Short-term changes in anaerobic oxidation of methane in response to varying methane and sulfate fluxes

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Abstract

A major role in global methane fluxes has been attributed to the process of anaerobic oxidation of methane, which is performed by consortia of methanotrophic archaea and sulfate reducing bacteria. An important question remains how these very slow growing microorganisms with generation times of 3–7 months respond to natural variations in methane fluxes at cold seeps. Here, we used an experimental flow-through column system filled with cold seep sediments naturally enriched in methanotrophic communities, to test their response to short-term variations in methane and sulfate fluxes. At stable methane and sulfate concentrations of ∼2 mM and 28 mM, respectively, we measured constant rates of anaerobic oxidation of methane (AOM) and sulfide production (SR) for up to 160 days of incubation. When percolated with methane-free medium, the anaerobic methanotrophs ceased to oxidize methane and to produce sulfide. After a starvation phase of 40 days, the addition of methane restored former AOM and SR rates immediately. At methane concentrations between 0–2.3 mM we measured a linear correlation between methane availability, AOM and SR. At constant fluid flow rates of 30 m yr$^{-1}$, ca. 50% of the methane was consumed by the ANME population at all concentrations tested. Reducing the sulfate concentration from 28 to 1 mM, a decrease in AOM and SR by 35% was observed. Hence, the marine anaerobic methanotrophs (ANME) are capable to consume substantial amounts of methane rising from the subsurface seabed to the hydrosphere over a wide range of fluxes of methane and sulfate.

1 Introduction

Between 5 to 10% of the organic matter deposited on the seafloor is converted to methane by a sequence of microbial processes in which methane production is the terminal degradation step (Canfield, 1993, Canfield et al., 2005). The dissolved concentrations of methane in the ocean range from a few nM in seawater to about hundred
mM in hydrate-bearing subsurface sediments (Reeburgh, 2007). With about $4 \times 10^{15}$ m$^3$ methane, submarine gas hydrates bind more methane than all other reservoirs on earth (Milkov, 2004). However methane emission from the ocean is rather low, contributing an estimated 3–5% of the atmospheric methane budget. This is due to the consumption of methane by anaerobic and aerobic microorganisms in the seabed which represent an effective filter against this potential greenhouse gas (Reeburgh, 1996, 2007).

The principles and regulation of aerobic methane consumption in terrestrial and marine systems are well understood (reviews: Hanson and Hanson, 1996; Trotsenko and Khmelenina, 2005). But due to the limited penetration of oxygen into the seabed, AOM is considered the globally more relevant sink for methane in the ocean (Hinrichs and Boetius, 2002, and literature therein). The net reaction for AOM with sulfate is:

$$\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O}$$ (R1)

This process is performed by consortia of methanotrophic archaea and sulfate reducing bacteria (Boetius et al., 2000; Orphan et al., 2001). Unfortunately, the biochemical pathways and the regulating factors of this important reaction are not fully known.

Sedimentary methane oxidation rates range from a few pmol cm$^{-3}$ day$^{-1}$ in diffusion driven deep sulfate methane transition zones (e.g., Blake Ridge; Wellsbury et al., 2000) up to 3 $\mu$mol cm$^{-3}$ day$^{-1}$ at cold seeps of Hydrate Ridge (Treude et al., 2003), and up to 10 $\mu$mol cm$^{-3}$ day$^{-1}$ in the methanotrophic microbial mats of the Black Sea (Treude et al., 2007). In vitro incubation studies with enriched ANME communities showed that AOM rates predominantly depend on methane concentrations, and suggested extraordinarily high apparent methane half-saturation constants ($k_M$-values) in the range of several mM (Nauhaus et al., 2002, 2005, 2007).

