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Sulfate- and iron-dependent anaerobic methane oxidation occurring side-by-side in freshwater lake sediment

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Running head: Sulfate- and iron-AOM in lake sediment

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Abstract

Anaerobic oxidation of methane (AOM) is a potentially important methane sink in lake sediments, but the biogeochemistry and microbial ecology of this process are understudied. Potential electron acceptors for AOM include Fe(III) and sulfate, however, it is not clear to which extent low sulfate concentrations constrain the coupling of AOM to sulfate reduction, nor if Fe(III) reduction drives AOM directly or via a cryptic sulfur cycle. We investigated AOM pathways in the sediment of iron-rich Danish Lake Ørn through anoxic sediment slurry incubations with additions of $^{13}$C-labeled methane as a substrate, sulfate and Fe(III) as potential electron acceptors, and molybdate as an inhibitor of sulfate reduction. The experiments demonstrated the co-occurrence of sulfate- and iron-dependent modes of AOM, with the former supported by recycling of sulfate coupled to iron reduction. Quantitative PCR analysis demonstrated the abundance of archaea of the ANME-2d clade (Ca. Methanoperedenaceae) as likely drivers of AOM. Our study demonstrates that sulfate-dependent AOM can consume methane at sulfate concentrations typical for freshwater systems and emphasizes the importance of sulfur- and iron cycling in the regulation of methane emission from freshwater sediments.
Introduction

Methane is a common byproduct of anaerobic decomposition of organic matter, and a greenhouse gas with high global warming potential. Freshwater systems such as lakes, wetlands, and rivers account for approximately half of global methane emissions to the atmosphere (Saunois et al. 2020; Rosentreter et al. 2021). High input of organic matter and low sulfate concentrations facilitate methanogenesis in freshwater systems, and methane production in these environments is predicted to increase in response to global warming, eutrophication, and urbanization (Yvon-Durocher et al. 2014; Rosentreter et al. 2021). Yet, a high variability in emissions from, and a limited mechanistic understanding of methane cycling in different ecosystems, hamper our ability to make robust quantitative predictions (Rosentreter et al. 2021).

One potentially important aspect is the role of methane oxidation in attenuating methane fluxes from freshwater systems. It has been estimated that as much as 30–99% of the methane produced in anoxic lake sediments can be oxidized to CO₂ by methanotrophic microbes (Bastviken et al. 2008 and citations therein). Until recently, most studies focused on aerobic oxidation of methane as the most efficient and thermodynamically favorable process, readily occurring in oxic water or within the thin oxic surface sediment layer (e.g., Frenzel et al. 1990; Bastviken et al. 2002). However, anaerobic oxidation of methane (AOM) may constitute another potentially important methane sink in freshwater systems (Zehnder and Brock 1980; Norði et al. 2013; Deutzmann et al. 2014). Despite progress in understanding AOM in freshwater settings (Segarra et al., 2015; Bar-Or et al. 2017; Martinez-Cruz et al., 2018), many aspects of this process require further clarification. Here, key issues include the mechanisms, electron acceptors, and microbes involved in AOM.
AOM coupled to sulfate reduction is the main sink for methane in marine sediments where sulfate is abundant and typically penetrates to several meters depth (Egger et al. 2018). In a wide variety of marine environments, this process is carried out by anaerobic methanotrophic archaea (ANME) often found in consortia with sulfate-reducing bacterial partners (Boetius et al. 2000; Orphan et al. 2001; Knittel and Boetius 2009). In freshwater sediments, which are commonly depleted in sulfate (typically 10–500 µM in overlying water, ≤10 cm sediment penetration depth; Holmer and Storkholm 2001), nitrite- and nitrate-dependent AOM have been demonstrated in nitrogen-rich environments (Deutzmann et al. 2014; Norði and Thamdrup 2014), and the involvement of Fe(III) and Mn(IV) minerals in AOM has been proposed in lake sediments (Sivan et al. 2011; Norði et al. 2013; Su et al. 2020). However, it is difficult to discern whether AOM is coupled to Fe(III) or Mn(IV) reduction directly or indirectly, with the oxides acting through re-oxidation of reduced sulfur species back to sulfate (Weber et al. 2016). So far, studies in natural freshwater sediments indicate some role of the indirect mechanism, known as a cryptic sulfur cycle, though a direct interaction has not been ruled out (Weber et al. 2016; Su et al. 2020). A cryptic sulfur cycle has also been proposed to operate below the sulfate-methane transition zone in marine sediments (Holmkvist et al. 2011).

Investigation of microbes involved in AOM in freshwater sediments have so far identified members of the bacterial NC10 clade (‘Ca. Methylomirabilis sp.’) and the methanotrophic archaea lineage ANME-2d (recently classified as ‘Ca. Methanoperedenaceae’) as likely conveyors of the process (Haroon et al. 2013; Deutzmann et al. 2014; Weber et al. 2017). NC10 bacteria couple methane oxidation to nitrite reduction and appear limited to the nitrate/nitrite-containing zone near the sediment surface (Ettwig et al. 2010; Deutzmann et al. 2014). By contrast, ANME-2d archaea were shown to catalyze AOM coupled to reduction of nitrate
(Haroon et al. 2013), iron (Ettwig et al. 2016), and manganese (Ettwig et al. 2016; Leu et al. 2020) in enrichment cultures. These organisms were enriched from freshwater sediments (Raghoebarsing et al. 2006; Haroon et al. 2013) and have been found in a variety of freshwater settings (Schubert et al. 2011; Ding et al. 2015; Narrowe et al. 2017). The clade was specifically linked to AOM in the sulfate-methane transition zone of the iron-rich Lake Ørn by means of RNA stable isotope probing (Weber et al. 2017). Likewise, Su et al. (2020) identified ANME-2d archaea (‘Ca. Methanoperedens sp.’) co-occurring with sulfate-dependent AOM in the sediments of meromictic, sulfate-rich Lake Cadagno. Typical marine ANME lineages were not detected in either of these lakes (Weber et al. 2017; Su et al. 2020). As ‘Ca. Methanoperedens’ does not appear to have capacity for sulfate reduction (Haroon et al. 2013; Ettwig et al. 2016), sulfate-reducing bacteria of the Desulfobulbaceae family were hypothesized as candidates for syntrophy with ‘Ca. Methanoperedens’ based on their highly similar depth distribution (Su et al. 2020).

In the sediments of the northern temperate Lake Ørn, AOM coupled to iron and/or sulfur cycling attenuates up to 90% of the diffusive methane flux (Norði et al. 2013; Weber et al. 2016). Thus, AOM may potentially exert important controls on methane emissions from freshwater environments. Yet, as outlined above, we lack a mechanistic understanding of iron- and sulfur-based AOM in such systems. With the aim of untangling the pathways involved, we conducted sediment slurry incubations with Lake Ørn sediment, adding $^{13}$C-labeled methane, sulfate and Fe(III) as potential electron acceptors, and molybdate as an inhibitor of sulfate reduction, and compared the depth-distribution of ANME-2d archaea to the distribution of AOM rates. Our results indicate that AOM in Lake Ørn is both sulfate- and iron-dependent, and under sulfate-limiting conditions can be sustained not only through a cryptic sulfur cycle but also through the coupling to Fe(III) reduction alone.
Materials and methods

Study site and sampling

Lake Ørn is a small (0.42 km²), iron-rich lake located in central Jutland, Denmark. The mean annual temperature in this area is 8.5°C, and the mean annual precipitation is 825 mm (2006–2015, Danish Meteorological Institute, http://dmi.dk/vejrarkiv). With a mean depth of 4 m and a maximum depth of 10.5 m, Lake Ørn experiences seasonal anoxia beneath 5 m depth (Skovgaard 2004). The lake is mainly fed by the river Funder Å, which, in turn, is supplied by groundwater. Predominant groundwater input and low water residence time (18 d) result in a high and steady supply of iron (37 t year⁻¹ in 2003) to the lake (Skovgaard 2004).

Sediment cores were collected on four occasions, in April, May, June, and October 2019. The cores were retrieved with a hand-operated gravity corer from 4.5 m depth, transported to the laboratory within 3 h and stored at approximate in situ temperature in a tank filled with lake water, under continuous aeration. A number of the stored cores were subsequently used for preparation of experiment slurries (see “Sediment chemistry manipulation experiments” section). On each sampling occasion, two cores were processed within 24 h after sampling to obtain geochemical profiles. In April, oxygen profiles were measured in three additional cores, and one core was sliced to determine physical characteristics (density, water content, porosity).

