Microbial Activity in Aquatic Environments Measured by Dimethyl Sulfoxide Reduction and Intercomparison with Commonly Used Methods

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A new method to determine microbial (bacterial and fungal) activity in various freshwater habitats is described. Based on microbial reduction of dimethyl sulfoxide (DMSO) to dimethyl sulfide (DMS), our DMSO reduction method allows measurement of the respiratory activity in interstitial water, as well as in the water column. DMSO is added to water samples at a concentration (0.75% [vol/vol] or 106 mM) high enough to compete with other naturally occurring electron acceptors, as determined with oxygen and nitrate, without stimulating or inhibiting microbial activity. Addition of NaN3, KCN, and formaldehyde, as well as autoclaving, inhibited the production of DMS, which proves that the reduction of DMSO is a biotic process. DMSO reduction is readily detectable via the formation of DMS even at low microbial activities. All water samples showed significant DMSO reduction over several hours. Microbially reduced DMSO is recovered in the form of DMS from water samples by a purge and trap system and is quantified by gas chromatography and detection with a flame photometric detector. The DMSO reduction method was compared with other methods commonly used for assessment of microbial activity. DMSO reduction activity correlated well with bacterial production in predator-free batch cultures. Cell-production-specific DMSO reduction rates did not differ significantly in batch cultures with different nutrient regimes but were different in different growth phases. Overall, a cell-production-specific DMSO reduction rate of 1.26 × 10−17 ± 0.12 × 10−17 mol of DMS per produced cell (mean ± standard error; R2 = 0.78) was calculated. We suggest that the relationship of DMSO reduction rates to thymidine and leucine incorporation is linear (the R2 values ranged from 0.783 to 0.944), whereas there is an exponential relationship between DMSO reduction rates and glucose uptake, as well as incorporation (the R2 values ranged from 0.821 to 0.931). Based on our results, we conclude that the DMSO reduction method is a nonradioactive alternative to other methods commonly used to assess microbial activity.

It is well documented that several microorganisms use dimethyl sulfoxide (DMSO) as a terminal electron acceptor (8, 31, 32, 41, 50), and the DMSO is subsequently reduced to dimethyl sulfide (DMS). The following enzyme systems have been reported to mediate DMSO reduction: the methionine sulfoxide reductase system (40), the aerobic trimethylamine reductase system (6), and the aerobic DMSO reductase system (50), the anaerobic DMSO reductase system (6), the anaerobic trimethylamine reductase system (6), and the aerobic DMSO reductase system (40).

In a recent paper, 65 bacterial species belonging to 33 genera and more than 300 strains were shown to reduce DMSO, whereas 26 bacterial species did not. Nevertheless, most of the species reported not to reduce DMSO are enteric bacteria belonging to the genera Salmonella, Shigella, and Proteus (18), organisms which are of minor importance in natural aquatic environments. DMSO-reducing organisms include aerobic microorganisms as well as anaerobic microorganisms and prokaryotic microorganisms as well as eukaryotic microorganisms.

DMSO reduction in eukaryotes has been demonstrated for several fungi (1, 50) and for yeasts (1, 5, 15, 50).

In addition to the biological pathway, slow chemical DMSO reduction to DMS takes place at high hydrogen sulfide (H2S) concentrations (49). Furthermore, a disproportionation (bimolecular redox reaction) of DMSO to DMS and dimethyl sulfone was observed in seawater and distilled water by Harvey and Lang (20).

DMSO is formed naturally from degradation of phytoplankton in marine environments (3), from microbially mediated oxidation of DMS (46, 47), and from photoxidation of DMS in water surface layers (9) and the atmosphere (3, 7). Natural DMSO concentrations in aquatic environments have been reported to range from <1 to 218 nM (21, 30, 36). DMSO has also been found in fruits and vegetables (33) and in wine (13) and is released into the environment by a number of anthropogenic activities as well, such as paper production. DMSO also has received attention because of its potential use in medicine as a solvent which can pass through lipid membranes (43).

Before a DMSO reduction assay can be widely used, it is essential to thoroughly compare the method with other commonly used methods. We compared the method described here with incorporation of radiolabeled thymidine into DNA (14), incorporation of radiolabeled leucine into cell proteins (26), and uptake, incorporation, and respiration of radiola-
beled glucose (22, 45). Natural bacterioplankton assemblages collected from surface water and groundwater were used in laboratory experiments in which the dilution culture approach was used (19).

The following questions are addressed in this paper. (i) Is the DMSO method described here applicable to microbial communities inhabiting the water columns of aquatic systems with different trophic states? (ii) Do the saturating DMSO concentrations used to measure DMSO reduction differ in different aquatic environments? (iii) Is the DMSO method applicable in the presence of different electron acceptors, such as oxygen or nitrate? (iv) Is there significant microbial DMS consumption during DMSO reduction? (v) Is the DMSO reduction method comparable to other assays commonly used to assess microbial activity?