First budgets for total methane fluxes, including microbial oxidation, at cold seep systems were calculated for Hydrate Ridge (Cascadia margin; Boetius and Suess 2004) and the Haakon Mosby Mud Volcano (Barents Sea; Niemann et al., 2006). At fluid flow rates of 0.1 to 2.5 m yr$^{-1}$, microbial methane oxidation removed about 50% of to-
tal methane flux in the sulfate penetrated surface sediments. In the transition from high to low fluid flow rates, the efficiency of the microbial filter increased at both sites, until no methane emission from the seabed was measured and all methane was consumed within the seabed (e.g., Acharax fields at Hydrate Ridge; Pogonophora fields, HMMV). Less is known about the control of AOM by sulfate fluxes. The apparent kinetics of AOM with regard to concentrations of methane and sulfate in the natural range are not yet known, but field data indicate that AOM decreases at sulfate concentrations <1 mM. The most significant problem in constraining budgets of methane emission at cold seeps is the lack of quantitative in situ methods to measure gaseous and dissolved methane emission to the hydrosphere, as well as subsurface transport processes of methane and sulfate. It is not possible obtain accurate measurements of methane and sulfate concentrations in interstitial porewaters of gassy sediment cores which degas substantially during retrieval from the seafloor (DeBeer et al., 2006). In addition, cold seeps show an extreme spatial and temporal variation in gas ebullition and fluid flow which is difficult to record (Tryon et al., 2002; Sauter et al., 2006). Finally, it is not known how the slow growing methanotrophs respond to variations in methane and sulfate supply.

Here we used continuous flow-through incubation of sediments from different cold seep ecosystems to test the response of ANME communities to short-term (2–40 days) variations in methane and sulfate fluxes. Flow-through reactors have been used previously for the study of growth patterns of ANME communities (Girguis et al., 2003, 2005). Our main questions were:

1. How does the availability of methane and sulfate influence AOM?

2. What is the efficiency of the ANME communities in consuming methane at high fluid flow rates?

3. Does the community retain its activity after starvation periods?
2 Material and methods

2.1 Sediments

Sediments from the Gullfaks (Heincke seep area; Hovland, 2007) in the northern North Sea (61°10.44′N, 2°14.65′E, 150 m water depth) were sampled on Heincke cruise 208 in May 2004 using a TV-guided multiple corer. The sandy sediments were widely covered with mats of sulfide oxidizing bacteria, which marked the area of methane-based sulfide production below the mats. The recovered sediments were highly permeable and consisted of medium to coarse sands. For the incubations we sampled the blackish sediment horizon between 2 and 15 cm, omitting the oxic top layer. Methane consumption rates, measured in in vitro incubations (using 14C-labeled methane according to Krüger et al., 2005), were on average 0.15 µmol g⁻¹ day⁻¹. Molecular analyses showed that the methanotrophic community was dominated by consortia of ANME-2a and -2c and their sulfate reducing partner bacteria of the Desulfosarciana/Desulfococcus cluster (Wegener et al., 2008).

Hydrate Ridge sediment (44°34.20′N, 125°08.77′E; 776 m water depth) was retrieved during RV Sonne cruise SO165-2 in 2002 via TV MUC sampling. Samples were obtained from seafloor covered with Beggiatoa indicating a high flux of sulfide from AOM (Treude et al., 2003).

Black Sea sediment was obtained from the Dniepr basin (44°46.41′N, 31°58.20′E, 326 m water depth) during R/V Poseidon cruise 317/3 in 2004 by pushcoring with the submersible JAGO. Samples were taken from the direct vicinity of a methane seep; degassing of methane during recovery and authigenic carbonate precipitates indicated a high AOM activity in the recovered sediments (Treude et al., 2005).

After recovery, all sediments were immediately transferred to gas tight Duran bottles and supplied with sulfate reducer medium (Widdel and Bak, 1992) as well as a methane headspace. Seawater medium was repeatedly replaced with new medium when sulfide concentrations exceeded 20 mM. All further handling of sediment was performed in an anaerobic glove box.
2.2 The flow-through setup

Sediments were filled into glass columns onto glass frits (Ochs Glasgerätebau, Bovenden, Germany) (Fig. 1). In the first setup, columns with a diameter of 40 mm were filled with sediments from Gullfaks to a height of 120 mm (total volume 151 ml, ca. 250 gram dry weight (gdw)). In the second setup, columns with a diameter of 40 mm were filled with different sediments to a height of 50 mm (total volume 63 ml), including inoculates from Gullfaks (sandy sediments, 95 gdw), Black Sea and Hydrate Ridge (clay sediments, 25 gdw). All concentrations and rates were normalized to dry weight of sediment.

