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Salinity as a Regulator of DMSP Degradation in *Ruegeria pomeroyi* DSS-3

Paula Salgado[1*], Ronald Kiene[2], William Wiebe[3], and Catarina Magalhães[1]

[1]*EcoBioTec Laboratory, Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Rua dos Bragas 289, P 4050-123 Porto, Portugal*
[2]*Department of Marine Sciences, University of South Alabama; 5871 USA Drive North, Mobile, Alabama, USA 36688 and Dauphin Island Sea Lab, Dauphin Island, Alabama 36528, USA*
[3]*Department of Marine Sciences; University of Georgia, Athens, GA 30602, USA*

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Dimethylsulfoniopropionate (DMSP) is an important carbon and sulfur source to marine bacterial communities and the main precursor of dimethylsulfide (DMS), a gas that influences atmospheric chemistry and potentially the global climate. In nature, bacterial DMSP catabolism can yield different proportions of DMS and methanethiol (MeSH), but relatively little is known about the factors controlling the pathways of bacterial degradation that select between their formation (cleavage vs. demethiolation). In this study, we carried out experiments to evaluate the influence of salinity on the routes of DMSP catabolism in *Ruegeria pomeroyi* DSS-3. We monitored DMS and MeSH accumulation in cell suspensions grown in a range of salinities (10, 20, 30 ppt) and with different DMSP amendments (0, 50, 500 μM). Significantly higher concentrations of DMS accumulated in low salinity treatments (10 ppt; $P < 0.001$), in both Marine Basal Medium (MBM) and half-strength Yeast Tryptone Sea Salts (½ YTSS) media. Results showed a 47.1% and 87.5% decrease of DMS accumulation, from salinity 10 to 20 ppt, in MBM and ½ YTSS media, respectively. On the other hand, MeSH showed enhanced accumulations at higher salinities (20, 30 ppt), with a 90.6% increase of MeSH accumulation from the 20 ppt to the 30 ppt salinity treatments. Our results with *R. pomeroyi* DSS-3 in culture are in agreement with previous results from estuarine sediments and demonstrate that salinity can modulate selection of the DMSP enzymatic degradation routes, with a consequent potential impact on DMS and MeSH liberation into the atmosphere.

Keywords: *Ruegeria pomeroyi*, DMSP degradation, salinity effect, dimethyl sulfide, methanethiol

*For correspondence. E-mail: psalgado@ciimar.up.pt; Tel.: 00351-223401 854

Introduction

Sulfur is a vital element in microorganism metabolism. Besides its assimilation into required biomolecules such as methionine (Cooper, 1983), marine algae also incorporate sulfur into the osmolyte dimethylsulfoniopropionate (DMSP; Stefels, 2000). DMSP is a precursor of the climatically active gas dimethylsulfide (DMS), which is a major contributor of organic sulfur emissions to the atmosphere from salt marshes, coastal wetlands and oceans (Steudler and Peterson, 1984; Charlson *et al.*, 1987; Aneja and Cooper, 1989; Liss *et al.*, 1997). DMS has a low residence time and is readily emitted to the atmosphere where it reacts with other species to produce a particulate-phase containing SO_4^{2-} which, in turn, composes most of the cloud condensation nuclei – CCN (Sievert *et al.*, 2007). Hence, an increase in marine DMS emissions would result in a CCN increase and clouds albedo, with a consequent decrease in incoming solar radiation to Earth's surface (Charlson *et al.*, 1987). However, the DMS connection to the earth's climate is currently under debate and may be more complex than previously believed. Quinn and Bates (2011) have reviewed several studies that tried to support the DMS-climate feedback loop theory and verified that CCN has other important sources such as wind-driven sea-salt particles and organic matter and it also depends on particle growth.