Geochemical profiling

The cores were sliced at 1 cm intervals down to 20 cm depth in a glove bag filled with N₂. For analysis of CH₄, 1 ml of sediment was transferred with a cut-off syringe into a 3 ml glass vial (Exetainer) containing 1 ml of 2.5% (w/v) NaOH. The vial was then quickly sealed with a chlorobutyl septum cap and stored upside down for at least 24 h until analysis. Headspace CH₄
was analyzed on a gas chromatograph with the flame-ionization detector (Thermo Scientific, Trace 1300), and the concentration of CH₄ in the sediment was subsequently calculated using the ideal gas law and equilibrium solubility equation (Wiesenburg and Guinasso 1979). Sediment samples for iron analysis were frozen at −18°C. Solid phase iron was extracted with HCl (0.1 g sediment in 5 ml 0.5 M HCl for 1 h at 25°C) targeting poorly crystalline Fe(III) oxides as well as authigenic Fe(II) phases, but not crystalline iron oxides (Kostka and Luther, 1994). The concentrations of Fe(II) and Fe(III) were determined spectrophotometrically using the Ferrozine method (Stookey 1970; Thamdrup et al. 1994).

For porewater extraction, sediment was transferred to 50 ml polypropylene centrifuge tubes and centrifuged at 3200 × g for 10 min. The supernatant was filtered inside the glove bag through a N₂-flushed 0.2 μm cellulose acetate syringe filter. Filtered porewater was stored in plastic vials at −18°C. Sulfate, nitrite, and nitrate concentrations in porewater were determined with ion chromatography (Dionex ICS-1500; detection limit 2 μmol L⁻¹). In October, nitrate was additionally determined with chemiluminescence after reduction to NO (Braman and Hendrix 1989). For analysis of sulfide, 1 ml of filtered porewater was fixed with 20% zinc acetate and frozen at −18°C. Sulfide was quantified spectrophotometrically with the methylene blue method (Cline 1969).

Oxygen profiles were measured with an optode coupled to a FireStingO2 optical oxygen meter (PyroScience, GmbH) at 100 μm depth resolution. The sediment density was determined from the weight of 1 cm³ of sample from each 1 cm-thick interval. The water content was determined as weight loss after drying the sediment at 105°C for 24 h.

The depth distributions of CH₄ and SO₄²⁻ were used to calculate rates of net production and consumption assuming steady state and using the curve-fitting software PROFILE (Berg et al.
Sediment chemistry manipulation experiments

Four manipulation experiments (further in the text referred to as Experiment I, II, III, and IV) were conducted, each on a separate sampling occasion (April, May, June, and October 2019, respectively). Each experiment included an unamended control as well as specific amendments, which differed between the experiments (except Experiment IV, which repeated parts of Experiments I and III). The amendments included ferric oxyhydroxide in the range of 20–100 µmol cm$^{-3}$, sulfate in the millimolar and micromolar range, elemental sulfur at 30 µmol cm$^{-3}$, 2 mM molybdate as inhibitor of sulfate reduction (Oremland and Capone 1988), and 2 mM molybdate in combination with either ferric oxyhydroxide or elemental sulfur. The concentrations of potential electron acceptors were chosen to cover the range found in freshwater sediments. Ferric oxyhydroxide was prepared by neutralizing 0.4 M FeCl$_3$ to pH 7 with NaOH and subsequently washed to remove NaCl (Lovley and Phillips 1986). A schematic of the treatment experiments, as well as the exact concentrations of the components of the amendments, are presented in Fig. 1.

Anoxic experimental slurries and incubation experiments were prepared in a temperature-controlled room at 18°C in the glove bag flushed with N$_2$. All the glassware used in the experiments was acid-washed in 1 M HCl. Material from the 8–12 cm depth interval, roughly corresponding to the sulfate-methane transition zone, was pooled from several cores, homogenized, and slurried 1:1 by adding an equal volume of anoxic ultrapure water (Experiments I, II, and III) or artificial freshwater medium (Experiment IV; anoxic ultrapure water

1998). The boundary conditions were set as measured concentrations at 0.5 and 19.5 cm depth for CH$_4$ and as concentration at 0.5 cm and no flux at 19.5 cm depth for SO$_4^{2-}$. The diffusivities of CH$_4$ and SO$_4^{2-}$ were calculated for the corresponding in situ temperatures (Boudreau 1997).
water, 0.5 mM MgCl₂, 0.5 mM CaCl₂, and 2 mM NaHCO₃). For amended slurries, amendments were made at this stage. When experimental amendments included molybdate in combination with other components, molybdate was added first.

Upon preparation, anoxic slurries were distributed into 20 ml serum vials (Supelco) that were filled to the top, and a headspace was made by removing 3.5 ml of slurry. The vials were then sealed with thick chlorobutyl rubber stoppers (Bellco Glass, Inc.) and crimped with aluminum crimp caps. The final headspace (1.8 ml) was flushed twice with N₂, after crimping and after 18 h of storage, respectively, to ensure anoxia and remove most of the ambient methane. To start the incubations, each vial received 350 µL of ¹³C-labeled CH₄ prepared by mixing 99.9% pure ¹³CH₄ (Sigma-Aldrich) with unlabeled CH₄ (final labeling ~ 5 atom %), yielding an aqueous concentration of ~ 180 µmol L⁻¹. The vials were then placed on a shaking table in the dark at 18°C and sampled in triplicates (duplicates in Experiment IV) after 0, 1, 3, 5, and 7 days of incubation. On day 3, a number of vials were additionally used for the determination of sulfate reduction rates (see below).

During sampling, 1 ml of gas was withdrawn from the headspace of the incubation vial after injection of 1 ml of N₂. Two 0.5 ml aliquots of the gas sample were transferred into sealed 3 ml Exetainers completely filled with saturated NaCl solution, while an outlet needle was inserted to let the displaced solution escape (Bastviken et al. 2010). The Exetainers were stored upside down for up to two weeks before analysis of CH₄ concentration and for up to two months before the carbon isotope analysis of CH₄ (Faust and Liebig 2018). All gas samplings and transfers were conducted using gas-tight syringes (Hamilton). After gas sampling, the sealed incubation vials were centrifuged at 1000 × g for 10 min and porewater samples were withdrawn with a syringe, with simultaneous supply of N₂ to compensate for the underpressure. Porewater was filtered
through an N2-flushed 0.2 µm cellulose acetate syringe filter (Q-Max GPF, Frisenette).

Porewater aliquots for analysis of dissolved inorganic carbon (DIC; concentration and carbon isotope composition) were stored in 2 ml glass vials (no headspace) on 10 µL of saturated HgCl₂ at 4°C (Torres et al. 2005). Porewater for sulfate analysis was stored at −18°C. A 0.5 ml aliquot for analysis of Fe²⁺ was mixed with 10 µL of 6 M HCl and stored at −18°C. For the experiment conducted in October, slurries in incubation vials were resuspended after porewater sampling, and the vials were opened to sample for analysis of total inorganic carbon (TIC; concentration and carbon isotope composition). For this, a 0.5 ml slurry sample was transferred into a 6 ml glass vial (Exetainer) prefilled with 250 µL of 0.1 M NaOH, quickly sealed with chlorobutyl septa cap, and stored at 4°C until analysis.

The CH₄ concentration in the headspace gas sample was determined as described above. The isotope composition of CH₄ was determined on an isotope ratio mass spectrometer with a pre-concentration unit (PreCon coupled to Delta V Plus, Thermo Scientific) using certified CH₄ isotope standards (Air Liquide) as reference material, and is reported as δ¹³C in per mil (‰) relative to Vienna Pee Dee Belemnite (VPDB). For analysis of porewater DIC, 250 µL sample was injected into a 12 ml He-flushed Exetainer prefilled with 50 µL of 85% phosphoric acid, and after 15 h of equilibration, concentration and isotopic composition of liberated CO₂ were measured by GC-IRMS (GasBench coupled to Delta V Plus, Thermo Scientific), using NBS 18 Calcite, IAEA-LSVEC Lithium Carbonate, and in-house bicarbonate standards as reference materials. For analysis of TIC, the headspace of the sample Exetainer was flushed with He, and 50 µL of 85% phosphoric acid was injected to liberate CO₂. Concentration and isotopic signature of the liberated CO₂ were analyzed after at least 15 h of equilibration by GC-IRMS (custom-built GC unit, Porapak-R column at room temperature coupled to Delta V Plus via
ConFloIII, Thermo Scientific). Porewater concentrations of Fe\(^{2+}\) were determined with the Ferrozine method (Stookey 1970; Thamdrup et al. 1994). Porewater SO\(_4^{2-}\) was analyzed with ion chromatography as described above.