The columns were closed with 2.5 cm thick butyl rubber stoppers and GL45 screw caps. Medical needles and tubing with lowest gas transmissibility (Viton; DuPont Performance Elastomers, Wilmington, US) connected the columns with the reservoir of 2 l (1 l in the short column experiment setup) artificial sea water medium. Oxygen transmissibility of the tubing was qualitatively tested with Resazurin (C_{12}H_{6}NO_{2}Na, 1mg/l) labeled seawater media and was not visible. The setup was operated as a closed system with medium recycling through a large reservoir. A high precision peristaltic pump (IP-N, Ismatec SA, Glattbrugg, Switzerland) circulated the seawater media between the reservoir and the sediment column. In all experiments a flow rate of 0.025 ml min^{-1} (36 ml d^{-1}) was adjusted.

2.3 Experimental procedure

The filled flow-through cells were mounted into the tubing system and sediment was allowed to settle for two days. The sediments were then percolated for at least 20 days with methane saturated media before starting the measurements. Samples were taken directly from in- and outflow of the columns to determine concentrations of methane and sulfide. Sulfide concentrations were determined by the copper sulfate method (Cord-Ruwisch, 1985). Briefly, 0.1 ml of the aliquot was added to 4 ml copper sulfate solution (5 mmol CuSO_{4} in 0.05 N HCl). The liquids absorption of monochromatic
light (wavelength of 480 nm) was measured immediately on a spectrometer. Absolute concentrations were determined by the calibration with sulfide standard solutions and blanks. Three replicate measurements were performed for each sample. An error value of 5% was reported for the copper sulfide method (Cord-Ruwisch, 1985). From the concentrations, sulfide production rates (SPR) per g dry weight are calculated according to Eq. (1),

$$SPR = \left( H_2S_{out} - H_2S_{in} \right) \times \frac{F_{day}}{d_w} \left[ \mu\text{mol gdw}^{-1} \text{d}^{-1} \right]$$

(1)

with the concentrations of $H_2S$ (µM) in the out- and inflow, the volume of percolated media per day (ml day$^{-1}$, $F_{day}$) and the dry weight (gdw). In all experiments we observed a consistent offset between sulfide production and methane oxidation in the presence of methane, most likely due to a contamination of the methane gas with other potential electron donors (see results).

For methane concentrations, subsamples of 0.5 ml media were added into gas tight 6 ml exetainers filled with 0.5 ml NaOH. Methane concentrations were determined from the 100 µl headspace triplicates using a GC-FID (Hewlett Packard 5890A, equipped with Porapak-Q column, 6 ft, 0.125\′, Agilent Technologies, Sta. Clara, CA), which was calibrated with methane standards. The AOM rate is calculated according to Eq. (2)

$$AOM = CH_4_{in} - CH_4_{out} \times \frac{F_{day}}{d_w} \left[ \mu\text{mol gdw}^{-1} \text{d}^{-1} \right]$$

(2)

with the methane concentrations of the in- and the outflow (CH$_4$$_{in/out}$), the flow rate per day $F_{day}$ as well as the dry weight gdw of the sediment in the column. Sulfate concentrations were measured using nonsuppressed ion chromatography according to Ferdelman et al. (1997).
3 Results and discussion

3.1 Sulfide production and methane consumption at stable conditions

The aim of the first experiment was to reach stable conditions in the two different flow-through setups with long and short columns, to obtain comparable measurements between columns and to constrain the temporal evolution of AOM in the incubations. In the experiment using five replicate long columns, Gullfaks sediments were percolated for more than 100 days with constant methane concentrations of around 1.6 mM. The flow rate was 36 ml d$^{-1}$, (32 m yr$^{-1}$), and the passage time for the medium entering at the bottom of the column (inflow) to the outflow was 36 h. A fluid flow velocity of 30 m yr$^{-1}$ is at the higher end of transport rates at cold seep ecosystems and was previously observed e.g. in active settings like those above gas hydrate at stability limits (Linke et al., 1994; Torres et al., 2002).

