Osmoprotective role of dimethylsulfoniopropionate (DMSP) for estuarine bacterioplankton

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ABSTRACT: Dimethylsulfoniopropionate (DMSP) is synthesized and used by marine phytoplankton as an osmolyte. Previous studies have shown that some of the dissolved DMSP (DMSPd) in seawater is taken up by bacterioplankton and not degraded. We tested the hypothesis that retention of DMSP provides some benefits to marine bacteria. In experiments with coastal seawater filtrates containing mainly bacteria, acute osmotic stresses of +5 and +10 ppt NaCl significantly inhibited bacterial production (BP) over 6 h, while the availability of 20 nM DMSPd relieved most of the BP inhibition. Partial relief of salt-induced inhibition of BP was observed with DMSPd concentrations as low as 2.5 nM, and DMSP was more effective at relieving osmotic stress than other compounds tested. Osmotic stresses resulted in a faster and greater overall uptake of DMSPd and accumulation of untransformed DMSP in bacterial cells (DMSP$_{cell}$). Retained DMSP reached osmotically-significant intracellular concentrations of 54 mM in salt-stressed bacterial populations. Retention of DMSP was accompanied by a lower production of methanethiol (MeSH), suggesting a down-regulation of the demethylation/demethiolation pathway under osmotic stress. These results show that estuarine bacterioplankton can use DMSP as an osmoprotectant, retaining up to 54% of the available dissolved DMSP untransformed in their cells. This benefit provided by DMSP may help explain why some DMSP is retained in bacteria in the ocean, even under unchanging salinity. This retention slows down the cycling of DMSP, with potential implications for the trophic transfer of DMSP and its contributions to sulfur and carbon fluxes in the ocean.

KEY WORDS: Osmolyte · Compatible solute · Salinity stress · Salt inhibition · Osmolarity · Sulfur cycle · DMSP retention · DMSP function

INTRODUCTION

Dimethylsulfoniopropionate (DMSP) is a tertiary sulfonium compound synthesized by many species of marine phytoplankton and some macroalgae. DMSP is an important component of the sulfur and carbon cycles within the microbial food web, and also has several ecophysiological functions within the microbial community. Produced at intracellular concentrations typically ranging from 1 to 400 mM (Keller et al. 1989, Stefels 2000), DMSP synthesis contributes up to 10% of all carbon fixed by primary production

(Matrai & Keller 1994, Archer et al. 2001, Howard et al. 2006). While the evolutionary drivers leading to the allocation of such a large fraction of the algae's resources into a single compound are still unclear, it is well known that several micro- and macroalgae use it as a compatible solute to cope with osmotic and thermal stresses (Dickson et al. 1980, Reed 1983, Dickson & Kirst 1986, 1987, Kirst et al. 1991, Karsten et al. 1992, Nishiguchi & Somero 1992). Additional functions for DMSP include protection against oxidative stress, with DMSP and its degradation products effectively scavenging reactive oxygen species

(Sunda et al. 2002), and overflow metabolism, allowing photosynthetic cells to dissipate excess reduced compounds and energy under nutrient limitation or excessive carbon fixation rates, preventing potential negative feedbacks on metabolic pathways (Stefels 2000). A few ecological functions have also been proposed for DMSP, such as being a grazing deterrent (Wolfe et al. 1997, Van Alstyne et al. 2001, Strom et al. 2003) and chemoattractant for marine bacteria (Zimmer-Faust et al. 1996, Miller et al. 2004, Seymour et al. 2010).

Most of the phytoplankton-DMSP loss (63 to 91%) is mediated by microzooplankton grazing (Archer et al. 2002, Simó et al. 2002). Ingested DMSP can either be released as dissolved DMSP or as dimethylsulfide (DMS) upon conversion by bacterial or algal DMSP lyases, or it can be retained and either passed up the food chain or assimilated to satisfy sulfur requirements. About 30% of the phytoplankton-DMSP ingested by herbivorous protists is retained in their cells (Simó et al. 2002, Tang & Simó 2003, Saló et al. 2009). This retention seems to be temporary, and DMSP-sulfur is eventually assimilated into the grazer's biomass (Belviso et al. 1990, Saló et al. 2009). However, some DMSP can be transferred to higher trophic levels, presumably when DMSP-bearing protists are eaten before completing DMSP transformation. DMSP has been measured in several omnivorous and carnivorous copepod species after gut clearance, suggesting that this DMSP was located in body tissues (Tang et al. 1999), making it more likely to be transferred to higher trophic levels. DMSP has also been found in tissues of several other animals (bivalves, gastropods, crustaceans and fish) that most likely acquired it from ingested food (Tokunaga et al. 1977, Iida & Tokunaga 1986, Dacey et al. 1994, Hill et al. 2000). The fact that DMSP has even been found in carnivorous fishes such as cod (Levasseur et al. 1994), salmon (Motohiro 1962) and mackerel (Ackman et al. 1972) further indicates that DMSP can be transferred through more than one trophic level.

When released to the extracellular environment, DMSP is rapidly taken up and degraded by heterotrophic prokaryotes for energy and biomass, using one of 2 alternative pathways. The demethylation/demethiolation pathway ultimately produces methanethiol (MeSH; Kiene & Taylor 1988), some of which is rapidly incorporated into macromolecules (González et al. 1999, Kiene et al. 1999). This pathway is widespread amongst bacterioplankton in the surface ocean (Reisch et al. 2011), highlighting the importance of DMSP degradation for assimilation of reduced carbon and sulfur by marine heterotrophs.

Moreover, biogeochemical data show that DMSP can satisfy as much as 15 and 100% of the bacterial requirements for carbon and sulfur, respectively (Kiene & Linn 2000a, Zubkov et al. 2001, Simó et al. 2002). The second pathway is mediated by DMSP lyases, which cleave DMSP to DMS and acrylate (Yoch 2002, Todd et al. 2007). DMS emissions from the ocean contribute between 0.55 and 1.1 Tmol sulfur yr^{-1} to the global atmosphere (Lana et al. 2011) and are hypothesized to have a cooling effect on the climate because DMS is oxidized to acidic products that either form or add on to aerosol particles (Charlson et al. 1987, Quinn & Bates 2011). DMS ventilation across the sea–air boundary is also a major mechanism for transferring sulfur from the oceans to the continents through the atmosphere (Lovelock et al. 1972).