Incubations for sulfate reduction rates were performed on day 3 of each experiment. Sealed triplicate vials from each treatment received 50 \(\mu\)L carrier-free \(^{35}\)SO\(_4^{2-}\) tracer (activity \(\sim 50\)kBq) and were incubated at 18°C in the dark for 1.5 h. To terminate the incubations, vials were decrimped and their slurry contents were transferred into 50 ml polypropylene centrifuge tubes prefilled with 10 ml of 20% zinc acetate. Killed controls were made by adding the radiotracer to the slurries after the activity was stopped by mixing with zinc acetate. All samples were subsequently stored at −18°C. Sulfate reduction rates were determined with the single-step hot chromium distillation method (Fossing and Jørgensen 1989). During the distillation, samples containing molybdate received reducing agent (CrCl\(_3\)) before the addition of acid.

**Evaluation of AOM potential**

Two sediment cores sampled in October 2019 were used in an experiment that tested the depth distribution of potential AOM rates. Each core was sliced in 2 cm intervals to 20 cm depth in a glove bag filled with N\(_2\). Each segment was used to prepare an anoxic slurry in artificial freshwater medium that was supplied with \(^{13}\)C-labeled CH\(_4\) and incubated as described above. Sulfate reduction rates could not be determined in most of the slurry samples because of low sulfate concentrations and are not discussed further for this experiment. Samples for biomolecular analysis were taken on day 0 (start of the incubations) by freezing \(\sim 1\) cm\(^3\) of slurry in liquid nitrogen and subsequently stored at −80°C.
AOM rate calculations

Rates of AOM were calculated from the concentration and $^{13}$C labeling of CO$_2$ liberated after acidification of porewater DIC or slurry TIC (see above) and $^{13}$C labeling of the headspace CH$_4$. For the calculation, $\delta^{13}$C was converted to an isotopic ratio, and then, fractional abundance $^{13}F = ^{13}$C:($^{12}$C + $^{13}$C) (Eq. 1,2):

$$R_{sample} = \left( \frac{\delta^{13}C_{sample}}{1000} + 1 \right) \times R_{standard} \quad (1)$$

$$^{13}F = \frac{R_{sample}}{1 + R_{sample}} \quad (2)$$

where $R_{sample}$ and $R_{standard} = 0.01118$ are the $^{13}$C:12C ratios of the sample and VPDB standard (Fry 2006), respectively. Excess $^{13}$C-CO$_2$ was then calculated for each time point according to Eq. 3:

$$E_t = (^{13}F_t - ^{13}F_0) \times [CO_2]_t \quad (3)$$

where $^{13}F_t$ is the fractional abundance of $^{13}$C-CO$_2$ at a given time point, $^{13}F_0$ is the fractional abundance of $^{13}$C-CO$_2$ at the beginning of the experiment (day 0), and [CO$_2$]$_t$ is the concentration of CO$_2$ at this time point. To evaluate the total production of CO$_2$ at each time point, excess $^{13}$C-CO$_2$ ($E_t$, Eq. 3) was divided by the corresponding fractional abundance of $^{13}$C-CH$_4$ in the headspace (Eq. 4):

$$(CO_2 \text{ produced})_t = \left( \frac{E_t}{^{13}F_{CH_4}} \right)_t \quad (4)$$

Finally, the AOM rate was calculated as the slope of linear regression of total CO$_2$ production over time.
Statistical analysis of treatment effects

Statistical analysis was used to confirm the AOM activity (i.e., non-zero rate), and to test the effects of experimental amendments by comparing them to unamended control. Linear regression slopes of δ^{13}C-DIC (i.e., δ^{13}C-CO₂ evolved from a porewater sample), total DIC-, and TIC production against time were tested for significant deviation from 0 with the analysis of variance (F-test at \( p < 0.05 \)). The change in concentration and δ^{13}C of headspace CH₄, as well as accumulation of SO₄^{2−} in the experimental treatments were tested using the same approach.

Statistical analysis of treatment effects on the aforementioned parameters was performed using linear mixed effect models, which allow to account for non-independence within data (Crawley 2013). Treatment and time were set as fixed effects (predictor variables), and replicates nested in time were set as a random effect (noise). The significance of the treatment effects was evaluated using analysis of variance (Crawley 2013). Simultaneous inference procedures were used to adjust for multiplicity (potential increase in the type I error rate as a result of multiple testing), and adjusted \( P \) values are reported (Hothorn et al. 2008). The analyses were conducted in R, v. 4.0.0 (R Development Core Team 2020) using packages “nlme” and “multcomp” and following the guidelines of Crawley (2013) and Hothorn et al. (2008).

Molecular analysis

Our molecular analysis targeted archaea of ANME-2d clade, as these were previously indicated as conveyors of AOM in the sediments of Lake Ørn, while no other known ANME lineages were detected (Weber et al. 2017). DNA was extracted from 0.25 g of the slurry using the PowerSoil Total DNA Isolation Kit (Qiagen), and the DNA concentration was quantified spectrophotometrically with a Nanodrop (mySPEC, VWR). The 16S rRNA genes of ANME-2d archaea were amplified using the primer pair AAA641F and AAA834R (Vaksmaa et al. 2017).
and quantified using quantitative PCR (qPCR), with DNA quantity normalized to 10 ng µL⁻¹. The qPCR reactions (25 µL) were prepared in triplicates and contained 12.5 µL RealQ Plus 2x Master Mix Green-Low ROX (Ampliqon, Denmark), 1 µL forward primer (20 ng µL⁻¹), 1 µL reverse primer (20 ng µL⁻¹), 8.5 µL of RT-PCR grade water, 1 µL of BSA and 1 µL template DNA. The amplification was performed on the Bio-Rad CFX Connect Real-Time System (Bio-Rad), and the data was analyzed with the Bio-Rad CFX Manager software.

The PCR products were separated using gel electrophoresis, extracted with the QIAquick Gel Extraction Kit (Qiagen), and subsequently purified with the QIAquick PCR Purification Kit (Qiagen). The purified PCR products were inserted into vectors with TOPO™ TA Cloning Kit for Sequencing (Invitrogen), following the manufacturer’s guidelines. E. coli TOP10 competent cells (Invitrogen) were transformed with the purified plasmid DNA using standard transformation procedures. The vector was extracted with GeneJET Plasmid Miniprep Kit (ThermoFisher), following the manufacturer’s instructions, and the presence of the targeted insert was confirmed by PCR. The PCR products were submitted for Sanger sequencing to the Institute of Clinical Molecular Biology (IKMB, Kiel). The obtained sequences were checked and trimmed using the program “4Peaks”, v. 1.8 (https://nucleobyt.es/4peaks/). The taxonomic affiliation to the ANME-2d cluster was confirmed based on the BLAST analysis (https://blast.ncbi.nlm.nih.gov) as well as by the phylogenetic analysis of sequences against the SILVA database using the MAFFT (v. 7; https://mafft.cbrc.jp/alignment/software/) and IQ-TREE (v. 1.6.12; http://www.iqtree.org) software (data not shown).
Results

Sediment characteristics

The sediment was anoxic below 2 mm depth, and nitrate was only detected in the upper 2–3 cm at concentrations below 5 \( \mu \text{mol L}^{-1} \) (detection limit ~ 2 \( \mu \text{mol L}^{-1} \)). Nitrite was not detected (data not shown). The distribution of poorly crystalline Fe(III) was similar across samplings, with HCl-extractable Fe(III) decreasing from 100–265 \( \mu \text{mol cm}^{-3} \) near the sediment surface and remaining consistently low (20 \( \mu \text{mol cm}^{-3} \) on average) below 10 cm depth (Fig. 2). Conversely, HCl-extractable solid phase Fe(II) increased from 70–200 \( \mu \text{mol cm}^{-3} \) at 0–1 cm to 400–730 \( \mu \text{mol cm}^{-3} \) at 20 cm depth with somewhat higher Fe(II) concentrations at 0–9 cm in October than on other sampling occasions (Fig. 2d). In April and May, \( \text{SO}_4^{2-} \) decreased gradually from 300–360 \( \mu \text{mol L}^{-1} \) at the surface (0–1 cm depth) and remained near or below the detection limit of ~ 2 \( \mu \text{mol L}^{-1} \) below 10 cm (Fig. 2a,b). The highest \( \text{SO}_4^{2-} \) concentrations were observed in June, with 380–420 \( \mu \text{mol L}^{-1} \) at the surface, and <5 \( \mu \text{mol L}^{-1} \) below 14 cm depth (Fig. 2c). The lowest surface concentrations (217–255 \( \mu \text{mol L}^{-1} \)) and shallowest penetration (8–9 cm) of \( \text{SO}_4^{2-} \) were observed in October (Fig. 2d). The concentration of \( \text{H}_2\text{S} \) (measured in April) was below 0.6 \( \mu \text{mol L}^{-1} \) throughout the sediment (data not shown).

