



# **Revisiting the dilution technique to quantify the role of microzooplankton in DMS(P) cycling: Laboratory and field tests**

Rafel Simo, Violeta Saló, Albert Calbet

## **► To cite this version:**

Rafel Simo, Violeta Saló, Albert Calbet. Revisiting the dilution technique to quantify the role of microzooplankton in DMS(P) cycling: Laboratory and field tests. *Journal of Plankton Research*, 2010, 32 (9), pp.1255. <10.1093/plankt/FBQ041>. <hal-00586124>

**HAL Id: hal-00586124**

**<https://hal.science/hal-00586124v1>**

Submitted on 15 Apr 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization



Journal of  
Plankton Research

**Revisiting the dilution technique to quantify the role of  
microzooplankton in DMS(P) cycling: Laboratory and field  
tests**

Journal:	<i>Journal of Plankton Research</i>
Manuscript ID:	JPR-2009-232.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	16-Mar-2010
Complete List of Authors:	Simo, Rafel; Institut de Ciències del Mar, CSIC, Marine Biology and Oceanography Saló, Violeta; Institut de Ciències del Mar, CSIC, Marine Biology and Oceanography Calbet, Albert; Institut de Ciències del Mar, CSIC, Marine Biology and Oceanography
Keywords:	DMS, DMSP, microzooplankton, dilution



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**Revisiting the dilution technique to quantify the role of microzooplankton  
in DMS(P) cycling: Laboratory and field tests**

Violeta Saló, Rafel Simó<sup>\*</sup>, Albert Calbet

Department of Marine Biology and Oceanography, Institut de Ciències del Mar, CSIC, Pg.  
Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalunya, Spain.

Keywords: dimethylsulfide, dimethylsulfoniopropionate, dilution technique,  
microzooplankton, grazing

<sup>\*</sup>Corresponding author: rsimo@icm.csic.es, phone: +34 932309590, fax : +34 932309555

For Peer Review

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**ABSTRACT**

With the aim of re-examining the suitability of the dilution technique in studies of dimethylated sulfur cycling in the sea, we conducted two types of experiments: one with a simplified food chain of cultured organisms, and secondly with field-sampled natural communities. In the case of *Isocrhysis galbana* being grazed by *Oxyrrhis marina*, algal growth ( $\mu$ ) and grazing mortality ( $m$ ) rates calculated using either chlorophyll *a* (chl*a*) or particulate dimethylsulfoniopropionate (DMSPp) were very similar. By means of a parallel incubation of prey with no predator, the regression-derived phytoplankton  $\mu$  was estimated. Net 24-h changes in DMS concentration showed a linear trend against DMSP grazing rates, with a slope that allowed estimation of the rate of DMS production due to grazing. When the method was applied to natural communities from coastal NW Mediterranean waters,  $\mu$  values of the bulk chl*a*-containing phytoplankton assemblage were higher than that of the DMSP-containing algae, but  $m$  were not significantly different. The grazing-mediated DMS production rate was successfully estimated, and parallel incubations with and without a specific inhibitor of bacterial DMS metabolism allowed us to estimate the gross DMS production by the whole community and the bacterial DMS consumption rate. A further experiment showed that bacterial consumption was not affected by dilution.

## INTRODUCTION

The dilution technique (Landry and Hassett, 1982; Landry et al., 1995) has been widely used to determine the impact of microzooplankton grazing on phytoplankton biomass and production in natural waters (Calbet and Landry, 2004; Calbet, 2008). This technique allows rates of grazing on, and growth of, natural phytoplankton assemblages to be obtained with minimal manipulation. It consists of a dilution series of whole seawater with particle-free seawater added to create a gradient of microzooplankton grazing pressure on phytoplankton. The technique is built upon the assumptions that phytoplankton growth is exponential and independent of the dilution if nutrients occur at non-limiting concentrations, whereas phytoplankton mortality is density-dependent and proportional to microzooplankton concentration.

The dilution technique has also proved to be useful to determine some of the biogeochemical fluxes associated with microzooplankton herbivory, such as ammonium uptake and regeneration (Neuer and Franks, 1993). Likewise, it has recently been explored for obtaining rates of dimethylsulfide (DMS) production derived from microzooplankton grazing (Wolfe et al., 2000; Archer et al., 2001a; Archer et al., 2003). DMS is the most abundant volatile sulfur compound in surface oceanic waters, where its concentration is supersaturated, so it tends to diffuse to the atmosphere (Andreae, 1990). This continuous emission of DMS from the ocean (estimated global flux of  $15\text{--}33 \cdot 10^{12}$  gS year<sup>-1</sup>; Kettle and Andreae, 2000; Simó and Dachs, 2002) is the most important natural source of sulfur to the global troposphere, with potentially large implications for aerosol acidity and cloud formation and microphysics (Charlson et al., 1987; Andreae, 1990). The concentration and emission of DMS from the surface ocean is driven by tight biogeochemical cycling involving physicochemical and biological processes.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

DMS is produced through the enzymatic cleavage of dimethylsulfoniopropionate (DMSP), a metabolite synthesized by many marine phytoplankton species (Keller et al., 1989; Stefels et al., 2007). The enzymes responsible for the conversion of DMSP into DMS (generally designated ‘DMSP lyases’) occur in most DMSP-producing phytoplankters and in some bacteria. The plankton community structure and dynamics have been shown to influence the rates and yields of the DMSP-to-DMS conversion, together with a number of meteorological (solar radiation spectrum and intensity, wind speed) and chemical (inorganic nutrients, colored dissolved organic matter) parameters (Simó, 2001; Simó, 2004; Stefels et al., 2007). A number of laboratory (Dacey and Wakeham, 1986; Malin et al., 1994; Christaki et al., 1996; Wolfe and Steinke, 1996) and field studies (Daly and DiTullio, 1996; Kwint et al., 1996; Archer et al., 2003) have demonstrated that zooplankton grazing enhances DMS production, probably by facilitating the mixing of algal DMSP and algal or bacterial DMSP lyases. Protozoans and other microzooplankton are major grazers in the ocean (Burkill et al., 1993) and, as such, are expected to play a central role in DMS production.

Several authors have examined the potential application of the dilution technique to quantify the growth and grazing-mediated mortality rates of DMSP-producing phytoplankton (Wolfe et al., 2000; Archer et al., 2001b; Archer et al., 2003; Fredrickson and Strom, 2009) and the grazing-mediated DMS production rate (Wolfe et al., 2000; Archer et al., 2001a; Archer et al., 2003) in plankton communities from temperate and subpolar waters. While growth and mortality rates were successfully measured, assays for DMS production did not always exhibit the significantly linear behavior required to reliably estimate production rates (Wolfe et al., 2000; Archer et al., 2001a), which prompts a further assessment of the method.

The limitations and pitfalls of the dilution approach that have been highlighted by several authors (Gallegos, 1989; Chavez et al., 1991; Evans and Paranjape, 1992; Landry et al., 1995; Dolan et al., 2000) might have hampered its applicability to studies of the DMS cycle. We aimed to test the utility of the method for quantifying DMSP consumption and DMS production through microzooplankton herbivory, with a simplified food chain of cultured organisms. For this purpose we used monospecific cultures of a DMSP-producing prey (the prymnesiophyte *Isochrysis galbana*) and an herbivorous protist (the heterotrophic dinoflagellate *Oxyrrhis marina*). A similar laboratory experiment with the same species has been carried out previously by Archer et al. (Archer et al., 2001a). The special feature and novelty of our own study is that we compared growth and grazing rates obtained from chlorophyll *a* (chl<sub>a</sub>), algal cell counts and DMSP measurements in our model system. Furthermore, replicate bottles with prey but no predator were prepared in order to calculate the specific growth rate of phytoplankton and compare it with that estimated from the regression analysis of the dilution series.

We report also on two identical dilution experiments conducted with natural plankton communities. Additional bottles were prepared to estimate gross DMS production and bacterial DMS consumption. Our goal was to test the applicability of this technique to quantify grazing-derived rates of DMSP consumption and DMS production and, hence, to assess the role of microzooplankton herbivory in the dynamics of dimethylated sulfur in meso- to oligotrophic surface waters.



METHOD

Laboratory experiment

The prymnesiophyte *Isocrhysis galbana* (obtained from the Provasoli-Guillard National Center of Culture of Marine Phytoplankton in 1988 ) was maintained in filtered, autoclaved seawater enriched with f/2 medium at 19°C in a 100  $\mu\text{E m}^{-2} \text{s}^{-1}$  14:10 hours light:dark cycle. The dinoflagellate *Oxyrrhis marina* (originally isolated from north-western Mediterranean coastal waters in 1995) was grown on *Rhodomonas salina* and incubated at the same temperature and light conditions as the prymnesiophyte. Stock cultures of both predator and prey were maintained under non-axenic conditions in 2 L Pyrex glass bottles. Before the experiment *O. marina* was transferred to the dark and starved for 3 days to allow grazers to remove and digest all prey.

