RESEARCH ARTICLE

Trace methane oxidation and the methane dependency of sulfate reduction in anaerobic granular sludge

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Abstract

This study investigates the oxidation of labeled methane (CH_4) and the CH_4 dependence of sulfate reduction in three types of anaerobic granular sludge. In all samples, ^{13}C-labeled CH_4 was anaerobically oxidized to ^{13}C-labeled CO_2, while net endogenous CH_4 production was observed. Labeled-CH_4 oxidation rates followed CH_4 production rates, and the presence of sulfate hampered both labeled-CH_4 oxidation and methanogenesis. Labeled-CH_4 oxidation was therefore linked to methanogenesis. This process is referred to as trace CH_4 oxidation and has been demonstrated in methanogenic pure cultures. This study shows that the ratio between labeled-CH_4 oxidation and methanogenesis is positively affected by the CH_4 partial pressure and that this ratio is in methanogenic granular sludge more than 40 times higher than that in pure cultures of methanogens. The CH_4 partial pressure also positively affected sulfate reduction and negatively affected methanogenesis: a repression of methanogenesis at elevated CH_4 partial pressures confers an advantage to sulfate reducers that compete with methanogens for common substrates, formed from endogenous material. The oxidation of labeled CH_4 and the CH_4 dependence of sulfate reduction are thus not necessarily evidence of anaerobic oxidation of CH_4 coupled to sulfate reduction.

Introduction

Upflow anaerobic sludge bed (UASB) reactors are widely applied for the treatment of organic-rich wastewaters and the concomitant production of biogas (Frankin, 2001). In sequences of microbial conversions, complex organic matter is degraded to H_2 and CO_2, formate and acetate. These compounds are subsequently used by methanogens (Table 1). The methanogenic communities are present in compact granules termed anaerobic granular sludge (Hulshoff Pol *et al.*, 2004). Many organic-rich wastewaters also contain sulfate, for example wastewaters from tanneries and the pulp and paper industry (Lens *et al.*, 1998). In those cases, part of the organic matter is used as an electron donor for sulfate reduction (Muyzer & Stams, 2008). The methanogenic substrates H_2 and CO_2, formate and acetate can also be used by sulfate reducers (Table 1). The presence of both methane (CH_4) and sulfate in those UASB reactors might allow the presence of microorganisms capable of mediating CH_4 oxidation coupled to sulfate reduction, especially because the long solid retention time in UASB reactors, commonly exceeding 6.5 months (Hulshoff Pol *et al.*, 2004), can support slow-growing microorganisms.

Anaerobic oxidation of methane (AOM) coupled to sulfate reduction according to Eqn. (1) occurs in anoxic marine sediments and is an important process in the global carbon cycle (Valentine & Reeburgh, 2000; Hinrichs & Boetius, 2002; Nauhaus *et al.*, 2002)

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad \Delta G^\circ = -16.6\,\mathrm{kJ\,mol^{-1}} \qquad (1)$$

Uncultured archaea, putatively called anaerobic methanotrophs (ANME) and distantly related to cultivated members from the methanogenic orders *Methanosarcinales* and *Methanomicrobiales*, are involved in AOM in marine sediments (Hinrichs *et al.*, 1999; Orphan *et al.*, 2002; Knittel *et al.*, 2005; Knittel & Boetius, 2009). The estimates of the

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Table 1. Stoichiometry and Gibbs-free energy changes of conversions that play a role in sulfate-reducing bioreactors

Eq.	Reaction equations, in which 8 e-mol are converted	$\Delta G^{\circ\prime}$ (kJ mol^{-1})
Sulfate reduction		
1	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	−48
2	$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	−152
3	$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$	−17
Methanogenesis		
4	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	−31
5	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−136

Gibbs-free energy changes were calculated from Thauer *et al.* (1977)

doubling time of ANME vary between 1 and 7 months (Girguis *et al.*, 2005; Nauhaus *et al.*, 2007; Krüger *et al.*, 2008; Meulepas *et al.*, 2009). ANME often occur in consortia with sulfate-reducing bacteria (Boetius *et al.*, 2000; Orphan *et al.*, 2001; Michaelis *et al.*, 2002; Elvert *et al.*, 2003; Knittel *et al.*, 2003). It has been suggested that a methanotrophic archeaon produces an electron carrier compound from CH_4, which is subsequently utilized by the sulfate-reducing partner (Zehnder & Brock, 1980; Alperin & Reeburgh, 1985; Hoehler *et al.*, 1994; DeLong, 2000). However, it remains unclear which electron carrier compounds are transferred from the methanotrophs to the sulfate-reducing bacteria. There is evidence that the ANME are mediating a form of reversed methanogenesis. ANME-1 contain nearly all genes typically associated with CH_4 production (Hallam *et al.*, 2004; Meyerdierks *et al.*, 2010), and two methyl-coenzyme M reductase analogs were found to make up to 10% of the extracted soluble proteins from AOM-mediating microbial mats from the Black Sea (Krüger *et al.*, 2003).

Pure cultures of methanogenic archaea also oxidize CH_4 to CO_2 anaerobically (Zehnder & Brock, 1979; Harder, 1997; Moran *et al.*, 2004). Unlike AOM in marine sediments, trace methane oxidation (TMO) is not coupled to sulfate reduction, but occurs during net methanogenesis. Moran *et al.* (2004, 2007) referred to this process as TMO. Quantification of CH_4 oxidation during net CH_4 production requires the use of isotopically labeled CH_4. Zehnder & Brock (1979) reported TMO in all of the nine methanogenic strains investigated, TMO occurred during hydrogenotrophic, methylotrophic and aceticlastic methanogenesis. The amounts of CH_4 oxidized varied between 0.001% and 0.36% of the amount of CH_4 produced. The biologically produced ^{14}C-labeled CH_4 used by Zehnder & Brock (1979) was likely contaminated with ^{14}C-labeled carbon monoxide, which might have resulted in an overestimation of the CH_4 oxidation (Harder, 1997). Using pure ^{14}C-labeled CH_4, Harder (1997) showed TMO by several methanogenic cultures growing on methanol or hydrogen/CO_2; the ratios between CH_4 oxidation and production were not determined. Moran *et al.* (2004) reported the highest CH_4 oxidation to CH_4 production ratio during methanogenic growth on trimethylamine ($0.36 \pm 0.05\%$).