On every sampling occasion, the \( \text{CH}_4 \) concentration increased with sediment depth, reaching ~ 600–900 \( \mu \text{mol L}^{-1} \) at 20 cm (Fig. 2). The concave shape of the profile suggested that net \( \text{CH}_4 \) consumption was especially pronounced above ~12 cm depth, in the layer containing reactive Fe(III) and typically also \( \text{SO}_4^{2-} \) (Fig. 2). Modeling of \( \text{CH}_4 \) and \( \text{SO}_4^{2-} \) profiles indicated that AOM consumed more than 80% of the diffusive \( \text{CH}_4 \) flux from the sediments and located net \( \text{CH}_4 \) consumption to the intervals 8–12, 0.5–17, 0.5–13, and 7–13 cm in April, May, June, and October, respectively (Fig. 2). The corresponding \( \text{CH}_4 \) consumption rates were 16, 4, 9, and
7.5 nmol cm\(^{-3}\) d\(^{-1}\) equivalent to depth-integrated rates of 0.64, 0.66, 1.13 and 0.45 mmol m\(^{-2}\) d\(^{-1}\).

The main zone of net SO\(_4^{2-}\) consumption was located at 4–8 cm in April and May, 5–10 cm in June, and 0.5–4 cm in October (Fig. 2). The corresponding modeled sulfate reduction rates were 9.8, 10.2, 8.2, and 21.5 nmol cm\(^{-3}\) d\(^{-1}\), or 0.39, 0.41, 0.41 and 0.75 mmol m\(^{-2}\) d\(^{-1}\). Thus, on average net sulfate reduction rates were 68% of the CH\(_4\) consumption rates.

**Sulfur and iron cycling in experimental incubations**

Sulfate in the unamended controls was either undetectable (Experiments I, IV), or below 10 \(\mu\)mol L\(^{-1}\) and did not change significantly during the incubations (Experiments II, III). By contrast, addition of Fe(III) resulted in the accumulation of sulfate at rates of 4.8–20 nmol cm\(^{-3}\) d\(^{-1}\) to final concentrations of 20–140 \(\mu\)M in all treatments except with 30 \(\mu\)mol cm\(^{-3}\) Fe(III) in Experiment IV where no net production was detected (Fig. 3) and the sulfate concentration remained at \(\sim\) 5 \(\mu\)M. The MoO\(_4^{2-}\)+Fe(III) treatments also all accumulated sulfate albeit at relatively lower rates of \(\sim\) 1–7.9 nmol cm\(^{-3}\) d\(^{-1}\) with final concentrations of 20–80 \(\mu\)M (Fig. 3c,d). Minor accumulation to 20–30 \(\mu\)M was also detected with S\(^0\) (\(\sim\) 3.7 nmol cm\(^{-3}\) d\(^{-1}\)) and MoO\(_4^{2-}\)+S\(^0\) (\(\sim\) 1.7 nmol cm\(^{-3}\) d\(^{-1}\)) (Fig. 3d), while no accumulation was observed with MoO\(_4^{2-}\) alone (Fig. 3c). In all treatments containing MoO\(_4^{2-}\), initial (day 0) sulfate concentrations were 10–50 \(\mu\)mol L\(^{-1}\) higher than in unamended controls (data not shown).

Treatments amended with sulfate in the millimolar range showed a decrease in sulfate concentration at rates of 18.5–67 nmol cm\(^{-3}\) day\(^{-1}\) (Fig. 3b), while no significant change was observed with lower sulfate addition (Fig. 3c).

The analysis of sulfate reduction rates confirmed complete inhibition of sulfate reduction in the treatments containing molybdate (Fig. 4c,d). In other treatments, the sulfate reduction rates were in the range of 20–70 nmol cm\(^{-3}\) d\(^{-1}\) (Fig. 4), which typically exceeded the estimated AOM.
rates by an order of magnitude or more (see below). While very high concentrations of Fe(III) (100 µmol cm⁻³ in Experiments I and IV) and SO₄²⁻ (5.9 mM in Experiment II) appeared to have inhibitory effect on sulfate reduction rates (Fig. 4a,b,d), the rates were similar between other treatments (Fig. 4). A reliable calculation of sulfate reduction rates in unamended control treatments was not possible due to low sulfate concentrations (below or close to the detection limit of ~ 2 µmol L⁻¹).

In the treatments that did not contain added Fe(III), initial solid phase Fe(III) was in the range of 20–45 µmol cm⁻³ across all experiments. Initial porewater Fe²⁺ ranged between 250–370 µmol L⁻¹ in unamended controls and 400–1000 µmol L⁻¹ in other treatments (data not shown). Continuous increase in porewater Fe²⁺ over the 7 days of incubation was detected in every treatment of every experiment (data not shown).

**Methanogenesis and AOM in experimental incubations**

In most incubations, methane concentrations either decreased or remained stable (Fig. 5). Net production of methane was only observed in the unamended control and molybdate treatments of Experiment III (sediment sampled in June) (Fig. 5c). Still, production of new, isotopically light methane was indicated by a decrease in δ¹³C-CH₄ in all other unamended controls (Experiments I, II, IV; p < 0.05; Fig. 5). Addition of Fe(III) or sulfate as electron acceptors suppressed this production of methane significantly compared to the unamended control in most or all cases in Experiments I–III (P < 0.05; Fig. 5). In Experiment IV, no significant dilution of δ¹³C-CH₄ (p < 0.05) was detected in any treatments.

AOM activity was indicated by increasing δ¹³C values of DIC in all incubations (Fig. 6; Table 1). The corresponding rates of DIC production from AOM varied widely between the unamended controls of the four experiments, from 0.15 nmol cm⁻³ d⁻¹ in Experiment IV to
4.6 nmol cm$^{-3}$ d$^{-1}$ in Experiment II (Table 1). While addition of millimolar sulfate levels only had a slight positive effect on the increase in $\delta^{13}$C and AOM rates compared to the unamended control in Experiment II, where the rate in the unamended control was already high, the addition of 75–175 µM sulfate in Experiment III stimulated DIC production by AOM 8-fold (Table 1). By contrast, AOM activity was not enhanced in the molybdate treatment of this experiment, which contained ~60 µM sulfate (Table 1).

In all incubations amended with Fe(III), with and without molybdate, we observed large drops in DIC concentration compared to the unamended controls, presumably due to adsorption of inorganic carbon to Fe oxides and/or precipitation of ferrous carbonate. As $^{13}$C-DIC produced by AOM would also be bound in the solid phase, we could not obtain reliable DIC production rates in these treatments. In Experiment IV, we addressed this issue by analyzing the total inorganic carbon (TIC) pool, but for Experiments I–III, $\delta^{13}$C-DIC served as measure of CO$_2$ production from $^{13}$CH$_4$ (Fig. 6, Table 1).

Additions of 20–80 µmol cm$^{-3}$ ferric oxyhydroxide induced a larger increase in $\delta^{13}$C-DIC compared to unamended controls, while the effect was not captured at the very high addition of 100 µmol cm$^{-3}$ Fe(III) in Experiment I (Fig. 6a, Table 1). Also the experiments with 20–100 µmol cm$^{-3}$ Fe(III) and molybdate exhibited higher $\delta^{13}$C than unamended controls (Fig. 6c, d), but when Fe(III) incubations with and without molybdate were run in parallel in Experiment IV, $\delta^{13}$C increased less in the presence of molybdate than without it (Fig. 6d). In Experiment IV, AOM rates calculated as rates of TIC production confirmed the stimulation of AOM in amendments with Fe(III), in this case at both 30 and 100 µmol cm$^{-3}$ (Fig. 7, Table 1). The TIC-based rates in amendments with MoO$_4^{2−}$+Fe(III) were not significantly higher than in the unamended control, with relatively large standard errors especially in unamended control
possibly obscuring the effect observed in δ¹³C-TIC (Fig. 7, Table 1). Likewise, the TIC-based analysis did not discern the effects of S⁰ and MoO₄⁻⁻^+S⁰ amendments indicated by the DIC-based analysis where both treatments had higher rates than unamended control (Table 1).

