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Research Paper

Assessment of DMSP turnover reveals a non-bioavailable pool of dissolved DMSP in coastal waters of the Gulf of Mexico

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Environmental context. DMSP is one of the most important substrates for marine bacteria and its cycling contributes substantially to fluxes of carbon and sulfur in the ocean. Accurate determination of the concentration of DMSP available to bacteria is essential to quantifying DMSP consumption rates, and this work improves those determinations by identifying non-bioavailable pools of DMSP that have previously gone unrecognised. Improved estimates of DMSP consumption rates will lead to better understanding of its role in ocean food web and biogeochemical dynamics.

Abstract. Dissolved dimethylsulfoniopropionate (DMSPd) is an important substrate for marine microbes and a precursor of sulfur gases. We compared DMSPd turnover flux rates in coastal seawater measured with a ^{35}S-DMSPd tracer to those obtained with the DMSP-uptake inhibitor glycine betaine (GBT). The ^{35}S-DMSP tracer method yielded DMSPd turnover fluxes (35.7–215 nM day^{-1}) that were 1.7 to 152 times higher than those obtained in parallel samples with the GBT inhibitor method (0.34–21.6 nM day^{-1}). Tests confirmed that GBT functioned as planned by strongly inhibiting DMSPd degradation and that ^{35}S-DMSPd gave accurate estimates of DMSPd loss rate constants. This left the initial DMSPd concentrations, determined by small volume drip filtration (SVDF) through Whatman GF/F filters (0.7-μm nominal retention) ([DMSPd]$_{SVDF}$), as a potential cause of the discrepancy in rate estimates. Indeed, GF/F filtrate incubations showed that the initial [DMSPd]$_{SVDF}$ overestimated the bioavailable DMSPd concentrations for at least two reasons: (1) a significant fraction (10–37 %) of DMSP passing through GF/F filters was in particles >0.2 μm (likely bacteria) and therefore not dissolved, and (2) a significant pool (0.44–1.0 nM) of operationally dissolved, non-particle DMSP ([DMSPd]$_{<0.2\,\mu m}$), comprising 40–99 % of [DMSPd]$_{SVDF}$, was refractory to degradation on a time scale of days. The nature of this refractory DMSP is currently unknown. Accounting for DMSP-containing particles and the refractory DMSP pool in GF/F filtrates is necessary to obtain the true bioavailable DMSPd concentrations, which we estimate to be very low (0.006–1.0 nM; mean of 0.41 nM) in the coastal waters examined, and to avoid overestimation of DMSPd turnover fluxes when using the ^{35}S-DMSP tracer technique.

Additional keywords: bacteria, bioavailability, carbon and sulfur fluxes, climate, dimethylsulfide, DMSPd, filtration, GBT, glycine betaine, organic sulfur, phytoplankton, refractory, uptake inhibitor.

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Introduction

The tertiary sulfonium compound dimethylsulfoniopropionate (DMSP) is an intracellular solute synthesised by a wide variety of marine phytoplankton and macroalgae.[1,2] It functions as an osmolyte in many phytoplankton species, with intracellular concentrations ranging from 10–250 mM.[3,4] It also may serve other important functions such as an antioxidant,[5] overflow metabolite,[2] cryoprotectant,[6] grazing deterrent[7] and interspecies signalling molecule.[8] DMSP is widely distributed in the euphotic zone with concentrations of total DMSP (DMSPt) ranging from 10 to 100 nM in most waters,[9] but with concentrations reaching 1000 nM during some phytoplankton blooms.[10]

The total DMSP pool in seawater consists of both particulate and dissolved fractions. Particulate DMSP (DMSPp) is often defined as that which is retained on filters such as Whatman GF/F (0.7-μm nominal retention), whereas dissolved DMSP (DMSPd)

is that which passes through a GF/F filter.[11–13] DMSPp is thought to be mainly contained in living phytoplankton cells, though it can also be found in grazers that have eaten phytoplankton prey.[14–16] DMSPp is released from phytoplankton into the dissolved pool through a combination of processes involving algal autolysis, viral attack and zooplankton grazing.[17–20] The operationally defined DMSPd is generally assumed to be free in solution, though no studies have directly tested this assumption. The DMSPd pool is rapidly utilised as a substrate by marine bacteria with turnover times on the order of hours in subtropical and temperate oceanic waters.[21,22] Current estimates suggest that DMSPd supports up to 15% of bacterial carbon demand[22,23] and provides most or all of the sulfur required for marine bacterial growth.[24,25] The importance of DMSP-sulfur to marine microbes is further illustrated by the fact that nearly all the phylogenetic groups of bacteria (including photosynthetic cyanobacteria) become labelled with ^{35}S when ^{35}S-DMSPd is supplied to seawater.[24,26–30] Even eukaryotic phytoplankton acquire DMSP-sulfur.[31]

In addition to its role as a carbon and sulfur source in the food web, DMSP is also important as a major precursor of dimethylsulfide (DMS),[32] a biogenic volatile sulfur gas that is ubiquitous in the photic zone of the ocean.[33,34] DMS emissions from the surface ocean to the atmosphere are estimated to be 0.5 to 1×10^{12} mol year^{-1}.[35,36] In the atmosphere, DMS oxidation products can generate aerosol particles and contribute to the growth of pre-existing aerosols. Aerosols influence the global climate system by backscattering solar radiation and by modifying cloud radiative properties, with potential for feedback on ocean communities that are originally responsible for DMSP and DMS production.[37] The fraction of DMSPd converted to DMS (the DMS yield) varies as a function of DMSPd availability, bacterial sulfur demand, and solar radiation exposure, and ranges from ~2–40%.[38–40] Even with low DMS yields, DMSPd is a significant precursor of DMS because the overall turnover flux of DMSPd is high.[21]

DMSPd turnover has been determined by inhibitor and tracer approaches that rely on accurate measurements of DMSPd concentrations. Kiene and Gerard[41] utilised micromolar additions of glycine betaine (GBT, $(CH_3)_3N^+CH_2COO^-$) to inhibit the uptake of DMSPd, and they measured the accumulation rate of DMSPd as sampled with a large volume (20 mL) gravity drip filtration through a GF/F filter. Several studies have added tracer levels of ^{35}S-DMSPd to seawater samples and quantified the loss rate constant of the DMSPd pool (k_{DMSPd}).[22,23,38,42] The turnover rate was then determined by multiplying k_{DMSPd} by the in situ DMSPd concentration determined typically by large volume (e.g. >10 mL) GF/F filtration. Filtration of more than a few millilitres of seawater, as done in the studies mentioned above, can cause DMSP release from plankton, leading to artificial increases in DMSPd concentrations.[12] Thus, turnover rates that depend directly on DMSPd concentrations could have been overestimated in previous studies. Because of these concerns and the availability of a more reliable sampling method for DMSPd based on small volume drip filtration (SVDF), in which only the first few millilitres of filtrate are collected for analysis,[12] an evaluation of the different approaches to quantify DMSPd turnover rates is warranted.

In the present study, we determined DMSPd consumption rates in coastal waters of Mobile Bay and the northern Gulf of Mexico by the tracer (^{35}S-DMSPd) and inhibitor (GBT) techniques, and we used the SVDF filtration procedure for collection of DMSPd samples and the base hydrolysis method for quantification of DMSP.[43] Discrepancies in the consumption rates obtained with the two techniques prompted us to evaluate the techniques and also the accuracy of DMSPd concentration obtained by the SVDF technique ($[DMSPd]_{SVDF}$). Our evidence suggests that the GBT inhibitor approach yielded reliable DMSPd turnover fluxes and the ^{35}S-DMSPd technique yielded reliable values for k_{DMSPd}. In contrast, the measured $[DMSPd]_{SVDF}$ over-estimated the truly dissolved DMSP concentration and the bioavailable pool in the coastal waters tested, resulting in overestimation of DMSP turnover if k_{DMSPd} was multiplied by $[DMSPd]_{SVDF}$. Additionally, we provide the first evidence for a significant pool of refractory DMSP (resistant to degradation on time scales of days), that is included in typical dissolved DMSP measurements.

