

## RESEARCH ARTICLE

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

Carsten J. Schubert<sup>1</sup>, Francisco Vazquez<sup>1</sup>, Tina Lösekann-Behrens<sup>2</sup>, Katrin Knittel<sup>2</sup>, Mauro Tonolla<sup>3</sup> & Antje Boetius<sup>4</sup>

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

Received 10 October 2010; revised 23 November 2010; accepted 27 November 2010. Final version published online 19 January 2011.

DOI:10.1111/j.1574-6941.2010.01036.x

Editor: Gary King

## Keywords

anaerobic methane oxidation; freshwater systems; archaea; sulfate-reducing bacteria; methanotrophs.

## Introduction

Methane is an effective greenhouse gas in the atmosphere. Although the methane concentration in the atmosphere is small compared with CO<sub>2</sub> (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

## 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 ( $\alpha = 1.031$ ) 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 ( $\delta^{13}\text{C} = -10\text{‰}$  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.

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 CO<sub>2</sub> and CH<sub>4</sub> 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 CH<sub>4</sub> year<sup>-1</sup> 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  $\text{SO}_4^{2-}$ ). 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  $2.4 \times 10^6 \text{ m}^3$  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 sub-aquatic 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  $\mu\text{M}$ ), but high sulfide (up to 280  $\mu\text{M}$ ) in the hypolimnion. Nitrate was not available below 12.5 m, ammonium concentrations increased to

40  $\mu\text{M}$  at 17.5 m depth (Halm *et al.*, 2009) and iron (II) was below 5  $\mu\text{M}$  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  $\text{CO}_2$  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  $-20^\circ\text{C}$ ) 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  $\mu\text{m}$  pore size (Whatman OE67) and analyzed using an ion chromatograph (150/4.0 mm ID column, eluent 3.2 mM  $\text{Na}_2\text{CO}_3$ , 1 mM  $\text{NaHCO}_3$ , detection limit 5  $\mu\text{mol L}^{-1}$ ; Metrosep A Supp 5, Metrohm 733; Herisau, Switzerland). Pore water samples for S(-II) analysis were filtered immediately after centrifugation through 0.45  $\mu\text{m}$  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  $\mu\text{mol L}^{-1}$ ; 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  $\text{CO}_2$  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 (BF<sub>3</sub>-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<sup>-1</sup> to 130 °C, and 4 °C min<sup>-1</sup> to 320 °C (20 min). The isotopic composition (δ<sup>13</sup>C against VPDB) of compounds of the fatty acid fraction was measured with a GC connected via a combustion interface to a mass spectrometer. δ<sup>13</sup>C 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:  $\delta^{13}\text{C} = \left( \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} - 1 \right) \times 1000\text{‰}$  and values are referenced against VPDB. The fractionation factor  $\alpha$  was calculated using the following formula:  $\alpha_{S/P} = R_S/R_P = (\delta_S + 1000)/(\delta_P + 1000)$ , 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]:

$$J = -\Phi \times D_s \frac{dc}{dx} \quad (1)$$

where  $J$  is the diffusive flux (mmol m<sup>-2</sup> a<sup>-1</sup>),  $\Phi$  is the porosity,  $D_s$  is the sediment diffusion coefficient (m<sup>2</sup> a<sup>-1</sup>),  $c$  is the concentration of methane (mmol m<sup>-3</sup>), 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  $D_{\text{methane}}$ :  $0.87 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$  [Eqn. (2)]:

$$D_s = \frac{D}{(1 + 3 \times (1 - \Phi))} \quad (2)$$

### 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 hexadecyltrimethylammonium 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=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  $15 \mu\text{g mL}^{-1}$  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  $10 \text{ mg mL}^{-1}$  lysozyme for 10 min at  $37^\circ\text{C}$ . All hybridizations were performed at  $46^\circ\text{C}$ . 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 1.** Oligonucleotide probes used in this study