Small aliquots of *O. marina* and exponentially growing *I. galbana* were mixed in 0.2  $\mu\text{m}$ -filtered and autoclaved seawater enriched with f/2 medium to produce an experimental stock with a prey to predator ratio of approximately 20:1. Once the initial experimental stock (termed 100%) was obtained, 75%, 50% and 25% dilution treatments were prepared by addition of filtered, autoclaved seawater enriched with f/2 medium. The actual level of dilution was determined from the initial concentration of *I. galbana* in each bottle relative to the concentration in the 100% treatment (undiluted bottle). All treatments were prepared in duplicate, 620 mL Pyrex bottles. The use of f/2 medium ensured that nutrients would not be limiting over the 24 hours experimental period. In addition, two bottles containing only *I. galbana* at the same concentration as that of the 100% bottles were prepared, and named 100iso. The latter bottles were used to directly obtain the net growth rate of the phytoplankter and the net DMS production rate in the absence of grazing.

All bottles were filled leaving no headspace, capped and rotated gently by hand prior to subsampling. Temperature and light conditions were the same as described above. The incubation time was 24 hours, with two sampling points;  $t_0$  and  $t_{24}$ . Subsamples of 350 mL from each dilution bottle were used for cell enumeration of prey, chl *a* measurements, and DMS(P) analyses. Predators from the undiluted water were enumerated at  $t_0$ .

### Experiments with natural communities

Seawater was sampled at 41.22° N, 02.13° E, 1.5 km off the north western Mediterranean coast, from a water-column depth of ca. 40 m. Water was collected from 5 meters depth at 11:00h local time on the 14 June and 31 July 2006, with a 15 L transparent hydrographic bottle and was gently siphoned into a carboy and rapidly transported to the laboratory. Light and temperature were measured at the sampling depth with a LI-COR LI-1400 data logger and a YSI 30 portable temperature meter.

A volume of the sampled water was gravity filtered through 0.2  $\mu$ m with a Pall Acropak 0.8/0.2 500 capsule previously rinsed thoroughly with HCl and distilled water. This water was used to dilute the natural whole water and prepare all the dilution bottles. Each dilution treatment (2.3 L, transparent polycarbonate bottles) was prepared in duplicate, except that the 100% was prepared in six replicates: two were used for the micrograzing dilution experiment and incubated in the light, two were spiked with dimethyldisulfide (DMDS) to a final concentration of 200 nM, and the other two were left as dark controls. The dilution bottles were amended with a nutrient mixture (15  $\mu$ M  $\text{NH}_4\text{Cl}$  and 1  $\mu$ M  $\text{Na}_2\text{HPO}_4$ ) in order to ensure nutrients would not be limiting algal growth, as such limitation would distort the results of the

dilution series. Two 100% bottles (i.e. undiluted seawater) were incubated in parallel without nutrient enrichment to be used as controls to correct for the natural algal growth.

Dilution bottles were incubated at the *in situ* temperature right under the water surface in a large (600 L) outdoor incubator with open-circuit water running from the coastal seawater intake at the Institut de Ciències del Mar, Barcelona. Natural sunlight was dimmed with one layer of neutral plastic mesh, which allowed ca. 60% of surface irradiance. The DMDS-amended and corresponding control bottles were incubated in the dark. The experiments lasted 24 hours. The initial chl<sub>a</sub> and DMS(P) concentrations were estimated from those measured in the whole and particle-free waters from which all the dilution bottles were prepared. The light incubated bottles were subsampled at  $t_{24}$  for chl<sub>a</sub> and sulfur analyses. The dark incubated bottles were subsampled at  $t_4$ ,  $t_6$ ,  $t_{10}$ ,  $t_{20}$  and  $t_{24}$  for sulfur analyses.

In order to test if bacterial DMS consumption is affected by dilution with 0.2  $\mu\text{m}$  filtered (low-bacteria) water, we conducted a further experiment on 6 July. Seawater was collected from the same location and a full series of dilution bottles was prepared in duplicate as described above. One replicate of each was spiked with DMDS, and both were incubated in the dark over 24 hours. Subsamples for DMS analysis were withdrawn at  $t_6$ ,  $t_{10}$  and  $t_{24}$ .

Cell counts

In the laboratory experiment, a Multisizer III Coulter Counter was used to obtain cell abundances of both predator and prey. Subsamples of 20 mL were analyzed in duplicate for each experimental bottle. When predator numbers were too low to be accurately measured with the Coulter Counter ( $<100 \text{ cells mL}^{-1}$ ), aliquots were preserved in 4% acidic Lugol's solution and counted in Sedgewick-Rafter counting chambers under the microscope.

The undiluted natural community samples were examined under microscopically to count microzooplankton and phytoplankton larger than 5  $\mu\text{m}$ . Bacteria and autotrophic pico- and nano-eukaryotes were counted following flow cytometry analysis procedures (Olson et al., 1993; Gasol and del Giorgio, 2000). Samples of 1.2 ml for bacterial abundance determination were preserved with 1% paraformaldehyde + 0.5% glutaraldehyde (final conc.), and kept frozen at  $-80^{\circ}\text{C}$  until analysis using a Becton-Dickinson FACSCalibur flow cytometer after staining with SybrGreen I (10,000x). Bacteria were detected and counted by their signature when plotting side scatter (SSC) versus green fluorescence (FL1) and FL1 vs. red fluorescence. *Synechococcus*, *Prochlorococcus* and photosynthetic picoeukaryote abundances were estimated from 2-mL samples preserved and stored as above, and analyzed in the FACSCalibur flow cytometer in unstained aliquots. The various phytoplankton groups were detected and counted by their signature when plotting side scatter (SSC) vs. red fluorescence (FL3) and versus orange fluorescence (FL2).

### Chlorophyll *a* analyses

Aliquots of 150 to 300 mL were filtered gently through a GFF filter and kept frozen at  $-20^{\circ}\text{C}$  until analysis. Chl *a* concentration was measured fluorometrically in 90% acetone extracts of the filters with and without acidification, following (Strickland and Parsons, 1972).

### Analysis of sulfur compounds

A 40 mL aliquot of seawater was withdrawn from each experimental bottle at  $t_0$  and  $t_{24}$  for sulfur analyses. DMS was measured immediately using a purge-and-trap/gas chromatography system modified from Simó et al. (1996). Volumes of 3 to 10 mL of GF/F filtered water were purged with He for 4 to 10 minutes, the stripped DMS was cryogenically trapped in a teflon

1  
2  
3 tube immersed in liquid nitrogen, injected by placing the trap in hot water, and separated and  
4  
5 quantified in a Shimadzu 14A gas chromatograph equipped with a flame photometric  
6  
7 detector. Daily calibrations were performed by injecting volumes of a gaseous DMS standard  
8  
9 obtained from a permeation tube. Total (particulate + dissolved) DMSP (DMSPt) was  
10  
11 measured by adding one pellet of NaOH to 10 mL of sample water gas tight vials, which were  
12  
13 stored in the dark for 1 to 5 days. In this way all the cells were disrupted and DMSP was  
14  
15 hydrolyzed into DMS. The samples were then analyzed as for DMS, and the result was  
16  
17 corrected for pre-existing DMS. Dissolved DMSP (DMSPd) was determined by gravity  
18  
19 filtering 3 mL of sample water through a GF/F filter, adding one NaOH pellet to the filtrate,  
20  
21 keeping it in the dark for 1 to 5 days, measuring the evolved DMS, and subtracting the pre-  
22  
23 existing DMS concentration. Particulate DMSP (DMSPp) was calculated by the difference  
24  
25 between DMSPt and DMSPd. Two replicates were run for each experimental bottle.  
26  
27  
28  
29  
30  
31  
32  
33

### 34 35 Calculation of growth and grazing rates

36  
37 Net growth rates ( $r$ ) of the measured algal variables (chl $a$ , *I. galbana* cell counts and  
38  
39 DMSPp), were determined from initial ( $C_{i0}$ ) and final ( $C_{i24}$ ) values assuming an exponential  
40  
41 growth model:  
42  
43  
44  
45

$$46 \quad r = \ln (C_{i24}/C_{i0})/t$$

47  
48  
49  
50  
51 The  $r$  values of duplicate bottles were plotted against the level of dilution (fraction of whole  
52  
53 water in the dilution treatment), and model I regression analysis was used to compute the  
54  
55 specific growth rate of the algae ( $\mu'$  = intercept) and the rate of mortality due to grazing ( $m$  =  
56  
57 slope). For the field experiment, because the intercept of the equation would provide an  
58  
59 overestimation of phytoplankton instantaneous growth rates (nutrients were added to these  
60

bottles), growth rates ( $\mu$ ) were obtained from net growth in unamended 100% bottles plus mortality rate  $m$  (Landry and Hassett, 1982) or, in other words, the intercept  $\mu'$  minus the net growth rate difference between nutrient amended and unamended 100% bottles. The errors associated with  $m$  and  $\mu$  were the standard errors of the slope and the intercept, respectively, provided by the regression analysis.

The net growth rate obtained in the laboratory experiment for the 100iso incubation bottles ( $r_{iso}$ :100% treatment with no predators) was compared to the value provided by the intercept of the regression analysis of the specific growth rates vs the dilution factor ( $\mu'$ ). This way the assumption that an infinite dilution would provide the growth rate of the prey in the absence of grazing was verified.

