncatula* may help to further progress in faba bean research (Link et al. 2010)—for example, advancing our knowledge about cold-related transcription factors (CBF and ICE) or of downstream frost-response genes, such as COR78 and MfCAS30 (cf. Tomashaw, 2001; Pennycooke and Stockinger, 2008).

Arbaoui et al. (2008b) identified the first putative quantitative trait loci (QTL) for aspects of frost tolerance in the faba bean. However, we still lack the tools required for marker-assisted breeding for abiotic stress tolerance of the faba bean. The current research aimed to develop data and expertise required for improvements of drought tolerance (Blum, 2014) and frost tolerance (Sakai and Larcher, 1987) in winter faba beans for growth in Germany—ultimately increasing and stabilizing its grain yield and thus promoting this highly valuable protein crop in our field rotations. A genome-wide association analysis was undertaken for this investigation (Pritchard and Przeworski, 2001).

MATERIALS AND METHODS

Genetic Materials

The genetic material used in this study included the so-called A-set (genotypes for association analysis) and the M-set (linkage mapping genotypes). For the A-set, 189 inbred lines were

available, and a number of check entries were added. These A-set lines have been bred via single-seed descent from the so-called Göttingen Winter Bean Population (GWBP), and were inbred to generations beyond F9. The GWBP was created in 1989 through the combination of 11 founder lines: Hiverna/1, Webo/1, Wibo/1, L79/79/1, L977/88/S1wn, L979/S1/1/1sn (German lines), Côte d'Or/1, Arrissot (French lines), Banner/1, Bourdon/1, and Bulldog/1 (UK lines). After the initial mixing, eight generations of natural reproduction were allowed. At Göttingen, such German winter beans reproduce with roughly 50–60% outcrossing (Gasim et al., 2004), and some mild natural selection for winter hardiness most likely occurred. Thereafter, the single-seed descent of the A-set lines was begun (Gasim, 2003).

For the M-set, the biparental RIL population of Arbaoui et al. (2008b) was used. Briefly, the parents of this M-set were the frost-tolerant pure line Côte d'Or/1 (a founder line of the GWBP) and the Chinese pure line BPL4328 (Duc and PetitJean, 1995). From this cross, a total of 101 pure lines (RIL) were derived via single-seed descent and were employed in the present study (Welna, 2014).

DNA Marker Assessment

Amplified fragment length polymorphism (AFLP) and single-nucleotide polymorphism (SNP) marker assays were conducted to genotype these entries. For each inbred line, one individual was used for AFLP detection and one different individual for SNP detection. For AFLP analyses, DNA was extracted from 100 mg leaf material per juvenile plant using the Illustra Nucleon Phytopure Genomic DNA Extraction Kit (GE Healthcare, Great Britain), applying mercaptoethanol and following the manufacturer's protocol with small modifications. AFLP analyses were performed following the protocol reported by Vos et al. (1995) using multiplex PCR (Ecke et al., 2010). In contrast to previous studies, we ran 19 cycles for pre-amplification, and the final AFLP amplification involved 11 cycles with an

143 annealing temperature of 64.2°C and 24 cycles with an annealing temperature of 56.0°C. A
144 total of 96 primer combinations were used, as described in detail by Welna (2014).
145 DNA fragments were detected using the ABI 3130xl Genetic Analyzer System
146 (Applied Biosystems), with GeneScan 500LIZ used to check fragment size. Actual scoring
147 was supported by GeneMapper 3.7 (Applied Biosystems). The resulting data was transformed
148 into a 0/1 matrix (Ecke et al., 2010). We performed marker loci definition and recording of
149 marker peak absence vs. presence for markers that were polymorphic between the M-set
150 parents, or that were polymorphic between three of the A-set lines (S_048, S_122; S_253; Ali
151 et al., in preparation). Hence, the GeneMapper-supported analysis was conducted jointly
152 across the M-set and A-set. Scored yet unmapped markers occurred for two reasons: they
153 were polymorphic in the M-set but could not be mapped, or they were monomorphic in the
154 M-set but polymorphic among the three A-set lines.
155 For SNP analyses, a sample of 50 mg freeze-dried leaf from each tested plant was sent
156 to LGC Genomics (Cambridge, UK) for DNA extraction and SNP analysis. Several hundred
157 assays for Kompetitive Allele Specific PCR (KASP) analysis were available from the work of
158 Cottage et al. (2012a, 2012b). SNPs were selected if they were simultaneously polymorphic in
159 the M-set and the A-set.
160
161 **Phenotyping**
162
163 The 189 A-set lines were analyzed for several physiological traits presumed to be associated
164 with drought tolerance, including contents of free proline, glycine betaine, and soluble sugars
165 (μmol g^{-1} in dry matter); leaf water content and membrane stability index (%); and
166 chlorophyll content (SPAD). Two replicates were realized, one from the 2011/12 season and
167 another from the 2012/13 season. For each entry and replicate, ten potted juvenile plants were
168 available. The plants were grown in a climate chamber, without stress, with 16-h light per

day, and at 20°C (day) and 17°C (night). Testing was performed using samples of the youngest unfolded (i.e., third and fourth) leaves at about 25 days after sowing. Traits were assessed from unstressed (control) and stressed leaflets or leaflet disks.

Leaflet discs were used to test proline, sugars, and membrane stability, whereas detached leaflets were used to examine chlorophyll, leaf water, and glycine betaine. Control proline and control sugar contents from unstressed plants were analyzed in the same sample of five discs, from ten independent leaflets per entry and replicate. Proline content was also analyzed in five stressed discs, and another five stressed discs were used for sugar content analysis. An additional five stressed and five unstressed discs were analyzed for membrane stability. Chlorophyll was measured in ten detached leaflets, directly after cutting and again after stress. The same leaflets were used to gravimetrically determine water content. Control glycine betaine was analyzed in ten independent detached leaflets from plants without stress. Glycine betaine after stress was measured in the same leaflets already used for measurements of chlorophyll and water content.

Throughout the experimental period, leaflet discs or leaflets were subjected to 48 h under continuous light, at 20°C and with 80% relative air humidity. For proline analysis, drought stress was induced using PEG (420 g PEG6000 per kg water) in HEPES buffer (25 mM KCl, 3.7 mM $CaCl_2$, and 5 mM HEPES). For analyses of other traits, stress was induced by wilting. Prior to analysis, the leaflet disks were incubated on a rotation shaker at 60 rpm. Analyses of sugar contents and membrane stability in leaflet discs, and analyses of chlorophyll, water, and glycine betaine contents in detached leaflets were each executed using a gossamer layer in sealed petri dishes.

Free proline content was assessed photometrically using freeze-dried material according to the method described by Bates et al. (1973). Soluble sugars contents were also analyzed photometrically from freeze-dried material, following the method of Yemm and Willies (1954). For membrane stability assessment, leaflet discs were shaken in 50-mL test

tubes with 20 mL deionized water for 24 h at 7°C. Electric conductivity (EC1) was measured. The samples were then boiled for 30 minutes followed by a second conductivity measurement (EC2). The Membrane Stability Index (MSI) was calculated as [1 – (EC1/EC2)] * 100, according to Chandrasekar et al. (2000). Chlorophyll content was determined indirectly using a SPAD502Plus (Konica Minolta) tool.

Glycine betaine content was analyzed in two steps. First ultra-performance liquid chromatography was conducted on 80 leaflet samples (UPLC-ESI-MS with a SeQuant ZIC®-HILIC column), using dried, milled, water-extracted, and 9:1 Acetonitrile-diluted material, after centrifugation (Spielmeyer and Pohnert, 2012, Gebser and Pohnert, 2013). In the second step, a NIRS calibration was developed based on these results and on NIRS spectra from the same samples. The ratio performance deviation (RPD) in the calibration was 2.29, and the RPD in cross-validation was 1.81. The calibration equation was used to predict the glycine betaine content of the remnant dried and milled leaflet samples.

During both seasons and for each trait, ten inbred lines were analyzed per calendar week. This established an incomplete block of a corresponding alpha-lattice design, with 20 incomplete blocks representing one replicate. The software PLABSTAT (Utz, 2004) was used throughout the analyses.

Phenotypic analysis of frost tolerance was conducted between September and April in 2011/12 and 2012/13 in a plant growth chamber with juvenile, hardened, potted plants (Sallam et al., 2015). The chamber was an accessible 4-m² Vötsch VB4018 that provided 200 µmol s^{-1} m^{-1} light, and could produce frost down to −20°C. Air humidity fluctuated freely. The 4-m² area could contain 54 pots (size 17 × 17 cm²), which represented one replicate. With two plants per entry and four entries per pot, we analyzed a total of 216 entries, establishing an alpha-lattice design with 12 entries per incomplete block (i.e., 3 pots) and 18 incomplete blocks. There were r = 2 per lattice experiment, and ten such experiments (five per each of two seasons) yielding a total of 20 replicates.

The utilized soil was a 3:1 mixture of local compost soil and sand, with the moisture gravimetrically maintained at approximately 70% of field capacity throughout the experimental period. Germination occurred under mild conditions. At the two-leaf stage, plants were transferred into the growth chamber for a ten-day hardening at 4–5°C and with a 10-h day length. To prepare for the frost test, each pot was plugged into an open-top polystyrene box. The actual test involved a sequence of three successive nights of frost down to −16, −18, and −19°C. The linear approach towards each frost level took 4 h, the frost temperature was maintained for 4 h, and the linear return to mild recovery conditions (about 5°C) took 6 h (cf. Roth und Link, 2010; Arbaoui et al., 2008a; Arbaoui and Link, 2008; Sallam et al., 2015). After these three nights of frost, the pots were transferred back to the greenhouse under mild conditions, maintaining their randomization. A recovery phase of four days and a regrowth phase of four weeks followed.

The following frost traits are reported here (cf. Sallam et al., 2015): plant height (cm) at end of hardening, loss of leaf turgidity due to frost (scale 1–9, with 9 representing the highest loss), loss of leaf color due to frost (scale 1–9), ultimate survival at end of the regrowth phase or number of days between frost test and death, and shoot regrowth (g per plant) at the end of the regrowth phase. Loss of turgidity and loss of color were each determined after the first, second, and third nights of frost and again after 4 days of the recovery phase. These eight scores were added to obtain an accumulated “loss of turgidity and color” score (Loss(T+C); scale 8–72). After the 4-day recovery phase, the main stems and tillers of these juvenile plants were chopped off at the second node. The remaining small trunk allowed us to make daily assessments of whether the plant was alive or dead, with any regrowth verifying that the plant was alive. After four weeks, all shoot biomass was harvested from living plants and weighed as fresh matter, and thus regrowth was assessed. For those plants that died, the number of days between the frost test and their death was transformed into a disposition to survive (0° to 90°; cf. Roth und Link, 2010; Sallam et al., 2015).

Disposition to survive was calculated as $\arctan(x_i / \mu_x)$, with x_i = number of days until death and μ_x = average number of days until death for those plants that died within the four-week regrowth phase. The surviving plants were accordingly scored as 90°.

The drought-related results were analyzed according to the alpha-lattice design of the experiments, resulting in lattice-adjusted means across the two replicates. These data (excluding checks) were employed to calculate Spearman rank correlations and repeatabilities (h^2).

The frost-related results were first analyzed according to the alpha-lattice design of the ten experiments. The resulting lattice-adjusted means (excluding checks) were utilized to analyze the series of experiments with the main effects "genotypes" and "experiments". Spearman rank correlations and h^2 were calculated accordingly. A covariance analysis was conducted with the GENOT command of PLABSTAT, to enable the development and analyses of a Smith-Hazel optimum selection index (Falconer and Mackay, 1996). All weight was given to the trait "disposition to survive," whereas the correlated traits of "plant height" and "loss of turgidity and color" were given weights of zero and were thus treated as auxiliary traits. The index was developed accordingly, and the resulting trait was named "frost tolerance index." The direct drought and frost traits and the frost tolerance index were then further subjected to association analyses.

Association Analysis

To study the potential associations between DNA markers and phenotypic expressions of traits, we analyzed 2018 polymorphic markers and all traits of the 189 inbred lines of the A-set, employing TASSEL 3.0 (Bradbury et al., 2007). Initially, 189 SNP loci and 1829 AFLP loci were available. After filtering for a minor allele frequency of 5% with 175 SNP and 1147 AFLP, a total of 1322 markers remained. The Mixed Linear Model mode was applied with an

optimum level of compression and a re-estimation of the variance component estimates after each marker. A kinship matrix was developed and employed. Again, the SNP and AFLP markers were used after filtering for a 5% minor allele frequency. SNP-based genetic similarities among the A-set lines were calculated using the simple-matching coefficient, and AFLP-based genetic similarities using the Jaccard coefficient (Piepho and Laidig, 1997).

Next, combined similarities were calculated, weighing the SNP-derived and the AFLP-derived coefficients according to their numbers (175/1322 and 1147/1322, respectively). With these combined similarities, a principal coordinate analysis was conducted (NTSYSpc, Rohlf 2000) to visualize potential structure among the A-set lines (Vilhjálmsson and Nordborg, 2013; Pearson and Manolio, 2008; D'hoop et al., 2010; Kollers et al., 2013). In the same way, genetic similarities were determined among the 11 founder lines of the GWBP. The average GS coefficient among the founders was utilized as a threshold to separate between kinship-relevant similarity and alike-in-state similarity among the A-set lines. All similarities below that threshold were set as zero, while all above the threshold were linearly transformed to a scale from zero to one. Following TASSEL, kinship values were constructed by adding a constant value of 1.0 to each resulting number. The resulting kinship matrix was utilized for association analyses.

