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# A novel inhibitory interaction between dimethylsulfoniopropionate (DMSP) and the denitrification pathway

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**Abstract** Dimethylsulfoniopropionate (DMSP) is an abundant organic sulfur compound in marine algae and denitrification influences nitrogen availability to primary producers, the key regulators of coastal eutrophication. In this study, we tested the effect of DMSP on the nitrous oxide ($N_2O$) reduction step of denitrification in sediments and biofilms from the Douro and Ave estuaries (NW Portugal) and in pure cultures of a denitrifying bacterium, *Ruegeria pomeroyi*. $N_2O$ accumulation rates were monitored in sediment slurries and bacterial cell suspensions amended with DMSP concentrations ranging from 0 to 5 mM. In these treatments $N_2O$ accumulation rates increased linearly with DMSP concentration ($R^2$ from 0.89 to 0.99, $p < 0.001$), suggesting an inhibitory effect of DMSP on the nitrous oxide reductase activity. The addition of DMSP to sediments and bacterial culture resulted in accumulation of dimethylsulfide (DMS) as well as $N_2O$. However, no direct inhibition on $N_2O$ reductase activity by DMS was observed. Natural concentrations of DMSP in the different estuarine sites were found to be linearly correlated to natural $N_2O$ effluxes ($R^2 = 0.64$, $p < 0.001$), suggesting that DMSP may negatively affect $N_2O$ reductase in situ. This newly identified interaction between DMSP and $N_2O$ emissions may have a significant ecological role as the inhibition of the nitrous oxide reduction enhances nitrogen loss via $N_2O$. Since $N_2O$ is a powerful greenhouse gas, the results from

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our study may be important for evaluating climate change scenarios.

**Keywords** Denitrification · DMSP · Estuarine sediments · Nitrogen · *Ruegeria pomeroyi* · Organic sulfur

## Introduction

The availability of fixed, inorganic nitrogen, particularly nitrate ($NO_3^-$), typically controls primary production in temperate zone estuaries and other coastal marine ecosystems (e.g. Jickells 1998). As such, anthropogenic nitrogen enrichment is commonly implicated in the eutrophication and subsequent disturbance of coastal marine systems (Ryther and Dunstan 1971; Codispoti and Christensen 1985; de Jonge and van Raaphorst 1995; Nixon et al. 1995). The anaerobic respiratory microbial process of denitrification plays an important role in nitrogen-enriched coastal ecosystems because it converts $NO_3^-$ to dinitrogen gas ($N_2$), thus reducing the amount of fixed N exported to the ocean. Thereby, denitrification mitigates the deleterious effects that excessive N inputs have on ecosystem health (Nixon 1981; Seitzinger 1988; Ogilvie et al. 1997; Seitzinger 2000; Seitzinger et al. 2006). Denitrification most often involves the oxidation of organic matter coupled to a series of nitrogen oxide reductions where $NO_3^-$ and/or nitrite ($NO_2^-$) are reduced to nitric oxide (NO), nitrous oxide ($N_2O$), and finally $N_2$. Nitrite reduction is typically closely coupled to the subsequent steps in the reduction sequence, so incomplete denitrification is relatively rare in nature and NO and $N_2O$ net production is small in most environments (Seitzinger 1988, 2000). Nontheless elevated $N_2O$ production does occur in many estuarine systems and estuaries are considered major sources of $N_2O$ to the atmosphere (Seiler and Conrad 1987; Bange et al. 1996). The factors that influence $N_2O$ production in these environments, however, are not fully understood.

The biological production of $N_2O$ is linked to the turnover of inorganic nitrogen by nitrifying and denitrifying microbes (Poth and Focht 1985; Ritchie and Nicholas 1972; Wrage et al. 2001; Casciotti and Ward 2005). Higher net production rates of $N_2O$ have been observed in highly eutrophic sediments and may be related to the low $O_2$ concentrations characteristic of these environments and/or $H_2S$ inhibition of $N_2O$ reduction during denitrification (Sørensen et al. 1980; Senga et al. 2006). The potential effects of anthropogenic activities on $N_2O$ release from aquatic sediments is evident by the concomitant increase in $N_2O$:$N_2$ with increased N loading to a system (Seitzinger and Nixon 1983; Magalhães et al. 2005a; Stow et al. 2005). $N_2O$ is an efficient greenhouse gas with a global warming potential >200 times that of carbon dioxide, on a per mole basis (Dickinson and Cicerone 1986), and the dominant stratospheric ozone-depleting substance (Ravishankara et al. 2009). Thus, understanding the factors controlling the production of $N_2O$ is essential to predict the influence of $N_2O$ on climate changes.

Dimethylsulfoniopropionate (DMSP) is an organic sulfur compound produced in large amounts by numerous species of marine phytoplankton (e.g. Stefels 2000), seaweed macroalgae (Reed 1983), and salt marsh grasses (Otte et al. 2004). In these organisms, DMSP function as an intracellular osmotic solute, antioxidant and cryoprotectant (Kirst 1989; Kirst et al. 1991; Sunda et al. 2002). Intracellular DMSP is released during physiological and mechanical stress, viral lysis or grazer attack (Stefels 2000). Much of this dissolved DMSP is rapidly degraded by marine bacteria (Kiene and Bates 1990; Kiene et al. 2000; Kiene and Linn 2000a, b) and satisfies a substantial part of the sulfur and carbon demand for many marine water column prokaryotes (Kiene et al. 2000; Zubkov et al. 2002). DMSP catabolism involves an enzymatic cleavage of DMSP to DMS via several pathways (e.g. DddL and DddD, Todd et al. 2007; Curson et al. 2008) and/or demethylation to 3-methiolpropionate (MMPA), which is further metabolized to 3-mercaptopropionate (MPA) or converted to methanethiol (MeSH). MeSH is assimilated into methionine and bacterial proteins or can be metabolized to mineralized end products such as $CH_4$, $CO_2$ and $H_2S$ (Kiene and Visscher 1987; Kiene and Taylor 1988; Kiene et al. 1999). Recently, genes encoding demethylation and enzymatic cleavage of DMSP have been identified providing new insights into these processes (Howard et al. 2006, 2008; Todd et al. 2009). Cleavage of DMSP results in the release of DMS and represents a major marine source of sulfur to the atmosphere (Schlessinger 1997). DMS

significantly affects atmospheric chemistry as well as the global climate system because it is oxidized to sulfate aerosols which lead to cloud formation, reducing the amount of solar radiation that is absorbed by the ocean (Charlson et al. 1987; Vallina and Simó 2007).

As sulfur and nitrogen are required by all living organisms, changes in the fluxes of these elements can alter the rate of fundamental biological processes. The cycling of these elements is mediated mainly by microbial processes and subjected to a multitude of interactions, many of which are complex and poorly understood. In particular, interactions between inorganic nitrogen and organic sulfur compounds in marine systems have not yet been described. In preliminary studies performed in DMSP-rich sandy sediments and rocky biofilms of the Douro estuary, we found evidence for incomplete denitrification, with high rates of $N_2O$ production. Thus, we hypothesized that this abundant organic sulfur compound may play a role in regulating the nitrous oxide reduction step of denitrification.

## Materials and methods

### Sample collection

Subtidal and intertidal sediments and rocky biofilm samples were collected between 2002 and 2009 from 24 locations in the Douro River estuary and two locations in the Ave River estuary (Fig. 1SI in Supplementary Material, Table 1). The Douro and Ave Rivers have mesotidal estuaries with average tidal ranges of 2.5 m (Vieira and Bordalo 2000), water column temperatures ranging from 7°C in winter to 22°C in summer, and salinities in the lower estuaries ranging from 0 to nearly 35 psu. Physical and chemical characteristics of these estuaries have been described previously (Gonçalves et al. 1992; Soares et al. 1999; Magalhães et al. 2002; 2005a; 2008; Mucha et al. 2003; Díez et al. 2005).