### Calculation of grazing-mediated DMS production

The difference between initial and final concentrations of DMS was used to calculate net DMS production in duplicate bottles at each dilution level. In parallel, DMSP grazing rates at each dilution were calculated by scaling DMSP mortality rates  $m_{DMSP}$  to the dilution factor and multiplying them by the mean DMSP concentration in each dilution bottle ( $\langle C_{DMSP} \rangle$ ), calculated according to the equation of Frost (1972):

$$\langle C_{DMSP} \rangle = C_{to} [e^{(\mu-m)(t_1-t_0)} - 1] / (t_1 - t_0) (\mu - m)$$

Net DMS production rate values were paired to the corresponding DMSP grazing rates, and a model I regression analysis was conducted. The slope provided the daily DMS production per grazed DMSP (nM DMS nM DMSP<sup>-1</sup>), which was multiplied by the mean DMSP concentration in the control (unamended and undiluted) bottles to obtain the rate of DMS production due to grazing ( $P_g$ ). The intercept ( $P_{ng}$ ) corresponded to the net DMS production that resulted from processes unrelated to grazing, namely algal exudation and cell lysis,

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

bacterial cleavage of initial DMSPd, and bacterial DMS metabolism. In the laboratory experiment the *P<sub>ng</sub>* value could be checked with the net DMS production in the bottles containing 100% prey and no predator (100iso). The error associated with *P<sub>ng</sub>* was the standard error of the intercept. The error of *P<sub>g</sub>* was obtained from those of the slope and the mean DMSP concentration in replicate controls.

**Calculation of gross DMS production and bacterial DMS consumption**

In the natural community experiments, DMDS was added to two replicate bottles of whole water as a selective inhibitor of bacterial DMS metabolism (Wolfe and Kiene, 1993). The slopes of DMS accumulation with time in each of the DMDS-amended bottles were averaged and taken as the rate of gross DMS production by the total plankton community in the dark. The slopes of DMS concentration in the corresponding duplicate controls were averaged to provide the net DMS production rate in the dark. The errors associated with gross and net DMS production rates were the averaged standard errors of the slopes. The difference between both rates provided the rate of bacterial DMS consumption at each dilution (Simó et al., 2000), with a propagated error.

The same calculations were applied to the results of the experiment on 6 July, where DMDS was added to one replicate of each pair of bottles throughout a full dilution series. In this case, the slope of DMS accumulation with time provided the rate of gross DMS production at each dilution level.

**RESULTS**

**Laboratory experiment**

The initial whole water pool from which all the dilutions were prepared contained  $10246 \pm 458$  (mean  $\pm$  standard error) *I. galbana* cells  $\text{mL}^{-1}$  and  $543 \pm 10$  *O. marina* cells  $\text{mL}^{-1}$ . The chl *a* concentration was  $1.83 \pm 0.01 \mu\text{g L}^{-1}$  and the DMSPp concentration was  $21.4 \pm 0.2 \text{ nM}$ , i.e., values similar to those commonly found in coastal and productive open-ocean waters. The 100iso bottle contained  $9833 \pm 163$  *I. galbana* cells  $\text{mL}^{-1}$ ,  $1.59 \pm 0.01 \mu\text{g chl } a \text{ L}^{-1}$  and  $23.3 \pm 0.3 \text{ nM DMSPp}$  at  $t_0$ . When plotting the net growth rate against the dilution factor, significant ( $p < 0.01$ ) negative slopes were obtained for algal cell abundances, chl *a* and DMSPp (Fig. 1).

Table 1 illustrates the growth rates of *I. galbana* ( $\mu'$ ) and the grazing rates ( $m$ ) obtained from the regression analyses applied to either cell counts, chl *a* concentrations, or DMSPp concentrations (Fig. 1). Grazing rates were very similar for chl *a* and DMSPp (no significant differences between slopes, ANCOVA test  $p = 0.699$ ), and differed from those obtained with cell counts ( $p < 0.001$ ). Growth rates based on chl *a*, cell counts and DMSPp were statistically different (intercept of the equations significantly different, ANCOVA,  $p < 0.001$ ). The algal growth rate calculated from the 100iso bottles (Table 1) fell between the rates estimated from the regression analyses of chl *a* and cell counts, and was very similar to that of DMSPp.

The initial concentrations of DMS were similar in all dilution bottles ( $0.3 \text{ nM}$ ), indicating that both the *I. galbana* culture and the  $0.2 \mu\text{m}$  filtered and autoclaved sea water contained some DMS. The initial whole water pool was prepared by highly diluting (1:125) the *I. galbana* culture with the filtered seawater + f/2, so, although the *I. galbana* culture contained more DMS than the dilution water the resulting mixture contained a DMS concentration very similar to that of the filtered seawater. The final ( $t_{24}$ ) DMS concentrations were higher than the initial ones in all cases, and this increase was lower as waters were more diluted.



When plotting net DMS production against DMSPp ingested, a straight line with significant positive slope ( $0.02 \text{ nM DMS nM DMSPp}^{-1}$ ;  $p < 0.001$ ) was obtained (Fig. 2). The product of this slope by the DMSPp ingestion rate in the undiluted sample yielded a rate of DMS production due to grazing ( $P_g$ ) of  $0.26 \pm 0.03 \text{ nM DMS d}^{-1}$  (Table 1). The intercept of the regression gave a  $P_{ng}$  estimate ( $0.02 \pm 0.02 \text{ nM DMS d}^{-1}$ , 95% confidence) that was indistinguishable from the value of net DMS production obtained in the 100iso bottles (Table 1).

### Experiments with natural communities

The plankton composition of the Mediterranean coastal waters sampled on 14 June and 31 July can be found in Calbet et al. (2008). Briefly, diatoms occurred in abundances of 24 diatom cells  $\text{mL}^{-1}$  in June (dominant genera: *Guinardia* and *Dactyliosolen*) and 131 cells  $\text{mL}^{-1}$  in July (*Nitzschia*, *Pseudo-nitzschia* and *Leptocylindrus*). Autotrophic flagellates (5-15  $\mu\text{m}$ ) occurred at about  $2 \cdot 10^3$  cells  $\text{mL}^{-1}$  and were dominated by haptophytes, a high-DMSP producing group. Picoeukaryotic phytoplankton amounted about  $3 \cdot 10^3$  cells  $\text{mL}^{-1}$ . Prokaryotic phytoplankton contained  $5 \cdot 10^4$  and  $20 \cdot 10^4$  *Synechococcus* cells  $\text{mL}^{-1}$  in June and July, respectively, and no *Prochlorococcus*. In June, the microbial grazers were mainly heterotrophic dinoflagellates ( $10 \mu\text{gC L}^{-1}$ ), followed by heterotrophic flagellates ( $7 \mu\text{gC L}^{-1}$ ) and some ciliates ( $5 \mu\text{gC L}^{-1}$ ). In July, heterotrophic flagellates ( $10 \mu\text{gC L}^{-1}$ ) dominated among microzooplankton, followed by lower biomass numbers of dinoflagellates and ciliates ( $2 \mu\text{gC L}^{-1}$  each).

Initial chl<sub>a</sub>, DMSPp and DMS concentrations of each dilution bottle were calculated from the measurements made in the whole and filtered waters: whole water contained  $0.49 \pm 0.03 \mu\text{g}$

chla  $L^{-1}$ ,  $55 \pm 2.1$  nM DMSPp and  $7.8 \pm 0.0$  nM DMS in June, and  $0.40 \pm 0.02$   $\mu g$  chla  $L^{-1}$ ,  $8.7 \pm 0.6$  nM DMSPp and  $5.2 \pm 0.2$  nM DMS in July. Filtered seawater contained  $9.2 \pm 0.3$  nM DMS in June and  $6.0 \pm 0.2$  nM DMS in July. It could be argued that calculating the initial DMS concentrations from those measured in whole and particle-free waters could introduce uncertainty owing to potential effects of water handling (and associated mechanical stress) on DMS metabolism. To test this, we conducted a further seawater sampling and prepared a dilution series by the same procedure employed in the regular experiments. Initial DMS concentrations were measured in each. DMS levels in the whole and the 0.2- $\mu m$  filtered seawater were  $4.5 \pm 0.1$  and  $3.4 \pm 0.0$  nM, respectively. In all dilution bottles, calculated DMS concentrations did not differ from measured concentrations: 4.2 vs.  $4.2 \pm 0.0$  nM (75%), 3.9 vs.  $3.9 \pm 0.1$  nM (50%), and 3.7 vs.  $3.6 \pm 0.1$  nM (25%). Wolfe et al. (2000) had already performed a similar test and obtained the same results.

Measurements conducted in each dilution bottle after 24 hours confirmed that grazing did occur at higher rates in the concentrated than in the diluted bottles. As a result, net growth rates of both chla and DMSPp showed a significant linear decrease with the dilution factor (Figs. 3 and 4). DMSPp producers, which were a subgroup of the chla-containing community, grew slower than the bulk phytoplankton assemblage in June ( $0.32$  vs.  $1.08 d^{-1}$ ) and July ( $0.59$  vs.  $0.96 d^{-1}$ ), yet the difference was not significant in the latter case (Table 2). Mortality rates ( $0.65$  vs.  $0.82 d^{-1}$  in June;  $1.45$  vs.  $0.99 d^{-1}$  in July) were not significantly different in either month (Table 2).