Adding to these well-documented functions, there is growing evidence that DMSP can serve as an osmolyte for bacteria. To maintain or restore their osmotic balance under high or changing salinity, marine prokaryotes need to accumulate intracellular compatible solutes, which they can either synthesize de novo or take up from their environment (Galinski 1995). Glycine betaine (GBT), a structural analogue of DMSP, is well recognized as one of the most efficient and widespread osmolytes used by animals, plants and bacteria (Yancey et al. 1982 and references therein, Larsen et al. 1987, Anthoni et al. 1991 and references therein), including natural bacterial populations (Kiene & Hoffmann Williams 1998). Most marine bacteria can take up GBT and DMSP from the environment at nanomolar concentrations, using the same high-affinity transport system (Kiene et al. 1998). Previous studies focusing on prokaryotes showed that under controlled conditions, availability of DMSP promotes growth in hypersaline medium for single strain cultures of *Escherichia coli* (Chambers et al. 1987, Cosquer et al. 1999) and some marine bacteria isolates (Diaz et al. 1992, Wolfe 1996). However, whether whole assemblages of marine bacterioplankton can take up and retain DMSP to cope with osmotic stress in natural systems has not been shown. To gain osmoprotection from DMSP, the marine bacterioplankton would have to retain the DMSP untransformed in their cells for at least a few hours, preventing or at least delaying its degradation through the normal pathways. In fact, during ^{35}S-DMSPd uptake experiments with marine bacterioplankton from the Gulf of Mexico, 2.2% of the added DMSP was found untransformed in cells 12 h after the tracer addition (Kiene & Linn 2000a). In

similar experiments with seawater collected near Halifax, Nova Scotia, Kiene & Linn (2000b) found 15% of added DMSP untransformed in particles after 23 h. In the Mediterranean Sea, Belviso et al. (1993) measured 9.5% of particulate DMSP+DMS in the 0.2 to 0.7 μm size fraction (corresponding to 3.6 nM DMSP+DMS). However, at the present time it is not clear whether retention of DMSP in bacterial cells provides any benefit to those cells.

Previous studies demonstrating the uptake and use of DMSP as an osmolyte by heterotrophic bacteria were conducted under somewhat artificial conditions, using monospecies cultures and high levels of DMSP (≥100 nM). In the present study, natural bacterial assemblages from coastal waters were tested for their ability to take up and retain DMSP untransformed in their cells under induced salinity stress. Bacterial production was measured to examine whether the retention of DMSP enabled the cells to alleviate the salt-induced inhibition and restore normal cell functioning. To our knowledge, our study is the first to test whether natural bacterial communities are able to use DMSP for osmoprotection purposes at the low nanomolar levels typically found in natural marine systems.

MATERIALS AND METHODS

Sample collection, preparation and salinity stress experiments

Surface water samples were collected from the Dauphin Island Sea Lab (DISL) pier in Mobile Bay (Gulf of Mexico) from February to August 2014. Salinity at the DISL pier over the sampling period was highly variable, and sampled water ranged from 10 to 25 parts per thousand (ppt) for the different experiments. Samples were collected using an acid-cleaned bucket and immediately brought back to the laboratory. For most experiments, seawater samples were gravity-filtered through 142 mm diameter Whatman GF/F filters using a polycarbonate in-line filter holder (Geotech). The GF/F filter removed nearly all of the phytoplankton community, but allowed passage of some bacteria (typically 50 to 80%; Lee et al. 1995). The filtrate was kept in the dark at room temperature for 1 to 4 d to allow for depletion of the bioavailable dissolved DMSP (DMSPd) to <1 nM by microbial consumption (see Li et al. 2015). Removal of DMSP-containing phytoplankton and endogenous DMSPd was necessary to avoid confounding effects of phyto-

plankton and grazer responses in our osmotic stress experiments. Upon depletion of endogenous labile DMSPd in the filtrate incubation, osmotic stress was induced by adding a 240 ppt NaCl solution to the samples, to reach +5 and +10 ppt increases from the ambient salinity. Addition of 5 and 10 ppt represents an increase in NaCl concentrations of 85.5 and 171 mM, which increased the osmolarity of the seawater samples by 171 and 342 mOsm l^{-1}, respectively. Salinity in the different treatments was measured using a hand-held refractometer. Different levels of DMSP (Research Plus), ranging from 0.5 to 20 nM (final concentration), were added within 1 h following the NaCl addition. In one experiment comparing the effect of different organic solutes on bacterial production under ambient and +10 ppt salinity, DMSP and other compounds (GBT, glutamate, trehalose, acrylate, glucose, valine) were each added to a final concentration of 20 nM to separate samples, within 1 h following the NaCl addition.

Bacterial production measurements

The rate of leucine incorporation into proteins, hereafter referred to as bacterial production (BP), was measured with the ^{3}H-leucine incorporation method (Kirchman et al. 1985) using the microcentrifuge modification described by Smith & Azam (1992). Quadruplicate 1.5 ml sub-samples (3 live samples and 1 killed control) were pipetted into acid-cleaned 2 ml microcentrifuge tubes (Axygen SCS-020X) containing ^{3}H-leucine (Perkin-Elmer, 144 Ci mmol^{-1}) added to a rate-saturating final concentration of 20 nM. The killed control tube also contained 75 μl 100% trichloroacetic acid (TCA), used to stop biological activity and account for abiotic residual radioactivity. Samples were then incubated at room temperature for 60 min, after which 75 μl of 100% TCA was added to the live samples and mixed to stop the incubation. The tubes were centrifuged at 13 000 × g for 15 min, and the supernatant was poured out. Each tube then received 1.5 ml 5% TCA and was centrifuged again for 5 min. TCA was poured out of each tube, and 1.5 ml 80% ethanol was added prior to centrifugation for 5 min. After removing the ethanol, the tubes were set to air-dry in a fume hood for several hours. The tubes then received 1.5 ml of Ecolume scintillation cocktail and were stored in the dark for at least 24 h. The ^{3}H-radioactivity in the samples was counted using a liquid scintillation counter (Tri-Carb 3110TR, Perkin Elmer).

Concentration of DMSP in the filtrate and in the cells

DMSP remaining dissolved in the water sample ($DMSP_{<0.2 \, \mu m}$) and accumulated into the bacterial cells ($DMSP_{cell}$) were measured by filtering duplicate 15 ml sub-samples from each treatment bottle through 0.2 µm Nylon filters (Magna) using a Hoefer 10-place filtration manifold. During filtration, particular attention was given to prevent the filters from drying, minimizing the release of DMSP from the cells. Tests showed that little or no DMSP loss occurs from bacterial cells filtered onto Nylon filters, which is consistent with previous results with other substrates taken up by marine bacteria (Kiene & Linn 1999). Each filter was transferred to a 14 ml serum vial containing 5 ml 3% HCl to preserve DMSP, and the serum vials were sealed with a Teflon-faced butyl rubber stopper. Filtrate was collected directly into a 50 ml centrifuge tube containing 450 µl of 50% HCl. Filters and filtrate samples were stored at room temperature until analysis for DMSP by gas chromatography (GC).

GC analysis

All DMSP samples were analyzed using a gas chromatograph (Shimadzu GC-2014 or GC-14A) with a Chromosil 330 column and a flame photometric detector (FPD) coupled with a purge and trap system (Kiene & Service 1991). Before analysis, DMSPd samples were hydrolyzed to DMS by pipetting a 5 ml sub-sample into a 14 ml serum vial and adding 1.7 ml of 5 M NaOH. The vials were immediately sealed with a Teflon-faced butyl rubber stopper. For $DMSP_{cell}$ samples, 2 ml of 5 M NaOH was added to the serum vials containing the filters. After vortexing the vials and waiting for 60 min to allow completion of the hydrolysis reaction, the vials were connected to the purge and trap GC system and sparged with ultrahigh purity (UHP) helium (90 ml min^{-1}) for 5 min before DMS was quantified by GC.

DMSP intracellular concentration

Intracellular DMSP concentrations were estimated from the amount of DMSP in bacteria-sized particles (in nmol DMSP per liter of sample) and the number of bacterial cells per liter of sample as determined by flow cytometry. The biovolume of each cell was assumed to be 0.07 µm^3 (Lee & Fuhrman 1987) and we further assumed that all cells contained DMSP.