**Depth distribution of potential AOM rates**

While the manipulation experiments all focused on the 8–12 cm interval, we explored the depth distribution of AOM in a set of ^¹³CH₄ labeling experiments with duplicate cores sampled in October. In these slurries, sulfate was only detected in the two shallowest sections at 0–4 cm depth, broadly consistent with the porewater profile (Fig. 2) considering the 1:1 dilution with sulfate-free water (Fig. 8b). The concentration of solid phase Fe(III) remained close to the in situ values and also declined with depth albeit with relatively large variability at depth (Fig. 8c). This experiment demonstrated highest AOM activity in the upper sediment sections, while AOM became undetectable below 14 cm (Fig. 8a). As the same methane concentration was applied at all depths, these rates can be viewed as potential rates with respect to methane. The rate of AOM in the slurries correlated with the concentrations of sulfate and Fe(III) (Spearman rₛ = 0.69 and 0.49, respectively; p < 0.05 in both cases).

The 16S rRNA gene qPCR analysis demonstrated the presence of anaerobic methane-oxidizing archaea of the ANME-2d clade across all depths with higher abundances detected at 7–11 cm (core I) and 5–7 and 15–19 cm (core II; Fig. 8d), mostly in agreement with the zones of methane consumption and sulfate reduction (Fig. 8d, Fig. 2).
Discussion

AOM and the geochemical zonation in Lake Ørn

The geochemical zonation in the sediment of Lake Ørn was similar to previous reports (Norði et al. 2013; Weber et al. 2016), with the progressive steepening of the concentration gradient of methane with depth to > 10 cm (Fig. 2) indicating its active consumption in the anoxic sediments well below the depth of oxygen and nitrate penetration. Sulfate and poorly crystalline Fe(III) were present in the zone of methane consumption as potential electron acceptors for AOM (Fig. 2). Sulfate profiles varied between samplings, likely reflecting seasonality, with the highest sulfate concentrations and penetration depth measured in June and the lowest in October (Fig. 2). Modeling of methane and sulfate profiles suggested that the zone of net sulfate consumption was located within the zone of AOM in May and June but was shifted upward in April and October (Fig. 2), and the integrated sulfate consumption rates accounted for ~ 68% of the AOM rate. These mismatches imply that if AOM in Lake Ørn is coupled to sulfate reduction, sulfate must to a large extent be sourced from internal, cryptic cycling. Alternatively, AOM could be coupled to another electron acceptor. Since poorly crystalline Fe(III) was detected throughout AOM zone and did not exhibit any clear seasonal variations (Fig. 2), this pool could potentially be involved in both sulfur cycling and direct, iron-dependent AOM. The contribution of crystalline Fe(III) oxides to these processes may also not be ruled out. While such Fe(III) forms are not targeted by the HCl extraction, their presence in the deeper sediment was indicated by a continued increase of HCl-extractable Fe(II) (Fig. 2) as also observed by Norði et al. (2013).

The likely involvement of sulfate and Fe(III) in AOM, inferred from the profiles, was also supported by the depth distribution of potential AOM rates, which decreased with depth in overall correlation with the SO_4^{2−} and Fe(III) concentrations (Fig. 8a,b,c). This rate distribution
contrasts with the zone of CH₄ consumption identified at 7–13 cm at the same time in October (Fig. 2), and with the previous observation of maximum AOM rates at approximately 10–20 cm depth measured with ¹⁴CH₄ tracer in November 2012 (Weber et al. 2016). The reason for the difference in the depth distribution of the rates is likely that AOM rates in situ depend on the ambient methane concentration, which increases with depth (see also Fig. 2), consistent with the observation of 1st order kinetics for AOM with respect to CH₄ in marine sediments (Knab et al. 2008). Here, we demonstrate that the metabolic potential to conduct AOM is actually higher in the sediment layers containing higher concentrations of suitable electron acceptors (Fig. 8a,b,c). In addition, the experiment demonstrated that while sulfate was undetectable in the slurries from depths below 4 cm, AOM was active down to ∼8–14 cm depth (Fig. 8a,b). This, as well as detectable AOM in the sulfate-depleted unamended controls of the four manipulation experiments (Table 1), further hints that AOM in Lake Ørn must rely on other processes than sulfate reduction alone.

**Sulfate- and Fe(III)-dependent AOM in Lake Ørn**

Our manipulation experiments demonstrate a direct coupling of AOM to sulfate reduction in the sediments of Lake Ørn. Thus, additions of sulfate within the in situ range (75 and 175 µM, Experiment III) resulted in a more than 8-fold increase in AOM rates relative to the sulfate-depleted unamended control, with no apparent lag phase, while no stimulation was observed at 60 µM sulfate in the presence of molybdate as inhibitor of sulfate reduction (Fig. 6c, Table 1). The similar response to the two different sulfate additions in Experiment III indicated saturation of sulfate-dependent AOM at these concentrations, and the similar sulfate reduction rates observed in the two treatments likewise indicated saturation of the bulk process (Table 1, Fig. 4). Though the kinetics of sulfate-dependent AOM in freshwater requires further quantification, this
aligns with previous reports of apparent half-saturation constants ($K_m$) for bulk sulfate reduction of 5–30 $\mu$M in freshwater cultures of sulfate-reducing bacteria (Holmer and Storkholm 2001 and citations therein), suggesting that the organisms involved in sulfate-dependent AOM in freshwater have at least as high affinity for sulfate as other freshwater sulfate reducers.

As discussed further below, the coupling to sulfate reduction was further supported by the inhibitory effect of molybdate on AOM in incubations with added Fe(III) relative to parallel incubations with Fe(III) only (Experiment IV; Fig. 7; Table 1). Conversely, there was only a weak or no response of AOM to sulfate additions in the millimolar range in Experiment II (Fig. 6b; Table 1). In culture, freshwater sulfate reducers are typically grown with millimolar sulfate (Widdel and Bak 1992) and we hence expect that sulfate reducers in Lake Ørn would be able to utilize sulfate at these concentrations. We suggest that the weak effect of sulfate in this experiment was related to the already high AOM rate measured in the unamended control (4.6 nmol cm$^{-3}$ d$^{-1}$ compared to $\leq$ 1 nmol cm$^{-3}$ d$^{-1}$ in the other experiments; Table 1), which indicates that the process on this occasion, in contrast to Experiment III (Fig. 6c), was not limited by sulfate. Thus, either sulfate-dependent AOM in the control proceeded with the low amounts of sulfate ($\leq$10 $\mu$M) detected throughout the experiment, or the microbes involved utilized another electron acceptor, most likely Fe(III), as discussed below.

Direct evidence for sulfate-dependent AOM in freshwater sediments is currently very scarce. Some studies did not see any effect of sulfate additions on AOM rates (Lake Constance; Deutzmann and Schink 2011) or even observed AOM inhibition (freshwater creek; Segarra et al. 2013). Likewise, a study from Lake Kinneret concluded that sulfate was not the electron acceptor for AOM, and inhibition of sulfate reduction with molybdate even had a positive effect on AOM (Bar-Or et al. 2017). So far, sulfate-dependent AOM was demonstrated in enrichment cultures...
prepared with the sediments from a freshwater natural gas source (Timmers et al. 2016) and in experiments with the sediments from Lake Cadagno (Su et al. 2020). However, both of these environments have relatively high sulfate concentrations, ~1.7 mM in the surface water of the natural gas source (Timmers et al. 2016), and ~0.9 mM in the upper sediment of Lake Cadagno where sulfate was detected down to ~30 cm depth (Su et al. 2020). Thus, our results demonstrate that sulfate-dependent AOM may be of general importance at sulfate concentrations in the 10–100 µM range typical for many freshwater systems (Holmer and Storkholm 2001), and emphasize the need for further investigations across systems.