With respect to DMS production, the slope of the plots of net DMS production against DMSPp consumption allowed estimation of rates of DMS production due to grazing ( $P_g$ ) of  $4.0 \pm 0.9$  nM DMS  $d^{-1}$  in June and  $3.6 \pm 1.3$  nM DMS  $d^{-1}$  in July. The amounts of DMSPp

1  
2  
3 ingested per day (calculated as the grazing mortality rate  $m$  times the 24h-mean DMSPp  
4 concentration in the whole seawater controls) were  $28.8 \text{ nM d}^{-1}$  in June and  $8.3 \text{ nM d}^{-1}$  in July,  
5  
6 which resulted in yields of grazing-mediated DMS production from the ingested DMSPp of  
7  
8 14% and 43%, respectively. The intercepts gave rates of net DMS production due to processes  
9  
10 other than grazing ( $P_{ng}$ ) of  $0.3 \pm 0.3 \text{ nM DMS d}^{-1}$  in June and  $-1.0 \pm 0.2 \text{ nM DMS d}^{-1}$  in July  
11  
12 (Table 2).  
13  
14  
15  
16  
17  
18  
19

20 The bottles incubated in the dark with and without added DMDS were used to obtain the rates  
21  
22 of gross DMS production by the total community and bacterial DMS consumption in the dark.  
23  
24 The DMDS amended bottles showed a faster DMS accumulation than the controls, indicating  
25  
26 that there was an active bacterial consumption of DMS (Fig. 5). In June, gross DMS  
27  
28 production was  $6.3 \pm 0.8 \text{ nM DMS d}^{-1}$ . Subtraction of the slopes gave a daily bacterial DMS  
29  
30 consumption of  $0.8 \pm 1.8 \text{ nM d}^{-1}$ . Comparison of  $P_g$  ( $4.0 \text{ nM DMS d}^{-1}$ ) and gross DMS  
31  
32 production ( $6.3 \text{ nM DMS d}^{-1}$ ) estimates indicates that a large fraction of the DMS produced  
33  
34 inside the experimental bottles was due to grazing. In July, the same calculations gave a gross  
35  
36 DMS production of  $5.0 \pm 0.5 \text{ nM DMS d}^{-1}$  and a bacterial consumption of  $5.0 \pm 1.0 \text{ nM d}^{-1}$ .  
37  
38 Again, most of the gross DMS production was due to grazing ( $3.6 \pm 1.3 \text{ nM d}^{-1}$ ). It must be  
39  
40 noted, though, that gross DMS production rates were measured in the dark, while grazing-  
41  
42 mediated DMS production rates were determined in polycarbonate bottles in the light. Effects  
43  
44 of presence or absence of visible light on DMS production are unclear but, should they occur,  
45  
46 the grazing-mediated DMS yields could vary from these reported.  
47  
48  
49  
50  
51  
52  
53  
54

55 The results of the experiment conducted to test if dilution affects bacterial DMS consumption  
56  
57 are shown in Fig.6. The time course of DMS concentrations in the incubations with and  
58  
59 without DMDS was determined for each dilution. Resulting bacterial DMS consumption rates  
60

1  
2  
3 averaged  $2.7 \pm 1.5$  nM DMS  $\text{d}^{-1}$ , with no differences between dilution treatments. These  
4  
5  
6 results strongly indicate that dilution did not affect the rates of DMS consumption by bacteria.  
7  
8  
9

## 10 11 12 DISCUSSION 13 14 15 16

17 We revisited the use of the dilution technique to assess its utility to quantify the role of  
18  
19 microzooplankton grazing in DMS(P) cycling in meso- to oligotrophic Mediterranean waters.  
20  
21 First, we wanted to corroborate experimentally the validity of the assumptions behind the  
22  
23 design of the dilution technique: (i) the apparent growth rate of phytoplankton shows a linear  
24  
25 variation with the dilution fraction, with an intercept that provides the growth rate of the  
26  
27 phytoplankton prey or, more precisely, the net growth of phytoplankton in the absence of  
28  
29 grazing and with excess nutrients; (ii) the slope of this curve provides the grazing rate on the  
30  
31 bulk phytoplankton assemblage, if the apparent growth is measured in biomass (cell counts,  
32  
33 chl $a$ ) units, or the grazing rate on the sub-assemblage of the DMSP producers, if the apparent  
34  
35 growth is measured in DMSP units.  
36  
37  
38  
39  
40  
41  
42

43 With this aim, we ran an experiment with a simplified food chain with cultured DMSP-  
44  
45 producing prey and predator. The observation that the algal growth rate measured from 100iso  
46  
47 (no predator) bottles fell within the 95% confidence intervals of the intercept of the regression  
48  
49 between cell counts and dilution level supported the first assumption of the dilution technique.  
50  
51 The same was observed with DMSPp, although chl $a$  based growth rates were slightly outside  
52  
53 the confidence interval. It is noticeable, however, the dissimilarity between growth rates  
54  
55 obtained based on cell counts and chl $a$ , while growth rates in 100iso bottles were almost  
56  
57 identical irrespective of the biomass indicator. *A priori* one would expect both approaches  
58  
59  
60

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

(cell based and chla based) to be equivalent and provide similar results. Accumulation of undigested chla and DMSPp concentrations in grazer food vacuoles may have contributed to increase the intercepts of apparent chla and DMSPp growth, but a simple calculation assuming that every *O. marina* might contain up to 5 undigested *I. galbana* cells showed this could only account for 25% of the observed difference and therefore cannot be invoked as the sole reason for this paradoxical result. We may find an alternative explanation when observing closely the size structure of the prey community in 100iso bottles (free of predators) and in the dilution series. After 24h incubation the *I. galbana* cells in the predator free bottles were ca. 15 % smaller in volume (at the 99% confidence level according to a Student t test) than the ones subjected to grazing. It seems therefore, that *O. marina* was selectively feeding on smaller cells and shaping in this way the size-structure of the prey community. This has consequences for the estimation of algal growth rates in the experiment (higher rates on chla-based results than on cell counts). Conversion of cell counts into algal carbon by using a conversion of cell carbon to the cell volume gave a growth rate of  $0.79 \pm 0.04 \text{ d}^{-1}$ , i.e., undistinguishable from that of chla. Obviously, the outcome of the experiment would have been the opposite if grazers had selected for larger cells.

The experiment also served to corroborate the second assumption of the dilution technique and support it's suitability to provide mortality rate measurements for the subgroup of the DMSP-producers. DMSP and chla-based estimates of grazing were very similar (Table 1, Fig. 1). In this case, where the algal assemblage was formed by a monoclonal DMSP-producing algal culture, the similarity of DMSP and chla-based estimates is indicative of the suitability of DMSP as a biomass marker, although both rate estimates were significantly different from those obtained from cell counts for the same reasons discussed above. Again, accumulation of undigested chla and DMSPp concentrations in grazer food vacuoles could hardly account for

more than 25% of the observed difference, the remaining being explained by apparent size-selective grazing.

The culture-based experiment, therefore, provided confidence in the fundamentals of the method. The subsequent experiments with natural (hence complex) plankton communities confirmed the potential of the dilution technique to provide growth and grazing-mediated mortality rates for a natural phytoplankton assemblage as well as the sub-assemblage of the DMSP-producers, as already reported by Archer and collaborators (Archer et al., 2001b).

The use of the dilution technique for measuring microzooplankton-mediated DMS production was first suggested by Wolfe and colleagues (Wolfe et al., 2000), and further developed and tested by Archer and colleagues (Archer et al., 2001a). The idea had been inspired by previous laboratory experiments which had shown that microzooplankton grazing on DMSP-containing phytoplankton leads to DMS formation (Wolfe et al., 1994; Christaki et al., 1996; Wolfe and Steinke, 1996). If grazing by microzooplankton results in the transformation of DMSPp to DMS, DMS production should decrease with decreasing grazing pressure, i.e., with dilution of the community with filtered seawater (Wolfe et al., 2000; Archer et al., 2001a). Actually, DMS production should decrease with decreasing DMSP ingestion rates with dilution. This prediction indeed matches our observations in both the lab and the natural community dilution experiments, where DMS production rates varied with the DMSP mortality rates throughout the dilution series in such a proportional way that a model I regression analysis could be applied (Figs. 2-4). The natural grazing-mediated DMS production rate could be calculated from the slope of the regression and the DMSP mortality in the undiluted and unamended bottle.

We consider that this approach to grazing-mediated DMS production is more appropriate than that employed by Archer et al. (2001a), who normalized net DMS production rates to the relative algal concentration in each incubation in order to account for the role of phytoplankton as the primary source of DMS. We argue that this normalization procedure yields erroneous numbers. Let us formulate the DMS budget in the 100% whole seawater bottles:

$$\text{daily net DMS production} = \text{DMS}_{t24} - \text{DMS}_{t0} = Pg + Png \quad (1)$$

where  $Pg$  is the gross DMS production due to grazing and  $Png$  is the net production not due to grazing, i.e., gross DMS production due to cell-associated and free DMSP lyases minus bacterial DMS consumption. The budget in a  $d$  dilution bottle (with  $d$  equal to either 0.75 or 0.50 or 0.25) will thus be:

$$\text{daily net DMS production}_{(d)} = \text{DMS}_{t24(d)} - \text{DMS}_{t0(d)} = (Pg \cdot d) + Png \quad (2)$$

with the assumption that only the gross DMS production due to grazing is reduced by the dilution. If we plot the daily net DMS production against the dilution factor  $d$ , the slope of the regression line equals the gross DMS production due to grazing ( $Pg$ ) and the intercept gives the net production not due to grazing ( $Png$ ). If we normalized the daily net DMS production by the algal fraction, following Archer et al. (2001), equation (2) would read:

$$\begin{aligned} \text{algal-specific daily net DMS production}_{(d)} &= (\text{DMS}_{t24(d)} - \text{DMS}_{t0(d)})/d = ((Pg \cdot d) + Png)/d = Pg \\ &+ (Png/d) \quad (3) \end{aligned}$$

The plot of the normalized net DMS production against the dilution factor  $d$  would have a hyperbolic shape  $y = a + (b/x)$  that might give the false impression of a linear behaviour, where the slope would not be  $P_g$  as intended, but would change sign depending on whether there would be net DMS production or consumption in the absence of grazing.