DMSP is an osmolyte and cryoprotectant produced by microalgae, macroalgae and a few higher plants (Kirst *et al.*, 1991; Tang *et al.*, 1999; Van Rijssel and Gieskes, 2002; Otte *et al.*, 2004; Van Alstyne, 2008). Also, DMSP holds other ecologically important functions by acting as herbivore deterrent (Van Alstyne *et al.*, 2001; Strom *et al.*, 2003; Fredrickson and Strom, 2009). Additionally, recent studies described the relevance of demethylated sulfur compounds (e.g. DMSP, DMS) as foraging cues and chemoattractants for predators to find and capture prey (Seymor *et al.*, 2010; Swan *et al.*, 2012; Garcés *et al.*, 2013; Garren *et al.*, 2014). Once released into the extracellular environment by viral lysis, algal senescence, zooplankton grazing on phytoplankton, or physiological stress (Hill *et al.*, 1998; Laroche *et al.*, 1999; Kiene *et al.*, 2000; Mulholland and Otte, 2002) the dissolved DMSP ($DMSP_d$) can be rapidly catabolized via two pathways which are microbially mediated: cleavage and demethylation/demethiolation (Visscher *et al.*, 1992; Yoch, 2002). Although the cleavage pathway represents a considerable source of DMS (Curson *et al.*, 2011), demethylation/demethiolation produces the highly reactive volatile sulfur compound methanethiol (MeSH). While many bacteria can select between producing more or less DMS and MeSH (Simó, 2001), relatively few investigations have addressed the environmental controls, such as

presence of oxygen, light and salinity, on the biological pathways of DMSP degradation and DMS/MeSH formation (Jonkers *et al.*, 1998; Van Duyl *et al.*, 1998; Zubkov *et al.*, 2001; Visscher *et al.*, 2003; Niki *et al.*, 2007; Magalhães *et al.*, 2012). Specifically, salinity was previously identified as an important environmental factor regulating DMS and MeSH net accumulations, with a potential impact on DMS and MeSH emissions in coastal ecosystems (Magalhães *et al.*, 2012). Coastal ecosystems contain a high range of physico-chemical gradients, including salinity, which can influence the recycling of key elements, such as carbon and sulfur (Dacey *et al.*, 1987; Iverson *et al.*, 1989; Smith and Hollibaugh, 1993; Gattuso *et al.*, 1998; Kiene *et al.*, 2000; Zindler *et al.*, 2012). Indeed, the high biological productivity of coastal areas, due for example to large inputs of nutrients through river discharges and coastal upwelling, raises questions of the importance of coastal zones in contributing to the sea surface production of DMS and DMSP and the sea-air exchange of DMS (Kettle and Andreae, 2000; Lana *et al.*, 2011; Zindler *et al.*, 2012).

This study was motivated by results obtained in our previous research where we identified salinity as an important environmental factor regulating ratios of natural DMS and MeSH emissions, but it was not possible to discriminate which specific processes were involved on the DMS and MeSH net fluxes measured in those natural complex communities, where several biological pathways and chemical reactions of DMS and MeSH production and loss may operate simultaneously (Holmer and Storkholm, 2001; Lomans *et al.*, 2002). Thus, in the present study we avoided the complexities inherent in sediment incubations by investigating the effect of salinity on the regulation of the two alternative DMSP degradation products (DMS and MeSH) in a pure culture of *Ruegeria pomeroyi* strain DSS-3. This bacterium possesses both the cleavage and a demethylation pathway for DMSP degradation (González *et al.*, 2003; Newton *et al.*, 2010) and since its whole-genome has been sequenced (Moran *et al.*, 2004), it became a model organism for DMSP degradation studies. *R. pomeroyi* DSS-3 was originally isolated from coastal Georgia (USA) seawater in a medium with a salinity of 14 ppt (González *et al.*, 1999). DSS-3 is a representative of the alphaproteobacteria taxon – *Roseobacter* group (Moran *et al.*, 2012) and is abundant in saltmarsh-estuarine ecosystems (González *et al.*, 1999), where salinity fluctuations define these systems. In culture, DSS-3 growth was tested in a salinity range of 100–400 mM NaCl (~6 – 23 ppt; González *et al.*, 2003). In the present study we hypothesized that salinity is a key factor in selecting the two competing pathways of bacterial DMSP degradation, and thus in controlling the relative production of DMS and MeSH compounds resulting from DMSP catabolism.

Materials and Methods

Bacterial strain

R. pomeroyi DSS-3 strain was obtained from the Spanish Type Culture Collection (CECT 7647^T) from the University of Valencia, Spain, and was maintained on half strength Yeast Tryptone Sea Salts (½ YTSS) solid medium containing 2 g yeast extract (Merck Millipore), 1.25 g tryptone peptone (OXOID), 20 g Sea salts (Sigma) and 15 g Agar powder (HIMEDIA) per L.

