Environ. Sci. Technol. **1999**, *33*, 571−579

Formation of Dimethyloligosulfides in Lake Kinneret: Biogenic Formation of Inorganic Oligosulfide Intermediates under Oxic Conditions

BORIS GINZBURG,† INKA DOR,† IRIT CHALIFA,† ORA HADAS,‡ AND OVADIA LEV*,†

Division of Environmental Sciences, Fredy and Nadine Herrmann School of Applied Science, The Hebrew University of Jerusalem, Jerusalem 91904, Israel, and Israel Oceanographic & Limnological Research, Ltd., Yigal Alon Kinneret Limnological Laboratory, P.O.B. 345, Tiberias 14201

The mechanism of formation of dimethyloligosulfides in Lake Kinneret was investigated by field and laboratory studies. The process was simulated under laboratory conditions using obligate aerobic and facultative bacteria that were isolated from Lake Kinneret and fed with different types of organo-sulfur nutrients. The lysis products of *Peridinium gatunense*—a dinoflagellate that dominates the phytoplankton population in Lake Kinneret during the winter−spring season—are the primary source of dimethyloligosulfides. Bacterial assimilation of the aged alga or algae lysis products yields inorganic oligosulfides, which are then methylated to form the dimethyloligosulfides. All the steps of this process are carried out under oxic conditions.

Introduction

Dimethyloligosulfides (DMOS), particularly those containing 2−4 catenated sulfur atoms are widely spread in food (*1*), soil (*2*), and water sources (*3*). These and other volatile sulfur compounds (VSC) often dominate the aroma of groundwater, freshwater, and wastewater effluents. Even trace concentrations of DMOS (0.01−1 ppb) impart strong putrid odor and taste in drinking water (*4*−*7*). DMOS emissions were associated with bacterial, algal, and chemical sources. Microbial synthesis of DMOS from algae, grass, and sulfur containing amino acids and other biochemicals has been demonstrated both under anaerobic (*8*, *9*) and aerobic conditions (*10*−*13*). Bacteria cultivation studies with methionine feed and other sulfur containing substrates showed the ability of a wide range of bacteria to produce DMOS (*14*, *15*), though in most cases only dimethyl disulfide (DM2S) was looked for. Hofbauer and Juttner (*16*) described DMOS and isopropylated oligosulfides production by axenic algal culture of *Microcystis flos aqua*. Nonbiogenic chemical production of DMOS was also reported, e.g., thermal decomposition of methionine (at 180 °C) forms a wide variety of volatile sulfur compounds including methanethiol and DMOS (*17*).

Despite the ubiquity of dimethyl sulfides in nature relatively little research efforts have been devoted to elucidate their formation mechanism. A possible pathway for the production of DMOS in food was suggested to be from *S*-methyl-L-cysteine sulfoxide via methyl methanethiosulfinate stimulated by the action of cysteine sulfoxide lyase (*18*). DM2S formation is often explained by oxidative dimerization of methanethiol (MSH). This mechanism, however, cannot account for the formation of higher dimethyl sulfides, and it is also less likely for very dilute—ppb-range—solutions, where second-order reactions are inherently slow. Formation of dimethyltrisulfide (DM3S) and dimethyltetrasulfide (DM4S) in Perth (Australia) water supplies was explained by methylation of inorganic oligosulfides formed under anoxic conditions in groundwater (*19*).

In a previous report we have described the appearance of dimethyl sulfide compounds in Lake Kinneret (*20*). The spatio-temporal distribution of the dimethyl sulfide compounds was found to correlate well with a bloom of a dinoflagellate alga, *Peridinium gatunense* in the lake. Lake Kinneret is the most important source of surface water in Israel, supplying some 25% of the water consumption of the State of Israel. A seasonal winter−spring bloom of *Peridinium gatunense* occurs every year and dominates the algal population and impacts the water quality of the lake. The dissolved organic carbon in the lake is more than doubled during the peak of the *Peridinium* bloom and reaches approximately 8−10 mg/L of DOC. The bloom is often associated with mild malodorous emissions. In a previous report we showed that dimethyl sulfide (DMS) is formed by cleavage of dimethylsulfoniopropionate (DMSP) which is stored by the *Peridinium* algae for osmoregulation purpose (*21*). In this report we describe the relationship between DMOS formation and the *Peridinium* bloom. We further show data suggesting that DMOS are formed by methylation of biogenically formed inorganic oligosulfide intermediates. As far as we know, this is the first report showing formation of inorganic oligosulfides by bacterial decomposition of sulfur-containing organic matter under aerobic conditions.

Experimental Details

Materials. Dimethyl sulfide and dimethyl sulfide-d_6 (DMS and DMS-d_6), methanethiol, dimethyl disulfide (DM2S), methyliodide and methyliodide-d_3, methionine and methionine-d_3, cysteine, and albumin were obtained from Aldrich. Dimethyltrisulfide (DM3S) and dimethyltetrasulfide (DM4S) were not available commercially. They were synthesized according to recently published procedures (*22*). Briefly, 1.5 g of sodium sulfide was boiled for 8 h with 7 g of elemental sulfur in 20 mL of distilled water. The resulting solution was mixed with 6 mL of methyliodide and allowed to stand overnight in ambient temperature. Fractional distillation (15 mmHg) yielded a pale yellow oil (bp 97 °C) giving a single GC/MSD peak of DM3S. Since DM4S decomposes at elevated temperatures during the vacuum distillation step, a mixture of DM4S with DM3S with an approximate ratio of 2:1 was obtained as a second fraction. Accurate content of the DM4S in this mixture could be determined by quantitation of the DM3S with the pure standards, so that the mixture could be used as standard stock for both DM3S and DM4S. Potassium salt of a mixture of inorganic oligosulfides was synthesized by reaction of hydrogen sulfide gas with potassium ethylate in dry ethanol (*23*). DMSP and DMSP-d_6 were synthesized according to a reported method (*24*). All solvents were of reagent grade, unless otherwise stated.

Analytical Methods. Semivolatile compounds were concentrated—prior to GC analysis—by a closed loop stripping apparatus (*25*) (CLSA) or by its open, one through, modification (*26*) (OSA). The closed loop apparatus exhibited >85%

* Corresponding author phone: 972-26585558; fax 972-26586155; e-mail ovadia@vms.huji.ac.il.

† The Hebrew University of Jerusalem.

‡ Israel Oceanographic & Limnological Research, Ltd.

10.1021/es980636e CCC: $18.00
Published on Web 01/05/1999

recovery of methylisoborneol and geosmin (using salting out technique) and 92% and 84% recovery of dimethyl disulfide and dimethyltrisulfide, respectively. The CLSA was inefficient for the determination of DMS and MSH because of solvent interference. Purge and trap preconcentrator (Tekmar LSC-2) coupled with GC/MSD analysis was used for the determination of the more volatile compounds such as dimethyl sulfide. CLSA was used for analysis of the natural water samples. OSA technique was used for algal and bacterial culture analysis, which were conducted with a smaller sample volume (0.1−0.2 l) and nitrogen gas purging. Henatsch and Juttner (*27*) demonstrated that nitrogen purging prevents artificial formation of DM3S by oxidation of hydrogen sulfide in the presence of methanethiol. A HP5890 gas chromatograph equipped with a temperature programmable on-column injector and an EI (electron ionization) mass selective detector (HP5971 MSD and NBS49K mass spectral library) was used for quantification and identification of the odorous compounds. A 30 m × 0.32 mm ID RTX-1 with 1 micrometer film thickness and a 25 m 0.32 mm coated Poraplot Q (Chrompack) capillary columns were used.

Lake Kinneret Sampling. Samples were collected from point A—the deepest point of the lake—by the sampling boat of the Yigal Alon Limnological Laboratory. The field sampling protocol and sample preservation were reported elsewhere (*20*).