Probe	Specificity	% FA	Probe Sequence (5'–3')	Reference
ARCH915	Most archaea	35	GTG CTC CCC CGC CAA TTC CT	Stahl & Amann (1991)
ANME-1-350	ANME-1	40	AGT TTT CGC GCC TGATGC	Boetius <i>et al.</i> (2000)
ANME-2-538	ANME-2	40	GGC TAC CAC TCG GGC CGC	Treude <i>et al.</i> (2005)
AAA-FW-641	AAA, freshwater cluster	60	GGT CCC AAG CCT ACC AGT	This study
AAA-FW-834	AAA, freshwater cluster	60	TGC GGT CGC ACC GCA CCT	This study
TPM666	Thermoplasmatales	*	CCC AGG AGT ACC TCT AGC	This study
EUB338 I–III	Most Bacteria	35	GCT GCC TCC CGT AGG AGT GCA GCCACC CGT AGG TGT GCT GCC ACC CGT AGG TGT	Daims <i>et al.</i> (1999)
Delta495a-c <sup>†</sup>	Most <i>Deltaproteobacteria</i> and most Gemmatimonadetes	25	AGT TAG CCG GTG CTT CCT AGT TAG CCG GCG CTT CCT AAT TAG CCG GTG CTT CCT	Loy <i>et al.</i> (2002), Lückner <i>et al.</i> (2007)
DSS658	<i>Desulfosarcina/Desulfococcus</i> branch of <i>Deltaproteobacteria</i>	50	TCC ACT TCC CTC TCC CAT	Manz <i>et al.</i> (1998)
SEEP1a-473	SEEP-SRB1a group	30	TTC AGT GAT ACC GTC AGT ATC CC	Schreiber <i>et al.</i> (2010)
HSEEP1a-453	Helper 1 for SEEP1a-473		RCG RTA TTR RCG CGG RAT AGG	Schreiber <i>et al.</i> (2010)
HSEEP1a-496	Helper 2 for SEEP1a-473		ACG GAG TTA GCC GGT GCT TCC	Schreiber <i>et al.</i> (2010)
SEEP1a-1441	SEEP-SRB1a group	45	CCC CTT GCG GGT TGG TCC	Schreiber <i>et al.</i> (2010)
NON338	Negative control	10	ACTCCTACGGGAGGCAGC	Wallner <i>et al.</i> (1993)
S*-DBACT-0193-a-A-18	NC10 bacteria	50	CGC TCG CCC CCT TTG GTC	Raghoebarsing <i>et al.</i> (2006)
S*-DBACT-0447-a-A-18	NC10 bacteria	50	CGC CGC CAA GTC ATT CGT	Raghoebarsing <i>et al.</i> (2006)

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

\*Stringent formamide concentration has not been determined; the probe was used at low stringency at 10% formamide.

<sup>†</sup>Used with competitors cDELTA495a-c according to Lückner *et al.* (2007).

<sup>‡</sup>Percent (vol/vol) formamide (FA) in hybridization buffer for hybridization.

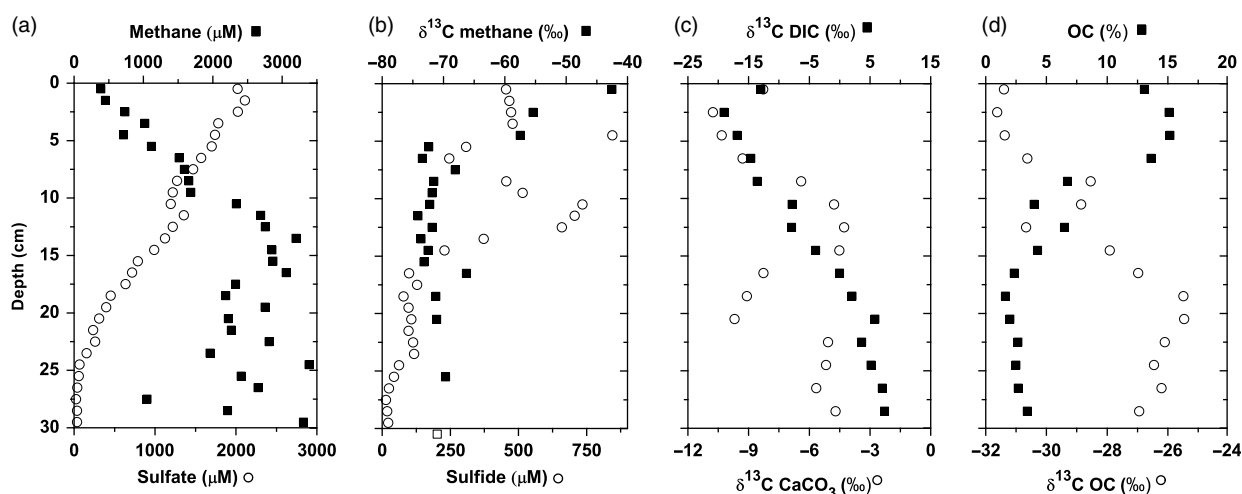
the genome of the dominant bacteria *Candidatus Methyloirabilis oxyfera* surprisingly showed that the apparently anaerobic, denitrifying bacterium encoded, transcribed, and expressed the well-established aerobic pathway for methane oxidation (Ettwig *et al.*, 2010).

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  $\mu\text{M}$ , 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  $\text{CH}_4$ ) 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  $\mu\text{M}$  below 25 cm sediment depth (Fig. 1a). The sulfide concentration profile showed two subsurface maxima reaching > 500  $\mu\text{M}$ , 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 ( $\delta^{13}\text{C}\text{-CH}_4$ ) 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  $\delta^{13}\text{C}\text{-CH}_4$  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  $\alpha$  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 ( $\alpha = 1.012\text{--}1.039$ ) or values measured in a boreal lake ( $\alpha = 1.037$ , Kankaala *et al.*, 2007). Another possibility for the rather heavy  $\delta^{13}\text{C}\text{-CH}_4$  values in the uppermost sediment layers would be acetoclastic 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 calculations for methane revealed an annual flux of 430  $\text{mmol m}^{-2}$



**Fig. 1.** Geochemical gradients in Lake Cadagno sediment core. (a) Methane and sulfate concentrations in  $\mu\text{M}$ . (b) Sulfide concentrations in  $\mu\text{M}$  and  $\delta^{13}\text{C}$  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).