R. pomeroyi DSS-3 growth curves

Several media have been used to study *R. pomeroyi* DSS-3 DMSP metabolism (González *et al.*, 1999, 2003; Bürgmann *et al.*, 2007; Todd *et al.*, 2012). To compare our results with previous studies, we grew *R. pomeroyi* DSS-3 in the two most widely used media, Marine Basal Medium (MBM) and ½ YTSS, so that we could evaluate whether the type of medium affected the sulfur compounds formed during DMSP degradation. *R. pomeroyi* DSS-3 cells streaked from plates were inoculated into 25 ml of liquid MBM and ½ YTSS, measured the optical density (OD) at 540 nm until reaching a starter culture (OD 0.2–0.4). MBM contained 50 mM Tris-HCl; pH 7.5, 19 mM NH_4Cl, 0.33 mM $K_2HPO_4{\cdot}3H_2O$ (Baumann and Baumann, 1981) and modified, according to González *et al.* (1999), with addition of 10 mM glucose and 0.1 mM $FeSO_4$ instead of FeEDTA. Different amounts of a sea salt mixture (Sigma) were included in both media to simulate salinities of 10, 20, and 30 ppt. Cell suspensions were incubated overnight at 28°C under aerobic conditions with rotary shaking (80 rpm). One milliliter of cell suspension was harvested and readings (OD_{540}) of each independent salinity treatment replicate were performed at successive intervals that varied between 6.3 and 15.5 h. Readings at each interval were normalized for the same OD.

Salinity treatments for *R. pomeroyi* DSS-3 cell suspensions

The salinity effect on the accumulation of volatile organosulfur compounds (DMS, MeSH) was evaluated in *R. pomeroyi* DSS-3 cells grown in 30 ml of liquid ½ YTSS and MBM media at three different salinities (10, 20, 30 ppt) for 16 h at 28°C on a rotary shaker (80 rpm) until near the end of the exponential phase (OD_{540} ~1.3). The experimental salinities were obtained by modifying the quantity of sea salts in each medium and measuring the corresponding salinity with a YSI Model 30 probe. Next, 3 ml aliquots of the grown *R. pomeroyi* culture were added to 12 ml crimp-topped serum vials and each vial was sealed with a Teflon-faced rubber stopper. Each salinity treatment was incubated in triplicate for 4 h at 26°C, with constant rotary shaking (80 rpm), under oxic (air headspace) conditions and with two different concentrations of DMSP (50, 500 μM) along with a non-amended control. Volatile sulfur compounds (DMS and MeSH) were measured in the headspace at the beginning (time 0 h) and end (time 4 h) of the incubation and analyzed by pulsed flame-photometric detection (GC/P-FPD) as described below. Negative controls with only sterile ½ YTSS and MBM media, without bacterial cells, and with DMSP amendments were run in triplicate for the different salinity treatments.

Analytical determinations

DMS and MeSH concentrations were measured by removing headspace subsamples (250 μl), after vigorous shaking for 30 sec, from the incubation vials with a glass gas-tight syringe and injecting them into a Varian gas chromatograph

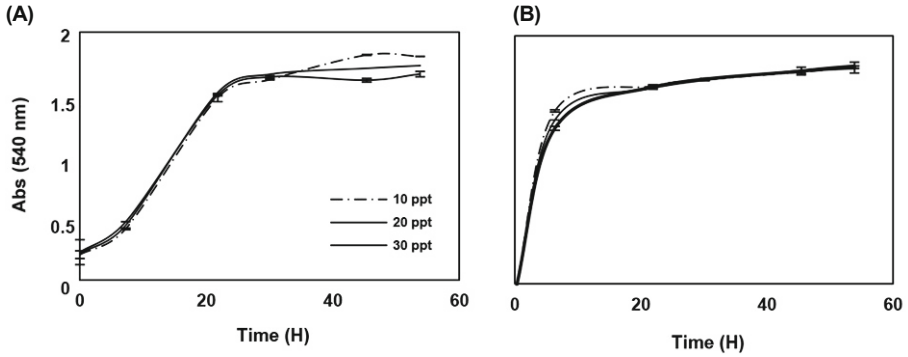

Fig. 1. Growth curves for *R. pomeroyi* cell cultures in MBM (A) and ½ YTSS (B) media of different salinities (10, 20 and 30 ppt). Values represent the average of normalized readings for the same OD and the range bars indicate standard deviations.