Flow cytometry

For bacterial counts, duplicate 1.8 ml sub-samples were pipetted into sterile 2 ml cryovials containing 26 µl of 35% electron microscopy (EM)-grade glutaraldehyde to a 0.5% final concentration. Samples were mixed well, flash frozen and stored at −80°C until analysis. Bacterial cell number analysis was performed on a FACSCalibur flow cytometer (BD Biosciences) according to the method described by Marie et al. (1999) and Brussaard (2004), and data were analyzed using the BD CellQuest Pro software. Prior to analysis, samples were melted in a 37°C water bath, diluted 10-fold in sterile 0.2 µm filtered TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) to avoid coincidence and stained with SYBR Green I. Yellow-green 1 µm beads were added in all samples as an internal standard.

^{35}S-DMSPd tracer incubations

Retention of the radioisotopic tracer ^{35}S-DMSP in cells, and production of ^{35}S-DMS and ^{35}S-MeSH were measured following the protocols of Kiene & Linn (2000b) and Slezak et al. (2007). For the retention experiment with whole seawater, a 50 ml centrifuge tube was filled with unfiltered estuarine (15 ppt salinity) water, spiked with ^{35}S-DMSPd to a final concentration of 1000 disintegrations per minute (dpm) ml^{-1} and incubated in the dark at room temperature. At several points during the 6 d incubation, duplicate 5 ml sub-samples were filtered through 0.2 µm Nylon filters that were rinsed with 3 × 1 ml of 0.2 µm-filtered seawater of the same salinity. All filters were transferred to 20 ml glass scintillation vials and processed as described below (in this section). For the DMSP and NaCl amendment experiment, four 250 ml polycarbonate bottles (2 treatments in duplicate) were filled with GF/F-filtered seawater that had been kept in the dark at room temperature for 36 h after filtration to allow bacteria to deplete endogenous DMSPd to <1 nM. NaCl was first added to 2 bottles to reach +10 ppt above ambient salinity, and then unlabeled DMSP was added to all bottles to a final concentration of 20 nM. ^{35}S-DMSPd was added 5 min later, to a final concentration of 10 000 dpm ml^{-1} to each treatment bottle. The ^{35}S-DMSP addition was at tracer levels (5.2 pM). After mixing gently, a 0.5 ml sub-sample from each bottle was transferred into a 7 ml plastic scintillation vial containing 4 ml Ecolume scintillation cocktail to count total added ^{35}S activity. At several points during the 3 h incubation, duplicate

5 ml sub-samples from each replicate bottle were filtered through 0.2 μm Nylon filters. The filtrates were collected directly into 50 ml polypropylene centrifuge tubes and removed before each filter was rinsed with 3 × 1 ml of 0.2 μm-filtered seawater (at ambient and +10 ppt salinity). All filters were transferred to 20 ml glass scintillation vials. At each time point, one of the duplicate filters from each replicate bottle was immediately covered with 8 ml of Ecolume scintillation cocktail to determine total ^{35}S taken up into the bacterial cells. The other replicate filter was treated with 0.5 ml of 1 M NaOH in the scintillation vial and placed on an orbital shaker at 100 rpm for at least 12 h to allow conversion of ^{35}S-DMSP to volatile ^{35}S-DMS, which was lost from the open vial. The 1 M NaOH addition was sufficient to cleave all DMSP in the samples and did not interfere with scintillation counting, whereas higher concentrations of NaOH caused interferences. These vials then received 8 ml of Ecolume scintillation cocktail, after which all vials were left to stabilize for at least 24 h in the dark before counting on a liquid scintillation counter. The difference in ^{35}S activity between filters treated with NaOH and those left untreated represented the untransformed ^{35}S-DMSPd in the cells.

For net DMS yield, at selected time points over a period of ~3 h, 5 ml sub-samples of the ^{35}S-DMSP-treated seawater filtrate were removed from the incubation bottles and transferred into 70 ml serum vials containing 0.1 ml of 10% sodium dodecyl sulfate (SDS), 0.5 ml of 100 μM unlabeled DMSP, and 0.05 ml of 5,5-dithiobis-2-nitrobenzoic acid (DTNB or Ellman's Reagent, 1 mg ml^{-1} of 50 mM TRIS-HCl, pH 8.0). The SDS and unlabeled DMSP were used to stop biological activity and further reactions of ^{35}S-DMSP, and DTNB was added to complex MeSH, a potential volatile product of DMSP degradation. DTNB does not react with DMS. To measure the total net volatile yield (i.e. DMS+MeSH), a second series of 5 ml sub-samples was pipetted into 70 ml serum vials containing SDS and unlabeled DMSP, but no DTNB. The vials were then sealed with a stopper fitted with a plastic cup holding a fluted wick soaked with 0.2 ml 3% H$_2$O$_2$ to trap the ^{35}S-volatiles. The vials were placed on an orbital shaker at 100 rpm for at least 6 h. The wick was then placed in a scintillation vial containing 4 ml of Ecolume scintillation cocktail, and counted. The difference between the total net volatile yield and the net DMS yield is hereafter referred to as the MeSH yield. At this time, a new stopper with plastic cup and H$_2$O$_2$-soaked wick was used to seal the vial, and 0.5 ml 5 M NaOH was injected through the stopper to convert the remain-

ing ^{35}S-DMSP in the sample to ^{35}S-DMS. After 6 h of shaking, the wick was placed in a scintillation vial with Ecolume scintillation cocktail, and counted for the remaining ^{35}S-DMSP as trapped DMS. The DMS and volatile yields were calculated as the amount of ^{35}S-DMS and ^{35}S-volatiles produced divided by the amount of ^{35}S-DMSP consumed (expressed as a percentage).

Statistical analysis

Student's *t*-tests, ANOVAs and Holm-Sidak pairwise comparison tests were used to explore the effect of different salinity, DMSP and other organic solutes amendments on BP, retention of DMSP in cells and fate of consumed ^{35}S-DMSP. All statistical tests were performed using SigmaPlot (Systat Software).

RESULTS

Retention of DMSP by marine bacterioplankton under ambient conditions

When a tracer level of dissolved ^{35}S-DMSP was added to an unfiltered estuarine (15 ppt salinity) water sample, ^{35}S rapidly accumulated in microbial cells (filterable particles >0.2 μm) with the total uptake reaching a maximum of about 48% of the added radiolabel after 0.8 d of incubation (Fig. 1). A significant fraction (19%) of the maximum total uptake, equivalent to about 9% of the added ^{35}S-DMSP, was retained untransformed in the cells for at least 2 d. Even after 6 d of incubation, there was still 2.4% of the added ^{35}S-DMSP remaining untransformed in the cells. These results indicate that a portion of the natural dissolved DMSP in seawater is taken up and retained in microbial cells for long periods, even when salinity is stable. To address the question of whether DMSP retention might have some benefits for heterotrophic bacteria, we carried out experiments with seawater filtrates in which acute salinity stress was imposed.