Algal Cultures. Medium ×6 proposed by Lindstrom (*28*) was used for cultivation. The cultures were subjected to periodic (12 h light and 12 h dark) illumination at constant temperature (18 °C). Algae enumeration of the *Peridinium* cells in the lake water was conducted by microscope assisted cell count (Hemocytometer) following algae centrifugating (3000−4000 rpm, Safeguard, U.S.A.) according to the "Standard methods" procedure (*29*).

Bacterial Cultures. Water samples were collected from the upper few centimeters of the Kinneret Lake near the shore. Bacteria colonies were cultivated on Nutrient agar plates colored with Brom Thymol blue. Different strains were distinguished by the different shape and color of the colonies. Fifty milliliters of Nutrient Broth medium were used for liquid cultivation of the selected bacteria strains in 100 mL Erlenmeyer flasks equipped with cotton wool stoppers. Enumeration of bacteria was conducted according to the Standard Methods procedure (*29*).

Substrate Spiking Tests. Bacteria cultures were cultivated for 15 h at 37 °C. Specified quantity of substrate was added to Lindstrom × 6 medium, and then nine volumes of the medium solution was mixed with one volume of bacteria culture. The mixed solution was kept under the same conditions used for algal cultivation i.e., 18 °C and periodic illumination. CLSA or purge and trap analyses were conducted at 0 time and 4 days after substrate addition, unless otherwise stated.

Results

Field Studies. Figure 1 depicts the contour maps of the time-depth concentration profiles of *Peridinium gatunense*, dimethyl disulfide and dimethyl trisulfide during 1995 (*20*). The concentration profiles demonstrate the good correlation between the number of the *Peridinium* cells and the two major organic dimethyl sulfides. Figure 2 shows the contour maps of the depth profiles of the pH, temperature, oxygen, and hydrogen sulfide taken over the same period at the same point in Lake Kinneret. The maps are based on data supplied by the Kinneret Limnological Laboratory. Comparison of Figures 1 and 2 reveals that the DMOS are originated in the oxic epilimnion, either in the oxic regions or in localized anoxic locations (though the second alternative is discarded based on the laboratory tests discussed below). It is highly unlikely that the dimethyloligosulfides originate from the

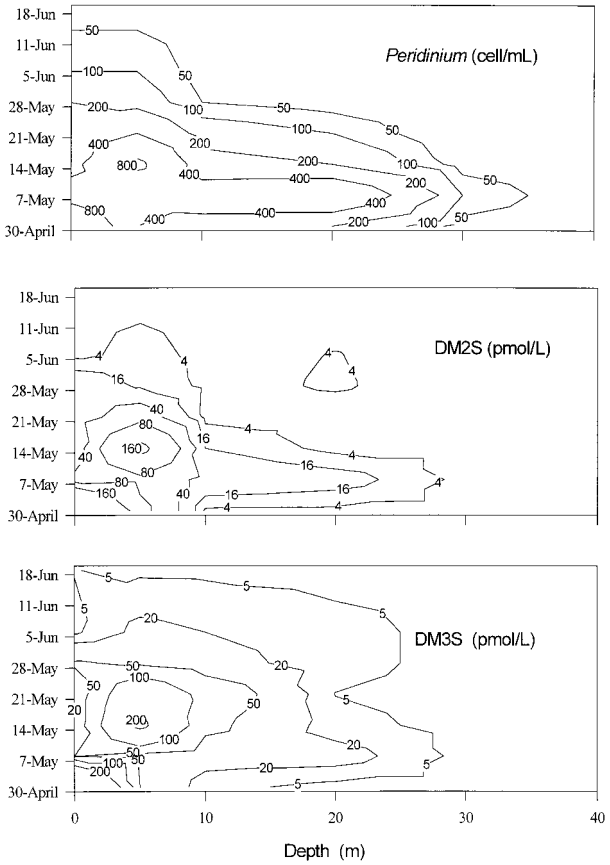


FIGURE 1. Spatio-temporal distribution of *Peridinium gatunense* cells, dimethyl disulfide (DM2S), and dimethyltrisulfide, sampled in 1995 in point A, the deepest point in Lake Kinneret.

anoxic hypolimnion or the sediment because of the following reasons: (1) There were good correlations between the DMOS and the algae cells, which do not prevail in the anoxic hypolimnion. (2) The algal bloom and the corresponding appearance of organic oligosulfides were initiated long before the stratification of the lake and the formation of anoxic hypolimnion. (3) Even after hydrogen sulfide was formed in the hypolimnion, there was negative correlation between this compound and each of the DMOS compounds. This points on unrelated, different formation mechanisms for hydrogen sulfide and DMOS. (4) The peak concentration of all DMOS occurred in the oxic epilimnion. The maximal concentration of compounds that originate in the hypolimnion or in the sediment should—at least part of the time—occur in the hypolimnion, but this was never encountered in our two-year study (1995, 1996). However, despite the good cross-correlation between the occurrence of the DMOS and *Peridinium* cells, it was impossible, based on the field studies alone, to prove that *Peridinium* cells are involved in DMOS formation. Good correlation between a biological species and a chemical compound can also stem from a codependent variable.

Laboratory Studies of *Peridinium gatunense* Cultures. Unialgal *Peridinium* cultures were cultivated and studied under laboratory conditions. Unfortunately, *Peridinium gatunense* is a rather delicate, slow-growing species, and true axenic cultures of *Peridinium* were never obtained by other groups nor ours. The alga was cultivated in a medium denoted Lindstrom ×6 (*28*). The alga reached up to 40 000 cells/mL after 30−60 days in this medium (Figure 3A). DMOS levels in this culture were either below the detection limit or rather low (<ca. 0.1 nmol/). In contrast, rather large concentrations of DMS prevailed throughout the growth cycle. This suggested

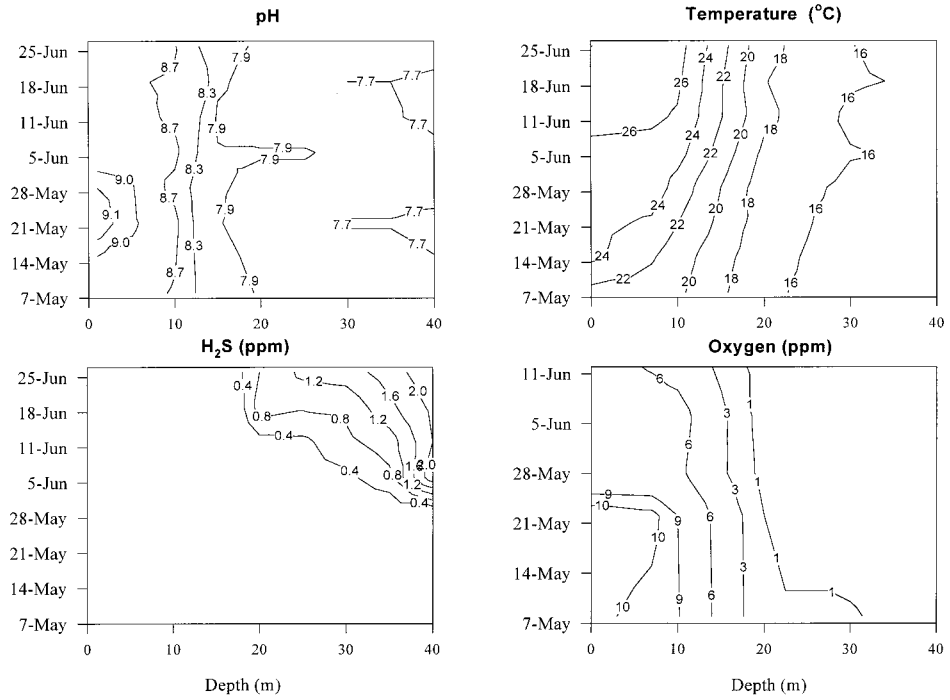


FIGURE 2. Spatio-temporal distribution map of pH, temperature, hydrogen sulfide ($HS^- + H_2S$), and dissolved oxygen as sampled in 1995 in point A by the Yigal Alon Kinneret Limnological Laboratory.

separate formation mechanisms for DMS and DMOS, and indeed a previous publication[21] showed that *Peridinium* cells store DMSP, which is cleaved to give DMS and acrylic acid in the Kinneret water.