For each trait, we tested the significance of associations between marker alleles and trait expression according to the methods of Benjamini and Hochberg (1995) and Benjamini and Yekutieli (2005). A false discovery rate of 20% was applied (Honsdorf et al., 2010). Phenotypic effects as calculated by TASSEL corresponded to the difference between the means of the two marker classes. The variance explained simultaneously by all significant markers of a given trait was obtained from multiple regression analysis employing the BASIC mode of PLABSTAT.

Analysis of overall gamete phase disequilibrium (also called linkage disequilibrium; LD) between marker pairs was carried out for the mapped markers (N = 805; 118 SNP and

687 AFLP) and repeated for all markers of the association analyses. Additionally, all markers found to be significantly associated with a trait were studied for mutual LD, whether mapped or not. Employing TASSEL, the selected LD type was "full matrix," and thus no sliding window was applied.

Mapping of Markers

A total of 130 SNP and 1415 AFLP were found to be polymorphic in the M-set and were used for mapping with Mapmaker 3.0. The Kosambi mapping function (Kosambi, 1944) was applied, with a maximum distance between markers of 35 cM and a LOD of 3.0 (Ecke et al., 2010). Further details are given in Welna (2014).

RESULTS

The genetic linkage map was used as a tool to study linkage disequilibrium among pairs of mapped markers and to compare the present data with previously published maps. Details of this map are reported by Welna (2014). Briefly, it spans 1633.2 cM and shows 1159 marker loci, including seven major linkage groups embracing 1140 loci. These groups can be associated with the six *Vicia faba* chromosomes *via* the consensus map of Cottage et al., 2012b (cf. Webb et al. 2015).

The average combined genetic similarity (GS) among the 189 A-set lines was 0.329, whereas the average GS among the 11 founder lines was 0.320. Among the founder lines, the lowest marker similarity was identified between Côte d'Or/1 and Banner/1 (GS = 0.281) and the highest was between Côte d'Or/1 and Wibo/1 (GS = 0.449). This pattern corroborated some findings of Kwon et al. (2010) based on TRAP markers. In the A-set, 9% of the pairwise marker similarities fell below the threshold of 0.320 set by the founders—hence, 9%

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of the kinship matrix data was deemed "unrelated"—while eight line pairs showed GS of >0.750.

With its first two axes, principal coordinate analysis (Figure 1) showed only 1.79% and 1.69% of the similarity variance in the data. Additional axes explained 1.44%, 1.31%, 1.22%, and so on down to 0.61% for the last one. The very even spread of lines across the area in Figure 1, and the very steady decay of the small portions explained by further axes in supplemental figure 1, indicate a lack of marked subgrouping among these lines.

Among the 11 founder lines, the overall mean LD (expressed as r^2) was 0.1135 (Hedrick, 1987). In contrast, the average r^2 in the A-set was very low: $r^2 = 0.0075$ for all pairs of the 118 mapped SNP markers, and $r^2 = 0.0074$ for the 687 mapped AFLP markers. Between all 1322 markers included in the genome-wide analysis, the average r^2 was 0.0077. Among the 21 markers that were significantly associated with a trait (see below), the average r^2 value was 0.0076, with a range of 0.000 to 0.043. For 210 pairs of the mapped set of markers, the two markers of each pair co-segregated in the 101 RIL that founded the linkage map (i.e., the distance was <1 cM). Altogether, 172 AFLP and 23 SNP markers were involved in these co-segregating marker pairs. Two of these pairs showed $r^2 = 1.0$, nine additional pairs had r^2 of >0.25, and 37 pairs showed r^2 of <0.001. The average in these extremely linked groups was $r^2 = 0.0522$, indicating a very fast decay of gamete phase disequilibrium.

Analysis of variance showed that all drought-related traits exhibited highly significant variation (F value, $\alpha = 1\%$) due to genotypes, due to the wilting treatment, and due to genotype × treatment interactions (details not given). Response to wilting was analyzed both as relative performance (stress in percent of control) and as absolute change due to wilting stress—with the version yielding higher repeatability (h^2) reported herein. For analyses of membrane stability, sugars, and water, relative performance yielded higher h^2, while the "stress minus control" analysis yielded higher h^2 values for glycine betaine, SPAD values, and proline (see below). The average of the latter was negative for SPAD values (Table 1).

Wilting reduced membrane stability to 36% of control, increased the sugar content by 2.3-fold, and reduced water content to 43% of control. Wilting increased glycine betaine content by 5.2 μmol g^{-1} and reduced the arbitrary SPAD values by 4.7 units, from 37.3 to 32.6 units. The average control proline content was nearly zero, at only 3.5 μmol g^{-1}; thus, the average proline content under stress of 425.6 μmol g^{-1} could also be reported as "stress minus control."

The frost tolerance index was constructed for each inbred line as follows: index = (0.7319 × disposition to survive) – (0.7017 × plant height) – (0.1945 × L(T+C)). All frost-related traits showed marked and highly significant variation (α = 1%) among the A-set lines. Regrowth showed an exceptionally high coefficient of variation of 52.3%.

Repeatabilities based on the two replicates varied between $h^2 = 0.40$ for unstressed membrane stability up to $h^2 = 0.91$ for SPAD values under unstressed conditions, as well as between $h^2 = 0.61$ for regrowth after frost test and $h^2 = 0.93$ for loss of turgidity and color. On average, the coefficients of variation were higher for response to wilting than for direct trait expression under control or under stress conditions.

We detected very few significant (α = 5%) correlations among the drought-related traits. Proline content after wilting was positively correlated (r = 0.34) with glycine betaine content after wilting and with gain of glycine betaine due to wilting (r = 0.41). Frost-related traits were all correlated among each other. Greater plant height of the lines during the hardening phase was associated with heavier loss of turgidity and color (r = 0.60) as well as with decreased disposition to survive, decreased frost tolerance index, and decreased regrowth ($-0.56 < r < -0.40$). Disposition to survive and frost tolerance index conveyed similar information (r = 0.99), whereas regrowth and loss of turgidity and color were only correlated with an r value of −0.50 (α = 1% for all of these correlations). No meaningful correlations were observed between frost and drought aspects ($-0.22 < r < 0.31$ for all coefficients).

Out of 21 reported traits, only 10 allowed the identification of significant QTL. For each of these 10 traits, between 1 and 8 putative QTL were identified (Tables 1 and 2).

For drought, we presently report only the QTL identified for performance under stressed conditions and for reaction to stress; we do not report QTL for performance under unstressed conditions. There were a total number of 21 markers with significant trait associations, three of which showed a signal in two traits. Gain of glycine betaine and gain of proline due to wilting shared a common AFLP marker locus on linkage group 7 (Table 2), with absence of the AFLP peak being associated with higher increases of both solutes. Multiple regression analysis indicated a multiple R^2 of 55.6% for %sugars and of 36.4% for proline.

Visual inspection of Q-Q-plots—i.e., observed *p* values of markers vs. expected *p* values of markers (Pearson and Manolia, 2008)—for drought-related traits supported the validity of data and results (cf. supplemental figure 2). Linear regression analysis of the proline phenotypes against the number of marker loci per A-set line with an "increase" marker allele yielded a correlation of $r = 0.57$ ($\alpha = 1\%$). This value increased slightly ($r = 0.58$) if the phenotypic values of the entries were correlated with values predicted based on the actual marker effects. Two lines were homozygous at all five detected QTL for the "increase" marker allele—including the line S150 (supplemental figure 3). Three drought-related markers were located on linkage group 7, at positions 27.3, 94.4, and 112.3 cM (Welna, 2014). With $0.000 < r^2 < 0.015$ among them, each of these three markers most likely marks a unique QTL rather than several of them marking the same QTL.

The disposition to survive and the frost tolerance index that was derived from it share one AFLP and one SNP marker (Table 2), with 7% and 8% of phenotypic variance explained per marker. Loss of turgidity and color was associated with eight marker loci, one of which was also shared with the other two frost traits (Vf_Mt086600, 9% variance explained, $p = 2.52\ 10^{-5}$). The "G" allele of this SNP was associated with a higher frost tolerance index and a

higher disposition to survive, and the "T" allele with higher loss of turgidity and color. The second common marker for disposition to survive and for the frost tolerance index, E40M458-369, did not show significant association with Loss(T+C), even when applying a false discovery rate as large as 30%.

Multiple regression analysis indicated a multiple R^2 value of 36.1% for the eight Loss(T+C) QTL jointly. The Q-Q-plots for frost-related traits again supported the data and results (cf. supplemental figure 4). Linear regression of Loss(T+C) against the number of marker loci per A-set line with an "increase" allele yielded a correlation of $r = 0.52$ ($\alpha = 1\%$). This value became $r = 0.53$ if phenotypic values were correlated with values predicted from the marker effects. Two lines were homozygous at seven or eight of the eight QTL for "decrease" of this trait, i.e., better frost tolerance (again including the line S150; supplemental figure 5). Three frost-related markers were located on linkage group 3, at positions 103.1, 128.8, and 142.3 cM. With $0.000 < r^2 < 0.030$ among them, each of these three markers most likely marks a unique QTL. The two markers for Loss(T+C) at linkage group 2 showed $r^2 = 0.0001$.

DISCUSSION

Winter faba beans are uncommon in Germany, since areas like Göttingen experience harsh enough winters to kill such crops about once per decade. The inclusion of winter faba beans in German rotations would be a novelty—and would probably attract more attention from farmers and policy-makers than any gradual yield improvement in spring faba beans. Climate change promises milder winters on average, and thus climate change and genetic improvement of winter hardiness may meet halfway to promote winter faba bean in Germany. Compared to spring beans, winter beans can better escape summer droughts (dehydration avoidance). Therefore, it is logical for breeders to work towards improving the dehydration

tolerance (Blum, 2014) of winter beans, to make their defense against abiotic stress more complete (Flores et al. 2012).

The present study is the first genome-wide association analysis of the faba bean focused on abiotic stress in winter beans. We phenotyped a set of 189 inbred winter bean lines based on physiological aspects that are mainly connected with dehydration tolerance (membrane stability, chlorophyll and water content after wilting stress, and solute accumulation in response to wilting). With the exception of glycine betaine accumulation, experience in assessing and interpreting these physiological parameters was available from previous experiments with spring faba beans (e.g., Venekamp and Koot, 1988; Balko et al., 1995, Stelling et al., 1994, Balko, 2005a; Balko, 2005b). Cromwell and Rennie (1953) previously detected relatively high amounts (1.1–1.5% in dry matter) of glycine betaine in *Vicia faba* shoots and roots. Glycine betaine accumulation is considered an adaptive response to drought stress (e.g., Jones and Storey, 1981, Gill et al., 2014, Lai et al., 2014), prompting our present assessment of glycine betaine. Stoddard et al. (2006) also proposed the investigation of glycine betaine as marker for osmotic stress response in the faba bean.

We additionally examined frost tolerance using the same set of inbred lines. The focus of these experiments was on frost symptoms after controlled frost stress in a plant growth chamber, based on prior experience with similar experiments (Arbaoui and Link, 2008; Roth and Link, 2010). Compared with the physiological experiments, there was greater input for the frost phenotyping, with 20 instead of 2 replicates.

There exist a variety of alternative approaches for both experiments, including wilting of whole plants instead of detached leaflets or leaflet discs, and a wide variety of different protocols for examining frost tolerance (Duc and Petitjean, 1995; Gehriger and Vullioud, 1982; Avia et al., 2013). It is possible that another approach could better fit the given situation and purpose. Moreover, future studies could include other or further measurements of physiological traits. A subset of 40 lines of our plant material was studied for field-based ^{13}C

discrimination as reported by Welna 2014 (cf. Ali et al., 2013). Although ^{13}C discrimination is a self-evident trait, this parameter did not yield promising correlations with field-based performance under drought. Leaf water potential and stomata resistance are common analyses (for faba bean, cf. Ricciardi et al. 2001). In another study, Ali (2015) performed an indirect yet rapid assessment of stomata conductance, using an infrared camera to measure the temperature depression of juvenile potted faba bean plants experiencing heavy drought, again utilizing a subset of our present faba bean lines. These results were significantly correlated ($r = 0.42$) with gain of dry matter after a life-threatening period of drought (about three weeks). Similar to glycine betaine, trehalose could be an interesting compound to assess as an indicator of abiotic stress response (López et al., 2006).

Several previous studies have presented promising correlations of both proline accumulation and chlorophyll fluorescence with field-based reactions of spring faba bean yield to drought, as well as among proline, membrane stability index, changes in leaf water content, and chlorophyll fluorescence (Balko et al., 1995; Balko, 2004, 2005a, 2005b; Stelling et al., 1994; Gadallah, 1999). Link et al. (2010) reported an r value of −0.41 between severity of frost injury symptoms (using growth chamber tests similar to those in the present study) and field-based winter hardiness. In contrast to in the study of Arbaoui (2008a), the pots in our present study were insulated with polystyrene such that the plant roots were kept at conditions milder than −5°C throughout. While both studies ensured that the shoots experienced full frost as programmed, the conditions in our present study should be more similar to the field situation. The inbred line S_048 was number 8 and 9 according to disposition to survive and frost tolerance index, respectively, and is proven to be a very winter-hardy line (Link and Martsch, unpublished). However, further validation is still necessary.

