Evidence for anaerobic oxidation of methane in sediments of a freshwater system (Lago di Cadagno)

Carsten J. Schubert¹, Francisco Vazquez¹, Tina Lösekann-Behrens², Katrin Knittel², Mauro Tonolla³ & Antje Boetius⁴

¹Department of Surface Waters-Research and Management, Swiss Federal Institute of Aquatic Science and Technology (Eawag), Kastanienbaum, Switzerland; ²Department of Molecular Ecology, Max Planck Institute for Marine Microbiology, Bremen, Germany; ³Istituto Cantonale di Microbiologia, Bellinzona, Switzerland; and ⁴Habitat group, Max Planck Institute for Marine Microbiology, Bremen, Germany

Correspondence: Carsten J. Schubert, Department of Surface Waters-Research and Management, Swiss Federal Institute of Aquatic Science and Technology (Eawag), Seestrasse 79, Kastanienbaum CH-6047, Switzerland. Tel.: +41 41 349 2195; fax: +41 41 349 2168; e-mail: carsten.schubert@eawag.ch

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Abstract

Anaerobic oxidation of methane (AOM) has been investigated in sediments of a high alpine sulfate-rich lake. Hot spots of AOM could be identified based on geochemical and isotopic evidence. Very high fractionation of methane (α = 1.031) during oxidation was observed in the uppermost sediment layers, where methane is oxidized most likely with sulfate-containing bottom waters. However, we could not exclude that other electron acceptors such as iron, or manganese might also be involved. Light carbon isotope values (Δ¹³C = −10‰ vs. Vienna Pee Dee Belemnite [VPDB]) of sedimentary carbonates at 16–20 cm sediment depth are indicative of a zone where methane was oxidized and the resulting bicarbonate ions were used for carbonate precipitation. 16S rRNA gene analysis revealed the presence of sequences belonging to the marine benthic groups B, C, and D and to the recently described clade of AOM-associated archaea (AAA). Catalyzed reporter deposition-FISH analysis revealed a high abundance of Deltaproteobacteria, especially of free-living sulfate-reducing bacteria of the Desulfosarcina/Desulfococcus branch of Deltaproteobacteria in the AOM zone. Here, loose aggregations of AAA cells were found, suggesting that AAA might be responsible for oxidation of methane in Lake Cadagno sediments.

Introduction

Methane is an effective greenhouse gas in the atmosphere. Although the methane concentration in the atmosphere is small compared with CO₂ (380 p.p.m.), methane’s impact as a greenhouse gas on a per mass unit is about 24 times higher (IPCC, 2001). Its atmospheric concentration has increased from 850 p.p.b. before industrialization to a level of 1.7 p.p.m. recently and it was estimated to contribute approximately 20% to the global warming effect (Wuebbles & Hayhoe, 2002; Dlugokencky et al., 2003).

New estimates showed that the ocean contributes only a small amount of 0.6–1.2 Tg methane to the atmosphere per year (<1% of the global budget, Rhee et al., 2009) due to very effective aerobic and anaerobic methane oxidation processes in the sediments and the water column (Reeburgh et al., 1991; Schubert et al., 2006). Although lakes and other freshwater systems cover a much smaller area of the Earth’s surface compared with the oceans (>3% of the earth continental ‘land’ surface, Downing et al., 2006), they have been made responsible for the release of significant amounts of CO₂ and CH₄ into the atmosphere (Bastviken et al., 2004; Juutinen et al., 2009). Considering both open water and plant-mediated fluxes, Bastviken et al. (2004) estimated global emissions to be in the range of 8–48 Tg CH₄ year⁻¹ based on measurements of over 70 lakes. If these estimates are correct, this amounts to 6–16% of the total natural methane emissions, much more than oceanic emissions and motivating this research.

Methanotrophic microorganisms play a major role in controlling methane fluxes from the ocean. The most important sink for methane in the ocean is the anaerobic oxidation of methane (AOM) with sulfate as an electron acceptor (Hinrichs & Boetius, 2002). Consortia consisting of methane-oxidizing archaea and sulfate-reducing bacteria are mediating this process in marine sediments and seawater.
(Knittel & Boetius, 2009). Recently, however, methane oxidation with manganese (birnessite) and iron (ferrihydrite) was also shown to occur in the ocean (Beal et al., 2009). Three different clades of anaerobic methanotrophic archaea have been identified and named ANME-1, -2, and -3. Their involvement in AOM has been shown by stable carbon isotope analysis (Orphan et al., 2001) and in vitro studies using sediment slurries enriched in AOM consortia (Nauhaus et al., 2007; Holler et al., 2009).

Recently, 16S rRNA gene sequences of ANME organisms have been retrieved from limnic and terrestrial environments (Eller et al., 2005; Alain et al., 2006; López-Archilla et al., 2007). One of the apparent differences between marine and limnic environments is the much lower concentration of sulfate in lake waters (< 0.2 mM sulfate compared with 28 mM in ocean waters, Mason & Moore, 1982). The limitation in sulfate is one of the main causes for the much higher methane emission from continental compared with marine aquatic systems. Here, we have studied the occurrence of AOM in Lake Cadagno, which has relatively high sulfate concentrations (approximately 2 mM SO\textsubscript{4}\textsuperscript{2-}). We have combined geochemical, isotopic, and molecular tools to identify the hot spots of AOM in Lake Cadagno sediments as well as providing information regarding the microorganisms potentially involved in this process.