Addition of 10% raw Kinneret water to a *Peridinium* rich culture (containing app. 10 000 cell/mL) resulted in a considerable increase in the level of DM2S, DM3S, and DM4S, while the level of the DMS remained virtually unchanged during the corresponding two day test (Figure 4). Spiking the *Peridinium* culture with sterilized (by a 120 °C autoclave treatment) Kinneret water did not increase the level of the oligosulfides. This test was conducted under aerobic conditions, and the *Peridinium* cells remained viable during the test period. These results demonstrated that the natural microorganism population of the lake can stimulate increased levels of DMOS in *Peridinium* cultures. This test also confirmed that DMS and dimethyloligosulfides are formed by different routes.

Next, it was interesting to observe whether the *Peridinium* cells are unique in their ability to stimulate production of DMOS in lake water. We have repeated the spiking tests with two other mature algae cultures that are abundant in the lake, *Chlorella*, a green algae, and *Aphanizomenon ovalisporum*, a cyanobacteria. These tests were conducted under aerobic conditions, using algal biomass containing the same level of chlorophyll *a*, 2 mg/L, in all tests. The number of the algae cells was not kept constant for all tests because of the size difference between these algae. The dry weight was not kept constant either. Chlorophyll *a* is a rough measure for viability; and because it can be easily determined, it was used as a common denominator for this qualitative test. The algae cultures were mixed with bacteria isolated from the Kinneret water, and the solution was kept for 2 days in an incubator. The level of DMOS was determined before and after the incubation period (Figure 5A). For all three algae, the bacteria significantly increased the DMOS levels. These tests showed that the *Peridinium* is not unique in its ability to stimulate DMOS production in bacteria rich environments. The focus of the investigation was accordingly shifted from the unialgal cultures to unibacterial cultures.

Studies with Specific Strains of Bacteria. Further investigation of the bacteria role in the production of DMOS was conducted using several bacteria lanes that were collected from the epilimnion. Six bacteria strains were isolated by multiple agar plate cultivation and then cultivated in Nutrient Broth medium. Spiking the *Peridinium* culture (containing approximately 5000 cells/mL) with each of the six bacteria strains resulted in an increased level of DMOS within 48 h, as shown in Figure 5B. Blank *Peridinium* culture mixed with the Nutrient Broth medium did not produce DMOS under similar test conditions.

Out of the six strains, only three were identified by Institute Pasteur, Paris by API Galerie and conventional characterization. Lane 1 is a gram negative, obligatory aerobe *Acinetobacter lwoffi*, lane 2 was identified as *Corynebacterium* a gram positive obligatory aerobe, and lane 6 was identified as *Bacillus circulans* a gram positive facultative strain. So, these three strains represent diversely different respiratory metabolism.

Public complaints on (mild) malodorous emissions from Lake Kinneret increase toward the end of the *Peridinium* bloom season. Therefore, it was interesting to investigate the DMOS yield during the different stages of the *Peridinium gatunense* growth cycle. Figure 3B,C demonstrate the yield of oligosulfides before and after addition of bacteria to unialgal *Peridinium* culture as a function of the *Peridinium* culture age. The figure compares the levels of DMOS taken at the time specified in the ordinate (the control samples) and the DMOS level after mixing the algal culture with *Acinetobacter lwoffi* stock and incubation at room temperature for an additional 2 days. The DMOS yield of the unialgal culture without added bacteria (Figure 3B,C) was increased as a function of culture age except for the first few weeks, when the old cells from the previous *Peridinium* stock were still dominant. The increase in DMOS was steeper during the declining growth phase after more than 60 days (Figure 3A). In fact, at the time corresponding to the maximal growth rate of the *Peridinium* the DMOS production rate exhibited a shallow minimum, which showed that the DMOS production is not connected to the viability of the algae. The increase

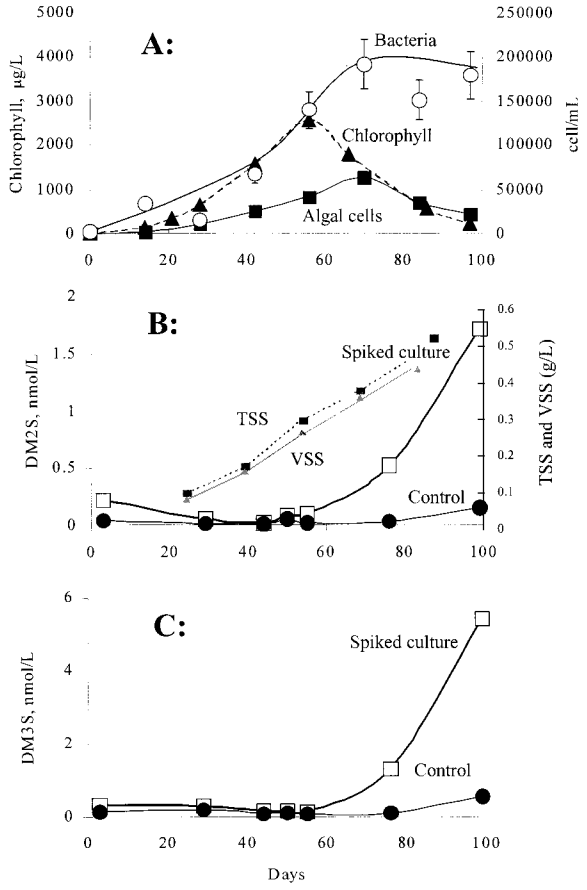


FIGURE 3. *Peridinium gatunense* growth curve. (A) Chlorophyll *a*, number of algae cells and total bacteria plate count. (B) DM2S production by bacteria spiked *Peridinium* culture as a function of culture age. Samples of the indicated age were spiked with bacteria, and the DM2S level was determined 2 days after spiking both in the control, unspiked culture and in the spiked sample. Total suspended solids (TSS) and volatile suspended solids (VSS) are also shown. (C) The same as (B) for DM3S production.

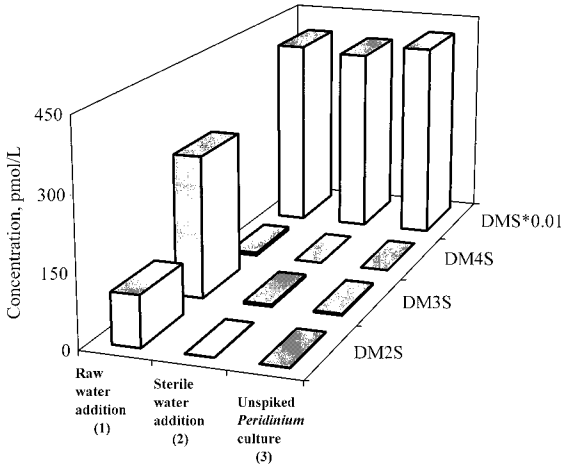


FIGURE 4. DMS and DMOS yield after addition of 1—raw lake water and 2—sterilized lake water to *Peridinium* culture samples compared to 3—unspiked culture.

in DMOS yield in *Peridinium* culture (without addition of bacteria) could not be attributed to the larger amount of *Peridinium* substrate, because the slope of the DMOS yield became much steeper (after 60 days) in the declining growth phase and not in the exponential phase, where biomass accumulation rate is maximal. Likewise, the increased