At the start of the present experiments, about 778 SNPs were known in the faba bean from the work of Cottage et al. (2012b). Our initial 189 SNPs comprised the polymorphic

subset of these known 778 SNPs for the given set of genotypes. These SNPs were detected based on BLAST correspondence to mRNA sequences of *Medicago truncatula* (Mt) and were developed as tools for exploitation of the Mt genome sequence for investigations of *Vicia faba* (Webb et al., 2015). We further added 1147 AFLP markers to the SNPs. Upon finding the unexpectedly low average LD ($r^2 = 0.0075$) and examining the low average LD of marker pairs with a distance of 1 cM or less ($r^2 = 0.0522$), the total number of utilized markers (N = 1322) was a limiting factor for this genome-wide association analysis. Obviously, several QTL must not yet be detected and should be searched for using additional markers. Meanwhile, an additional 622 SNPs have now been identified by Kaur et al. (2014), and many more are in the pipeline (e.g., Höfer et al., 2014; Webb et al., 2015).

LD among the 11 founder lines was r = 0.1135. In 1985, Hudson showed that the r^2 caused by sampling N pure lines from a basic pool with zero LD amounts to approximately 1/N. Accordingly, with 11 founder lines, the expected gamete phase disequilibrium would be $r^2 = 1/11 = 0.091$. However, Hudson (1985) considered a range of N = 50 to N = 200, so the current case of N = 11 was below his scale. We thus simulated 364 samples of 11 haplotypes with two bi-allelic loci each, drawn from a basic pool with zero LD and allele frequencies of 20% and 80% throughout, which resulted in an average LD of $r^2 = 0.1031$. With allele frequencies of 50% throughout, the average LD became $r^2 = 0.0972$. This is similar to both the measured LD among the 11 founder lines and to what Hudson (1985) predicted. Thus, it is likely that the 11 founder lines represent a basic germplasm pool with a very low or zero LD—probably similar to the very low LD in our A-set of lines ($r^2 = 0.0075$). Seed exchange between different geographic locations over the past millennia, along with the partial allogamy of the faba bean, may have caused this seemingly very low gamete phase disequilibrium. This is a disadvantage given the limited numbers of available markers, as QTL may remain undetected. However, when more markers are identified, this high

resolution will increase the probability that informative markers will remain useful for several generations and across different germplasm pools.

Of the analyzed markers, 88% (N = 1159) represented an independent map position. The map size of 1633.2 cM (cf. Welna, 2014) is in reasonable agreement with the map sizes of 1216.8 cM, 1403.8 cM, and 1685.8 cM presented by Kaur et al. (2014), Webb et al. (2015), and Ellwood et al. (2008), respectively. The first version of the 1403.8-cM map, as presented at Hyderabad in 2012 (Cottage et al., 2012b), was based on 643 SNPs; a subset of which (N = 111 SNPs) was included in the current work. Very high colinearity was detected between our map and the map of Cottage et al. (2012b), with the exception of only five SNPs (Welna, 2014; page 29 and appendix pages XXXII–XXXV).

We found a rather low repeatability of glycine betaine values and of glycine betaine accumulation under wilting ($0.425 < h^2 < 0.614$, Table 1). This was likely because a portion of the data was predicted via NIRS calibration. Hence, the true relationship between proline and glycine betaine might be stronger than r = 0.34. The highest h^2 values after stress were found for water content ($h^2 = 0.884$) and %water ($h^2 = 0.880$)—yet, no QTL was discovered. Similarly, no QTL was detected for membrane stability.

The weakest correlation between two frost traits was found between regrowth and plant height (r = −0.40), and the strictest correlation was between regrowth and disposition to survive (r = 0.76). The frost tolerance index was correlated with plant height (r = −0.56), with regrowth (r = 0.76), with Loss(T+C) (r = −0.69), and with disposition to survive (0.99, being the main trait of this index; all significances with α = 1%). The very high repeatabilities—for example, of Loss(T+C) ($h^2 = 0.929$)—reflected the high total number of replications employed. Still, no QTL were detected for regrowth and plant height.

The positive weak connection (r = 0.31**) between the soluble sugars under stress and plant height at the start of hardening was the only notable correlation between drought and frost traits. In particular, drought-provoked proline accumulation and frost traits were

uncorrelated ($-0.06 < r < 0.08$), despite the fact that proline is also a physiological aspect of frost tolerance (Arbaoui et al., 2008a).

One has to be cautious about interpreting QTL data. Due to the risks of false-positive results, a newly identified QTL should be considered putative (Göring et al., 2001). The AFLP locus E36M48-279 was, with its allele "0," putatively associated with glycine betaine increase of 1.02 µmol g^{-1} and a proline increase of 57.1 µmol g^{-1}. This may corroborate the correlation between these two traits. The major putative QTL for frost tolerance (SNP Vf_Mt3g086600) was shared with disposition to survive and Loss(T+)C, and seemingly explained 7–9% of the phenotypic variance. Three of the 189 inbred lines were homozygous at seven or eight of the Loss(T+C) markers for the allele associated with low frost symptoms. One of these favorable lines (S_150) was also one of two lines that showed homozygosity at all five proline marker for the favorable allele (supplemental figure 3).

To examine the plausibility of the present findings, association analyses were repeated with the GLM mode of TASSEL 3.0 (instead of MLM). Most of the results were very similar to those presented in Table 2; the same marker allele was associated with trait increase. When comparing the effect sizes estimated with MLM with their GLM-estimated counterparts, the differences ranged from between 0.7% and 16% of the MLM effect size—except for the putative QTL of chlorophyll after wilting, for which the MLM effect was 45% larger than the GLM estimate. For the relative content of soluble sugars (stress in percent of control; %sugars), different results were obtained when analyzed via GLM instead of MLM. All five significant MLM effect-size estimates were larger than their GLM counterparts, with differences ranging from 35% to 377% (average of 2.3-fold larger). Moreover, none of the MLM-detected markers showed significance under GLM. The phenotypic distribution of %sugars was skewed towards higher values. While most A-set lines had relative contents of 148–330%, four lines showed exceptionally high values of >400% (i.e., more than a four-fold increase due to wilting; cf. Table 1). Upon removal of these four A-set lines from the data set,

no significant QTL for %sugars remained in MLM mode. A corresponding procedure for proline led to the loss of only the weakest of the five QTL for that trait. Further investigations are needed to determine whether such MLM-GLM comparison is worthwhile in cases like the present, where population structure does not seem to be a major issue.

The model legume *M. truncatula* shows genetic variation for freezing tolerance (Pennycooke et al., 2008; Avia et al., 2013), which may be of interest for winter faba bean research. Avia et al. (2013) studied a biparental cross with 178 RIL, and found a number of convincing QTL—the most important one being on Mt chromosome 6. Tayeh et al. (2013a) revealed colinearity of this QTL with a *Pisum sativum* freezing tolerance QTL previously detected by Dumont et al. (2009). With considerable input, Tayeh et al. (2013b) narrowed down the set of candidate genes for this QTL to a few C-repeat binding factors/dehydration-responsive element binding protein1 that are well known for involvement in frost tolerance (Thomashow, 2001). For frost tolerance, the QTL identified in our experiments (Table 2) showed syntenic locations on the Mt genome on Mt chromosomes 3, 4, 5, 7, and 8 (with none on Mt chromosome 6). For two of our QTL, markers could not be mapped; thus, no syntenic location is known. To date, none of our putative QTL appear to be identical to that identified by Tayeh et al. (2013a) in Mt chromosome 6.

With regards to drought tolerance, little information is available about QTL in *Medicago truncatula* (Arraouadi et al., 2012), or about candidate genes in the faba bean (Abid et al., 2015) and in *Medicago truncatula* (Wang et al., 2013). We propose a first glimpse candidate gene search for two of our putative QTL: AFLP locus E36M48-279 (associated with proline and glycine betaine) and SNP locus Vf_Mt3g086600 (associated with frost tolerance). Our proposals rely on the tight connection between the current map and the map of Cottage et al. (2012b; cf. Webb et al., 2015), as well as the macro-synteny of both to *M. truncatula* (Webb et al. 2015; Satovic et al., 2013*)*. The position of E36M48-276 at 94.4 cM (linkage group 7; Table 2; Welna 2014) corresponds to a position between Vf_Mt4g088010

and Vf_Mt4g091530 on *Vicia faba* (Vf) chromosome 6 (9.5-cM distance between these two SNP; Webb et al., 2015). This part of Vf chromosome 6 is syntenic to a large region of Mt chromosome 4. Based on seven SNPs in the direct vicinity of our AFLP locus, its position is projected from our map to a position at a 4.2-cM distance from Vf_Mt4g091530 (map of Webb et al. 2015). It is furthermore projected to its putative syntenic location on Mt chromosome 4 at approximately 31,266,000 Bp (3.5 Hapmap; http://www.medicagohapmap.org). Given the very fast decay of LD in our A-set lines, it may be appropriate to search the frame from 0.5 cM to the left and right—corresponding to about 63,800 Bp to each side in the Mt sequence. This region contains about 26 genes, as displayed by 3.5 Hapmap, with the R2R3-MYB transcription factor located between 31,322,350 Bp and 31,323,683 Bp. Cominelli et al. (2005) reported this transcription factor to be involved in the regulation of stomatal movements in *Arabidopsis thaliana*, and expressed in stomatal guard cells. A null mutation in the R2R3-MYB transcription factor resulted in reduced stomatal movements. This small region on Mt chromosome 4 also includes other candidate genes with possible connections to drought.

The locus of SNP Vf_Mt3g086600 can directly be found on Mt chromosome 3 at position 28,532,000 Bp (annotated as “unknown protein”); an appropriate search frame could be 80 Kb to the left and right of it. At 28,570,300 Bp lies a gene for glycine aldehyde dehydrogenase, an enzyme belonging to the glycine betaine pathway. This osmotically important solute may well play a role in frost tolerance. However, this is speculative and needs further investigation. Obviously the genome sequences of *Medicago truncatula*, as well as of *Lotus japonicas*, *Glycine max*, *Lens culinaris*, and *Cicer arietinum* (Webb et al, 2015; Satovic et al., 2013; Varshney et al., 2014) have become very important tools for future faba bean research.

606 Here we reported findings from the first association analysis in the faba bean focused
607 on abiotic stress. The present results markedly improve the prospects for introducing adapted
608 winter beans in German field rotations.
609
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869 Figure caption; Tables.

870 Figure 1. First two axes of a principal coordinate analysis, based on genetic similarity

871 estimates from 1322 genome-wide markers.

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Table 1. Phenotypic results of drought-related and frost-related traits.

Trait	Average	Minimum	Maximum	Coefficient of variation	LSD (5%)	h^2	# QTL
DROUGHT STRESS							
Control							
Membrane stability [%]	82.87	78.73	85.08	0.013	2.38	0.400	1*
Glycine betaine [µmol g^{-1} in DM]	10.07	8.56	11.55	0.058	1.02	0.614	1*
Sugars [µmol g^{-1} in DM]	437.95	169.32	865.70	0.286	198.2	0.678	0
Chlorophyll [SPAD units]	37.31	26.73	47.15	0.098	3.12	0.906	0
Water [%]	89.94	86.90	91.37	0.008	0.85	0.826	2*
Stress							
Membrane stability [%]	29.72	9.65	46.78	0.253	15.91	0.426	0
Glycine betaine [µmol g^{-1} in DM]	15.25	12.37	18.69	0.078	2.39	0.477	0
Sugars [µmol g^{-1} in DM]	959.10	532.0	1395.5	0.163	219.3	0.745	0
Chlorophyll [SPAD units]	32.63	21.86	42.66	0.107	4.15	0.818	1
Proline [µmol g^{-1} in DM]	425.60	203.3	609.10	0.144	66.77	0.846	5
Water [%]	38.06	15.20	53.49	0.210	7.59	0.884	0
Relative performance (stress in % of control) or absolute difference between treatments (Δ)							
%Membrane stability	36.18	11.73	57.32	0.258	18.73	0.481	0
ΔGlycine betaine	5.18	2.42	7.97	0.206	2.26	0.425	2
%Sugars	233.08	148.4	441.00	0.205	94.75	0.492	5
ΔChlorophyll	−4.68	+1.29	−10.00	0.421	3.46	0.602	0
%Water	42.63	17.09	59.56	0.208	8.58	0.880	0
FROST STRESS							
Regrowth (g per plant)	0.66	0.08	1.66	0.523	0.60	0.614	0
Disposition to survive (°)	66.01	27.35	83.81	0.150	11.03	0.838	2
Frost tolerance index (arbitrary units)	37.36	4.30	52.94	0.224	8.42	0.869	2
Plant height (cm)	5.76	4.14	8.06	0.145	0.70	0.907	0
Loss(T+C) (scale 8–72)	35.48	22.57	58.73	0.165	4.34	0.929	8

*Not reported here.

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Table 2. Association analyses results for drought- and frost-related traits (minor allele frequency 5%; N = 189 inbred lines; mixed linear model, Kinship-matrix, FDR 20%).