Materials and methods

Study sites and sample collection

Most water samples used in the present study were collected from the Dauphin Island Sea Lab pier (30°15′N, 88°05′W) located near the mouth of Mobile Bay, a large, shallow, highly turbid estuary that empties into the northern Gulf of Mexico. Samples collected from the Sea Lab pier had salinities ranging from 8.0 to 31.6 ppt, and temperatures ranging from 12.6 to 16.9 °C (Tables 1, 2). One offshore shelf water sample with low turbidity and high salinity (35.2 ppt) was collected in the Gulf of Mexico south of Mobile Bay (29°25′48″N, 87°58′48″W). All water samples were collected from the surface with an acid-rinsed bucket, avoiding disturbance of the water as much as possible. After collection, water samples were stored in the dark at the in situ temperature (±1 °C) during transport to the laboratory (<5 min for pier samples and ~1 h for the shelf sample). The surface-water temperature was measured in situ with a thermometer, and salinity was obtained by using a hand-held refractometer. Immediately upon return of the water samples to the laboratory, sub-samples were collected for DMSPt and DMS concentration measurements. For DMSPt, 10 mL of water from a well-mixed sample was pipetted into a 15-mL centrifuge tube containing 50 µL of 50% H_2SO_4. Preserved samples were stored for >24 h before 0.5-mL subsamples were analysed for DMSP content as described below. For dissolved DMS analysis, seawater samples were taken up into a glass syringe and filtered through a 25-mm diameter Whatman GF/F filter (GE Life Sciences, Pittsburg, PA, USA) directly into a purge and trap-gas system as described below. The small volume drip filtration procedure (SVDF) of Kiene and Slezak[12] was used for determination of dissolved DMSP. Briefly, ~30 mL of water sample was gently poured into a polysulfone magnetic filter tower (Pall Gelman, Port Washington, NY, USA), holding a 47-mm diameter Whatman GF/F filter (0.7-µm nominal retention). The water was allowed to drip by gravity pressure and the initial 3.5 mL of filtrate was collected into a 15-mL centrifuge tube that contained 17.5 µL of 50% H_2SO_4 (0.25% final H_2SO_4 concentration). The acid in the receiving tube served to stop any biological activity in the filtrate, thereby preserving the DMSP that passed through the filter. The concentration of DMSP in these filtrates is referred to as $[DMSPd]_{SVDF}$ and was quantified as described below.

The GBT inhibitor approach for whole water DMSPd consumption

We used the technique of Kiene and Gerard[41] wherein GBT (anhydrous, Sigma–Aldrich, St Louis, MO, USA) was added to

Table 1. Sampling dates, locations and initial characteristics of coastal seawater water samples used for whole water incubations with tracer levels of ^{35}S-DMSPd or 5 µM glycine betaine (GBT)

Also shown are the rate constants for ^{35}S-DMSPd loss ($k_{^{35}S\text{-}DMSPd}$), the DMSPd loss rates calculated from $k_{^{35}S\text{-}DMSPd} \times [DMSPd]_{SVDF}$, and DMSPd loss calculated from the accumulation of $[DMSPd]_{SVDF}$ in the presence of 5 µM GBT. The ratio of the ^{35}S-DMSP-determined loss rate to the GBT-determined loss rate is shown in the far right column for each experiment. Time courses of $[DMSPd]_{SVDF}$ and ^{35}S-DMSP for three of the experiments (18 November, 3 December and 10 March) are shown in Fig. 1. $[DMSPd]_{SVDF}$, dissolved DMSP concentration by small volume drip filtration through a GF/F filter (see text); DMSPp, particulate DMSP; [DMS], dissolved dimethylsulfide concentration; Sea Lab Pier, Dauphin Island Sea Lab; N.D., not determined

Sampling location	Date	In situ temperature (°C)	Salinity (ppt)	$[DMSPd]_{SVDF}$ (nM)	[DMSPp] (nM)	[DMS] (nM)	$k_{^{35}S\text{-}DMSPd}$ (day^{-1})	DMSPd loss rate by ^{35}S-DMSPd (nM day^{-1})	DMSPd loss rate by GBT inhibition (nM day^{-1})	Ratio of loss rates (^{35}S-DMSPd : GBT inhibition)
Sea Lab Pier	11 November 2008	16.9	30.3	3.79	N.D.	N.D.	35.0	133	15.6	8.56
Sea Lab Pier	18 November 2008	15.2	31.6	2.61	44.2	1.61	82.3	215	7.89	27.3
Sea Lab Pier	3 December 2008	12.6	29.7	2.86	87.8	1.39	27.1	77.5	12.7	6.11
Sea Lab Pier	14 January 2009	14.5	9	0.90	12.0	1.15	57.4	51.6	0.34	152
Gulf of Mexico shelf	10 March 2009	20.6	35.2	1.68	N.D.	N.D.	21.4	35.7	21.6	1.65
Average				2.37			44.6	103	11.6	39.1

Table 2. Characteristics of coastal seawater used to prepare GF/F filtrate incubations and the initial pools of DMSP in the filtrate incubations

Also shown are the concentrations of $DMSPd_{SVDF}$, DMSPp and DMS in the whole water used to prepare the filtrate incubation, initial $DMSPd_{<0.2\,\mu m}$ in the filtrate incubation, the initial particle-associated DMSP in the GF/F filtrate ($DMSP_{0.2–0.7\,\mu m}$), the fraction of whole water $[DMSPd]_{SVDF}$ that was particle-associated ($[DMSP]_{0.2–0.7\,\mu m}$: $[DMSPd]_{SVDF}$) and the refractory DMSPd remaining at the end of the filtrate incubation. Refractory DMSPd is the $[DMSPd]_{<0.2\,\mu m}$ remaining at the end of the filtrate incubation time. Sea Lab Pier, Dauphin Island Sea Lab; N.D., not determined

Sampling location	Date	In situ temperature (°C)	Salinity (ppt)	Filtrate incubation time (h)	Whole water $[DMSPd]_{SVDF}$ (nM)	Whole water [DMSPp] (nM)	Whole water [DMS] (nM)	Initial $[DMSPd]_{<0.2\,\mu m}$ in GF/F filtrate (nM)	Initial $[DMSP]_{0.2–0.7\,\mu m}$ in GF/F filtrate (nM)	$[DMSP]_{0.2–0.7\,\mu m}$: $[DMSPd]_{SVDF}$	Refractory DMSPd (nM)
Sea Lab Pier	16 December 2008	18.0	13	22	N.D.	N.D.	N.D.	10.6	1.11		0.58
Sea Lab Pier	6 January 2009	18.6	17	24	1.45	N.D.	N.D.	4.38	0.45	0.31	0.97
Sea Lab Pier	14 January 2009	14.5	9	46	0.90	12.0	1.15	1.68	0.33	0.37	N.D.
Sea Lab Pier	27 January 2009	14.5	8	46	1.16	4.38	5.88	2.65	0.12	0.10	1.15
Sea Lab Pier	6 Febuary 2009	13.1	20	62.5	2.27	70.4	3.94	4.93	0.66	0.29	N.D.
Gulf of Mexico shelf	10 March 2009	20.6	35.2	24	1.68	N.D.	N.D.	2.21	0.28	0.17	0.44
Average								4.41	0.54	0.25	

a final concentration of 5 μM to unfiltered seawater to inhibit DMSPd uptake and therefore the consumption of the natural pool of DMSPd. GBT is a structural analogue of DMSP and these two compounds are mutual inhibitors of their respective uptake.[44] The rate of change of $[DMSPd]_{SVDF}$ in inhibited samples, corrected for changes in non-inhibited controls, was taken as the natural DMSPd consumption rate.[41] The only difference from the approach used by Kiene and Gerard[41] was that here we used the small volume drip filtration protocol[12] to collect DMSPd samples (see above). We confirmed that GBT remained effective during our experiments by including tracer additions of dissolved ^{35}S-DMSP and following its concentration during the incubations (see below for details regarding ^{35}S-DMSP measurements).

For the GBT inhibitor experiments, unfiltered seawater was carefully transferred into a series of 2-L Teflon bottles. GBT from an aqueous stock solution (20 mM, prepared from anhydrous reagent, Sigma–Aldrich) was added to a final concentration of 5 μM to duplicate bottles and a separate set of duplicate bottles were left untreated as controls. Both experimental treatments and controls were incubated in the dark at the in situ temperature. At selected time points, a 30-mL sub-sample was removed from each bottle and processed for $[DMSPd]_{SVDF}$.

The ^{35}S-DMSPd tracer approach for whole water DMSPd consumption

In parallel with the GBT inhibitor-$[DMSPd]_{SVDF}$ accumulation experiments, we measured the DMSPd loss rate constant in whole water samples using the ^{35}S-DMSP tracer approach.[38] Duplicate 60-mL Teflon bottles were filled with unfiltered seawater and treated with tracer levels of ^{35}S-DMSP to a concentration of 1000 dpm mL^{-1} (specific activity: 2.4–6.3 × 10^5 dpm pmol^{-1}). The Teflon bottles were closed, mixed gently and then incubated in the dark in a water bath at the in situ temperature. At selected time points, a 5-mL sub-sample from each bottle was withdrawn by pipette and filtered using gentle vacuum (<1300 Pa) through a 0.2-μm nylon filter held on a 10-place Hoefer filtration manifold (Hoefer Inc., Holliston, MA, USA). The filtrate from each sample was collected into a separate polyethylene vial containing 50 μL of 50 % sulfuric acid to stop biological activity and preserve the DMSP. After 24 h of storage, a 4-mL sub-sample from each preserved filtrate sample was transferred into a 60-mL glass serum bottle and the bottle was sealed with a rubber stopper fitted with a suspended plastic cup (Kimble Chase, Vineland, NJ, USA). The plastic cup held a Pall AE glass fibre filter soaked with 0.2 mL of 3 % H_2O_2. At this point, 0.5 mL of 5 N NaOH was injected through the stopper to quantitatively cleave ^{35}S-DMSP in the sample to ^{35}S-DMS, which was oxidised and subsequently trapped in the H_2O_2-soaked filter. The bottles were then placed on a rotary shaker for >10 h to allow ^{35}S-DMS to be quantitatively trapped in the filter wick. After the trapping period, filter wicks were placed into 5-mL plastic scintillation vials with 4-mL Ecolume scintillation fluid (MP Biomedicals, Santa Ana, CA, USA). The scintillation vials were held for >12 h to allow counts to stabilise before they were quantified with a Packard Tri-Carb model 2500 TR scintillation counter (Perkin Elmer, Waltham, MA, USA).[21] This procedure gives a measure of the amount of ^{35}S-DMSP remaining in the dissolved (<0.2-μm size) pool after incubation. Loss of particulate ^{35}S during filtration through 0.2-μm nylon filters was assumed to be negligible based on a previous investigation that showed that this type of filter retained particulate (i.e. cellular) materials derived from uptake of low molecular weight organic substrates even when the cellular material was cytosolic and would be released if cells leaked on filtration.[45] The DMSPd loss rate constant ($k_{^{35}S\text{-}DMSPd}$) was calculated as the slope of the natural log of the fraction of remaining ^{35}S-DMSPd *v*. time. The total initial added amount of ^{35}S was determined by pipetting a 0.5-mL sub-sample from each bottle directly into a scintillation vial containing 4-mL scintillation fluid (Ecolume) and measuring ^{35}S-activity by liquid scintillation counting.