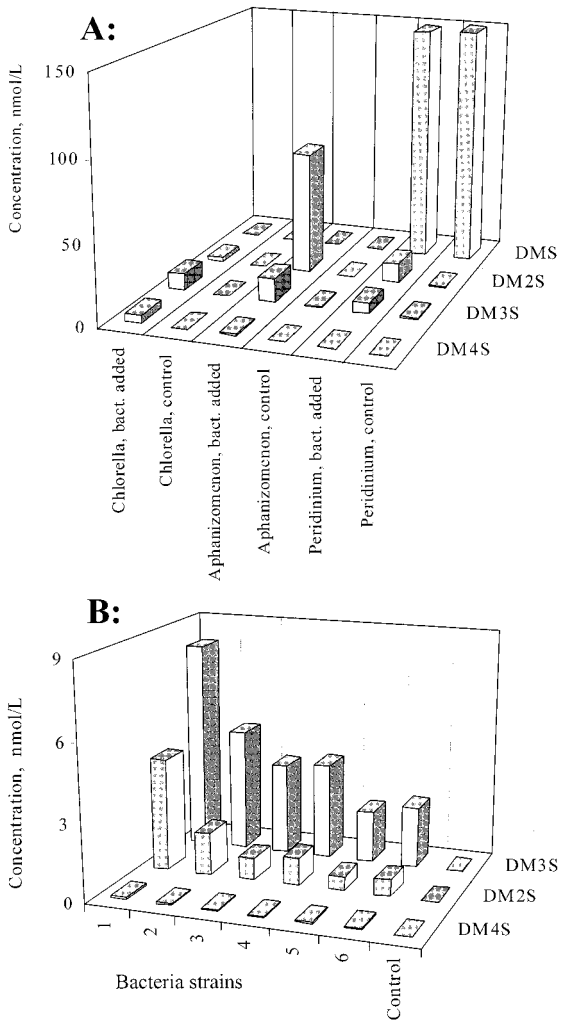


FIGURE 5. (A) DMS and DMOS produced before and after the addition of bacteria to different algae cultures (the algae cultures contained 2 mg/L chlorophyll *a*). (B) DMOS production by *Peridinium* culture spiked with bacteria lanes 1—6, respectively (see text). Row 7 represents DMOS production by blank culture without added bacteria.

bacteria count in the unspiked culture (observed in Figure 3A) can only partly account for the increased DMOS yield, because the specific yield of DMOS by the bacteria in the unialgal culture (i.e., DMOS/bacteria cell count) was also increased in the declining growth phase.

The trends in the DMOS production as a function of algae age became more pronounced in the samples that were collected from the *Peridinium* culture and spiked with bacteria (Figure 3B,C). The increase in DMOS concentration became even steeper during the declining growth phase (compare Figure 3A—C). The *Peridinium* growth cycle was accompanied by a corresponding—almost linear—increase in the total suspended solids (TSS) and volatile suspended solids (VSS) (Figure 3B). The accumulation of TSS and VSS continued even in the declining growth phase because *Peridinium* contains a fraction of refractory, hard to biodegrade polyglucan theca, which continues to contribute to the volatile and total suspended solids of the culture long after the cells' death. So, it was postulated that the increased amount of the refractory substrate is the source of the DMOS. However, the ratio of DMOS/TSS and DMOS/VSS also increased steeply during the declining growth phase and therefore this hypothesis was abandoned. These observations indicate that the bacteria can efficiently synthesize DMOS from sulfur—containing organic matter of dead algae or their

TABLE 1. Production of VSC by *Acinetobacter lwoffii* Culture Spiked with Sulfur-Containing Substrates[a]

conditions		av concentrations nmol/L (number of experiments)									
substrate	addition	methanethiol	DMS	DM2S	DM3S	DM4S	methanethiol-d_3[b]	DMS-d_6	DM2S-d_6	DM3S-d_6	DM4S-d_6
nutrient broth	*e*	nd	nd[d]	0.05 (3)	0.09 (3)	nd[d]					
Peridinium gatunense[b]	*e*	*e*	148 (6)	11.8 (6)	8.61 (6)	0.34 (6)					
Chlorella[b]	*e*	*e*	nd[d]	4.47 (2)	10.5 (2)	0.76 (2)					
Aphanizomenon[b]	*e*	*e*	nd[d]	89.9 (2)	17.2 (2)	1.08 (2)					
albumin	*e*	*e*	*e*	167 (2)	49.2 (2)	5.18 (2)					
methionine-d_3		nd[d]	*e*	nd[d]	0.05 (4)	nd[d]	14.1 (4)	10.2 (4)	31.4 (4)	16.3 (4)	0.76 (4)
methionine	O_2 satd	*e*	*e*	24.9 (2)	27.4 (1)	1.71 (2)					
methionine	*e*	17.5 (8)	6.22 (8)	39.1 (8)	20.9 (8)	0.64 (8)					
cysteine	*e*	nd[d]	nd[d]	0.05 (3)	0.04 (3)	nd[d]					
K_2S_n	*e*	nd[d]	nd[d]	0.21 (2)	0.33 (2)	0.63 (2)					
DMSP[c]	*e*	*e*	*e*	−6.7 (3)	2.02 (3)	0.13 (2)					
DMSP-d_6[c]	*e*	*e*	*e*	0.18 (2)	0.08 (2)	nd[d]			4.04 (2)	1.1 (2)	0.04 (2)
DMS	*e*	*e*	*e*	0.05 (1)	0.04 (1)	nd[d]					
cysteine	CD_3I	nd[d]	nd[d]	0.08 (3)	0.11 (3)	nd[d]	15.4 (3)	nd[d]	20.2 (3)	25.0 (3)	1.95 (3)
methionine	CD_3I			3.62 (3)	12.2 (3)	0.25 (3)			2.87 (3)	5.01 (3)	0.32 (3)
nutrient broth	CD_3I	nd[d]	nd[d]	0.13 (2)	0.08 (2)	nd[d]	nd[d]	nd[d]	0.05 (2)	0.06 (2)	nd[d]

[a] 5 mmol/L S substrate concentration, 4 day exposition time, exept for ~ marked rows were 0.67 mmol/L and 3 days exposure. [b] 2 mg/L chlorophyll *a* concentration (instead of S) was used to standartize the experiments. [c] 21 day exposition time. [d] nd, not detected. [e] Not measured.

lysis products and less efficiently from living algae cells. This explains the increased ratio of DMOS/TSS for high culture age, and it is in agreement with the observations of malodor emissions from the lake during the late *Peridinium* bloom season.

To investigate the role of the substrate feed in the production of DMOS, we fed pure cultures of specific bacteria strains with different types of sulfur-containing substrates, as depicted in Table 1. In all cases, the nutrient feed contained 5 mmol sulfur/(liter sample). For the algal spikes where sulfur content was unknown, we added an algae culture quantity corresponding to 2 mg/L chlorophyll *a* to the bacteria cultures. Methionine, DMSP, albumin (taken as a model for methionine containing proteins), and three algae strains that are abundant in Lake Kinneret (*Chlorella*, *Peridinium*, and *Aphanizomenon*) initiated considerable production of DMOS as compared to a blank Nutrient Broth feed. In contrast, cysteine, DMS, and inorganic oligosulfide substrates—consisting of a combination of potassium hydrogen disulfide, potassium trisulfide, and potassium tetrasulfide—did not increase the level of DMOS. Repeating the bacteria feed test with methionine-d_6 feed yielded exclusively DMOS-d_6, which confirmed that methionine can function as a sulfur source as well as a methyl donor. All these tests were conducted under aerobic conditions, with air diffusing through the cotton wool stoppers of the Erlenmeyer flasks. To confirm aerobic conditions, the methionine feed test was repeated also with oxygen bubbling, which eliminated the DMS and gave a somewhat lower level of DM2S due to evaporation. Otherwise, the results of the oxygen bubbling test agreed well with the other methionine spiking tests (Table 1).