	DNA marker	LG	Position (cM)	Trait	*p* value	Effect*	"Increase Allele"	R^2 (%)
				DROUGHT				
1	E36M48-279	7	94.4	ΔGlycine betaine	4.72×10^{-5}	1.017	"0"	9.31
2	E41M58-139	-	-		1.81×10^{-4}	0.726	"0"	7.55
3	E40M48-432	-	-	%Sugars	9.59×10^{-8}	24.23	"1"	14.66
4	E40M62-295	-	-		1.36×10^{-7}	23.41	"0"	14.49
5	E32M51-178	-	-		4.02×10^{-6}	22.47	"0"	11.17
6	E42M48-357	4	154.0		2.25×10^{-4}	21.58	"1"	8.43
7	E40M48-310	-	-		3.57×10^{-4}	15.97	"0"	6.86
8	E41M60-310	7	27.3	Chlorophyll (Stress)	8.99×10^{-5}	2.37	"0"	8.19
9	E42M51-135	3	125.9	Proline	3.80×10^{-6}	44.28	"0"	12.32
10	E40M55-194	1	33.0		3.83×10^{-5}	42.04	"1"	8.71
1	E36M48-279	7	94.4		8.51×10^{-5}	57.14	"0"	9.06
11	E41M62-459	2	211.9		3.38×10^{-4}	44.03	"0"	6.94
12	E36M48-304	7	112.3		7.46×10^{-4}	76.67	"1"	6.60
				FROST				
1	E40M58-369	3	128.8	Disposition to survive	1.02×10^{-4}	9.742	"0"	8.11
2	Vf_Mt3g086600	2	97.0		2.77×10^{-4}	8.859	"G"	7.14
2	Vf_Mt3g086600	2	97.0	Frost tolerance index	9.66×10^{-5}	8.014	"G"	8.16
1	E40M58-369	3	128.8		1.05×10^{-4}	8.235	"0"	8.08
2	Vf_Mt3g086600	2	97.0	Loss (T+C)	2.52×10^{-5}	5.964	"T"	9.47
3	Vf_Mt5g046030	4	170.3		2.90×10^{-4}	3.635	"C"	9.00
4	E41M48-064	-	-		4.86×10^{-4}	2.867	"1"	6.56
5	E42M59-217	3	142.3		5.81×10^{-4}	4.816	"0"	6.42
6	E40M55-221	2	62.9		7.62×10^{-4}	3.381	"1"	6.15
7	E42M58-219	3	103.1		8.24×10^{-4}	2.946	"1"	6.31
8	E36M56-320	6	74.0		9.06×10^{-4}	3.452	"0"	7.39
9	E41M51-174	-	-		9.71×10^{-4}	2.989	"1"	5.98

*Difference of impact of "increasing allele" versus "decreasing allele"; cf. Table 1.

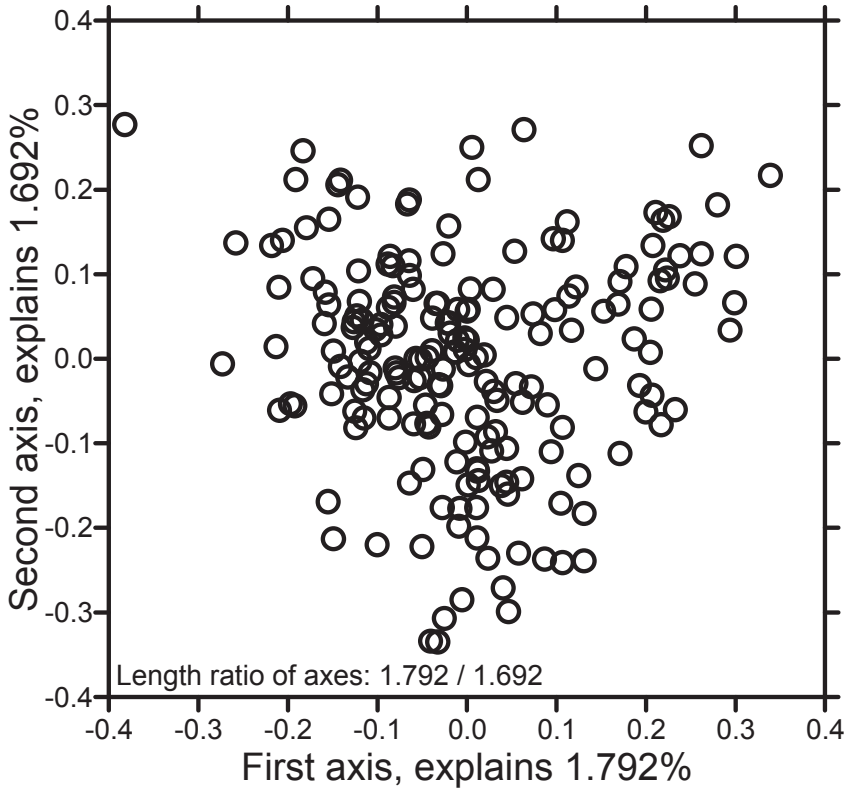
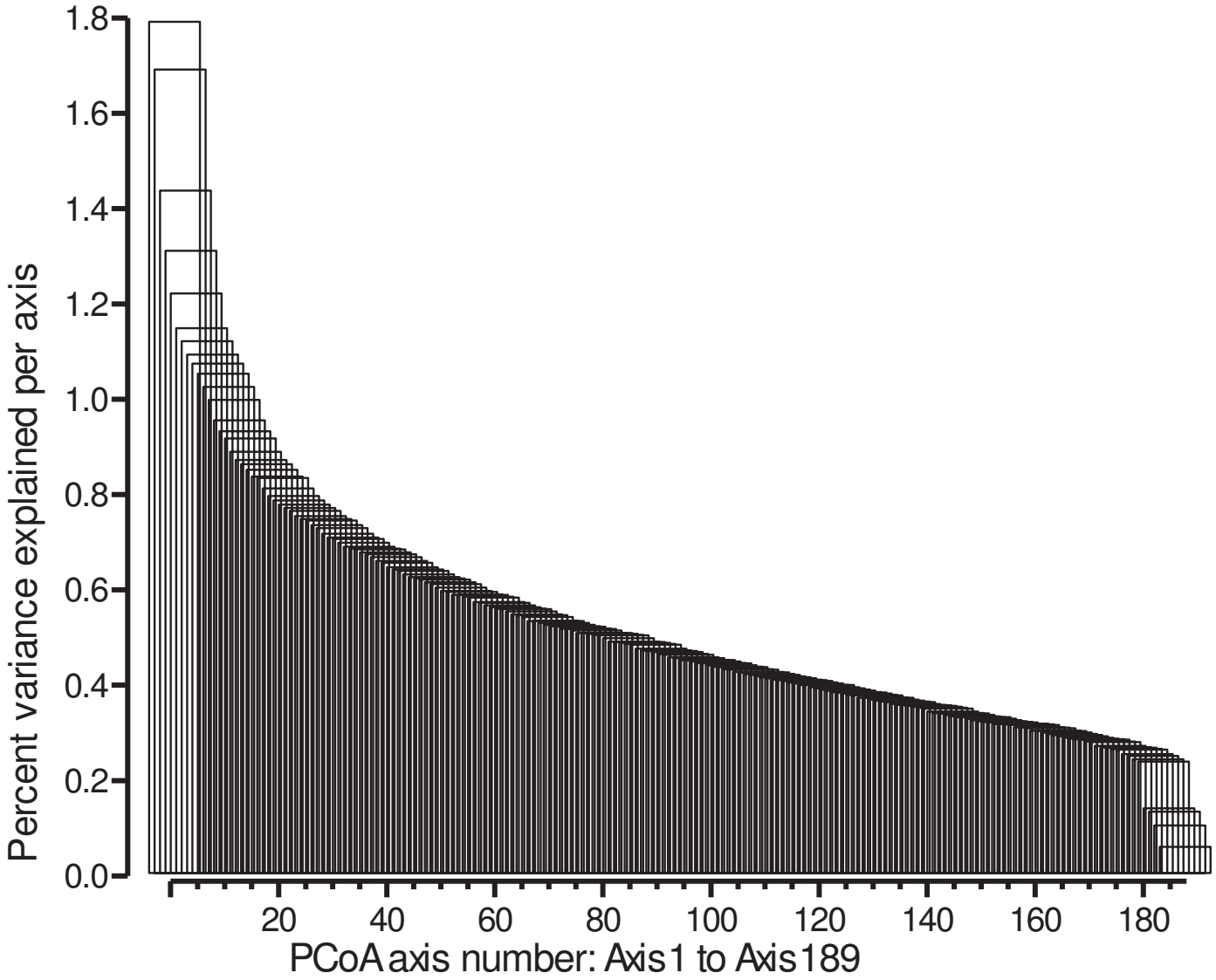
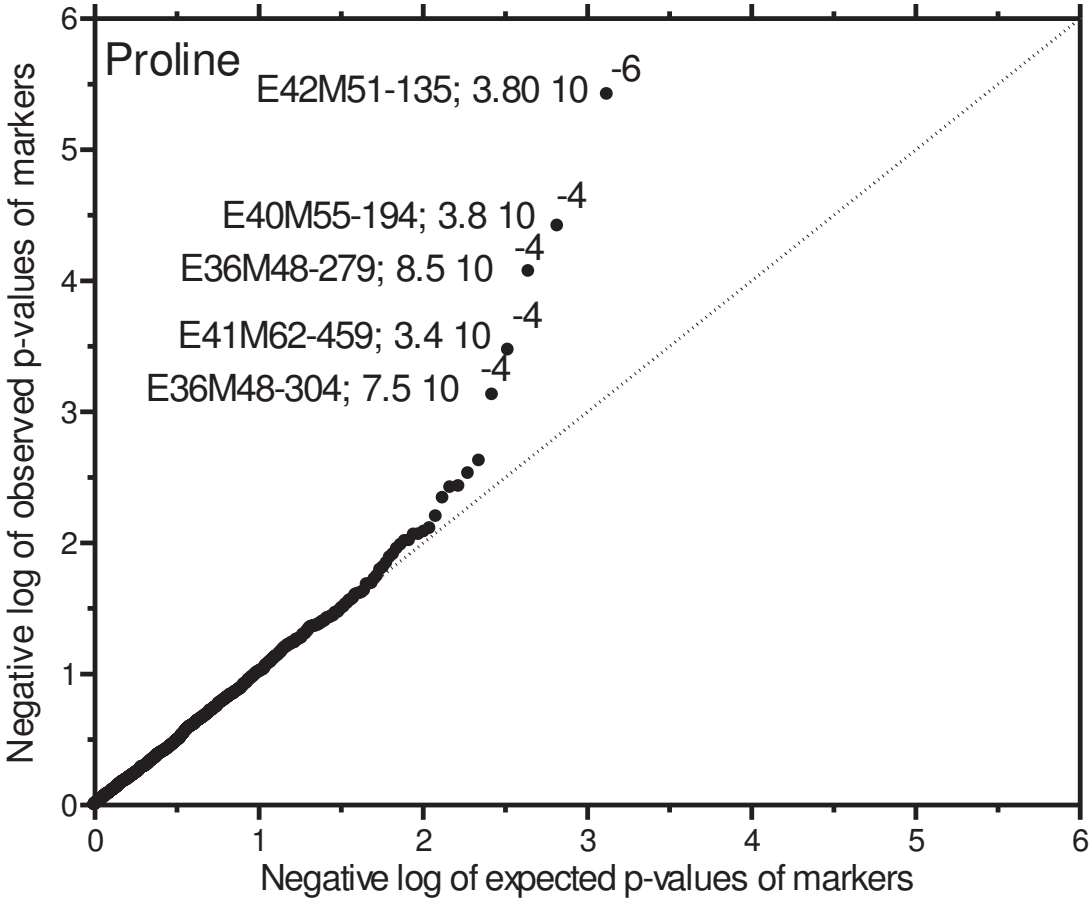


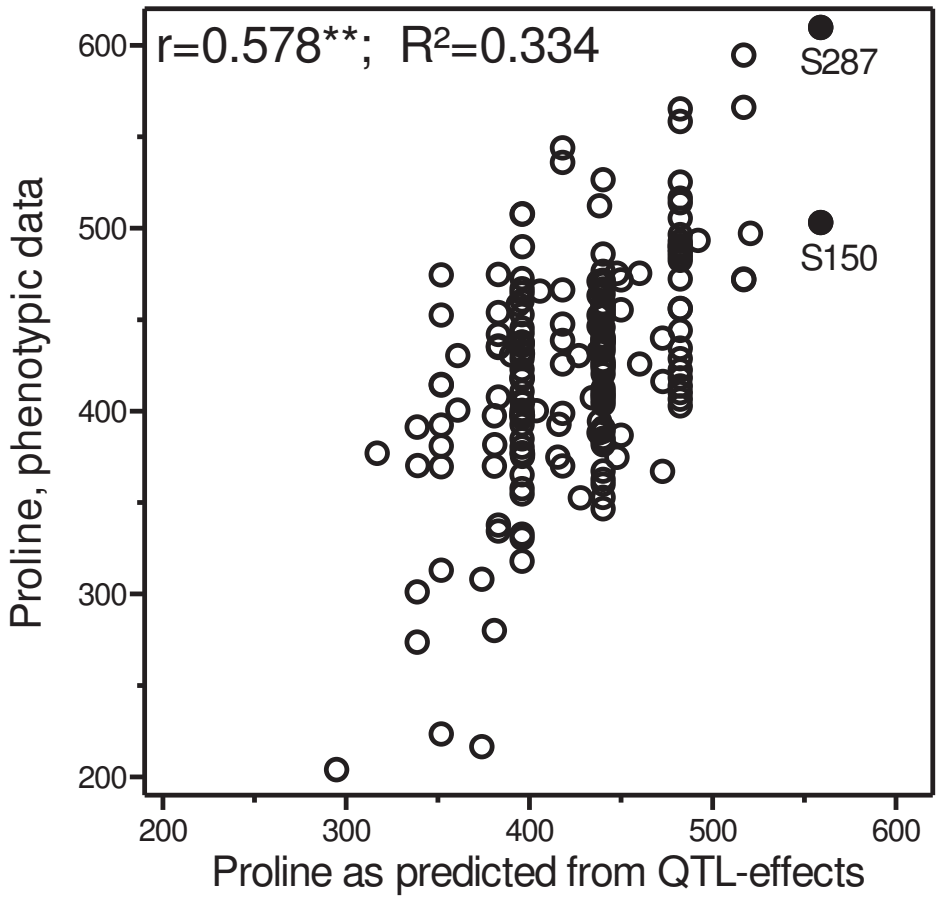
Figure 1. First two axes of a principal coordinate analysis, based on genetic similarity estimates from 1322 genome-wide markers.