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MATERIALS AND METHODS

Sampling sites and sampling. The water samples used originated from various freshwater habitats. Groundwater was withdrawn from monitoring wells in the Lobau area (GWA-85; 48°09′N, 16°32′E) and at St. Lorenz, Austria (GW-SL; 47°50′N, 13°21′E), with a piston pump. Surf ace water samples were obtained from the River Danube in Vienna, Austria (48°13′N, 16°24′E); from the channel of the River Danube in Vienna (48°13′N, 16°22′E); from Lake Mondsee in Mondsee, Austria (47°51′N, 13°21′E); and from a small pond on the island of Texel, The Netherlands (52°59′N, 4°34′E). Marine samples were collected in the Wadden Sea (Texel, The Netherlands; 52°59′N, 4°34′E).

Water samples were obtained by filtering acid-rinsed carboys 10 cm below the water surface. Samples were transported to the laboratory and either used for experiments within 2 h or filtered (Whatman GF/C glass fiber filters) and stored at 4°C in the dark for at least 1 week. Before use, water was again filtered through a Whatman GF/C glass fiber filter and a 0.8 µm-pore-size polycarbonate filter (Millipore Corp.) to remove larger particles and the majority of bacterial predators. Samples from the Wadden Sea were filtered only by gravity (pore size, 0.8 µm) to prevent destruction of phytoplankton cells and subsequent release of large amounts of dimethyl sulfoniopropionate (DMSP).

Saturating concentrations of DMSO and effects on microbial activity. Prefiltered sample water from various habitats were amended with DMSO at concentrations ranging from 0.001 to 20% (vol/vol) (140 µM to 2.8 M), and DMS production was determined after 12 h of incubation at room temperature in order to determine saturating DMSO concentrations. In addition, samples from the same bodies of water were incubated at the in situ temperature for 1 to 2 h. The microbial activities in these samples were measured via bacterial incorporation of tritiated thymidine ([3H]TdR) and tritiated leucine ([3H-Leu) in the presence of DMSO at the concentrations mentioned above to check for stimulating effects of DMSO concentrations on microbial activity.

Is DMSO reduction an exclusively biological activity? Prefiltered water samples from different freshwater environments were incubated with DMSO at final concentrations ranging from 0.005 to 20% (vol/vol) (700 µM to 2.8 M) at ambient temperatures in the dark for several hours. Controls were treated with potassium cyanide (KCN) (final concentration, 0.25%) and sodium azide (NaN3) (final concentration, 0.25%), two commonly used blockers of the cytochrome oxidase in microbes. Additional samples were autoclaved (120°C, 20 min), or formaldehyde (final concentration, 3.7%) was added. Subsamples for DMS analysis were taken at the beginning of incubation in order to determine the natural background concentration of DMS. An additional set of samples, containing Milli-Q water amended with DMSO, was prepared to check for DMS contamination originating from the DMSO reagent. For subsequent routine measurements, NaN3 was chosen as the most suitable inhibitor for experimental blanks, as well as for termination of the samples after incubation. Formaldehyde worked fine as a fixative but interfered with gas chromatography of DMS, whereas autoclaving was found to be inconvenient for measurements in the field.

DMSO reduction versus time. Samples from various aquatic environments that differed in trophic status and therefore in microbial activity were amended with DMSO at a saturating concentration (0.75%, vol/vol; 106 mM), and DMS production was monitored over time by analyzing subsamples at 1- to 3-h intervals for up to 12 h to check for a linear relationship or any other type of relationship between DMSO reduction and incubation time.

Microbial consumption of DMS. Duplicate flasks (500 ml) were filled with filtered sample water, 0.8 µM-pore polycarbonate filtered, and amended with DMSO at a final concentration of 0.75% (vol/vol). To one flask chloroform (CHCl3) (final concentration, 500 µM) was added to inhibit microbial DMSO consumption (42). DMSO production was monitored for about 45 h. A similar experiment was carried out with seawater, and in this experiment we determined the DMSO content by using a protocol of Kiene (24).

Effect of DMSO on the reduction of other electron acceptors. Bacterial oxygen consumption was determined in the presence of a saturating DMSO concentration. Prefiltered sample water was poured into two precleaned Schott flasks (250 ml). Both flasks were added to a flask containing about 20% (vol/vol) of sample water from the River Danube or the channel of the River Danube (49°C) with a saturating concentration of 0.75% (vol/vol; 106 mM), while the second flask remained unamended as a reference. The volume of DMSO added was accounted for in the reference bottle by adding bacterium-free filtered (pore size, 0.2 µm; Millipore Corp.) sample water. The flasks were enriched with nutrients (6 mg of yeast [Merck Corp.] per liter and 16 mg of peptone [Serva Corp.] per liter) to initiate substantial growth of bacteria and pronounced oxygen consumption. The decrease in oxygen concentration (percent saturation) was monitored with a respirometer equipped with two oxygen sensors (Cyclobios Corp.), which were introduced into the glass flasks through Teflon septa. The flasks were closed without headspace and placed on magnetic stirrers to obtain homogeneous distribution of the microorganisms in the bottles. The respirometer was connected to a personal computer, which recorded the oxygen saturation twice per minute for each bottle. Experiments were run for about 12 h. Occasionally, bottles were subsampled to determine the DMS content at the beginning and end of incubation. Right before each DMSO experiment, a control run (oxygen was measured in two samples without the addition of DMSO) was conducted to check for drift of the oxygen sensors.