Effect of salinity stress and DMSP availability on bacterial production

In a 2 d pre-incubated seawater filtrate containing bacteria and very low dissolved DMSP concentrations (<1 nM), increasing the salinity by 10 ppt had a rapid inhibitory effect on BP, decreasing it by 43%

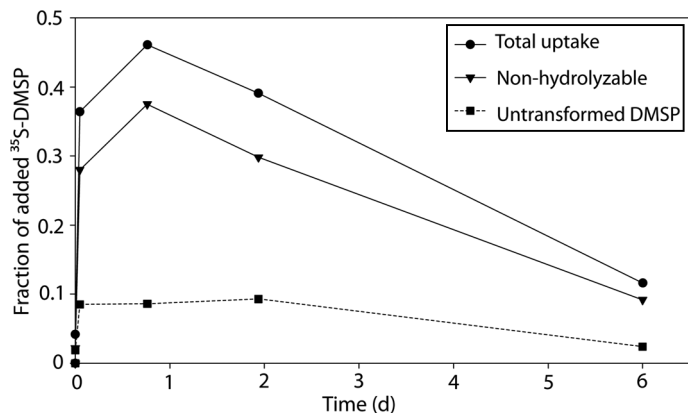Fig. 1. Uptake of tracer-level dimethylsulfoniopropionate (^{35}S-DMSP) into particles >0.2 µm in unfiltered estuarine water from Mobile Bay (15 ppt salinity). Total uptake represents the ^{35}S-activity in particles retained on 0.2 µm Nylon filters. Parallel filters at each time point were treated with 1 M NaOH, and the non-hydrolyzable fraction represents the remaining ^{35}S activity on the filters after alkaline treatment (non-DMSP ^{35}S). The difference between total uptake and non-hydrolyzable ^{35}S represents untransformed DMSP in particles >0.2 µm

after 2 h when compared to the ambient salinity control treatment (ANOVA, p < 0.005; Fig. 2A). At 6 h after NaCl addition, BP in both the +5 and +10 ppt treatments was inhibited by 36 and 34%, respectively (ANOVA, p < 0.005; Fig. 2B). Addition of 20 nM DMSP, within 1 h following NaCl addition, relieved most of the inhibition of BP caused by 10 ppt salinity stress (BP was 55% higher with DMSP than without, p < 0.01), decreasing this inhibition to −11% of the control after 2 h. After 6 h in the +5 and +10 ppt salt treatments, samples with 20 nM DMSP had higher BP than those without added DMSP, but the effect was statistically significant only at +5 ppt (t-test, p < 0.01; p = 0.069 for +10 ppt), decreasing the BP inhibition to −9% of the control.

To explore the effectiveness of DMSP against osmotic stress, we compared it to other recognized osmolytes typically used by marine bacteria (GBT, glutamate, trehalose). We also tested some organics that are not generally considered osmolytes but can readily be used as a carbon source by marine bacteria (glucose, acrylate, valine) to confirm that the increase in bacterial production was due to the salinity stress relief rather than a direct stimulation from the addition of substrate. BP was measured 1 and 4 h after the different solutes were added at a final concentration of 20 nM under ambient and +10 ppt salinity (Fig. 3). For all solutes, addition of 10 ppt NaCl effectively inhibited BP (significantly lower than ambient salinity; t-test, p < 0.01). After 1 h, DMSP provided better osmoprotection than any of the other solutes tested. GBT also conferred some osmoprotection, but to a lesser extent than DMSP. After 4 h, BP for the DMSP addition was significantly higher in the +10 ppt treatment than in the control (t-test, p < 0.01; Fig. 3B). The GBT treatment almost completely relieved the inhibition, whereas BP for all the other additions was still significantly lower at high salinity compared to the ambient salinity treatment (t-test, p < 0.005).

Uptake and retention of DMSP under osmotic stress

To confirm that bacteria were taking up DMSP and accumulating it untransformed in their cells, the concentrations of DMSP remaining dissolved in the filtrate (i.e. DMSP$_{<0.2 \text{ µm}}$) and accumulated in the cells (i.e. DMSP$_{cell}$) after a 20 nM DMSP addition were measured over time. Higher salinity treatments resulted in faster consumption of dissolved DMSP by bacteria (Fig. 4A), with 3.53 and 2.22 nM DMSP$_{<0.2 \text{ µm}}$ remaining after 6 h for the +5 and +10 ppt treatments, respectively, compared to 6.88 nM in the control (ANOVA; p < 0.01). Bacteria also retained more DMSP untransformed in their cells when salinity was increased, and more at +10 ppt salinity than at +5 ppt
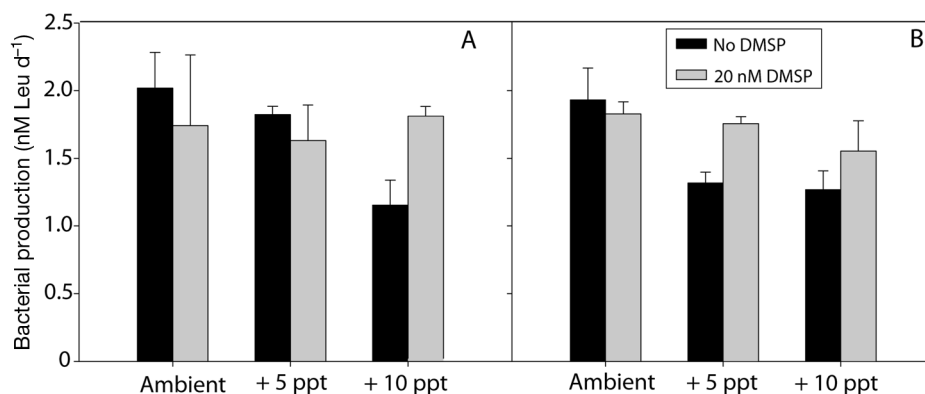

Fig. 2. Bacterial production rate at ambient salinity (10 ppt), +5 and +10 ppt salinity in a GF/F filtrate incubation without dimethylsulfoniopropionate (DMSP) and with 20 nM DMSP added (A) 2 h and (B) 6 h after salt and DMSP additions. Error bars represent SD
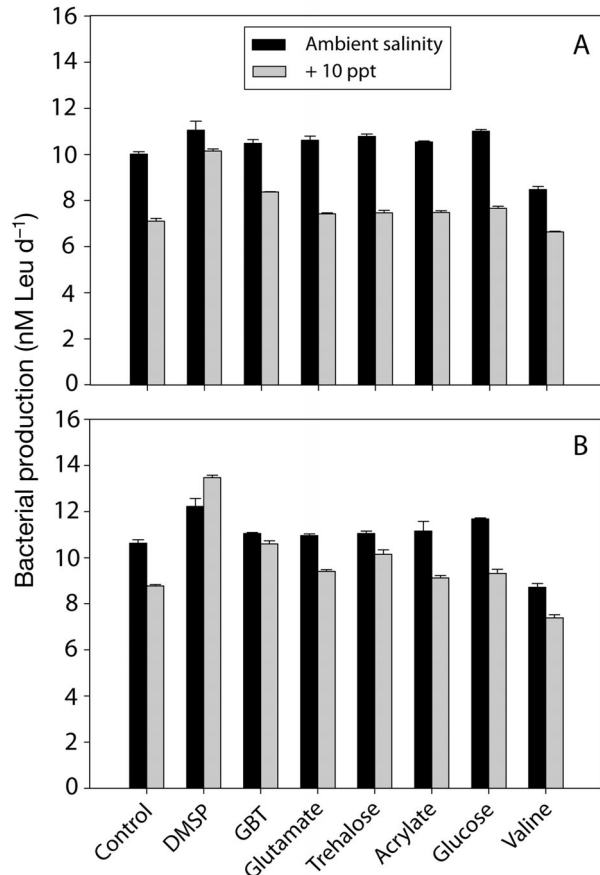
Fig. 3. Bacterial production rate at ambient (25 ppt) and +10 ppt salinity in a GF/F filtrate incubation without (control) and with a 20 nM addition of different organic solutes at (A) 1 h and (B) 4 h after salinity change. Error bars represent SD