Filtrate incubation experiments

To focus on the consumption of DMSPd without the confounding factor of simultaneous DMSP production, or phytoplankton DMSP release, we used seawater filtrate incubations that contained mainly bacteria. Filtrate incubations were started by filtering surface seawater from a 10-L carboy through a 142-mm Whatman GF/F filter (>0.7-μm nominal retention) using gravity pressure. For each experiment, ~9 L of water was filtered and only one filter was used. The GF/F filtration removed essentially all the phytoplankton and zooplankton while allowing a fraction (typically 10–30 % of the natural bacterioplankton) to pass through the filter.[44,46] The GF/F filtrate incubations were incubated for up to 48 h in the dark in 2-L Teflon bottles held within 2 °C of the in situ temperature. With removal of the phytoplankton and incubation of the samples in the dark, DMSP production was assumed to be negligible. This assumption was tested by addition of 5 μM GBT to some filtrate incubations. GBT blocked DMSP consumption in the filtrate incubations and there was no additional accumulation of DMSP in the presence of GBT suggesting no DMSP production (data not shown). The large volume filtration used to prepare the filtrate incubations caused some release of DMSPd from the particulate DMSP pool such that the initial concentrations of DMSPd in the filtrate incubations was significantly above the $[DMSPd]_{SVDF}$ obtained from whole water. This was expected based on previous results[12] and, in fact, was desired in the present context to achieve a 'natural' addition of DMSPd to concentrations above the background level (see Results).

Immediately after the GF/F filtration, the filtrate was homogenised by gentle mixing and then split into two sets (with duplicate Teflon bottles in each set). One set was left untreated whereas the other set was treated with ^{35}S-DMSPd (see below). This allowed comparison of the loss rates of DMSPd as measured by conventional gas chromatography techniques with that obtained by following ^{35}S-DMSPd spiked into the samples at time zero. For the unlabelled GF/F filtrate incubations, DMSP concentrations in three different size fractions were measured at selected time points. A 10–20-mL sample of the filtrate incubation water was pipetted directly into a storage tube containing 50 μL of 50 % sulfuric acid (0.13 to 0.25 % final concentration) to preserve the total DMSP. We refer to this concentration as $[DMSP]_{<0.7\,\mu m}$ because it was from a GF/F filtrate (i.e. <0.7 μm) and to distinguish it from [DMSPt], which is the conventional term for total concentration of DMSP in whole, unfiltered seawater.[47] At the same time point, a separate 10–20-mL sample was filtered through a 25-mm diameter nylon membrane (0.2-μm pore size) under low vacuum (1300 Pa). The filtrate was collected directly into a 50-mL polypropylene centrifuge tube containing 50 μL of 50 % sulfuric acid to preserve DMSP in the less than 0.2-μm size fraction ($[DMSPd]_{<0.2\,\mu m}$). The difference between $[DMSP]_{<0.7\,\mu m}$ and $[DMSPd]_{<0.2\,\mu m}$ represents the concentration of DMSP contained in particles >0.2 μm in the filtrate incubation ($[DMSP]_{0.2–0.7\,\mu m}$). Independent determination of particulate DMSP in the filtrate incubations by measuring DMSP retained

on 0.2-μm nylon filters gave similar results to the difference calculation (data not shown). The acidified sub-samples were left for at least 24 h so that any endogenous DMS was removed by ventilation or acid catalysed oxidation.[12] The DMSP content in each unlabelled acid-preserved sample was measured after alkaline hydrolysis to DMS with analysis of the resulting DMS by gas chromatography as described below. In some cases, sub-samples of the collected volume were analysed, but at some of the later time points, analysis of the entire 10–20-mL volume was required to detect DMSP in the <0.2-μm filtrate.

A second set of filtrate incubation samples, run in parallel with the unlabelled filtrates, was treated with ~1000 dpm mL^{-1} of ^{35}S-DMSP at time zero of the unlabelled DMSP measurement time course. The loss of the ^{35}S-DMSP from the <0.2-μm size fraction was monitored by filtering the labelled samples through 0.2-μm nylon membranes and capturing the filtrate in tubes containing 50 μL of 50 % H_2SO_4. These samples were analysed for remaining ^{35}S-DMSP by the procedures outlined above.

In most cases the filtrate incubation experiments were monitored for 24 h. In two experiments, unlabelled filtrate incubations were pre-incubated for 24 h to allow the endogenous $[DMSPd]_{<0.2\,\mu m}$ to reach low levels before ^{35}S-DMSP was added. These experiments allowed for a comparison of the rate of change of the remaining unlabelled dissolved DMSP (i.e. $[DMSPd]_{<0.2\,\mu m}$) pool, as measured by GC, with that of the added radioactive tracer.

A comparison of the bioavailability of unlabelled exogenous DMSP with the ^{35}S-DMSP tracer was made by adding 37 nM DMSPd from a reagent stock (DMSP · HCl; obtained from Selact, Groningen) to one set of a 24-h pre-incubated GF/F filtrate incubation prepared from Gulf of Mexico shelf seawater. The incubated filtrate had a $[DMSPd]_{<0.2\,\mu m}$ of 0.44 nM at the time of the DMSP addition. A parallel set of filtrate incubation samples received the same 37 nM DMSP reagent addition, plus 1000 dpm mL^{-1} of ^{35}S-DMSP. The loss rates of the unlabelled $[DMSPd]_{<0.2\,\mu m}$ and the ^{35}S-DMSP were compared. Incubation, sampling and analyses for these bioavailability tests were as described above. The first sub-sample for measurement of $[DMSPd]_{<0.2\,\mu m}$ was taken within 2 min of the experimental additions and this time point was designated as the initial time point.

Sulfur analyses

DMS in seawater or that produced from DMSP by alkaline hydrolysis was analysed using a purge and trap system coupled to a Shimadzu GC-14A gas chromatograph (GC) (Shimadzu Scientific Instruments, Columbia, MD, USA) with a Chromosil 330 column and a flame photometric detector, as described in detail by Rellinger et al.[48] For dissolved DMS, water samples (2 mL) were introduced into the purge vessel by syringe filtration through Whatman GF/F filters. For analysis of the acidified DMSPt and $DMSPd_{SVDF}$ samples, sub-samples ranging from 0.5 to 3.3 mL were transferred by pipette to a serum vial. One millilitre of 5 M NaOH was added and the vial was quickly sealed with a Teflon-faced butyl rubber serum stopper. After allowing at least 15 min for reaction, the sample was connected to the purge and trap system and the entire contents of the vial purged with helium and trapped in a Teflon loop immersed in liquid N_2. All DMSP and DMS samples were analysed in duplicate, with analytical precision <10 %. The absolute detection limit of the GC system was 1 pmol of DMS per injection. Thus, for a 2-mL sample the detection limit for DMS was 0.5 nM. The volume analysed of acidified DMSP samples from the filtrate incubations was adjusted depending on expected concentrations. For $[DMSP]_{<0.7\,\mu m}$ and $[DMSPd]_{<0.2\,\mu m}$ in some filtrate incubations, samples as large as 20 mL were analysed in 60-mL vials with 5 mL of 5 M NaOH, translating to a detection limit of 0.05 nM DMSP. Blanks consisting of water that was pre-sparged with He routinely gave no detectable DMS.

Results

Comparison of DMSPd turnover rates: GBT inhibitor v. ^{35}S-DMSP approach

Addition of 5 μM of GBT to three different unfiltered coastal water samples caused linear ($P < 0.01$) increases of $[DMSPd]_{SVDF}$ in 2–6-h dark incubations, whereas $[DMSPd]_{SVDF}$ remained steady in non-inhibited controls (Fig. 1a–c). The inhibitory effect of GBT on DMSPd consumption was confirmed in parallel incubations of unfiltered seawater with ^{35}S-DMSP that showed that GBT blocked the loss of the tracer, whereas the tracer was consumed rapidly ($k_{^{35}S\text{-}DMSPd} = 21.4$ to 82.3 day^{-1}), and nearly completely, in non-inhibited samples (Fig. 1d, e). Rates of DMSPd consumption, based on differences in the slopes of $[DMSPd]_{SVDF}$ *v.* time between GBT-treated and non-treated samples, ranged from 0.34 to 21.6 nM day^{-1} for these and two additional experiments (Fig. 1, Table 1). In these same experiments, DMSP consumption rates obtained by multiplying $k_{^{35}S\text{-}DMSPd}$ (Fig. 1d–f) by the initial $[DMSPd]_{SVDF}$ ranged from 35.7 to 215 nM day^{-1} (Table 1). The ^{35}S tracer-determined rates exceeded the GBT-determined rates in the same experiments by an average factor of 39.1, with factors ranging from 1.65 in a high salinity (35.2 ppt) shelf water sample to 152 in a low salinity (9 ppt) sample from Mobile Bay (Table 1).