Materials and methods

Study site

Lake Cadagno is a meromictic lake located in the catchment area of a dolomite vein rich in gypsum in the Piora valley in the southern Alps of Switzerland (46°33′N, 8°43′E). The lake lies at 1923 m above sea level and has a maximum water depth of 21 m in summer. It covers an area of 26 ha, with a volume of 2.4 x 10\textsuperscript{6} m\textsuperscript{3} and a water temperature of 4–6 °C below 13 m (Del Don et al., 2001). The water column is characterized by a high-salinity monimolimnion and a permanent chemocline moving during the year between a depth of 9 and 14 m, separating the aerobic epilimnion from the anaerobic, sulfidogenic hypolimnion. The lake bottom below the redox transition zone is anoxic all the time (Wagener et al., 1990). Because of the infiltration of subaquadic spring water flowing through gypsum-rich dolomites, Lake Cadagno water contains relatively high concentrations of sulfate (1.5 mM), hydrogen-carbonate, calcium, magnesium, and sulfide (1 mM) resulting from sulfate reduction in the hypolimnion and sediments (Hanselmann & Hutter, 1998; Del Don et al., 2001; Dahl et al., 2010). We detected no oxygen below 12 m (the detection limit of the oxygen sensor was 1 μM), but high sulfide (up to 280 μM) in the hypolimnion. Nitrate was not available below 12.5 m, ammonium concentrations increased to 40 μM at 17.5 m depth (Halm et al., 2009) and iron (II) was below 5 μM in the bottom water and not available in the uppermost sediments (Hanselmann & Hutter, 1998).

Sampling

Sediment samples were recovered in August 2002 in 21 m water depth using a sediment corer. All analyses were performed on one core. Before splitting the core, 2 mL of sediment were sampled every 1 cm sediment depth from holes in the side of the plastic core that were covered by tape and only removed shortly before the syringe was introduced to avoid loss of methane. These 2 mL of sediment were transferred into a 15 mL vial prefilled with 4 mL of 2 M NaOH, to trap the CO\textsubscript{2} and preserve the samples for methane measurements. After slicing the core into 1-cm-thick disks (30 samples in total), sediment samples were divided for pore water, geochemical (samples were frozen at −20 °C) and molecular analyses (for preservation, see below). One half of each sediment slice was centrifuged to extract the pore water (stored at 4 °C; for treatment and analysis of pore waters, see below).

Pore water analysis

Pore water samples for sulfate and calcium analysis were filtered in the lab with cellulose acetate filters of 0.45 μm pore size (Whatman OE67) and analyzed using an ion chromatograph (150/4.0 mm ID column, eluent 3.2 mM Na\textsubscript{2}CO\textsubscript{3}, 1 mM NaHCO\textsubscript{3}, detection limit 5 μmol L\textsuperscript{-1}; Metrosep A Supp 5, Metrohm 733; Herisau, Switzerland). Pore water samples for S(-II) analysis were filtered immediately after centrifugation through 0.45 μm membrane disc filters (FP30/0.45CA; Whatman) and 1 mL of Zn acetate solution (4% in 2% acetic acid) was added to 1 mL of sample. Photometric analysis (Hitachi U2000) was performed the following day using standard methods (665 nm wavelength, 1 cm cuvette, detection limit 2 μmol L\textsuperscript{-1}; DEW 2004).

Total, organic, and inorganic carbon, and lipid analysis

Total carbon (TC) was measured with a precision of 0.2% using a CN analyzer (Heraeus) after samples had been freeze-dried. The total inorganic carbon (TIC) concentrations of sediment samples were measured using a CO\textsubscript{2} coulometer (5011; Coulometric Inc.). The precision of TIC measurements was 0.2%. TOC in sediments was calculated as the difference of TC and TIC.

Sediments were extracted three times for 20 min ultrasonically in an ice bath using methanol, methanol:dichloromethane (1:1), and dichloromethane to obtain the total lipid extracts. Aliquots of the total extracts were saponified after the addition of internal standards and separated into
fatty acid and neutral (nonsaponifiable) lipid fractions. The fatty acid fractions were methylated (BF₃-MeOH, Sigma) and the neutral fractions were silylated (BSTFA, Sigma). Both fractions were analyzed by GC and GC-MS for the quantification and identification of biomarkers, respectively. The chromatographic column in both systems was a DB5 (50 m, 0.25 mm, 25 μm) and the temperature program was 80 °C (1 min), 20 °C min⁻¹ to 130 °C, and 4 °C min⁻¹ to 320 °C (20 min). The isotopic composition (δ¹³C against VPDB) of compounds of the fatty acid fraction was measured with a GC connected via a combustion interface to a mass spectrometer. δ¹³C values were corrected for the carbon atoms introduced during derivatization.