The experiments with Fe(III) amendments showed that Fe(III) can actively contribute to sulfate production in the sediments of Lake Ørn (Fig. 3a,d). This confirms the conclusion of Weber et al. (2016) who analyzed the isotopic composition of sulfate produced anaerobically after homogenization of sediment from the lake and inferred the oxidation of reduced sulfur compounds coupled to Fe(III) reduction as the likely source of sulfate (Weber et al. 2016). Thus, the increase in δ¹³C-DIC and AOM rates relative to unamended controls in incubations with added Fe(III) (Figs. 6a,d, 7; Table 1) could either be driven by direct, Fe(III)-dependent AOM or by a cryptic sulfur cycle that includes abiotic and/or biologically catalyzed reactions (Weber et al. 2016) and ultimately drives AOM coupled to sulfate reduction. The possibility of iron-mediated AOM independent of sulfate reduction was tested by the addition of molybdate, a competitive inhibitor of sulfate reduction. While the lower rates in incubations with MoO₄²⁻+Fe(III) compared to Fe(III) alone confirm that a large part of the effect of Fe(III) on AOM was indeed indirect via a stimulation of sulfur cycling, the pronounced increase in δ¹³C-DIC in the MoO₄²⁻+Fe(III) treatments relative to unamended control (Experiments III, IV; Fig. 6c,d; Table 1) validated the existence of a parallel, direct coupling of AOM to iron.
reduction. This result contrasts with results from Lake Cadagno, where addition of manganese or iron oxides only stimulated AOM after 2 weeks of incubation, and where no significant stimulation was observed when molybdate was added together with the metal oxides, leading to the conclusion that the oxides acted solely through stimulation of sulfur cycling (Su et al. 2020). There, however, manganese and iron oxides were added at lower concentration (10 $\mu$mol cm$^{-3}$), which together with the sulfidic conditions may potentially have obscured a direct effect. In freshwater, direct evidence of AOM coupled to Fe(III) reduction has so far been obtained in an enrichment culture containing ANME-2d archaea (Ettwig et al. 2016) and in long-term incubation experiments of Lake Kinneret sediment, where methane consumption was only detected three months after the addition of magnetite and hematite (Bar-Or et al. 2017). Thus, our observation of AOM stimulation by Fe(III) and MoO$_4^{2-}$+Fe(III) additions within just one week, provides novel evidence of the existence of a freshwater sediment microbial community immediately capable of utilizing Fe(III) as an electron acceptor for AOM.

Our results demonstrate that sulfate- and iron-dependent AOM occur side-by-side in Lake Ørn sediment. The TIC production rates in the MoO$_4^{2-}$+Fe(III) treatments with different Fe(III) amendments were nearly identical (Table 1, Fig. 7, Experiment IV), indicating that AOM driven directly by iron reduction was not affected by differences in Fe concentrations within the range of additions (30–100 $\mu$mol cm$^{-3}$). By contrast, the TIC production rates with Fe(III) alone increased with increasing Fe(III) addition (Table 1, Fig. 7). This indicates different kinetics for the direct and indirect effects of Fe(III) on AOM with the latter likely being controlled by the rate of sulfate production (Fig. 3). Thus, sulfate remained at $\sim$ 5 $\mu$M with 30 $\mu$mol cm$^{-3}$ Fe(III) while it accumulated to 40 $\mu$M with 100 $\mu$mol cm$^{-3}$ Fe(III). From a thermodynamic perspective, Fe(III) is a more favorable electron acceptor than sulfate, and iron reducers are expected to
outcompete sulfate reducers in the presence of readily-available forms of Fe(III) (Lovley and Phillips 1987). Thus, it may seem surprising that sulfate-dependent AOM was in fact stimulated by increasing Fe(III) additions. However, previous observations suggest that rather high Fe(III) concentrations are required for dissimilatory Fe(III) reduction to dominate bulk carbon metabolism in sediments (Thamdrup 2000; Roden and Wetzel 2002). Reported Fe(III) concentrations required for 90% of total carbon metabolism to occur via Fe(III) reduction range from 30 µmol cm⁻³ in marine (Thamdrup 2000) to >50 µmol cm⁻³ in freshwater sediments (Roden and Wetzel 2002). Our data showed active sulfate reduction even in the 100 µmol cm⁻³ Fe(III) treatments of Experiments I and IV (Fig. 4a,d). Competitive suppression of sulfate reduction is, thus, most likely restricted to the upper few cm of the Lake Ørn sediment containing highest amounts of poorly crystalline iron oxides (up to 270 µmol cm⁻³ Fe(III); Fig. 2), which is consistent with the sulfate reduction rate profiles reported by Norði et al. (2013). Furthermore, while a weaker dilution of ¹³C-CH₄ in the treatments amended with SO₄²⁻ and Fe(III) compared to unamended controls indicated some inhibition of methanogenesis (Experiments I–IV, Fig. 5), the observed dilution of ¹³C-CH₄ in the unamended control treatments, as well as in some SO₄²⁻- and Fe(III)-amended treatments (Fig. 5) points to the possibility for co-occurrence of methanogenesis with sulfate and iron reduction. While this biogeochemical complexity in the AOM zone of the Lake Ørn sediment complicates elucidation of the kinetics of the individual processes, our results demonstrate that sulfate- and Fe(III)-dependent AOM pathways coexist, and that Fe(III) does not only contribute by supporting the sulfate-dependent pathway.

To address the role of possible cryptic sulfur cycle intermediates, i.e., reduced forms of sulfur, in Experiment IV we used amendments with zero-valent sulfur (S⁰) and MoO₄²⁻+S⁰. These amendments investigated two possibilities: S⁰ as an electron acceptor for AOM, and S⁰ as an
intermediate supporting sulfate-dependent AOM trough disproportionation to sulfate and sulfide (Weber et al. 2016). We indeed observed slow accumulation of sulfate in the S⁰ and MoO₄²⁻+S⁰ treatments (Fig. 3d), however, in the latter case it likely resulted from the reaction between FeOOH and S⁰, as molybdate is known to inhibit disproportionation of elemental sulfur (Finster et al. 1998). Although in both treatments (S⁰ and MoO₄²⁻+S⁰) the DIC production rates were higher than in unamended control, they were low relative to the Fe(III) treatments (Table 1). Hence, there was no strong evidence of S⁰ as an electron acceptor for AOM, which gives further support to AOM mediated directly by Fe(III) reduction.

**Microbial mechanisms of AOM in Lake Ørn**

A previous study using ¹³C-CH₄ and ¹³C-CO₂ stable isotope probing of rRNA pinpointed anaerobic methanotrophic archaea of ANME-2d clade, currently classified as *Ca. Methanoperedenaceae*, as likely methane oxidizers in the sediments of Lake Ørn and found no evidence of other known ANME clades (Weber et al. 2017). In our study, 16S rRNA qPCR demonstrated the presence of the ANME-2d clade throughout the sediment, with a broad abundance maximum that coincided with the SO₄²⁻ and Fe(III)-containing methane oxidation zone (Fig. 8d, Fig. 2). This clade was also identified as the likely conveyor of AOM in the sediments of Lake Cadagno though there both the process and the organisms were focused in a narrow zone at 15–20 cm depth (Schubert et al. 2011; Su et al. 2020). As this zone was further characterized by high abundances of putative sulfate reducers of the *Desulfobulbaceae* family, sulfate-dependent AOM was suggested to rely on the interdependency of these groups in analogy to the ANME-sulfate-reducing bacteria relationships known from marine systems (Boetius et al. 2000; Orphan et al. 2001; 2002). In our study, the identity of sulfate reducers potentially involved in AOM remains unknown, yet recently acquired amplicon sequencing data indicates
that *Desulfobulbaceae* are not abundant in the sediments of Lake Ørn (Rousteau et al. unpubl.). The capacity to reduce sulfate has been hypothesized for ANME-2 archaea (Milucka et al. 2012), but does not seem to be present in ANME-2d clade (Ettwig et al. 2016). Thus, interaction with unidentified sulfate reducers is still expected (Fig. 9).

While more comprehensive analysis is required to understand the exact spectrum of functions performed by ANME-2d in these sediments and the potential presence of other unknown methanotrophs, the group could potentially be involved in both the sulfate- and iron-dependent AOM demonstrated by our incubations (Fig. 9). Iron-dependent AOM has been demonstrated in Ca. Methanoperedens (Ettwig et al. 2016) and it has been proposed that archaea of ANME lineages, including ANME-2d, are capable of direct extracellular electron transfer using large multi-haem cytochromes, where the donated electrons can be transferred to either a syntrophic bacterial partner, metal oxides, humic substances, or potentially any other suitable electron acceptor (Arshad et al. 2015; McGlynn et al. 2015).

**Broader biogeochemical implications**

Our experiments demonstrate that both sulfur and iron cycling are important for AOM in the sediments of Lake Ørn. These sediments house the metabolic potential to support AOM coupled to sulfate reduction, partially supported by sulfate regeneration via reduction of Fe(III) oxides, as well as AOM directly coupled to Fe(III) reduction. Therefore, our study not only demonstrates sulfate-dependent AOM at sulfate concentrations which are widespread in freshwater systems but also highlights important interactions between the sulfur and iron cycle in freshwater sediments.