(CP-3800). Each volatile sulfur gas was separated with a Mega-Bore silica plot column at 189°C and detected with a P-FPD, where nitrogen was the carrier gas at 3 ml/min. DMS concentrations were calculated by using standards generated from DMSP into DMS, after alkaline hydrolysis with a 5 M NaOH solution and with a conversion efficiency of 100% (Kiene and Service, 1991). MeSH concentrations were estimated using the standard curve for DMS since the slopes of the standard curves of MeSH and DMS have been previously demonstrated to agree (Kiene, 1996). Detection limit for both sulfur gases was 10 nM in the dissolved phase. DMS and MeSH concentrations in solution were determined from measured headspace concentrations, liquid- and gas-phase volumes and empirical distribution coefficients of the selected organosulfur compounds according to respective salinity (Przyjazny *et al.*, 1983).

Data analysis

Before testing the statistical significance of data, the Leven's test for the equality of variances and Kolmogorov-Smirnov test for normality were applied to all salinity experimental data. After data passed the tests for homoscedasticity and normality, an one-away ANOVA was used to evaluate the statistical differences between salinity treatments. All data analyses were performed at the 95% confidence level ($P < 0.05$), unless otherwise stated. QI Macros SPC Software 2013 was applied for statistical analysis.

Results

R. pomeroyi growth curves at different salinities

The growth behavior of *R. pomeroyi* DSS-3 strain in MBM and ½ YTSS media at different salinities (10, 20, 30 ppt) showed typical bacterial growth in both media, while the lag phase was undetectable in ½ YTSS medium (Fig. 1B). These differences are most likely due to the fact that *R. pomeroyi* cells were maintained on solid ½ YTSS medium and the consequent adaptation to the liquid ½ YTSS was more rapid. While *R. pomeroyi* DSS-3 growth curves for the two media differed, the growth curves were found to be similar to each other for each specific salinity within each medium. These results confirmed that the range of salinities tested (10, 20, 30 ppt) did not substantially affect *R. pomeroyi* DSS-3 growth performance in MBM and ½ YTSS media.

The absence of an effect of different salinities on DSS-3 growth curves for each medium during the 16 h of growth ensured that differences between the DMSP degradation observed among the different salinity treatments were not influenced by differences in DSS-3 growth rates.

Salinity effect on *R. pomeroyi* DSS-3 DMS and MeSH production

In order to quantify the sulfur volatiles originated from the DMSP degradation pathways in the presence of dense cell suspensions of DSS-3, it was necessary to add DMSP concentrations well above the nanomolar levels common in seawater. We chose 50 and 500 μM as a suitable range of DMSP concentrations for the culture conditions. Higher concentrations of DMSP (500 μM) added to cultures of *R. pomeroyi* DSS-3 resulted in progressively higher DMS production in all treatments (Fig. 2), and changes in salinity clearly affected the magnitude of DMS produced in both media. Results showed that highest DMS productions were always observed at the lowest salinity tested in both media and for any of the DMSP amendments, except when no DMSP

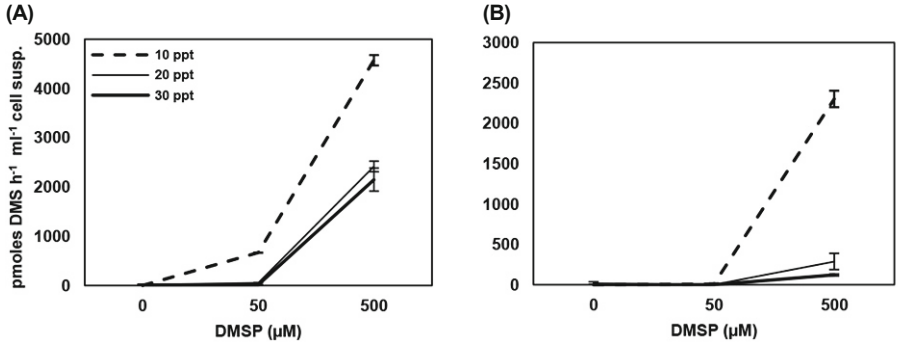

Fig. 2. Effect of different salinity conditions on net DMS production rates in *R. pomeroyi* DSS-3 cell suspensions incubated under different DMSP concentrations in oxic conditions in MBM (A) and ½ YTSS (B) media. All cultures were amended with 10 mM glucose.