Methane concentrations and carbon isotopes

Methane concentrations were measured on a 5160 Mega Series gas chromatograph (Carlo Erba Instruments) using a flame ionization detector, a GS-Q P/N 115-3432 column (J & W Scientific), and hydrogen as a carrier gas. Oven temperature was 40 °C and detector temperature was 200 °C. Standards (100, 1000, and 10 000 p.p.m.) were used for calibration (Scotty Specialty Gases). The methane isotopic composition was determined with a preconcentration unit (Precon, Finnigan) connected to a mass spectrometer (Delta XL, Finnigan). Units are in δ notation: δ¹³C = ((¹³C/¹²C_sample)/(¹³C/¹²C_standard)−1) × 100‰ and values are referenced against VPDB. The fractionation factor α was calculated using the following formula: αS/P = RS/RP = (δS+1000)/(δP+1000), where S is the substrate, P the product, and R the ratio of the heavy isotope to the light isotope.

Diffusive flux calculation

The diffusive flux of methane was calculated from linear pore water concentration gradients between the 0.5 and the 3.5 cm sediment core according to Fick's first law assuming steady-state conditions [Eqn. (1); e.g., Berner, 1980]:

\[ J = -\Phi \times D_s \frac{dc}{dx} \]  

(1)

where \( J \) is the diffusive flux (mmol m⁻² a⁻¹), \( \Phi \) is the porosity, \( D_s \) is the sediment diffusion coefficient (m² a⁻¹), \( c \) is the concentration of methane (mmol m⁻³), and \( x \) is the depth (m). The sediment diffusion coefficient, \( D_s \) of methane was calculated according to Iversen & Jørgensen (1993) using the porosity of 0.92 (Birch et al., 1996) and a molecular diffusion coefficient of \( D_{\text{methane}} = 0.87 \times 10^{-5} \) cm² s⁻¹ [Eqn. (2)]:

\[ D_s = \frac{D}{(1 + 3(1 - \Phi))} \]  

(2)

DNA extraction, PCR amplification, and clone library construction

For DNA extraction, cores were sectioned and frozen at −20 °C. Total community DNA was directly extracted from 2 g of wet sediments (pools from 2 to 4 cm and 9 to 15 cm sediment depth, respectively) according to the method of Zhou et al. (1996). The protocol encompassed three cycles of freezing and thawing, chemical lysis in a high-salt extraction buffer (1.5 M NaCl) by heating of the suspension in the presence of sodium dodecyl sulfate (SDS) and hexadecltrimethylammonium bromide, and a proteinase K step. Crude DNA was purified using the Wizard DNA Clean-Up Kit (Promega, Madison, WI). Domain-specific primers Arch20F (Massana et al., 1997) and Arch958R (DeLong, 1992) were used to amplify archaeal 16S rRNA genes from the extracted chromosomal DNAs. PCRs were performed and products were purified as described previously (Ravenschlag et al., 1999). DNA was ligated in the pCR4 TOPO vector (Invitrogen, Carlsbad, CA) and transformed into Escherichia coli TOP10 cells (Invitrogen) according to the manufacturer’s recommendations.

Sequencing and phylogenetic analysis

Sequencing was performed by Taq cycle sequencing using a model ABI377 sequencer (Applied Biosystems). Using the CHIMERA_CHECK program of the Ribosomal Database Project II (Center for Microbial Ecology, Michigan State University, http://rdp8.cme.msu.edu/cgi/chimera.cgi?su=S SU) and the program Bellerophon (Advanced Computational Modelling Center, University of Queensland, Australia, http://foo.maths.uq.edu.au/~huber/bellerophon.pl), potential chimeric sequences were not detected in the clone libraries. Sequence data were analyzed using the ARB software package (Ludwig et al., 2004). Phylogenetic trees of 16S rRNA gene sequences were calculated by parsimony, neighbor-joining, and maximum-likelihood analysis with different sets of filters. For tree calculation, only almost full-length sequences were considered. Partial sequences from Lake Cadagno were inserted into the reconstructed tree by parsimony criteria without allowing changes in the overall tree topology.

Catalyzed reporter deposition FISH (CARD-FISH)