Labeled-CH_4 oxidation during net CH_4 production was also observed in anoxic sediments, digested sewage sludge and anaerobically stabilized sewage sludge, but at much higher CH_4 oxidation to CH_4 production ratios compared with pure cultures (Zehnder & Brock, 1980; Harder, 1997). The CH_4 oxidation was 90% of the CH_4 production in digested sewage sludge at a CH_4 partial pressure of 2.0 MPa and in the presence of 10 mM ferrous sulfate (Zehnder & Brock, 1980). According to Schilov *et al.* (1999), acetoclastic methanogenesis can even be reversed at a CH_4 pressure of 10 MPa in granular sludge, consisting of *Methanosarcina* and *Methanosaeta* spp.-dominated mixed cultures.

This study investigates the capacity of anaerobic granular sludge from UASB reactors to oxidize CH_4 anaerobically. To assess whether CH_4 oxidation in anaerobic granular sludge can contribute to sulfate reduction, $^{13}CH_4$ oxidation, CH_4 production and sulfate reduction rates in the presence and absence of sulfate, and in the presence and absence of 20 mM bromoethanesulfonate (an inhibitor for methanogenesis) were quantified. In addition, the effect of the CH_4 partial pressure on sulfate reduction, $^{13}CH_4$ oxidation and methanogenesis was evaluated.

Materials and methods

Biomass sources

Granular sludge samples were obtained from three full-scale mesophilic UASB reactors: a methanogenic reactor treating wastewater from paper mills (Industriewater Eerbeek, Eerbeek, the Netherlands, June 2005), a methanogenic reactor treating distillery wastewater (Nedalco, Bergen op Zoom, the Netherlands, July 2005) and a sulfate-reducing reactor fed with ethanol (Emmtec, Emmen, the Netherlands, May 2006). Additionally, a mix of crushed methanogenic (Eerbeek) and sulfate-reducing (Emmtec) sludge was used. The granules (2–4 mm) were crushed by pressing granules sequentially through needles with diameters of 1.2, 0.8 and 0.5 mm. All incubations were started within 3 months after sludge collection. The sludges were stored anaerobically at 4 °C and washed four times with anoxic medium before inoculation.

Medium

The basal medium consisted of: NaCl ($7\,g\,L^{-1}$), $MgCl_2 \cdot 6H_2O$ ($1.2\,g\,L^{-1}$), KCl ($0.5\,g\,L^{-1}$), NH_4Cl ($0.3\,g\,L^{-1}$), $CaCl_2$ ($0.15\,g\,L^{-1}$), Na_2SO_4 ($2.8\,g\,L^{-1}$), KH_2PO_4 ($0.43\,g\,L^{-1}$), $K_2HPO_4 \cdot 3H_2O$ ($1.56\,g\,L^{-1}$), a trace element solution ($1\,mL\,L^{-1}$), a $0.5\,g\,L^{-1}$ resazurine solution ($1\,mL\,L^{-1}$) and

demineralized water (Weijma *et al.*, 2000a). The trace element solution contained: $FeCl_2 \cdot 4H_2O$ (1500 mg L^{-1}), $CoCl_2 \cdot 2H_2O$ (190 mg L^{-1}), $MnCl_2 \cdot 4H_2O$ (100 mg L^{-1}), $ZnCl_2$ (70 mg L^{-1}), H_3BO_3 (62 mg L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (36 mg L^{-1}), $NiCl_2 \cdot 6H_2O$ (24 mg L^{-1}), $CuCl_2 2H_2O$ (17 mg L^{-1}) and HCl 37% (7 mL L^{-1}). The final pH of the medium was 7.2. Resazurine was added to check whether the conditions were anaerobic; it becomes colorless at a redox below −110 mV and becomes pink at a redox above −51 mV. The medium was boiled, cooled down under a nitrogen (N_2) flow and transferred to stock bottles with an N_2 headspace. For control incubations, a stock was made with medium from which the sodium sulfate was omitted.

Ambient pressure sulfate reduction, methanogenesis and ^{13}C-CH_4 oxidation assays

To assess AOM, and the potential coupling with sulfate reduction, by anaerobic granular sludge, ambient pressure incubations were performed with ^{13}C-labeled CH_4 ($^{13}CH_4$) in 120-mL serum bottles. The $^{13}CH_4$ gas was supplied by Campro (Veenendaal, the Netherlands) and had a purity of 99%, 1.0% $^{12}CH_4$ being the sole impurity. After inoculation, the bottles were closed with butyl rubber stoppers sealed with crimp seals and flushed with N_2. Subsequently, the bottles were partly vacuated and filled with 90 mL medium from an anaerobic stock using syringes and needles. Finally, the headspaces of the bottles were made vacuum again (to a residual pressure of *c.* 5 kPa) and filled with 0.17 MPa N_2 or $^{13}CH_4$. The bottles were incubated at 30 °C in an orbital shaker controlled at 100 r.p.m. Liquid (2.5 mL) and headspace (100 μL) samples were taken weekly for pH, sulfate, sulfide, fatty acids, alcohols and gas composition analyses. In addition, the headspace pressure and the weight of each bottle (as a measure for liquid and headspace volume) were measured.

Incubations were performed in duplicate with Eerbeek sludge (0.00, 0.05, 0.1, 0.2 and 0.3 g volatile suspended solids; VSS), Nedalco sludge (0.2 g_{VSS}), Emmtec sludge (0.2 g_{VSS}) and a mix of crushed Eerbeek (0.1 g_{VSS}) and Emmtec (0.1 g_{VSS}) sludge. Each sludge type was incubated with an N_2 headspace, a $^{13}CH_4$ headspace or a $^{13}CH_4$ headspace with a sulfate-free medium. The following control incubations with $^{13}CH_4$ and sulfate were carried out: no biomass, autoclaved Eerbeek sludge (0.2 g_{VSS}) and Eerbeek sludge inhibited by 20 mM bromoethanesulfonate.