Fig. 4. Effect of +5 and +10 ppt salinity treatments on the time course of (A) dissolved dimethylsulfoniopropionate ($DMSP_{<0.2\,\mu m}$) concentration remaining and (B) untransformed DMSP in cells ($DMSP_{cell}$) after a 20 nM DMSPd addition to a 10 ppt (ambient salinity) GF/F filtrate incubation. Error bars represent SD

(Fig. 4B). The amount of $DMSP_{cell}$ (expressed in nM dissolved equivalents) after 6 h was 1.13 nM for the ambient salinity control, and increased to 3.19 and 4.56 nM for the +5 and +10 ppt treatments (ANOVA, p < 0.001; Fig. 4B). While the DMSP levels in the cells decreased somewhat after 6 h, the enhanced retention of DMSP in cells caused by the elevated salinity treatments was largely maintained through at least 24 h (Fig. 4B).

To assess whether DMSP is accumulated in the cells at a concentration high enough to act as an osmolyte, we estimated the intracellular concentration using cell abundance after 6 h in each treatment, and assuming a cell volume of 0.07 μm^3. Estimated intracellular concentrations were 12.9, 37.9 and 54.3 mM for the ambient, +5 and +10 ppt treatments, respectively (Table 1). These concentrations are osmotically significant and could partially offset the increase in extracellu-
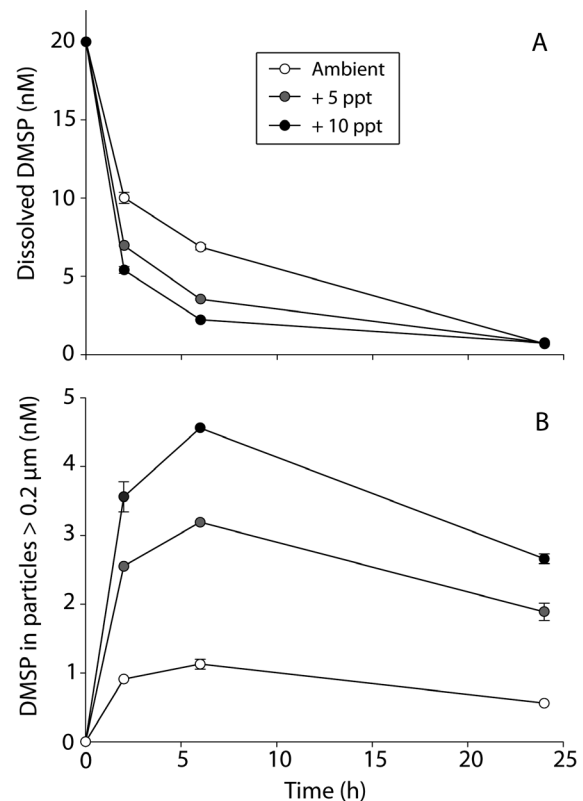
lar osmolarity. For example, the increase in the +5 ppt treatment over the ambient salinity samples was 25 mM DMSP, which was 15% of the osmolarity contributed by the +5 ppt NaCl treatment. The increase for the +10 ppt treatment was 41.1 mM DMSP, which was 12% of the osmolarity of the 10 ppt NaCl addition. These estimates assume that all cells were taking up DMSP. If only a fraction of the cells accumulated DMSP, the intracellular DMSP concentration in

Table 1. Estimates of the intracellular dimethylsulfoniopropionate (DMSP) concentration in bacteria in GF/F filtrate incubations after adding 20 nM of dissolved DMSP under ambient (15 ppt), +5 and +10 ppt salinity. Cell volume was assumed to be 0.07 μm^3

	Salinity treatment		
	Ambient	+5 ppt	+10 ppt
DMSP in bacteria-sized particles (nM)	1.13	3.19	4.56
Heterotrophic abundance (cells l^{-1} × 10^9)	1.26	1.20	1.20
Intracellular [DMSP] (mM)	12.9	37.9	54.3

those cells would be higher, and the accumulation might have offset a larger fraction of the imposed osmotic stress.

In a different experiment, several concentrations of DMSPd were supplied to a natural bacterial community to assess the ambient level required to provide osmoprotection against a 10 ppt NaCl osmotic stress. Without added DMSP, the 10 ppt NaCl addition caused a 40% inhibition of BP (2-way ANOVA; p < 0.001; Fig. 5). For a DMSPd addition of 0.5 nM, BP was still significantly inhibited by +10 ppt NaCl (2-way ANOVA; p < 0.001), whereas for additions of 2.5 nM and above, the decrease in BP due to salt amendment was not significant, indicating that DMSP at and above 2.5 nM concentrations were sufficient to provide bacteria with effective protection against this sudden salinity stress (Fig. 5). The relief of BP inhibition was also progressively stronger as more DMSP became available. $DMSP_{cell}$ increased with the concentration of DMSP added ($r^2 = 0.999$ and 0.996 at ambient and +10 ppt salinity, respectively, p < 0.001; Fig. 6), and there was greater retention of DMSP in the cells in the +10 ppt treatment (2-way ANOVA; p < 0.001).

Fate of ^{35}S-DMSPd under osmotic stress

To test whether bacteria under salinity stress process DMSP differently, we used ^{35}S-DMSP to follow the fate of a 20 nM unlabeled DMSP addition and the net production of DMS and MeSH. The results show an increased retention of DMSP in the +10 ppt salinity treatment (48% higher than the control 3 h after
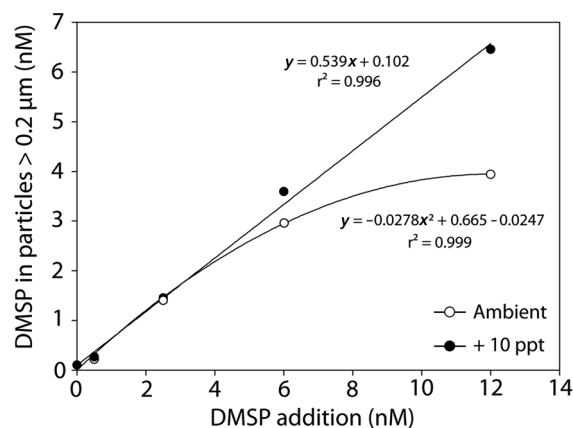
Fig. 6. Concentration of untransformed dimethylsulfoniopropionate in bacterial cells ($DMSP_{cell}$; nM equivalent) at ambient and +10 ppt salinity in a GF/F filtrate incubation amended with different levels of DMSP, 1.5 h after increasing salinity. The GF/F filtrate was prepared from coastal seawater from Mobile Bay, with an ambient salinity of 11 ppt

salt addition; t-test, p < 0.01; Fig. 7A), as well as a lower net production of MeSH at higher salinity (35% lower than the control; t-test, p < 0.05; Fig. 7C). There was no difference in net production of DMS from DMSP between the 2 treatments when expressed as a fraction of the DMSP consumed from the dissolved pool (Fig. 7B). These results suggest a lower overall degradation of DMSP by the demethylation/ demethiolation pathway caused by an enhanced retention in the cells under salinity stress.