Sediment subsamples (pools from 2 to 4 cm and 9 to 15 cm sediment depth, respectively) were fixed with 50% ethanol and treated by sonication as described previously (Ravenschlag et al., 2000). In situ hybridizations with horseradish peroxidase (HRP)-labelled probes, followed by tyramide signal amplification (also known as CARD) were carried out as described by Pernthaler et al. (2002). For
permeabilization of rigid archaeal cell walls, filters were incubated in 15 μg mL⁻¹ proteinase for 2 min at room temperature and finally washed in MilliQ water and dehydrated in absolute ethanol. For permeabilization of bacterial cell walls, filters were incubated in 10 mg mL⁻¹ lysozyme for 10 min at 37 °C. All hybridizations were performed at 46 °C. Hybridized samples were examined using an epifluorescence microscope (Axiophot II; Carl Zeiss, Jena, Germany). For each probe and sample, 700–1000 DAPI-stained cells in 10–20 independent microscopic fields were counted. Micrographs of microbial aggregates were taken using a confocal laser scanning microscope (LSM510; Carl Zeiss). Probes were purchased from biomers.net (Ulm, Germany). Probe sequences and formamide concentrations required for specific hybridization are given in Table 1. The specificity of new probes was evaluated by Clone-FISH as described by Schramm et al. (2002). As a positive control clone Cad24-26 was selected for probes AAA-FW-641 and -834. As a reference having one mismatch clone GoM_SMBush4593_Arch26 (Luley and colleagues, database release; accession numbers AM404409) was used for AAA-FW641.

Nucleotide sequence accession numbers

The sequence data reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers AM851080–AM851088.

Results and discussion

Whereas AOM has been described for many marine environments (Knittel & Boetius, 2009) including pelagic settings (Schubert et al., 2006), the occurrence of this process in terrestrial aquatic systems has been rarely reported (Eller et al., 2005; Alain et al., 2006; Schubert et al., 2010). In marine sediments, AOM depending on sulfate entering the seafloor via diffusion was first described by Boetius et al. (2000). However, most lakes and rivers contain very little sulfate; hence, it is commonly believed that methane oxidation coupled to sulfate reduction is not of significance in freshwater systems. A study by Raghoebarsing et al. (2006) and Ettwig et al. (2008) reported AOM coupled to denitrification (a process that would produce more energy than coupling to sulfate reduction) in a bioreactor inoculated by an environmental sample from a Dutch canal. Sequencing of

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>% FA</th>
<th>Probe Sequence (5’–3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARCT915</td>
<td>Most archaea</td>
<td>35</td>
<td>GTG CCC CGC CAA TTC CT</td>
<td>Stahl &amp; Amann (1991)</td>
</tr>
<tr>
<td>ANME-1-350</td>
<td>ANME-1</td>
<td>40</td>
<td>AGT TTT CGC GCC TGATGC</td>
<td>Boetius et al. (2000)</td>
</tr>
<tr>
<td>ANME-2-538</td>
<td>ANME-2</td>
<td>40</td>
<td>GGC TAC CAC TCG GGC CGC</td>
<td>Treude et al. (2005)</td>
</tr>
<tr>
<td>AAA-FW-641</td>
<td>AAA, freshwater cluster</td>
<td>60</td>
<td>GGT CCC AAG CTT ACC AGT</td>
<td>This study</td>
</tr>
<tr>
<td>AAA-FW-834</td>
<td>AAA, freshwater cluster</td>
<td>60</td>
<td>TGC GGT CGC ACC GCA CCT</td>
<td>This study</td>
</tr>
<tr>
<td>TPM666</td>
<td>Thermoplasmatales</td>
<td>*</td>
<td>CCC AGG AGT ACC TCT AGC</td>
<td>This study</td>
</tr>
<tr>
<td>EUB338-I-III</td>
<td>Most Bacteria</td>
<td>35</td>
<td>GCT GCC TCC GTG AGG AGT</td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>Delta495a-c1</td>
<td>Most Deltaproteobacteria and most Gemmatimonadetes</td>
<td>25</td>
<td>AGT TAG CCG GTG CCT CT</td>
<td>Loy et al. (2002), Lücker et al. (2007)</td>
</tr>
<tr>
<td>DSS658</td>
<td>Desulfosarcina/Desulfococcus branch of Deltaproteobacteria</td>
<td>50</td>
<td>AGT TAG CCG GCC CT CTT</td>
<td></td>
</tr>
<tr>
<td>SEEP1a-473</td>
<td>SEEP-SRB1a group</td>
<td>30</td>
<td>TCC ACT TCC TCT CTC CAT</td>
<td>Schleifer et al. (2010)</td>
</tr>
<tr>
<td>HSEEP1a-453</td>
<td>Helper 1 for SEEP1a-473</td>
<td>45</td>
<td>ACG GAG TTA GCC GGT GCT TCC</td>
<td>Schleifer et al. (2010)</td>
</tr>
<tr>
<td>HSEEP1a-496</td>
<td>Helper 2 for SEEP1a-473</td>
<td>10</td>
<td>ACTCTACGGGAGCAGG</td>
<td>Schleifer et al. (2010)</td>
</tr>
<tr>
<td>SEEP1a-1441</td>
<td>SEEP-SRB1a group</td>
<td>45</td>
<td>CCC CTT GCC GGT TGG TCC</td>
<td></td>
</tr>
<tr>
<td>NON338</td>
<td>Negative control</td>
<td>10</td>
<td>ACTCTACGGGAGCAGG</td>
<td>Wallner et al. (1993)</td>
</tr>
<tr>
<td>S-D8ACT-0193-a-A-18</td>
<td>NC10 bacteria</td>
<td>50</td>
<td>CGC TGG CCC CCT TTG GTC</td>
<td></td>
</tr>
<tr>
<td>S-D8ACT-0447-a-A-18</td>
<td>NC10 bacteria</td>
<td>50</td>
<td>CGC CAG CAA GTC ATT CTT</td>
<td></td>
</tr>
</tbody>
</table>

Specificity (% vol/vol) formamide (FA), probe sequence, and references for the probes.