Because GBT appeared to be effective at blocking DMSPd consumption (Fig. 1d–f), as intended, it seemed that the likely source(s) of the large discrepancy between GBT- and tracer-determined rates would be the values obtained for $k_{^{35}S\text{-}DMSPd}$ or $[DMSPd]_{SVDF}$ (or both). To test the validity of the $k_{^{35}S\text{-}DMSPd}$ and $[DMSPd]_{SVDF}$ determinations we carried out a series of experiments with dark-incubated seawater filtrates.

Particulate DMSP in GF/F filtrates

Because the GF/F filtrate incubations contained bacteria, and bacteria can take up and retain DMSP,[45,49] we determined the >0.2-μm 'particulate' DMSP in the <0.7-μm filtrate incubations ($[DMSP]_{0.2–0.7\,\mu m}$). The initial concentrations of $[DMSP]_{0.2–0.7\,\mu m}$ in the filtrate incubations ranged from 0.12 to 1.11 nM (Table 2) which represented 10–37 % of the $[DMSPd]_{SVDF}$ determined in the whole water used to prepare the filtrate incubations (Table 2). Thus, $[DMSPd]_{SVDF}$ overestimates the true DMSPd concentration because a significant fraction of $[DMSPd]_{SVDF}$ is in particles and not dissolved. Multiplication of $k_{^{35}S\text{-}DMSPd}$ by $[DMSPd]_{SVDF}$ would therefore estimate DMSPd consumption rates and this could partially explain why ^{35}S-DMSP-determined rates were higher than those obtained by GBT inhibition. However, after correcting the $[DMSPd]_{SVDF}$ values in Table 1 by subtracting the $[DMSP]_{0.2–0.7\,\mu m}$ values in Table 2, the tracer rates still exceeded the inhibitor rates by 1.4 to 96-fold. Therefore additional factors must be responsible for the discrepancy in the rates.

Assessing the accuracy of $k_{^{35}S\text{-}DMSPd}$

The accuracy of $k_{^{35}S\text{-}DMSPd}$ was assessed by comparing the loss rates of dissolved $DMSP_{<0.2\,\mu m}$ and tracer spikes of ^{35}S-DMSPd in GF/F filtrate incubations. The initial $[DMSPd]_{<0.2\,\mu m}$ in the filtrate incubations was 1.68 to 10.6 nM

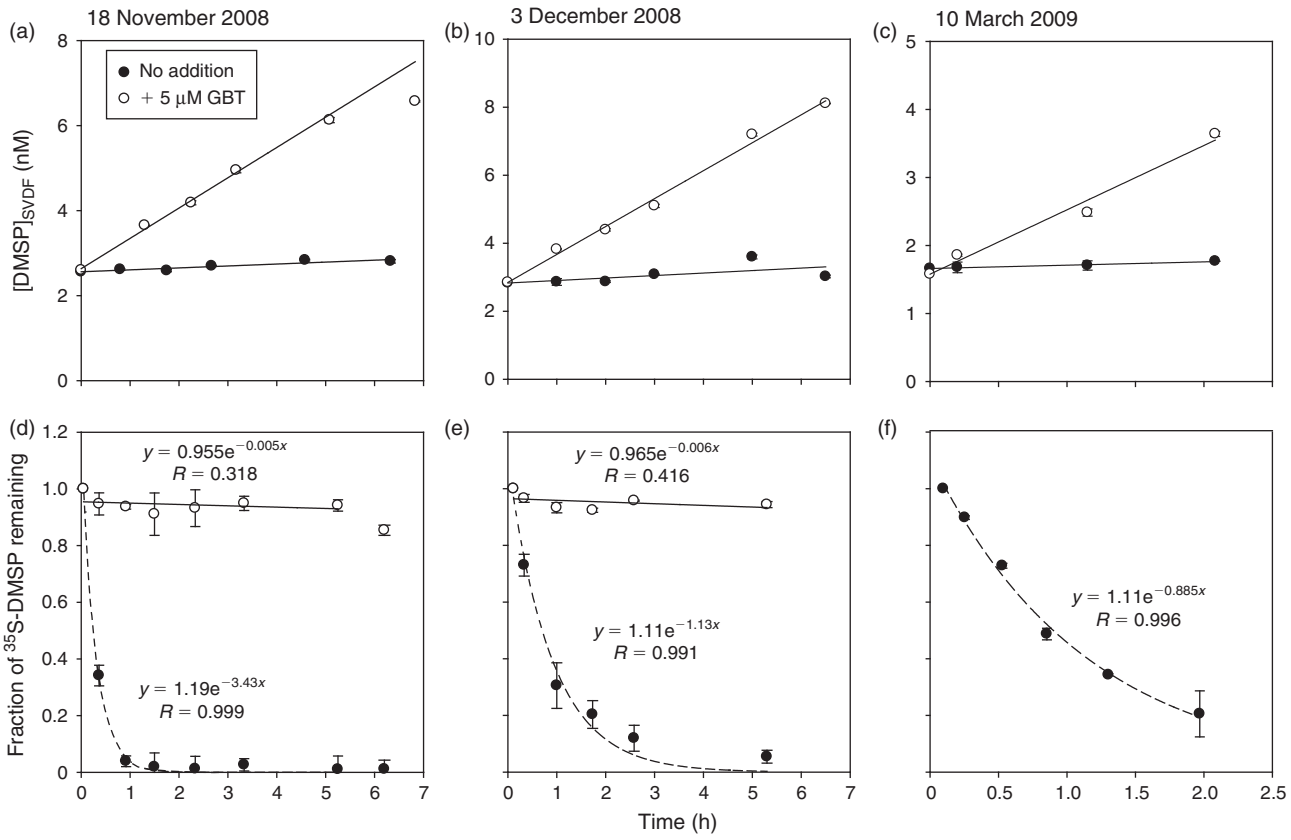

Fig. 1. Time courses of dissolved dimethylsulfoniopropionate (DMSPd) concentration by small volume drip filtration ($[DMSPd]_{SVDF}$, upper three panels) and the fractional loss of tracer levels of ^{35}S-labelled DMSPd (^{35}S-DMSPd) (lower three panels) for three different seawater samples treated with either 5 µM unlabelled glycine betaine (GBT) (open circles) or no GBT addition (closed circles). Samples were unfiltered seawater from the coastal Gulf of Mexico collected on 18 November 2008 (a, d), 3 December 2008 (b, e) and 10 March 2009 (c, f). Incubations were conducted in the dark at the in situ temperature. Vertical bars denote the range of duplicate measurements. Solid lines in upper panels represent a linear least-squares fit to the data and solid and dashed curves in the bottom panels are exponential fits to the data with the equations shown.

(Table 2, Fig. 2) and was 1.3 to 3.0 times higher than the corresponding $[DMSPd]_{SVDF}$ from the whole water used to prepare each filtrate incubation (Table 2). This can be attributed to filtration-induced release of DMSP from particles (plankton) during the fairly large volume filtration (9 L) used to prepare the filtrate incubations. As expected, $[DMSPd]_{<0.2\ \mu m}$ decreased over time due to uptake and metabolism of the DMSP by bacteria in the filtrate incubations. The decreases of $[DMSPd]_{<0.2\ \mu m}$ initially followed a quasi-exponential pattern but concentrations levelled off at between 0.5 to 1.0 nM, which was 26–67 % of the $[DMSPd]_{SVDF}$ concentrations in the whole water used to prepare the filtrate incubations (horizontal dashed lines in Fig. 2b, c). These stable concentrations were well above our detection limit of 0.05 nM for these experiments. In parallel filtrate incubations spiked with tracer levels of ^{35}S-DMSP at time zero, the tracer declined exponentially (Fig. 2d–f) and decreased to nearly undetectable levels (<2 % of initial) when incubations were carried out to 24 h (Fig. 2e; see also Fig. 1d, e). The nearly complete consumption of the tracer was in contrast to the unlabelled $[DMSPd]_{<0.2\ \mu m}$ that levelled off at ~6–22 % of the initial concentrations in the filtrate incubations by 24 h (open triangles in Fig. 2d–f). If we subtract the asymptotic, 24 h value of $[DMSPd]_{<0.2\ \mu m}$ (i.e. the apparently non-reactive pool) from the concentrations at each time point, then recalculate the fractions of the initial concentrations (closed triangles and dotted line fits in Fig. 2d–f), then the loss kinetics of both the ^{35}S tracer and the $[DMSPd]_{<0.2\ \mu m}$ agreed almost exactly (Fig. 2d–f).

