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Microbial conversion of inorganic carbon to dimethyl sulfide in anoxic lake sediment (Plußsee, Germany)

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Abstract. In anoxic environments, volatile methylated sulfides like methanethiol (MT) and dimethyl sulfide (DMS) link the pools of inorganic and organic carbon with the sulfur cycle. However, direct formation of methylated sulfides from reduction of dissolved inorganic carbon has previously not been demonstrated. When studying the effect of temperature on hydrogenotrophic microbial activity, we observed formation of DMS in anoxic sediment of Lake Plußsee at 55 °C. Subsequent experiments strongly suggested that the formation of DMS involves fixation of bicarbonate via a reductive pathway in analogy to methanogenesis and engages methylation of MT. DMS formation was enhanced by addition of bicarbonate and further increased when both bicarbonate and H_2 were supplemented. Inhibition of DMS formation by 2-bromoethanesulfonate points to the involvement of methanogens. Compared to the accumulation of DMS, MT showed the opposite trend but there was no apparent 1:1 stoichiometric ratio between both compounds. Both DMS and MT had negative δ^{13}C values of −62‰ and −55‰, respectively. Labeling with $NaH^{13}CO_3$ showed more rapid incorporation of bicarbonate into DMS than into MT. The stable carbon isotopic evidence implies that bicarbonate was fixed via a reductive pathway of methanogenesis, and the generated methyl coenzyme M became the methyl donor for MT methylation. Neither DMS nor MT accumulation were stimulated by addition of the methyl-group donors methanol and syringic acid or by the methyl-group acceptor hydrogen sulphide. The source of MT was further investigated in a $H_2^{35}S$ labeling experiment, which demonstrated a microbially-mediated process of hydrogen sulfide methylation to MT that accounted for only <10% of the accumulation rates of DMS. Therefore, the major source of the ^{13}C-depleted MT was neither bicarbonate nor methoxylated aromatic compounds. Other possibilities for isotopically depleted MT, such as other organic precursors like methionine, are discussed. This DMS-forming pathway may be relevant for anoxic environments such as hydrothermally influenced sediments and fluids and sulfate-methane transition zones in marine sediments.

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1 Introduction

Among volatile organic sulfur compounds, the methylated sulfides – dimethyl sulfide (DMS) and, to a lesser extent, methanethiol (MT, or methyl mercaptan) – are the most abundant components. The biogeochemical processes involving methylated sulfides in ocean surface waters have received particular attention because of the connection between DMS and climate (Charlson et al., 1987). In anoxic environments, DMS and MT link carbon and sulfur cycles in diverse ways. In contrast to complex organic sulfur compounds formed during early diagenesis that are refractory to biodegradation (Ferdelman et al., 1991), DMS and MT remain reactive and available for microbial processes. Their role as intermediates during remineralization of organic matter has been elaborated in earlier studies and is briefly summarized below and in Fig. 1. Decomposition of S-methyl compounds (compounds with methyl groups bonding to a sulfur atom) such as dimethylsulfoniopropionate (DMSP) and methionine initially yields DMS or MT (Kiene et al., 1990), both of which can be further catabolized by microbes that respire anaerobically. Isolated microorganisms that are

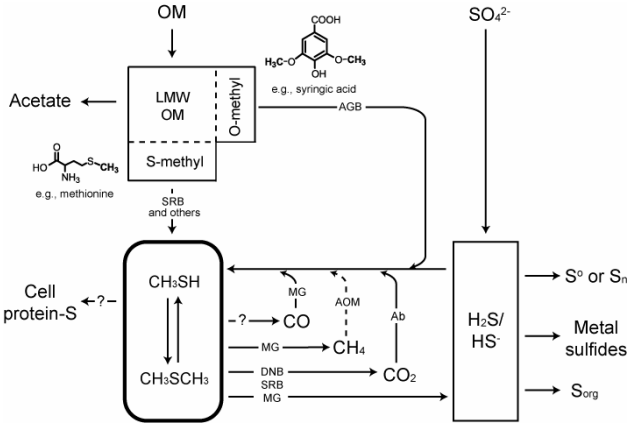

Fig. 1. A schematic diagram summarizing geochemical processes linked by methylated sulfides in anoxic environments. Arrows with dashed line: hypothesized processes; arrows with a question mark: processes that have not been examined. See text for detailed discussion. The structures of syringic acid and methionine are also shown as examples of compounds with O- and S-methyl groups, respectively. Abbreviations: Ab, abiotic process; AGB, acetogenic bacteria; AOM, anaerobic oxidation of methane; DNB, denitrifying bacteria; LMW, low molecular weight; MG, methanogens; OM, organic matter; S_{org}: sulfurized OM formed during early diagenesis; SRB, sulfate reducing bacteria.

known to oxidize methylated sulfides include denitrifying bacteria (Visscher and Taylor, 1993), sulfate reducing bacteria (Tanimoto and Bak, 1994) and methanogens (Lomans et al., 1999a). During degradation of DMS, MT usually accumulates transiently as an intermediate (Lomans et al., 1999b). Whether methylated sulfides can be used to synthesize protein-sulfur during anabolism in anoxic environments is not clear, but evidence exists that pelagic marine bacterioplankton preferentially assimilate methylated sulfides over sulfate or hydrogen sulfide (Kiene et al., 1999).

In addition to processes whereby methylated sulfides are produced from organic sulfur compounds, other processes exist in which methylated sulfides are derived by linking organic or inorganic carbon to H_2S/HS^- (hydrogen sulfide hereafter), either biologically and/or abiotically (Fig. 1). First, O-methyl groups (methyl groups bonding to an oxygen atom) can be transferred microbially to hydrogen sulfide to form MT, with additional methylation under certain circumstances yielding DMS (Lomans et al., 2002). Known O-methyl donors include methanol (van Leerdam et al., 2006) and methoxylated aromatic compounds such as lignin monomers (Lomans et al., 2002). This process is proposed to be the main mechanism contributing to methylated sulfides in DMSP-limited freshwater sediments, as evidenced by the strong correlation between concentrations of methylated sulfides and hydrogen sulfide in lake sediment (Lomans et al., 1997). Second, based on the study of trace methane oxidation of *Methanosarcina acetivorans*, Moran et al. (2007) hypothesized that during anaerobic oxidation of methane (AOM), methane is converted to methylated sulfides, which are subsequently oxidized by sulfate reducers. It is currently not known whether and exactly how such coupling proceeds in AOM organisms. Third, conversion of carbon monoxide (CO) to MT and DMS has also been observed in *M. acetivorans* (Moran et al., 2008), which represents the first finding of microbially mediated incorporation of inorganic carbon into methylated sulfides. The process probably proceeds via stepwise reduction of CO as coenzyme-bound intermediates to form a methyl group and could be coupled to energy conservation via a chemiosmotic mechanism. However, this process has not yet been studied using environmental samples, and the lack of knowledge on CO abundance in aquatic environments hampers evaluation of the significance of this process in natural settings. Last, small alkyl-S molecules, except DMS, can be abiotically synthesized from carbon dioxide (CO_2) and hydrogen sulfide in the presence of iron monosulfide under anoxic conditions (Heinen and Lauwers, 1996). This process is accelerated at temperatures higher than 50 °C. The abiotically synthesized alkylated sulfides are considered as building blocks for larger organic molecules in the primitive earth (Huber and Wächtershäuser, 1997). Jointly, these biological and abiotic reactions compete for hydrogen sulfide with other reactions such as the formation of elemental sulfur, polysulfur, metal sulfides and complex organic sulfur compounds.