Effect of the CH_4 partial pressure on sulfate reduction

The effect of the CH_4 partial pressure on sulfate reduction by methanogenic sludge was investigated by incubating Eerbeek sludge (0.5 g_{VSS}) under a headspace of 0.17 MPa N_2, 0.17 MPa CH_4, 1.1 MPa N_2 or 1.1 MPa CH_4. The 0.17 MPa incubations were performed in 1-L serum bottles closed with butyl rubber stoppers, and the 1.1 MPa incubations were performed in 0.60-L pressure vessels (Parr, Moline, IL). After adding the sludge, the bottles or vessels were closed and flushed with N_2 gas. Subsequently, 500 mL anaerobic medium from the stock bottle was added and the headspaces of the serum bottles and vessels were flushed again and filled with N_2 or CH_4. The bottles were incubated at 30 °C in an orbital shaker controlled at 100 r.p.m., whereas the pressure vessels were controlled at 30 °C and equipped with a stirrer operated at 100 r.p.m. Three times a week, liquid samples (2.5 mL) were taken for pH, sulfate and sulfide analyses.

Effect of the CH_4 partial pressure on CH_4 production and ^{13}C-CH_4 oxidation

The effect of the CH_4 partial pressure on CH_4 production and $^{13}CH_4$ oxidation rates was assessed in triplicate incubations with Eerbeek sludge (0.02 g_{VSS}) and Nedalco sludge (0.02 g_{VSS}) at atmospheric (0.101 MPa) and elevated (10.1 MPa) pressure. These tests were performed in glass tubes (18 mL), sealed with a butyl rubber stopper and a cap at one site and equipped with a piston at the opposite site (Fig. 1; De Glasinstrumentenmakerij, Wageningen, the Netherlands). Because the plunger was able to move freely, the pressure inside the tube was the same as outside. The top part of the piston was made from rubber and precisely fitted the tube. The glass tubes did not leak: in blank incubations, the total volume, measured at ambient pressure, did not change. The glass tubes were filled with sludge, closed, flushed with N_2 and filled with 9 mL medium. After removing the N_2 gas (with a syringe and needle), 3 mL $^{13}CH_4$ was added. The glass tubes were incubated unshaken at 30 °C in a nonpressurized incubator or in a 2.0-L pressure vessel (Parr) filled with 1.8 L water. The pressure vessel was

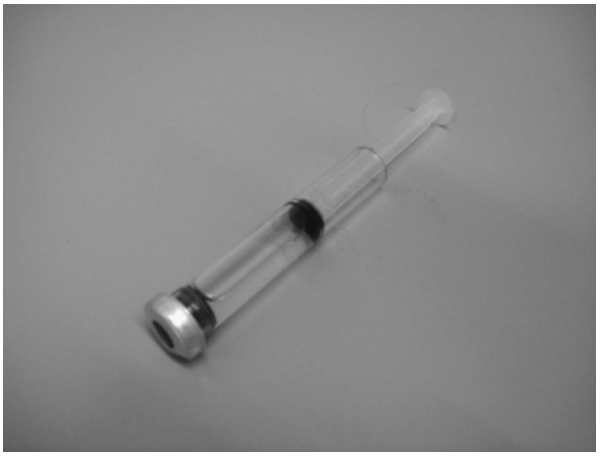

Fig. 1. Photograph of a tube with a piston used for the high-pressure incubations.

pressurized with N_2 gas. The pH, liquid volume, gas volume and gas composition were measured weekly. To do so, the pressure vessel had to be depressurized. Both pressurization and depressurization were performed gradually (over a period of 2 h).

Analyses

Before analysis, liquid samples were filtered over a 0.2-μm cellulose acetate membrane filter (Schleicher & Schuell OE 66, Schleicher & Schuell, Dassel, Germany). Sulfide was measured photometrically using a standard kit (LCK 653) and a spectrophotometer (Xion 500) both from Hach Lange (Düsseldorf, Germany). This method accounted for all dissolved sulfide species (H_2S, HS^- and S^{2-}). Sulfate was measured on a DX-600 ion chromatograph (Dionex Corporation, Salt Lake City) as described previously (Sipma *et al.*, 2004). Volatile fatty acids, methanol and ethanol were analyzed on an HP 5890A gas chromatograph (Hewlett Packard, Palo Alto) according to Weijma *et al.* (2000b).

The headspace composition was measured on a GC-MS from Interscience (Breda, the Netherlands). The GC-MS system was composed of a Trace GC equipped with a GC-GasPro column (30 m × 0.32 mm; J & W Scientific, Folsom, CA) and an Ion-Trap MS. Helium was the carrier gas at a flow rate of 1.7 mL min^{-1}. The column temperature was 30 °C. The fractions of CH_4 and CO_2 in the headspace were derived from the peak areas in the gas chromatograph. The fractions of ^{13}C-labeled CH_4 ($^{13}CH_4$) and ^{13}C-labeled CO_2 ($^{13}CO_2$) were derived from the mass spectrum as done by Shigematsu *et al.* (2004). The method was checked using standards with known mixtures of $^{12}CO_2$, $^{13}CO_2$, $^{13}CH_4$ and $^{12}CH_4$.

The pressure in the bottles and tubes was determined using a portable membrane pressure unit, WAL 0–0.4 MPa absolute (WalMess und Regelsysteme, Oldenburg, Germany). The pH was checked by means of pH paper (Macherey-Nagel, Düren, Germany). The VSS and total suspended solids contents of the wet sludge were analyzed according to standard methods (American Public Health Association, 1995).

A previously constructed archaeal clone library of Eerbeek sludge (Roest *et al.*, 2005) was used to perform a similarity search against sequences deposited in publicly available databases till January 2010. The search was performed using the NCBI BLAST search tool (BLASTN; http://www.ncbi.nlm.nih.gov/BLAST/).

Calculation of absolute amounts and specific rates

The total amounts of SO_4^{2-}, sulfide, $^{13}CH_4$, $^{12}CH_4$, $\sum ^{13}CO_2$ ($^{13}CO_2$ and $H^{13}CO_3^-$) and $\sum ^{12}CO_2$ ($^{12}CO_2$ and $H^{12}CO_3^-$) per bottle or $^{12}CH_4$ and $\sum ^{13}CO_2$ per tube were calculated according to:

$$SO_4^{2-} = [SO_4^{2-}] \times V_{liquid}$$

$$\text{sulfide} = [\text{sulfide}] \times V_{liquid}$$

$$^{13}CH_4 = f^{13}CH_4 \times P \times V_{gas} \quad (\text{same for } ^{12}CH_4)$$

$$\begin{aligned}\sum ^{13}CO_2 &= {}^{13}CO_2 + H^{13}CO_3^- \\ &= f^{13}CO_2 \times P \times (V_{gas} + V_{liquid}/k \\ &\quad \times (1 + K_a/[H^+]) \quad (\text{same for } ^{12}CO_2)\end{aligned}$$

The symbols indicate the following: V_{liquid} is the liquid volume in the serum bottle or tube, V_{gas} the gas volume in the serum bottle or tube, k the Henry's law constant for CO_2 at sampling temperature (20 °C): 0.0388 mol L^{-1}, K_a the dissociation constant of H_2CO_3: 4.5 10^{-7}, P the pressure at sampling temperature and f the fraction.