*Percent (vol/vol) formamide (FA) in hybridization buffer for hybridization.
the genome of the dominant bacteria Candidatus *Methylo-
mirabilis oxyfera* surprisingly showed that the apparently
anaerobic, denitrifying bacterium encoded, transcribed, and
expressed the well-established aerobic pathway for methane
oxidation (Ettwig et al., 2010).

Thermodynamic considerations, however, would not
only favor nitrate but also iron and manganese over sulfate
as an electron acceptor in AOM. This process has just
recently been described in sediments of the Eel River Basin,
where microorganisms were capable of using manganese
(birnessite) and iron (ferrihydrite) to oxidize methane (Beal
et al., 2009). However, because nitrate is not present at all
and iron (Dahl et al., 2010) and manganese only in very
small (Fe, Mn < 5 μM, Hanselmann & Hutter, 1998) and
hence not in sufficient amounts for quantitative methane
oxidation in the sediments of Lake Cadagno, those pathways
seem not very likely.

**Geochemical evidence for AOM**

The main purpose of this study was to investigate the
occurrence of AOM in a freshwater system containing
considerable amounts of sulfate (2 mM). Methane became
oversaturated with regard to atmospheric pressure, forming
free gas bubbles below 13 cm sediment depth, at a water
depth of 21 m in the center of Lake Cadagno. Gas escape
upon retrieval prevented precise quantification (see scatter
in data, Fig. 1a). A linear profile indicating diffusive
methane transport was obtained between 15 cm (2.9 mM
CH₄) and the sediment surface (0.5 mM). The sulfate profile
behaved inversely to the methane profile, starting with
values of 2.1 mM sulfate at the sediment surface and
decreasing to < 40 μM below 25 cm sediment depth
(Fig. 1a). The sulfide concentration profile showed two
subsurface maxima reaching > 500 μM, indicating
sulfide production at above 5 cm sediment depth and
around 12 cm sediment depth (Fig. 1b) and can be taken as
further support for AOM (Boetius et al., 2000), although a
fraction of the sulfide production might be related to
organoclastic sulfate reduction. A clear indication
for methane oxidation can be seen in the carbon isotopic
composition profile of methane. The carbon isotopic
composition of methane (δ¹³C-CH₄) was relatively uniform
at −71.8‰ between 30 and 5 cm depth (Fig. 1b). Above
5 cm, the isotope values of methane became increasingly
heavier (i.e. less negative) from −57.5 to −42.6‰ at the
sediment surface, indicating strong methane oxidation. The
fractionation against the heavier isotope was demonstrated
for aerobic (Barker & Fritz, 1981) as well as anaerobic
microbial oxidation of methane (Holler et al., 2009).
The increase of δ¹³C-CH₄ in Lake Cadagno, from an average
value of −71.8‰ below 5 cm to values of −42.6‰ in
the top 5 cm of the sediment (Fig. 1b), can be described
with a fractionation factor α of 1.031. This value is well in
the range found by Holler et al. (2009) for AOM taking
place in different cultures in the lab (α = 1.012–1.039) or
values measured in a boreal lake (α = 1.037, Kankaala et al.,
2007). Another possibility for the rather heavy δ¹³C-CH₄
values in the uppermost sediment layers would be aceto-
lastic methanogenesis; however, this is rather unlikely
because sulfate-reducing bacteria would outcompete
methanogens under the occurring sulfate concentration in
this zone (Martens & Berner, 1974). Diffusive flux calcula-
tions for methane revealed an annual flux of 430 mmol m⁻²

![Fig. 1. Geochemical gradients in Lake Cadagno sediment core. (a) Methane and sulfate concentrations in μM. (b) Sulfide concentrations in μM and δ¹³C of methane (in ‰ vs. Vienna Pee Dee Belemnite, VPDB). (c) Carbon isotope values (in ‰ vs. VPDB) of dissolved inorganic carbon (DIC) and carbonate. (d) Organic carbon concentrations (% of dry weight) and carbon isotopic composition (in ‰ vs. VPDB).](image-url)
in the uppermost sediment layer (0.5–3.5 cm) that is higher than the flux values for AOM zones from Treude et al. (2005) in sediments off the coast of Chile. Higher values in limnic systems are reasonable because methanogenesis is the main pathway for organic material breakdown compared with sulfate reduction in marine systems (Jørgensen, 1982).