The finding of formation of methyl sulfides from CO by *M. acetivorans* raises the question whether CO_2 (or bicarbonate at neutral pH), the most abundant dissolved inorganic carbon species in natural waters, can be converted to MT and DMS by microbial activity. Although CO_2 is not a growth substrate of *M. acetivorans*, it is produced in vivo via oxidation of CO and then introduced into the reductive steps by methanofuran (Oelgeschläger and Rother, 2008), a coenzyme shared by autotrophic methanogens. In anoxic environments, reduction of CO_2 to a methyl group is mostly coupled to hydrogenotrophy, such as autotrophic methanogenesis and acetogenesis. Previous studies of the effect of molecular hydrogen (H_2) on methylated sulfides have yielded inconclusive results. Lomans et al. (1999b) found that after prolonged incubation of anoxic lake sediment under H_2, degradation of methylated sulfides decreased dramatically. In contrast, the addition of H_2 did not affect thiol methylation potential in wetland sediments (Stets et al., 2004).

In an experiment in which we studied hydrogenotrophic reactions as a function of temperature in the anoxic sediment of the eutrophic Lake Plußsee, we detected DMS production after addition of H_2 and bicarbonate at 55 °C. This observation points to the potential presence of a hitherto unrecognized pathway of DMS formation that may be relevant to certain ecosystems functioning at higher temperatures. Our study tested whether this reaction is in fact mediated biologically and further examined the link among CO_2, H_2, hydrogen sulfide, and methylated sulfides. We performed

experiments to characterize the mechanism and to identify the microbial group responsible for the production of methylated sulfides in sediment slurry incubations. The effect of H_2 and bicarbonate on methylated sulfides, methane and acetate was examined, followed by tests aimed at verifying whether the observed DMS formation is related to methyl transfer during O-demethylation from organic substrates. We used a specific inhibitor to investigate the involved enzymatic pathway, which was subsequently supported by a study of the natural carbon isotopic abundance in methylated sulfides. The flow of C- and S-atoms into MT and DMS was further tracked by isotope labeling using ^{13}C- and ^{35}S-labeled compounds.

2 Materials and methods

2.1 Study site and sampling

Lake Plußsee (54°10′ N, 10°23′ E) is a well-studied eutrophic lake located in northern Germany. It has a stable thermal stratification in summer and regularly occurring anoxia in the hypolimnion, leading to high hydrogen sulfide concentrations in the bottom water (up to 50 µmol L^{-1}; Eller et al., 2005). Sediment samples used for this study were collected in July 2006 from the deepest part of the lake, i.e., at 28 m using a small multicorer from a rowboat. After sampling, the upper 15-cm portion of triplicate sediment cores was transferred into an air-tight bottle without gas headspace and stored in the dark at 4 °C for three months before being used for incubation experiments.

2.2 Slurry preparation

Sediment slurries were prepared anoxically by homogenizing approximately one volume of sediment with one volume of sterilized distilled water. After autoclaving, the distilled water was sparged with nitrogen (N_2) for at least one hour to remove dissolved oxygen. $NaHCO_3$ was added as buffer after sparging (final concentration = 1 to 3 mmol L^{-1}) and $Na_2S{\cdot}9H_2O$ as reducing agent (final concentration = 50 µmol L^{-1}). Aliquots of 8 or 10 mL of sediment slurry were dispensed into 16 mL Hungate tubes and sealed with butyl rubber stoppers. The tubes were evacuated three times and flushed with N_2 or H_2, and were finally pressurized to 200 kPa. All the tubes were incubated at 55 °C in the dark without shaking.

2.3 Substrate and inhibitor amendments

Substrate and inhibitor concentrations are reported for a liquid phase that is ca. 95% (w/w) of the sediment slurry. $NaHCO_3$ was added to 10 mmol L^{-1} from a CO_2-stabilized stock solution. Other tested substrates (reported in final concentrations) include: $Na_2S{\cdot}9H_2O$, 200 µmol L^{-1} MT, 50 µmol L^{-1}, methanol, 800 µmol L^{-1}; syringic acid, 100 µmol L^{-1}. 2-Bromoethanesulfonic acid (BES, 20 mmol L^{-1}) was applied to inhibit methanogenesis.

2.4 Stable isotope labeling

$NaH^{13}CO_3$ was spiked in the middle of an experiment, which was initiated by addition of both H_2 and 10 mmol L^{-1} $NaHCO_3$ at time zero. Assuming that the consumption kinetics of bicarbonate was identical to that of the parallel incubations with substrate amendments, we added 80 µmol L^{-1} $NaH^{13}CO_3$ (99.9% ^{13}C) to achieve a final ^{13}C abundance of ∼2% of the estimated bicarbonate concentration remaining at this time point (∼4 mmol L^{-1}). The exact ^{13}C content of the spiked $NaHCO_3$ pool was not confirmed by stable carbon isotope analysis.

Before stable carbon isotopic analysis of DMS and MT, Hungate tubes were stored after incubation for one month at −20 °C. To improve the transfer of DMS and MT vapors into the headspace, the tubes were heated to 60 °C for 20 min prior to analysis.