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  $\mu\text{M}$  at 20 cm to 2  $\mu\text{M}$  (data not shown), indicating the utilization of calcium ions to form calcium carbonates. The carbon isotopic composition of the solid carbonate phase varied between  $-4.3\text{‰}$  and  $-6.4\text{‰}$  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  $-10.8\text{‰}$  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  $^{13}\text{C}$ -depleted carbonates.

Additionally, the carbon isotopic composition of the dissolved inorganic carbon ( $\delta^{13}\text{C}$ -DIC) was measured. The  $\delta^{13}\text{C}$ -DIC values decreased from  $-13.0\text{‰}$  at the top to  $-19.0\text{‰}$  at 2.5 cm sediment depth, but then increased steadily to  $7.4\text{‰}$  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 ( $\delta^{13}\text{C}$ -DIC of  $-15$  to  $-16\text{‰}$ , Bernasconi pers. commun.), the DIC derived from the breakdown of organic material settled from above ( $\delta^{13}\text{C}$ -Corg of organic material at the sediment surface is  $-31.4\text{‰}$  and organic carbon concentrations reached 15.4%, Fig. 1d) and DIC derived from the oxidation of methane ( $\delta^{13}\text{C}$ - $\text{CH}_4 = -71.8\text{‰}$  to  $-42.6\text{‰}$ ). The  $^{13}\text{C}$  enrichment of the DIC pool with increasing sediment depth is due to hydrogenotrophic microbial methane production, in which the lighter  $^{12}\text{C}$  isotope is used for  $\text{CH}_4$  production, leaving the heavier  $^{13}\text{C}$  isotope behind in the DIC pool.

To summarize, we observed (1) the production of light  $\delta^{13}\text{C}$ -DIC ( $-19\text{‰}$ ) combined with isotopically heavy values for the residual methane ( $\delta^{13}\text{C}$ - $\text{CH}_4 = -42.6\text{‰}$ ) in the uppermost sediments, (2) the precipitation of isotopically light carbonate ( $\delta^{13}\text{C}$ - $\text{CaCO}_3 = -10.8\text{‰}$ ) and a depletion of  $\text{Ca}^{2+}$  in the pore-water above 6 cm sediment depth, and (3)

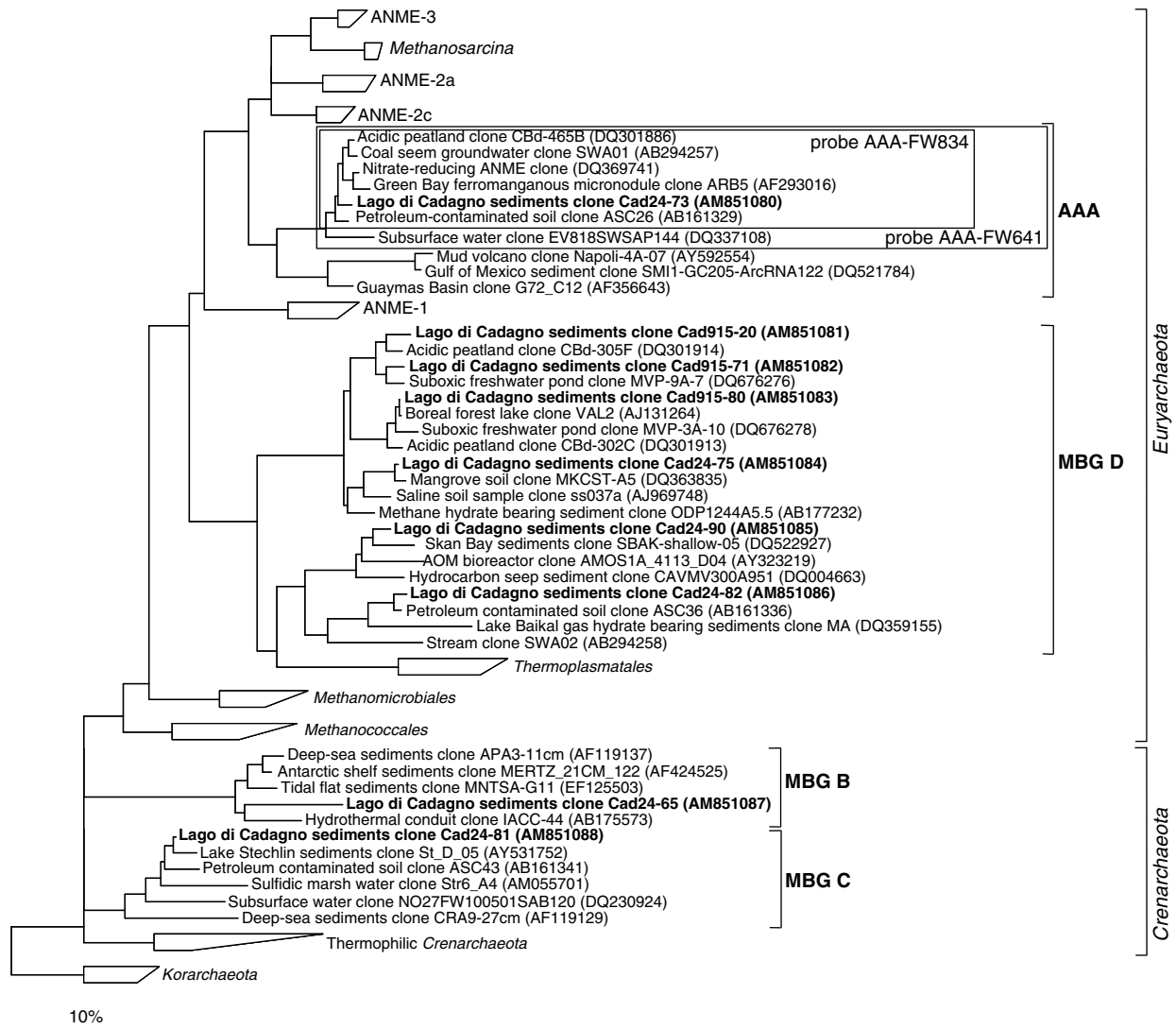
the production of sulfide in this layer. Hence, based on all these findings, we propose that AOM takes place in Lake Cadagno sediments.