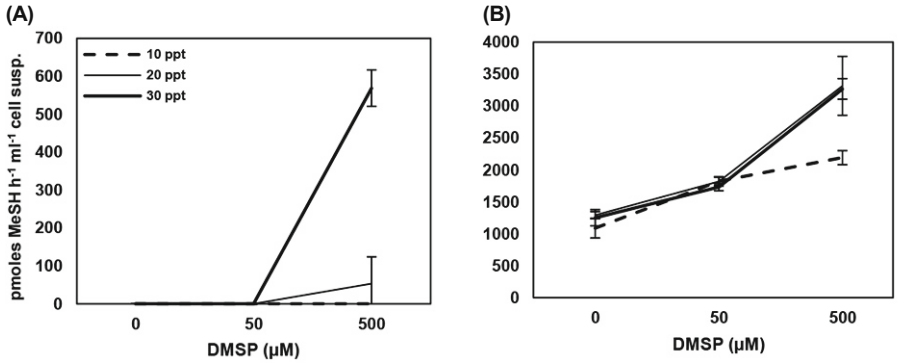

Fig. 3. Effect of different salinity conditions on net MeSH production rates in *R. pomeroyi* DSS-3 cell suspensions incubated under different DMSP concentrations in oxic conditions in MBM (A) and ½ YTSS (B) media. All cultures were amended with 10 mM glucose.

was added in both media (Fig. 2) and for 50 μM DMSP of ½ YTSS medium, where no significant differences were registered between the three salinities (P >0.05; Fig. 2B). In MBM with the 500 μM DMSP amendment, 89.1% more DMS was produced in the treatment with salinity of 10 ppt compared with the salinity of 20 or 30 ppt (Fig. 2A). Additionally, in the ½ YTSS medium amended with 500 μM DMSP, 77.3% lower DMS production was observed at 20 ppt salinity compared to the 10 ppt treatment and 57.9% less DMS production was registered in the 30 ppt treatment compared to 20 ppt (Fig. 2B).

Interestingly, increasing salinity had the opposite effect on MeSH production from DMSP, with higher concentrations of MeSH produced in treatments with higher salinities. In the MBM, MeSH was detected only in the 500 μM DMSP amendment, with the highest value occurring at 30 ppt salinity (P <0.001; Fig. 3A). MeSH production was 90.6% higher in the 30 ppt MBM medium compared to the 20 ppt medium and no detected production in the 10 ppt MBM medium (Fig. 3A). While there was a background of MeSH production in the ½ YTSS medium without DMSP addition (Fig. 3B), likely from sulfur amino acids in the medium, DMSP additions stimulated further MeSH production with an increase at higher salinities. For the 500 μM DMSP addition, MeSH production was 33.9% lower in the 10 ppt salinity treatment compared to the 20 and 30 ppt (P < 0.05; Fig. 4B). There were no statistically significant differences in MeSH production between the different salinities for treatments amended with 50 μM DMSP (P > 0.05; Fig. 3B).

Potential chemical production of DMS and MeSH was evaluated in parallel trials prepared according to the aforementioned conditions between salinities of 10, 20, and 30 ppt and with 50 μM DMSP and 500 μM DMSP amendments, in both MBM and ½ YTSS media without bacterial cells. Results confirmed the absence of chemical production of DMS or MeSH, with no detectable DMS or MeSH accumulation in treatments incubated without cells (data not shown).

Discussion

Methylated sulfur compounds such as DMS and, to a much lesser extent, MeSH provide important contributions to the sulfur transfer between aquatic environments and the atmosphere (Charlson *et al.*, 1987; Aneja and Cooper, 1989; Howard *et al.*, 2006).