2.5 Radioisotope labeling

In the labeling experiment with $H_2^{35}S$, $Na_2S{\cdot}9H_2O$ was not added to the sterilized and purged distilled water in order to maintain sufficient specific activity of dissolved inorganic sulfide. Instead, the distilled water was blended with the redox indicator resazurin (final concentration = 1 mg L^{-1}) and reduced slowly with freshly-prepared sodium dithionite solution until the pink color of the liquid indicative for oxic conditions had cleared out completely. Aliquots of sediment slurry of 25 mL were poured through a funnel into 120 mL serum vials and amended with H_2 and 10 mmol L^{-1} $NaHCO_3$. Autoclaved slurries (120 °C, 25 min) were used as control to account for non-biological reaction and/or processing artifacts.

Radioactive sulfide was produced biologically and purified to remove S-bearing byproducts (T. Holler, unpublished data). The specific activity was 50.9 MBq $mmol^{-1}$ in the form of $Zn^{35}S$. Each serum vial was supplemented with $H_2^{35}S$ gas generated by acidifying a defined volume of $Zn^{35}S$ suspension (equivalent to 59.5 kBq). The injected $H_2^{35}S$ should have resulted in a final hydrogen sulfide concentration of 37 µmol L^{-1} ignoring partitioning into solid S phases.

To allow for equilibration of $H_2^{35}S$ in the serum vials, the time-zero sampling was performed after two hours of incubation. The clear supernatant of the sediment slurry was collected with a plastic syringe fitted with a hypodermic needle, filtered (0.2 µm), and the filtrate was injected directly into a 5% zinc acetate solution for determination of radioactivity in the dissolved fraction and concentration of hydrogen sulfide. The samples for determining hydrogen sulfide concentration were stored at −20 °C until analysis. The remaining sediment slurry was treated with 20% of zinc acetate and 1 mmol L^{-1} NaOH and left for two hours at 4 °C in order to trap H_2S, MT and carbon disulfide vapors (Adewuyl and Carmichael, 1987) into the liquid or solid phase. We interpreted the remaining volatile radioactivity to represent

DMS, which was trapped using the method slightly modified from Kiene and Linn (2000). The original butyl stopper on the serum vial was exchanged for a butyl stopper that had an Eppendorf centrifuge vial (1.5 mL) attached to it. Inside the Eppendorf vial was a strip of Gelman AR glass fiber filter treated with freshly prepared 3% H_2O_2 solution. The serum vials were placed in the dark at room temperature and the sediment slurries were stirred for >6 h. The traps were then removed, and the strips placed in 5 mL scintillation vials with scintillation fluid for determination of ^{35}S-radioactivity. Tests with 50 µmol L^{-1} DMS showed that after trapping, DMS decreased to a level below the detection limit of the flame ionization detector. ^{35}S-DMS was not available to determine the exact trapping efficiency, but the trapping efficiency for $H_2^{35}S$ in bicarbonate-buffered solution was higher than 90%. The remaining sediment slurries were subjected to a two-step cold distillation (Fossing and Jørgensen, 1989) to investigate the distribution of radioactivity in acid volatile sulfide (AVS: H_2S+FeS) and chromium reducible sulfur (CRS: S^0+FeS_2). *N*, *N*-dimethylformamide was applied in the second step to improve the yield of elemental sulfur (Kallmeyer et al., 2004).

Assuming that only the dissolved fraction could be responsible for formation of methylated sulfides, we calculated the average production rate of DMS derived from doubly methylated hydrogen sulfide ($\mathrm{Rate}_{\Sigma H_2S+2Me}$) during a labeling period using the following equation

$$\mathrm{Rate}_{\Sigma H_2S+2Me} = \frac{a_{\mathrm{trap}}}{a_{\mathrm{dis}}} \times \{H_2S\} \times \frac{1}{t}. \quad (1)$$

Here $\mathrm{Rate}_{\Sigma H_2S+2Me}$ is expressed as µmol (L of slurry)$^{-1}$ d^{-1}. a_{trap} is the radioactivity of the trapped pool, a_{dis} is the activity in the dissolved pool of the sediment slurry, $\{H_2S\}$ is the concentration of hydrogen sulfide per volume of sediment slurry (µmol (L of slurry)$^{-1}$), and t is the number of days during which the reaction was run. Since $H_2^{35}S$ is likely to be oxidized into aqueous sulfur oxyanions even under anoxic conditions (Elsgaard and Jørgensen, 1992), a_{dis} can be an overestimation of the $H_2^{35}S$ pool, leading to underestimated values of $\mathrm{Rate}_{\Sigma H_2S+2Me}$.

2.6 Analytical techniques

Concentrations of methane and methylated sulfides were determined by headspace analysis. Care was taken to maintain the incubation temperature during gas sampling. An aliquot of 100 µL gas was taken from a headspace of 6 to 8 mL using a Hamilton gas-tight syringe for on-column injection via a programmable temperature vaporizing inlet. A gas chromatograph (Trace GC Ultra, ThermoFinnigan) equipped with a CP-PoraBOND Q (Varian Inc.) column and a flame ionization detector was used to quantify the compounds. The column temperature was programmed from 60 °C (1 min isothermal) to 240 °C (2 min isothermal) at a rate of 40 °C min^{-1}. The distribution coefficients for DMS and MT at 55 °C are 6.9 and 4.3, respectively (Przyjazny et al., 1983). Calibration was made with standards prepared anoxically from chemicals. The limits of detection (LOD) were 0.4 µmol L^{-1} for MT and 0.5 µmol L^{-1} for DMS under the described analytical conditions.

For carbon isotope analysis, the same model of GC was coupled to a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer via a Finnigan combustion interface-III. One milliliter of gas was injected into the split/splitless inlet. A column and a temperature program identical to those described above were used. Values of $\delta^{13}C$ relative to that for Vienna-PeeDee Belemnite are defined by the equation $\delta^{13}C$ (‰) = $(R_{\mathrm{sample}}/R_{\mathrm{standard}} - 1) \times 1000$ with $R = {}^{13}C/{}^{12}C$ and $R_{\mathrm{standard}} = 0.0112372 \pm 2.9 \times 10^{-6}$. Internal precision of $\delta^{13}C$ was better than ± 0.1‰ (one standard deviation). We used two types of standards to evaluate the isotopic accuracies of methylated sulfides at low concentrations: (1) gaseous standards were prepared by injecting 5 to 550 µL of pure standards into helium-flushed, water-free headspace vials that were allowed to equilibrate at 60 °C. Isotopic values of the gaseous standards were not influenced by partition of methylated sulfides into aqueous solution and were taken as "real" values. (2) Solution standards of different concentrations were prepared under a helium headspace with a defined volume of water. By comparing the isotopic values of the solution standards with those of the gaseous standards, we found that at concentrations lower than 5 µmol L^{-1}, there was a positive shift for MT (up to 4.5‰) and a negative shift for DMS (up to 2.5‰). Since a strict relationship between the correction factor and concentration was not established, we did not correct the $\delta^{13}C$ values of MT, which had concentrations lower than the threshold during the whole course of the incubation experiments.