At each intertidal sediment site, a total of 20 sediment cores of 10 cm depth were collected with sterile, truncated 50 ml plastic syringes (3 cm diameter). Sediment from the subtidal sites was retrieved with a Petit Ponar grab sampler (Wildco 1728, Wildlife Supply, Saginaw, MI, USA; 225 $cm^2$). A total of 20 sub-cores of 10 cm depth were collected from the sediment retrieved with the grab sampler, using the procedure described above. Intertidal rocky biofilms were removed by scraping the rocks in the field with a sterile scalpel (approximately 80 g of material). In addition, 5 liters of overlying estuarine water were collected at each site and stored in acid-cleaned polyethylene bottles. Immediately after collection, samples were homogenized, stored in sterile plastic bags and transported in the dark to the laboratory in refrigerated ice chests. In the laboratory, the estuarine water from each site was immediately filtered (0.2 μm, Schleicher & Schuell membrane filters), and samples were processed for the slurry experiments.

### Slurry experiments

A cross-site comparison of the effects of DMSP on $N_2O$ release via denitrification pathway was tested by adding 1.5 mM DMSP to triplicate slurries prepared from 26 different sites, including intertidal and subtidal sediments and rocky biofilms (Fig. 1SI in Supplementary Material; Table 1). The effect of different DMSP additions (0, 5, 50, 100, 500, 1500 and 5000 μM) on $N_2O$ accumulation was also tested at sites ISS1, ISS2, IRB1, IMS1, ISS10, ISS12, and ISS13 (Fig. 1SI in Supplementary Material; Table 1). In addition, at sites IMS1, ISS10, ISS12 and ISS13 the concentrations of sulfur compounds (DMS, MeSH and hydrogen sulfide [$H_2S$]) and $N_2O$ were monitored in slurry treatments with the same seven progressive concentrations of DMSP (from 0 to 5 mM) and five different concentrations of DMS ranging from 0 to 100 μM. The DMS used in these experiments was produced from the hydrolysis of pure DMSP (Kiene 1988).

Slurries were prepared by adding 10 ml of incubation water (overlying water from sampling site, amended with 300 μM $KNO_3$ and 2 mM glucose) to 30 ml serum bottles containing homogenized sediments (ca. 5 g) or scraped rocky biofilm (ca. 1 g). Glucose and $NO_3^-$ were added so that C and N would not be limiting during the period of incubation (4 h). Thus, these measurements reflect potential rates of the processes under these particular conditions. Serum bottles were hermetically sealed with Teflon stoppers and aluminum crimp seals. Each serum bottle, with the sample and incubation water, was purged with $N_2$ for 15 min to remove oxygen

**Table 1** Characterization of the study sites used for sediment and biofilm incubation experiments. Shown are salinity, DMSP, Chl *a*, total cell number (TCN), total organic matter (OM), grain size, $N_2O$ accumulation and denitrification rates from all sampling sites (mean ± SD, three replicates)

| Date | Location | Type of sample | Site code | Salinity (psu) | DMSP (μmoles $l^{-1}$ sed) | Chl *a* (μg $l^{-1}$ sed) | TCN (cells $g^{-1}$ sed) | OM (%) | Grain size (% dry weight) | | $N_2O$ (nmoles $h^{-1}$ $g^{-1}$ sed) | Denitrification (nmoles $N_2$ $h^{-1}$ $g^{-1}$sed) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | <63 μm | >2000 μm | | |
| Oct. 02 | Douro | Intertidal sandy sediment | ISS1 | 32.1 | 2.8 ± 0.4 | 24.4 ± 17.8 | $3.8 \times 10^7 \pm 1.3 \times 10^6$ | 1.3 ± 0.7 | 1.28 | 15.90 | 0.6 ± 0.3 | 4.1 ± 0.5 |
| Jul. 04 | Douro | Intertidal sandy sediment | ISS2 | 15.8 | 6.5 ± 0.9 | 36.0 ± 18.2 | $8.2 \times 10^7 \pm 5.5 \times 10^6$ | 0.2 ± 0.5 | 0.31 | 9.08 | 1.7 ± 0.1 | 16.2 ± 1.1 |
| Jul. 04 | Douro | Intertidal rocky biofilms | IRB1 | 15.3 | 66.4 ± 13.5 | 600.6 ± 247.6 | $1.7 \times 10^9 \pm 1.0 \times 10^8$ | 14.9 ± 3.2 | – | – | 21.6 ± 7.6 | 139.0 ± 6.6 |
| Aug. 04 | Douro | Intertidal sandy sediment | ISS3 | 19.7 | 5.0 ± 0.1 | 29.6 ± 3.2 | $1.6 \times 10^8 \pm 1.2 \times 10^7$ | 2.0 ± 1.6 | 0.38 | 12.15 | 5.4 ± 0.2 | 10.3 ± 5.0 |
| Aug. 04 | Douro | Intertidal sandy sediment | ISS4 | 19.7 | 5.2 ± 0.7 | 19.8 ± 2.2 | $5.5 \times 10^7 \pm 1.5 \times 10^6$ | 2.6 ± 0.1 | 0.54 | 14.67 | 1.7 ± 0.3 | 11.0 ± 3.3 |
| Aug. 04 | Douro | Intertidal rocky biofilms | IRB2 | 19.7 | 247.3 ± 86.2 | 197.9.0 ± 158.2 | $1.8 \times 10^9 \pm 1.9 \times 10^6$ | 74.7 ± 9.3 | – | – | 34.8 ± 5.2 | 140.9 ± 41.5 |
| Aug. 04 | Douro | Intertidal rocky biofilms | IRB3 | 19.7 | 97.2 ± 14.8 | 1015.0 ± 81.2 | $3.49 \times 10^9 \pm 8.1 \times 10^7$ | 51.5 ± 26.8 | – | – | 62.8 ± 11.3 | 145.3 ± 32.4 |
| Aug. 04 | Douro | Intertidal rocky biofilms | IRB4 | 19.7 | 73.5 ± 15.0 | 650.8 ± 81.6 | $7.8 \times 10^8 \pm 3.6 \times 10^6$ | 27.3 ± 12.1 | – | – | 19.7 ± 2.2 | 137.7 ± 15.6 |
| Sep. 04 | Douro | Intertidal sandy sediment | ISS5 | 6.5 | 4.4 ± 1.0 | 10.6 ± 1.2 | $5.0 \times 10^7 \pm 1.3 \times 10^6$ | 7.9 ± 1.5 | 0.00 | 11.04 | 0.2 ± 0.0 | 2.5 ± 0.0 |
| Sep. 04 | Douro | Intertidal sandy sediment | ISS6 | 7.0 | 4.4 ± 1.0 | 43.0 ± 2.2 | $2.0 \times 10^8 \pm 6.4 \times 10^6$ | 5.9 ± 0.9 | 0.00 | 9.30 | 2.8 ± 2.0 | 29.3 ± 0.8 |
| Sep 04 | Douro | Intertidal sandy sediment | ISS7 | 6.3 | 4.1 ± 0.3 | 23.6 ± 3.8 | $2.4 \times 10^8 \pm 1.9 \times 10^6$ | 1.3 ± 0.5 | 0.13 | 16.59 | 0.7 ± 0.1 | 15.7 ± 2.9 |
| Sep. 04 | Douro | Intertidal sandy sediment | ISS8 | 6.3 | 3.4 ± 1.2 | 19.1 ± 4.6 | $3.9 \times 10^7 \pm 1.0 \times 10^6$ | 2.4 ± 1.2 | 0.99 | 16.21 | 4.0 ± 0.3 | 7.4 ± 1.9 |
| Sep. 04 | Douro | Intertidal sandy sediment | ISS9 | 6.3 | 5.3 ± 0.7 | 19.6 ± 3.2 | $4.2 \times 10^7 \pm 5.9 \times 10^5$ | 2.1 ± 2.1 | 0.00 | 16.76 | 1.5 ± 0.3 | 7.9 ± 0.9 |
| Nov. 04 | Douro | Subtidal sandy sediment | SSS1 | 13.87 | 4.6 ± 1.2 | 18.8 ± 1.4 | $8.1 \times 10^7 \pm 4.7 \times 10^5$ | 0.5 ± 0.1 | 0.10 | 67.59 | 0.2 ± 0.1 | 18.7 ± 0.9 |
| Nov. 04 | Douro | Subtidal sandy sediment | SSS2 | 8.24 | 8.3 ± 1.0 | 8.0 ± 2.4 | $2.3 \times 10^8 \pm 1.3 \times 10^6$ | 1.1 ± 0.3 | 3.03 | 56.31 | 0.3 ± 0.0 | 8.2 ± 0.2 |
| Nov. 04 | Douro | Subtidal muddy sediment | SMS1 | 9.55 | 39.2 ± 10.6 | 30.2 ± 8.2 | $2.5 \times 10^8 \pm 1.0 \times 10^5$ | 10.9 ± 0.2 | 29.75 | 3.27 | 0.4 ± 0.1 | 8.5 ± 1.3 |
| Nov. 04 | Douro | Subtidal sandy sediment | SSS3 | 0.12 | 2.5 ± 0.6 | 1.2 ± 0.4 | $1.9 \times 10^7 \pm 9.4 \times 10^4$ | 0.3 ± 0.0 | 0.04 | 44.02 | 0.0 ± 0.0 | 0.4 ± 0.1 |
| Mar. 05 | Douro | Subtidal sandy sediment | SSS4 | 24.57 | 8.7 ± 1.9 | 12.2 ± 5.0 | $1.6 \times 10^8 \pm 3.3 \times 10^5$ | 2.3 ± 0.1 | 5.02 | 67.16 | 0.7 ± 0.2 | 6.4 ± 0.4 |
| Mar. 05 | Douro | Subtidal sandy sediment | SSS5 | 11.83 | 8.6 ± 1.6 | 4.6 ± 0.4 | $1.2 \times 10^8 \pm 3.9 \times 10^5$ | 2.1 ± 0.2 | 3.15 | 17.98 | 0.3 ± 0.1 | 11.6 ± 0.5 |
| Mar. 05 | Douro | Subtidal muddy sediment | SMS2 | 16.96 | 28.4 ± 18.6 | 15.1 ± 4.1 | $2.1 \times 10^8 \pm 1.4 \times 10^6$ | 11.0 ± 0.1 | 36.77 | 3.44 | 0.6 ± 0.3 | 6.4 ± 0.5 |
| Mar. 05 | Douro | Subtidal sandy sediment | SSS6 | 1.19 | 2.6 ± 0.5 | 0.4 ± 0.1 | $9.8 \times 10^6 \pm 1.9 \times 10^5$ | 0.3 ± 0.0 | 0.05 | 51.10 | 0.0 ± 0.0 | 0.2 ± 0.1 |
| Jan. 08 | Ave | Intertidal sandy sediment | ISS10 | 22 | 2.1 ± 0.7 | 1.1 ± 0.3 | $3.0 \times 10^7 \pm 1.9 \times 10^5$ | 0.8 ± 0.1 | 0.46 | 42.88 | 1.2 ± 0.1 | 26.7 ± 17.1 |
| Jan. 08 | Ave | Intertidal muddy sediment | IMS1 | 0 | 0.0 ± 0.0 | 1.0 ± 0.2 | $3.2 \times 10^8 \pm 5.5 \times 10^6$ | 13.1 ± 1.1 | 36.72 | 1.19 | 9.5 ± 2.1 | 61.2 ± 10.5 |
| Feb. 08 | Douro | Intertidal sandy sediment | ISS11 | 22 | 2.8 ± 0.0 | 39.1 ± 7.5 | $3.0 \times 10^7 \pm 1.6 \times 10^5$ | 0.4 ± 0.1 | 0.44 | 50.27 | 0.3 ± 0.1 | 8.1 ± 2.8 |
| Feb. 08 | Douro | Intertidal sandy sediment | ISS12 | 0 | 7.7 ± 0.9 | 5.4 ± 1.5 | $2.1 \times 10^8 \pm 3.0 \times 10^6$ | 1.1 ± 0.1 | 1.34 | 19.89 | 2.1 ± 0.3 | 18.6 ± 3.4 |
| Apr. 09 | Douro | Intertidal sandy sediment | ISS13 | 16 | 2.5 ± 0.3 | 7.4 ± 1.2 | $2.9 \times 10^8 \pm 4.2 \times 10^6$ | 1.3 ± 0.2 | 0.91 | 14.90 | 0.5 ± 0.1 | 6.0 ± 0.5 |