Methionine was selected for further investigation as a DMOS precursor that is also widely abundant. To verify that our investigation does not relate only to *Acinetobacter,* we have exposed methionine and *Peridinium* cells to the three bacteria strains that were identified, *Acinetobacter lwoffii, Corynebacterium*, and *Bacillus circulans.* In all cases, methionine and *Peridinium* additions initiated emissions of DMOS (data not shown). The production of DMOS by Acinetobacter *lwoffii* was always somewhat higher as compared to the other two bacteria strains, despite the fact that it is an obligate aerobe.

Figure 6 depicts dynamic production curves of volatile sulfur compounds from methionine fed *Acinetobacter lwoffii* cultures. The test conditions were identical to that of Table 1. The production of the three DMOS followed the typical modified Monod assimilation curve with substantial lag phase

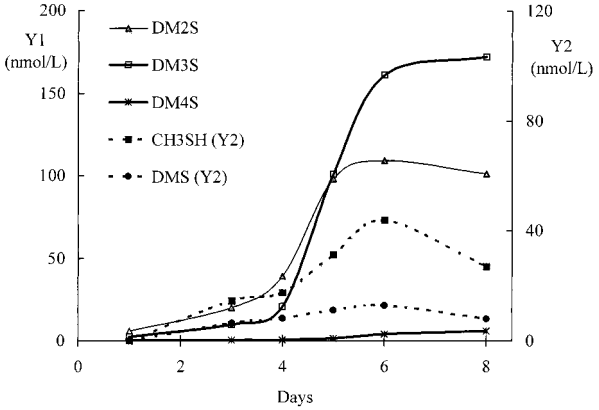
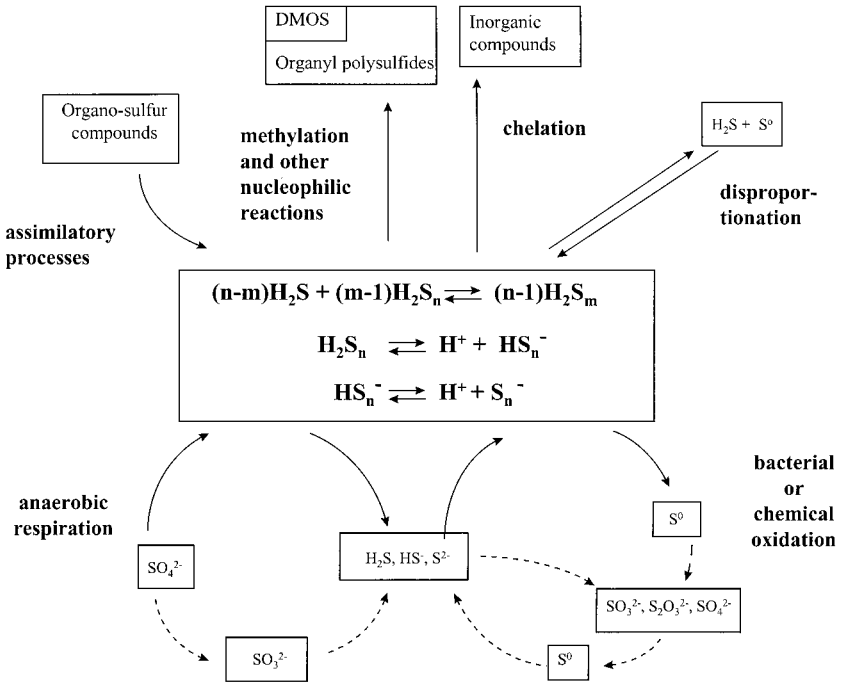


FIGURE 6. Volatile sulfur compounds produced during methionine decomposition by *Acinetobacter lwoffii.* Initial concentration of the substrate 5 mmol/L (Y1 axis presents levels of DM2S, DM3S, DM4S; Y2 axis presents the coordinate for CH_3SH and DMS).

periods. Methanethiol and DMS production curves exhibited also a declining phase. The levels of methanethiol and DMS were found to be 10−30 nmol/L which falls within the reported range (*11*, *14*). The concentrations of the DMOS were somewhat higher, about 50−100 nmol/L. The bacteria converted approximately 0.01% of the methionine sulfur to DMOS during an 8-day batch test. The decrease in concentrations of DMS and methanethiol after 6 days can be attributed to slow evaporation of these compounds, since the vials were not sealed (to allow oxygen supply). Air oxidation probably also contributed to the disappearance of methanethiol. The lag phase period in the production of all four major organosulfur compounds may be attributed to the production of C−S lyase enzymes that are required in order to assimilate methionine. The similar duration of the lag phase for all of the volatile sulfur compounds supports the view that none of these compounds is an intermediate product in a series of chemical steps leading to the formation of others. The possibility that DMS is a precursor for DMOS biosynthesis was rejected also based on the data of Table 1. Indeed, most of these compounds are stable under ambient conditions, and it is difficult to conceive how they can transform chemically to DMOS. A possible exception is the methanethiol, which can produce DM2S by oxidative dimerization.

Production of Inorganic Oligosulfides. The very different yield of DMOS from methionine and cysteine feeds (Table

SCHEME 1. Formation and Fate of Inorganic Oligosulfides and Dimethyloligosulfide Production



1), despite their similar structure, point to a major role that is played by the sulfur methyl group. Wajon et al. (*19*) proposed that inorganic oligosulfides, which were produced in anoxic groundwater, act as precursors for DMOS formation in drinking water. Methylation of the inorganic oligosulfides produces the DMOS, which are more stable and prevail longer under oxic conditions. To check the postulate that inorganic oligosulfides are intermediates in the production of DMOS and in order to verify that all these steps are carried out under strictly aerobic conditions, we have conducted a set of experiments with the obligatory aerobe *Acinetobacter lwoffii*.

Currently, there is only one method for speciation of inorganic oligosulfides: a spectrophotometric method, which is based on slight difference in the UV absorption spectrum of the different inorganic oligosulfides (*30*). Other methods give only lumped attributes (*31*). These methods were not applicable for the current studies, because the turbidity and color of the bacteria culture interfere with the spectrophotometric determination and the high concentration of dissolved oxygen and other chemical interferences limit the usefulness of electrochemical methods. Therefore, we have used a semiquantitative method for the determination of inorganic oligosulfides that was based on methylation by deuterated methyliodide and subsequent GC/MS determination of the DMOS-d_6. Preliminary studies revealed that methylation of inorganic sulfides follow a second-order reaction rate with first-order dependence on the inorganic oligosulfide concentration. The rate constant ranged between 0.23 and 0.51 h^{-1} in 2 mmol/L methyliodide in room temperature, comparable to literature data (*32*). So the oligosulfides were quantitated by methylation with 2 mmol/L methyliodide-d_3, followed by GC/MSD analysis. At the current stage of development, the method cannot ensure accurate speciation, since interconversion between different forms of inorganic oligosulfides (Scheme 1) during the methylation can distort the exact distribution of oligosulfides. Moreover, the method could not be used for quantitation of dimethylpentasulfide and higher DMOS. However, the presence of oligosulfides is clearly reflected by this method.

Cysteine, which failed to produce DMOS and methionine which successfully produced DMOS (Table 1), was fed

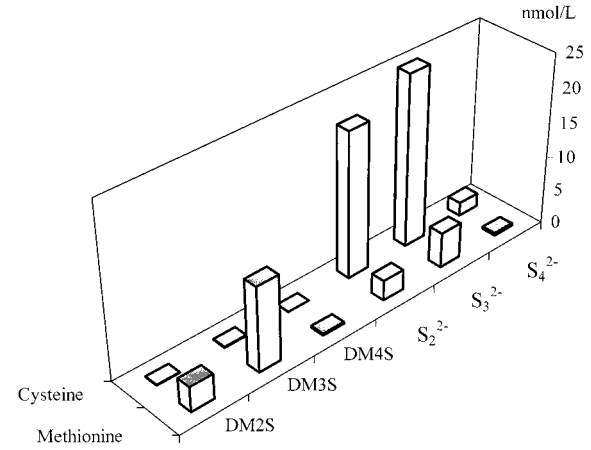


FIGURE 7. Distribution of organic and inorganic oligosulfides produced by addition of methionine and cysteine to *Acinetobacter lwoffii* culture. Initial concentration 0.67 mmol/L; 3 days cultivation (S_n^{2-} denotes the sum of polysulfanes and their deprotonated forms i.e., S_n^{2-}, HS_n^- and H_2S_n).