Radioactivity was determined by liquid scintillation counting (Packard 2500 TR) with a counting window of 4 to 167 keV without luminescence correction. The scintillation cocktail Lumasafe Plus (Lumac BV, Holland) was mixed with the zinc acetate-fixed dissolved fraction and the glass fiber strips. The counting efficiency was higher than 95%. Counting time was 10 min for all samples. The concentration of hydrogen sulfide was determined colorimetrically by the methylene blue method (Cline, 1969).

For acetate analysis, sediment slurries in Hungate tubes were centrifuged at 800 × g for 10 min, and 1 mL supernatant was removed with a plastic syringe fitted with a 21-gauge needle and filtered through a 0.45-µm Rotilabo Teflon syringe filter. The filtrates were stored at −20 °C until analysis. Acetate was measured using a high performance liquid chromatograph equipped with a Nucleogel Column (Machery-Nagel Inc.) and a photodiode array detector. Calibration curves were generated using standards prepared gravimetrically from sodium acetate. The detection limit for acetate was 10 to 15 µmol L^{-1}.

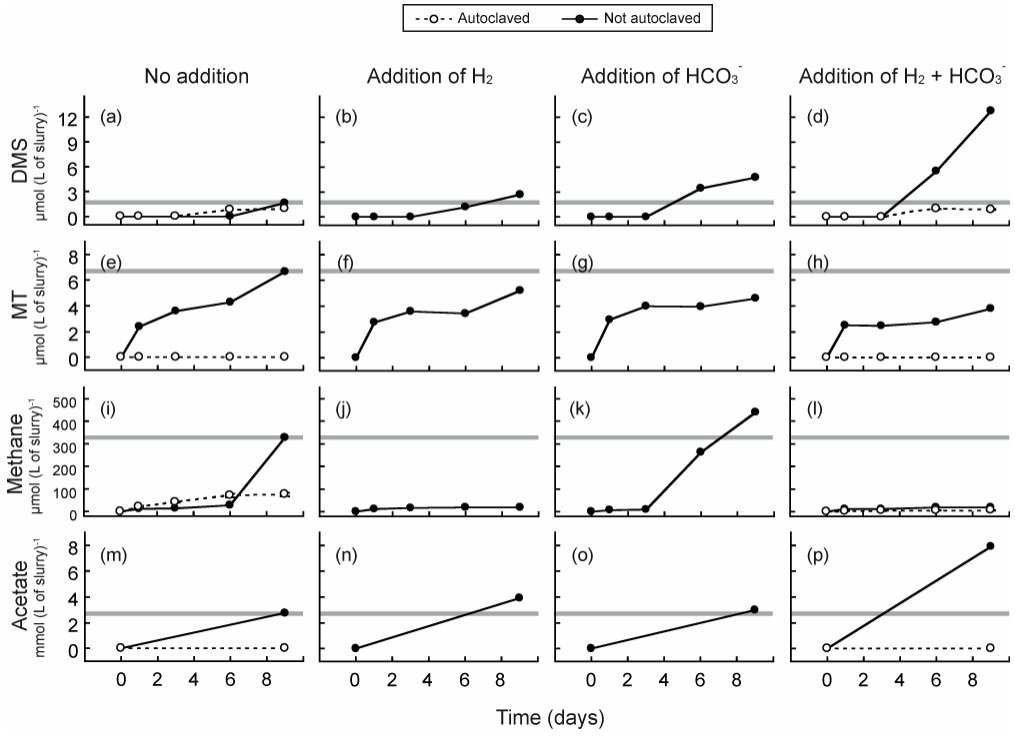

Fig. 2. Effects of H_2 and bicarbonate on the formation of dimethyl sulfide (DMS), methanethiol (MT), methane, and acetate in the sediment slurries of Lake Plußsee. Gray lines mark the final concentration of each compound in parallel with the concentration when no substrate was added. Data are from a single sample tube but the pattern is reproducible in similar incubations.

2.7 Statistical analysis

The LOD of instrumental methods was assessed statistically by the equation LOD$= Y_B + 3\times S_{y/x}$, where Y_B represents the signal of instrumental background and was estimated by the intercept of a calibration curve, and $S_{y/x}$ stands for the deviation of Y values (see Miller and Miller, 2005, for detailed description). The effect of individual treatments was tested using a one- or two-tailed t test with α set at 0.05.

3 Results

3.1 Addition of substrates

In the first experiment, we examined the effects of H_2 and bicarbonate on the dynamics of DMS and MT formation at 55 °C. We also monitored the concentrations of methane and acetate, the major carbon pools whose formation can potentially compete for H_2 and bicarbonate in this system. Compared to the control without any addition (Fig. 2a), there was no stimulation of DMS accumulation when H_2 was added as the single substrate (Fig. 2b). However, addition of bicarbonate resulted in an increase of DMS (Fig. 2c), and simultaneous addition of both H_2 and bicarbonate further enhanced DMS accumulation (Fig. 2d). The average accumulation rate was 2.1 μmol DMS (L of slurry)$^{-1}$ d^{-1} between days 3 and 9. Subsequent tests with the same treatment yielded a range of maximal accumulation rates from 1.3 to 2.4 μmol DMS (L of slurry)$^{-1}$ d^{-1}. Compared to the accumulation of DMS, MT showed the opposite trend. After nine days of incubation the final MT concentration was highest in the control without any substrate addition (Fig. 2e) and lowest when both H_2 and bicarbonate were added (Fig. 2h). However, there was no apparent 1:1 stoichiometric ratio for the relationship between MT and DMS. In autoclaved dead controls, MT concentrations were below LOD (Fig. 2e, h), and DMS became detectable only after six days of incubation in the presence of H_2 and bicarbonate, but with concentrations being significantly lower than in the active sediment (Fig. 2d). Methane accumulation was suppressed by addition of H_2 (Fig. 2j, l) but slightly enhanced by amendment with bicarbonate (Fig. 2k). The acetate concentrations at time zero in the active and autoclaved sediments were both below 10 μmol L^{-1}. The effect of H_2 and bicarbonate when added separately on acetate formation was minor and negligible, respectively (Fig. 2n, o). In combination, H_2 and bicarbonate resulted in a 2.7-fold increase of acetate (Fig. 2p). Note that the concentration of acetate was much higher than DMS, MT and methane by one to three orders of magnitude at the end of incubation. Although addition of bicarbonate alone already enhanced DMS formation (Fig. 2c), in the following