All samples were incubated in the dark for 4 h at constant temperature (20°C) with rotary shaking (70 rpm). At 0 h and 4 h, gas samples were collected and quantified as described below. For each site, the accumulations of $N_2O$ in DMSP-treated and control samples were compared with samples in which $N_2O$ reductase was completely blocked by addition of 20% (vol:vol) acetylene ($C_2H_2$).

## Culture studies

Cultures of *Ruegeria pomeroyi* DSS-3, previously named *Silicibacter pomeroyi* DSS-3 (Yi et al. 2007), was grown for 48 h in marine basal media, modified by addition of 10 mM glucose and 200 μM $NO_2^-$ and substitution of 0.1 mM FeEDTA for $FeSO_4$ (González et al. 1999, 2003). As the gene for $NO_3^-$ reduction was not identified in the genome sequence for this strain (Moran et al. 2004), nitrogen was added as $NO_2^-$. Three and a half milliliters of a stationary-phase culture ($OD_{540}$ 0.7–1.0) were placed in a 12 ml crimp-toped serum vial, sealed with a Teflon stopper and purged with $N_2$ for 15 min. The accumulation of $N_2O$, DMS, MeSH and $H_2S$ was measured in triplicate vials receiving progressive concentrations of DMSP (0–5 mM) and DMS (0–785 nM), as described for the slurry experiments. Total inhibition of nitrous oxide reductase was achieved by adding $C_2H_2$ (20% vol:vol) to parallel triplicate serum vials containing *R. pomeroyi* as described above. Incubations were performed for 4 h in the dark at 26°C and with constant rotary shaking speed (80 rpm). $N_2O$ and volatile sulfur gases (DMS, MeSH and $H_2S$) were measured at the beginning and end of the incubation as described below. Linearity of the processes during the incubation period was confirmed in selected tests (data not shown).

Nitrous oxide accumulations were calculated in all slurries and pure culture experiments (with and without DMSP and DMS additions) as the difference in $N_2O$ concentrations at 0 h and 4 h. Slurries with added $C_2H_2$ and no DMSP were run as positive controls for the complete inhibition of nitrous oxide reductase (Yoshinari and Knowles 1976) and to calculate denitrification rates. Denitrification rates were determined as the difference between the concentration of $N_2O$ produced with and without $C_2H_2$ (Joye et al. 1996). $N_2O$ accumulations in DMSP and DMS treated slurries were compared to those in parallel $C_2H_2$ treatments to calculate the percentage of nitrous oxide reductase inhibition in each treatment. The percentage of nitrous oxide reductase inhibition was calculated as: ($N_2O$ produced in treatments receiving DMSP or DMS)/($N_2O$ produced in treatments with $C_2H_2$) × 100.