(separately) to *Acinetobacter lwoffii* cultures (0.67 mmol/L was added to 200 mL of culture). After 3 days of incubation the samples were exposed to 2 mmol/L methyliodide-d_3. Both the dimethyl sulfides and the deuterated dimethyl sulfides were determined by OSA-GC-MSD analysis. The distribution of organic and inorganic oligosulfides that were produced from the methionine and cysteine feeds is depicted in Figure 7. Methionine feed produced—under these conditions—predominantly methylated oligosulfides, while the cysteine yielded almost exclusively inorganic oligosulfides, which appeared as deuterated dimethyloligosulfides in the GC/MSD analysis. These results are presented in the last three rows of Table 1. Note that the distribution of the DMOS and the deuterated DMOS for the methionine feed were very similar supporting the view that DMOS were produced by methylation of formerly produced inorganic oligosulfides. Likewise, the distribution of DMOS-d_6 for the methionine and cysteine feeds supports the view that the distribution of the DMOS was not determined by the rate of methylation.

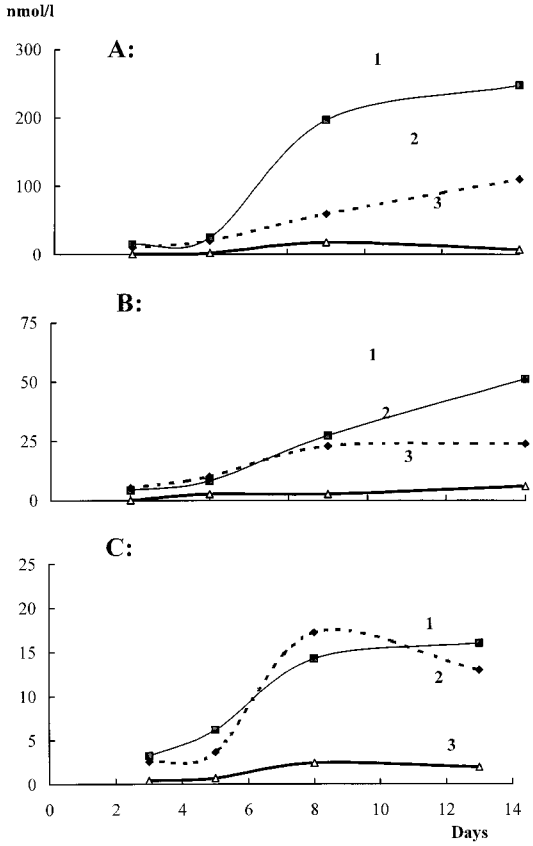


FIGURE 8. Inorganic oligosulfide yields, after addition of 5 mmol/L cysteine substrate to bacteria cultures (derivatization was carried out with methyliodide-d_3): A: *Acinetobacter lwoffii*, B: *Corynebacterium*, C: *Bacillus circulans*; 1: DM2S, 2: DM3S, 3: DM4S.

Variation of the initial methionine concentration and the duration of the feed test (i.e., the time of CD_3I addition) changed significantly the ratio between the inorganic oligosulfides and the DMOS. For example, addition of 0.67 and 5 mmol/L methionine gave ratio of 0.21 and 0.03 between the total inorganic oligosulfides and DMOS. This shows that larger availability of the methyl donor shifts the products from the intermediate inorganic oligosulfides toward the methylated forms. When the feed test duration was shortened from 4 to 3 days (for 0.67 mmol/L methionine feed) the ratio between the inorganic oligosulfides and DMOS was increased from 0.21 to 0.51 again pointing on inorganic sulfides as intermediates in a series of steps leading to DMOS.

Kinetic studies of the production of the inorganic oligosulfides by *Acinetobacter lwoffi*, *Corynebacterium*, and *Bacillus circulans* that were fed with cysteine are presented in Figure 8A−C. The DMOS-d_6 yield curves for all three target bacteria (Figure 8A−C) are very similar, and they are also practically identical to the kinetics of the DMOS yield from methionine feed (Figure 6). Note that DMOS were not produced from cysteine. In all cases, DM3S-d_6 was the dominant DMOS-d_6, and DM4S-d_6 exhibited the lowest abundance. This again shows that methylation is not the rate determining step in DMOS production from methionine.

The distribution of DMOS as a function of pH in absolute and relative values is depicted in Table 2. Disulfide and trisulfide were the dominant compounds for pH 4−10. The average number of sulfur atoms in the oligosulfides was increased at higher pH. Thus, the relative level of DM2S goes down from 65% (of the total DMOS) for pH 3.9 to 10% at pH 10.2, while the relative fraction of DM4S is tripled by this pH change. Comparison of the distribution of the oligosulfides with literature data is rather confusing, because of the wide discrepancy between reported data and the uncertainties involved in the methods for determination of oligosulfides. Several authors claim (*30*, *33*) that pentasulfane and its deprotonated moieties are the dominant oligosulfide compounds in the relevant pH range and that at higher pH the lower oligosulfides become more dominant. This is not commensurate with the distribution of Table 2. Resolving the controversy about the speciation of inorganic oligosulfides in water is beyond the scope of this paper, but Appendix A shows that a simple material balance, taking into account the diprotic nature of the oligosulfanes, predicts larger abundance of higher oligosulfides at higher pH in qualitative accord with Table 2.

TABLE 2. Distribution of Inorganic Oligosulfides Produced by *Acinetobacter lwoffii* Fed on Methionine at Different pH

	concn, nmol/L (percentage of the total DMOS)		
compound	pH = 3.9	pH = 5.8	pH = 10.2
DM2S	12.3 (65%)	9.5 (29%)	1.45 (10%)
DM3S	6.15 (33%)	21.7 (68%)	11.7 (84%)
DM4S	0.3 (2%)	0.95 (3%)	0.8 (6%)

Our field studies suggest predominance of the low DMOS, which do not agree with previous speciation studies of inorganic polysulfides that suggest dominance of tetrasulfide and pentasulfide moieties (*30*, *31*, *33*). To elucidate the reason for this observation, we have conducted a semiquantitative stability study of DMOS in oxygen saturated water in dark beakers and under solar irradiation. The DMOS were very stable, at least for several weeks in the dark, which agrees well with previous studies on the stability of DMOS in organic solvents (*34*, *35*). However, the large DMOS were converted to lower DMOS within several days of exposure to sun light which also agree well with previous data on the relative instability of larger organic polysulfides (*34*, *35*).

Discussion

Formation of Inorganic and Organic Oligosulfides. This investigation that originally focused on the source of DMOS in Lake Kinneret led to several general conclusions, which may apply for other oxic aquatic systems.

(1) DMOS can be formed by biogenic assimilation of organo-sulfur compounds. In Lake Kinneret—the subject of the current study—biodegradation of aged *Peridinium* cells or their lysis products form DMOS.

(2) The whole process of DMOS formation can be accomplished in oxygen rich water.

(3) Inorganic oligosulfides can be formed by biogenic processes under oxic conditions.

(4) DMOS in Kinneret are probably formed by methylation of inorganic oligosulfide intermediates that are formed under oxic conditions.