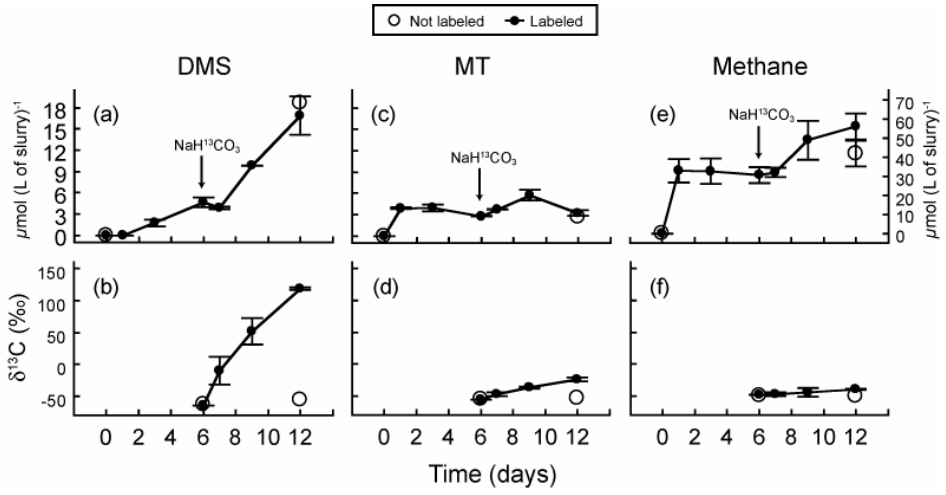

Fig. 3. Concentrations and stable carbon isotopic values of dimethyl sulfide (DMS), methanethiol (MT) and methane in sediment slurries under non-labeling and labeling conditions. $NaH^{13}CO_3$ was added on day 6 of the incubation. Error bars represent ± 1 standard error of duplicate tubes.

experiments we added bicarbonate together with H_2 in order to obtain higher yields of DMS and to facilitate labeling studies.

To test whether the observed DMS formation was related to the activity of O-demethylation, we examined the effects of sodium sulfide, MT, methanol and syringic acid under either a N_2 or a H_2 headspace (Table 1). Experimental concentrations of each of these compounds were in the range that stimulated production of DMS and MT in previous studies of freshwater sediment (Lomans et al., 1997; Stets et al., 2004). The results indicated that the presence of H_2 and bicarbonate was the major factor leading to increased DMS formation; none of the other four substrates significantly increased DMS formation after 12 days of incubation. The effects of sodium sulfide, methanol or syringic acid on MT formation were either minor or insignificant. The added MT decreased over time and reached 30% of the initial values after 12 days of incubation. An autoclaved control confirmed that the disappearance of MT was driven by abiological processes (data not shown).

3.2 Inhibition tests

Reduction of bicarbonate with H_2 in anoxic sediment is commonly associated with activities of either acetogens or methanogens. Acetogenesis was very active in our incubated sediment but could not be specifically inhibited to examine the corresponding effects on formation of methylated sulfides. However, the role of methanogens can be evaluated by BES, an inhibitor for methanogenesis (Chidthaisong and Conrad, 2000). Relative to the positive control (Table 2), DMS formation was 60% inhibited by 20 mmol L^{-1} BES ($P = 0.024$, one-tailed t-test), whereas MT accumulation increased slightly ($P = 0.044$, one-tailed t-test). Since methane production was already suppressed in the presence of high H_2 partial pressure (Fig. 2i–l), BES did not significantly lower the methane level ($P = 0.182$, one-tailed t-test) in this experiment in which 200 kPa H_2 was applied. BES had no effect on the formation of acetate.

3.3 Stable carbon isotopic compositions under non-labeling and ^{13}C labeling conditions

The stable carbon isotopic compositions of methylated sulfides provide additional evidence on the enzymatic pathways involved in their formation. Under the non-labeling condition, DMS was ^{13}C-depleted with δ-values as negative as -62‰ on day 6, and increased slightly to -56‰ at the end of incubation (Fig. 3b). The analyzed δ^{13}C values of MT were -55‰ and -53‰ on days 6 and 12 (Fig. 3d), respectively, but the actual δ^{13}C values of MT should have been more negative, since the MT concentrations were below the threshold value of 5 μmol L^{-1} (cf. Materials and Methods). Methane production was low and the δ^{13}C values remained around -50‰ during the course of incubation (Fig. 3f).

In parallel, we performed a ^{13}C-labeling experiment with $NaH^{13}CO_3$ to investigate the incorporation of bicarbonate into DMS. If bicarbonate was used directly to form one of the methyl groups in DMS rather than exerting an indirect effect that favored DMS formation (e.g., via buffering the aqueous solution), we would expect rapid labeling after $NaH^{13}CO_3$ was added. The experimental results showed that DMS became enriched in ^{13}C by nearly 40‰ already one day after label addition. The ^{13}C enrichment of DMS increased to 181‰ after 12 days with DMS having a final δ^{13}C-value of +119‰ (Fig. 3b). Assuming no consumption of DMS, we

Table 1. Concentrations of DMS and MT after 12 days of incubation with inorganic and organic substrates. Results are presented as means and standard errors from duplicate tubes.

Treatment	Headspace	DMS µmol (L of slurry)$^{-1}$	MT µmol (L of slurry)$^{-1}$
No addition	N_2	3.7±1.7	3.0±1.7
Methanol, 800 µmol L^{-1}	N_2	1.5±0.1	2.6±0.2
Syringic acid, 100 µmol L^{-1}	N_2	1.1±0.4	2.7±0.1
No addition	H_2 (+HCO_3^-)	11.1±0.3	2.8±0.1
Sodium sulfide, 200 µmol L^{-1}	H_2 (+HCO_3^-)	9.6±1.0	4.4±1.6
Methanethiol, 50 µmol L^{-1}	H_2 (+HCO_3^-)	9.1±0.6	14.8±4.2
Methanol, 800 µmol L^{-1}	H_2 (+HCO_3^-)	3.7±0.4	4.3±0.1
Syringic acid, 100 µmol L^{-1}	H_2 (+HCO_3^-)	6.8±0.5	3.8±0.4

Table 2. Effects of 20 mmol L^{-1} 2-bromoethanesulfonate (BES) on formation of dimethyl sulfide, methanethiol, methane and acetate after 12 days of incubation. The sediments slurries were added with 200 kPa H_2 and 10 mmol L^{-1} bicarbonate. Results are presented as means and standard errors from duplicate tubes.