## Analytical techniques

For $N_2O$ analysis, 6 ml of gas sample was collected from serum vials by simultaneously adding 6 ml of 3 M sodium chloride (NaCl) (Joye et al. 1996). A separate set of time zero samples were sacrificed for $N_2O$ analysis immediately after being purged with $N_2$. $N_2O$ was quantified using a Varian gas chromatograph (CP-3800) equipped with an electron-capture detector (ECD) with two Hay Sep D columns at 100°C and an automatic backflush system to prevent $C_2H_2$ from passing to the detector. The detector and injector temperatures were 250°C and 125°C, respectively (Joye et al. 1996; Magalhães et al. 2005b). For the ECD detector, a mixture of 5% methane in argon was used as the carrier gas. The retention time of $N_2O$ was 1.6 min and its concentration was calculated using a standard curve generated from certified gas standards ($N_2O$ in He, Scott Specialty Gas). The volatile sulfur gases were monitored by removing 50 μl headspace subsamples from the incubation vials with a glass gas-tight syringe and injecting these into the GC. DMS, MeSH and $H_2S$ were separated with a Mega-Bore silica plot column at 189°C and detected with a pulse flame photometric detector (P-FPD). Nitrogen was used as a carrier gas at 3 ml $min^{-1}$, and the retention times were 1.13, 1.43 and 2.53 min, respectively for $H_2S$, MeSH and DMS. DMS concentrations were determined using DMSP standards converted to DMS by sodium hydroxide (NaOH) treatment (Kiene and Service 1991). Standard curves were linear ($R^2 = 0.99$) over 5 orders of magnitude. Repeated injections of standards and samples yielded coefficients of variation between 4-8%. 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 1996a). The detection limit for both DMS and MeSH was 10 nM. The detection limit for $N_2O$ was 15 nM. $H_2S$ was measured on a relative peak area basis since no standard curve was available

for this compound. Dissolved concentrations of DMS and MeSH were calculated from measured headspace concentrations and empirical solubility coefficients, taking into account variations in temperature and salinity (Przyjazny et al. 1983; Dacey et al. 1984).

To measure DMSP concentrations in sediment and rocky biofilm samples, subsamples were cold alkali treated and the amount of DMS released was measured by gas chromatography as described above. While this procedure quantitatively converts DMSP to DMS (White 1982), the production of DMS from other sources may lead to an overestimate of DMSP in some samples (Kiene 1996b). For the sake of simplicity, however, we refer to the base-hydrolysable DMS pool in sediments and biofilms as DMSP. Sediment and biofilm DMSP was measured in triplicate sediment subcores (1 cc) from each sampling site. Samples were placed in 12 ml serum vials and sealed with Teflon septa and aluminum crimp seals. After sealing, 1 ml of 5 M NaOH was added and total DMS was measured in the headspace after 12 h of incubation at room temperature as described above. Sediment samples for chlorophyll *a* (Chl *a*) were collected by syringe core (1 cc) from the homogenized sediment and biofilm samples. Extraction was performed for triplicate subcores in a mixed solution of acetone, methanol and water (45:45:10) according to Joye et al. (1996), and Chl *a* concentration was determined spectrophotometrically (Strickland and Parsons 1972). The percent organic matter content of sediment was determined by drying the sediment at 60°C to a constant weight, followed by ignition in a muffle furnace at 550°C for 4 h and reweighing. Sediment particle size distribution was determined by sieving pre-combusted sediments (500°C for 4 h for the organic removal) in a sieve shaker device (<0.063 mm, >0.063 mm; >0.125 mm; >0.25 mm; >0.5 mm; >1 mm; >2 mm).

For total counts of microbial cells, 1 g of homogenized sample was added to 2.5 ml of saline solution (0.2 µm-filtered, 9 g $l^{-1}$ NaCl, 200 µl of 0.2 µm-filtered, 12.5% [v/v]Tween 80) and fixed with 1 ml of formaldehyde (0.2 µm-filtered, 4% [v/v]). The slurries were stirred at 150 rpm for 15 min followed by sonication for 20–30 s at low intensity (0.5 cycle, 20% amplitude). Sub-samples of the slurries were then stained with 4′,6′-diamidino-2-phenylindole (DAPI), and incubated in the dark for 12 min (Porter and Feig 1980). Samples were filtered onto black Nucleopore polycarbonate filters (0.2 µm pore size, 25 mm diameter, Whatman, UK) under gentle vacuum and washed with autoclaved, 0.2 µm-filtered distilled water. Membranes were placed on glass slides and cells were counted at 1875× with an epifluorescence microscope (Laphot, Nikon, Japan).

### Reagents and chemicals

Stock solutions of L-glucose, $NaNO_2$, $KNO_3$, $FeSO_4$, NaOH, NaCl, Tris HCl, $K_2HPO_4$ and $NH_4Cl$ were prepared in deionised water. All chemicals were obtained from Sigma-Aldrich or Merck and were of ACS grade (>99% purity). DMSP-Cl was purchased from Selact (Netherlands) and had a purity of >98%.

### Statistical analysis

Hierarchical cluster analysis (HCA) and two-dimensional principal component analysis (PCA) were used to detect inter-site differences in the environmental data collected (Table 1). For HCA, Euclidean distances were calculated from a 4th root transformation of the data. ANOSIM was used to test the statistical significance of differences between clusters generated by HCA. For ANOSIM, the R statistic is an absolute measure of how well groups separate and ranges between 0 (indistinguishable) and 1 (well separated). PRIMER version 6 (Clarke and Warwick 1994) was used to perform multivariate statistical analysis. Linear regression models were also applied to the environmental variables measured according to Zar (1996).

## Results

### Site characterizations

The characteristics of each sampling site in terms of total organic matter, Chl *a* content, grain size distribution, DMSP concentration (total hydrolysable DMS) and total cell counts are provided in Table 1. Sandy sediments of the Douro and Ave estuaries were composed mainly of highly permeable coarse sand and gravel (>0.5 mm), with low organic matter content. Muddy and rocky biofilm sites were characterized by higher values of organic matter content. Rocky biofilm sites had the highest Chl *a* and DMSP concentrations (Table 1).

Hierarchical cluster analysis of the environmental data for all sites show that samples from rocky sites were most distinct from those of the sandy and muddy sites. However, the sandy and muddy sites were still distinguishable from one another (Fig. 1) and the separation of these three sample types was statistically significant (ANOSIM, $R = 0.98$, $p = 0.001$). In the two-dimensional principal component analysis (PCA), the two axes together explained 80% of the variation in the variables measured for all sites (Fig. 2a). While PCA 1 was closely related to Chl *a* content, DMSP concentrations, total cell numbers and net $N_2O$ fluxes and denitrification rates (this axis explained 66% of the total variability), PCA 2 was more related to salinity and grain size. Of all three environments, the rocky sites had the highest Chl *a,* DMSP concentrations and net $N_2O$ flux rates (Fig. 2b–d). For all data, DMSP concentrations were strongly correlated with Chl *a* concentration ($R^2 = 0.96$, $p < 0.01$; Fig. 2SIa in Supplementary Material), and this was also the case when data from the muddy samples and rocky biofilms were considered separately ($R^2 = 0.93$ and 0.98, $p < 0.05$; Fig. 2SIb, c in Supplementary Material). DMSP concentrations were found to be positively correlated to the natural $N_2O$ effluxes measured for all sites (Fig. 3a); this relationship was strongest for the sandy subtidal sediment samples (Fig. 3b).

## Effect of DMSP on $N_2O$ emissions

The effect of DMSP on $N_2O$ fluxes was first studied by amending triplicate slurries from each site with 1.5 mM DMSP. At 24 out of 26 sites there was an increase of $N_2O$ accumulation in the slurries with DMSP addition compared to no amendment controls (Fig. 4a), suggesting the occurrence of an inhibitory effect of DMSP on the enzyme that catalyzes the reduction of $N_2O$. Based on comparisons to samples treated with 20% acetylene (i.e. 100% inhibition of nitrous oxide reductase), the addition of 1.5 mM of DMSP caused between 0 and 100% inhibition (with an average of $37 \pm 31\%$) of the nitrous oxide reductase step of denitrification (Fig. 4b). Total inhibition of the nitrous oxide reductase step of denitrification by 1.5 mM DMSP was achieved only from sandy site sediments, while no inhibition was evident for samples from two of the three muddy sediment sites (SMS1, IMS1; Fig. 4b).