Figure 2 shows the results for one replicate column of Gullfaks seep sediments. Both sulfide production and methane oxidation were relatively stable. Methane oxidation was on average 0.16±0.04 µmol per gram dry weight (gdw$^{-1}$). The offset between sulfide production and methane oxidation in the experiments of ca 30% was consistently observed when medium was saturated with methane. Background sulfide production in the sediments incubated without methane was as low as 0.01 µmol gdw$^{-1}$ day$^{-1}$. Hence, this low background cannot explain the offset between SR and AOM of ca. 0.07 µmol gdw$^{-1}$ day$^{-1}$ coinciding with saturation of the medium with methane. Most likely, the methane supplied was contaminated with another electron donor for sulfate reduction such as carbon monoxide or higher hydrocarbons.

In the short column setup, inoculates from the Black Sea, from Hydrate Ridge and Gullfaks were tested in parallel incubations. The sulfate reduction and methane oxidation rates stayed constant for a period of 160 days. We measured average methane consumption rates of 0.42 (±0.15), 0.34 (±0.15) and 0.08 (±0.03) µmol gdw$^{-1}$ d$^{-1}$ and sulfate reduction rates of 0.58 (0.18), 0.47 (±0.016) and 0.07 (±0.03) µmol gdw$^{-1}$ d$^{-1}$ for Black Sea, Hydrate Ridge and Gullfaks, respectively. The AOM rates match well
with measurements directly obtained after sampling for the sediment horizons used as inoculate for the flow through columns (data not shown).

At the given methane and sulfate concentrations, we did not observe a significant increase of metabolic activity over time, which would have indicated population growth (Nauhaus et al., 2007). We can exclude that a lack of nutrients has limited growth since they were added to the medium in substantial amounts (Widdel and Bak, 1992). Similar observations of constant AOM rates over long incubation times were published by Girguis et al. (2003, 2005). In their investigation, seep sediments were percolated with methane-saturated seawater at atmospheric pressure for 24 weeks, however no growth-related increase in AOM activity was observed. Reasons for this stagnation of population size may be energy limitation by methane supply at atmospheric pressure. In high pressure batch incubations (∼1.4 MPa CH₄), Nauhaus et al. (2007) observed an increase in sulfide production by a factor of ten within almost two years related to growth of the ANME community (growth rate of 0.021 week⁻¹). It was found that the growth of anaerobic methanotrophs is extremely slow, probably with generation times of >7 months at atmospheric pressure (Girguis et al., 2005; Nauhaus et al., 2007).

In conclusion, we could show that flow through columns can be used as a stable set up for short (days to weeks) and long term experiments (months) for physiological experiments using seep sediments naturally enriched in ANME populations from a variety of locations with different sediment grain sizes. The total mass balance calculated either via accumulated rate measurements (methane oxidation), or via the difference in sulfide concentrations in the reservoir at beginning and end of the experiments match rather well (ca. 15 µmol H₂S gdw⁻¹ sediment in 83 days).

3.2 Reaction of SR and methane consumption to a methane pulse

The results of a 40 days methane pulse experiment are presented in Fig. 3. We only show the results of one column (C1) filled with sediments from Gullfaks, the other 4 replicate columns with Gullfaks sediments gave similar results. The columns were run for 120 days at 2 mM methane and 28 mM sulfate, before methane was removed for 36
days (Fig. 3a). After 36 days of starvation, methane concentration in the medium was increased to 2 mM within 2 days. The outflow showed increasing sulfide concentrations immediately after methane was percolated through the column (day 38, Fig. 3b). Accordingly, AOM (Fig. 3c) and SR (Fig. 3d) returned to similar rates as before the starvation phase, with 0.19±0.03 μmol gdw⁻¹ d⁻¹ and 0.24±0.05 μmol gdw⁻¹ d⁻¹, respectively. During the 36 days starvation phase, background SR dropped to rates as low as ~0.04 μmol g⁻¹ day⁻¹. After 40 days of exposure to methane, the medium was again degassed. Within 6 days, the methane concentration in the outflow fell below 50 μM and both methane oxidation and sulfate reduction rates decreased almost completely to zero. This proves the direct coupling between methane oxidation and sulfide production in AOM. However, it remains unknown whether the electron transfer from methane to sulfide is carried out within one or between two organisms.