Our study casts new light on the mechanisms that drive AOM in freshwater systems and on the environmental conditions that may support the process and thereby mitigate methane
emissions. It demonstrates that relatively low sulfate concentrations are not an impassable obstacle for sulfate-dependent AOM, with low half-saturation constants of sulfate reduction being a potential microbial adaptation to sulfate deprivation. Relatively high concentration of Fe(III) and Mn(IV) are common in freshwater systems (Roden and Wetzel 1996; Thomsen et al. 2004; Giblin 2009), and thus, favourable conditions for metal-dependent AOM likely exist in many locations, be it direct or via sulfur cycling. Recent studies also indicate that sulfate can be efficiently recycled by freshwater cable bacteria (Sandfeld et al. 2020) which therefore could act as an alternative source of sulfate for AOM in freshwater sediments. Hence, the AOM pathways suggested in our study may be of broader relevance across limnic environments, and sulfate- and Fe(III)-dependent AOM should be taken into account in mechanistic analyses of factors controlling freshwater methane fluxes.
References


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Table 1. Change in δ\textsuperscript{13}C-DIC with time (‰ d\textsuperscript{−1}), and AOM rates calculated as rates of DIC- (Experiments I–IV) and TIC (Experiment IV) production (nmol cm\textsuperscript{−3} d\textsuperscript{−1}). P values are included in parentheses for those treatments where the rate was higher than in the corresponding unamended control (0.05 significance level).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Change in δ\textsuperscript{13}C-DIC with time (‰ d\textsuperscript{−1})</th>
<th>DIC production rate (nmol cm\textsuperscript{−3} d\textsuperscript{−1})</th>
<th>TIC production rate (nmol cm\textsuperscript{−3} d\textsuperscript{−1})</th>
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</thead>
<tbody>
<tr>
<td>I (April)</td>
<td>Unamended Control</td>
<td>6.09 ± 0.43</td>
<td>1.03 ± 0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fe(III) 20 µmol cm\textsuperscript{−3}</td>
<td>11.24 ± 0.67 (P &lt; 0.001)</td>
<td>NA\textsuperscript{a}</td>
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<tr>
<td></td>
<td>Fe(III) 80 µmol cm\textsuperscript{−3}</td>
<td>10.74 ± 0.43 (P &lt; 0.001)</td>
<td>NA\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fe(III) 100 µmol cm\textsuperscript{−3}</td>
<td>7.64 ± 1.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II (May)</td>
<td>Unamended Control</td>
<td>11.57 ± 0.97</td>
<td>4.64 ± 0.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SO\textsubscript{4}\textsuperscript{2−} 1.3 mM</td>
<td>14.53 ± 0.58 (P &lt; 0.01)</td>
<td>5.46 ± 0.17 (P = 0.073)</td>
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<tr>
<td></td>
<td>SO\textsubscript{4}\textsuperscript{2−} 3.7 mM</td>
<td>13.17 ± 0.48</td>
<td>4.84 ± 0.16</td>
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<td></td>
<td>SO\textsubscript{4}\textsuperscript{2−} 5.9 mM</td>
<td>12.16 ± 0.49</td>
<td>3.64 ± 0.12</td>
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<td>III (June)</td>
<td>Unamended Control</td>
<td>0.56 ± 0.25\textsuperscript{b}</td>
<td>0.99 ± 0.13</td>
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<tr>
<td></td>
<td>MoO\textsubscript{4}\textsuperscript{2−} 2 mM</td>
<td>0.46 ± 0.28\textsuperscript{b}</td>
<td>0.89 ± 0.08</td>
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<td></td>
<td>MoO\textsubscript{4}\textsuperscript{2−}+Fe(III) 20 µmol cm\textsuperscript{−3}</td>
<td>7.82 ± 0.39 (P &lt; 0.001)</td>
<td>3.21 ± 0.23 (P &lt; 0.001)</td>
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<td>MoO\textsubscript{4}\textsuperscript{2−}+Fe(III) 80 µmol cm\textsuperscript{−3}</td>
<td>7.51 ± 0.15 (P &lt; 0.001)</td>
<td>NA\textsuperscript{a}</td>
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<td></td>
<td>SO\textsubscript{4}\textsuperscript{2−} 75 µM</td>
<td>19.37 ± 1.2 (P &lt; 0.001)</td>
<td>8.78 ± 0.32 (P &lt; 0.001)</td>
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<td>SO\textsubscript{4}\textsuperscript{2−} 175 µM</td>
<td>18.88 ± 0.81 (P &lt; 0.001)</td>
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<td>IV (October)</td>
<td>Unamended Control</td>
<td>0.31 ± 0.08</td>
<td>0.15 ± 0.04</td>
<td>0.75 ± 0.67\textsuperscript{b}</td>
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<td>Fe(III) 30 µmol cm\textsuperscript{−3}</td>
<td>3.58 ± 0.5 (P &lt; 0.001)</td>
<td>NA\textsuperscript{a}</td>
<td>4.76 ± 0.56 (P &lt; 0.001)</td>
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<td>Fe(III) 100 µmol cm\textsuperscript{−3}</td>
<td>NA</td>
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<td>6.97 ± 0.55 (P &lt; 0.001)</td>
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<td></td>
<td>MoO\textsubscript{4}\textsuperscript{2−}+Fe(III) 30 µmol cm\textsuperscript{−3}</td>
<td>3.35 ± 0.09 (P &lt; 0.001)</td>
<td>NA\textsuperscript{a}</td>
<td>1.47 ± 0.32</td>
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<td>MoO\textsubscript{4}\textsuperscript{2−}+Fe(III) 100 µmol cm\textsuperscript{−3}</td>
<td>2.18 ± 0.18 (P &lt; 0.001)</td>
<td>NA\textsuperscript{a}</td>
<td>1.48 ± 0.41</td>
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<td>SO\textsuperscript{0} 30 µmol cm\textsuperscript{−3}</td>
<td>1.76 ± 0.12 (P &lt; 0.001)</td>
<td>0.68 ± 0.06 (P &lt; 0.001)</td>
<td>1.87 ± 0.74</td>
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<td></td>
<td>MoO\textsubscript{4}\textsuperscript{2−}+SO\textsuperscript{0} 30 µmol cm\textsuperscript{−3}</td>
<td>1.43 ± 0.09 (P &lt; 0.001)</td>
<td>0.34 ± 0.03 (P &lt; 0.05)</td>
<td>1.46 ± 0.32</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Treatment with added Fe(III) where adsorption/precipitation of DIC did not allow reliable calculation of AOM rates (see text).

\textsuperscript{b}Slope is not significantly different from 0 (F-test, \(p > 0.05\)).
Fig. 1. Outline of the slurry incubation experiments. Experiments I, II, III, and IV were conducted with the sediment sampled in April, May, June, and October, respectively. In each experiment, material pooled from the 8–12 cm segment of several sediment cores was used to prepare anoxic slurries. Each experiment included amended slurries (composition shown in boxes) and a live, unamended control. Gas-tight incubations were conducted for 7 days at 18°C and in the dark.

Fig. 2. Geochemical profiles of methane, sulfate and HCl-extractable solid phase iron in Lake Ørn sediment (mean, n=2). Bottom water concentrations are plotted at −0.5 cm. Horizontal panels a, b, c, and d correspond to April, May, June, and October, respectively. In methane and sulfate profiles, lines correspond to modeled concentrations, and grey areas correspond to the zones of consumption derived from the distribution of the corresponding chemical species. Error bars represent the span in concentration between replicate cores. Points without error bars indicate that the measurement was available from only one core.

Fig. 3. Rate of change in porewater sulfate concentrations in different treatments of four slurry incubation experiments. Panels a, b, c, and d correspond to Experiment I (April), II (May), III (June), and IV (October), respectively. The rates are calculated based on measurements from triplicate (a, b, c) or duplicate (d) vials at five time points (days 0, 1, 3, 5, 7). Error bars indicate the standard error of the slope. Treatments where accumulation (positive change) or consumption (negative change) were significant (p < 0.05) are marked with an asterisk (*).

Fig. 4. Sulfate reduction (SR) rates in different treatments of four slurry incubation experiments. Panels a, b, c, d correspond to Experiments I (April), II (May), III (June) and IV (October),
respectively. Each bar represents the mean of three replicate vials. Flat bars indicate the rates below detection. The error bars correspond to the standard deviation of the mean. The rates in unamended controls could not be determined due to low sulfate concentrations (see text) and are marked with “NA” (not available).