$^{12}CH_4$ production, $\sum ^{13}CO_2$ production, $\sum ^{13}CO_2$ production and sulfate reduction rates were obtained from a line plotted through the first five successive data points.

Results

CH_4 dependence of sulfate reduction by methanogenic sludge

Figure 2 compares the development of sulfate and sulfide with time for incubations with Eerbeek granular sludge at different N_2 and CH_4 partial pressures. All four incubations showed sulfate removal coupled to sulfide production at a more or less constant rate. However, sulfate reduction was faster at a higher CH_4 partial pressure. The increased sulfate reduction was a result of the increased CH_4 partial pressure rather than the increased total pressure because an elevated N_2 pressure did not result in an increased sulfate reduction rate.

AOM by anaerobic granular sludge

A series of incubations were performed to assess the ability of anaerobic sludge to oxidize $^{13}CH_4$ anaerobically (Figs 3–5). In all incubations with $^{13}CH_4$ and 0.2 g_{VSS} non-autoclaved methanogenic sludge (Eerbeek or Nedalco sludge) between 0.04 and 0.22 mmol $\sum ^{13}CO_2$ was produced (Fig. 5e, f, h, i, j and l). The fraction of $\sum ^{13}CO_2$ of the total $\sum CO_2$ in these incubations was between 5% and 23%. In controls without $^{13}CH_4$, the amount of $\sum ^{13}CO_2$ formed remained below 0.01 mmol and the fraction $\sum ^{13}CO_2$ of the total $\sum CO_2$ was always equal to the natural abundance of 1.07 (±0.1)% (Fig. 5a–d). In all incubations, the volatile fatty acids, methanol and ethanol were measured, but not presented if the absolute amounts were lower than 0.01 mmol.

Oxidation of $^{13}CH_4$ by molecular oxygen is unlikely, because in all incubations, the liquid remained colorless,

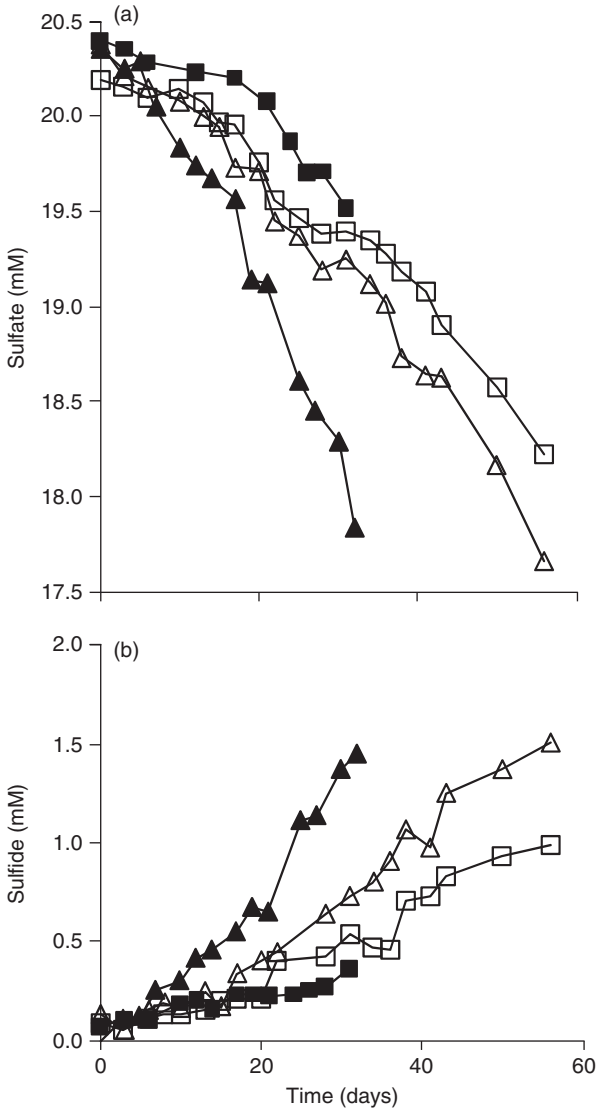

Fig. 2. Effect of the methane partial pressure and total pressure on sulfate removal (a) and sulfide production (b) in batch incubations with 0.5 g_{VSS} Eerbeek sludge. The headspaces of the different incubations contained: 0.00 MPa CH_4 and 0.16 MPa N_2 (□), 0.00 MPa CH_4 and 1.1 MPa N_2 (■), 0.16 MPa CH_4 (△) and 1.1 MPa CH_4 (▲).

indicating that the redox was lower than -51 mV (at which resazurine turns pink) and an overpressure of N_2 or CH_4 was maintained in the bottles. In addition, no oxygen or intermediates of aerobic CH_4 oxidation, such as methanol and formaldehyde, could be detected.