An anaerobic respiration experiment was performed with a mixed bacterial culture, which was prepared from a groundwater sample obtained from a hypoxic aquifer (GW A-85). Water was poured into precleaned glass bottles (10 liters), enriched with nutrients by adding 10 ml of liquid medium (6 mg of yeast and 16 mg of peptone per liter) and filtered (pore size, 0.2 µm) sample water, and continuously sparged with pure nitrogen. Development of a bacterial community in the anoxic tank was monitored by microscopic examination (see below). After 48 h, the culture was split and dispensed into two precleaned sterile glass bottles (5 liters), and nitrate was added to a concentration of about 40 mg liter1 by adding calcium nitrate (Ca(NO3)2) (Merck Corp.). One of the two bottles was also amended with DMSO at a saturating concentration (0.75%, vol/vol; 106 mM). Both bottles were sampled at 15- to 30-h intervals by using sterile syringes inserted through septa in Teflon-lined silicon septa for up to 100 h. Filter-able subsamples were stored in sterile glass vials (2.2 ml) and stored frozen at –80°C until they were used for nitrate analysis (photometric nitrate reductase test; Boehringer-Mannheim Corp.). The volume of each subsample was replaced by nitrogen gas.

Experimental setup of batch cultures for intercomparision of methods. Two different types of batch cultures (an enrichment culture and two dilution cultures) were prepared in order to determine DMSO reduction rates in different aquatic environments. The enrichment culture was established by adding liquid medium (1 g of yeast extract [Merck Corp.] and 5 g of peptone [Serva Corp.] in 1 liter of filtered [pore size, 0.2 µm] sample water) to filtered (pore size, 0.8 µm) groundwater at a ratio of 1:9. For a second set of experiments, dilution cultures were prepared, in which 9 parts of filtered (pore size, 0.2 µm) sample water from either the River Danube or the channel of the River Danube (19). DMSO was always added at a saturating concentration (0.75% [vol/vol] or 106 mM), except when the noncumulative approach was used (see below). All microcosms were prepared in combination (45°C for 4.5 h) glass bottles (2 to 5 liters) that were closed with minimum headspace by using plastic lids with acid-rinsed, Teflon-lined silicon septa and were incubated in the dark at room temperature (20°C) for up to 52 h. Subsamples for DMS, bacterial abundance, and respiration measurements were withdrawn at irregular intervals.

Cumulative approach versus noncumulative approach. Microcosm experiments were also performed to compare a cumulative approach (i.e., DMSO was added to a batch culture at the beginning of a growth experiment and the accumulation of DMS was monitored over time) with a noncumulative approach used for most common microbial activity assays (i.e., no DMSO was added to the batch culture), but DMSO reduction rates were determined in subsamples by short-term incubation.

A dilution culture with water from the channel of the River Danube was prepared as described above, split, and placed into two microcosms. One microcosm was amended with DMSO at a saturating concentration (106 mM), and the...
and Hobbie (45). Incubation was stopped by adding 5 ml of 6 NH2SO4. The concentration of 10.2 nM in glass bottles for 4 h by using the protocol of Wright 100-W HBO lamp (Osram Corp.). Nonfluorescent immersion oil (Cargille type A; Cargille Corp.). Direct counting in scintillation vials. Eight milliliters of scintillation cocktail (Insta Gel; Packard) was added to 5% (wt/vol) ice-cold trichloroacetic acid, and the filters were placed in scintillation vials. Samples from batch cultures were transferred to combusted glass vials (25 ml), and DMSO was added to a final concentration of 0.75% (vol/vol) (106 mM). The vials were closed by using lids with Teflon-lined silicon septa without headspace and were incubated at room temperature in the dark for 2 h. Activity measurements were carried out in duplicate, and a sample fixed with NaN3 (final concentration, 100 μM) served as a control (for the current standard protocol see below).

DMS analysis. DMS analysis was performed by using a modified purge and trap system (4, 25), in which volatile sulfur compounds are stripped from water samples (1 to 5 ml) with a purged stream of nitrogen gas, cryotrapped in liquid nitrogen, and subsequently injected into a gas chromatograph (model 5890 Series II; Hewlett-Packard) equipped with a flame photometric detector and a Porapak Q column (Supelco Corp.). Water vapor was removed by a glass tube filled with potassium carbonate (K2CO3), which was and mounted between the bubbling chamber and the cryotrap (4). The oven temperature was 190°C, and the carrier gas flow rate was 50 ml min⁻¹. Ethyl methysulfide served as an internal standard; this compound was injected into the bubbling chamber in appropriate amounts and was stripped together with the water samples. Under these conditions, the retention times for DMS and ethyl methysulfide were 2 and 3.4 min, respectively. Standards were prepared gravimetrically with ethylene glycol. The analytical precision was always <5%. The detection limit was about 3 pmol of cryotrapped DMS.