### 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 bioreactor coupling methane oxidation to denitrification (Raghoebarsing *et al.*, 2006). The archaea were later lost from the bioreactor 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



**Fig. 2.** Phylogenetic tree showing the affiliations of Lake Cadagno 16S rRNA gene sequences to selected sequences of the domain *Archaea*. There are 9 sequences shown in the tree. These are representatives of the nine different phylotypes found in the clone libraries. The tree was calculated with nearly full-length sequences (> 1200 bp) by maximum-likelihood analysis in combination with a filter, which considers only 50% conserved regions of the 16S rRNA gene of *Archaea*. Branching orders that were not supported by all calculation methods are shown as multifurcations. Partial sequences were subsequently inserted into the reconstructed consensus tree by parsimony criteria, without allowing changes in the overall tree topology. Clone sequences from Lake Cadagno sediments are in boldface type: clones Cad24-\* were retrieved from sediments of 2–4 cm depth and clones Cad915-\* were retrieved from sediments of 9–15 cm depth. The bar indicates 10% estimated phylogenetic divergence. Boxes indicate the specificity of FISH probes used in this study. MBG: marine benthic group, AAA.

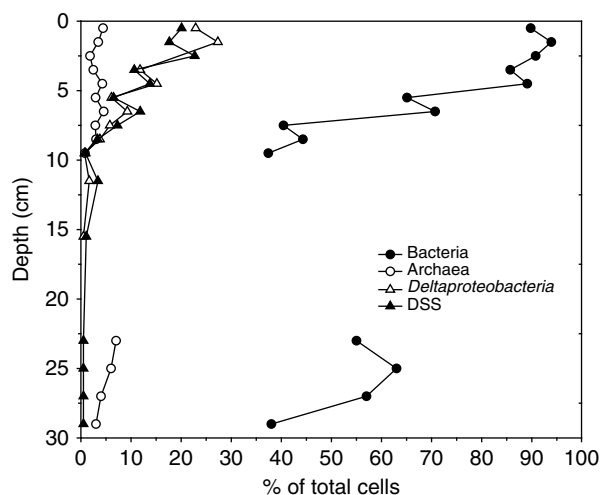
clone and three clones, respectively, from the 2- to 4-cm-depth horizon. MBGB, alternatively designated as deep-sea archaeal group (DSAG), and MBGC, a subgroup of the Miscellaneous Crenarchaeotal Group (MCG; Teske & Sørensen, 2008), are often found at seeps and in the deep biosphere (Biddle *et al.*, 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

**Table 2.** 16S rRNA gene libraries from Cadagno sediments

Affiliation	Representative clone in phylogenetic tree	Number of clones (2–4 cm depth)	Number of clones (9–15 cm depth)
AAA	Cad24-73	5	0
MBGD, Cadagno phylotype 1	Cad915-20	0	13
MBGD, Cadagno phylotype 2	Cad915-71	3	15
MBGD, Cadagno phylotype 3	Cad915-80	7	15
MBGD, Cadagno phylotype 4	Cad24-75	2	0
MBGD, Cadagno phylotype 5	Cad24-90	1	0
MBGD, Cadagno phylotype 6	Cad24-82	19	0
MBGB	Cad24-65	1	0
MBGC	Cad24-81	3	0
Total		41	43



**Fig. 3.** Relative abundance of bacteria, archaea, *Deltaproteobacteria*, and sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* branch (DSS) of *Deltaproteobacteria* as detected by CARD-FISH along the vertical profile in Lake Cadagno sediments. Total cells were counted using DAPI.

(Figs 3 and 4a, b). Cell numbers that could be detected by CARD-FISH decreased from 86% to 94% of the total DAPI-stained cells ( $1.8\text{--}3.3 \times 10^9$  cells mL<sup>-1</sup>) in 0–5 cm depth to only 37% in 10 cm ( $6.6 \times 10^8\text{--}1.7 \times 10^9$  cells mL<sup>-1</sup>). The relative abundance of archaea was low for all layers and ranged between 1% and 5% of the total cells ( $1.4 \times 10^7\text{--}1.1 \times 10^8$  cells mL<sup>-1</sup>). The background signal of samples, observed with the probe NON338, was negligible (0–0.1% of the total cells).