DISCUSSION

Natural marine microbes take up dissolved DMSP and retain some of it untransformed in their cells (Belviso et al. 1993, Kiene & Linn 2000a,b, Vila-Costa et al. 2014). Our results showed that 9% of the ^{35}S-DMSP added as a tracer to an unfiltered, estuarine water sample (15 ppt natural salinity) was found untransformed in the cells after 2 d of incubation in the dark. Even though this retained fraction decreased with time, it persisted for several more days (2.4% of ^{35}S-DMSP was still untransformed after 6 d; Fig. 1). This suggests that DMSP retention could provide some benefit to bacteria. We investigated this possibility by testing whether DMSP retention could relieve stress imposed by a rapid increase in salinity. Using seawater GF/F filtrates that contained natural bacterioplankton assemblages but no phytoplankton, and which had been pre-incubated to allow endogenous dissolved DMSP to be consumed to <1 nM, we
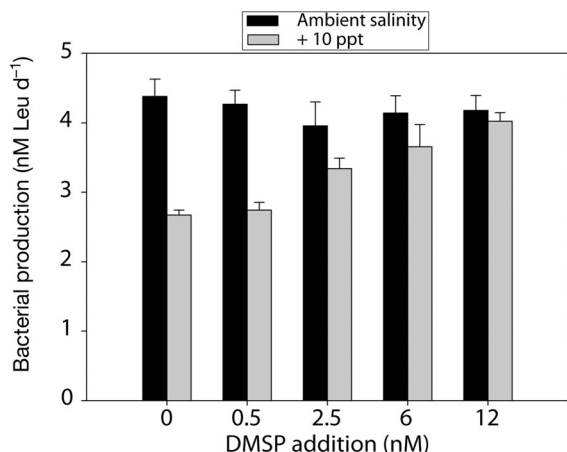

Fig. 5. Bacterial production rate at ambient salinity (11 ppt) and +10 ppt salinity in a GF/F filtrate incubation with increasing levels of dimethylsulfoniopropionate (DMSP) added 1.5 h after salt addition. Error bars represent SD
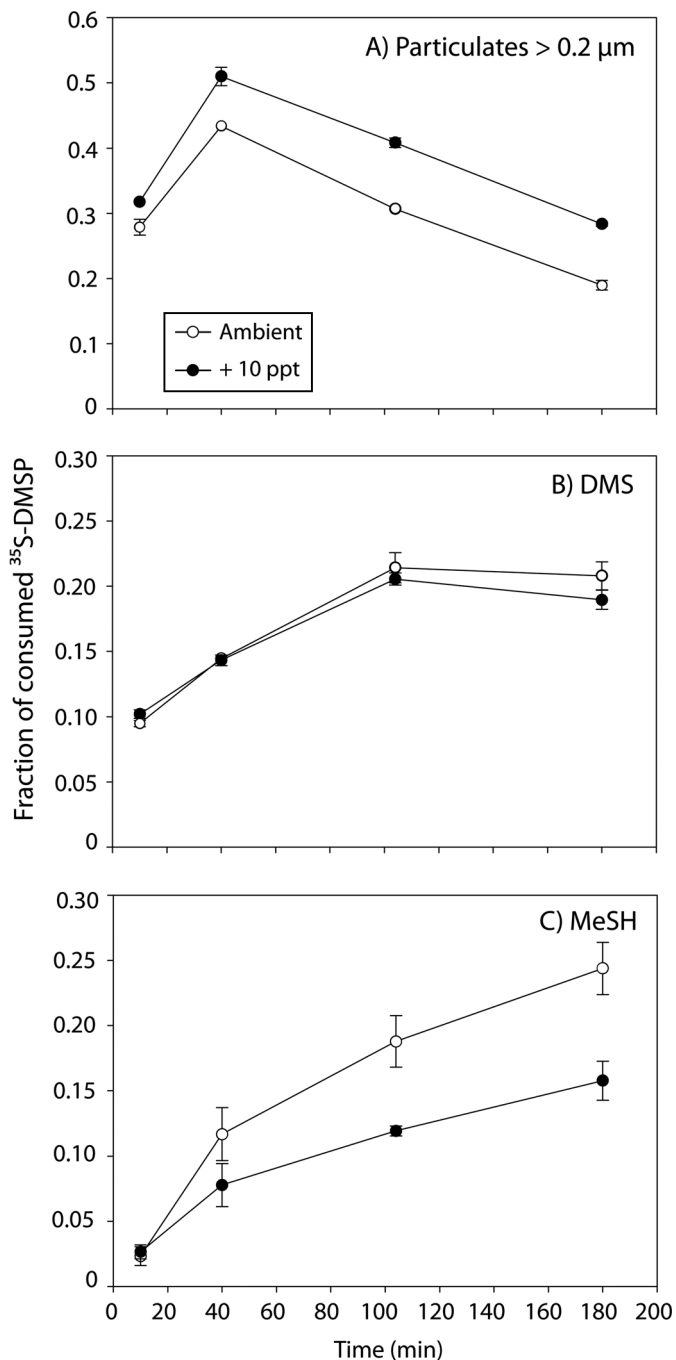
Fig. 7. The fate of 20 nM dissolved dimethylsulfonio-propionate (DMSPd) (traced with ^{35}S-DMSP) in bacteria-containing seawater filtrates incubated at either ambient salinity (25 ppt) or with +10 ppt NaCl. (A) Fraction of consumed ^{35}S retained as untransformed DMSP in particles >0.2 μm; (B) fraction of consumed DMSP converted to dimethylsulfide (DMS); (C) fraction of consumed DMSP converted to methanethiol (MeSH). Error bars represent SD. The seawater filtrate was pre-incubated to allow endogenous DMSP to be consumed to low levels (<1 nM). The time course started with the addition of NaCl, 20 nM DMSP, and a spike of tracer-level ^{35}S-DMSP

observed a marked inhibition of BP (protein synthesis as measured by ^{3}H-leucine incorporation) after imposing a sudden salinity stress of +5 and +10 ppt compared to ambient salinity (Fig. 2). This osmotic stress was mostly relieved when nanomolar concentrations of DMSP were provided, showing that even low levels of DMSP help bacteria to alleviate salinity stress, implying an osmoprotective role of DMSP for marine bacterioplankton.