Determination of bacterial abundance. Portions (5 to 10 ml) of samples fixed with formaldehyde (final concentration, 3.7%) were stained either with acridine orange (Merck Corp.) (48) or with 4',6'-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.) (34). Samples were filtered onto black polycarbonate filters (pore size, 0.2 μm; Millipore Corp.), placed onto glass slides, and embedded in nonfluorescent immersion oil (Cargille type A; Cargille Corp.). Direct counting was carried out with a Zeiss Axiosplan epi-epifluorescence microscope fitted with a 100-W HBO lamp (Oslam Corp.).

Bacterial [3H]TdR and [3H]Leu incorporation. Incorporation of radiolabeled tracers into trichloroacetic acid-insoluble precipitates was used to estimate bacterial cell and biomass production. Ten-milliliter subsamples (triplicates) from the microcosms and one formaldehyde-killed control were incubated in test tubes (14 ml; Greiner Corp.) with either [methyl-3H]TdR (specific activity, 70 Ci mmol⁻¹; Amersham) or [methyl-3H]Leu (specific activity, 131 Ci mmol⁻¹; Amersham) at a final concentration of 10 nM for 1 to 2 h (14, 26). Incubation was stopped by adding 1 ml of formaldehyde (37%). Samples were filtered onto cellulose membrane filters (pore size, 0.2 μm; Millipore) and rinsed twice with 5% (wt/vol) ice-cold trichloroacetic acid, and the filters were placed in scintillation vials. Eight milliliters of scintillation cocktail (Insta Gel; Packard) was added to each vial, and after 12 h the radioactivity was counted with a liquid scintillation counter (Tri-Carb 2000; Packard). Quenching was corrected by using an external standard ratio.

Uptake, incorporation, and respiration of [14C]glucose. Radiolabeled glucose was used to determine bacterial uptake and respiration rates in natural bacterial communities. Forty-milliliter subsamples from the microcosms were incubated with [D-14C]glucose (specific activity, 291 mCi mmol⁻¹; Amersham) at a final concentration of 10.2 nM in glass bottles for 4 h by using the protocol of Wright and Hobbie (45). Incubation was stopped by adding 5 ml of 6 N H2SO4. The [14C]CO2 released from the liquid phase was trapped overnight on filter paper (Whatman no. 1) soaked with 100 μl of β-phenylethylamine (Riedel-de-Haen) and placed in plastic wells attached to the rubber stoppers sealing the bottles (22). Subsequently, the acidified liquid phase was filtered onto cellulose membrane filters (pore size, 0.2 μm; Millipore) and rinsed with ultrapure water (MQ; Millipore). The filters containing the radiolabeled bacteria and the filters with the adsorbed [14C]CO2 were placed in scintillation vials and counted with the scintillation counter as described above. The sum of the amount of [14C]incorporated and the amount of [14C]CO2 released accounted for the total glucose uptake.

RESULTS

DMSO reduction mediated by microorganisms. Samples amended with DMSO showed pronounced DMS production in all experiments, whereas experiments in which the respiratory activity of the microbial community was inhibited by cyanide and azide or was terminated by using formaldehyde resulted in no significant DMSO reduction (data not shown). The DMS detected in the controls either originated from the DMSO reagent or represented the natural background levels in the different habitats.

Effects of DMSO addition on microbial activity. Bacterial activity, as determined by [3H]TdR and [3H]Leu incorporation, was not affected by DMSO concentrations up to 1% (vol/vol) (140 mM). The results of a typical experiment with different DMSO concentrations are shown in Fig. 1B. In general, bacterial activity started to decrease at DMSO concentrations greater than 5% (vol/vol). The [3H]TdR incorporation rates declined rapidly as DMSO concentrations of ≥10% (vol/vol) (≥1.4 M). As expected, the total number of bacteria was not affected (Fig. 1B). The results indicate that DMSO at high concentrations (≥10%) may inhibit microbial activity, but no effects were detectable at concentrations of <1% after short incubation periods.

Saturating DMSO concentrations. Independent of the trophic states of the freshwater samples tested, the highest DMSO reduction rates were always observed at DMSO concentrations between 0.5 and 1% (vol/vol) (70 and 140 mM); the results of experiments performed with samples from the River Danube and groundwater samples from the Lobau wetland,
which represent two very different environments, are shown in Fig. 1A. The highest rates of DMSO reduction for the River Danube and the Lobau groundwater were $1.22 \pm 0.04$ and $0.11 \pm 0.008$ nmol liter$^{-1}$ h$^{-1}$, respectively. Based on these and similar measurements, a DMSO concentration of 0.75% (106 mM) was chosen and used for the standard protocol.

**Microbial DMS consumption.** No measurable loss of DMS and therefore no difference in DMS production rates could be observed in comparative experiments performed with freshwater and seawater samples in which DMS consumption was artificially inhibited in control samples by CHCl$_3$; thus, DMS consumption was negligible in our sets of experiments (Fig. 2).

High DMSP concentrations, such as those observed for the Wadden Sea samples, were readily degraded into DMS during incubation; the resulting DMS could be clearly distinguished from DMS originating from bacterial DMSO reduction (Fig. 2B).