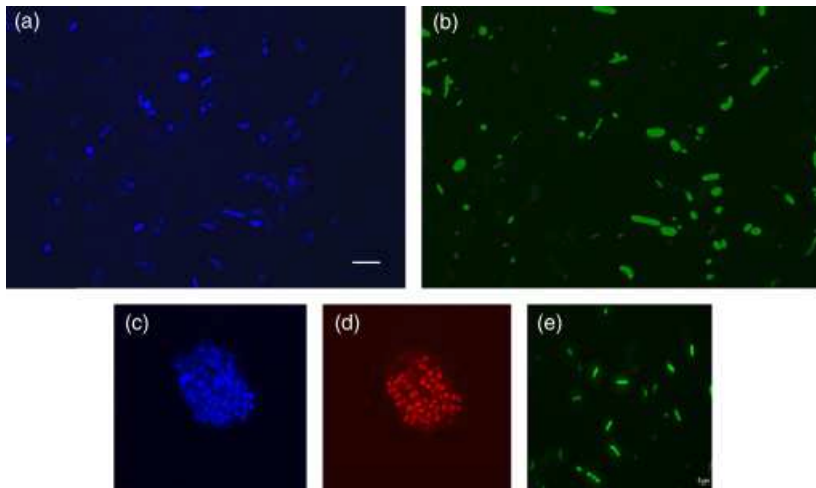
Based on the results of our clone library, we designed two new oligonucleotide probes for the archaeal cluster AAA (Table 1) to study their *in situ* abundance in Lake Cadagno sediments. Using the new probes, AAA-FW-641 and AAA-FW-834, monospecific consortia were detected in all layers between 1 and 10 cm depth. Here, these consortia are referred to as AAA aggregates. Typical consortia consist of approximately 10–100 loosely aggregated AAA cells (Fig. 4c and d). The abundance of AAA aggregates was very low and could not be exactly quantified ( $< 1 \times 10^6$  aggregates mL<sup>-1</sup>). 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 *deltaproteobacteria* 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.

The *in situ* abundance of representatives of MBGD (Thermoplasmatales), the dominant clone group, was low, with  $\ll 1\%$  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





**Fig. 4.** Single cells and cell aggregates in sediments from Lake Cadagno, visualized by CARD-FISH. Scale bars = 5  $\mu\text{m}$ . All panels show confocal laser scanning micrographs. (a) DAPI staining showing all cells in surface sediments. (b) Corresponding CARD-FISH image of bacteria (probe EUB338 I-III). (c,d) DAPI staining and corresponding FISH image of AAA aggregates in 5–6 cm sediment depth (probe AAA-FW-641). (e) FISH image of DSS bacteria in 1–2 cm sediment depth (probe DSS 658).

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  $^{13}\text{C}$  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  $\text{C}_{15}$  fatty acids (Taylor & Parkes, 1983). These fatty acids showed  $\delta^{13}\text{C}$  values at 2.5 and 11 cm of down to  $-44.8\text{‰}$  VPDB (for comparison, the average  $\delta^{13}\text{C}$  value for these fatty acids below both oxidation zones is  $-32.9\text{‰}$  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

sediments 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 archaeological 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 (Heijs *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  $-71.8\text{‰}$  to  $-42.6\text{‰}$ ,  $\alpha = 1.031$ ) 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 ( $-10.8\text{‰}$ ) 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 methanotrophs 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.

## Acknowledgements

We would like to thank Jutta Niggemann for helping with the sampling, Ruth Stierli and Martin Märki for porewater analyses, and Matthias Saurer and Rolf Siegwolf for the possibility to measure methane carbon isotopes at PSI. We acknowledge Stephan Wehling for help with cloning and Wiebke Rentzsch and Nicole Rödiger for help with FISH analysis. Michelle Botinelle is thanked for the logistic support necessary for performing the fieldwork and Helmut Brandl for discussion of pore water data. Funding has been provided by the Swiss Federal Institute of Aquatic Science and Technology (Eawag) and the Max Planck Society.

## References

- Alain K, Holler T, Musat F, Elvert M, Treude T & Krüger M (2006) Microbiological investigation of methane- and hydrocarbon-discharging mud volcanoes in the Carpathian Mountains, Romania. *Environ Microbiol* **8**: 574–590.
- Barker JF & Fritz P (1981) Carbon isotopic fractionation during microbial methane oxidation. *Nature* **293**: 289–291.
- Bastviken D, Cole J, Pace M & Tranvik L (2004) Methane emissions from lakes: dependence of lake characteristics, two regional assessments, and a global estimate. *Global Biogeochem Cy* **18**: GB4009.
- Beal EJ, House CH & Orphan VJ (2009) Manganese- and iron-dependent marine methane oxidation. *Science* **325**: 184–187.
- Berner RA (1980) *Early Diagenesis: A Theoretical Approach*. Princeton University Press, Princeton.
- Biddle JF, Lipp JS, Lever MA *et al.* (2006) Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. *P Natl Acad Sci USA* **103**: 3846–3851.
- Birch L, Hanselmann K & Bachofen R (1996) Heavy metal conservation in Lake Cadagno sediments: historical records of anthropogenic emissions in a meromictic lake. *Water Res* **30**: 679–687.
- Boetius A, Ravensschlag K, Schubert CJ *et al.* (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**: 623–626.
- Cadillo-Quiroz H, Brauer S, Yashiro E, Sun C, Yavitt J & Zinder S (2006) Vertical profiles of methanogenesis and methanogens in two contrasting acidic peatlands in central New York State, USA. *Environ Microbiol* **8**: 1428–1440.