In this study, we addressed the importance of salinity as a modulating factor on the magnitude of DMS and MeSH accumulation during DMSP degradation and tested this hypothesis in a simplified biological model system consisting of a pure culture of *R. pomeroyi* DSS-3. Overall, our findings suggest an enhanced MeSH production at high salinities (20 and 30 ppt) and greater DMS production at the lowest salinity tested (10 ppt), suggesting that salinity can select the preferential route of DMSP degradation within *R. pomeroyi.* These findings confirm previous results on complex estuarine sediments communities where highest net DMS production rates were observed in the lowest estuarine salinity treatments (Magalhães *et al.*, 2012). In fact, high DMS production under low-salinity conditions has been also previously observed in natural environments (Visscher *et al.*, 2003; Niki *et al.*, 2007; Yang *et al.*, 2011; Magalhães *et al.*,

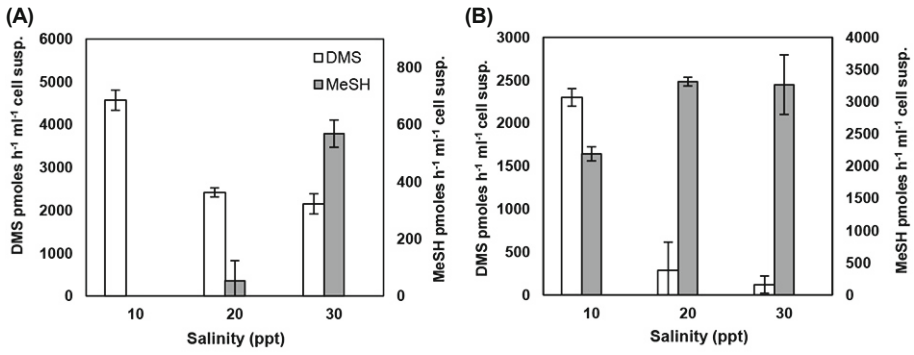

Fig. 4. Net DMS and MeSH production rates for the different salinity treatments amended with 500 μM DMSP in *R. pomeroyi* cell suspensions under oxic conditions on MBM (A) and ½ YTSS (B) media with 10 mM glucose addition.

2012). In our salinity experiments with *R. pomeroyi* DSS-3 cells we demonstrated that DMS production from the cleavage pathway is stimulated at lower salinity treatments. However, the reason for higher DMS production under low salinity and lower DMS production at high salinity is not entirely clear. *R. pomeroyi* is capable of accumulating DMSP to high intracellular concentrations for osmoprotection (70 mM; Reisch *et al.*, 2008). So it is possible that at high salinity more DMSP is retained in cells undegraded, leading to less DMSP degradation and, consequently, lower overall DMS production. In fact, accumulation of glycine betaine, an intracellular osmolyte, has been previously observed in an estuarine water filtrate culture to a gradient of increased salinities (Kiene and Hoffmann Williams, 1998). MeSH production, on the other hand, showed the opposite pattern indicating that the demethylation pathway was probably operating at a relatively high level even if more DMSP was retained for osmotic purposes. Additionally, González *et al.* (1999) verified that DMSP availability might also control the differential production between DMS and MeSH. Thus, if there isn't limitation of C and S supply and DMSP is available in excess of bacterial sulfur demand, a larger fraction of the DMSP can be degraded to DMS which is subsequently lost by diffusion through the bacterial cell membrane (Kiene *et al.*, 2000; Moran *et al.*, 2012).