DMOS Formation by an Assimilatory Process. Despite the strong spatial and temporal correlations between the DMOS and *Peridinium*, only indirect connection between these two variables was found in this study. *Peridinium* cultures that were not spiked with Kinneret water failed to produce substantial levels of DMOS, whereas *Aphanizomenon* cyanobacteria and *Chlorella* green algae were equally efficient sources of DMOS after addition of bacteria culture or raw Kinneret water. Based on these tests and specific organo-sulfur (e.g., methionine and albumin shown in Table 1) feed tests, it can be concluded that the *Peridinium* stimulate the formation of DMOS by increasing the organic load. The high concentration of *Peridinium* also increases the level of bacteria that can biodegrade the lysis products of *Peridinium*,

which further increase the rate of DMOS production. The ability to stimulate DMOS production is found to be rather common for bacteria and all six obligatory aerobe and facultative bacteria that were isolated from Lake Kinneret produced DMOS from *Peridinium* algae and from other organo-sulfur compounds.

The Whole Process of DMOS Formation Is Carried Out under Oxygen Rich Conditions. This conclusion is supported by both field and laboratory studies. The field studies showed unequivocally the ubiquity of DMOS in the epilimnion of Lake Kinneret. Moreover it has been proven that DMOS were also generated in the epilimnion and not in the hypolimnion or the sediment. As discussed in the field studies section, the second conclusion was supported by the location of the DMOS peak concentration, the formation of DMOS in unstratified lake, and the good correlation between the algae and the DMOS.

Additional support for the conclusion regarding the formation of DMOS in oxic conditions was obtained from laboratory studies that confirmed that DMOS were formed after addition of Kinneret water to oxic *Peridinium* culture or by addition of organo-sulfur compounds to obligatory aerobic bacteria cultures that were isolated from the lake.

Neither the field study alone nor the laboratory studies by themselves were sufficient to prove unequivocally that the DMOS can be formed in freshwater under oxic conditions. One could argue that the DMOS were formed in anoxic patches in the epilimnion or that the laboratory experiments do not reflect natural environments. But the combination of the field studies with the laboratory studies of field samples of specific strains of bacteria provides sound basis for this conclusion. Although the production of DMOS under oxic conditions is counterintuitive, it is not entirely surprising. Aerobic production of reduced sulfur compounds was reported before, including, for example, sulfate reduction under oxic conditions (*36*). The formation of hydrogen sulfide, DMS, DM2S, and other reduced sulfur compounds in oxic marine environments was also reported (*37*).

Biogenic Formation of Inorganic Oligosulfides under Oxic Condition. Oligosulfides are rather abundant in oxic and anoxic water sources, and their function as DMOS precursors was established before (*18*). However, as far as we know the biogenic formation of inorganic oligosulfides in oxic environments has been proven here for the first time. The evidence for the biogenic formation of inorganic oligosulfides comes from laboratory studies with pure cultures of specific strains of facultative and aerobic bacteria that were carried out under aerobic conditions. Cysteine as well as methionine nutrients led to biogenic production of inorganic oligosulfides (Figures 7 and 8).

DMOS Formation from Inorganic Oligosulfide Intermediates. Laboratory studies confirmed formation of oligosulfides by three different bacteria (including an obligatory aerobe strain) that were fed on cysteine under oxic cultivation conditions (Figure 8). In the absence of methyl donor the oligosulfides do not form DMOS and accumulate in the water, while in the presence of methyl donors they were readily transformed to DMOS. Moreover, the ratio between the DMOS and the inorganic oligosulfides was increased when excess methionine or another methyl donor (e.g., methyl iodide or ethyl iodide) was present. Likewise, the ratio between the DMOS and inorganic oligosulfides was increased for higher exposure time as expected for the ratio between final products and their intermediates.

"Paper chemistry" suggests alternative routes for the production of DMOS—involving CH_3S transfer instead of methylation. For example, Chin and Lindsay (*38*) proposed that methylmethanethiosulfinate serves as CH_3S donor (to an organosulfide moiety, CH_3SSH) increasing chain length and yielding CH_3SOH byproduct. However, in our laboratory tests we never found the corresponding dimethyl sulfone or dimethylsulfine though they were carefully looked for in the GC/MS-SIM studies.

We demonstrated the formation of inorganic oligosulfides only for the two sulfur amino acids, but these results suggest a general pattern. We believe that this information also suggests that DMOS are formed in the Kinneret through inorganic polysulfide intermediates though we still did not look for these compounds in the lake.

Production and Fate of Inorganic Oligosulfides in Aquatic Systems. Scheme 1 outlines the reported ways for the production of inorganic oligosulfides along with the biogenic assimilatory pathway that was described in this article. The most common mechanism for the production of inorganic oligosulfides is by reaction of elemental sulfur with bisulfide. This is the source for the ubiquity of oligosulfides in hydrogen sulfide and sulfur rich groundwater. Inorganic oligosulfides are often produced by respiratory reduction of sulfate, which yields oligosulfides, either directly or by formation of colloidal sulfur and bisulfide (*39*). Oxidative formation of inorganic sulfides by chemical or biogenic processes (*40*) is also possible. Often, oxidation or exposure of anoxic groundwater containing hydrogen sulfide to air gives elemental sulfur, which combines with hydrogen sulfide to give the inorganic oligosulfide (*41*).

This research illuminates yet another pathway for biogenic formation of oligosulfides, through assimilatory processes involving sulfur containing amino acids and other sulfur-organic compounds. Here, the microorganisms cleave the C—S bond and utilize the organic moieties, and the surplus sulfur species is discarded. So, unlike the respiratory redox pathways and the chemical transformations, the assimilatory process does not require a transition of the water from oxic to anoxic conditions or vice versa, and it can proceed—all the way—under strictly oxic conditions.

Inorganic oligosulfides are strong nucleophiles in their deprotonated forms (HS_n^- and S_n^{2-}) (*32*). Thus, possible sinks for oligosulfides include chelation by metal ions (e.g., Fe-(III)) or nucleophilic reactions with organic species. The formation of DMOS is only one such pathway (*42*), and in other environments dialkylmoieties (*15*) and other organic oligosulfide compounds were reported (*43*). When a methyl donor is available, then methylation takes place. Indeed, this was demonstrated for methyliodide, methionine, or protein nutrients. In the absence of methyl donors, the process was terminated at the inorganic oligosulfide stage.

The fate of DMOS was only briefly addressed in this research, and more studies are needed in order to understand the distribution and fate of DMOS in aquatic systems. However, the fact that higher DMOS are readily converted to smaller ones under solar irradiation and the generality of the DMOS formation process (for different substrates and different bacteria) suggest that it is possible that marine DM2S may also form through inorganic polysulfide intermediates. DM2S is rather abundant in marine water and its source is not yet fully resolved.

Acknowledgments

The financial help of the KFK-BMBV, Germany and the Ministry of Science, Israel, and the Water Commissioner, Israel is gratefully acknowledged. The authors thank Prof. Z. Aisenshtat for helpful discussions.

Appendix A

Polysulfanes obey the following acid dissociation and disproportionation equilibria

$$H_2S_n \rightarrow H^+ + HS_n^- \quad (A1)$$

$$HS_n^- \leftrightarrow H^+ + S_n^{2-} \quad (A2)$$

$$H_2S_m \leftrightarrow S + H_2S_n \quad (A3)$$

where, for simplicity $m = n + 1$. The total concentration of the oligosulfides of specific sulfur length, n, is given by

$$C_{Sn} = [H_2S_n] + [HS_n^-] + [S_n^{2-}] \quad (A4)$$

[ ] and { } denote concentrations and activities, respectively. $[H_2S_n]$ is given by

$$[H_2S_n] = C_{Sn}\{H^+\}^2({}^cK_{a1,Sn}\,{}^cK_{a2,Sn} + \{H^+\}{}^cK_{a1,Sn} + \{H^+\}^2)^{-1} \quad (A5)$$