It is to be noted that the grazing-mediated DMS production,  $P_g$ , derived from DMSP mortality, does not refer only to DMSP-to-DMS conversion during the very process of grazing, e.g., by the mix of algal DMSP and DMSP lyase upon ingestion or in grazer's vacuoles. It may include also processes dependent on grazing but indirectly related, such as the bacterial DMS production from the dissolved DMSP released during grazing. Over the 24 h period of the incubations, this process will also vary with dilution as dilution affects grazing and the associated release of dissolved DMSP (Archer et al., 2001). Being a very labile substrate, which generally turns over in a matter of hours (Kiene et al., 2000; Vila-Costa et al., 2008), most DMSP released by grazing will be consumed (and partly converted to DMS) during the 24 hours of incubation, at all dilution levels. For our purpose of assessing the role of grazing in DMS production, this secondary production also counts as 'grazing-mediated'.

As discussed above, the intercept of the regression,  $P_{ng}$ , provides the net DMS production due to processes other than grazing. The fact that, in the laboratory experiment, the intercept of the dilution series was not significantly different from the net DMS production in the bottle with *I. galbana* only (a tiny net production) supports the use and meaning of the intercept. In the experiment with the natural community on 31 July, the intercept  $P_{ng}$  was negative ( $-0.95 \pm 0.24 \text{ nM d}^{-1}$ ). That is, without grazing there was a net DMS loss. Within the capped, glass experimental bottles, none or very little headspace was left, so, DMS removal due to ventilation was negligible. DMS photolysis, if any because of the absence of UVB radiation,



would have been a constant proportion of the DMS production through the dilution series and, if so, would have had little effect on estimates of algal-specific DMS production (Archer et al., 2001; Toole et al., 2003). Thus, processes associated with bacterial activity must be the main reason responsible for DMS consumption. The intercept  $P_{ng}$ , hence, is expected to correspond to bacterial net DMS consumption, that is, the net result of bacterial DMS consumption and production from the DMSPd already present in the initial community. Underlying this interpretation of the intercept, though, there is the assumption that bacterial consumption is not largely affected by dilution. This has been raised as the major pitfall of the application of the dilution technique to the DMS cycle (Wolfe et al., 2000), because changes in bacterial activity due to dilution could confound the estimates of grazing-mediated DMS production rates or even prevent them by breaking any linearity (Wolfe et al., 2000).

Our experiment on 6 July also served to test the behavior of bacterial DMS consumption with dilution. The experiment yielded indistinguishable DMS consumption rates (Fig. 6), indicating that bacterial DMS consumption was not significantly affected by dilution. This could seem to be quite a striking result, because dilution should have decreased bacterial abundances and activities proportionally. However, there are two hypothetical explanations for such an apparently null effect: (a) By diluting with filtered seawater, we dilute flagellate numbers along with bacterial numbers. The probability of bacterivore-bacterium encounter decreases with dilution, and we should expect bacterivory and bacterial mortality to decrease accordingly. (b) Since dissolved organic matter (DOM) is released during seawater filtration, the bottles with more filtered water (more diluted) will have more DOM. Most probably DMS is not the limiting factor for bacterial growth, and most of the DMS is consumed through co-metabolism with other DOM components (Vila-Costa et al., 2006; del Valle et al., 2007). Therefore, we can expect specific DMS consumption to increase with dilution. We argue that

these factors altogether may help compensate for the initial dilution of the DMS consumers and eventually yield similar consumption rates over the 24-hours period. Finally, we want to point out that the initial DMS concentration was not reduced with the dilution factor because filtered seawater contained a little more DMS than whole seawater.

The use of DMDS as an inhibitor of bacterial DMS metabolism to determine gross DMS production in parallel to dilution experiments is based upon the assumption that DMDS efficiently blocks bacterial DMS metabolism throughout the duration of the experiments. In a study in North Atlantic waters, Simó et al. (2000) reported that DMS accumulation after addition of 260 nM DMDS did not remain linear over a 20-h period, as though the action of DMDS as inhibitor were only effective in the shorter term (8 h). In experiments, however, DMDS amended incubations gave linear DMS production curves throughout the 24-h period, and duplicate bottles replicated very well (Fig. 5), so that community gross DMS production rates could be measured quite precisely. The further estimation of bacterial DMS consumption by subtraction of the slopes of gross and net DMS productions carries a much larger statistical error. An alternative approach would be the  $^{35}\text{S}$ -DMS radiotracing method, where radio-labeled DMS is added in trace amounts and its disappearance is measured with time to obtain very precise rates of bacterial DMS consumption (del Valle et al., 2007). Then, to derive gross DMS production rates DMS consumption must be added to net DMS production, again increasing the statistical uncertainty by error propagation. The choice of one approach or the other is, therefore, dependent on whether the emphasis is placed on the gross production or the bacterial consumption. There is also the possibility of combining both. In any case, the otherwise desirable use of  $^{35}\text{S}$ -DMS is limited by its availability because this is not yet a commercially available radiotracer.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

With this study we have revisited the dilution technique suggested by Wolfe, Archer and collaborators (Wolfe et al., 2000; Archer et al., 2001a; Archer et al., 2001b; Archer et al., 2003). The main assumptions of the method have been tested, and the data treatment to derive grazing-mediated DMS production rates has been revised. We have also complemented the method with estimates of the gross DMS production by all processes in the whole planktonic community, to be compared with grazing-mediated DMS production. Altogether, the proposed protocol (Table 3) is intended to provide comprehensive information on the role of microzooplankton grazing in DMS(P) dynamics in natural plankton communities, at least in meso- to oligotrophic (Mediterranean-like) waters: (1) growth and grazing rates of both the bulk phytoplankton assemblage and the sub-assemblage of the DMSP-producers; (2) rates of community gross DMS production and DMS production due to grazing; and (3) bacterial DMS consumption. It is important to notice, however, that there are some processes that the dilution technique as applied here cannot discriminate and measure yet, such as bacteria and virus mediated DMS production. Further studies are required to achieve a sound quantification of these processes.

**Acknowledgements**

We acknowledge support from the Spanish Ministry of Science and Innovation (former Education and Science) through a PhD fellowship to V.S. and through the project MICROROL (CTM2004-02575/MAR). We thank J. Movilla for helping with field sampling, E. Saiz for assistance with the setup of the dilution experiment, I. Trepas and J. Felipe for chlorophyll *a* analyses, and two anonymous reviewers whose comments helped to improve the manuscript.

## REFERENCES

- Andreae, M.O. (1990) Ocean-atmosphere interactions in the global biogeochemical sulfur cycle. *Mar. Chem.*, **30**, 1—29.
- Archer, S.D., Stelfox-Widdicombe, C.E., Burkill, P.H. and Malin, G. (2001a) A dilution approach to quantify the production of dissolved dimethylsulphoniopropionate and dimethyl sulphide due to microzooplankton herbivory. *Aquat. Microb. Ecol.*, **23**, 131—145.
- Archer, S.D., Widdicombe, C.E., Tarran, G.A., Rees, A.P. and Burkill, P.H. (2001b) Production and turnover of particulate dimethylsulphoniopropionate during a coccolithophore bloom in the northern North Sea. *Aquat. Microb. Ecol.*, **24**, 225-241.
- Archer, S.D., Stelfox-Widdicombe, C.E., Malin, G. and Burkill, P.H. (2003) Is dimethyl sulphide production related to microzooplankton herbivory in the southern North Sea? *J. Plankton Res.*, **25**, 235—242.
- Calbet, A. (2008) The trophic roles of microzooplankton in marine systems. *ICES J. Mar. Sci.*, **65**, 325—331.
- Calbet, A. and Landry, M.R. (2004) Phytoplankton growth, microzooplankton grazing, and carbon cycling in marine systems. *Limnol. Oceanogr.*, **49**, 51—57.
- Calbet, A., Trepát, I., Almeda, R., Saló, V., Saiz, E., Movilla, J.I., Alcaraz, M., Yebra, L. and Simó, R. (2008) Impact of micro- and nanograzers on phytoplankton assessed by standard and size-fractionated dilution experiments. *Aquat. Microb. Ecol.*, **50**, 145-156.
- Charlson, R.J., Lovelock, J.E., Andreae, M.O. and Warren, S.G. (1987) Oceanic phytoplankton, atmospheric sulphur, cloud albedo and climate. *Nature*, **326**, 655—661.