- Coolen MJL, Hopmans EC, Rijpstra WIC, Muyzer G, Schouten S, Volkman JK & Sinninghe Damsté JS (2004) Evolution of the methane cycle in Ace Lake (Antarctica) during the Holocene: response of methanogens and methanotrophs to environmental change. *Org Geochem* **35**: 1151–1167.
- Dahl TW, Anbar AD, Gordon GW, Rosing MT, Frei R & Canfield DE (2010) The behavior of molybdenum and its isotopes across the chemocline and in the sediments of sulfidic Lake Cadagno, Switzerland. *Geochim Cosmochim Acta* **74**: 144–163.
- Daims H, Brühl A, Amann R & Schleifer KH (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434–444.
- Davis J & Yarbrough H (1966) Anaerobic oxidation of hydrocarbons by *Desulfovibrio desulfuricans*. *Chem Geol* **1**: 137–144.
- Del Don C, Hanselmann KW, Peduzzi R & Bachofen R (2001) The meromictic alpine Lake Cadagno: orographical and biogeochemical description. *Aquat Sci* **63**: 70–90.
- DeLong EF (1992) Archaea in coastal marine environments. *P Natl Acad Sci USA* **89**: 5685–5689.
- Dlugokencky EJ, Houweling S, Bruhwiler L, Masarie KA, Lang PM, Miller JB & Tans PP (2003) Atmospheric methane levels off: temporary pause or a new steady-state? *Geophys Res Lett* **30**. DOI: 10.1029/2003GL018126.
- Downing JA, Prairie YT, Cole JJ et al. (2006) The global abundance and size distribution of lakes, ponds, and impoundments. *Limnol Oceanogr* **51**: 2388–2397.
- Eller G, Kanel LK & Krüger M (2005) Cooccurrence of aerobic and anaerobic methane oxidation in the water column of Lake Plusssee. *Appl Environ Microb* **71**: 8925–8928.
- Ettwig K, Shima S, van de Pas-Schoonen KT et al. (2008) Denitrifying bacteria anaerobically oxidize methane in the absence of Archaea. *Environ Microbiol* **10**: 3164–3173.
- Ettwig KF, Butler MK, Le Paslier D et al. (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**: 543–548.
- Galand PE, Fritze H & Yrjala K (2003) Microsite-dependent changes in methanogenic populations in a boreal oligotrophic fen. *Environ Microbiol* **5**: 1133–1143.
- Hallam SJ, Putnam N, Preston CM, Detter JC, Rokhsar D, Richardson PM & DeLong EF (2004) Reverse methanogenesis: testing the hypothesis with environmental genomics. *Science* **305**: 1457–1462.
- Halm H, Musat N, Lam P et al. (2009) Co-occurrence of denitrification and nitrogen fixation in a meromictic lake, Lake Cadagno (Switzerland). *Environ Microbiol* **11**: 1945–1958.
- Hanselmann K & Hutter R (1998) Geomicrobiological coupling of sulfur and iron cycling in anoxic sediments of a meromictic lake: sulfate reduction and sulfide sources and sinks in Lake Cadagno. *Lake Cadagno: a meromictic Alpine lake* (Peduzzi R, Bachofen R & Tonolla M, eds), Documenta Ist. Ital. Idrobiol., **63**: 85–98.
- Heijs SK, Sinninghe Damste JS & Forney LJ (2005) Characterization of a deep-sea microbial mat from an active cold seep at the Milano mud volcano in the Eastern Mediterranean Sea. *FEMS Microbiol Ecol* **54**: 47–56.
- Hinrichs K-U & Boetius A (2002) The anaerobic oxidation of methane: new insights in microbial ecology and biogeochemistry. *Ocean Margin Systems* (Wefer G, Billett D, Hebbeln D, Jørgensen B, Schlüter M & Van Weering T, eds), pp. 457–477. Springer-Verlag, Berlin.
- Holler T, Wegener G, Knittel K, Boetius A, Brunner B, Kuypers M & Widdel F (2009) Substantial  $^{13}\text{C}/^{12}\text{C}$  and D/H fractionation during anaerobic oxidation of methane by marine consortia enriched *in vitro*. *Environ Microbiol Rep* **1**: 370–376.
- Inagaki F, Nunoura T, Nakagawa S et al. (2006) Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. *P Natl Acad Sci USA* **103**: 2815–2820.
- IPCC (2001) Climate change 2001: the scientific basis. *Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change* (Houghton JT, Ding Y, Griggs DJ, Noguer M, van der Linden PJ, Dai X, Maskell K & Johnson CA, eds), Cambridge University Press, Cambridge.
- Iversen N & Jørgensen B (1993) Diffusion coefficients of sulfate and methane in marine sediments: influence of porosity. *Geochim Cosmochim Acta* **57**: 571–578.
- Jørgensen BB (1982) Mineralization of organic matter in the sea bed – the role of sulfate reduction. *Nature* **296**: 643–645.
- Juutinen S, Rantakari M, Kortelainen P, Huttunen JT, Larmola T, Alm J, Silvola J & Martikainen PJ (2009) Methane dynamics in different boreal lake types. *Biogeosciences* **6**: 209–223.
- Kankaala P, Taipale S, Nykänen H & Jones RI (2007) Oxidation, efflux, and isotopic fractionation of methane during autumnal turnover in a polyhumic, boreal lake. *J Geophys Res* **112**: G02003.
- Kendall MM, Wardlaw GD, Tang CF, Bonin AS, Liu Y & Valentine DL (2007) Diversity of Archaea in marine sediments from Skan Bay, Alaska, including cultivated methanogens, and description of *Methanogenium boonei* sp. nov. *Appl Environ Microb* **73**: 407–414.
- Knittel K & Boetius A (2009) Anaerobic oxidation of methane: progress with an unknown process. *Ann Rev Microbiol* **63**: 311–334.
- Knittel K, Boetius A, Lemke A, Eilers H, Lochte K, Pfannkuche O & Linke P (2003) Activity, distribution, and diversity of sulfate reducers and other Bacteria in sediments above gas hydrate (Cascadia Margin, Oregon). *Geomicrobiol J* **20**: 269–294.
- Knittel K, Lösekann T, Boetius A, Kort R & Amann R (2005) Diversity and distribution of methanotrophic archaea at cold seeps. *Appl Environ Microb* **71**: 467–479.
- Lloyd KG, Lapham L & Teske A (2006) An anaerobic methane-oxidizing community of ANME-1b Archaea in hypersaline Gulf of Mexico sediments. *Appl Environ Microb* **72**: 7218–7230.
- López-Archilla AI, Moreira D, Velasco S & Lopez-Garcia P (2007) Archaeal and bacterial community composition of a pristine