Compounds	Positive control	+ BES
Dimethyl sulfide, µmol (L of slurry)$^{-1}$	11.7±0.3	4.2±2.2
Methanethiol, µmol (L of slurry)$^{-1}$	3.1±0.1	4.7±0.8
Methane, µmol (L of slurry)$^{-1}$	48.2±16.9	32.8±8.2
Acetate, mmol (L of slurry)$^{-1}$	6.4±0.3	6.7±0.4

calculated the ^{13}C abundance of the newly fixed carbon into DMS based on the measured size and δ-values of the DMS pool at different time points. The results showed that (1) if both methyl groups were derived from bicarbonate, the average ^{13}C abundance of the newly fixed carbon was 1.3% during the period after labeling addition, and (2) if only one methyl group was involved, the average ^{13}C abundance of the newly fixed carbon would raise to 2.6%. These estimates are consistent with at least one methyl group being derived from the $NaH^{13}CO_3$ spiked DIC pool (∼2% ^{13}C abundance).

MT showed a much slower but significant ($P = 0.002$, one-tailed t-test) enrichment, and reached −24‰ by the end of the experiment. ^{13}C-enrichment of methane was merely 9‰ (Fig. 3f), a finding that agrees with our observation that methanogenesis via reduction of CO_2 was low.

3.4 Labeling with $H_2^{35}S$

The result of the ^{13}C-labeling experiment indicated incorporation of bicarbonate with MT, but the source of MT remains unclear. The effects of sodium sulfide, methanol and syringic acid on MT formation, if any, might have been too small to be resolved by changes in MT concentration (Table 1), and the slight ^{13}C enrichment of MT in the labeling experiment is not conclusive proof for methylation of hydrogen sulfide by reduced bicarbonate. With the radiotracer experiment with $H_2^{35}S$, we sought to better characterize the reaction of formation of DMS – via the intermediate MT – from hydrogen sulfide. The MT pool targeted in this experiment may derive from the reactions of hydrogen sulfide with inorganic carbon or non-S-methyl compounds such as O-methyl pools (Fig. 1).

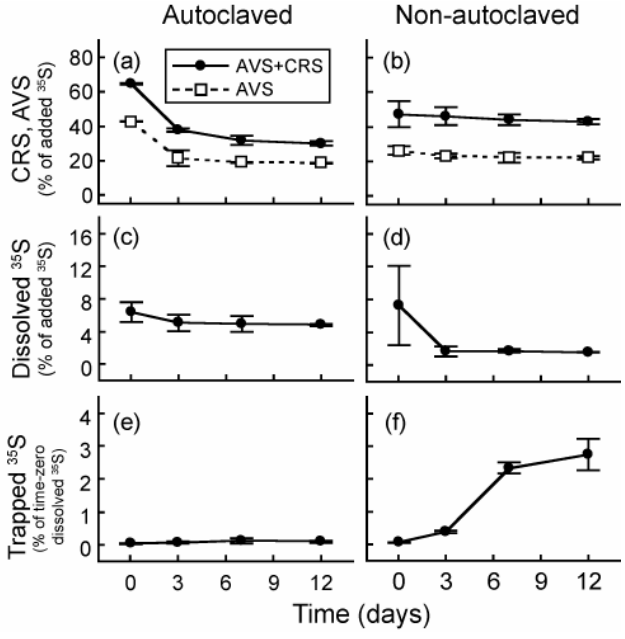

Fig. 4. Distribution of radioactivity in the sediment slurries of Lake Plußsee during incubation with H_2, bicarbonate and $H_2^{35}S$. Time-zero values were measured two hours after addition of radiotracer. AVS: acid volatile sulfide (H_2S + FeS); CRS: chromium reducible sulfur (S^0 + FeS_2). The dissolved ^{35}S was measured from filtered slurry supernatant. The trapped ^{35}S was interpreted to represent labeled dimethyl sulfide. Error bars represent ± 1 standard error of duplicate bottles.

At time zero (after two hours of incubation), the dissolved fraction contained less than 10% of total added radioactivity (Fig. 4c, d). A significant amount of $H_2^{35}S$ entered the solid phase AVS (most likely FeS) and CRS fractions via isotope exchange (Figs. 4a, 4b; Fossing et al., 1992). The trapped radioactivity in non-autoclaved samples was much higher than in the autoclaved control (Fig. 4e, f; $P = 0.008$, one-tailed t-test), suggesting that DMS formed biologically. In the non-autoclaved samples, the trapped radioactivity increased most rapidly between days 3 and 7 and finally reached 2.5% of the dissolved radioactivity. We obtained an average $\text{Rate}_{\Sigma H_2S+2Me}$ of 0.06 µmol (L of slurry)$^{-1}$ d^{-1} over the whole period of incubation and a higher rate of 0.1 µmol (L of slurry)$^{-1}$ d^{-1} between days 3 and 7. The rates are in the range of reported sulfide-dependent MT production rates in freshwater sediment at low hydrogen sulfide concentrations (Lomans et al., 1997), but represent <10% of the ambient DMS production rate determined by the kinetics of total DMS formation in this study.

4 Discussion

Our experimental data showed that both DMS and MT were of biogenic origin and depleted in ^{13}C. However, both compounds responded differently to addition of substrates and inhibitors, and exhibited different patterns of ^{13}C incorporation. Therefore, DMS and MT formation will be discussed separately, followed by a note on the implication of these processes for natural anoxic environments.

4.1 Microbial DMS formation

Our observation indicates that dissolved inorganic carbon in anoxic aquatic environments can be incorporated into DMS: H_2 alone did not stimulate DMS formation while bicarbonate alone did, and both together stimulated DMS even more; MT accumulated slightly when DMS production was hindered by the lack of H_2 and bicarbonate or by the addition of BES; ^{13}C-labeled bicarbonate was incorporated much more rapidly into DMS than into MT. These facts suggest the following reaction:

$$HCO_3^- + H^+ + 3H_2 + CH_3SH \rightarrow CH_3SCH_3 + 3H_2O \qquad (2)$$

$$\Delta G^{\circ\prime} = -159.8\,\text{kJ per reaction}$$

The standard-state free energy for the proposed reaction was calculated using thermodynamic data for aqueous species listed in Scholten et al. (2003). The production rate is consistent with the previously observed kinetics in other freshwater environments (Lomans et al., 1997; Stets et al., 2004). Addition of MT did not further stimulate DMS formation (Table 1), suggesting that this process is not limited by MT. Since addition of methanol or syringic acid failed to stimulate DMS production, this process is not related to O-demethylation.