In the incubations without sludge or with autoclaved sludge, no $\sum {}^{13}CO_2$ was formed in the presence of $^{13}CH_4$ (Fig. 4a and b). From the 10 incubations with different amounts of Eerbeek sludge and with 0.16 MPa $^{13}CH_4$ and 20 mM sulfate, a linear relationship between the $^{13}CH_4$ oxidation rate and the biomass concentration of 11.14 μmol g_{VSS}^{-1} day^{-1} was found ($R^2 = 0.98$, Fig. 3). This

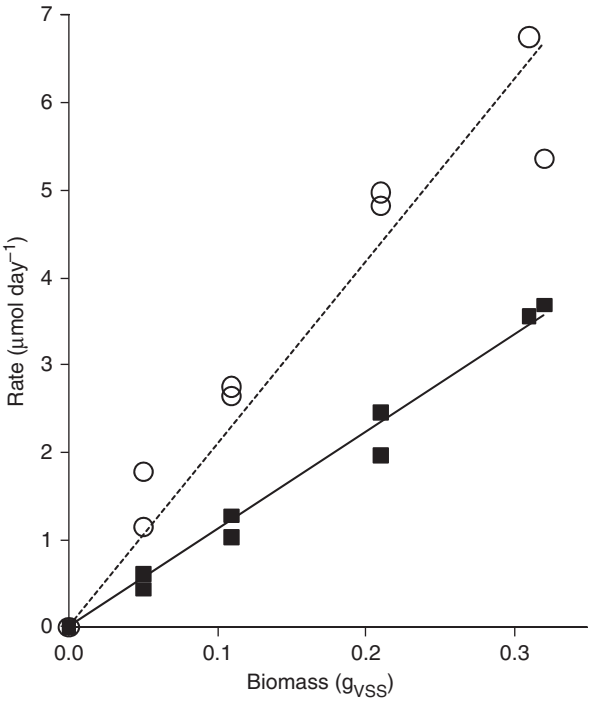

Fig. 3. The $^{13}CH_4$ oxidation rate (■) and sulfate removal rate (○) during the first 20 days of incubation with different amounts of Eerbeek sludge.

shows that living methanogenic granular sludge mediates the oxidation of $^{13}CH_4$ under anoxic conditions.

Endogenous methanogenesis and sulfate reduction in anaerobic granular sludge

No electron donor, other than $^{13}CH_4$, was added to any of the incubations. However, the $^{13}CH_4$ oxidation was too low to account for the observed sulfate reduction (Figs 3 and 5e–h). In addition, sulfate was reduced even when no CH_4 was added (Fig. 5a–d). Moreover, $^{12}CH_4$ production took place in almost all bottles (Fig. 5). Likely, organic compounds present in or released from the inocula are used as substrates for sulfate reduction and methanogenesis. Indeed, the VSS in the bottles with 0.2 g_{VSS} Eerbeek sludge decreased by 23.2 (± 3.2) mg ($N = 4$) during 30 days of incubation, indicating that the sludge slowly decomposed. When complete oxidation of the organic matter (CH_2O) is assumed, 23 mg organic matter can account for 0.39 mmol sulfate reduction or CH_4 production. This fits reasonably well with the sum of the sulfate reduction and $^{12}CH_4$ production after 30 days of incubation (Fig. 5a, e and i).

Potential coupling between CH_4 oxidation and sulfate reduction in anaerobic granular sludge

To find a possible coupling between the observed $^{13}CH_4$ oxidation and sulfate reduction, incubations with and

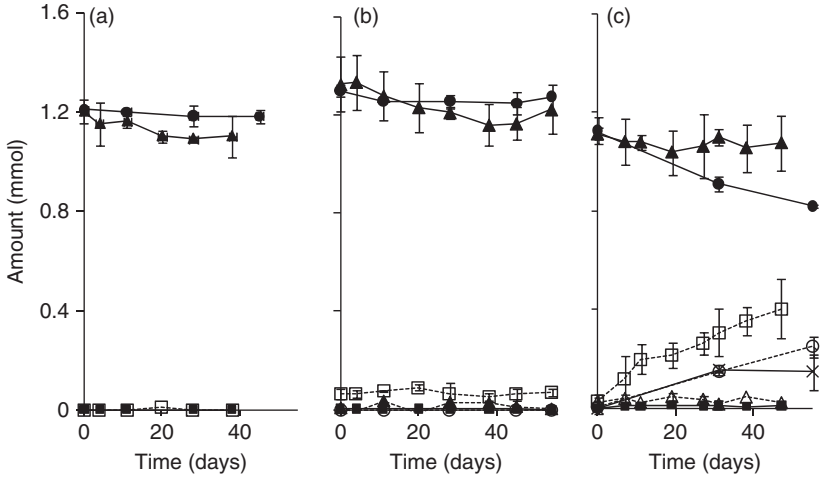

Fig. 4. SO_4^{2-} (●) reduction to sulfide (○), $^{12}CH_4$ (△), $\sum {}^{12}CO_2$ (□) and acetate (×) production, and $^{13}CH_4$ (▲) oxidation to $\sum {}^{13}CO_2$ (■) in bottles with no sludge blank (a), 0.20 g_{VSS} autoclaved granular Eerbeek sludge (b) and 0.20 g_{VSS} granular Eerbeek sludge in presence of 20 mM bromoethanesulfonate (c). Error bars indicate the SDs of two independent incubations.

without sulfate were compared. In the incubations with sulfate and $^{13}CH_4$, 0.25, 0.37, 0.46 and 0.83 mmol sulfate was reduced during the incubations (Fig. 5e–h, respectively). In the incubations without sulfate, there was no sulfide production and instead additional $^{12}CH_4$ was produced (Fig. 5i–l), indicating that in the absence of sulfate, the methanogens were able to utilize the endogenous substrates otherwise utilized by sulfate reducers. Like the $^{12}CH_4$ production, the $\sum {}^{13}CO_2$ production was also higher in the absence of sulfate (Fig. 5i–l). In addition, $^{12}CH_4$ and $\sum {}^{13}CO_2$ production always proceeded simultaneously (Fig. 5e–l), suggesting that the $^{13}CH_4$ oxidation was associated with methanogenesis, but not with sulfate reduction. When 20 mM bromoethanesulfonate was added, both $^{12}CH_4$ and $\sum {}^{13}CO_2$ production were completely inhibited (Fig. 4c).

To assess whether $^{13}CH_4$ oxidation can be coupled to sulfate reduction by mixing methanogenic and sulfate-reducing sludge, a series of incubations were performed with crushed Eerbeek and Emmtec sludge (Figs 5d, h and l). Also in these incubations, $^{13}CH_4$ oxidation was inhibited by the presence of sulfate, and the ratio between $^{13}CH_4$ oxidation and $^{12}CH_4$ production was not increased (mixed sludge: 0.18; Eerbeek sludge: 0.19; Emmtec: 0.13).