Previous investigations found that anaerobic methanotrophs produce large amounts of methyl-coenzyme M reductase (MCR), which is most likely the enzyme responsible for the first step in methane oxidation. For example, this MCR constituted 7% of the total environmental protein extract in methanotrophic mats of the Black Sea (Krüger et al., 2003). It may be a good strategy to maintain a high amount of functional proteins in the extremely slow growing cells, to utilize a wide range of methane concentrations. For example, in our experiment, the present methanotrophic population utilized the same fraction of methane over 2 orders of magnitude in substrate availability without any delay.

Furthermore, the results of the methane pulse experiment support previous observations on the longevity of seep methanotrophs kept under anoxic conditions at in situ temperature without substrate. Even after storage of months to years, immediate sulfide formation can be observed directly after methane addition, reaching similar rates as in the field, at the time of sampling. The ability of anaerobic methanotrophs to survive long starvation periods could be an important advantage, especially with regard to the high spatial and temporal variability of methane fluxes at seeps, and also when considering their slow growth.
3.3 Sulfate reduction and methane oxidation at different methane and sulfate concentrations

At the low energy yield of AOM, efficient use of the natural range of methane and sulfate concentrations is critical to the anaerobic methanotrophs. In nature, AOM is often limited to a narrow zone where methane and sulfate overlap (SMTZ), in which both reactants show very low concentrations. At seeps, methane concentrations and fluxes may be extremely high, but very often sulfate is depleted within the top few cm, and its penetration from the overlying bottom water can be suppressed by high upward fluxes of sulfate free subsurface fluids (Niemann et al., 2006). Previous environmental observations suggested a strong dependence of AOM rates on the fluxes of sulfate (Treude et al., 2003). To examine the kinetic effect of AOM reactant availability, we incubated two replicate columns from Gullfaks with a series of different methane and sulfate concentrations. After an adjustment time of ≥8 days for each concentration, three to five measurements of methane and sulfide concentrations were performed within about ten days. Figures 4 and 5 show AOM and SR rates at different concentrations of methane and sulfate and constant flow velocities of 30 m yr⁻¹.

In Fig. 4 the metabolic activities without methane and at different methane concentrations between 0.3 and 2.3 mM are plotted. In the absence of methane, a background sulfide production of about 0.02 µmol gdw⁻¹ d⁻¹ was determined. Sulfide production increased to 0.17 µmol gdw⁻¹ d⁻¹ at 1.35 mM CH₄ and to 0.21 µmol gdw⁻¹ day⁻¹ at methane concentrations of 2.3 M. Methane oxidation followed the trend of sulfide production with rates of 0.02, 0.10, 0.17 µmol gdw⁻¹ day⁻¹ at 0.33 mM, 1.35 mM and 2.3 mM CH₄, respectively. The relation between methane concentration and methane consumption as well as sulfide production was linear in this range. The 20–30% offset between SR and AOM is explained as above.

A methane saturation effect (indicated by a declining slope) was not observed in our experiments, which suggests AOM $K_M$-values beyond 2 mM. Nauhaus et al. (2002) observed a linear relationship between methane oxidation and sulfide production be-
low methane pressures of 0.1 MPa (about 1.5 mM), and suggested a high methane $K_M$ in the range of several mM for AOM and methane-fueled SR. In comparison, for hydrogenotrophic sulfate reduction, half saturation constants for $H_2$ are as low as 141 Pa ($\sim 1 \mu$mol; Lovley et al., 1982), but the energy yield of this process is orders of magnitudes higher than in AOM. The high half saturation constant for methane in AOM may be due to predicted reverse operation of the methanogenic methyl-coenzyme M reductase, producing a methyl radical as a first step in AOM (Krüger et al., 2003; Hallam et al., 2004; Shima and Thauer, 2005).