- coastal aquifer in Donana National Park, Spain. *Aquatic Microbial Ecol* **47**: 123–139.
- Lösekan T, Knittel K, Nadalig T *et al.* (2007) Diversity and abundance of aerobic and anaerobic methane oxidizers at the Haakon Mosby Mud Volcano, Barents Sea. *Appl Environ Microb* **73**: 3348–3362.
- Loy A, Lehner A, Lee N *et al.* (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl Environ Microb* **68**: 5064–5081.
- Lücker S, Steger D, Kjeldsen KU, MacGregor BJ, Wagner M & Loy A (2007) Improved 16S rRNA-targeted probe set for analysis of sulfate-reducing bacteria by fluorescence *in situ* hybridization. *J Microbiol Meth* **69**: 523–528.
- Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.
- Manz W, Eisenbrecher M, Neu TR & Szewzyk U (1998) Abundance and spatial organization of gram negative sulfate-reducing bacteria in activated sludge investigated by *in situ* probing with specific 16S rRNA targeted oligonucleotides. *FEMS Microbiol Ecol* **25**: 43–61.
- Martens CS & Berner RA (1974) Methane production in the interstitial waters of sulfate-depleted marine sediments. *Science* **185**: 1167–1169.
- Mason B & Moore CB (1982) *Principles of Geochemistry*. John Wiley, London.
- Massana R, Murray AE, Preston CM & DeLong EF (1997) Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. *Appl Environ Microb* **63**: 50–56.
- Meyerdieks A, Kube M, Kostadinov I, Teeling H, Glöckner FO, Reinhardt R & Amann R (2010) Metagenome and mRNA expression analyses of anaerobic methanotrophic archaea of the ANME-1 group. *Environ Microbiol* **12**: 422–439.
- Mills HJ, Hodges C, Wilson K, MacDonald IR & Sobecky PA (2003) Microbial diversity in sediments associated with surface-breaching gas hydrate mounds in the Gulf of Mexico. *FEMS Microbiol Ecol* **46**: 39–52.
- Munson MA, Nedwell DB & Embley TM (1997) Phylogenetic diversity of Archaea in sediment samples from a coastal salt marsh. *Appl Environ Microb* **63**: 4729–4733.
- Musat N, Halm H, Winterholler BR *et al.* (2008) A single-cell view on the ecophysiology of anaerobic phototrophic bacteria. *P Natl Acad Sci USA* **105**: 17861–17866.
- Nauhaus K, Albrecht M, Elvert M, Boetius A & Widdel F (2007) *In vitro* cell growth of marine archaeal–bacterial consortia during anaerobic oxidation of methane with sulfate. *Environ Microbiol* **9**: 187–196.
- Niemann H, Fischer D, Graffe D *et al.* (2009) Biogeochemistry of a low-activity cold seep in the Larsen B area, western Weddell Sea, Antarctica. *Biogeosci Discuss* **6**: 5741–5769.
- Orphan VJ, House CH, Hinrichs K-U, McKeegan KD & DeLong EF (2001) Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. *Science* **293**: 484–487.
- Orphan VJ, House CH, Hinrichs KU, McKeegan KD & DeLong EF (2002) Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. *P Natl Acad Sci USA* **99**: 7663–7668.
- Peckmann J & Goedert JL (2005) Geobiology of ancient and modern methane-seeps. *Palaeogeogr Palaeoclimatol* **227**: 1–5.
- Pernthaler A, Pernthaler J & Amann R (2002) Fluorescence *in situ* hybridization and catalyzed reporter deposition (CARD) for the identification of marine Bacteria. *Appl Environ Microb* **68**: 3094–3101.
- Pernthaler A, Dekas AE, Brown CT, Goffredi SK, Embaye T & Orphan VJ (2008) Diverse syntrophic partnerships from deep-sea methane vents revealed by direct cell capture and metagenomics. *P Natl Acad Sci USA* **105**: 7052–7057.
- Raghoebarsing AA, Pol A, van de Pas-Schoonen KT *et al.* (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* **440**: 918–921.
- Ravenschlag K, Sahn K, Pernthaler J & Amann R (1999) High bacterial diversity in permanently cold marine sediments. *Appl Environ Microb* **65**: 3982–3989.
- Ravenschlag K, Sahn K, Knoblauch C, Jørgensen BB & Amann R (2000) Community structure, cellular rRNA content and activity of sulfate-reducing bacteria in marine Arctic sediments. *Appl Environ Microb* **66**: 3592–3602.
- Reeburgh WS, Ward BB, Whalen SC, Sandbeck KA, Kilpatrick KA & Kerkhof LJ (1991) Black Sea methane geochemistry. *Deep-Sea Res* **38** (suppl 2): S1189–S1210.
- Rhee TS, Kettle AJ & Andreae MO (2009) Methane and nitrous oxide emissions from the ocean: a reassessment using basin-wide observations in the Atlantic. *J Geophys Res* **114**: D12304.
- Schramm A, Fuchs BM, Nielsen JL, Tonolla M & Stahl DA (2002) Fluorescence *in situ* hybridization of 16S rRNA gene clones (Clone-FISH) for probe validation and screening of clone libraries. *Environ Microbiol* **4**: 713–720.
- Schreiber L, Holler T, Knittel K, Meyerdieks A & Amann R (2010) Identification of the dominant sulfate-reducing bacterial partner of anaerobic methanotrophs of the ANME-2 clade. *Environ Microbiol* **12**: 2327–2340.
- Schrenk MO, Kelley DS, Delaney JR & Baross JA (2003) Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. *Appl Environ Microb* **69**: 3580–3592.
- Schubert CJ, Coolen MJL, Neretin LN *et al.* (2006) Aerobic and anaerobic methanotrophs in the Black Sea water column. *Environ Microbiol* **8**: 1844–1856.
- Schubert CJ, Lucas FS, Durisch-Kaiser E, Stierli R, Diem T, Scheidegger O, Vazquez F & Mueller B (2010) Oxidation and emission of methane in a monomictic lake (Rotsee, Switzerland). *Aquat Sci* **72**: 455–466.
- Stahl DA & Amann RI (1991) Development and application of nucleic acid probes in bacterial systematics. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 205–248. John Wiley & Sons Ltd, Chichester, UK.