Effect of the CH_4 partial pressure on the ratios between CH_4 oxidation and CH_4 production

Table 2 compares the incubations conducted at ambient pressure with incubations performed at 10 MPa, each time with 100% $^{13}CH_4$ in the headspace. The elevated $^{13}CH_4$ partial pressure slightly inhibits methanogenesis and stimulates $^{13}CH_4$ oxidation. As a result, the $^{13}CH_4$ oxidation to $^{12}CH_4$ production ratios increased from 0.18 and 0.16 at ambient pressure (0.10 MPa $^{13}CH_4$) to 0.45 and 0.48 at elevated pressure (10 MPa $^{13}CH_4$), respectively, for Eerbeek and Nedalco sludge.

Archaeal clone library from Eerbeek sludge

The results presented in Figs 2–5a, e and i were obtained with Eerbeek sludge. The archaeal community in Eerbeek sludge has been investigated previously (Roest *et al.*, 2005). A summary of the composition of the archaeal 16S rRNA gene clone library is reprinted with permission and provided in Table 3. In brief, four of the 12 clones grouped closely (> 93% sequence similarity) with *Methanosaeta concilii*, a cultivated methanogen that grows on acetate. Three other clones grouped closely with *Methanobacterium beijingense*, a methanogen capable of growth on formate and H_2 and CO_2. Two of the clones did not group with cultivated species, grouping most closely with uncultivated Crenarchaeota and Euryarchaeota found in other bioreactors.

Discussion

Multiple lines of evidence in this study support the conclusion that $^{13}CH_4$ oxidation in anaerobic granular sludge is related to methanogenic activity and not to sulfate reduction, similar to that reported for pure cultures of methanogens (Zehnder & Brock, 1980; Harder, 1997; Moran *et al.*, 2004). Firstly, the CH_4 oxidation during each incubation was always coinciding with $^{13}CH_4$ oxidation. Secondly, both methanogenesis and $^{13}CH_4$ oxidation were completely inhibited by bromoethanesulfonate (a specific inhibitor for methanogenesis). Thirdly, the presence of sulfate decreased both the CH_4 production and the $^{13}CH_4$ oxidation. Fourthly, clones from Eerbeek sludge grouped closely with methanogens, but not to ANME archaea (Roest *et al.*, 2005; Table 3). The nature of the $^{13}CH_4$ oxidation in anaerobic granular sludge is discussed in the subsequent paragraph.

Trace CH_4 oxidation in anaerobic granular sludge

Because $^{13}CH_4$ oxidation takes place in the absence of inorganic electron acceptors other than $\sum CO_2$, and no

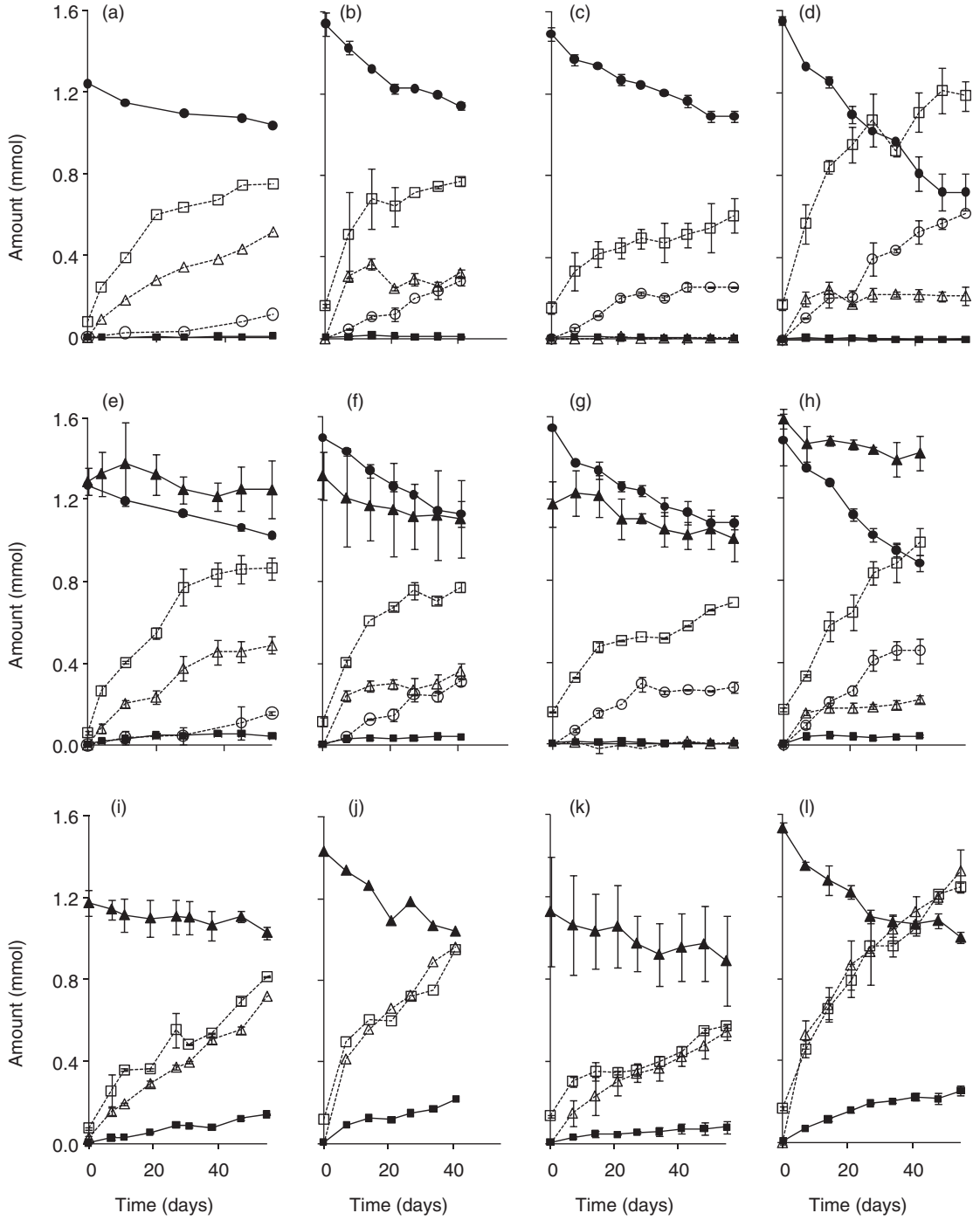

Fig. 5. SO_4^{2-} (●) reduction to sulfide (○), endogenous $^{12}CH_4$ (△) and $\sum {}^{12}CO_2$ (□) production and $^{13}CH_4$ (▲) oxidation to $\sum {}^{13}CO_2$ (■) in the presence of sulfate and in the absence of $^{13}CH_4$ (a–d), in the presence of sulfate and $^{13}CH_4$ (e–h) and in the absence of sulfate and in presence of $^{13}CH_4$ (i–l) in bottles with 0.20 g_{VSS} granular Eerbeek sludge (a, e, i), Nedalco sludge (b, f and j), Emmtec sludge (c, g and k) and mixed crushed Eerbeek (0.10 g_{VSS}) and Nedalco (0.10 g_{VSS}) sludge (d, h and l). Error bars indicate the SDs of two separate incubations; there were no replicates of incubations a and j.