DMS under the non-labeling condition was ^{13}C-depleted with δ-values as negative as −62‰ (Fig. 3b). According to Eq. (2), one of the methyl groups was from MT and the other from reduced bicarbonate. Since the measured $\delta^{13}C$ value of MT in experiments without label addition was around −54‰, a simple mixing model provides a rough estimate of −70‰ for the $\delta^{13}C$ value of the methyl group from reduced bicarbonate. Compared with the $\delta^{13}C$ value of the added $NaHCO_3$ (−2.2‰; data not shown), the strong ^{13}C-depletion of the methyl group is consistent with a kinetic isotopic effect typical for H_2/CO_2 metabolizers such as autotrophic methanogens (Whiticar, 1999) or acetogens (Gelwicks et al., 1989).

Suppression of DMS production by BES further suggests that methanogens are involved in DMS formation. Moran et al. (2008) previously discussed the enzymatic steps of *M. acetivorans* to produce methylated sulfides. We hypothesize that the same pathway is active with CO_2 rather than CO as the direct precursor (Fig. 5). Normally, coenzyme M (CoM-SH) receives the methyl group from N^5-methyl-H_4MPT:CoM-SH methyltransferase (Mtr) complex to form methyl-CoM, which releases methane by forming heterodisulfide with coenzyme B (CoB-SH) by the catalysis of methyl-CoM reductase (Mcr). Under the condition of DMS production, the methyl group is transferred from methyl-CoM to MT, a reaction that is the reverse of the CoM methylation step employed by DMS-consuming methanogens (Hedderich and Whitman, 2006). As Moran et al. (2008) pointed out, such a shortcut to regenerate CoM-SH is feasible, owing to the low energy barrier in the activation step of methanogenic DMS consumption by methylthiol:CoM methyltransferase (Mts) ($\Delta G^{\circ\prime} = 0.35$ kJ per reaction; Tallant et al., 2001). The overall energy yield for the methanogenic DMS production is theoretically lower than normal methanogenesis, as the H^+-pumping step of heterodisulfide reduction is bypassed and energy conservation is restricted to the Na^+-pumping Mtr complex (Hedderich and Whitman, 2006). From an energetic point of view, therefore, DMS might be considered as a by-product of inefficient methanogenesis.

DMS formation may also be a response to environmental stress. For instance, Moran et al. (2008) attributed the production of methylated sulfides by *M. acetivorans* to the high CO concentration (300 kPa) in their cultures. CO likely inhibits Mcr, causing CoM-SH to be sequestered and energy production stopped. Transferring the methyl group to hydrogen sulfide or MT can be a shortcut to regenerate CoM-SH while bypassing Mcr. In our incubation, the headspace CO concentrations were lower than 0.5 Pa (data not shown) and were unlikely to have a marked inhibitory effect. Nevertheless, the methane accumulation rates at 55 °C in Lake Plußsee sediment were indeed much lower by one to two orders of magnitude than rates at 27° and 40 °C (data not shown). This suggests that, while the mesophilic community was under high temperature stress, an activation of a

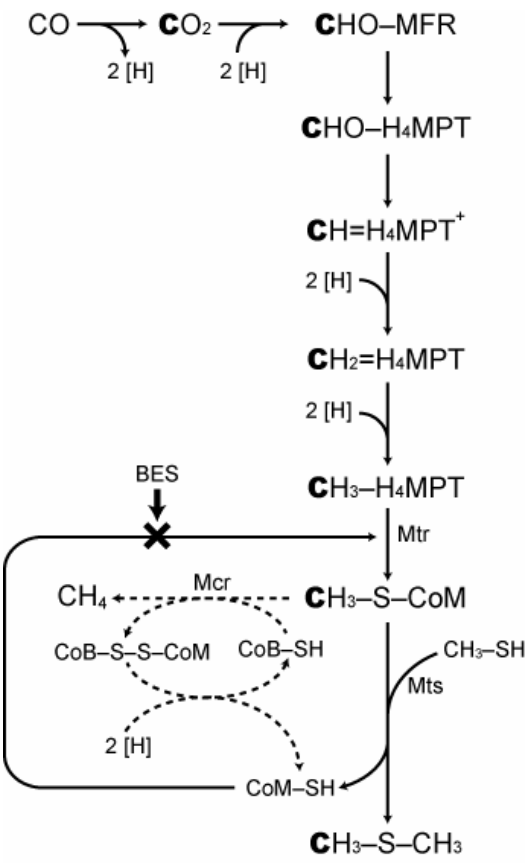

Fig. 5. A simplified scheme of methanogenic dimethyl sulfide (DMS) formation. The formation of DMS from carbon monoxide (CO) is reported in Moran et al. (2008). The carbon atoms highlighted in bold type refer to the process proposed in this study, i.e., formation of DMS from CO_2. The dashed arrows are pathways that are bypassed when DMS rather than methane is produced. The enzymatic step inhibited by 2-bromoethanesulfonic acid (BES) is marked with a cross. Abbreviations: MFR, methanofuran; H_4MPT, tetrahydromethanopterin; CoM-SH, coenzyme M; CH_3-S-CoM, methyl coenzyme M; CoB-SH, coenzyme B; Mtr, N^5-methyl-H_4MPT:CoM-SH methyltransferase; Mcr, methyl-CoM reductase; Mts, methylthiol:CoM methyltransferase.

thermophilic population of methanogens did not occur. The elevated temperature may have also improved the availability of substrates such as MT, CO_2 and H_2 via acceleration of organic matter breakdown, making the DMS-forming pathway thermodynamically feasible for methanogens. Nevertheless, growth by this mechanism remains to be determined, even if it is thermodynamically feasible.

We note that high H_2 partial pressure, which favored DMS formation when added together with bicarbonate, failed to stimulate methanogenesis during our incubation (Fig. 2). A similar lack of methanogenesis was also found in previous incubation experiments with Lake Plußsee sediments that used H_2 rich gas mixture (80:20 H_2/CO_2) but lower temperatures (4° and 25 °C) (Nüsslein and Conrad, 2000; Heuer et al., 2010), while incubation under 4% H_2 headspace enhanced methanogenic activity (Nüsslein and Conrad, 2000). The reasons why methanogenesis is stimulated successfully with addition of low H_2 concentrations but not with high concentrations remain to be explored. Although our data do not provide evidence that would help to explain this phenomenon, we demonstrated that under the condition of high H_2 partial pressure and elevated temperature, a fraction of H_2 and CO_2 still enter the methanogenic pathway but with DMS as the final product.