**Incubation time and linearity in DMSO reduction.** Microbially mediated DMS production was found to be linear over time for several hours. Typical results for regression analysis and DMSO reduction rates obtained from a wide spectrum of freshwater habitats are shown in Fig. 3. The DMSO reduction rates spanned a range of 3 orders of magnitude, from 7.4 pmol liter$^{-1}$ h$^{-1}$ in pristine groundwater to 1.43 nmol liter$^{-1}$ h$^{-1}$ in a sample from a pond that was eutrophic at the time of sampling (Fig. 3).

**DMSO as a terminal electron acceptor.** In all aerobic respiration experiments, oxygen consumption was significantly lower in the presence of DMSO than in controls to which no DMSO was added. The reduced oxygen consumption in DMSO-amended samples coincided with DMS production. Occasional DMS measurements in the respiration experiments (closed respirometry) indicated an electron balance, but this balance has to be proved more precisely. Typical time courses for the oxygen concentrations obtained with nutrient-enriched water samples from different freshwater habitats (Lake Mondsee and GW-SL) are shown in Fig. 4. A lag phase for oxygen consumption and DMSO reduction occasionally resulted from artificially high incubation temperatures which differed from the in situ conditions (Fig. 4C). Oxygen sensor drift

![FIG. 2. DMS production over time in samples from a small eutrophic freshwater pond on the island of Texel (A) and in samples from the Wadden Sea (B). One sample, treated with CHCl$_3$ (500 mM), served as a control for microbial DMS consumption. The values are means ± standard deviations (n = 3).](image)

![FIG. 3. Linear regression analysis of the DMS production rate (in nanomoles per liter per hour) versus time in samples from different freshwater environments. Samples were obtained from the Danube River (DR), from the channel of the River Danube (CRD), from a pristine groundwater site (GW A-85), and from a freshwater pond on Texel (The Netherlands) on two consecutive days (TP 1 and TP 2).](image)

![FIG. 4. Decrease in the oxygen concentration over time in samples from different bodies of water with and without DMSO amendment. (A) Control experiment without added DMSO to check for drift of the oxygen sensors. (B through D) Oxygen consumption in DMSO-amended samples (+ DMSO) and in the unamended controls (− DMSO). Samples originated from Lake Mondsee (LM) and from a nearby groundwater site (GW-SL).](image)
was tested in samples that were not amended with DMSO (Fig. 4A). An anaerobic respiration experiment comparable to these aerobic respiration studies was also conducted, which resulted in reduced nitrate consumption in the presence of DMSO (Fig. 5).

Relationship between bacterial growth and DMSO reduction. In the enrichment culture prepared from groundwater, accumulation of DMS over time resulting from microbial DMSO reduction was closely related to the increase in cell number (Spearman rank correlation coefficient $|R| = 0.996; P < 0.001$) (Fig. 6A). The initial DMS concentration (0.9 nM) resulted from traces of DMS present in the DMSO reagent and from the natural DMS background in the body of water sampled. From the increase in cell number over time, the actual cell production was calculated and related to the DMSO reduction rates (calculated from the increase in DMS) (Fig. 6B). Cell production and DMS production did not show a linear relationship for the whole growth cycle (see below).

Cell-production-specific DMSO reduction rates in different growth phases. To check for variations during the different growth stages, the DMSO reduction rate was related to cell production for the lag, exponential, and stationary growth phases of the enrichment culture. The slope of the linear regression analysis line represented the cell-production-specific DMSO reduction rate in terms of DMS production per newly produced bacterial cell. The cell-production-specific DMSO reduction rate was highest in the stationary phase ($2.22 \times 10^{-17} \pm 0.75 \times 10^{-17}$ mol of DMS per produced cell), lower in the lag phase ($1.79 \times 10^{-17} \pm 0.17 \times 10^{-17}$ mol of DMS per produced cell), and lowest in the exponential phase ($0.87 \times 10^{-17} \pm 0.05 \times 10^{-17}$ mol of DMS per produced cell) (Fig. 6B). The regression coefficients for the lag and exponential phases were high (0.97 and 0.99, respectively), but the regression coefficient for the stationary phase was rather low (0.75).

Cell-production-specific DMSO reduction rates in different environments. The cell-production-specific DMSO reduction rates were calculated for the exponential phases in various bacterial growth kinetic experiments performed with our enrichment and dilution cultures. The enrichment culture prepared from groundwater (GW A-85), for example, represented a nutrient-rich environment, whereas the dilution cultures (River Danube and channel of the River Danube) contained much lower nutrient concentrations. The latter two sites are characterized by very similar natural nutrient concentrations. Despite the different nutrient regimes in our experiments, the cell-production-specific DMSO reduction rates for the enriched culture (Fig. 7A) and dilution cultures did not differ significantly (Fig. 7B through D) ($P > 0.25$, as determined by analysis of covariance [ANCOVA]). Linear regression analysis of pooled data from all of the experiments conducted resulted in a cell-production-specific DMSO reduction rate of $1.26 \times 10^{-17} \pm 0.12 \times 10^{-17}$ mol of DMS per produced cell ($n = 35; R^2 = 0.78; P < 0.001$).