${}^cK_{a1,Sn}$ and ${}^cK_{a2,Sn}$ are the first and second dissociation constants for the polysulfanes (in concentration units). The values of ${}^cK_{a1,Sn}$ and ${}^cK_{a2,Sn}$ were reported to be (5.0, 9.7); (4.2, 7.5); (3.8, 6.3); (3.5, 5.7) for $n = 2-5$ respectively.[33] Thus, higher polysulfanes are stronger acids. From equilibrium relationship eq A3 is manifested in

$$K_{Sn/Sm} = \{S\}\{H_2S_n\}/\{H_2S_m\} \quad (A6)$$

Introducing eq A5 into A6 and rearrangement gives

$$C_{Sm}/C_{Sn} = (\{S\}/K_{Sn/Sm})({}^cK_{a1,Sm}\,{}^cK_{a2,Sm} + \{H^+\}{}^cK_{a1,Sm} + \{H^+\}^2)/({}^cK_{a1,Sn}\,{}^cK_{a2,Sn} + \{H^+\}{}^cK_{a1,Sn} + \{H^+\}^2) \quad (A7)$$

Thus, for unit activity of sulfur (or for constant dissolved sulfur concentration) C_{Sm}/C_{Sn} in eq A7 is a monotonically decreasing function of $\{H^+\}$ since the ${}^cK_{a1,Sm}$ and ${}^cK_{a2,Sm}$ are larger than ${}^cK_{a1,Sn}$ and ${}^cK_{a2,Sn}$ for $m > n$. For example, for very low pH the value $[C_{Sm}/C_{Sn}]/[\{S\}/K_{Sn/Sm}] = 1$ while for very basic pH (i.e. pH $\gg$ p${}^cK_{a2,Sm}$) it is given by ${}^cK_{a1,Sm}{}^cK_{a2,Sm}$-$({}^cK_{a1,Sn}{}^cK_{a2,Sn})^{-1}$ which always is >1 (for $m > n$). So, higher pH increases the average molecular weight of the polysulfanes in accordance with the pattern observed in Table 2.

Literature Cited

(1) *Sulfur compounds in Foods*; Mussinan, C. J., Keelan, M. E., Eds.; ACS Symposium Series 564; American Chemical Society: Washington, DC, 1993.
(2) Aisenshtat, Z.; Lipiner, G.; Cohen, Y. In *Microbial mats: stromatolites*; Cohen, Y., Casteholz, R. W., Halvorson, H. O., Eds.; Alan R. Liss: New York, p 471.
(3) Richards, S. R.; Rudd, J. W. M.; Kelly, C. A. *Limnol. Oceanogr.* **1994,** *39*, 562−572.
(4) Monzcvinz, J. T.; Ainsworth, L. D. *J. AWWA* **1974**, 537−539.
(5) Wnorowski, A. U.; Scott, W. E. *Water Sci. Technol.* **1992**, *25*, 225−232.
(6) Wajon, J. E.; Alexander, R.; Kagi, R. I. *Chemosphere* **1985**, *14*, 85−89.
(7) Campbell A. T.; Reade, A. J.; Warburton, I.; Whiteman, R. F. *J. Inst. Water. Environ. Man.* **1994**, *8*, 52.
(8) Khiari, D.; Suffet, I. H.; Barrett, S. E. *Water Sci. Technol.* **1995,** *31*, 93.
(9) Krasner, S. W.; Barret, S. E.; Dale, M. E.; Hwang, S. G. *J. AWWA* **1989**, 86−93.
(10) Bechard, R. G.; Rayburn, W. R. *J. Phycol.* **1979**, *15*, 379−383.
(11) Segal, W.; Starkey, R. L. *J. Bacteriol.* **1969**, *98*, 908−913.
(12) Juttner, F. *Z. Naturforsch.* **1984,** *39C*, 867−871.
(13) Jenkins, D.; Medseker, L. L.; Thomas, J. F. *Environ. Sci. Technol.* **1967**, *1*, 731−735.
(14) Kadota, H.; Ishida, Y. *Annu. Rev. Microbiol.* **1972**, *26*, 127−138.
(15) Wajon J. E. *Water Qual. Bull.* **1988,** *13*, 90−101.
(16) Hofbauer, B.; Juttner, F. *FEMS Microbiol. Ecol.* **1988,** *53*, 113−122.
(17) Yu, T. H.; Ho, C.-T. *J. Agric. Food Chem.* **1995**, *43*, 1641−1646.
(18) Ames, J. M. In *Sulfur compounds in Foods*; ASC Symposium Series 564; Mussinan, C. J., Keelan, M. E., Washington, DC, 1993; p 104.
(19) Wajon, J. E.; Heitz, A. *Water Sci. Technol.* **1995**, *31*, 87.
(20) Ginzburg, B.; Chalifa, I.; Zohari, T.; Hadas, O.; Dor, I.; Lev, O. *Water Res.* **1998**, *32*, 1789.
(21) Ginzburg, B.; Dor, I.; Chalifa, I.; Hadas, O.; Lev, O. *Environ. Sci. Technol.* **1998**, *32*, 2130.
(22) Korchevin, N. A.; Turchaninova, L. P. *Zh. Obshch. Khim.* **1989**, *59*, 1785.
(23) Clarke, E. T.; Solouki, T.; Russel, D. H.; Martell, A. E.; McManus, D. *Anal. Chim. Acta* **1994**, *299*, 97−111.
(24) Dickson, D. M.; Wyn Jones, R. G.; Davenport, J. *Planta* **1980**, *150*, 158−165.
(25) Grob, K.; Zurcher, F. *J. Chromatogr.* **1976**, *117*, 285−294.
(26) Boren, H.; Grimvall, A.; Palmborg, J.; Savenhed, R. *J. Chromatogr.* **1985**, *348*, 67−78.
(27) Henatsch, J. J.; Juttner, F. *Arch. Hydrobiol.* **1990**, *119*, 315−323.
(28) Lindstrom, K. *Hydrobiol.* **1985**, *70*, 77−85.
(29) Eaton, A. D.; Cleseceri, L. S.; Greenberg, A. E. *Standard methods for the examination of water and wastewater*, 19th ed.; American Pub. Health Association: Washington, DC, 1995.
(30) Giggenbach, W. *Inorg. Chem.* **1972**, *11*, 1201−1207.
(31) Boulegue, J. *Electron. Acta* **1978**, *42*, 1439−1445.
(32) Haag, W. R.; Mill, T. *Environ. Toxicol. Chem.* **1988**, *7*, 917−984.
(33) Schwarzenbach, G.; Fisher, A. *Helv. Chim. Acta* **1960**, *169*, 1365−1383.
(34) Pickering, T. L.; Saunders, K. J.; Tobolsky, A. V. *J. Am. Chem. Soc.* **1967**, *89*, 2364−2367.
(35) Grant, D.; Van Wazer, J. R. *J. Am. Chem. Soc.* **1968**, *86*, 3012−3017.
(36) Canfield, D. E.; Des Marais, D. J. *Science* **1991**, *251*, 1471−1473.
(37) Elliot, S.; Lu, E.; Sherwood, R. In *Biogenic Sulfur in the Environment*; Saltsman, E., Cooper, W. J., Eds.; American Chemical Society: Washington, DC, 1988.
(38) Chin, H. W.; Lindsay, R. C. In *Sulfur compounds in foods*; Mussinan, C. J., Keelan, M. E., Eds.; ACS Symposium Series, 564; American Chemical Society: Washington, DC, 1993; p 90.
(39) Boulegue, J. *Geochim. Cosmochim. Acta* **1977**, *41*, 1751−1758.
(40) Chen, K. Y.; Morris, J. G. *Environ. Sci. Technol.* **1972**, *6*, 529−537.
(41) Steudel, R. *Ind. Eng. Chem. Res.* **1996**, *35*, 1417−73.
(42) Drotar, A.; Burton, G. A.; Tavernier, J.; Fall, R. *Appl. Environ. Microbiol.* **1987**, 1626−1631.
(43) Tolstikov, G. A.; Shul'ts, E. E.; Tolstikov, A. G. *Russ. Chem. Rev. (Engl. Transl.)* **1997**, *66*, 813−826.

Received for review June 22, 1998. Revised manuscript received November 9, 1998. Accepted November 13, 1998.

ES980636E