Sequences retrieved from fresh Lake Plußsee sediment using the *mcr*A-gene primer showed the greatest homology with members of the *Methanomicrobiales* (Heuer et al., 2010), which are strict H_2/CO_2 metabolizers and are unlikely to possess Mts required for the transfer of methylated sulfides (Fig. 5). Therefore, it is likely that the observed DMS formation was performed by a minor population of methanogens that, like some members of the *Methanosarcinales*, are capable of using both H_2/CO_2 and methylated sulfides (Kendal and Boone, 2006).

4.2 Microbial MT formation

Autoclaved controls confirmed that MT in our system is also of biological origin, and the natural δ^{13}C-values are very negative (<-50‰). In contrast to some previous studies suggesting that methyl transfer from methoxylated aromatic compounds to hydrogen sulfide is a main source of MT in freshwater sediment (Lomans et al., 1997, 2002), addition of sodium sulfide, methanol and syringic acid to our microcosm failed to stimulate MT formation. By labeling with $H_2^{35}S$ we were able to quantify the inventory of methylated hydrogen sulfide that finally entered the DMS pool. The minor supply of MT derived directly from hydrogen sulfide does not support the accumulation rate of DMS that is supposed to derive from MT (Eq. (2)). Taken together, our experiments suggest that the DMS production proceeded via MT derived mostly from organic-S pools. However, from these experiments we can not yet identify the mechanism of MT formation.

Nevertheless, the carbon isotopic composition of MT at natural abundance levels is intriguing and deserves further discussion. The main question is: How can we explain the ^{13}C depletion of MT that we observed under the non-labeling condition? Hydrogenotrophic bicarbonate reduction is usually considered the main process that generates the ^{13}C-depleted methyl group. If the slight enrichment of MT in the ^{13}C-labeling experiment reflects the signal of bicarbonate incorporation (Fig. 3), the contribution of bicarbonate reduction must be very minor, otherwise the small carbon pool of MT and its inferred rapid turnover should have allowed a pronounced labeling signal. Alternatively, demethylation of ^{13}C-labeled DMS may also explain the slight enrichment of MT if there was a tight coupling between production and consumption of MT. However, since a substantial fraction of

MT is lost to other reactions (cf. Sect. 3.1) there must have been some other major source. A second possible source of a ^{13}C-depleted methyl group would be methoxylated aromatic compounds: Keppler et al. (2004) reported that the methyl pool in lignin has δ^{13}C values as negative as −66‰. This possibility is nevertheless not supported by our results from the substrate tests and $H_2^{35}S$-labeling experiment. A last potential source that has received little attention but cannot be ruled out is the S-methyl pool of amino acids. MT accumulated rapidly after addition of methionine in the Plußsee sediment (data not shown), but we have no information on the pool size of free methionine and its endogenesis from enzymatic hydrolysis of macromolecules. According to a study on the carbon isotopic pattern in purine alkaloids in higher plants (Weilacher et al., 1996), the methyl pool in S-adenosylmethionine, a coenzyme with its methyl group derived from methionine, showed a moderate ^{13}C-depletion ($\delta^{13}C < -39$‰). If methionine has an identical carbon isotopic composition in its methyl group, it could be another source for a moderately ^{13}C-depleted methyl pool. Additionally, methionine and S-adenosylmethionine are involved in biosynthesis of many O- and S-methyl pools in organic matter, including DMSP and lignin monomers. Direct isotopic analysis of the methyl group in methionine will be essential to better constrain the propagation and distribution of δ^{13}C signatures of C1 compounds in nature, including methylated sulfides.

4.3 Implications for anoxic environments

Although our experimental temperature hinders implications for the local biogeochemistry of the lake, the incorporation of bicarbonate into DMS may be relevant to several anoxic environments. The fact that this process was more easily observed at elevated temperature in our incubations makes geothermally influenced subsurface sediments and hydrothermal vents a potential target for future studies on this topic. For example, as summarized in Fig. 1, abiotic synthesis of alkylated sulfides has been demonstrated in the laboratory and is considered relevant for hydrothermal environments. A recent study on in situ measurements further demonstrated considerable amounts of MT in a hydrothermal area (Reeves and Seewald, 2009). The supply of MT, the usually high chemical potential of H_2 and bicarbonate, and, possibly, elevated temperature, qualify hydrothermal systems as candidate ecosystems in which the DMS-forming process characterized in this study could take place.

However, the possibility to detect such a process at lower temperatures should not be excluded. We suggested that the high temperature during incubation facilitates this reaction by increasing the availability of substrates (MT, CO_2 and H_2) to methanogens. If this is true and elevated temperature is not a prerequisite, there are low-temperature environments with similar chemical conditions in the real world that deserve further examination. One setting is the sulfate-methane transition zone (SMTZ) in marine sediments associated with AOM activity (e.g., Hoehler et al., 2000). It is a particular zone where sulfate is exhausted and methane from underlying sediment is consumed. Additionally, this zone is often accompanied by peak hydrogen sulfide and bicarbonate concentrations. Laboratory experiments demonstrated that during the transition from sulfate reduction to methanogenesis, there is a decoupling of H_2 production and consumption and hence a temporary accumulation of H_2 (Hoehler et al., 1999). Isotopic evidence for acetogenesis via CO_2 reduction in an extended sediment interval just below the SMTZ at the Cascadia Margin is also consistent with elevated H_2 concentration in situ (Heuer et al., 2009). If there are sources of MT, e.g., transmethylation from lignin monomer to hydrogen sulfide, the SMTZ qualifies as an additional environment where DMS formation by methanogens is thermodynamically favorable.

5 Conclusions

In this study, we investigated the formation of DMS and MT in anoxic lake sediment at elevated temperature. On the one hand, we provided multiple lines of evidence for a novel microbial pathway of DMS production in anoxic lake sediment. This pathway connects DMS to bicarbonate and H_2 and is mediated by methanogens. Subsequent investigations will have to characterize the physiological conditions under which methanogens favor production of DMS and other methylated sulfides rather than methane, and to explore the environmental relevance of this novel pathway. On the other hand, our data could not identify the mechanism of MT formation despite various experimental attempts. This illustrates a more complicated biogeochemistry of MT, which will remain a great challenge for future research.

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