When ~37 nM unlabelled $[DMSPd]_{<0.2\ \mu m}$ from a reagent stock was added to a GF/F filtrate incubation prepared from shelf water (pre-incubated for 24 h in the dark at the in situ temperature so the endogenous DMSPd was nearly completely consumed), the added DMSPd was consumed over the next 24 h with a small, transient accumulation (2 nM above initial) in the bacteria-sized particulate ($[DMSP]_{0.2–0.7\ \mu m}$) pool (Fig. 3a). As was found with the excess $[DMSPd]_{<0.2\ \mu m}$ resulting from filtration (Fig. 2d–f), the loss kinetics of exogenously added reagent $[DMSPd]_{<0.2\ \mu m}$ agreed very closely with that of ^{35}S-DMSP in parallel samples (Fig. 3b). These findings suggest that the ^{35}S-DMSP tracer approach provides accurate estimates of DMSPd loss from a reactive pool that is above some background level of non-reactive DMSPd that does not appear to be consumed on a time scale of 24 h (i.e. the asymptotic value of $[DMSPd]_{<0.2\ \mu m}$ reached after 24-h incubation).

Evidence for a refractory DMSPd pool

Further evidence for a non-reactive, i.e. refractory, $[DMSPd]_{<0.2\ \mu m}$ pool was obtained from filtrate incubations that were pre-incubated for ~24 h to allow initial $[DMSPd]_{<0.2\ \mu m}$ to reach very low (<1.3 nM), quasi-steady levels. Incubation for a further 24 h in the dark showed that $[DMSPd]_{<0.2\ \mu m}$ remained reasonably steady at 1.0–1.3 nM and 0.44–0.54 nM in two

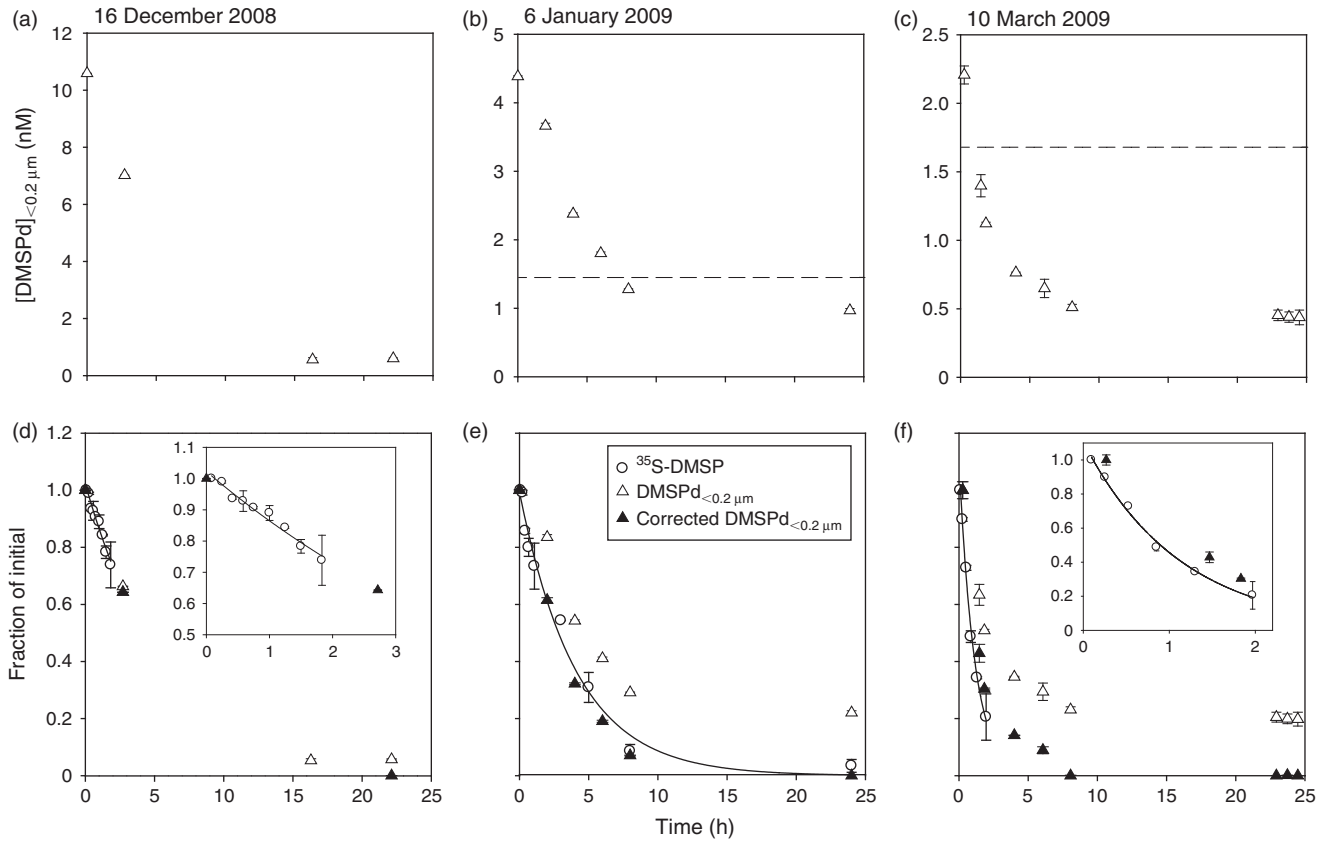

Fig. 2. Results from three filtrate incubation experiments showing changes with time of concentration of dissolved dimethylsulfoniopropionate (DMSPd) that passed through a 0.2-µm filter ($[DMSPd]_{<0.2\ \mu m}$) (top panels) and the fraction of initial ^{35}S-labelled DMSPd (^{35}S-DMSPd) remaining in parallel incubations (bottom panels, open circles). Also shown in the bottom panels are the fraction of initial $[DMSPd]_{<0.2\,\mu m}$ remaining (open triangles) and corrected $[DMSPd]_{<0.2\,\mu m}$ fractions (closed triangles) obtained by subtracting the final concentration of $[DMSPd]_{<0.2\,\mu m}$ from each time point (see text). Insets in panels d and f show the early time courses in greater detail; for these experiments ^{35}S-DMSPd data (open circles) were available only for the first 2 h. In bottom panels, solid and dotted lines represent least-squares regression fits (first order exponential loss) for the fraction of remaining ^{35}S-DMSPd and corrected fractions of $[DMSPd]_{<0.2\,\mu m}$ respectively. Symbols and vertical bars represent the mean ± range ($n = 2$). GF/F filtrate incubations were prepared from Gulf of Mexico seawater on 16 December 2008 (a, d), 6 January 2009 (b, e) and 10 March 2009 (c, f). In panels b and c, the horizontal dashed lines represent the concentration of DMSPd determined by small volume drip filtration ($[DMSPd]_{SVDF}$) in whole seawater used to prepare the filtrate incubations; $[DMSPd]_{SVDF}$ data were not available for 16 December 2008.

separate experiments (Fig. 4a, b). In contrast, tracer levels of ^{35}S-DMSP (1–4 pM) added to these filtrates were consumed extremely rapidly (>90 % in 2 h) and almost completely in both experiments (Fig. 4c, d).

Discussion

Dissolved DMSP is a widely used substrate for marine microbial populations[26,50] and it is potentially a globally significant source of the climatically active trace gas, DMS.[51,52] Previous estimates of the DMSPd turnover flux have been based on measurements of DMSPd concentrations that may have been overestimated because of filtration-induced release of DMSP from particulate material.[12] In response to these problems, Kiene and Slezak developed a small volume drip filtration (SVDF) protocol that minimised filtration artefacts and appeared to give much lower estimates of DMSPd than previous studies and that produced results that agreed with in situ deployed dialysis samplers.[12] With $[DMSPd]_{SVDF}$ providing seemingly more reliable estimates of DMSPd concentrations, we sought to reassess the turnover flux of this pool. As a first step in this reassessment, we compared two different methods to estimate DMSPd turnover, the GBT inhibitor approach and the ^{35}S-DMSP tracer approach, both of which rely on determination of the DMSPd concentration. In comparisons with several different water samples from the coastal Gulf of Mexico, we found that DMSPd turnover estimates by the ^{35}S-tracer technique were from 1.7 to 152 times higher than rates obtained with the GBT inhibitor technique (Table 1). To investigate the reasons for this discrepancy, we evaluated each of the approaches and looked more closely at the DMSPd pool itself.