DMSO reduction and multiple-assay comparison. The rate of DMSO reduction was also compared to the rates of incorporation of $[^3H]$Tdr and $[^3H]$Leu, as well as to the rates of uptake, incorporation, and respiration of $[^14C]$glucose in a growth kinetic experiment performed with a dilution culture (channel of the River Danube). The production of DMS was linearly related to the incorporation of TdR and leucine (Fig. 8) with regression coefficients of 0.90 and 0.94, respectively. The relationship of the DMSO reduction rate to the rates of uptake and incorporation of glucose was exponential, with regression coefficients of 0.90 and 0.93, respectively (Fig. 9A and B), whereas the relationship of DMSO reduction to respiration of glucose was rather uncoupled (Fig. 9C). Glucose respiration did not show close relationship to either incorporation of TdR and Leu or the cell production rate. When data from similar experiments were pooled, DMS production was highly correlated with TdR and leucine incorporation and with glucose uptake and incorporation (Table 1) but not with glucose respiration. For comparison, all rates normalized to the other activity parameters are listed in Tables 2 (linear relation-
ship) and 3 (exponential relationship). The DMSO reduction rates normalized to the TdR and leucine uptake rates varied by a factor of up to 5 between the River Danube and the channel of the River Danube ($P < 0.001$, as determined by ANCOVA) (Table 2). A similar variation (two- to fivefold) was also observed for the cell-production-specific rates of incorporation of TdR and leucine (Table 2).

**Cumulative approach versus noncumulative approach.** The growth responses and activity patterns of the bacterial communities in a DMSO-amended dilution culture and a DMSO-free dilution culture were highly correlated. A comparison of the individual activity parameters, determined for both batch cul-

**TABLE 1.** Regression coefficients from linear regression analysis of DMS production versus TdR and leucine incorporation and from exponential regression analysis of glucose uptake and incorporation in the multiple-assay comparison

<table>
<thead>
<tr>
<th>Sample</th>
<th>TdR incorporation</th>
<th>Leucine incorporation</th>
<th>Glucose uptake</th>
<th>Glucose incorporation</th>
<th>Glucose respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD</td>
<td>0.861</td>
<td>0.874</td>
<td>0.821</td>
<td>0.889</td>
<td>NS</td>
</tr>
<tr>
<td>CRD-A</td>
<td>0.783</td>
<td>0.934</td>
<td>0.913</td>
<td>0.930</td>
<td>NS</td>
</tr>
<tr>
<td>CRD-U</td>
<td>0.902</td>
<td>0.944</td>
<td>0.902</td>
<td>0.931</td>
<td>NS</td>
</tr>
</tbody>
</table>

*The relationship between the rate of DMSO reduction and glucose respiration did not reveal any consistent pattern.*

*RD, water from the River Danube; CRD-A, water from the channel of the River Danube, amended with DMSO; CRD-U, water from the channel of the River Danube, not amended with DMSO. Rates of DMSO reduction were determined by short-term incubation of subsamples.

*NS, not significant.*
tires, resulted in Spearman rank correlation coefficients between 0.85 ($P < 0.01$) and 0.98 ($P < 0.001$) (Table 4). Furthermore, rates normalized to all other activity parameters did not differ significantly when the cumulative approach and the noncumulative approach were compared ($P > 0.05$, as determined by ANCOVA) (Tables 2 and 3).

**DISCUSSION**

The ability to reduce DMSO has been shown to be widespread in microorganisms. About 100 microbial species have been reported to reduce DMSO (18), and these species include prokaryotes as well as eukaryotic and aerobic organisms as well as anaerobic organisms. The eukaryotes are represented mainly by yeasts and fungi. Whether other eukaryotic groups (e.g., protozoa) are also able to reduce DMSO remains to be investigated. To study a large variety of aquatic systems in order to test the use of DMSO reduction as a measure of microbial activity, samples were taken from lentic and lotic surface waters having different trophic states, as well as from two pristine groundwater sites. The protocol of the DMSO reduction method reported for freshwater sediment samples (17) had to be modified substantially by including a purge and trap system for gas chromatographic determination of DMS (4, 25). This step allows very precise DMSO reduction measurements to be obtained even in oligotrophic environments (e.g., groundwater sites) with low microbial activities.

Experiments in which the organisms in environmental samples were killed by autoclaving or adding formaldehyde before amendment with DMSO and incubating the samples for several hours (data not shown) resulted in no relevant DMSO reduction. This is in agreement with studies of soil and freshwater sediments (1, 17). In the most recently described protocol, water samples are fixed by increasing the pH to 13 with 6 N NaOH (37), which is less harmful than NaN₃ to humans and the environment and is suitable for sample storage for at least several days (for a description of the protocol see below). Abiotic reduction of DMSO to DMS, as well as disproportionation of DMSO, seems negligible in alkaline media. As shown in this study, DMSO reduction is almost exclusively mediated by biological activity. Caution must be taken in the presence of high H₂S concentrations, at which chemical reduction of DMSO may take place (49).