Progressive additions of DMSP tested at seven different sites (ISS1, ISS2, IRB1, IMS1, ISS10, ISS12, ISS13) resulted in a proportional increase in $N_2O$ accumulation in the majority of the experiments performed ($R^2$ from 0.89 to 0.99, $p < 0.001$; Fig. 5). The exception was the muddy site (IMS1), for which an accumulation of $N_2O$ was only detected at the highest DMSP concentration tested (5 mM DMSP; Fig. 5d). The magnitude of $N_2O$ accumulation for a given concentration of DMSP added varied between the different environments studied (Fig. 5). The effect of DMSP was also tested in cultures of the bacterium *R. pomeroyi*. The results confirmed that DMSP reduced nitrous oxide reductase activity, with proportional increases of $N_2O$ in treatments with progressively higher concentrations of added DMSP ($R^2 = 0.72$, $p < 0.01$; Fig. 6a).

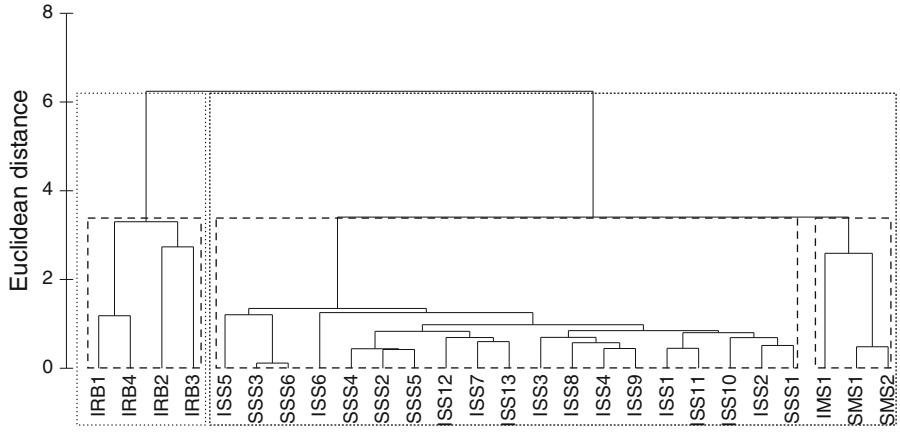


**Fig. 1** Dendogram for hierarchical cluster analysis of the 26 samples based on group-average linking of Euclidean distances calculated for 4th root-transformed environmental data presented in Table 1. Two or three clusters of samples were generated at the Euclidean distance level of 6.2 and 3.4, respectively

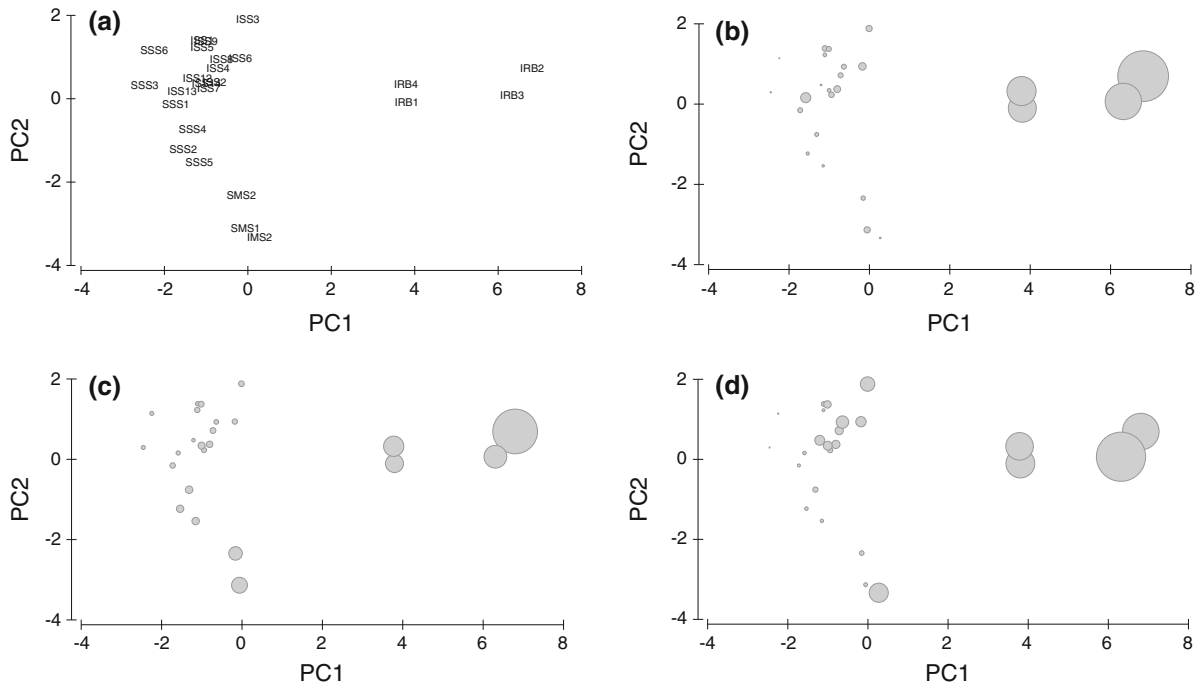


**Fig. 2** Two-dimensional principal components analysis (PCA) of the 26 samples calculated for 4th root-transformed environmental data presented in Table 1. Values of environmental variables for each sample were represented as circles of diameter scaled linearly to the magnitude of the value (**b–d**). Chlorophyll *a* concentration (**b**); DMSP concentration (**c**); $N_2O$ accumulation rates (**d**)

### DMS vs. $N_2O$ accumulation

DMSP additions to sediment slurries and cultures of *R. pomeroyi* resulted in net DMS accumulations. In all cases rates of DMS accumulation were roughly proportional to the concentration of DMSP added (Figs. 6a, 7). With the exception of samples from the muddy site (Fig. 7a), DMS and $N_2O$ accumulation rates showed a similar pattern up to 1.5 mM of DMSP added ($R^2$ from 0.93 to 0.97, $p < 0.001$; Fig. 7b–d). DMS and $N_2O$ concentrations were correlated across the full range of DMSP additions in *R. pomeroyi* cultures ($R^2 = 0.74$, $p < 0.01$) (Fig. 6a). MeSH and $H_2S$ were below detection limits for all sediment samples (data not shown).

Because DMSP was degraded to DMS (Fig. 7), we carried out separate tests to determine whether DMS alone had any inhibitory effects on nitrous oxide reductase activity in sediment slurries and *R. pomeroyi* cultures. For all sediments tested, progressively higher DMS additions did not cause a progressive accumulation of $N_2O$ in the sediment slurries (Fig. 8). This was also true for the *R. pomeroyi* cell suspensions, where $N_2O$ accumulation was only evident at the highest DMS amendment (Fig. 6b). These findings suggest that DMS, the main enzymatic hydrolysis product of DMSP, likely had no direct inhibitory effect on the nitrous oxide reductase step of denitrification.