The demethiolation of methylmercaptopropionic acid (MMPA; a transient DMSP degradation product), leads sequentially to MeSH production, which in our experiments with *R. pomeroyi* DSS-3 cell cultures was favored with the highest salinity treatments. This is in agreement with previous studies that mentioned a bacterial preference (>50%) for the MeSH-producing pathway in most high salinity environments (Kiene and Linn, 2000; Kiene *et al.*, 2000; Howard *et al.*, 2008). Moreover, Kiene and Linn (2000) averaged quantitative ^{35}S-DMSP$_d$ partitioning flows by using ^{35}S-DMSP tracer and found that in seawater samples, where salinity was of 29 ppt, MeSH was the dominant sulfur volatile product (~75%) of initial DMSP metabolism. It is also well established that the dominant process in ocean waters is demethylation/demethiolation (Moran *et al.*, 2012) most likely due to the incorporation of some of the MeSH into methionine by bacterioplankton (Kiene *et al.*, 1999; Simó *et al.*, 2000). In our salinity trials we can corroborate that MeSH produced from DMSP demethylation/demethiolation is enhanced at higher salinities. This is also in agreement with previous work in sediments collected along an estuarine salinity gradient which revealed a prevalence of MeSH accumulations at high salinity sites and in elevated salinity treatments (Magalhães *et al.*, 2012). Because of the complex nature of sediments, it was not possible in that earlier study to conclude that salinity shifts caused or facilitated changes in the relative DMS and MeSH production rates. For example, salinity changes could have affected the binding of MeSH to sediment particles (Kiene, 1991) which, in turn, could have influenced the measurement of gaseous MeSH. Our present work with pure cultures of *R. pomeroyi* DSS-3, on the other hand, clearly demonstrates that salinity indeed can influence the relative prevalence of the different DMSP$_d$ degradation pathways, with low salinity favoring the cleavage pathway over the demethylation/demethiolation pathway, resulting in a significant influence on production of DMS vs. MeSH.

We cannot exclude the possible MeSH formation from other precursors besides DMSP in the cultures. Indeed the higher MeSH accumulations in the ½ YTSS compared with MBM registered in all salinity treatments, may have originated from degradation of ½ YTSS medium organic sulfur compounds, as Bürgmann *et al.* (2007) noticed in their control samples. The yeast extract-tryptone complex in ½ YTSS medium can provide some methionine that could be degraded to produce extra MeSH. Despite the high MeSH background signal in ½ YTSS medium, our results revealed a significant salinity influence on MeSH production at the highest DMSP amendment (500 μM DMSP). While MeSH could also be a potential degradation product of DMS, González *et al.* (1999) did not detect MeSH in *R. pomeroyi* DSS-3 cell suspensions grown in the presence of DMS, which suggests that in our cell suspensions the biological formation of MeSH most likely was derived by demethylation/demethiolation of DMSP.

In the MBM medium, in contrast to what was observed for the ½ YTSS medium, DMS accumulation was favored over MeSH, independent of the salinity treatments. In fact, in a further study, González *et al.* (2003) verified that net MeSH accumulation was always lower than DMS accumulation in stationary-phase cultures of DSS-3 grown in MBM, confirming our observations of highest DMS production in MBM compared to MeSH. In addition, very low production of MeSH during DMSP degradation can result from rapid MeSH turnover through continuous demethylations or conversions (Suylen *et al.*, 1987; Bürgmann *et al.*, 2007; Dickshat *et al.*, 2010; Reisch *et al.*, 2011) which might explain the low MeSH production rates in our treatments performed in this medium. Also, the free iron in MBM (from $FeSO_4$) could bind to MeSH and, therefore, reduce the final MeSH concentrations (Butler *et al.*, 1992). Additionally, *R. pomeroyi* DSS-3 is able to degrade MeSH up to 40% after some hours of incubation (González *et al.*, 1999).

In conclusion, our results, confirmed that *R. pomeroyi* DSS-3 is flexible in metabolizing DMSP when facing different salinity conditions. These findings are consistent with the conclusion that *R. pomeroyi* DSS-3 possesses several strategies and characteristics for adaptations in marine environments (Moran *et al.*, 2004; Christie-Oleza *et al.*, 2012). Our study with *R. pomeroyi* DSS-3 provides insights into the potential effect of salinity on the two DMSP degradation pathways (cleavage and demethylation/demethiolation). Suspensions of *R. pomeroyi* DSS-3 cells showed an enhanced MeSH production resulting from DMSP demethylation/demethiolation in higher salinities as opposed to higher DMS production from the cleavage pathway in the lowest salinity treatments. These results together provide one mechanistic explanation for how salinity influences DMS and MeSH production in complex natural sediment communities (Magalhães *et al.*, 2012) by demonstrating that salinity can indeed influence the relative prevalence of the different DMSP$_d$ degradation pathways, with a significant influence on DMS *vs.* MeSH net production. Salinity, therefore, should be considered as a possible regulator of DMS emissions, an important contributor to the global sulfur cycle and, consequently, in shaping earth's atmosphere and climate.

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