Evaluation of the GBT inhibitor method

This approach is based on the competitive inhibition of transmembrane transport of DMSP by high concentrations of the structural analogue GBT.[41,44] In the present study we found that additions of 5µM GBT (~5000 times higher concentration than the endogenous DMSPd) inhibited loss of ^{35}S-DMSP by more than 99 % during 5–6 h incubations and resulted in easily measured, linear accumulations of DMSPd as measured by SVDF (Fig. 1). Because GBT inhibits uptake of DMSP,[44] and because DMSP degrading enzymes are not inhibited by GBT,[53,54] these findings suggest that the location of DMSP-degrading enzymes (e.g. DMSP lyases and demethylases) was mainly intracellular in the natural seawater microbial populations studied here. Cell surface DMSP lyase activity has been reported in bacteria[54] and a phytoplankter,[55] but this mechanism did not

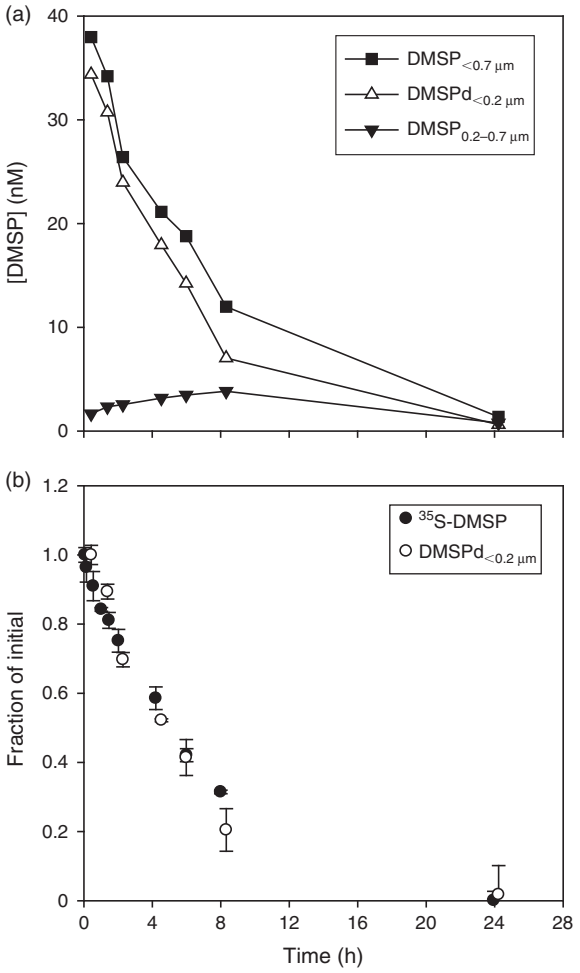

Fig. 3. Time courses of the concentration of dimethylsulfoniopropionate (DMSP) that passed through a 0.7-µm filter ($[DMSP]_{<0.7\ \mu m}$), concentration of dissolved DMSP (DMSPd) that passed through a 0.2-µm filter ($[DMSPd]_{<0.2\ \mu m}$) and [DMSP] contained in >0.2-µm 'particulate' DMSP in the <0.7-µm filtrate incubations ($[DMSP]_{0.2-0.7\ \mu m}$) (upper panel) in a GF/F filtrate incubation amended with exogenous unlabelled dissolved dimethylsulfoniopropionate (DMSPd) (~37 nM) and tracer levels (<0.01 nM) of ^{35}S-DMSPd and incubated in the dark. The filtrate incubation was prepared from Gulf of Mexico shelf water on 10 March 2009 and pre-incubated for 24 h in the dark before the amendments of unlabelled and labelled DMSPd. The bottom panel shows the time courses of the fraction of initial $[DMSPd]_{<0.2\ \mu m}$ and ^{35}S-DMSPd remaining. Vertical bars denote the range from duplicate bottles.

seem to be important in DMSPd degradation by natural populations in our samples. Because GBT was effective at preventing DMSPd degradation, the pool of $DMSPd_{SVDF}$ increased over time owing to continued natural production processes (Fig. 1). In contrast, $[DMSPd]_{SVDF}$ concentrations in non-inhibited control samples were nearly steady, reflecting a balance between natural production and loss processes. DMSPd production can arise from several mechanisms, including viral lysis of phytoplankton cells,[18] grazing[56] and phytoplankton senescence.[57] The rate of $[DMSPd]_{SVDF}$ accumulation in the presence of GBT represents gross DMSPd production. The difference in $[DMSPd]_{SVDF}$ accumulation between GBT-inhibited and non-inhibited control samples should represent the natural removal rate (e.g. turnover flux) for DMSPd.[41] In the experiments performed here, which used the SVDF technique to collect DMSPd samples, DMSPd consumption rates by GBT-inhibition ranged from 0.34 to 21.6 nM day^{-1} (Table 1). These rates are very close to those obtained in an earlier study utilising the GBT inhibitor approach in the same area of the coastal Gulf of Mexico (4 to 27 nM day^{-1}).[41]

In the present study, the SVDF technique yielded highly reproducible $[DMSPd]_{SVDF}$ concentrations and a nearly perfect pattern of inhibition by the GBT treatment (i.e. divergent linear accumulation time course for $[DMSPd]_{SVDF}$, and common intercept) (Fig. 1a–c). In a study with the ciliate *Favella* sp., GBT did not affect grazing,[58] therefore it seems unlikely that grazing-mediated DMSPd release would have been altered from the natural situation in our GBT treatments. With this information, and the fact that GBT inhibited dissolved ^{35}S-DMSP loss by >99 % (Fig. 1d, e), there is no evidence that the GBT-determined DMSPd consumption rates are seriously underestimated, such that this could explain why the GBT-determined rates are so much lower (39-fold on average) than the ^{35}S-DMSP-determined rates. If anything, the GBT rates could be overestimates if the 5 µM GBT treatment caused faster DMSP release from the particulate DMSP pool, but there is no evidence for this phenomenon in limited tests with cultured phytoplankton.[41] We therefore conclude that the GBT-determined DMSPd consumption rates are valid estimates of the true rates.

Evaluation of the ^{35}S-DMSP tracer method for $k_{^{35}S\text{-}DMSPd}$

When tracer levels of dissolved ^{35}S-DMSP (<0.01 nM) were added to unfiltered seawater the ^{35}S-DMSPd disappeared exponentially from the dissolved pool, with first order rate constants ($k_{^{35}S\text{-}DMSPd}$) ranging from 21.4 to 82.3 day^{-1} (Fig. 1d–f; Table 1). These rate constants are high compared with those measured with ^{35}S-DMSP in the temperate North Sea (2.1–11.4 day^{-1}),[22] but comparable to those measured in productive shelf waters of the Gulf of Mexico (3.0–51.4 day^{-1}).[21] The microbial populations consumed >97 % of the added ^{35}S-DMSPd when incubations were carried out over 5 h or more (Fig. 1d, e). As mentioned above, the competitive inhibitor of DMSP uptake, GBT, prevented the loss of ^{35}S-DMSP tracer (Fig. 1a, b), suggesting that the loss was mediated by an active uptake process, as reported previously.[38] Because the concentration of DMSPd (as measured by SVDF) in these whole water incubations was in approximate steady-state, multiplication of the $k_{^{35}S\text{-}DMSPd}$ by $[DMSPd]_{SVDF}$ should give the turnover flux of the dissolved DMSP pool. Turnover flux rates calculated in this way ranged from 35.7 to 215 nM DMSP day^{-1}, which are on the high end of what has been measured previously.[21–23] The ^{35}S-DMSP-determined turnover fluxes were 1.7 to 152 times higher than GBT-determined fluxes from parallel incubations (Table 1). An important clue that the ^{35}S-DMSP-determined turnover fluxes might be overestimated is that they would represent daily consumption of 88 to 487 % of the DMSPp standing stock in the three water samples for which DMSPp data are available (calculated from data in Table 1). Such high turnover of the particulate DMSP pool seems unreasonable given that phytoplankton populations i.e. the source of most DMSPp and ultimately DMSPd) are likely to turn over less than once per day.[59] In contrast to the ^{35}S-DMSP results, the DMSPd turnover fluxes estimated with the GBT technique would have consumed only 3 to 18 % of the DMSPp pools per day, which may be more reasonable fractions of the DMSPp passing through the dissolved pool given that some DMSP-sulfur is retained or degraded by grazers.[14,15,60]

If the ^{35}S-DMSP technique overestimated the DMSPd turnover flux, it does not appear to result from a problem with the DMSPd loss rate constants ($k_{^{35}S\text{-}DMSPd}$). In several experiments with dark-incubated GF/F filtrate incubations (i.e. with no DMSP