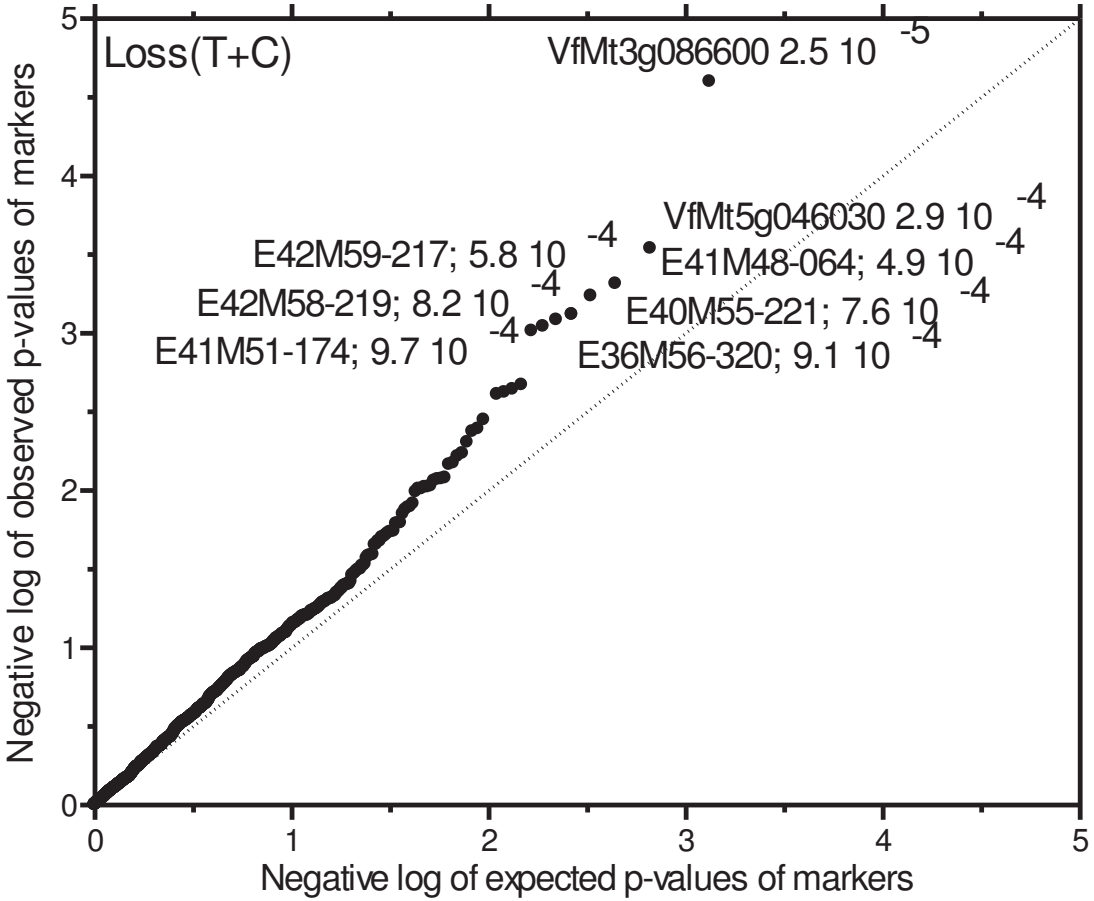
Supplemental figure 1: Steady decay of variance explained per axis of Principal Coordinate Analysis.



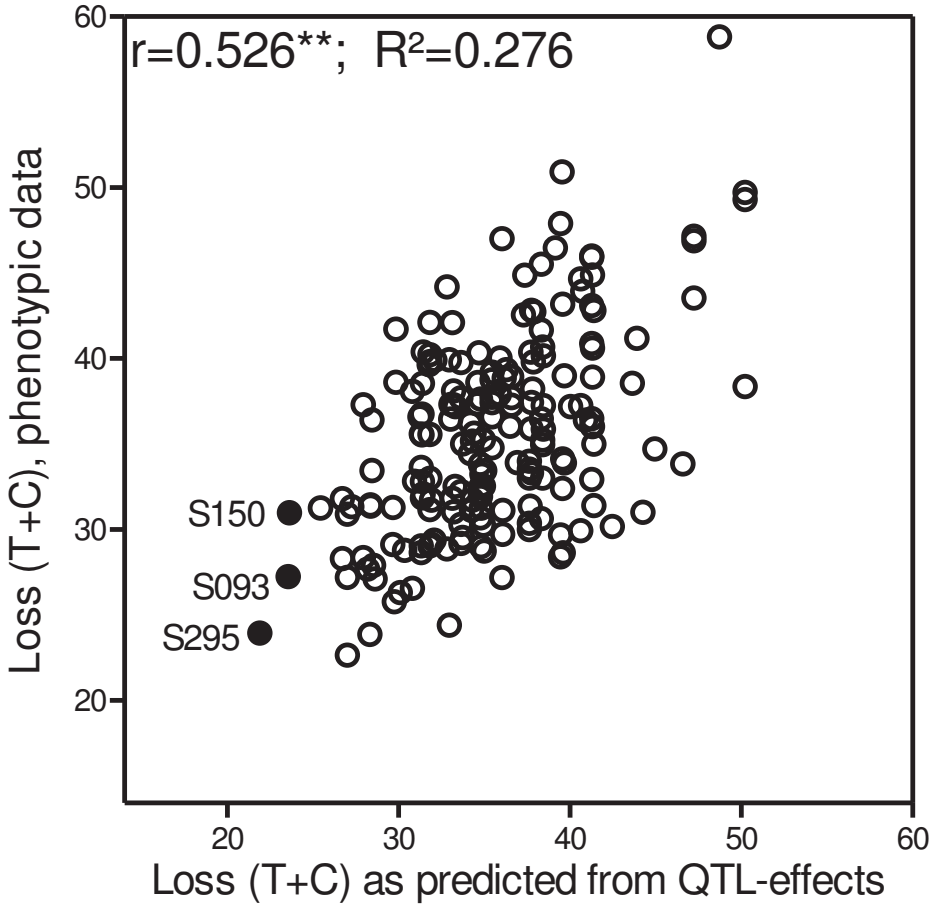
Supplemental figure 2: Q-Q plot for proline; the 5 significant QLT are indicated.



Supplemental figure 3: Prediction of phenotypic proline results from estimated QLT effects.



Supplemental figure 4: Q-Q plot for Loss(T+C); the 8 significant QLT are indicated.



Supplemental figure 5: Prediction of phenotypic Loss(T+C) results from estimated QLT effects.

Discussion

Non-DMSP DMS-precursors

In fact, DMSP is a key molecule in the marine environment. The possible osmoregulative functions of DMSP in macroalgae from tidal waters were recognized in the mid-20th century. The alkaline mediated release of the highly volatile DMS from DMSP and subsequent GC analysis provides a straightforward method to quantify DMSP without interference of other common trimethylammonium betaines like GBT. As the released DMS is usually measured in the headspace of the samples, this method is applicable to diverse matrices such as seawater **[Kiene and Slezak 2006]**, microalgae cultures **[van Rijssel and Gieskes 2002]**, plant material **[Husband and Kiene 2007]** and sediments **[van Bergeijk 2002, van Bergeijk 2006]**. However, due to the fact that DMSP is quantified indirectly, as released DMS, a simple back-calculation of the determined DMS concentrations to DMSP in the samples has to be treated with caution. It is not negligible that also other algal metabolites release DMS under alkaline conditions, which would cause a systematic error in calculation of DMSP concentrations. Therefore, an overestimation of DMSP via indirect quantification is quite likely. This is underlined by Sciuto *et al*. who showed that 4-dimethylsulfonio-2-methoxybutyrate and 5-dimethylsulfonio-4-methoxy-2-aminopentanoate, which were isolated from marine red algae, can release DMS under alkaline conditions (2M NaOH, 90°C, 15 min) **[Sciuto 1982, Sciuto 1988]**. First evidence for a systematic error of an indirect quantification of DMSP were obtained by Colmer *et al*. who compared a direct quantification approach for DMSP in sugar cane (*Sacchanum officinarum* L.) based on ion chromatography (IC) with the indirect, gas chromatography based, method. The causes for the discrepancies between IC and GC measurements of 14-65% were not clarified, but possible other DMS precursors or an insufficient extraction of DMSP prior to IC measurements were discussed **[Colmer 2000]**. In this context, we could determine differences between direct and indirect DMSP quantification of 3% to 14% in various marine microalgae cultures by application of an improved UPLC method based on the work of Wiesemeier and Pohnert **[Wiesemeier and Pohnert 2007] (Manuscript A)**. Here, DMSP was quantified in methanolic extracts, which were previously spiked with isotope labeled D_6-DMSP as internal standard. For comparison between direct and indirect quantification, the corresponding extracts were split and measured via UPLC-MS (after derivatization with pyrenyldiazomethane) and indirectly after base treatments via GC-MS. The very high reproducibility and comparability of the measurements was achieved by the utilization of the same extracts for direct and indirect measurements as well as the use of D_6-DMSP as isotope labeled internal standard. In this case, differences in extraction of DMSP, which were discussed by

Colmer *et al*., as reason for the above mentioned discrepancies can be excluded **[Colmer 2000]**. However, the chemical structures of the non-DMSP DMS-sources remained elusive. Considering the work of Gage and Rhodes who suggested 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB) as a possible DMS precursor, this compound could explain a fraction of the determined differences between direct and indirect methods **[Gage and Rhodes 1997]**. As antecedent in DMSP biosynthesis in marine algae, the concentration of DMSHB is closely related to DMSP production in the cells. Hence, an increased DMSP synthesis could also lead to a higher intracellular concentration of the different precursor molecules of the biosynthetic pathway, including DMSHB as possible DMS precursor. This seems plausible in consideration of the experiments of Spielmeyer and Pohnert who found larger differences between direct and indirect DMSP quantification methods in growth phases of the diatom *Skeletonema marinoi* with elevated DMSP production **[Spielmeyer and Pohnert 2012]**. Additionally, DMSP synthesis and also concentration of other DMS precursors was shown to be highly dependent on nitrate availability in cultures of the diatom *S. marinoi* **[Spielmeyer and Pohnert 2012]**. It is assumed that nitrate limited conditions lead to a shift in microalgal metabolism which results in an increased synthesis of DMSP and replacement of other nitrogen containing compatible solutes. Through the transamination reaction in DMSP biosynthesis, the valuable nitrogen can be redistributed into new amino acids **[Stefels 2000]**. Hence, increased synthesis of DMSP is a feasible mechanism for nitrogen recycling under nitrate limited conditions. Furthermore, nitrate deficiency might also lead to a substitution of nitrogen containing choline by dimethyl sulfocholine in polar phospholipids, which are important to maintain a certain cell membrane polarity in microalgae. Such phosphatidyl sulfocholine containing phospholipids were first identified in the diatom *Nitzschia alba*, but it is assumed to have a widespread distribution in marine phytoplankton **[Anderson 1978]**. Due to the dimethylsulfonio-moiety of sulfocholine, it is very likely that this compound may cleave DMS under alkaline conditions. These polar lipids may therefore contribute to alkaline labile DMS precursors and could partially explain the difference between direct and indirect DMSP quantification in phytoplankton cultures under nitrate limited conditions **(Manuscript A) [Spielmeyer and Pohnert 2012]**. Since DMSP is regarded as the major source of DMS and alternative DMS-precursors are mainly discussed as part of the compatible solutes in microalgae, future experiments should also deal with lipid extraction from marine algae and determination of possible DMS-cleavage from this fraction. In particular in respect to their ecological and geochemical relevance, the direct quantification of non-DMSP DMS-precursors may provide important information since these compounds may account for around 120 Gmol of biogenic sulfur emissions per year **(Manuscript A)**.

Osmoregulation/osmoadaption processes in marine phytoplankton

In order to investigate osmoregulative processes, the determination of DMSP alone provides just a very simplistic image. Thus, methods for efficient and selective analysis of highly polar substances are necessary. Based on the method of Wiesemeier and Pohnert **[Wiesemeier and Pohnert 2007]**, improvements of the derivatization procedure, sample preparation and the use of a phenyl-column instead of a C_{18}-column led to a higher sensitivity, a lower limit of detection, a better peak shape and less complex sample preparation **(Manuscript A)**. Wiesemeier and Pohnert used a Bligh and Dyer treatment for lysis and extraction of the microalgae cells with subsequent drying of the extracts and dissolution of the residue in MeOH. Through simple filtration of the algae and transfer of the filter into MeOH **(Manuscript A)**, one extraction and a drying step during sample preparation could be omitted. Because of the possible degradation of DMSP during prolonged sample handling, which can be observed by the typical smell of DMS, a simpler sample preparation may also reduce quantification errors and improve the comparability of the individual measurements. Furthermore, the simultaneous quantification of DMSP and GBT and detection of other metabolites like DMS-Ac and SMM allows a more detailed view on the osmoregulation of marine phytoplankton.