- Chavez, F.P., Buck, K.R., Coale, K.R., Martin, J.H., DiTullio, G.R., Welschmeyer, N.A., Jacobson, A.C. and Barber, R.T. (1991) Growth rates, grazing, sinking, and iron limitation of equatorial Pacific phytoplankton. *Limnol. Oceanogr.*, **36**, 1816—1833.
- Christaki, U., Belviso, S., Dolan, J.R. and Corn, M. (1996) Assessment of the role of copepods and ciliates in the release to solution of particulate DMSP. *Mar. Ecol. Prog. Ser.*, **141**, 119—127.
- Dacey, J.W.H. and Wakeham, S.G. (1986) Oceanic dimethylsulfide: Production during zooplankton grazing on phytoplankton. *Science*, **233**, 1314—1316.
- Daly, K.L. and DiTullio, G.R. (1996) Particulate dimethylsulfoniopropionate removal and dimethylsulfide production by zooplankton in the Southern Ocean. In: Kiene, R.P., Visscher, P.T., Keller, M.D., Kirst, G.O. (eds) *Biological and environmental chemistry of DMSP and related sulfonium compounds*. Plenum Press, New York, 223—238.
- del Valle, D.A., Kieber, D.J. and Kiene, R.P. (2007) Depth-dependent fate of biologically-consumed dimethylsulfide in the Sargasso Sea. *Mar. Chem.*, **103**, 197-208.
- Dolan, J.R., Gallegos, C.L. and Moigis, A. (2000) Dilution effects on microzooplankton in dilution grazing experiments. *Mar. Ecol. Prog. Ser.*, **200**, 127—139.
- Evans, G.T. and Paranjape, M.A. (1992) Precision of estimates of phytoplankton growth and microzooplankton grazing when the functional response of grazers may be nonlinear. *Mar. Ecol. Prog. Ser.*, **80**, 285—290.
- Fredrickson, K.A. and Strom, S.L. (2009) The algal osmolyte DMSP as a microzooplankton grazing deterrent in laboratory and field studies. *J. Plankton Res.*, **31**, 135-152.
- Frost, B.W. (1972) Effects of size and concentration of food particles on the feeding behaviour of the marine planktonic copepod *Calanus pacificus*. *Limnol. Oceanogr.*, **17**, 805—815.

- Gallegos, C.L. (1989) Microzooplankton grazing on phytoplankton in the Rhode River, Maryland: nonlinear feeding kinetics. *Mar. Ecol. Prog. Ser.*, **57**, 23—33.
- Gasol, J.M., and del Giorgio, P.A. (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci. Mar.*, **64**, 197—224.
- Keller, M.D., Bellows, W.K. and Guillard, R.L. (1989) Dimethyl sulfide production in marine phytoplankton. In Saltzman, E.S. and Cooper, W.J. (eds), *Biogenic Sulfur in the Environment*. American Chemical Society, Washington, DC, 183—200.
- Kettle, A.J. and Andreae, M.O. (2000) Flux of dimethylsulfide from the oceans: A comparison of updated data sets and flux models. *J. Geophys. Res.*, **105**, 793—808.
- Kiene, R.P., Linn, L.J. and Bruton J.A. (2000). New and important roles for DMSP in marine microbial communities. *J. Sea Res.* **43**, 209-224.
- Kwint, R.L.J., Quist, P., Hansen, T.A., Dijkhuizen, L. and Kramer, K.J.M. (1996) Turnover of dimethylsulfoniopropionate and dimethylsulfide in the marine environment: a mesocosm experiment. *Mar. Ecol. Prog. Ser.*, **145**, 223—232.
- Landry, M.R., Kirshtein, J. and Constantinou, J. (1995) A refined dilution technique for measuring the community grazing impact of microzooplankton, with experimental tests in the central equatorial Pacific. *Mar. Ecol. Prog. Ser.*, **120**, 53—63.
- Landry, M.R., Hassett, R.P. (1982) Estimating the grazing impact of marine microzooplankton. *Mar. Biol.*, **67**, 283—288.
- Malin, G., Liss, P.S. and Turner, S.M. (1994) Dimethyl sulphide: Production and atmospheric consequences. J. C. Green and B. S. C. Leadbeater (eds). *The haptophyte algae*. Clarendon Press, Oxford, pp. 303—320.
- Neuer, S. and Franks, P.J.S. (1993) Determination of ammonium uptake and regeneration rates using the seawater dilution method. *Mar. Biol.*, **116**, 497—505.

- Olson, R.J., Zettler, E.R., DuRand, M.D. (1993) Phytoplankton analysis using flow cytometry. P.F. Kemp, B.F. Sherr, E.B. Sherr and J.J.Cole (eds.). *Handbook of methods in aquatic microbial ecology*. Lewis Publishers, CRC Press, pp. 175-186.
- Simó, R. (2001) Production of atmospheric sulfur by oceanic plankton: biogeochemical, ecological and evolutionary links. *Trends Ecol. Evol.*, **16**, 287—294.
- Simó, R. (2004) From cells to globe: approaching the dynamics of DMS(P) in the ocean at multiple scales. *Can. J. Fish. Aquat. Sci.*, **61**, 673—684.
- Simó, R. and Dachs, J. (2002) Global ocean emission of dimethylsulfide predicted from biogeophysical data. *Global Biogeochem. Cycles*, **16**, 1078, doi: 10.1029/2001GB001829.
- Simó, R., Grimalt, J.O. and Albaigés, J. (1996) Sequential method for the field determination of nanomolar concentrations of dimethyl sulfoxide in natural waters. *Anal. Chem.*, **68**, 1493—1498.
- Simó, R., Pedrós-Alió, C., Malin, G. and Grimalt, J.O. (2000) Biological turnover of DMS, DMSP and DMSO in contrasting open-sea waters. *Mar. Ecol. Prog. Ser.*, **203**, 1—11.
- Stefels, J., Steinke, M., Turner, S., Malin, G. and Belviso, S. (2007) Environmental constraints on the production and removal of the climatically active gas dimethylsulphide (DMS) and implications for ecosystem modelling. *Biogeochemistry*, **83**, 245—275.
- Strickland, J.H.D. and Parsons, T.R. (1972) A practical handbook of seawater analysis. *Bull. Fish. Res. Board Can.*, **167**, 1—310.
- Toole, D., Kieber, D.J., Kiene, R.P., Siegel, D.A. and Nelson, N.B. (2003) Photolysis and the dimethylsulfide (DMS) summer paradox in the Sargasso Sea. *Limnol. Oceanogr.*, **48**, 1088—1100.

- Vila-Costa, M., del Valle, D.A., González, J.M., Slezak, D., Kiene, R.P., Sánchez, O. and Simó, R. (2006) Phylogenetic identification and metabolism of marine dimethylsulfide-consuming bacteria. *Environ. Microbiol.*, **8**, 2189-2200.
- Vila-Costa, M., Kiene, R.P. and Simó, R. (2008) Seasonal variability of the dynamics of dimethylated sulfur compounds in a coastal northwest Mediterranean site. *Limnol. Oceanogr.* **53**, 198-211.
- Wolfe, G.V. and Kiene, R.P. (1993) Radioisotope and chemical inhibitor measurements of dimethyl sulfide consumption rates and kinetics in estuarine waters. *Mar. Ecol. Prog. Ser.*, **99**, 261—269.
- Wolfe, G.V., Levasseur, M., Cantin, G. and Michaud, S. (2000) DMSP and DMS dynamics and microzooplankton grazing in the Labrador Sea: application of the dilution technique. *Deep-Sea Res.*, **47**, 2243—2264.
- Wolfe, G.V., Sherr, E.B. and Sherr, B.F. (1994) Release and consumption of DMSP from *Emiliana huxleyi* during grazing by *Oxyrrhis marina*. *Mar. Ecol. Prog. Ser.*, **111**, 111—119.
- Wolfe, G.V. and Steinke, M. (1996) Grazing-activated production of dimethyl sulfide (DMS) by two clones of *Emiliana huxleyi*. *Limnol. Oceanogr.*, **41**, 1151—1160.
- Wolfe, G.V., Steinke, M. And Kirst, G.O. (1997) Grazing activated chemical defence in a unicellular marine algae. *Nature*, **387**, 894—897.



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

Table 1: Results of the laboratory dilution experiment with *O. marina* grazing on *I. galbana*. Values for grazing mortality ( $m$ ) and algal growth ( $\mu$ ) rates were determined with cell counts, chlorophyll  $a$ , and DMSPp after model I regression analyses of the curves of Fig. 1. The directly measured algal growth rate in the bottle with *I. galbana* only ( $r_{iso}$ ) is also shown. The bottom line shows the relationship between DMS production and dilution fraction (grazing pressure) as calculated from model I regression analyses of the curves of Fig. 2.  $P_g$  refers to DMS production due to grazing (slope), and  $P_{ng}$  is for DMS production due to factors other than grazing (intercept). The directly measured DMS production in the bottle with *I. galbana* only (100iso) is also shown. Values are presented with the associated standard error.

Biomass descriptor	$m$ (d <sup>-1</sup> )	$\mu$ (d <sup>-1</sup> )	$r^2$	$r_{iso}$ (d <sup>-1</sup> )
cell counts	0.66 ± 0.05	0.65 ± 0.04	0.96	0.73 ± 0.05
chl $a$	0.48 ± 0.02	0.80 ± 0.02	0.98	0.70 ± 0.07
DMSPp	0.46 ± 0.04	0.87 ± 0.03	0.95	0.80 ± 0.10
	$P_g$ (nM DMS d <sup>-1</sup> )	$P_{ng}$ (nM DMS d <sup>-1</sup> )	$r^2$	100iso (nM DMS d <sup>-1</sup> )
	0.26 ± 0.03	0.02 ± 0.02	0.95	0.06 ± 0.03

Table 2: Summary of the growth ( $\mu$ ) and grazing mortality ( $m$ ) rate estimates obtained with the natural community experiments. Note the comparison between the bulk phytoplankton community (chl<sub>a</sub> measurements) and the sub-community of the DMSP producers (DMSPp measurements). The bottom lines show the rates of DMS production due to grazing ( $P_g$ ) and not due to grazing ( $P_{ng}$ ). Also included is the coefficient of model I linear regressions ( $r^2$ ). Values are presented with the associated standard error.