## Discussion

### Effect of DMSP on the denitrification $N_2O$ reduction step

In this study, the effect of DMSP on the nitrous oxide reduction step of the denitrification process was investigated in different benthic estuarine environments and in pure cultures of *R. pomeroyi,* a marine bacterium that can both denitrify (Moran et al. 2004) and demethylate and cleave DMSP (González et al. 2003). We found that progressively higher additions of DMSP to intertidal sandy sediments and rocky biofilms of the Douro and Ave estuaries resulted in greater inhibition of the reduction of nitrous oxide

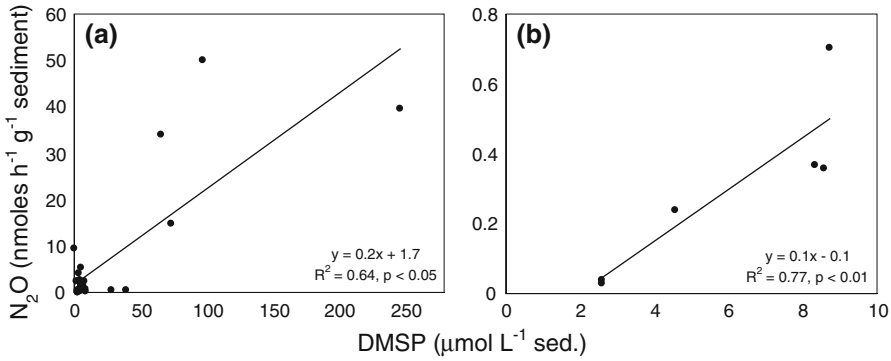


**Fig. 3** Linear relationship between DMSP concentration and rates of $N_2O$ accumulation for all sampling sites (**a**) and for sandy subtidal samples (**b**)

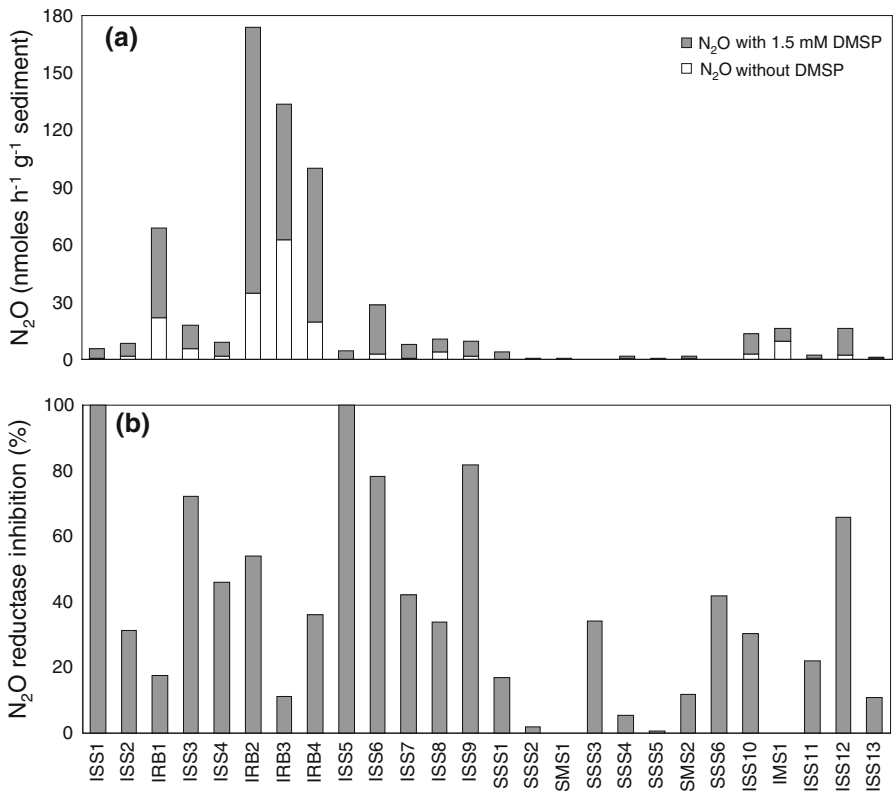


**Fig. 4** Variation among sampling sites of $N_2O$ accumulation rates for treatments with and without 1.5 mM of DMSP (**a**) and percentage of inhibition of nitrous oxide reductase caused by DMSP addition (**b**)

($N_2O$) to atmospheric nitrogen ($N_2$). The occurrence of this inhibitory effect was also confirmed in cell suspensions of *R. pomeroyi*.

Our results from the cross-site comparison revealed that the percent inhibition (magnitude of $N_2O$ accumulation) of nitrous oxide reductase varied between 0 and 100% for the same added DMSP concentration (1.5 mM), with total inhibition achieved at the sandy sites, and no inhibition registered at two of the three muddy sediment sites (Fig. 4b). The variability observed in the different environments and the absence of a perfect linear relationship between added DMSP concentrations and $N_2O$ accumulation for all sites at which progressive DMSP

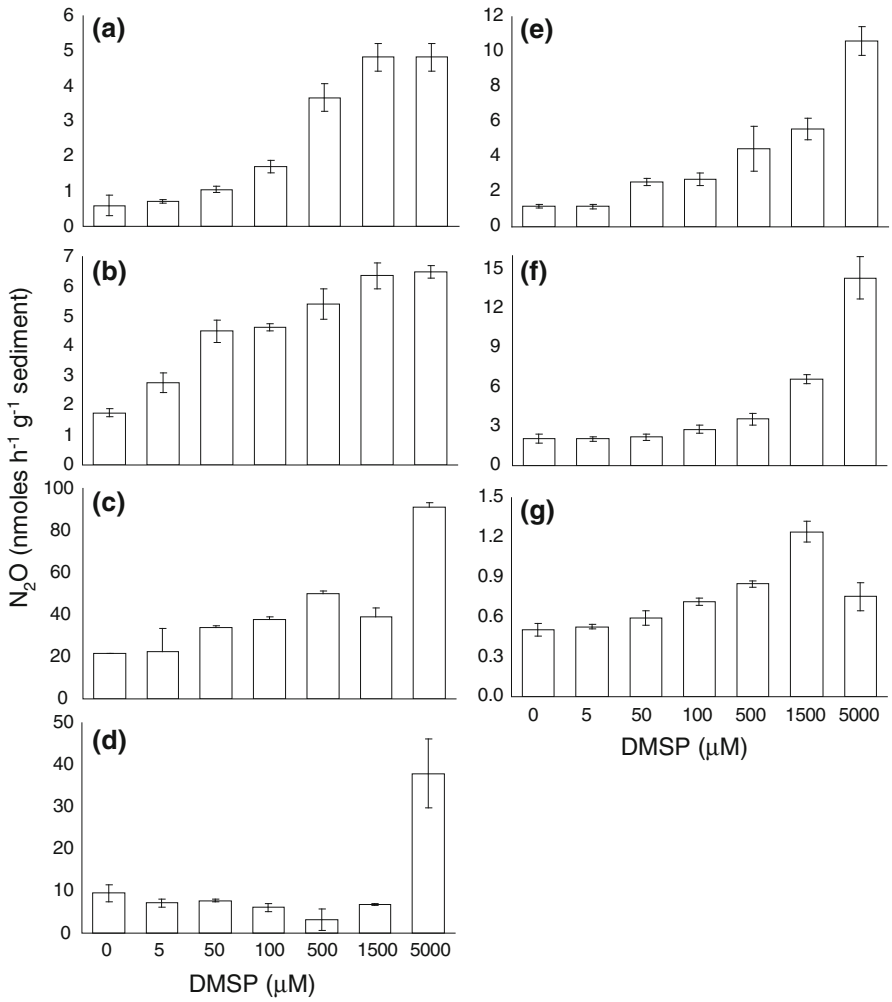


**Fig. 5** Evolution of $N_2O$ accumulation with progressive concentrations of DMSP in different intertidal sediment and rocky biofilm sites: **a** ISS1, **b** ISS2, **c** IRB1, **d** IMS1, **e** ISS10, **f** ISS12, **g** ISS13

concentrations were tested, suggest that DMSP per se may not be the agent that directly inhibits nitrous oxide reduction. However, at this stage, it is unclear if the inhibition of the nitrous oxide reductase step of denitrification was directly caused by DMSP itself or by one of the intermediate breakdown products, other than DMS, known to accumulate during its degradation (Kiene et al. 2000).