- Takai K & Horikoshi K (1999) Genetic diversity of archaea in deep-sea hydrothermal vent environments. *Genetics* **152**: 1285–1297.
- Taylor J & Parkes R (1983) The cellular fatty acids of the sulphate reducing bacteria, *Desulfobacter* sp., *Desulfobulbus* sp., and *Desulfovibrio desulfuricans*. *J Gen Microbiol* **129**: 3303–3309.
- Teske A & Sørensen KB (2008) Uncultured archaea in deep marine subsurface sediments: have we caught them all? *ISME J* **2**: 3–18.
- Thiel V, Peckmann J, Richnow HH, Luth U, Reitner J & Michaelis W (2001) Molecular signal for anaerobic methane oxidation in Black Sea seep carbonates and microbial mats. *Mar Chem* **73**: 97–112.
- Treude T, Niggemann J, Kallmeyer J, Wintersteller P, Schubert CJ, Boetius A & Jørgensen BB (2005) Anaerobic oxidation of methane and sulfate reduction along the Chilean continental margin. *Geochim Cosmochim Acta* **69**: 2767–2779.
- Vetriani C, Tran HV & Kerkhof LJ (2003) Fingerprinting microbial assemblages from the oxic/anoxic chemocline of the Black Sea. *Appl Environ Microb* **69**: 6481–6488.
- Wagener S, Schulz S & Hanselmann K (1990) Abundance and distribution of anaerobic protozoa and their contribution to methane production in Lake Cadagno (Switzerland). *FEMS Microbiol Ecol* **74**: 39–48.
- Wallner G, Amann R & Beisker W (1993) Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganism. *Cytometry* **14**: 136–143.
- Wuebbles DJ & Hayhoe K (2002) Atmospheric methane and global change. *Earth-Sci Rev* **57**: 177–210.
- Yan B, Hong K & Yu ZN (2006) Archaeal communities in mangrove soil characterized by 16S rRNA gene clones. *J Microbiol* **44**: 566–571.
- Zhou J, Brunns MA & Tiedje JM (1996) DNA recovery from soils of diverse composition. *Appl Environ Microb* **62**: 316–322.