DMSO concentrations up to 1% (vol/vol) (140 mM) did not affect microbial activity in our sets of experiments (Fig. 1B). DMSO concentrations between 0.5 and 1% (vol/vol) (70 and 140 mM) resulted in the highest DMSO reduction rates (Fig. 1A), and therefore, a DMSO concentration of 0.75% (vol/vol) (106 mM) was chosen for the standard protocol (this was designated the saturating concentration). At DMSO concentrations higher than 5% (vol/vol) (700 mM), inhibitory effects on the microbial community were observed (Fig. 1B). Such inhibiting effects were also shown for sewage sludge samples at a DMSO concentration of 15% (37) and for freshwater sediment samples at concentrations of more than 10% (17). The DMSO concentrations required for the highest reduction rates in freshwater samples were about 1 order of magnitude lower than those reported for sediments and sewage sludge samples (1, 17, 37).

An important point when DMSO reduction is used to measure the overall microbial activity is that DMS consumption seems to be negligible. This was true for all of our freshwater samples (Fig. 2A). Nevertheless, substantial microbial consumption of DMS may occur in marine waters; this consumption ranges from <1 to 16 nmol liter⁻¹ day⁻¹ (29, 42). In addition, extended incubation in the presence of DMSO and DMS may have a strong selective effect on the microbial community. In our samples from the Wadden Sea, which were characterized by a high initial concentration of DMSP, which was quantitatively turned into DMS by cleavage during the first few hours of incubation, a high potential to reduce DMSO and no significant loss of DMS were observed (Fig. 2B).

Incubation times between 1 and 5 h were found to be sufficient to obtain a measurable increase in DMS. There was a linear relationship between DMSO reduction and incubation time (Fig. 3) up to 5 to 12 h for a wide spectrum of aquatic environments and incubation temperatures tested, indicating that there were no relevant shifts in the microbial community and/or its activity under this set of conditions. The results also show the great range in microbial activity (as determined by DMSO reduction) present in aquatic environments that differ in trophic status.

As DMSO is suggested to serve as an electron acceptor and DMSO reduction therefore represents a respiratory activity, we conducted respiration experiments under oxic and anoxic conditions. In the presence of DMSO at the saturating concentration (0.75%, vol/vol), much lower oxygen consumption was regularly observed than that in samples incubated without DMSO (Fig. 4). An experiment with an oxygen-free enrichment culture showed a similar pattern for reduction of nitrate (Fig. 5). Nevertheless, oxygen and nitrate reduction continued to some extent, even at the saturating DMSO concentration.
Thus, DMSO only partially replaced the other, thermodynamically more favorable electron acceptors. Sporadic DMS measurements in our respiration experiments indicated that the number of electrons which moved from oxygen to DMSO was in balance. Nevertheless, this hypothesis has to be proved more precisely. Apparently, microorganisms can conserve energy when they couple oxidation of organic matter with the reduction of DMSO, because bacterial growth was not inhibited by DMSO and proceeded as it did in non-DMSO-amended experiments (Fig. 1; Tables 2 and 3). At saturating DMSO concentrations, DMSO reduction is apparently based not only on specific DMSO reductases, which have been described for several microorganisms (6, 16, 40, 44, 49), but also on nonspecific enzyme systems. Another explanation for the competitive reduction of DMSO might be its ability to pass through lipid membranes readily (43); thus, it probably does not need active transport to allow it to penetrate to the site of electron transfer. DMSO was also reduced efficiently at nonsaturating concentrations in anoxic marine sediments with high concentrations of sulfate (23), an electron acceptor which is from a thermodynamic point of view energetically less favorable than DMSO.

In order to test the potential of the DMSO reduction approach to replace other commonly used methods, we performed a series of comparative batch culture experiments. The DMSO reduction rate assay was compared to other commonly used bacterial activity assays, such as the assays for incorporation of radiolabeled TdR (14) and leucine (26) and the assays for uptake, incorporation, and respiration of radiolabeled glucose (22, 45).

Accumulation of microbially produced DMS in batch cultures followed closely the increases in bacterial cell numbers through all growth phases (Fig. 6A), producing typical bacterial growth curve patterns (2). This indicates that DMSO reduction is tightly linked to cell production during growth phases. As shown in Fig. 6, different growth phases showed some variability in the cell-production-specific DMSO reduction rate, with the highest rates calculated for the stationary phase and the lowest rates calculated for the exponential phase, indicating that there was low respiratory activity but high cell production activity when the increase in cell number was greatest. Some uncoupling of respiratory activity to cell production is obvious in the stationary phase, as indicated by the lower R² values (Fig. 6B). A loose relationship to cell production in the stationary phase was also detected for TdR incorporation, as follows: \( R^2 = 0.97 \pm 0.03 \).
and leucine incorporation (data not shown). Uncoupling of cell and biomass production was also observed by Chin-Leo and Kirchman (10) and has been interpreted as an adjustment of the cells to changing environmental conditions. Kirchman and Hoch (28) found for TdR and leucine incorporation changes in conversion factors from high values in the lag phase to lower values in the exponential phase (in $10^{-18}$ cells/mol incorporated). This is in contrast to the cell-production-specific DMSO reduction rates which we obtained (reciprocal values have to be used for comparison). Riemann et al. (35) found no significant differences in conversion factors calculated separately for the exponential phase and the near stationary phase. Because of the differences in the growth phases and because the regression coefficients in the stationary phases were rather low, all calculations of cell-production-specific rates for DMSO reduction and uptake and incorporation of TdR, Leu, and glucose have been determined only for the exponential growth phase. Although the experiments conducted differed considerably in their nutrient regimes and therefore in their bacterial growth patterns (maximum bacterial abundance for the enrichment culture, $5 \times 10^7$ cells ml$^{-1}$; maximum bacterial abundance for the dilution cultures, $4 \times 10^6$ cells ml$^{-1}$), the cell-production-specific DMSO reduction rates for all experiments varied within a small range ($0.87 \times 10^{-17}$ to $1.34 \times 10^{-17}$ mol of DMS per produced cell) and did not differ significantly (Fig. 7).