reduced compound other than $^{12}CH_4$ was produced (Fig. 5i–l), the formation of $\sum {}^{13}CO_2$ from $^{13}CH_4$ in anaerobic granular sludge must be accompanied by the formation of $^{12}CH_4$ from $\sum {}^{12}CO_2$, additional to the endogenous $^{12}CH_4$ production. A one-to-one exchange between the two isotopes of CH_4 during methanogenesis implies that the actual net endogenous CH_4 production in anaerobic granular sludge is the $^{12}CH_4$ production minus the $^{13}CH_4$ oxidation.

Table 2. $^{12}CH_4$ production and $^{13}CH_4$ oxidation rates at 0.10 and 10 MPa $^{13}CH_4$ by Eerbeek and Nedalco sludge

	Eerbeek sludge		Nedalco sludge	
$\mu mol\ g_{VSS}^{-1}\ day^{-1}$	0.10 MPa $^{13}CH_4$	10 MPa $^{13}CH_4$	0.10 MPa $^{13}CH_4$	10 MPa $^{13}CH_4$
$^{12}CH_4$ production rate	47.1 (±1.9)	36.6 (±7.3)	18.9 (±0.4)	15.3 (±2.8)
$\sum {}^{13}CO_2$ production rate	8.6 (±0.9)	16.3 (±6.2)	3.0 (±0.24)	7.3 (±2.3)

Table 3. Identity of archaeal cloned 16S rRNA gene amplicons retrieved from the anaerobic wastewater treatment system at Eerbeek, the Netherlands (%, percentage of similarity between cloned 16S rRNA gene sequences, the closest relative and the closest cultured relative in the NCBI database; BLASTN)

Clone	Accession number clone	Closest relative in the database (BLASTN)	%	Accession number closest relative	Closest cultured relative in the database (BLASTN)	%	Accession number cultured relative
1A3	AY426474	Uncultured archaeon clone R2-A1 from granular sludge	100	FJ971746	*Methnosaeta concillii*	99	NR028242
1A7	AY426475	*Methnosaeta concillii*	100	NR028242	NR	NR	NR
1A8	AY426476	Uncultured Crenarchaeotes archaeon involved in anaerobic sludge digestion	96	CU916760	NA	NA	NA
1B7	AY426477	Uncultured archaeon from a expanded granular sludge bed	97	AB447760	*Methnosaeta concillii*	93	NR028242
1C11	AY426478	Uncultured archaeon clone R2A-4 from granular sludge	99	FJ167436	*Methanobacterium beijingense* strain 8-2	99	NR028202
1E4	AY426479	Uncultured archaeon clone R2-A1 from granular sludge	99	FJ971746	*Methnosaeta concillii*	99	NR028242
1G1	AY426480	Uncultured archaeon clone CG-4 from a methanogenic digester	99	AB233294	*Methanobacterium* sp. strain 169	99	AB368917
1H10	AY426481	Uncultured archaeon clone from a expanded granular sludge bed	97	AB447845	*Methanothrix soehngenii*	92	X51423
2B5	AY426482	Uncultured archaeon clone T64 from manure pit sludge (China)	99	EU662696	NA	NA	NA
2C2	AY426483	Uncultured archaeon from a expanded granular sludge bed	96	AB447760	*Methanobacterium beijingense* strain 8-2	96	NR028202
2C4	AY426484	Uncultured archaeon clone R2A-4 from granular sludge	99	FJ167436	*Methanobacterium beijingense* strain 8-2	99	NR028202
2H1	AY426485	Uncultured bacterium clone HnA32fl from granular sludge	99	AB266905	*Methanomethylovorans* sp. Z1	98	EF174501

Modified after Roest *et al*. (2005) and updated.
NR, nor relevant; NA, not available

Such isotopic exchange could be the result of the reversibility of enzymes involved in methanogenesis (Hallam *et al*., 2004; Thauer & Shima, 2008). Probably, $^{12}CH_4$ oxidation also takes place during endogenous methanogenesis; however, it is not possible to measure this. Because the bottles contained only $^{13}CH_4$ initially, the $^{12}CH_4$ oxidation can be expected to have been much lower than the $^{13}CH_4$ oxidation. Because of the difference in the isotopic composition between CH_4 and CO_2, an incorporation of ^{13}C into CO_2 was observed during the incubations. The finding that labeled CH_4 production (from labeled CO_2) coincides with net AOM in ANME-dominated marine sediments (Orcutt *et al*., 2005, 2009; Treude *et al*., 2007) supports the hypothesis that the enzymes involved in AOM and methanogenesis are similar and operate simultaneously in both ways.

In methanogenic pure cultures, CH_4 oxidation to CH_4 production ratios up to 0.36% were obtained (Zehnder & Brock, 1979; Harder, 1997; Moran *et al*., 2004). Even at ambient pressure, much higher ratios were obtained in anoxic sediment (2%; Zehnder & Brock, 1980), digested sewage sludge (8%; Zehnder & Brock, 1980) and methanogenic granular sludge (16–19%; Fig. 5i and j and Table 2). An important difference is that for the pure culture studies, an electron donor other than CH_4 (hydrogen, formate, acetate or methanol) was added and this was not done for the sediment and sludge studies. In sediment and sludge, hydrogen or acetate is released from endogenous compounds. However, these compounds are immediately consumed again by the methanogenic- or sulfate-reducing communities present in the sludge. A lower

hydrogen pressure and acetate concentration makes the reversed conversion of methanogenesis (Table 1) more favorable. Also, an elevated CH_4 partial pressure favors reversed methanogenesis, which can explain the positive effect of the $^{13}CH_4$ partial pressure on the ratio between $^{13}CH_4$ oxidation and CH_4 production (Zehnder & Brock, 1980; Table 2).