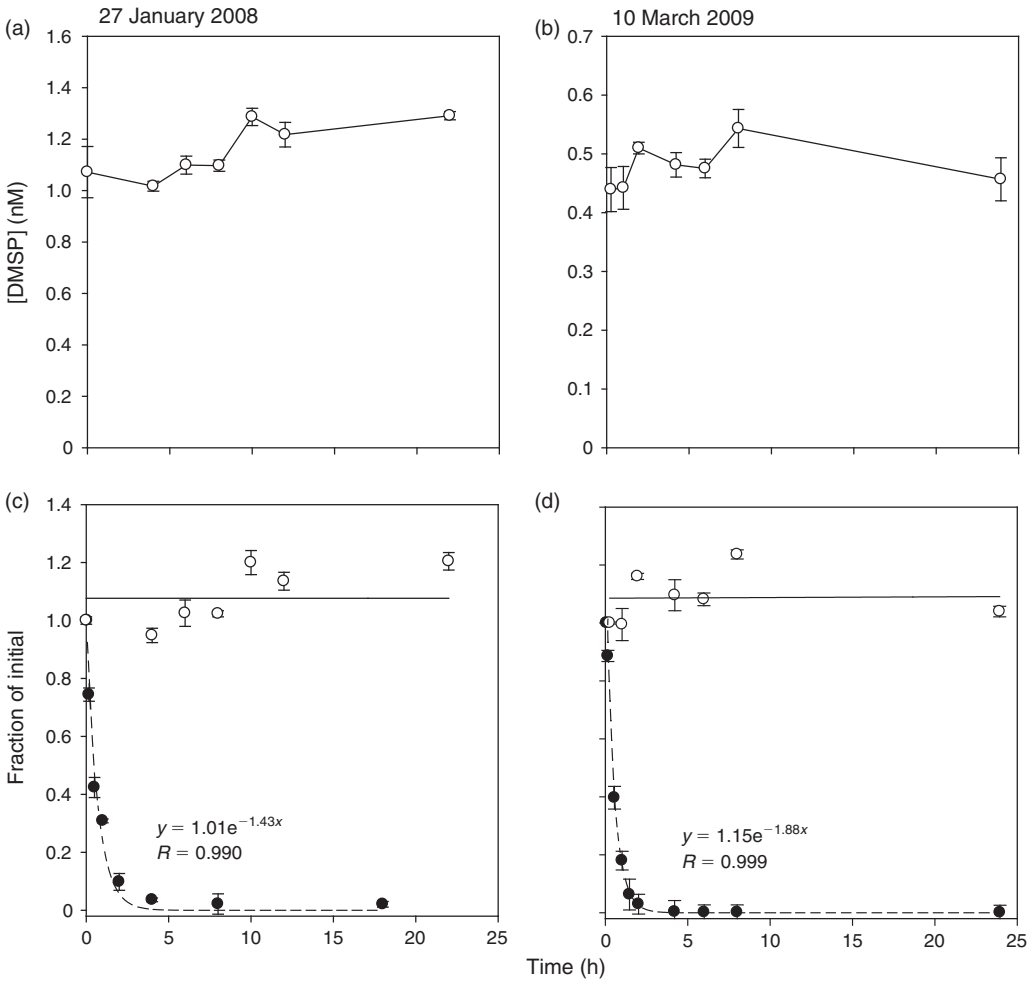

Fig. 4. Time course of the concentration of dissolved dimethylsulfoniopropionate (DMSPd) that passed through a 0.2-µm filter ($[DMSPd]_{<0.2\,\mu m}$) loss (upper panels) in 24-h pre-incubated GF/F filtrate incubations from the Gulf of Mexico, prepared on 27 January 2009 (panels A and C) and 10 March 2009 (panels B and D). Bottom panels show the fraction of initial $[DMSPd]_{<0.2\,\mu m}$ (open circles) and ^{35}S-labelled DMSPd (^{35}S-DMSPd) tracer (closed circles) plotted as a function of incubation time in the dark; the ^{35}S-tracer was added at a concentration of 4 pM at time zero on the scale shown, which was 24 h after the preparation of the filtrate incubations. In the bottom panels, solid lines show linear regression fits ($P < 0.05$) for the fraction of initial $[DMSPd]_{<0.2\,\mu m}$ *v.* time, and dashed lines are exponential fits of the fraction of ^{35}S-DMSP remaining (equations are shown for the exponential fits; $P < 0.05$ for both experiments). Values are the average from duplicate bottles and vertical bars represent the range of the data.

production), the ^{35}S-DMSP loss pattern very closely matched that of unlabelled dissolved DMSP ($[DMSPd]_{<0.2\,\mu m}$) measured by the conventional base hydrolysis-gas chromatography method (Figs 2, 3). This was true whether the initial elevated DMSPd in the filtrate incubations was from filtration-induced releases (i.e. a natural addition from ruptured plankton; Fig. 2) or from addition of reagent DMSP (Fig. 3). During the later time points of the incubations, however, the ^{35}S-DMSP and the $[DMSPd]_{<0.2\,\mu m}$ diverged somewhat, with the tracer being nearly completely consumed (<2 % remaining) whereas the unlabelled $[DMSPd]_{<0.2\,\mu m}$ stopped decreasing after a few hours at concentrations of ~0.5 or ~1.1 nM (Fig. 2a–c). When this apparently refractory pool of $[DMSPd]_{<0.2\,\mu m}$ was subtracted from each time point, the agreement between the ^{35}S-DMSP and unlabelled $[DMSPd]_{<0.2\,\mu m}$ loss patterns (expressed as a fraction of the initial concentrations) was improved, especially for later time points (Fig. 2d–f).

These findings lead to two conclusions. First, the ^{35}S-DMSP was an excellent tracer of the bioavailable $[DMSPd]_{<0.2\,\mu m}$ pool and its loss kinetics should therefore give accurate estimates of $k_{^{35}S\text{-}DMSPd}$ in unfiltered seawater. Second, there was a significant pool of non-bioavailable $[DMSPd]_{<0.2\,\mu m}$ in the filtrate incubations that in some cases represented a significant fraction (26–67 %) of $[DMSPd]_{SVDF}$ (dotted lines in Fig. 2b, c) measured in whole water used to prepare the filtrate incubations. The existence of the non-bioavailable DMSP pool was even more clearly demonstrated by the experiments where the filtrate incubations were pre-incubated for 24 h in the dark, leaving pools of $[DMSPd]_{<0.2\,\mu m}$ of ~0.5 and 1.1 nM in the two experiments that did not change appreciably during the next 24 h incubation. The stability of the residual $[DMSPd]_{<0.2\,\mu m}$ pool was in marked contrast to the picomolar-levels of ^{35}S-DMSP added during this period, which was consumed rapidly and nearly completely, indicating that it was bioavailable and that the bacteria were not limited in their ability to consume low levels of DMSPd (Fig. 4). In these dark-incubated filtrate incubations we found no evidence for significant DMSP production, as expected because phytoplankton, the main source of DMSP in marine waters,[3,14] were removed by the GF/F

filtration, and because DMSP production is generally light dependent.[52,61] Thus, the steady-state concentrations of $[DMSPd]_{<0.2\,\mu m}$ in >24 h-old filtrate incubations did not represent a balance of production and loss, but rather simply lack of loss of this apparently refractory DMSPd pool.

What is the nature of the refractory DMSPd pool?

To our knowledge, this is the first study to observe a biologically non-reactive DMSPd pool in seawater. Two general hypotheses are consistent with our observations. One is that some fraction of the DMSPd pool is protected from rapid biodegradation by physicochemical mechanisms, and the other is that what we measure as DMSP is not DMSP at all, but some other compound that yields DMS upon addition of base to the sample. Below we briefly discuss each of these hypotheses.

It is now well recognised that interaction between organic matter and mineral surfaces can protect otherwise labile organic compounds from microbial degradation.[62–64] To our knowledge this has not been shown definitively for DMSP, but it is worth noting that a previous study found a sizable pool (13 μmol L^{-1}) of 'base-hydrolysable DMS' in salt marsh sediments that was stable for at least 12 days of dark incubation, despite the fact that the same sediments could readily degrade exogenous DMSP.[65] Release of such material in a colloidal form (which is operationally dissolved; <0.2 μm) from sediments to the water column, could explain our results because all of our samples were from coastal areas where sediment resuspension is common.

Another protection mechanism for DMSPd might be binding to colloidal or dissolved materials in the water column. There is evidence that adsorption of the amino acids glycine[66] and leucine[67] to high molecular weight dissolved organic matter (DOM) can greatly decrease the bioavailability of these otherwise labile compounds. Preliminary tests with samples of refractory DMSP from Mobile Bay have shown that it passes through 3000 molecular weight cut-off ultrafilters, and is not retained on hydrophobic C18 resin (J. Motard-Côté and R. P. Kiene, unpubl. data). This would argue that the refractory DMSPd pool is hydrophilic and not associated with high molecular weight material. Additionally, the fact that >97 % of added ^{35}S-DMSPd was consumed during 12–24h incubations (Figs 2–4) suggests that there was not a significant short-term exchange of the tracer into the refractory pool. Additional work will be needed to test whether DMSP can be protected by interaction with colloidal particles or DOM and if this can explain our observations of a refractory pool of DMSPd in coastal seawater.

What if the apparent refractory DMSPd is not DMSP at all? It is important to realise that our measurements, like those of nearly all studies on DMSP to date, utilised an indirect method for quantification, namely the alkaline conversion of DMSP into DMS at room temperature, with subsequent measurement of the released DMS. This approach is quantitative for DMSP,[43] but it is possible that other DMS precursors also could release DMS under alkaline conditions. White[43] showed that S-methylmethionine could be present in some marine macroalgae, but alkaline conversion of S-methylmethionine to DMS was insignificant at 23 °C, so this is not likely to explain our findings. Several intermediates in the phytoplankton DMSP synthesis pathway, such as dimethylsulfonium hydroxybutyrate,[68] could be present in seawater but virtually nothing is known about the lability of these compounds or their concentrations in seawater. The dimethylsulfonium compounds, gonyauline and gonyol are produced by some dinoflagellates such as *Lingulodinium polyhedrum* and *Prorocentrum minimum*,[69,70] and dimethylsulfocholine is present in the diatoms *Nitzschia alba*[71,72] and *Phaeodactylum tricornutum*,[73] but again nothing is known about the concentrations of these dimethyl sulfur compounds in seawater, or whether they release DMS under the alkaline conditions of our assays. Yet another possibility for the observed refractory 'DMSPd' pool is that it is a DMS complex of some kind that releases the DMS under strong basic conditions.