DMS is a major degradation product of DMSP and its formation could involve different cleavage pathways (Cantoni and Anderson 1956; Todd et al. 2007; Curson et al. 2008). Recent evidence, however, favors the non DMS-producing demethylation/demethiolation pathway as the major fate of DMSP in seawater and sediments. This pathway produces 3-methylmercaptopropionate, MeSH or 3-mercaptopropionate (Kiene and Taylor 1988; Kiene 1996a; Howard et al. 2008). Taking into account the several possible routes of bacterial DMSP degradation, we hypothesize that differences in the fate of DMSP in each environment, perhaps due to differences in bacterial communities that inhabit each site, could also modulate the inhibition of $N_2O$ reductase observed in our experiments. Differences among sites in chemical and physical parameters may also influence sediment adsorption capacities, which, in turn, could be responsible for differences in the availability of the compound(s) responsible for the inhibition of the nitrous oxide reductase step of denitrification. Interestingly, muddy sites were found to be less affected by DMSP additions in comparison to the rocky biofilms and sandy sediments. Thiols produced during DMSP catabolism are largely associated with the particle phase of sediments (Kiene

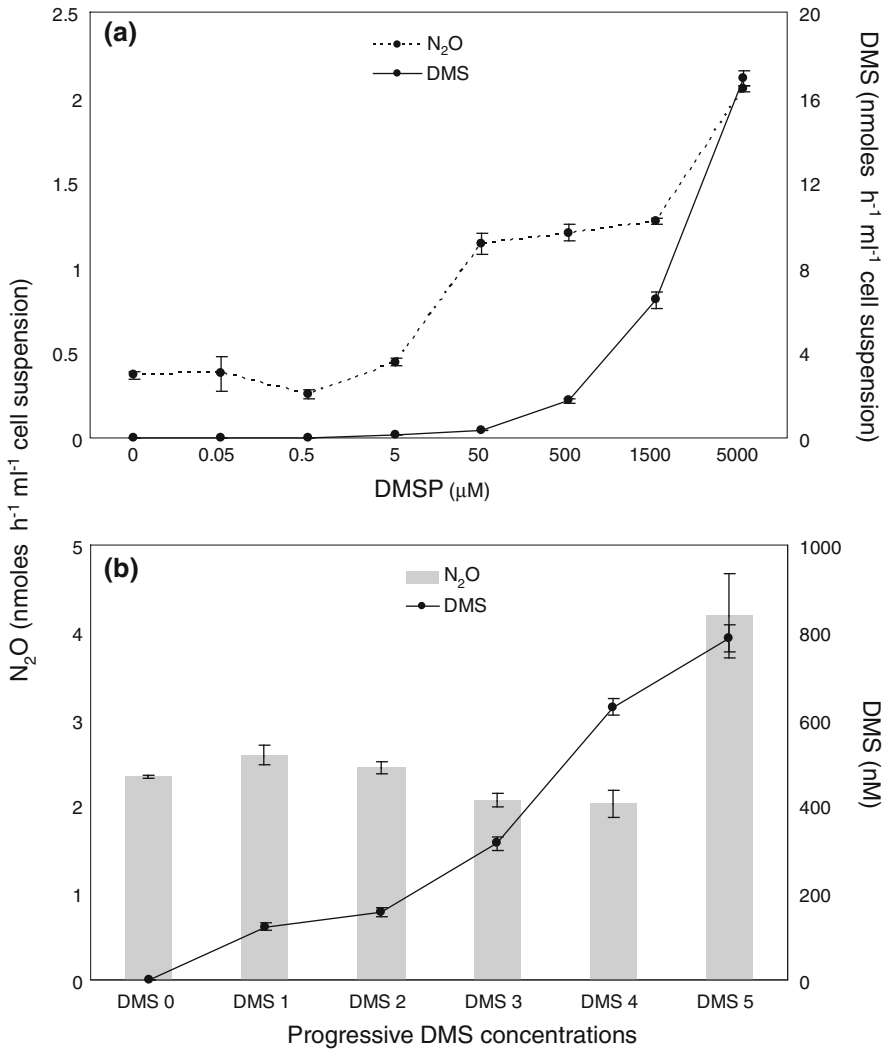


**Fig. 6** Evolution of $N_2O$ and DMS accumulation with progressive concentrations of DMSP in cell suspensions of *Ruegeria pomeroyi* (**a**) and evolution of $N_2O$ in cell suspensions of *R. pomeroyi* in treatments with five different volumes of DMS produced from the hydrolysis of pure DMSP achieving time zero DMS concentrations in the serum vials from 0 to 785 nM (**b**). Error bars = standard deviation of the mean of three replicates

1991) and muddy sediments likely have a very high capacity for thiol adsorption (Mopper and Taylor 1986). Thus, we hypothesize that the high tendency of the muddy sediments to adsorb or bind thiols was likely responsible for the reduced effect of DMSP on $N_2O$ accumulation in these samples. Only with the highest DMSP addition (5 mM) was clear response of $N_2O$ accumulation observed in muddy sediments. We interpret these results to indicate that the adsorption capacity was saturated at these DMSP concentrations.

While MeSH and $H_2S$ were also monitored in our experiments, these compounds did not accumulate at measurable concentrations in either the sediment slurries or in *R. pomeroyi* cell suspensions. Instead, DMS accumulated in concentrations that were proportional to DMSP additions, suggesting that lyase pathways were favored in our experiments. This is in agreement with previous studies based on observations from both sea water (Van Duyl et al. 1998) and bacterial cultures (Kiene et al. 2000), which demonstrated that DMSP availability controls DMS production. In *R. pomeroyi* the differential production of DMS and MeSH was previously found to be driven by DMSP availability as well as the growth rate of the bacterium (González et al. 1999, 2003; Kiene et al. 2000). Thus, inter-site differences in DMS patterns and magnitudes of accumulation between our different experiments may, in part, be explained by variation in microbial biomass and growth rates (Kiene et al. 2000). In addition, differences in the production and consumption of DMS by different microbial populations can also play important roles in

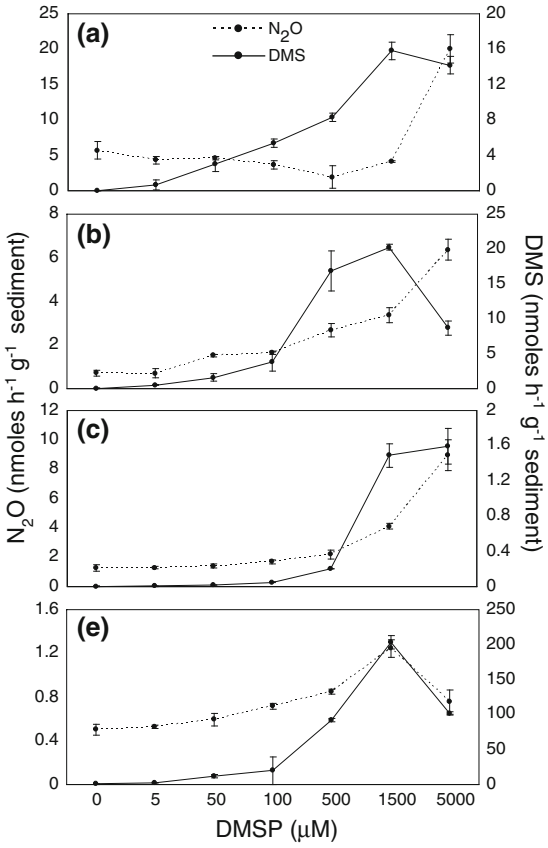


**Fig. 7** Evolution of $N_2O$ and DMS accumulation with progressive concentrations of DMSP in different intertidal sediment sites: **a** IMS1, **b** ISS10, **c** ISS12, **d** ISS13. Error bars = standard deviation of the mean of three replicates