AOM substantially increases alkalinity by producing isotopically light bicarbonate and causes the precipitation of carbonate (Hinrichs & Boetius, 2002), which is often used as a fossil signature of AOM (Thiel et al., 2001; Peckmann & Goedert, 2005). The same scheme could be observed in the retrieved sediment core: carbonate concentrations were in general very low (below 0.2%), but increased slightly toward the surface and reached approximately 0.6% at 5 cm sediment depth (data not shown). Calcium ion concentrations on the other hand decreased toward the top of the core from about 5 μM at 20 cm to 2 μM (data not shown), indicating the utilization of calcium ions to form calcium carbonates. The carbon isotopic composition of the solid carbonate phase varied between −4.3% and −6.4% below 20 cm and between 15 and 8 cm sediment depth. Between 20 and 16 cm and above 7 cm, a stronger depletion of down to −10.8% was observed (Fig. 1c). The latter layers could thus indicate zones in which dissolved inorganic carbon (DIC) derived from methane oxidation has led to precipitation of 13C-depleted carbonates.

Additionally, the carbon isotopic composition of the dissolved inorganic carbon (δ13C-DIC) was measured. The δ13C-DIC values decreased from −13.0‰ at the top to −19.0‰ at 2.5 cm sediment depth, but then increased steadily to 7.4‰ toward the bottom of the core (Fig. 1c). The very light values at the sediment surface could be explained by a mixture of inorganic carbon from the exchange with bottom water DIC (δ13C-DIC of −15 to −16‰, Bernasconi pers. commun.), the DIC derived from the breakdown of organic material settled from above (δ13C-Corg of organic material at the sediment surface is −31.4‰ and organic carbon concentrations reached 15.4%, Fig. 1d) and DIC derived from the oxidation of methane (δ13C-CH4 = −71.8‰ to −42.6‰). The 13C enrichment of the DIC pool with increasing sediment depth is due to hydrogenotrophic microbial methane production, in which the lighter 12C isotope is used for CH4 production, leaving the heavier 13C isotope behind in the DIC pool. To summarize, we observed (1) the production of light δ13C-DIC (−19‰) combined with isotopically heavy values for the residual methane (δ13C-CH4 = −42.6‰) in the uppermost sediments, (2) the precipitation of isotopically light carbonate (δ13C-CaCO3 = −10.8‰) and a depletion of Ca2⁺ in the pore-water above 6 cm sediment depth, and (3) the production of sulfide in this layer. Hence, based on all these findings, we propose that AOM takes place in Lake Cadagno sediments.

**Archaeal diversity in Lake Cadagno sediments**

To study the archaeal community involved in methane production or degradation in sediments of Lake Cadagno, two 16S rRNA gene clone libraries were constructed from 2 to 4 cm (42 clones) and 9 to 15 cm (43 clones) sediment depth (Fig. 2). Most Lake Cadagno 16S rRNA gene sequences showed the highest similarity to sequences of uncultivated microorganisms from soil or other freshwater habitats and archaeal diversity was low. Based on a 97% 16S rRNA gene sequence similarity criterion, only nine different phylotypes were detected (Table 2). The majority of 16S rRNA gene sequences belonged to the euryarchaeotal marine benthic group D (MBGD), found in a variety of limnic and marine habitats (e.g. Munson et al., 1997; Takai & Horikoshi, 1999; Galand et al., 2003; Mills et al., 2003; Vetriani et al., 2003; Coolen et al., 2004). In total, six distinct MBGD phylotypes were retrieved, three of which were only found in 2–4 cm sediment depth. One phylotype represented by 13 clones was unique to the 9–15 cm-depth interval and grouped with a clone sequence from acidic peatland (Cadillo-Quiroz et al., 2006), and two additional phylotypes included sequences from both sediment depth intervals.

The second archaeal lineage detected in lake Cadagno sediments is the AOM-associated archaea (AAA) clade of Euryarchaeota, which is most closely related to group GoM Arc1 repeatedly found in Gulf of Mexico cold seep sediments (Lloyd et al., 2006) and the known anaerobic methanotrophs of group ANME-2 (86–90% sequence similarity). Five sequences have been retrieved, but only from the 2- to 4-cm-depth interval (>98% sequence similarity to each other, one phylotype). The closest relative of these Cadagno clones is an archaeal clone sequence (accession number DQ369741; 95–99% sequence similarity), which has been retrieved from a bio reactor coupling methane oxidation to denitrification (Raghoebarsing et al., 2006). The archaea were later lost from the bio reactor biomass and the methane-based denitrification was assigned to NC10 bacteria (Ettwig et al., 2008). Other sequences in this clade were retrieved from different marine and freshwater habitats, such as an extinct Antarctic methane seep (Niemann et al., 2009), deep-sea sulfide chimneys (Schrenk et al., 2003), ground water (Nedelkova, database release, accession numbers AJ583380–AJ583384), and acidic peatland (Cadillo-Quiroz et al., 2006).

As third and fourth archaeal lineages, the crenarchaeotal marine benthic groups B and C (MBGB and MBGC) were identified (Table 2). These groups were represented by one
clone and three clones, respectively, from the 2- to 4-cm-depth horizon. MBGB, alternatively designated as deep-sea archaeal group (DSAG), and MBGC, a subgroup of the Miscellaneous Crenarchaeotal Group (MCG; Teske & Sørensen, 2008), are often found at seeps and in the deep biosphere (Biddle et al., 2006; Inagaki et al., 2006). Members of the marine ANME groups (ANME-1, ANME-2, ANME-3) were not found in the clone libraries from Lake Cadagno sediments.