Using the above described LC-MS method (extraction with methanol, derivatization with pyrenyldiazomethane, UPLC-MS with phenyl column, **Manuscript A**),a cellular concentration of DMSP of ca. 35 pg/cell and around 7 pg/cell GBT could be determined in cultures of the dinoflagellate *Prorocentrum minimum* **(Manuscript A)**. The latter finding is very interesting as Keller *et al.* reported that another strain, *P. minimum* (CCMP1329), does not produce GBT **[Keller 1999]**. This suggests that the production of GBT is not just species, but also strain specific in *P. minimum*. Strain specific differences in algal metabolism are not unlikely, as also reported by Yost and Mitchelmore, who compared the enzyme activity of five different strains of the symbiotic dinoflagellate *Symbiodinium microadriaticum*. This species exhibits partially very high differences in the DMSP lyase activity (DLA) between the investigated strains. While the strain CCMP 829 showed a four times higher activity (DLA : chlorophyll a) than the strains CCMP 421, CCMP 828 and CCMP 1633, there was no detectable DMSP lyase activity in the strain CCMP 830 **[Yost and Mitchelmore 2009]**. Yost and Mitchelmore suggest that DMSP lyase is not a universal enzyme in *S. microadriaticum*. Further strain specific differences in DMSP and DMS production were also reported by Steinke *et al*. in the dinoflagellate in *Symbiodinium* sp. **[Steinke 2011]**. Another factor, which could cause the above mentioned differences in intracellular GBT in *P. minimum,* is the reported high variability of GBT concentration over the growth phases of marine phytoplankton **[Keller 1999]**. Therefore, the differences in determined GBT concentration

in *P. minimum* between our findings **(Manuscript A)** and previous works **[Keller 1999]** might also result from different sampling points during the exponential growth phase of this species.

A more sophisticated approach for the analysis of DMSP and other zwitterionic osmolytes without previous time consuming derivatization relies on hydrophilic interaction liquid chromatography with a zwitterionic stationary phase (ZIC®-HILIC). This technique allows a good interaction between the column material and the zwitterionic analytes (Figure 6) and, therefore, a good separation of different highly polar zwitterionic osmolytes can be achieved. By the application of mass spectrometry and monitoring of the ion traces of the corresponding compounds, a simultaneous quantification of even coeluting substances is possible. This method was first applied on the investigation of the DMSP-uptake by marine phytoplankton **(Manuscript B)**.

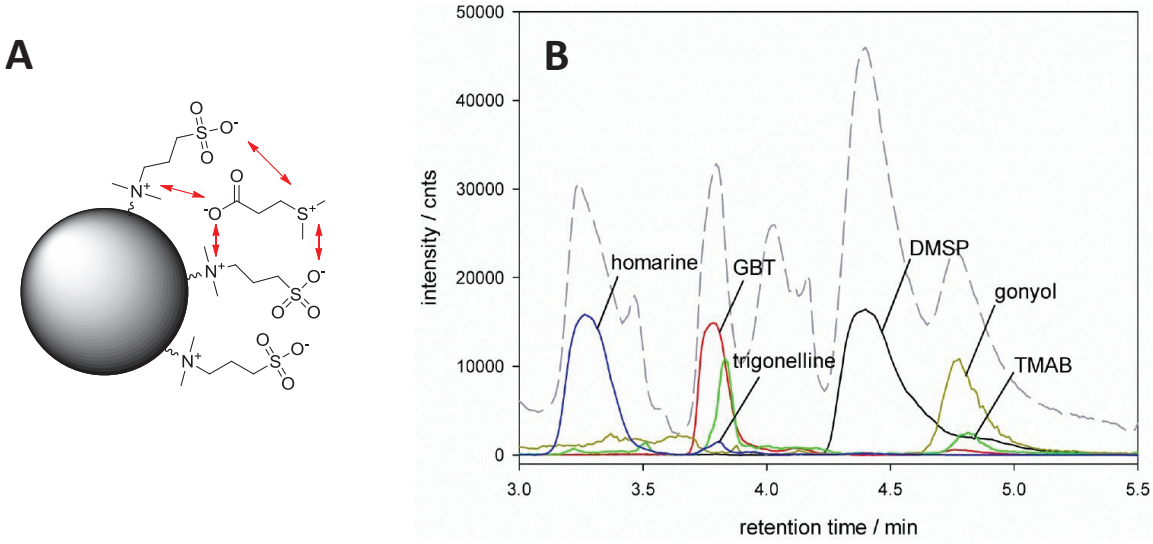


Figure 6: A: Schematic picture of DMSP interacting with a ZIC®-HILIC stationary phase; particle surface is derivatized with zwitterionic gamma-alkyl-dimethylammonium sulfonate. Arrows represent possible electrostatic interactions between the stationary phase and DMSP as analyte. B: Example chromatogram of a methanolic extract of *Emiliania huxleyi* measured via LC-MS on a ZIC®-HILIC column with ion traces of the main zwitterionic osmolytes. The dashed line represents the total ion count (TIC) of the measurement. Ion traces of GBT, gonyol, TMAB and trigonelline are 10-times amplified. Figure taken from (Manuscript D).

Through application of this method to methanolic phytoplankton extracts, a wide set of different zwitterionic compounds could be separated and simultaneously analyzed via mass spectrometric detection using three different isotope labeled internal standards (D_6-DMSP, D_6-DMS-Ac and D_3-gonyol). This allowed a more detailed view on the mechanisms to adapt to different seawater salinities in the coccolithophore *Emiliania huxleyi* and the dinoflagellate *Prorocentrum minimum* **(Manuscript D)**. It could be shown that these two species have completely different osmoadaption strategies. *E. huxleyi* cells, which were grown at higher salinity exhibit a significant

lower average cell size while the summed up amount of investigated zwitterionic metabolites (DMSP, GBT, homarine, trigonelline, trimethylammonium propionate (TMAP) and trimethylammonium butyrate (TMAB)) per cell remained constant or even slightly decreased. Only at the highest observed salinity, the cellular osmolyte concentration increased dramatically. Furthermore, there could be no further cell size reduction of *E. huxleyi* observed at the highest tested salinity of 38‰ compared to cultures grown at 34‰. In contrast to the behavior of *E. huxleyi*, cultures of *P. minimum* showed a constant increasing concentration of overall analyzed zwitterionic osmolytes under elevated medium salinities. Cell sizes of *P. minimum*, however, were not affected by the salt concentration of the culture medium. As also known from other phytoplankton species, their cell sizes can be affected very differently by the medium and seawater salinity. Like it was shown in *E. huyleyi* **(Manuscript B)**, a negative correlation between salinity and cell size was also observed in the diatom *Thalassiosira weissflogii* **[Garcia 2012]**, the dinoflagellate *Prorocentrum reticulatum* **[Röder 2012]** and even in the marine macroalgae *Porphyra purpurea* **[Reed 1980]**. However, for other species like the benthic diatom *Attheya ussurensis* **[Aizdaicher and Markina 2011]** and the pelagic diatom *Skeletonema* sp. **[Balzano 2011]**, there were no salinity-dependent cell size variations reported. Additionally, under more extreme conditions like a very low salinity below 5‰, a positive correlation between cell size and salinity can be observed. Although, this effect seems to be rather dependent on the nutrient availability in such environments **[Svensson 2014]**. The observed remarkable higher osmolyte concentration in *E. huxleyi* at a salinity of 38‰ compared to 34‰ suggests a critical cell size at which metabolism of *E. huxleyi* changes from cell size adaptation to increased synthesis of zwitterionic osmolytes **(Manuscript D)**. At lower salinities, cell size adjustment seems more beneficial to the algae to adapt to different salt concentrations. Only at higher salinities, at which a further cell size reduction becomes unfavorable, algae invest energy into synthesis of new osmolytes. Cell size dependent changes in algal metabolism are also known from reproduction mechanisms of diatoms. Since walls of diatoms are built of two overlapping rigid biomineralized halves, named epitheca and hypotheca, respectively, the resulting construction is comparable, in a very simplistic point of view, to a petri dish. During mitosis, each new daughter cell occupies one of these halves. The new missing silicate shells are then synthesized inside the parental shell, which results in a decrease of mean cell size of the population. Below a certain cell size, which is designated as sexual size threshold (SST), a differentiation into sexual mating types, the release of pheromones and sexual reproduction occurs **[von Dassow 2006, Gillard 2013, Scalco 2014]**. This shows that cellular features, like a certain critical cell size, can trigger major physiological changes in marine phytoplankton, which may also include an elevated biosynthesis of zwitterionic

metabolites as observed in our experiments **(Manuscript D)**. In consequence, the synthesis of different zwitterionic osmolytes including DMSP follows not in any case a linear relationship to the corresponding environmental factors. As it could be shown in **Manuscript D**, medium salinity can indirectly, trigger an overproportional high DMSP synthesis, which makes predictions of DMSP production in different habitats and under changing environmental conditions very difficult.

Interestingly, changes in medium salinity in *P. minimum* cultures affected the overall composition of intracellular zwitterionic osmolytes. Between salinities of 20‰ and 32‰, the intracellular DMSP concentration remained almost constant, whereas concentrations of GBT and DMS-Ac increased dramatically. Only at the highest and lowest investigated salinity of 16‰ and 36‰, respectively, changes in the DMSP concentration could be observed **(Manuscript D)**. This suggests a fundamental compensatory effect of DMSP and a potential fine tuning of the osmotic pressure via GBT and DMS-Ac in this species. In contrast to our findings, Zhuang *et al*. reported, in a salinity range between 22‰ and 34‰, a positive correlation between medium salinity and intracellular concentration of DMSP in *P. minimum* **[Zhuang 2011]**. Due to the fact that the authors calculated the DMSP concentration per cell and had not measured the cell volumes, it is unclear whether this effect relies on increased DMSP synthesis or a possible cell size reduction. Similar to the observed strain specific DMSP lyase activity in *Symbiodinium microadriaticum* **[Yost and Mitchelmore 2009]**, strain specific differences might explain the diverse response of *P. minimum* to salinity changes **(Manuscript D) [Zhuang 2011]**. This may include different enzyme activities, responsible for DMSP biosynthesis, or corresponding enzyme expressions. Further evidence for major physiological differences in this species are the different cell shapes (round, oval, triangular), which are reported for *P. minimum* **[Pertola 2005]**. However, the factors which are responsible for the observed morphological differences are still unknown. In this context, the physiological state of the algae cells, e.g. cell turgor, or environmental factors are discussed **[Hajdu 2000]**. Furthermore, changes in algal metabolism with respect to changes in medium salinity are not just limited to osmolyte biosynthesis **(Manuscript D)** but include also other metabolites in *P. minimum* which might have no obvious osmoregulative function. It is reported that seawater salinity largely affects the total concentration of toxic metabolites and also the toxin profile in marine dinoflagellates **[Hwang and Lu 2000, Engström-Öst 2011, Errera and Campbell 2011]**. Furthermore, other environmental factors like water temperature or the growth conditions of the algae, especially their nutrient supply are respective influencing factors **[Hwang and Lu 2000, Röder 2012]**. The possible physiological variations in marine microalgae, even within the same species, make universal predictions about their response to environmental factors very difficult.

Therefore, further research should also address strain specific differences in marine phytoplankton.

Additionally to the long term osmoadaption processes that we reported in our study **(Manuscript D)**, it was shown that DMSP is very important for the short term osmoregulation, over few hours, of marine micro- and macroalgae after rapid changes in seawater salinity **[Dickson 1980, Van Bergeijk 2003]**. As the biosynthesis of DMSP from methionine follows a four-step mechanism with involvement of several enzymes, it seems plausible, that the achievement of a steady state DMSP concentration after salinity downshocks by simple excretion of DMSP is faster than the biosynthetic response to rapidly increasing salinities **[Dickson 1980, Van Bergeijk 2003]**. However, the contribution of minor abundant osmolytes and metabolites, which are more difficult to quantify, like DMS-Ac or gonyol, to these short term osmoregulation processes is still unknown and should be the focus of further research.

An interesting approach to investigate the multiple metabolic changes during osmoregulation and acclimation was published by Lyon *et al*. who surveyed the variability in the proteome of the sea-ice diatom *Fragilariopsis cylindrus* **[Lyon 2011]**. The measured up-regulation of certain proteins (aminotransferases, NADPH dependent reductases, methyltransferases and decarboxylases) further reinforces the DMSP synthesis pathway, proposed by Gage *et al*. (confer Figure 4, page 21) **[Gage and Rhodes 1997]**. Furthermore, the authors suggest that the S-adenosyl methionine (SAM) dependent methyltransferases and the pyridoxyl dependent decarboxylases are essential enzymes in the biosynthetic pathway of DMSP. This is supported by the fact that these enzymes are absent in some DMSP non-producing strains of *Phaeodactylum tricornutum* **[Lyon 2011]**. Osmotic adaption of phytoplankton is therefore a highly complex system with regulation of cell morphology and concentration of a wide set of different polar metabolites.

Although the different direct LC-MS based quantification methods provide valuable information about the distribution of a large set of zwitterionic osmolytes in marine phytoplankton **(Manuscripts A,B and D)**, their disadvantage compared to indirect methods is the relatively high detection limit of DMSP. Therefore, when it comes to the quantification of dissolved DMSP in seawater, which mostly has a concentration of less than 2.8 nM **[Kiene and Slezak 2006]**, high sensitive indirect measurements, which provide detection limits of down to 5 pM **[Yassaa 2006]**, are still vital. For such applications, an additional concentration step during sample preparation using ZIC®-HILIC cartridges might help to overcome this drawback of LC-MS based methods to a certain extent. A reduction of the detection limit of direct quantification methods could also deliver information about the global distribution of GBT, DMS-Ac and other zwitterionic phytoplankton metabolites in natural seawater, which are not available to the present day.