Date	Biomass descriptor	$m$ (d <sup>-1</sup> )	$\mu$ (d <sup>-1</sup> )	$r^2$	$m/\mu$
14 June	chl <sub>a</sub>	0.82 ± 0.16	1.08 ± 0.11	0.83	0.76
	DMSPp	0.65 ± 0.06	0.32 ± 0.04	0.94	2.03
31 July	chl <sub>a</sub>	0.99 ± 0.24	0.96 ± 0.16	0.76	1.03
	DMSPp	1.45 ± 0.47	0.59 ± 0.33	0.74	2.46
		$P_g$ (nM DMS d <sup>-1</sup> )	$P_{ng}$ (nM DMS d <sup>-1</sup> )	$r^2$	
14 June		3.97 ± 0.88	0.30 ± 0.34	0.88	
31 July		3.57 ± 1.34	-0.95 ± 0.24	0.93	

Table 3: Recommended protocol for the application of the dilution technique to quantify the growth of DMSP-producing algae, the DMSPp consumption and DMS production by micro-herbivores, the community gross DMS production, and the bacterial DMS consumption in plankton communities. All treatments to be prepared at least in duplicates.

Bottle label	% of whole seawater	Amendment	Incubation conditions	Subsampling times (h) <sup>d</sup>	Measurements	Data analysis	Process rate calculations
25	25	+ nutrients	light <sup>b</sup>	24	chl <sub>a</sub> , DMSPp, DMS	1. Regression of chl <sub>a</sub> or DMSPp apparent growth vs dilution factor  2. Regression of DMS production vs DMSP grazed in each dilution (scaled $m_{DMSP}$ x mean DMSP conc.)	1.1. Algal growth ( $\mu_{chl_a}$ or $\mu_{DMSP}$ ) from intercepts corrected for growth without nutrients  1.2. Mortality by grazing ( $m_{chl_a}$ or $m_{DMSP}$ ) from slopes  2.1. Net DMS production not due to grazing ( $P_{ng}$ ) from intercept  2.2. DMS production due to grazing ( $P_g$ ) from slope x mean DMSP conc. in controls
50	50	+ nutrients	light	24	chl <sub>a</sub> , DMSPp, DMS		
75	75	+ nutrients	light	24	chl <sub>a</sub> , DMSPp, DMS		
100	100	+ nutrients	light	24	chl <sub>a</sub> , DMSPp, DMS		
100c	100	none	light	24	chl <sub>a</sub> , DMSPp, DMS		
100d	100	none	light/dark <sup>c</sup>	8, 24 <sup>e</sup>	DMS	Regression of DMS concentration vs time	Community net DMS production from slope
100i	100	+ DMDS <sup>a</sup>	light/dark <sup>c</sup>	8, 24 <sup>e</sup>	DMS	Regression of DMS concentration vs time	Community gross DMS production from slope  Bacterial DMS consumption from subtraction of the above slopes <sup>f</sup>

a: DMDS (dimethyl disulfide) concentration typically  $\approx 200$  nM, or  $\geq 20$  times the endogenous DMS concentration.

b: Light intensity as similar as possible to that at sampling depth. Light quality to be decided; in our case we used PAR+ part of UVA (polycarbonate bottles).

c: Ideally to be incubated in the same light regime as the dilution bottles. We incubated them in the dark.

d: Time zero concentrations are calculated from measurements in whole and  $0.2 \mu\text{m}$  filtered according to the dilution factors.

e: At least one intermediate time point is convenient for the regression analysis. In our case we sub-sampled after 4, 6, 10, 20 and 24 hours.

f: Alternatively, the  $^{35}\text{S}$ -DMS radiotracing method can be used to determine DMS consumption more precisely and to derive community gross DMS production by addition to community net DMS production.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**FIGURE LEGENDS**

Fig. 1. Plots of the apparent growth of *Isochrysis galbana* versus the dilution factor in the laboratory grazing experiment with *Oxyrrhis marina*. Algal biomass measured as cell counts (filled circles), chlorophyll *a* (open circles), and DMSPp (open squares). Parallel datapoints correspond to replicate incubation bottles. The results of the linear regression analyses are reported in Table 1.

Fig. 2. Relationship between DMS produced and DMSP grazed in each dilution level in the laboratory experiment with *O. marina* feeding on *I. galbana*. Parallel datapoints correspond to replicate incubation bottles. The results of the linear regression analyses are reported in Table 1.

Fig. 3. Dilution experiment with a natural plankton community sampled on 14 June in coastal NW Mediterranean waters. Top: Apparent growth of phytoplankton vs the dilution factor, with phytoplankton biomass measured as chlorophyll *a* (open circles) and DMSPp (open squares). Grey circles and squares correspond to the incubations without nutrient additions. Bottom: net DMS production vs DMSP grazed. In all cases, parallel datapoints correspond to replicate incubation bottles. The results of the linear regression analyses are reported in Table 2.

Fig. 4. Dilution experiment with a natural plankton community sampled on 31 July in coastal NW Mediterranean waters. Top: Apparent growth of phytoplankton vs the dilution factor, with phytoplankton biomass measured as chlorophyll *a* (open circles) and DMSPp (open squares). Grey circles and squares correspond to the incubations without nutrient additions.

Bottom: net DMS production vs DMSP grazed. In all cases, parallel datapoints correspond to replicate incubation bottles. The results of the linear regression analyses are reported in Table 2.

Fig. 5. Time course of DMS concentrations in dark incubations of whole natural communities with (filled circles) and without (open circles) 200 nM of DMDS on 14 June (top) and 31 July (bottom). Duplicate symbols correspond to duplicate bottles, and error bars correspond to the standard error of duplicate analyses in each bottle. The lines show the average of the regression analyses for each pair of bottles. Regression analyses of the 14 June data give an average slope of the DMDS-amended treatments (gross DMDS production) of  $6.33 \pm 0.77 \text{ nM d}^{-1}$ ,  $r^2 = 0.94$ , and an average slope of the controls (net DMS production) of  $5.50 \pm 1.04 \text{ nM d}^{-1}$ ,  $r^2 = 0.86$ . Regression analyses of the 31 July data give an average slope of the DMDS-amended treatments of  $4.98 \pm 0.53 \text{ nM d}^{-1}$ ,  $r^2 = 0.96$ , and an average slope of the controls (net DMS production) not significantly different from 0 at the 95% confidence level, with an associated error of  $0.50 \text{ nM d}^{-1}$ .

Fig. 6. Bacterial DMS consumption rates estimated in dilution bottles prepared with a natural plankton community sampled on 6 July in coastal NW Mediterranean waters. Error bars correspond to 1 standard error as calculated by error propagation of the incubation slopes.