**In situ quantification of bacteria and archaea**

*In situ* quantification of the total bacterial and archaeal cell numbers (cells were counted under the microscope, for details see 'Experimental procedures') in Lake Cadagno sediments using CARD-FISH and the general probes (ARCH915 and EUB338 I–III, targeting almost all bacteria and archaea) indicated an active microbial population. Bacteria strongly dominated the sediment in all depth layers.
Cell numbers that could be detected by CARD-FISH decreased from 86% to 94% of the total DAPI-stained cells (1.8–3.3/10^9 cells mL^-1) in 0–5 cm depth to only 37% in 10 cm (6.6–1.7/10^8 cells mL^-1). The abundance of AAA aggregates was very low and could not be exactly quantified (< 1 × 10^6 aggregates mL^-1). In Lake Cadagno sediments, dual hybridizations with probes for AAA and the domain Bacteria (probe EUB338 I–III) did not show the presence of any physically attached bacterial partners. We observed signals of single cells in the 0–3 cm depth layers, with one (S-D-BACT-0193-a-A-18) (Table 1, Fig. 4e) of the two probes used by Raghoebarsing et al. (2006) to detect NC10 bacteria, but could not detect any signal with the second probe (S-D-BACT-0447-a-A-18), indicating the first probe to be unspecific. Other known anaerobic methanotrophs, such as ANME-1 and ANME-2, were not detected by CARD-FISH.

Deltaproteobacteria were highly abundant and accounted for > 20% of the total cells in 0–3 cm sediment depths (Fig. 3). In 5 cm depth, the relative abundance was still high, with 15% of the total cells, but strongly decreased below that depth and only 1% of the total cells were detected between 15 and 29 cm depth. The morphology of detected cells was rod-shaped and uniform. Using probe DSS658, almost all deltaproteobacterial cells could be assigned to sulfate-reducing bacteria of the Desulfosarcina/Desulfococcus branch, which has been repeatedly reported to contain the ANME-partner bacteria (Fig. 3). To further resolve the phylogeny of detected DSS cells, we used two specific probes for SEEP-SRB1a, a DSS subgroup that has been shown to form consortia with ANME-2 archaea (Schreiber et al., 2010). Except for one cluster of roughly 40 cells in 2–3 cm depth, none of the detected DSS could be assigned to SEEP-SRB1a, thus indicating the presence of DSS cells different from those often associated with ANME-2 archaea at marine seeps.

In situ abundance of representatives of MBGD (Thermoplasmatales), the dominant clone group, was low, with ≪ 1% of the total cells in the AOM horizon above 5 cm depth.

**Microorganisms associated with the AOM zone of Lake Cadagno**

To explore the diversity of microorganisms potentially responsible for AOM, we investigated upper sediments from the AOM peak (2–4 cm), the bottom of the AOM zone (9–15 cm), and a deep horizon (23–29 cm). We could not find evidence for the occurrence of known ANME-1, -2, -3
consortia in the 16S rRNA clone libraries. Instead, sequences from group AAA were retrieved. The AAA clade is related to ANME-2 and consists only of uncultivated representatives from mainly limnic and terrestrial habitats, but also few marine sequences were included (Knittel & Boetius, 2009). Here, we present the first microscopic evidence for cell aggregates belonging to the AAA clade, which may be responsible for the observed AOM in Lake Cadagno sediments. Lake Cadagno bottom water and sediments do not contain any oxygen, nitrate, or nitrite, but high sulfate concentrations and thus, we propose that AOM is coupled to sulfate reduction in Lake Cadagno sediments. However, we cannot rule out a partial coupling to iron or manganese reduction.

Support that AOM might occur in the uppermost sediment layer using sulfate comes from co-existing sulfate-reducing bacteria (SRB) in the same sediment layers. About 20% of all bacteria belong to the Deltaproteobacteria and almost all of them belong to the DSS group (Fig. 3), of which subgroup SEEP-SRB1a is known to be involved in AOM (Knittel et al., 2003; Schreiber et al., 2010). However, the use of specific CARD-FISH probes to address the AOM-associated subgroup SEEP-SRB1a failed, indicating DSS other than SEEP-SRB1 being present in Lake Cadagno. Assuming that AAA are the AOM-mediating organisms, no physical contact between the archaea and any bacterial partner existed. Such monospecific aggregations of ANME cells have been repeatedly reported for marine seep sediment (e.g. Orphan et al., 2001, 2002; Knittel et al., 2005; Lösekann et al., 2007). These ANME-1, -2, or -3 aggregations have been shown to be as depleted in $^{13}$C as ANME/SRB aggregates suggesting that they are actively involved in AOM (Orphan et al., 2002). So far, neither any cultured SRB or methanogen has ever been shown to be capable of anaerobic growth on methane as a sole carbon source nor have genes for dissimilatory sulfate reduction been found on ANME-metagenomes (Hallam et al., 2004; Pernthaler et al., 2008; Meyerdierks et al., 2010). However, almost five decades ago, the sulfate-reducing bacterium *Desulfovibrio desulfuricans* was isolated and its ability to co-oxidize small amounts of methane when growing on other carbon sources like lactate was reported (Davis & Yarbrough, 1966). This indicates that it may possess the enzymatic machinery needed for AOM and shows the possibility that other microorganisms might have developed an efficient strategy for coupling AOM to sulfate reduction.