**Fig. 5.** Changes with time in concentration and $\delta^{13}C$ of methane (‰ vs. VPDB) in different treatments of four slurry incubation experiments. Panels a, b, c, and d correspond to Experiment I (April), II (May), III (June), and IV (October), respectively. The rates are calculated based on measurements from triplicate (a, b, c) or duplicate (d) vials at five time points (days 0, 1, 3, 5, 7). Error bars indicate the standard error of the slope. Rates of change significantly different from 0 ($p < 0.05$) are marked with an asterisk (*). Significant difference from unamended control ($P < 0.05$) is denoted with a plus sign (+).

**Fig. 6.** Change in $\delta^{13}C$-DIC in different treatments of four slurry incubation experiments. Panels a, b, c, d correspond to Experiments I (April), II (May), III (June) and IV (October), respectively. Each data point represents the mean of two replicate vials and error bars correspond to the span in replicate measurements. Points without error bars had a span smaller than the symbol size. Data for the Fe(III) 100 $\mu$mol cm$^{-3}$ treatment (Experiment IV, panel d) was not available. Note the different scale of the y-axis in the panel d.

**Fig. 7.** AOM rates calculated as the TIC production rates in different treatments of Experiment IV (October). The rates of TIC production were calculated based on concentration and $^{13}C$ labeling of slurry TIC and $^{13}C$ labeling of the headspace CH$_4$ in the duplicate vials. Error bars indicate the standard error of the regression slope.
Fig. 8. Depth distribution of potential AOM rates, sulfate, reactive Fe(III), and microbial abundances of ANME-2d archaea in a $^{13}$CH$_4$ slurry incubation experiment. Here, AOM rates are the TIC production rates ± standard error of the slope. Sulfate and Fe(III) correspond to single measurements from day 0 of the incubation. ANME-2d counts are the mean of three qPCR replicates ± standard deviation (often smaller than the symbol size and not visible). Filled and open symbols represent two replicate cores used to prepare two separate sets of slurries.

Fig. 9. Model of microbial and abiotic processes potentially involved in AOM in the sediments of Lake Ørn. ANME-2d archaea perform the methane-oxidizing step of sulfate-dependent AOM, while sulfate reduction is performed by an unidentified syntrophic bacterial partner. Sulfate may be regenerated through sulfide reoxidation: 1) via abiotic reaction with iron oxides, followed by microbial disproportionation of elemental sulfur, or 2) via coupling to microbial iron reduction. Additionally, ANME-2d couple methane oxidation directly to the reduction of ferric iron.

ANME-2d, anaerobic methanotrophic archaea; SRB, sulfate-reducing bacteria; SDB, sulfur-disproportionating bacteria; IRB, iron-reducing bacteria.
### Experiment I (April)

- **Unamended Control**
- Fe(III) 20 μmol cm\(^{-3}\)
- Fe(III) 80 μmol cm\(^{-3}\)
- Fe(III) 100 μmol cm\(^{-3}\)

### Experiment II (May)

- **Unamended Control**
- \(\text{SO}_4^{2-} 1.3 \text{ mM}\)
- \(\text{SO}_4^{2-} 3.7 \text{ mM}\)
- \(\text{SO}_4^{2-} 5.9 \text{ mM}\)

### Experiment III (June)

- **Unamended Control**
- \(\text{MoO}_4^{2-} 2 \text{ mM}\)
- \(\text{MoO}_4^{2-} 2 \text{ mM} \text{ Fe(III)} 20 \mu\text{mol cm}^{-3}\)
- \(\text{MoO}_4^{2-} 2 \text{ mM} \text{ Fe(III)} 80 \mu\text{mol cm}^{-3}\)
- \(\text{SO}_4^{2-} 75 \mu\text{M}\)
- \(\text{SO}_4^{2-} 175 \mu\text{M}\)

### Experiment IV (October)

- **Unamended Control**
- Fe(III) 30 μmol cm\(^{-3}\)
- Fe(III) 100 μmol cm\(^{-3}\)
- \(\text{MoO}_4^{2-} 2 \text{ mM}\)
- \(\text{MoO}_4^{2-} 2 \text{ mM} \text{ Fe(III)} 30 \mu\text{mol cm}^{-3}\)
- \(\text{S}^0 30 \mu\text{mol cm}^{-3}\)
- \(\text{MoO}_4^{2-} 2 \text{ mM}\)

Dark incubations for 7 days
CH$_4$ ($\mu$mol L$^{-1}$)  |  SO$_4^{2-}$ ($\mu$mol L$^{-1}$)  |  HCl-extractable Fe(III) ($\mu$mol cm$^{-3}$)  |  HCl-extractable Fe(II) ($\mu$mol cm$^{-3}$)

Sediment depth (cm)

(a)  

(b)  

(c)  

(d)
Change in $\text{SO}_4^{2-}$ (nmol cm$^{-3}$ d$^{-1}$)

- Unamended Control
- Fe(III) 20 μmol cm$^{-3}$
- Fe(III) 80 μmol cm$^{-3}$
- Fe(III) 100 μmol cm$^{-3}$
- $\text{SO}_4^{2-}$ 1.3 mM
- $\text{SO}_4^{2-}$ 3.7 mM
- $\text{SO}_4^{2-}$ 5.9 mM
- $\text{MoO}_4^{2-}$ 2 mM
- $\text{MoO}_4^{2-}$ 75 μM
- $\text{MoO}_4^{2-}$ 100 μmol cm$^{-3}$
- $\text{MoO}_4^{2-}$ 175 μM
- $\text{MoO}_4^{2-}$ + Fe(III) 20 μmol cm$^{-3}$
- $\text{MoO}_4^{2-}$ + Fe(III) 80 μmol cm$^{-3}$
- $\text{MoO}_4^{2-}$ + Fe(III) 100 μmol cm$^{-3}$
- $\text{MoO}_4^{2-}$ + S$^0$ 30 μmol cm$^{-3}$
Unamended Control

Fe(III) 20 μmol cm\(^{-3}\)

Fe(III) 80 μmol cm\(^{-3}\)

Fe(III) 100 μmol cm\(^{-3}\)

SO\(_4^{2-}\) 1.3 mM

SO\(_4^{2-}\) 3.7 mM

SO\(_4^{2-}\) 5.9 mM

Unamended Control

MoO\(_4^{2-}\) 2 mM

MoO\(_4^{2-}\)+ Fe(III) 20 μmol cm\(^{-3}\)

MoO\(_4^{2-}\)+ Fe(III) 80 μmol cm\(^{-3}\)

MoO\(_4^{2-}\)+ Fe(III) 100 μmol cm\(^{-3}\)

SO\(_4^{2-}\) 75 μM

SO\(_4^{2-}\) 175 μM

Unamended Control

MoO\(_4^{2-}\) 2 mM

MoO\(_4^{2-}\)+ Fe(III) 20 μmol cm\(^{-3}\)

MoO\(_4^{2-}\)+ Fe(III) 80 μmol cm\(^{-3}\)

MoO\(_4^{2-}\)+ Fe(III) 100 μmol cm\(^{-3}\)

S\(^0\) 30 μmol cm\(^{-3}\)

MoO\(_4^{2-}\)+ S\(^0\) 30 μmol cm\(^{-3}\)
TIC production rate
(nmol cm$^{-3}$ d$^{-1}$)

Unamended Control
Fe(III) 30 μmol cm$^{-3}$
MoO$_4^{2-}$ 100 μmol cm$^{-3}$
MoO$_4^{2-}$ + Fe(III) 30 μmol cm$^{-3}$
MoO$_4^{2-}$ + S$^0$ 30 μmol cm$^{-3}$
MoO$_4^{2-}$ + S$^0$ 30 μmol cm$^{-3}$
AOM rate (nmol cm\(^{-3}\) d\(^{-1}\))

\[ \text{SO}_4^{2-} \] (µmol L\(^{-1}\))

HCl-extractable Fe(III) (µmol cm\(^{-3}\))

ANME-2d 16S rRNA gene copies cm\(^{-3}\) slurry
S-AOM

ANME-2d

CH$_4$

CO$_2$

Sulfide reoxidation

Fe(II)

Fe(III)

Fe(II)

Fe(III)

SRB

HS$^-$

S$^0$

SO$_4^{2-}$

IRB

SDB

ANME-2d

CH$_4$

CO$_2$

Fe-AOM