Linear regression analysis of pooled data from all of the experiments conducted resulted in a cell-production-specific DMSO reduction rate of $1.26 \times 10^{-17}$ ± $0.12 \times 10^{-17}$ mol of DMS per produced cell, which accounted for almost 80% of the variance of the data. Riemann et al. (35) and Coveney and Wetzel (11) also found no differences in cell-production-specific incorporation rates for TdR under different nutrient conditions. Further support for our findings was provided by Kirchman (27), who determined cell-production-specific rates of incorporation for TdR and leucine with and without nutrient addition and found that different nutrient concentrations did not have a significant effect on the cell-production-specific activity rates. A multiple-assay comparison also proved that there is a high-level correlation between DMSO reduction and TdR incorporation, as well as Leu incorporation, throughout all growth phases (Fig. 8). The comparison of DMSO reduction with the uptake and incorporation of glucose also indicated that there is tight coupling but an exponential type of relationship (Fig. 9A and B). The same relationship was also observed for glucose-specific TdR and leucine incorporation, as well as for glucose-specific cell production (Table 3). At low rates, glucose uptake and incorporation increased faster than TdR and Leu incorporation, cell production, and DMSO reduction. The relatively high level of glucose metabolism at the beginning of the experiments indicated that maintenance metabolism was occurring, whereas later in the bacterial growth kinetics the community switched to Leu and TdR incorporation to support cell growth (12). In contrast to all other activity parameters determined, glucose respiration (release of CO$_2$) was only poorly related to DMSO reduction (Fig. 9C; Table 1). A probable reason for this is that production of CO$_2$ in our study was based on only a single substrate (limitation effects), whereas reduction of DMSO measured a substrate-independent respiratory activity.

There could be various reasons for the differences in the cell-production-specific TdR and Leu incorporation rates obtained for the River Danube and channel of the River Danube cultures (Table 2). Coveney and Wetzel (11) found that integrated TdR incorporation is more tightly coupled to the increase in biovolume than to the increase in cell abundance. Biovolume was not determined in our experiments. Different cell-production-specific activity rates might also reflect interspecific variability between different members of the bacterial community (38).

To demonstrate that adding DMSO does not influence bacterial growth, we designed a set of experiments to compare the cumulative effect and noncumulative effect of DMS resulting from microbial DMSO reduction. The time courses of all activity parameters in both batch cultures exhibited the same pattern and were highly correlated (Table 4). Additionally, the specific activity rates obtained with the cumulative approach did not differ significantly from those obtained with the non-cumulative approach, as tested by slope comparison (Tables 2 and 3) ($P > 0.05$ for all comparisons, as determined by ANCOVA). A close relationship between DMSO reduction and electron transport system activity, a comparable respiratory activity, was reported by Griebler (17), who found high correlations for freshwater sediments ($R^2 = 0.81; P < 0.001; n = 16$). The latter study also mentions a close relationship between DMSO reduction and bacterial TdR incorporation, as well as $\alpha$-glucosidase activity. Further relationships supporting the notion that DMSO reduction is tightly coupled to microbial activity have been demonstrated by comparisons of DMSO reduction activity with heat output, organic carbon content, and microbial biomass in soil samples (1, 39).

Based on the results obtained in this study, we propose that DMSO reduction can be used as a tool to measure microbial activity. The following standard protocol for DMSO reduction measurements is suggested. Natural water samples (25 ml) are amended with DMSO to a final concentration of 0.75% (vol/vol) (106 mM) and placed in sterile glass vials without headspace; the vials are capped with screw lids having Teflon-coated silicon septa. A prekilled sample (in which the organisms are killed by adding 200 $\mu$L of 6 N NaOH 10 min before incubation) serves as a control. Samples are gently mixed and incubated at the in situ temperature in the dark for up to 5 h. Incubation is stopped (see above), and samples may be stored at 4°C in the dark for several days. For marine samples, killing organisms in the samples with NaOH leads to conversion of DMSP, which might be present at high concentrations (30), to DMS and therefore may result in trouble distinguishing between DMS originating from DMSP and DMS originating from microbial DMSO reduction. Thus, in the case of marine samples, the fates of DMSP and DMS have to be monitored.

In this study we evaluated use of the DMSO reduction method as a sensitive and reliable tool to measure microbial activity in freshwater systems. This method is a nonradiolabeling, relatively inexpensive technique that allows rapid analysis (about 5 min) of a sample. Therefore, this method has the potential to be an alternative method or to complement other methods used to assess microbial activity.

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