DMSP is an efficient osmoprotectant compared to other organic solutes

DMSP is an important substrate for bacteria, and it is possible that its availability could provide enough carbon, sulfur and energy to stimulate BP despite the salinity stress, masking the effects of the salt inhibition. The addition of alternative substrates allowed us to distinguish between the substrate influence and the potential osmotic effect. DMSP, and to a lesser extent GBT, were the only solutes tested that conferred rapid osmoprotection only 1 h after the salt and solute additions (Fig. 3a). It is noteworthy that DMSP was more effective at relieving the salinity inhibition than GBT, which is well recognized as one of the most efficient and widespread natural osmolytes (Yancey 2005). Addition of glutamate, trehalose, acrylate, glucose and valine did not alleviate the salt inhibition after 1 h, suggesting the absence of a quick osmotic effect from these solutes. The presence of high-affinity uptake systems for DMSP and GBT in marine microorganisms (Kiene et al. 1998) results in the rapid uptake of DMSP and GBT, which could explain the quick osmotic response of these compounds compared to the other solutes tested here. DMSP additions resulted in a higher overall stimulation of BP and a faster relief of salt inhibition than when alternative carbon sources and osmoprotectants were added, indicating that DMSP is used by bacteria not only as a labile substrate, but also as an efficient osmolyte.

Bacteria accumulate DMSP to high intracellular concentrations

To be used as an osmoprotectant, DMSP must be accumulated and retained untransformed in the cells for several hours. We found that intracellular DMSP content increased with external salinity in our experiments with seawater filtrates containing bacteria only (Fig. 4B). Even at ambient salinity (control), a

significant fraction of the DMSP taken up was retained untransformed in the cells. This observation is consistent with results from Kiene & Linn (1999), who showed that after incubating GF/F-filtered seawater with ^{35}S-DMSP for 25 h, 11% of the ^{35}S recovered in the particulate fraction was untransformed DMSP. Increasing salinity resulted in a faster and greater overall uptake of DMSP after 6 h (Fig. 4), and caused the bacteria to accumulate additional DMSP and keep it intact in their cells. The fact that bacteria subjected to salinity stress accumulate DMSP (Fig. 4) and that DMSP alleviates salt inhibition of bacterial production (Figs. 1 & 2) represents strong evidence that DMSP is used as an osmolyte by natural heterotrophic prokaryotes.

The intracellular DMSP concentration accumulated under salt addition was high enough (13 to 54 mM) to provide bacterial cells with some level of osmoprotection (Table 1). During similar experiments done with GBT, Kiene & Hoffmann Williams (1998) also found increasing retention of GBT in the cells as the salinity increased, with intracellular GBT concentrations of 16, 28 and 43 mM for 7, 14 and 25 ppt salinity treatments, respectively, in close agreement with our intracellular DMSP concentrations. Our estimation of the intracellular concentration of DMSP assumes that 100% of cells counted by flow cytometry were accumulating DMSP, which is unlikely to happen in a natural microbial assemblage. A more reasonable assumption might be that ca. 50% of the microbial cells in the natural system are actually taking up DMSP (see below), in which case the intracellular concentration estimated in Table 1 would be doubled, and could reach 109 mM for the +10 ppt treatment. Also, in the possible event of shrinkage of the cytoplasmic volume due to the increase in external osmolarity (Csonka 1989 and references therein), the intracellular DMSP concentration would be even higher. For example, if the hyperosmotic shock caused the bacterial biovolume to decrease to 0.04 µm^3 from the assumed value of 0.07 µm^3, the intracellular DMSP concentration would be 95 mM in the +10 ppt treatment if 100% of the cells took up the DMSP, and 190 mM if only 50% of the cells took up DMSP. These intracellular concentrations are in the range produced by phytoplankton, which use DMSP as an osmolyte (Reed 1983, Dickson & Kirst 1986, 1987, Karsten et al. 1992).

That not all bacterioplankton cells would participate in DMSP uptake is supported by several lines of evidence. Firstly, a significant fraction of the bacterioplankton is not metabolically active, and thus not likely to participate in the uptake of DMSP. Based on single-cell assays using fluorescence *in situ* hybridization, it is estimated that about 60% of the bacterial cells in marine waters are metabolically active, although this fraction is highly variable both seasonally and spatially (Lennon & Jones 2011 and references therein). Secondly, several studies looking at the single-cell uptake of ^{35}S-DMSP in different environments showed that typically, ca. 50% of the heterotrophic prokaryote community is able to take up and assimilate sulfur from DMSP (Malmstrom et al. 2004, Vila et al. 2004, Motard-Côté et al. 2012). This number should, however, be considered as a maximum estimate, since these long incubations (ca. 24 h) can allow for the conversion of ^{35}S-DMSP to ^{35}S-MeSH, which could then be assimilated by organisms lacking the capacity to import DMSP. Additionally, the uptake of DMSP requires specific membrane transporters, whose distribution among marine bacterioplankton is not well known, and might not be found in all taxa. Transporters importing DMSP in the cells belong to 2 superfamilies: the ATP-binding cassette (ABC)-type (Kempf & Bremer 1998) and the betaine-choline-carnitine transporter (BCCT)-type (Todd et al. 2010, Ziegler et al. 2010). The ABC transport systems OpuA and OpuC identified in *Bacillus subtilis* are similar to the ProP and ProU systems in *Escherichia coli* (Boncompagni et al. 2000, Sleator et al. 2001). OpuA has only a low affinity for DMSP (inhibition constant, K_i = 912 µM), whereas OpuC has a high affinity for DMSP (K_i = 39 µM) as well as several of its derivatives (Broy et al. 2015). One BCCT-type transporter mediating DMSP import is called OpuD in *B. subtilis*, similar to BetP in *Corynebacterium glutamicum* and CaiT in *E. coli*. Recently, another BCCT-type transporter identified from *Marinomonas* MWYL1, DddT, has been shown to mediate uptake of DMSP. Genes similar to *dddT* were also found in the 2 marine species *Halomonas* HTNK1 and *Sagittula stellata* E37 (Todd et al. 2007, 2010, Johnston et al. 2008) and 2 isolates from the gut of Atlantic herring, *Pseudomonas* and *Psychrobacter* (Curson et al. 2010). Interestingly, the genes for the DddT uptake system are often found clustered with other *ddd* genes involved in DMSP degradation. Only these 4 transport systems (OpuA, OpuC, OpuD and DddT) and their homologs are known to mediate uptake of DMSP in cells. A better knowledge of their distribution among the marine bacterioplankton taxa would improve our understanding of the potential global importance of the DMSP retention mechanism and its effect on the global cycling of DMSP.

DMSP provides osmoprotection at low ambient concentration

DMSP provided significant osmoprotection at 2.5 nM (Fig. 5), a concentration within the range that is typically measured in different environments (Kiene & Slezak 2006). More DMSP provided more protection, and inhibition of BP by acute +10 ppt salinity increase was almost completely relieved by a 12 nM DMSP addition, a concentration that could be found during bloom conditions or in microenvironments around leaking cells (Mitchell et al. 1985, Wolfe 2000). Our results (e.g. Fig. 6) suggest that retention of DMSP in natural systems could be enhanced in oceanic or estuarine waters, where salinity is high or subject to strong and rapid fluctuations. Retention could also be promoted at higher environmental DMSPd levels, which are likely to happen in relatively productive environments and where intense grazing activity might release more DMSPd (Archer et al. 2002, Simó et al. 2002). However, these results also show that some DMSP is retained in the microbial cells even at very low ambient DMSPd levels (0.5 nM in this study; Fig. 6). Thus, DMSP accumulation is likely to occur in less productive environments as well. As mentioned above, DMSP retention in bacterial cells has been observed in open ocean samples with presumably stable salinity and low steady state DMSPd concentrations (1.7 nM) (Kiene & Linn 2000a).