Fig. 1

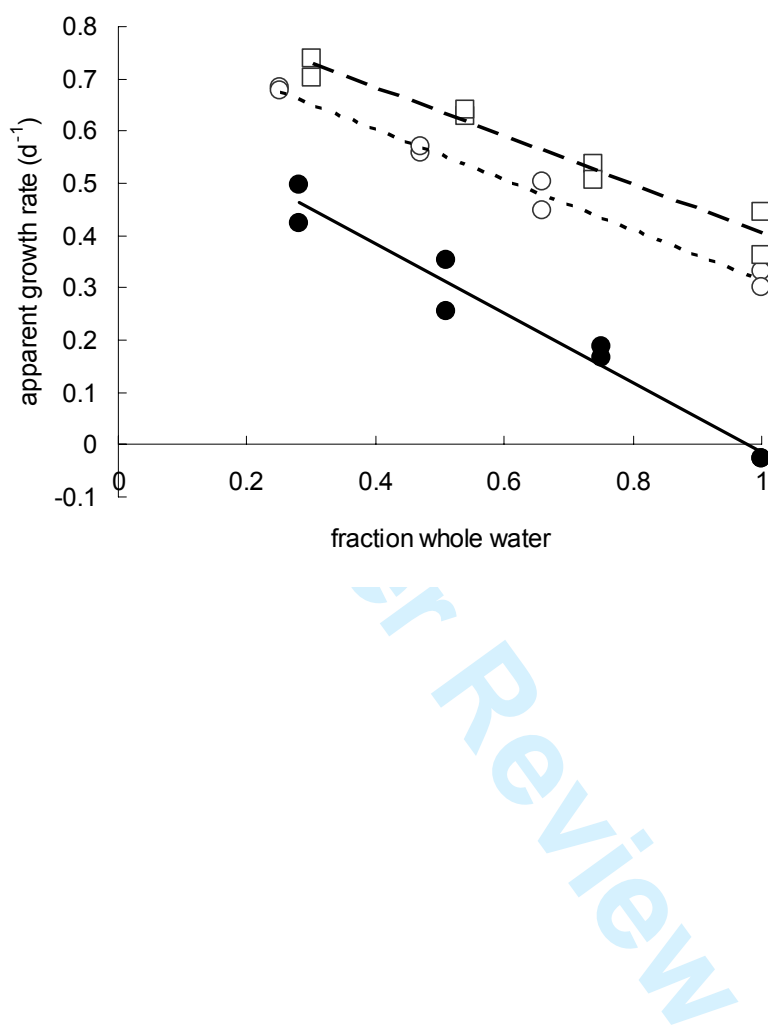


Fig. 2

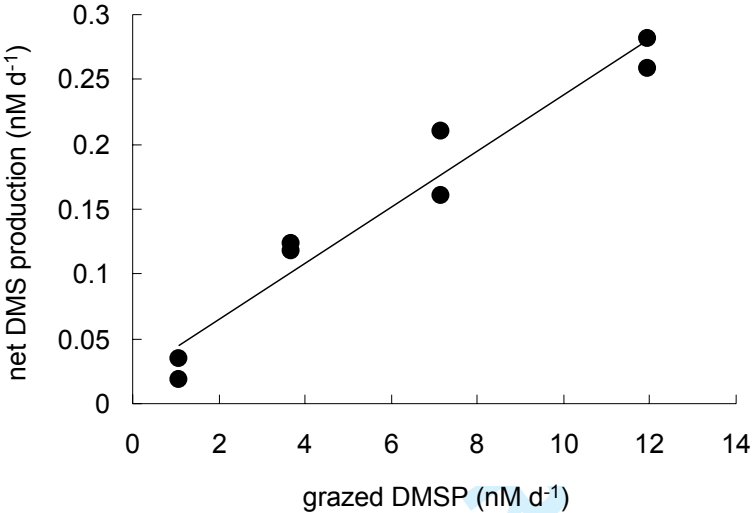




Fig. 3

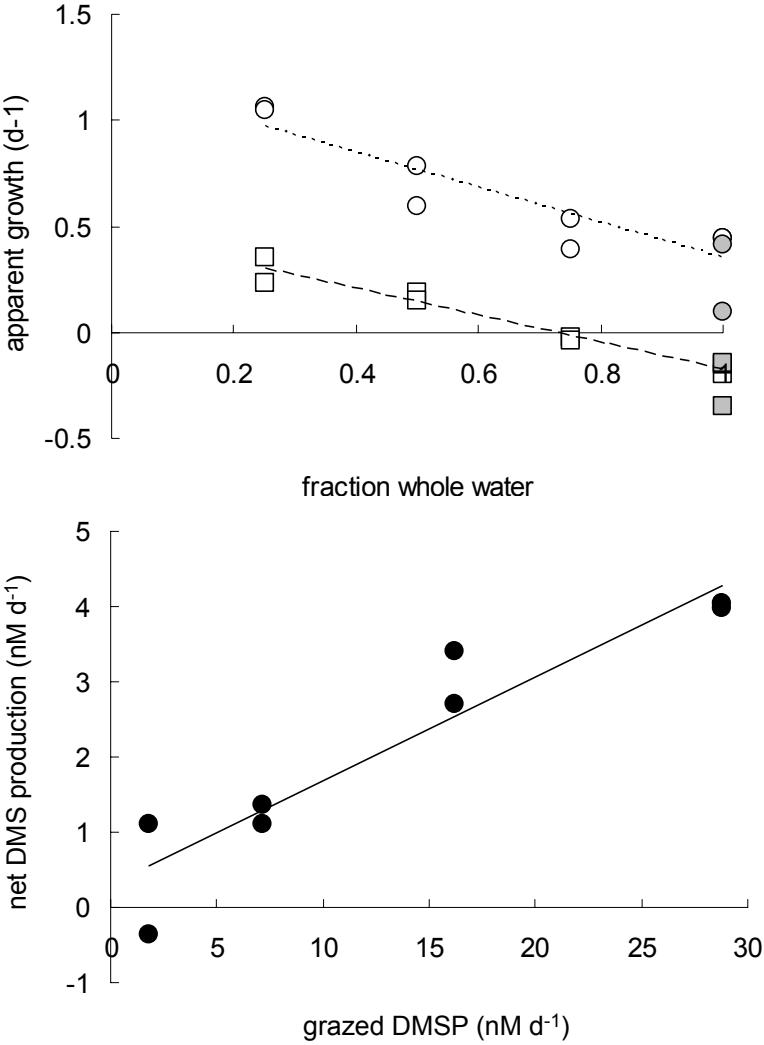


Fig. 4

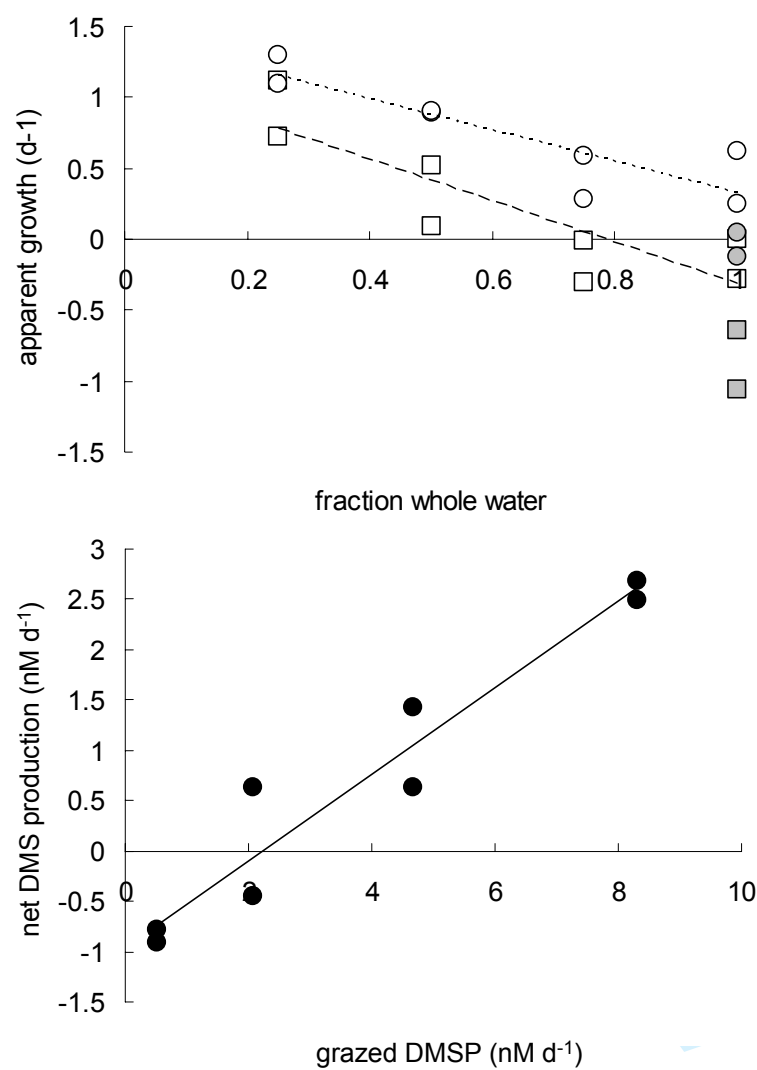


Fig. 5

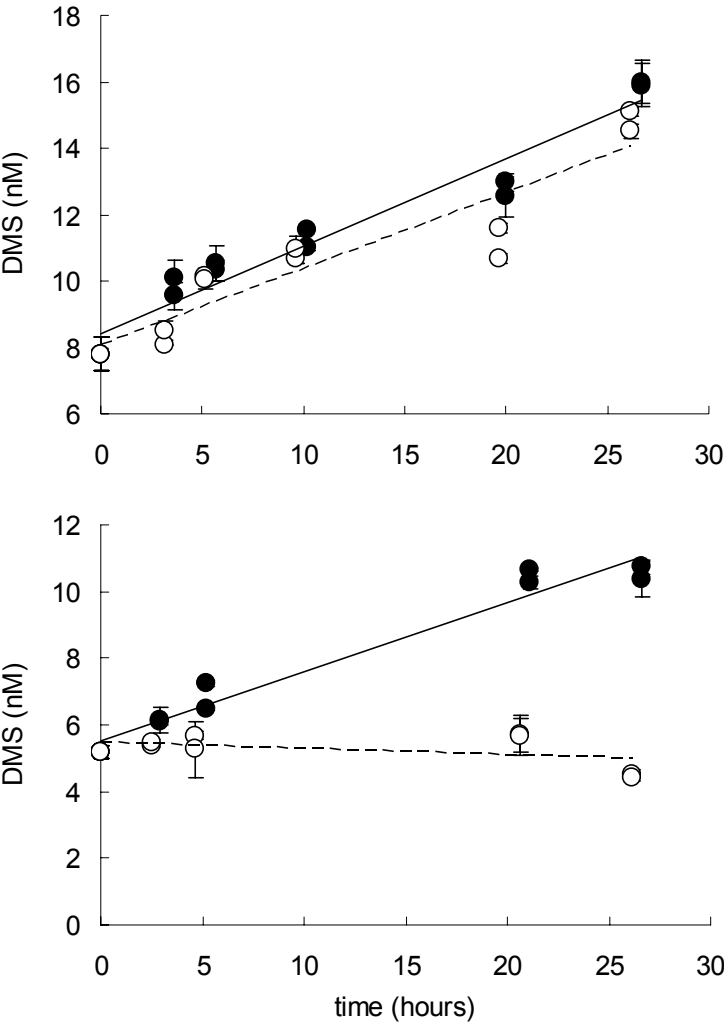


Fig. 6