In Lake Cadagno sediments, DSS cell abundances strongly decreased with depth to almost zero at 9 cm depth (Fig. 4). This decrease is in line with the geochemical parameters suggesting that AOM takes place in the uppermost sediment layers. An additional hint for an involvement of those SRB in AOM comes from the isotopic signature of SRB-specific anteiso and iso $C_{15}$ fatty acids (Taylor & Parkes, 1983). These fatty acids showed $\delta^{13}$C values at 2.5 and 11 cm of down to $-44.8\%$ VPDB (for comparison, the average $\delta^{13}$C value for these fatty acids below both oxidation zones is $-32.9\%$ VPDB). These relatively light values measured in the oxidation zones indicate that sulfate-reducing bacteria are actively involved in methane oxidation. In contrast to the findings of fatty acids indicative of SRB, we could not find archaeal lipids (archaeol, hydroxy-archaeol) or only in such small amounts that prevented isotopic measurements. Interestingly, we did not detect sequences of known methanogenic and methanotrophic clades in the archaeal clone libraries in the two sediment horizons investigated. Other archaeal sequences retrieved from the AOM zone were affiliated with the crenarchaeotal marine benthic groups B and C (MBGB, MBGC), both of which are usually present in marine cold seep sediments and the deep biosphere. MBGB archaea, which have only been found in methane-rich
sedi-ments so far, have been reported to benefit directly or indirectly from AOM in marine sediments (Biddle et al., 2006; Inagaki et al., 2006; Teske & Sørensen, 2008). Biddle et al. (2006) suggested that members of the MBGB oxidize methane, but do not assimilate its carbon. Instead, they could use auxiliary carbon substrates and complex organic nutrients for the synthesis of cell material and growth. MBGC archaea of the MCG showed a much wider habitat range (based on clone libraries) including terrestrial sites and marine cold and hot environments (for a review, see Teske & Sørensen, 2008). A linkage to methane has not been shown so far, but archaeal carbon-isotopic signatures and polar lipids from MCG-dominated sediment layers indicate the utilization of buried organic carbon (Biddle et al., 2006).

Remarkably, neither sequences from the AAA clade, nor MBGB, nor MBGC sequences were found in the clone library from the bottom of the AOM zone. Here, members of the euryarchaeotal marine benthic group D were found exclusively. Usually, this group is rarely found in clone libraries, but nevertheless widely distributed in cold marine sediments (Kendall et al., 2007), seeps (Orphan et al., 2001; Knittel et al., 2005), deep sea sediments (Vetriani et al., 2003), deep subsurface sediments (Biddle et al., 2006), hydrothermal vents (Takai & Horikoshi, 1999), and mud volcanoes (Hejs et al., 2005), but also in mangrove and saline soils (Yan et al., 2006), estuary sediments, and salt marshes (e.g. accession number AF015978, database release).

Conclusions

Anaerobic methane oxidation occurs in freshwater sediments of Lake Cadagno. The shift toward a heavy carbon isotopic composition of methane (from $-71.8\%$ to $-42.6\%$, $\alpha = 1.031$) hints to AOM in the uppermost centimeters of the sediments. Further support comes from sulfide production and light $\delta^{13}\text{C}_{\text{CaCO}_3}$ values ($-10.8\%$) of precipitated carbonates in this horizon. AOM is most likely linked to sulfate reduction in this strong sulfidic environment because no nitrate was present in the sediments and the overlying anoxic water. Although only minor amounts of iron and manganese were present, we cannot exclude AOM linked to iron and manganese reduction. No anaerobic methano-trophs of clades ANME-1, -2, -3 responsible for AOM in marine systems could be detected. Instead, we present the first microscopic evidence for cell aggregates belonging to the AAA clade, which may be responsible for the observed AOM together with (or without) sulfate-reducing bacteria of the DSS branch. To prove this hypothesis, further experiments are needed such as Halogen In Situ Hybridization-Secondary Ion Mass Spectroscopy (HISH-SIMS, Musat et al., 2008), a method that has been successfully applied on Lake Cadagno planktonic samples. The process described here has implications for AOM taking place in environments where elevated sulfate concentrations occur in freshwater systems such as lakes in karst areas, volcanic lakes, and fjords where fresh and sea water merge. Furthermore, high methane emissions out of limnic systems like that proposed by Bastviken et al. (2004) are taking place in systems where aerobic methane oxidation is low and where AOM is not or only mildly taking place.

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