In our experiments with pre-incubated filtrates containing only bacteria, there seemed to be a threshold at which the availability of DMSP was high enough that the cells could afford to keep some of it intact for a certain time. For DMSP additions of 2.5 nM and below (Fig. 6), the amount of DMSP in cells was the same at ambient and +10 ppt salinity. With these lower additions, most of the DMSP is probably degraded and used to meet bacterial energy, carbon and sulfur demands, leaving little excess DMSP to be accumulated. This threshold, or in other words the level of DMSP required to achieve a significant accumulation and osmoprotective effect, probably depends on the bacterial activity level (hence the bacterial demands), and is thus expected to be lower in less productive environments. Additional studies will be needed to test this hypothesis.

Decreased production of MeSH under salinity stress

Experimental increases in salinity resulted in a lower net production of MeSH, suggesting a down-regulation of the demethylation/demethiolation path-way, while a larger fraction of the DMSP taken up was retained and accumulated in the cells for osmoprotection. Consistent with our results, Kiene & Hoffmann Williams (1998) also saw a decrease in $^{14}CO_2$ production along with a higher accumulation of ^{14}C-GBT in the cells at higher salinity, indicating a retarded degradation of GBT. Salgado et al. (2014) reported a different pattern with cultures of *Ruegeria pomeroyi*, where salinity increases resulted in lower DMS but enhanced MeSH productions. However, their DMSP amendments were 50 and 500 µM, which is 3 and 4 orders of magnitude higher than what was added in this study, and could explain the different response observed.

Significance and implications of bacterial retention for the DMSP cycle

Retention of untransformed DMSP in prokaryote cells for osmotic purposes is very likely to happen under natural conditions. Previous studies showing this phenomenon added high levels of DMSP to dense bacterial cultures (Wolfe 1996), which in most cases were enteric bacteria that do not naturally encounter DMSP in their environment (Mason & Blunden 1989, Diaz et al. 1992, Cosquer et al. 1999). Here, we showed that the natural bacterial assemblage from GF/F-filtered coastal seawater retained a significant fraction of DMSPd in their cells, even at extremely low ambient DMSPd concentrations (0.5 nM; Fig. 5). This microbially-retained pool increased with both the availability of DMSPd and the level of osmotic stress, and did provide osmoprotection to the cells at DMSPd concentration as low as 2.5 nM, which is in the range of concentration occurring in most marine environments. While our experiments focused on salinity stress, it remains possible that DMSP retention could be protective against other stresses experienced by bacteria, such as oxidative stress, nutrient starvation, and thermal shock.

To understand the impacts of this accumulation mechanism on the DMSP cycling in marine waters, we first need to establish whether this pool can represent a significant fraction of the total and dissolved DMSP pools. Fig. 5 shows strong relationships between the accumulation of intracellular DMSP and the external DMSP concentration, at ambient and increased salinity. Under +10 ppt salinity, this accumulation was linear, and 54 % of the DMSPd initially provided was found in the cells after 1.5 h. The ambient salinity treatment followed the same trend at lower DMSPd levels. While retention continued to increase

above 3.5 nM added DMSPd in the ambient salinity treatment, the fraction of added DMSPd accumulated decreased above 3.5 nM, and was ca. 33% of the DMSP addition at the 12 nM level. The microbially retained pool thus ranged from 33 to 54% of the extracellular pool of dissolved DMSP, which is higher than previous estimates (10 to 15% of the DMSPd pool) from whole seawater (Fig. 1) and in 1 d old filtrate incubations (10 to 37%; Li et al. 2015). Low levels of labile organic nutrients in our 3 to 4 d old GF/F filtrate likely resulted in a low bacterial growth rate (McManus et al. 2004). A faster growing community would probably degrade DMSP faster, leading to a lower retention of untransformed DMSP in the cells. Nevertheless, the microbially-retained pool of DMSP can be a significant fraction of the operationally-defined DMSPd pool, and could impair the measurement of truly dissolved DMSP since the traditional methods for DMSPd involve filtration through GF/F-type filters, which let some bacteria pass through (Lee et al. 1995). Therefore, the operationally-defined DM-SPd will overestimate the 'dissolved' DMSP pool if these bacteria have accumulated DMSP in their cells (see also Li et al. 2015). Such an overestimation could also indirectly affect the DMSP turnover rate calculations that are based on the DMSPd concentration.

Whether bacteria significantly affect the turnover of DMSP by taking up and retaining DMSP within their cells is another important question. With a residence time of days, the microbially-retained pool appears to turn over much more slowly than the extracellular DMSP pool, slowing down the overall DMSP cycling and the fluxes of sulfur and carbon within the microbial food web. Accumulation of DMSP in bacterial cells could also enhance the transfer of DMSP-sulfur to higher trophic levels through bacterivory. The fate of ingested untransformed DMSP via grazing is still poorly understood. Most studies addressing this question have focused on the assimilation of phytoplankton-DMSP by herbivores. Saló et al. (2009) found that 32% of the DMSP-sulfur ingested by the dinoflagellate *Oxyrrhis marina* preying on the diatom *Thalassiosira pseudonana* was assimilated into biomass, while the biggest fraction was either converted to DMS or released as DMSPd to the extracellular environment. In natural conditions however, grazers might be eaten by predators before digesting all the DMSP, passing a fraction of it up to higher trophic levels (Tang & Simó 2003). No study so far has looked at the fate of DMSP ingested by protists (ciliates and flagellates) feeding on bacteria. It is generally assumed that the major impact of microzooplankton grazing on the DMSP cycle is to enhance the overall production of DMS (Wolfe & Steinke 1996) by desegregating the DMSP and DMSP-lyase during the digestive process, and thus reducing the overall assimilation of DMSP-sulfur by the trophic web. This DMS production via grazing could be further increased if DMSP retention by bacteria somehow makes them more appealing to micrograzers, for example by allowing them to attain greater size (via greater osmotic volume). Overall, the major impact of accumulation and retention of DMSP into bacterial cells is likely to be a general slowing down of the turnover, and potentially a higher trophic transfer to grazers. On the other hand, the retention of DMSP provides bacteria with a quick and efficient protection against osmotic stresses, which allows them to process organic matter and recycle nutrients under a wider range of osmotic conditions, maintaining healthy ecosystem functioning.

CONCLUSIONS

DMSP is well recognized as a major source of reduced sulfur and carbon for bacteria. It is most often regarded as an extremely labile compound in the dissolved organic matter pool, with a fast turnover time. Our results suggest an osmoprotective role for DMSP in marine bacterioplankton, expanding the role of marine bacteria in DMSP cycling beyond their ability to degrade DMSP. We have shown that DMSP is accumulated in the cells and used as an osmolyte by prokaryotes in a natural marine system, relieving acute osmotic stress even at low DMSPd concentrations typically found in these natural systems. DMSP can be retained in bacterial cells for several days, leading to a slow turnover of this pool compared to extracellular labile DMSP. Enhanced retention of DMSP in bacteria under salinity stress appears to be due, in part, to the inhibition of the demethylation/demethiolation degradation pathway. By diverting DMSP from faster degradation pathways, this microbially-retained pool, which can represent a significant fraction of the operationally-defined DMSPd standing stock, can slow down the overall cycling of DMSP and allow trophic transfer of DMSP to bacterial grazers.

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