Competition of methanogens and sulfate reducers for endogenous substrates

Methanogenesis and sulfate reduction occurred simultaneously in incubations with methanogenic sludge and sulfate, even when no electron donor was added (Fig. 5a, b and d). Thus, both processes must have been fueled by substrates released from an endogenous source. Methanogenesis increased when sulfate was omitted (Fig. 5i–l) and sulfate reduction increased when methanogenesis was inhibited (Fig. 4c), indicating that sulfate reducers in methanogenic sludge compete for common substrates with methanogens (McCartney & Oleszkiewicz, 1993; Stams, 1994; Stams *et al.*, 2005; Muyzer & Stams, 2008).

During degradation of particular organic matter, fatty acids, alcohols and hydrogen are produced as intermediates (Stams, 1994). Fatty acids and alcohols can subsequently be further degraded to acetate and hydrogen by acetogenic bacteria or used by sulfate reducers. Acetate and hydrogen are substrates for both sulfate reducers and methanogens (Stams *et al.*, 2005; Muyzer & Stams, 2008). However, sulfate reducers can obtain more energy from the utilization of acetate and hydrogen than methanogens under standard conditions (Table 1). In incubations with Nedalco sludge, sulfate reduction became dominant after 2 weeks of incubation (Fig. 5b and f), and in incubations with Emmtec sludge sulfate reduction was dominant from the start (Fig. 5c and g). However, methanogenesis was not suppressed during the 55 days of incubation with Eerbeek sludge (Fig. 5a and e). The sulfate-reducing microbial community in Eerbeek sludge is apparently not abundant and versatile enough to win the competition for the endogenous substrate. This is supported by the inability of sulfate reducers in Eerbeek sludge to utilize the acetate that accumulated when bromoethanesulfonate was added (Fig. 4c). It has often been observed that in anaerobic sludge from UASB reactors, acetate is predominantly degraded by methanogens in the presence of sulfate (Visser *et al.*, 1993; van Bodegom & Stams, 1999; Stams *et al.*, 2005). Acetate-degrading sulfate reducers have only slightly better growth kinetic properties than *Methanosaeta* (dominant in anaerobic sludge). Therefore, it may take years before acetoclastic methanogens are outcompeted by acetate-degrading sulfate reducers, especially when the relative cell number of the acetate-degrading sulfate reducers is initially low (Stams *et al.*, 2005).

CH_4 dependence of sulfate reduction in Eerbeek sludge

Sulfate reduction by Eerbeek sludge was positively influenced by the CH_4 partial pressure (Fig. 2). Because $^{13}CH_4$ oxidation by Eerbeek sludge was not coupled to sulfate reduction (Fig. 5), it is unlikely that the additional sulfate reduction at an elevated pressure was due to the utilization of CH_4 as an electron donor. A more likely explanation is that the CH_4 partial pressure confers a competitive advantage to the sulfate reducers due to product inhibition of the methanogens. Thermodynamics can help to demonstrate this. The Gibbs-free energy change of a conversion is calculated according to the following equation:

$$\Delta G' = \Delta G^\circ + RT \ln([\text{products}]/(\text{substrates}]) \quad (2)$$

The difference in $\Delta G'$ (of any CH_4 production process) between a CH_4 partial pressure of 1 and 100 bar is therefore: $(\Delta G^\circ + RT \quad \ln([1]/(1])) - (\Delta G^\circ + RT \quad \ln([100]/(1])) = RT \ln 100 = 8.3145 \times 313.15 \times \ln 100 = 12.10^3$ J. Thus, the $\Delta G'$ of any methanogenic process at 30 °C is decreased by 12 kJ mol^{-1} CH_4 when the CH_4 partial pressure is increased by a factor 100. The $\Delta G^{\circ\prime}$ of sulfate reduction is only 17 kJ mol^{-1} higher than the $\Delta G^{\circ\prime}$ of methanogenesis (Table 1); therefore, the 12 kJ mol^{-1} can affect the competition considerably. A slight repression of methanogenesis was indeed observed in incubations with sulfate at an elevated CH_4 partial pressure (Table 2).

Consequences for AOM in marine sediments

The TMO rates in anaerobic sludge, even in the presence of sulfate and at ambient pressure, are in the same order of magnitude as the highest AOM rates found in marine sediments. Eerbeek granular sludge oxidizes CH_4 at 11.4 μmol g_{VSS}^{-1} day^{-1} or 10.3 μmol $g_{\text{dry weight}}^{-1}$ day^{-1}, whereas the highest reported AOM rates thus far are 2–8 and 8–21 μmol $g_{\text{dry weight}}^{-1}$ day^{-1} for Hydrate Ridge sediment (Krüger *et al.*, 2005) and Black Sea microbial mats (Treude *et al.*, 2007), respectively. In several marine sediment studies, the oxidation of isotopically labeled CH_4 is taken as a measure for the AOM (Alperin & Reeburgh, 1985; Iversen & Jørgensen, 1985; Gal'chenko *et al.*, 2004; Kallmeyer & Boetius, 2004; Krüger *et al.*, 2005; Niemann *et al.*, 2006; Knab *et al.*, 2008; Beal *et al.*, 2009) or the CH_4-dependent sulfate reduction is taken as a measure for AOM coupled to sulfate reduction (Krüger *et al.*, 2005; Nauhaus *et al.*, 2005; Treude *et al.*, 2007). This paper shows that this approach is not correct in CH_4-producing granular sludge. Note that CH_4 production also takes place in many marine sediments: all of eight tested AOM-mediating sediments endogenously produced CH_4, at rates between 0.005 and 0.4 μmol $g_{\text{dry weight}}^{-1}$ day^{-1} (Krüger *et al.*, 2005). Moreover, a decoupling of AOM from sulfate reduction (Alperin & Reeburgh,

1985; Hoehler *et al.*, 1994; Hansen *et al.*, 1998; Orcutt *et al.*, 2005) and the contemporaneous occurrence of net CH_4 production (Hoehler *et al.*, 1994) were reported in marine sediments, indicating that TMO may also contribute to the oxidation of labeled CH_4 in some marine sediments.

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