DMSP uptake by marine phytoplankton

Due to the fact that DMSP is a ubiquitous molecule in the euphotic zone of the oceans, marine microalgae can not only satisfy their DMSP demand via biosynthesis, but also through uptake from the surrounding seawater. First experiments by Vila-Costa *et al.* using radiolabeled ^{35}S-DMSP could show an effective uptake of radioactive sulfur by DMSP low- and non-producers, respectively, like *Thalassiosira pseudonana* and *Thalassiosira oceanica*. Based on the inhibition of ^{35}S-uptake after addition of unlabeled DMSP and GBT, respectively, they suggested the use of the same transport mechanism for DMSP and GBT **[Vila-Costa 2006]**. Due to the DMSP degradation activity of phytoplankton, it could not be excluded that the observed sulfur assimilation resulted from the uptake of other labeled sulfurous molecules such as MeSH or DMS after extracellular cleavage of DMSP. Besides the different cytoplasmic DMSP cleaving enzymes like dddD from the marine bacterium *Halomonas* sp., it is known that some DMSP-lyases in marine bacteria are also bound to the cell membrane **[Yoch 1997, Curson 2011a]**. These enzymes are able to degrade DMSP on the surface of the cell to acrylate and DMS. These non-polar substances are, in contrast to the zwitterionic DMSP, able to diffuse through the cell membrane without a specific transport mechanism and can therefore cause misinterpretations when radiolabeled ^{35}S-DMSP is used as tracer of direct DMSP uptake in marine phytoplankton and bacteria. The synthesis of an eightfold stable isotope labeled $^{13}C_2D_6$-DMSP (confer Figure 7A) and the determination its uptake via mass spectrometric analysis of cell extracts allowed the verification of the proposed direct uptake mechanism of DMSP by marine phytoplankton **(Manuscript B)**. Through deuteration at the dimethylsulfonio terminus and ^{13}C-labeling of the 1- and 2-positions of the carboxylic acid of $^{13}C_2D_6$-DMSP, possible transformation processes during DMSP assimilation could be excluded via mass spectrometric measurements (confer Figure 7B).

In contrast to Vila-Costa *et al.* **[Vila-Costa 2006]**, it was revealed that also algal species, which are known as DMSP high-producers, possess a specific transport system for uptake of DMSP **(Manuscript B)**. The DMSP non-producer *Thalassiosira weissflogii* showed an extraordinary fast uptake of ca. 60% of the initial concentration of $^{13}C_2D_6$-DMSP of 125 nM within 2.5 minutes of incubation. Finally, the intracellular concentration reached a steady state of 70% of the initially added $^{13}C_2D_6$-DMSP after 20 minutes. The development of such highly effective transport systems could enable respective marine microalgae to take up the necessary DMSP from the surrounding seawater. Thus, these microalgae do not need to invest energy and nutrients into DMSP biosynthesis. Due to the fact that DMSP is ubiquitous in the marine environment, these algae would gain an evolutionary advantage towards DMSP-producers.

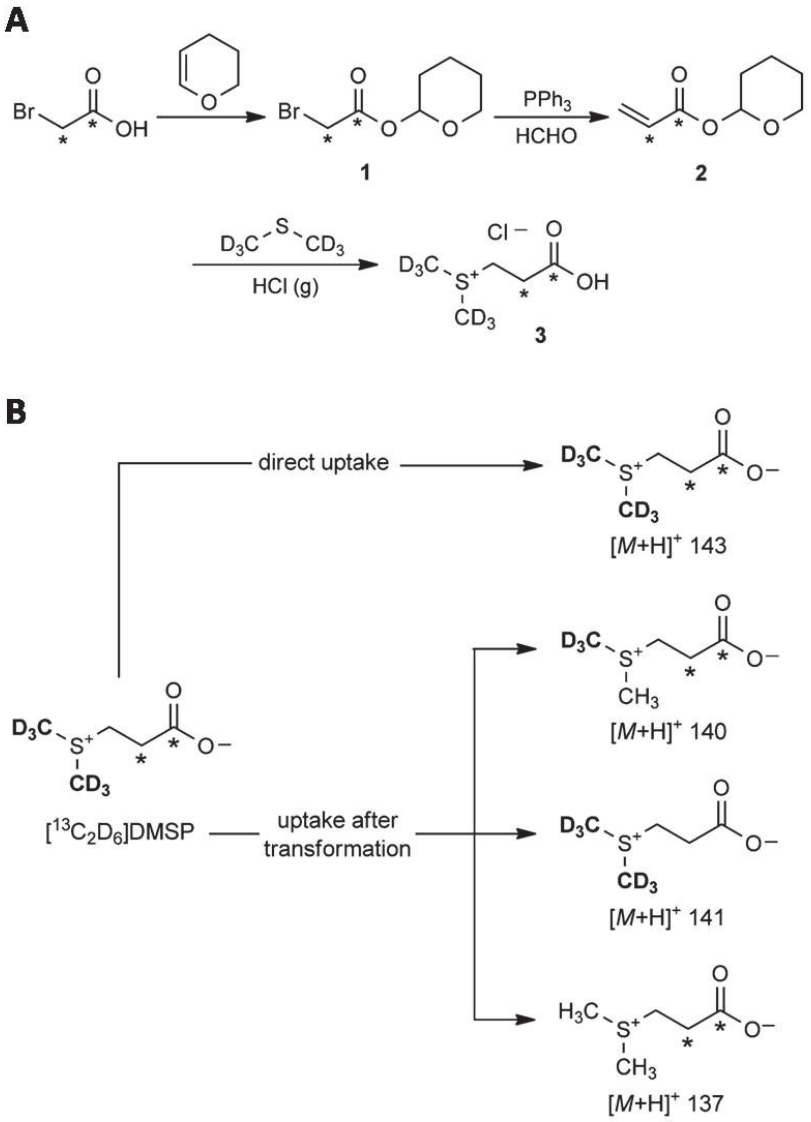


Figure 7A: Synthetic pathway to isotope labeled $^{13}C_2D_6$-DMSP (3) with $^{13}C_2$-bromoacetic as starting compound. "*" represent ^{13}C isotopic labelings of the corresponding carbon atoms. B: Possible transformations of $^{13}C_2D_6$-DMSP during uptake by marine phytoplankton with ion traces $[M+H]^+$ of the corresponding compounds. Figures taken from (Manuscript B).

Also the DMSP producing coccolithophore *E. huxleyi* exhibited a high DMSP assimilation rate, which reached a plateau after 20 minutes with uptake of 30% of the supplied $^{13}C_2D_6$-DMSP **(Manuscript B)**. This is rather surprising while *E. huxleyi*, as DMSP high-producer, should have no demand of an external DMSP supply. However, also for DMSP producing phytoplankton species, it is energetically favorable to take up these molecules from the surrounding seawater than to invest into its biosynthesis. Previous studies used short time incubations of 5-10 min for the calculation of uptake rates per hour **[Kiene 1998]**. Due to the observed steady state in DMSP uptake after 20 minutes, calculated DMSP uptake rates might be overestimated. These specific uptake systems for DMSP underline its high relevance for marine phytoplankton for several

physiological processes. Furthermore, the determined direct uptake of DMSP by several phytoplankton species proves that marine microalgae are not only its major source, but can act as a considerable sink for DMSP in the oceans with a major impact on the marine sulfur cycle.

Effect of DMS-Ac and gonyol on microbial DMSP/DMS sulfur cycling

The numerically most important sink for DMSP is marine bacterioplankton. The abundance of marine bacteria is closely associated with the presence of phytoplankton. Due to the strong increase in photosynthetic activity and primary production during phytoplankton blooms, the abundance of heterotrophic bacteria typically follows this trend with a certain delay **[Buchan 2014]**. Typically after decline of a bloom, the level of bacteria remains high as they use organic matter, which is released by dying phytoplankton cells, as easy available nutrients. In this context, DMSP plays a major role as a source for reduced sulfur and as high energy nutrient for marine bacteria. It could be shown that the composition of zwitterionic osmolytes in marine phytoplankton can be very complex and is highly species specific **(Manuscript D)**. Therefore, marine bacteria are exposed to many different zwitterionic compounds, which can possibly be used as alternative sulfur or energy source. In this context, the distribution of the dimethylsulfonio-metabolites DMS-Ac and gonyol in different phytoplankton species was investigated. Through a survey of 13 different microalgae cultures from different taxonomic groups (7 diatoms, 3 dinoflagellates, two haptophytes and one cryptophyte), we could find that DMS-Ac contributes to zwitterionic osmolytes in a relative abundance of 0.1% to 1990%, compared to DMSP. Furthermore, gonyol, which was up to now described only in the dinoflagellate *Gonyaulax polyedra*, was found in all investigated dinoflagellates and haptophytes with a relative contribution of 4.5% to 121%, compared to DMSP **(Manuscript E)**. This shows that phytoplankton associated marine bacteria are usually exposed to a complex mixture of dimethylsulfonio-metabolites. Due to their similar structure, the zwitterionic microalgae metabolites DMS-Ac and gonyol may also contribute to cover the sulfur requirements of marine bacterioplankton. However, they might also interfere with different DMSP degradation pathways. In order to investigate the potential of DMS-Ac and gonyol to act as alternative substrate for the two main DMSP degradation pathways, cleavage of DMS and the demethylation/demethiolation pathway, respectively, the four well known bacteria species *Ruegeria pomeroyi* (DSS-3), *Sulfitobacter* sp. (EE-36), *Alcaligenes faecalis* (M3A) and *Halomonas* sp. (HTNK1) were chosen as model organisms **(Manuscript E)**. Previous works with these species were focused on the identification of different enzymes and their encoding genes which catalyze the release of DMS **[Curson 2008, Todd 2009, Todd 2010, Curson 2011a, Todd 2011, Todd 2012]** and MeSH **[Miller**

and Belas 2004, Reisch 2011b] from DMSP. Reisch *et al*. showed that the purified DMSP dependent demethylation enzyme (DmdA) does not recognize DMS-Ac as alternative substrate **[Reisch 2008]**. This enzyme catalyzes the first reaction step of the demethylation/demethiolation pathway, which ultimately results in the release of MeSH. By incubation of *R. pomeroyi* with DMS-Ac and measurement of the released sulfurous gases via GC-FPD, a very high concentration of MeSH was determined in the headspace of this species that exceeded the amount produced from DMSP **(Manuscript E)**. This indicates a hitherto unidentified demethylation/demethiolation pathway in this species that accepts DMS-Ac as substrate. Furthermore, release of MeSH in a demethylation/ demethiolation like pathway from gonyol was also observed in *A. faecalis*. Incubation of the above mentioned model organisms with different combinations of DMSP, DMS-Ac and gonyol showed species-specific inhibitory effects of DMS-Ac and gonyol on DMSP degrading enzymes. The most dramatic effect was monitored in *R. pomeroyi* cultures, where the presence of gonyol completely inhibited DMSP dependent DMS production and the demethylation/demethiolation pathways. An inhibitory effect of gonyol on DMS release was also observed in *Sulfitobacter* sp. and *A. faecalis*, whereas *Halomonas* sp. showed no such effect. This might be ascribed to the structurally different type of enzymes involved in DMS production from DMSP in this species. While the DddD enzyme from *Halomonas* sp. leads to 3-hydroxypropionate as by-product **[Todd 2010]**, DMSP lyases from all other investigated species co-produce acrylate **[Curson 2008, Todd 2009, Curson 2011a, Todd 2011, Todd 2012]**.

By quantification of the remaining substrates after incubation via LC-MS, we could determine the ability of marine bacteria to metabolize DMSP, DMS-Ac and gonyol, respectively. This gives the general utilization of these compounds regardless to the used conjugation or degradation pathway(s). Interestingly, except in *A. faecalis*, which exhibits very high DMSP-lyase activity, there was no discrimination in bacterial utilization of the different substrates (DMSP, DMS-Ac and gonyol) of ca. 45% of the initial substrate concentration in *Sulfitobacter* sp. and *Halomonas* sp. Due to the total conversion of all supplied substrates in *Ruegeria pomeroyi*, possible preferences in substrate utilization could not be detected in this species **(Manuscript E)**. The different degradation processes of DMSP in bacteria that result in release of DMS or MeSH are well studied and have been frequently reviewed **[Groene 1995, Kiene 2000, Curson 2011b, Reisch 2011a]**. However, information about bacterial metabolism of gonyol and DMS-Ac is very rare. Previous works on the osmoprotective properties of DMS-Ac in bacteria have not investigated further breakdown processes **[Chambers 1987, Randall 1996, Cosquer 1999]**. However, investigations of DMS-Ac and other related dialkylsulfonio-metabolites in rats showed that these substances may play a major role in methionine biosynthesis through provision cleavable methyl groups for

transmethylation reactions **[Dubnoff and Borsook 1948, Maw and Duvigneaud 1948, Maw 1953]**. In marine bacteria, which are exposed to DMS-Ac and gonyol, respectively, as easily available nutrients, these substances might act as substrate for transmethylation reactions as well. Pichereau *et al*. showed that wild type strains of *Sinorhizobium meliloti* catabolize DMS-Ac through a demethylation pathway, which is also used for GBT metabolism. During its enzymatic degradation, GBT undergoes a stepwise demethylation to N,N-dimethylglycine, sarcosine and finally glycine. However, the demethylation products of DMS-Ac, methylthioacetate (MTA) and thioglycolic acid (TGA) possess toxic properties to wild type *S. meliloti*. Thus, the high excretion rates of MTA and TGA are probably a type of detoxification mechanism of the cells **[Pichereau 1998]**. Due to the fact, that the synthetic gonyol, which was supplied to the bacteria, was a racemic mixture of the natural occurring *S*-gonyol and the corresponding *R*-isomer, the total conversion of gonyol by *R. pomeroyi* **(Manuscript E)** revealed, that the responsible enzymes in this species show no stereospecificity for this substrate. The lover overall utilization of gonyol by the other investigates species of ca. 45% allow no definite statement about the enantioselectivity in other bacteria. However, the similar utilization of DMSP, DMS-Ac and gonyol in *Sulfitobacter* sp. and *Halomonas* sp. may suggest a metabolic pathway without stereochemical preference.