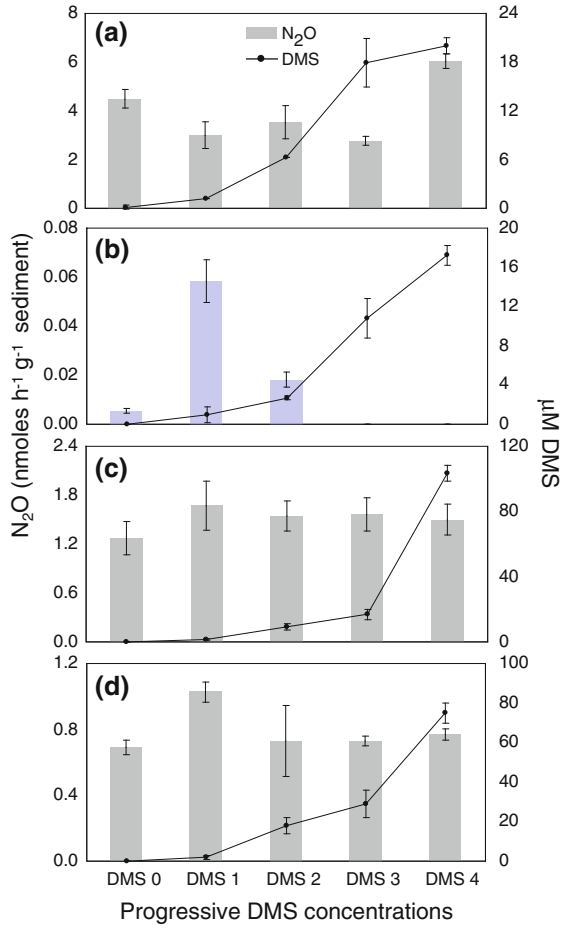


**Fig. 8** Rates of $N_2O$ accumulation with progressive concentrations of DMS in different intertidal sediments. Different volumes of DMS produced from the hydrolysis of pure DMSP achieved time zero DMS concentrations in slurries from 0 to 100 µM. **a** IMS1, **b** ISS10, **c** ISS12, **d** ISS13. Error bars = standard deviation of the mean of three replicates

determining DMS concentration in different environments (Vila-Costa et al. 2006; Del Valle et al. 2007; Schäfer 2007).

The increasing production of DMS with progressive DMSP additions followed the pattern of $N_2O$ accumulation. However, when DMS was added directly to the same sediment samples, we found no evidence for an inhibitory effect of DMS on the nitrous oxide accumulation. This was also true for *R. pomeroyi* cell suspensions, where $N_2O$ accumulated only in the highest DMS treatment (800 nM). The fact that DMS accumulation decreased for the highest DMSP concentration (5 mM) and was not followed by a decrease of $N_2O$ accumulation at sites ISS10 and ISS12 (Fig. 7b, c) also agrees with at most a minimal effect of DMS on $N_2O$ reductase. While no measurable concentrations were detected for MeSH and $H_2S$ during our experiments, we cannot discount the possibility of an effect of these compounds on the observed inhibition as we were limited by the detection limit of our quantification methods for these two compounds. In fact, $H_2S$ has been previously identified as an inhibitor of the nitrous oxide reductase (Sørensen et al. 1980; Senga et al. 2006). However, the concentrations of $H_2S$ that were reported to affect $N_2O$ reduction to $N_2$ during denitrification were much higher than the detection limit of the analytical method used to quantify $H_2S$ in this study. Other degradation products of DMSP, such as 3-mercaptopropionate, acrylate were not monitored in this study, but are generated during the different DMSP degradation pathways (e.g. Kiene

and Visscher 1987; Kiene and Taylor 1988; Taylor and Gilchrist 1991; Visscher and van Gemerden 1991; Yoch 2002; Todd et al. 2007). It will be the objective of our future research to discover the exact compound(s) involved in this inhibitory interaction.

## DMSP concentrations and $N_2O$ effluxes under natural conditions

DMSP is a widespread and important organic sulfur compound in the environment and it occurs at high concentrations in many marine micro- and macroalgae and a few higher plants (e.g. Yoch 2002). Intracellular concentrations of DMSP in marine phytoplankton range from 1–50 mM in diatoms to 100–300 mM in prymnesiophytes and dinoflagellates (Keller et al. 1989; Yoch 2002). In addition, marine sediments can contain approximately 1000-fold more DMSP than overlying water, with published values ranging between 0.5 and 897 μmol $l^{-1}$ of sediment (Nedwell et al. 1994; Jonkers et al. 1998; Yoch 2002). In this study, DMSP concentrations for all samples analyzed varied between 0 and 247 μmol $l^{-1}$ of sediment. These values are within the range found in previous studies (Nedwell et al. 1994; Jonkers et al. 1998; Yoch 2002). Given its biological source, a correlative relationship between Chl *a* and DMSP is commonly observed in marine systems (Yang et al. 2005), but is not always significant (e.g. Barnard et al. 1984 and Turner et al. 1988). In our study, Chl *a* correlated significantly with DMSP concentrations at the rocky biofilms and muddy sites, but no clear relationship between these two variables was observed for the intertidal and subtidal sandy sites. A lack of a significant correlation could be a reflection of the differences in primary producer communities present in the different sites as not all algae contain DMSP (Keller et al. 1989).

Our data show that higher $N_2O$ accumulation rates were observed for the rocky biofilm sites with higher Chl *a* and DMSP concentrations (Fig. 2). While not conclusive, these data do suggest that the inhibitory interactions identified in experimental incubations may be occurring under natural conditions. The significant linear and positive relationship observed between $N_2O$ accumulation rates and DMSP concentrations (Fig. 3) supports this hypothesis. This inhibitory interaction could also explain the previously measured high values of net $N_2O$ effluxes from undisturbed rocky biofilm samples under in situ conditions, with $N_2O$:$N_2$ ratios up to 40% (Magalhães et al. 2005a, b). These $N_2O$:$N_2$ ratios are much higher than are typical for natural coastal sediment systems (ca 5%; Seitzinger 1988). Higher $N_2O$ net effluxes are usually attributed to high $NO_3^-$ availability in eutrophic systems and consequently to higher denitrification rates (Kieskamp et al. 1991; Robinson et al. 1998; Seitzinger 2000; Trimmer et al. 2000; Magalhães et al. 2005a, b). However, in this study we identified DMSP as another possible factor that could affect net $N_2O$ production. Interestingly, our previous studies (Magalhães et al. 2005a, b) revealed no significant relationship between rates of $N_2O$ accumulation and $NO_3^-$ availability, suggesting that water column $NO_3^-$ concentration per se did not control the previously observed $N_2O$ production rates in rocky biofilm sites reported in Magalhães et al. (2005a, b).

## Ecological implications

The ecological implications and the environmental importance of the inhibitory effect of DMSP on denitrification need to be rigorously evaluated to provide a realistic view of the global significance of this process. We believe, however, that marine and estuarine micro- and macroalgae blooms provide ideal conditions for the inhibitory interaction described in this study to occur. In nutrient-enriched aquatic systems, blooms of micro and/or macroalgae are followed by dystrophic events during which micro- and macroalgae degrade, releasing large amounts of particulate and dissolved organic matter, dissolved inorganic nitrogen (DIN) and dissolved organic nitrogen (DON) (Tyler et al. 2001; Sundback et al. 2003). Respiration of this organic matter often results in hypoxia in both sediments and the water column. The shift to hypoxia or anaerobic conditions facilitates fixed nitrogen removal from the environment via denitrification (Seitzinger 1988; Ogilvie et al. 1997; Seitzinger 2000). Another consequence of eutrophication-linked micro- and macroalgae blooms (Jonkers et al. 1998) is higher DMSP production (van Duyl et al. 1998), which is released during algal lysis or senescence (Stefels and van Boekel 1993). Thus, eutrophic conditions likely maximize the activity of bacterial communities with critical roles in nitrogen

metabolism and DMSP decomposition. Despite significant research efforts, the global $N_2O$ budget remains poorly understood (Dickinson and Cicerone 1986; Ravishankara et al. 2009). Thus, a more comprehensive understanding of the interaction between DMSP and $N_2O$ may help resolve the $N_2O$ budget in marine systems. It would also provide insight into the potentially offsetting effects of this interaction on climate change through the promotion of climate cooling (DMS) (Charlson et al. 1987; Lawrence 1993; Vallina and Simó 2007) and warming ($N_2O$) gas emissions (Dickinson and Cicerone 1986). Our data suggest that the effects of DMSP on denitrification may be more complex than the specific inhibition identified here. As such, future research is necessary to identify additional interactions between organic sulfur compounds and the denitrification process for a better understanding of the marine N and S cycles.

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