Whether the refractory 'DMSPd' pool that we observed in coastal seawater is protected DMSP or some other DMS precursor(s) remains to be determined. Identifying the compound(s) responsible for the refractory DMSPd pool will present analytical challenges because the concentrations of this pool appear to be one nanomolar or less (Figs 3, 4). Direct methods for DMSP determination[74–76] would be advantageous in this endeavour but these approaches do not presently have the necessary sensitivity to detect sub-nanomolar concentrations of dissolved DMSP in a seawater matrix.

Particulate DMSP in the $DMSP_{SVDF}$ fraction

Our findings from the GF/F filtrate incubation experiments suggest that some of the DMSP that passes through GF/F filters is in particles that can be captured on 0.2-μm pore-size filters (Fig. 3). This sub-micron particulate DMSP pool is probably heterotrophic bacteria that pass through GF/F filters,[46] and that retain some DMSP in their cells.[49] From the initial time points in the filtrate incubation experiments, we estimated that the $[DMSP]_{0.2–0.7\,\mu m}$ pool represented 10–37 % of the independently determined $[DMSPd]_{SVDF}$ from the original seawater used to prepare the filtrate incubations (Table 2). These are probably upper limits to the contributions of 'particulate' DMSP to the $[DMSPd]_{SVDF}$ pool because the large volume filtrations used to prepare the filtrate artificially increased the initial $[DMSPd]_{<0.2\,\mu m}$ concentrations (Table 2, Fig. 2) and the filtration took ~30 min, during which time the bacteria may have taken up some of this DMSP before the first time points were collected. Additional tests (not shown) conducted by filtering a much smaller volume (25 mL) through a GF/F filter and immediately filtering the filtrate through a 0.2 μm nylon filter, produced similar results (i.e. 12 % of $[DMSPd]_{SVDF}$ is in bacterial cells), suggesting the bacterial pool of DMSP is not an artefact of the larger volume filtrations presented here. Therefore $[DMSPd]_{SVDF}$ (or any similar GF/F filtrate fraction) will overestimate the truly dissolved, and hence still bioavailable, DMSPd pool because some of the DMSP that passes through these filters is already associated with >0.2-μm particles, probably bacteria. This overestimation only partly explains the discrepancy in the DMSPd consumption rates yielded by GBT-inhibition and ^{35}S-DMSP approaches, which is due also to the presence of the refractory $DMSPd_{<0.2\,\mu m}$ pool, as previously discussed.

Estimation of bio-available DMSPd in the coastal seawater

Our results indicate that $[DMSPd]_{SVDF}$ contains at least two pools of DMSP ($DMSP_{0.2–0.7\,\mu m}$ and refractory $DMSPd_{<0.2\,\mu m}$) that are not available for further biological uptake on the time scale of ^{35}S-DMSP tracer experiments. So what is the true bioavailable DMSPd concentration ($[DMSPd]_{bio\text{-}avail}$)? We estimated $[DMSPd]_{bio\text{-}avail}$ for our whole water incubation samples by applying the following equation for DMSPd turnover flux rates[77]:

Table 3. Estimated bioavailable dissolved dimethylsulfoniopropionate (DMSPd) concentration ($[DMSPd]_{bio\text{-}avail}$) in whole water samples, calculated by dividing the DMSPd consumption rate obtained from the glycine betaine (GBT) inhibition experiment by $k_{^{35}S\text{-}DMSPd}$ obtained in a parallel incubation

Also shown is the percentage of [DMSPd] as determined by small volume drip filtration ($[DMSPd]_{SVDF}$) that was bioavailable

Sampling location	Date	$[DMSPd]_{bio\text{-}avail}$ (nM)	$[DMSPd]_{bio\text{-}avail}$: $[DMSPd]_{SVDF}$ (%)
Sea Lab pier	11 November 2008	0.44 ± 0.038	12
Sea Lab pier	18 November 2008	0.96 ± 0.013	3.7
Sea Lab pier	3 December 2008	0.47 ± 0.029	16
Sea Lab pier	14 January 2009	0.0059 ± 0.099	0.66
Gulf of Mexico shelf	10 March 2009	1.0 ± 0.034	60
Average		0.41	18.6

$$\frac{[DMSPd]}{dt} = k_{DMSPd}[DMSPd]_{bio\text{-}avail}$$

For [DMSPd]/dt we used the GBT-determined DMSPd consumption rates (Table 1) and for k_{DMSPd} we used $k_{^{35}S\text{-}DMSPd}$ (Table 1), both of which were assumed to be accurate based on results discussed above. We then calculated $[DMSPd]_{bio\text{-}avail}$ as follows:

$$\frac{\left(\frac{[DMSPd]}{dt}\right)_{GBT}}{k_{^{35}S\text{-}DMSPd}} = [DMSPd]_{bio\text{-}avail}$$

The resulting estimates of $[DMSPd]_{bio\text{-}avail}$ ranged from 0.006 to 1.0 nM, with the lowest value obtained from a brackish water sample (9 ppt) and the highest value from a shelf water sample (35.2 ppt) (Table 3). The mean $[DMSPd]_{bio\text{-}avail}$ from the five experiments we conducted was 0.41 nM, which was on the lower end of the range of concentrations of DMSPd obtained by SVDF and dialysis sampling in the Sargasso and Ross Seas.[12] Importantly, the calculated $[DMSPd]_{bio\text{-}avail}$ for each sample represented, on average, only 18 % (range 0.66–60 %) of the $[DMSPd]_{SVDF}$ measured in the same samples (Table 3). This indicates that $[DMSPd]_{SVDF}$ substantially overestimates the bioavailable DMSP in coastal Gulf of Mexico waters. Such an overestimate can help to explain the large discrepancy between the GBT- *v.* ^{35}S-DMSP-determined DMSPd consumption rates that we observed (Table 1). Although the GBT inhibitor method also relies on $[DMSPd]_{SVDF}$, the rates calculated from this approach are probably not sensitive to the presence of the non-bioavailable pool (both DMSP in particles and the refractory pool) because they are calculated from the accumulation of $[DMSPd]_{SVDF}$ above the background pool. Variations in $[DMSPd]_{bio\text{-}avail}$ in the different water samples could be attributed to the presence of different microbial communities[29] or to variations in the abundance of DOM and colloidal material,[78] which might affect the bioavailability of DMSPd as discussed previously. Further research will be needed to learn what factors control the bioavailability of DMSPd in seawater.

Implications for DMSP biogeochemistry research

If the true bioavailable DMSPd concentrations are substantially lower than what is routinely measured by SVDF and other filtration approaches, then DMSP turnover flux measurements, which depend directly on measured [DMSPd], likely have been overestimated. Previous studies using the ^{35}S-DMSP technique to determine k_{DMSPd} have obtained turnover flux rates ranging from 1 to 129 nM day^{-1} in diverse coastal and oceanic waters.[21,22,42,79] At these rates DMSPd contributed up to 15 % of the bacterial carbon demand and 25 to >100 % of the bacterial sulfur production.[21,22,24,79] In addition, previous studies concluded that despite DMS yields of only 5–20 % from DMSPd metabolism, the high turnover fluxes of DMSPd could still be a substantial source of climatically active DMS.[21] At the present time it is not possible to ascertain how far off earlier estimates of these biogeochemical contributions might be. In some cases offsetting errors might have occurred. For example, even though the $[DMSPd]_{bio\text{-}avail}$ was almost certainly overestimated, some of these earlier studies may have underestimated k_{DMSPd} because they measured only uptake or uptake + volatiles production rather than loss of DMSP from the dissolved pool.[21,22] Additionally, it may be that the overestimations of the bioavailable DMSP are less significant in oceanic waters compared with the mostly estuarine-coastal waters studied here. This is suggested by the fact that our sample with the highest salinity, collected on the shelf outside of Mobile Bay, had the smallest discrepancy (1.6-fold) between the ^{35}S-DMSP and GBT-determined rates, and the highest estimated $[DMSPd]_{bio\text{-}avail}$ (Table 3). More recent measurements made in the open Gulf of Mexico, off the coast of Louisiana, found that rates of DMSPd turnover measured with ^{35}S-DMSP were overestimated by 5.7–14.7 % if the refractory pool of DMSP (0.1 to 0.23 nM) was not subtracted from the $DMSPd_{<0.2\,\mu m}$ concentrations used in the calculations.[80] The refractory DMSPd concentration and the $DMSP_{0.2\text{–}0.7\,\mu m}$ that passes through GF/F in the $DMSPd_{SVDF}$ protocol, should be accounted for whenever the true bioavailable DMSPd concentration is required. Obtaining these corrections requires considerable effort and may add additional uncertainties. Without a direct analytical method, estimating the refractory pool requires a 24-h or longer bioassay. Estimating the bacterial DMSP fraction requires a double filtration, GF/F by gravity, then 0.2-μm nylon with vacuum, because direct filtration of seawater through 0.2-μm membrane filters generally causes artificial increases in DMSPd concentrations.[12] Nonetheless, including these corrections will improve estimates of DMSPd turnover fluxes that depend on accurate estimates of the bioavailable DMSPd concentration.

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