DMSP and proline in biofilm formation and structuring

Regarding the multiple functions of DMSP in the marine environment, previous publications showed also that DMSP plays an important role in structuring the bacterial environment of corals by provision of favorable surroundings (nutrient supply) for certain bacteria species **[Raina 2010, Raina 2013]**. For marine macroalgae, it is crucial to control and structure their bacterial environment to prevent degradation of the algal tissue and fouling processes. This can be achieved by excretion of antibacterial metabolites **[Steinberg 1997, Nylund 2005]** or attraction of specialized bacteria species, which prevent their host from colonization of other bacteria and settlement of algae and larvae of marine invertebrates **[Armstrong 2001, Dobretsov 2013]**.

In this context, it was shown that the algal metabolite DMSP and the amino acid proline are very effective compounds against bacterial attachment on the marine brown algae *Fucus vesiculosus* **(Manuscript C)**. It is not obvious that these highly polar substances are used as active compounds on the surface of the algae. Due to the high polarity and solubility of DMSP and proline in water, these compounds are likely to diffuse from the algal surface into the surrounding seawater when released by algae cells. Therefore surface concentrations of such polar metabolites are usually much lower compared to non-polar compounds which persist on the surface of marine algae over prolonged time periods **[Jennings and Steinberg 1997]**. This is substantiated by the fact, that the

surface concentrations of proline and DMSP on *F. vesiculosus* which range between 0.09 ng/cm^2 and 1.25 ng/cm^2 **(Manuscript C)** are significantly lower than concentrations of non-polar deterrents such as fucoxanthine (700-9000 ng/cm^2) from *F. vesiculosus* **[Saha 2011]** or 1,1,3,3-tetrabromo-2-heptanone (3600 ng/cm^2) from *Bonnemaisonia hamifera* **[Nylund 2008]**. However, the very low natural surface concentrations of DMSP and proline were effective to reduce bacterial attachment even though no complete inhibition could be determined. Interestingly, bacteria which were isolated from *F. vesiculosus* (*Rheinheimera baltica* and *Shewanella baltica*) were affected to a lesser extent than species isolated from other algae or sediments. The same effect was previously examined for another deterrent, fucoxanthine, from *F. vesiculosus* **[Saha 2011]**. It could be shown that DMSP even promoted the surface attachment of *R. baltica* **(Manuscript C)**. The very low anti-attachment activity of DMSP, proline and fucoxanthine on bacteria strains which were isolated from *F. vesiculosus* indicates that these substances can play a major role in structuring the bacterial environment in the vicinity of marine macroalgae. The observed inhibition of bacterial attachment by DMSP represents a new interaction mechanism with high ecological importance. Previously, DMSP was regarded mainly as high energy nutrient, sulfur source **[Zubkov 2001]** and infochemical (chemoattractant) for marine bacteria **[Seymour 2010]**. These findings show that the influence of DMSP on bacterial biofilm formation and structuring is based on positive feedbacks through nutrient provision **[Raina 2010]** as well as negative feedbacks through bacterial deterrence **(Manuscript C)**. An inverse relationship between DMSP concentration and the abundance of epiphytic microalgae was also observed on the cordgrass *Spartina alterniflora* **[Jackson and Stuckey 2007]**. However, the authors suggest that this effect does not depend on released DMSP by *S. alterniflora*, but on abiotic factors or grazing. The formation of a bacterial biofilm is a major promoter for colonization of other macrofoulers **[Unabia and Hadfield 1999]** and further degradation processes, which result in destruction of algal tissue. Therefore, the modulation of bacterial biofilm formation and composition largely affects growth and health of marine macroalgae. In this context, the relevance of DMSP is widely unknown and requires further research.

GBT in frost tolerance of faba beans

Another interesting topic, where information about the concentration of zwitterionic osmolytes are of high interest, is the cultivation of crops. Since zwitterionic metabolites like DMSP and GBT increase enzyme and membrane stability under extreme environmental conditions, for example high salinities or very low temperatures **[Otte 2004, Ashraf and Foolad 2007]**, specific cultivation of crops with higher content of such compounds could reduce crop loss due to weather extremes.

Since the current IPCC report predicts, due to global climate change, more frequent high temperature weather conditions with occasional occurrence of extreme cold winters **[IPCC 2014]**, the cultivation of drought and frost resistant crop species will become more and more important. In this context, direct quantification methods using LC-MS system with zwitterionic ZIC®-HILIC stationary phase **(Manuscripts A, B and D)** are a useful tool to obtain information about possible stress tolerance mechanisms of plants. The ability to use isotope labeled internal standards allowed us to quantify GBT in aqueous extracts of German winter faba bean (*Vicia faba* L.) leaflet extracts without changes in instrument parameters regarding the different sample matrices **(Manuscript F)**. These measurements were used for calibration of corresponding near infrared spectroscopy (NIRS) measurements for further GBT quantification in order to identify quantitative trait loci (QTL) in *V. faba* responsible for frost tolerance. Furthermore, the simple sample preparation procedure (dilution of the extract with acetonitrile/water 9:1 and centrifugation) makes this method suitable for experiments with large numbers of individual samples, as needed for QTL analysis. Thus direct LC-MS methods for quantification of zwitterionic metabolites can also provide useful information for breeders with respect to selective cultivation of more robust crops, which could withstand possible weather extremes caused by the global climate change.

Conclusion and outlook

The recent advances in analytical instrumentation and techniques for determination of highly polar substances provide suitable methods for investigation and quantification zwitterionic substances. In this context, the coupling of liquid chromatography with highly sensitive mass spectrometry and the improvements of polar stationary phases (e.g. zwitterionic ZIC®-HILIC phases) made these methods suitable for simultaneous analysis of a variety of different zwitterionic phytoplankton metabolites with high environmental relevance. Important factors for the focus on the roles of DMSP and DMS in the marine network are, besides its ecological and environmental relevance, the simple and robust analytical methods for these compounds. These methods are based on the gas chromatographic (GC) determination of DMS and DMSP as released DMS after alkaline hydrolysis.

The application of novel LC-MS techniques, which were used in this thesis, now enables a more detailed look on the complex osmoregulation/osmoadaption processes of phytoplankton, the interactions between marine algae and bacteria and the influence of different dimethylsulfonio-metabolites on the marine sulfur cycle.

The simultaneous quantification of a wide spectrum of different zwitterionic osmolytes, aliphatic dimethylsulfonio- and trimethylammonium compounds as well as aromatic metabolites, in the cosmopolitan microalgae *E. huxleyi* and *P. minimum* allowed us to distinguish between two completely different osmoadaption strategies of these species. While the coccolithophore *E. huxleyi* responded with a significant cell size reduction to increased medium salinities, the dinoflagellate *P. minimum* maintained a constant cell size over the whole range of salinity (16‰ - 38‰), but reacted with a higher concentration of zwitterionic osmolytes to overcome osmotic stress. As reported, the osmolyte composition of *P. minimum* is highly dependent on the salinity of the growth medium. Variations in osmolyte composition may also affect the biogenic DMS release from bacterial DMSP metabolism trough interactions of other osmolytes with DMSP degrading enzymes or inhibition of DMSP uptake. However, due to the lack of information about the composition of zwitterionic osmolytes in marine phytoplankton and its alteration dependent on different environmental factors, the influence of DMS-Ac, gonyol and other known, and yet unknown, zwitterionic metabolites on the biogenic DMS release in the marine environment remain elusive. In this context, it could be shown that gonyol largely affects the release of the volatile DMSP cleavage products DMS and MeSH in *Ruegeria pomeroyi*. Furthermore, the minor influences on emission of volatile sulfur species in *Sulfitobacter* sp., *Halomonas* sp. And *Alcaligenes faecalis* indicate a high species specificity regarding such metabolic modulations mediated by small zwitterionic compounds like gonyol. These results provide a first insight into

the different interactions of phytoplankton derived zwitterionic osmolytes with the microbial DMSP metabolism. Furthermore it suggests an indirect effect of gonyol, and potentially other metabolites, on the marine sulfur cycle by modulating the release of volatile sulfurous compounds.

The direct LC-MS based analysis of zwitterionic osmolytes has not just the advantage to analyze a very broad spectrum of different metabolites simultaneously but also a more accurate quantification of DMSP by determination of its corresponding ion trace. Since commonly used, indirect, DMSP quantification methods rely on the alkaline treatment of the samples with subsequent GC analysis of the released DMS, there is no differentiation between DMS derived from DMSP or other sources, respectively possible. The comparison between direct and indirect quantification with isotope labeled internal standards showed a possible overestimation of DMSP of up to 14%, depending on the investigated phytoplankton species, growth stages and nutrient supply. However, the responsible metabolites for this non-DMSP derived DMS are still not identified and should be focus of further research. As preliminary experiments showed that the small zwitterionic compounds DMS-Ac, gonyol and gonyauline do not cleave DMS under alkaline conditions, also other compound classes like sulfocholine containing phospholipids should be taken into account as alternative DMS sources.

Furthermore, the quantification of DMSP via LC-MS techniques allowed the investigation of uptake mechanisms in marine phytoplankton. For incubation experiments with five different microalgae species, a synthetic 6-fold stable isotope labeled $^{13}C_2D_6$-DMSP was used. The deuteration of the dimethylsulfonio-terminus and the ^{13}C labeling of positions 1 and 2 of the propionic acid moiety allowed the determination of possible transformations of $^{13}C_2D_6$-DMSP during the uptake. Here, it could be shown that all investigated species were able to take up the labeled DMSP directly without previous molecular modifications. The very fast uptake of about 60% of the initial concentration of 125 nM of$^{13}C_2D_6$-DMSP within 2.5 minutes by the DMSP non-producer *Thalassiosira weissflogii* suggest the development of a highly effective DMSP uptake mechanisms in this species, which would allow *T. weissflogii* to take up the needed DMSP from the surrounding seawater. Therefore, they would not need to invest energy and nutrients for DMSP synthesis, which could be a possible evolutionary advantage of this species toward DMSP producing phytoplankton. Since also the strong DMSP producer *Emiliania huxleyi*, which should not be reliant on an external DMSP supply, took up 30% of the provided $^{13}C_2D_6$-DMSP in addition to the present intracellular DMSP, it can be stated that marine microalgae are not just the main source but can also act as a substantial sink for DMSP in marine environment.

Additionally to the widely discussed relevance of DMSP in structuring of microbial biofilms and support of bacterial growth by provision of an easily available energy and sulfur source, it could be shown that it can also inhibit the attachment of certain bacteria on the surface of the brown algae *Fucus vesiculosus*. This fact, together with the previously mentioned studies, illustrates the complexity of the marine sulfur cycle which seems in its various interactions just roughly understood.

Regarding the relevance of marine phytoplankton as major source of DMSP, future experiments should deal with the combination of LC-MS measurements for osmolyte quantification and proteomic techniques. This could provide new perspectives on osmoregulation mechanisms the involved enzymes and a possible prediction of the behavior of marine phytoplankton to changing environmental conditions. Furthermore, to enhance the numerous data about the environmental relevance of DMSP and its distribution on regional and even global scales it is of high interest to investigate the abundance of other phytoplankton derived zwitterionic osmolytes that might interfere with the biogeochemical cycles of DMSP and DMS. Due to the just recently upcoming direct LC-MS based quantification methods for DMSP and other zwitterionic osmolytes, there is a high potential for investigation of new zwitterionic substances and their roles in the marine environment. Contemporary information of the relevance of DMSP and structurally related substances seem to be just the tip of the iceberg since many of these compounds have been overlooked in many studies over the last decades. Last but not least, the novel LC-MS based methods for direct quantification of zwitterionic metabolites provide valuable information about their contribution to stress resistance mechanisms in economic plants, which may be a vital topic with regard to manmade global climate change.

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Conference contributions

- ASLO Aquatic Sciences Meeting 2011, Feb 13-18, 2011, San Juan, Puerto Rico
  Poster: "Dimethylsulfoniopropionate (DMSP): How to unravel roles of a multifunctional molecule"

- 2nd International Student Conference on Microbial Communication (MiCom 2011), Sep 13-16, 2011, Jena, Germany
  Talk: "That extra something – Studies of DMSP uptake mechanism with isotope labeled compounds"

- ASLO Summer Meeting 2012, Jul 8-13, 2012, Lake Biwa, Otsu, Shiga, Japan
  Talk: "New insights in osmoregulative function of DMSP and other zwitterionic substances"

Curriculum vitae

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11/2010-11/2013	Stipend from the International Max Planck Research School for Global Biogeochemical cycles (IMPRS gBGC), Jena

01/2013 - 02/2013	Research stay in the group of Prof. David J. Kieber at State University of New York – College for Environmental Science and Forestry (SUNY-ESF), Syracuse NY; works about DMSP oxidation in microalgae
05/2013 – 06/2013	Research stay in the group of Dr. Michael Steinke at the University of Essex, School of Biological Sciences, Colchester, UK; works about the degradation and interactions of DMSP, DMS-Ac and gonyol in marine bacteria
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Declarations

Selbstständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbstständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

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Einverständniserklärung des Betreuers

Ich bin mit der Abfassung der Dissertation als publikationsbasiert, d.h. kumulativ, einverstanden und bestätige die vorstehenden Angaben. Eine entsprechend begründete Befürwortung mit Angabe des wissenschaftlichen Anteils des Doktoranden/der Doktorandin an den verwendeten Publikationen werde ich parallel an den Rat der Fakultät der Chemisch-Geowissenschaftlichen Fakultät richten.

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Prof. Dr. Georg Pohnert

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