The influence of sulfate concentration on the oxidation of methane was examined by percolating two other replicate columns with reduced seawater medium containing 28, 3, 2 and 1 mM sulfate (Fig. 5). Within the tested low sulfate concentrations from 3 to 1 mM a weak decline of metabolic rates was determined. However the scattering within the data was quite strong. We conclude that the half saturation for sulfate in methanotrophy is below the examined concentrations, around 0.5 mM. In comparison, organoclastic sulfate reducers show half saturation constants between 70 $\mu$M ($Desulfovibrio salexigens$) and 200 $\mu$M ($Desulfobacter postgatei$) (Ingvorsen and Jørgensen, 1984a, b). In flow-through incubations of undisturbed marine sediments Pallud and Van Capellen (2006) found $K_M$-values 100 to 300 $\mu$M. These $K_M$-values were significantly lower than results from simultaneously performed batch incubations ($\sim 1000 \mu$M), which underlines advantages of flow-through incubations for $K_M$-determinations, since they can better simulate in situ conditions of the sediment.

Sulfate reducers have different strategies to achieve low $K_M$-values; e.g., $Desulfovibrio desulfuricans$ shows intracellular sulfate enrichment up to the factor of 5000 compared to its environment (Cypionka, 1989). Compared to the organoclastic sulfate reducers, the organism performing sulfate reduction in anaerobic methanotrophy investigated here appear to have a relatively high $K_M$ for sulfate. This might be due to the low energy yield of methane dependent sulfate reduction which limits energy intense sulfate enrichment by the organisms. Furthermore the microorganisms in the investigated sediments might be adapted to high sulfate concentrations since they were collected...
from active cold seeps in which relatively high concentrations of methane and sulfate overlap. It remains unknown if the methanotrophic populations of the ubiquitous sulfate methane transition zones in the seabed are better adapted to low sulfate concentrations than their relatives inhabiting the cold seeps.

4 Conclusions

At a constant methane supply of about 2 mM, different marine methanotrophic communities enclosed in continuous flow through columns resulted in a stable rate of anaerobic oxidation of methane during 160 days. A tight link between methane oxidation and sulfate reduction was clearly shown by providing pulses of methane to environmental methanotrophic communities. An interruption of the methane supply led to an immediate decline of sulfate reduction. After percolation with methane free media for more than 40 days, former methane oxidation and sulfate reduction rates were reached immediately without a lag phase. Hence, the methanotrophic populations seem to be able to survive relatively long starving periods. Rates of anaerobic oxidation of methane were strongly regulated by methane concentrations. Between 0.3 and 2.3 mM CH$_4$ we found an almost linear increase of methane oxidation and sulfide production. This suggests half saturations ($K_M$-values) for methane of several mM in AOM. Sulfate concentrations below 3 mM decreased methane oxidation rates, the $K_M$ for sulfate is estimated at around 0.5 mM. Apparently, the high level of functional proteins maintained by the anaerobic methanotrophs allows for immediate responses to a wide range of concentrations of both electron donor and acceptor in the anaerobic oxidation of methane.

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Fig. 1. Flow-through system with the sediment column (A), medium reservoir (B) and the peristaltic pump (C). System pressure is stabilized via a second medium reservoir (D) which is pressurized by nitrogen (E). The medium was sampled at the inflow (F) and outflow (G).
Fig. 2. Comparison of methane oxidation and sulfide production in a long-term continuous flow-through experiment. The data shown are from one of five similar replicates (Gullfaks seep sediments). (a) The development of methane and (b) of sulfide (inflow concentration (filled circles); outflow concentrations (open circles)) (c) the calculated methane oxidation (grey bars) and sulfide production rates (black bars).
Fig. 3. Changing activities of a methanotrophic community in response to a methane pulse. (A) Methane concentrations, (B) sulfide concentrations. Filled circles represent the measurements at the inflow and open circles those at the outflow; (C) the resulting methane oxidation rates and (D) sulfide production.
Fig. 4. Effect of different methane concentrations on rates of methane oxidation (open symbols) and sulfate reduction (filled symbols). Data are shown for two replicate columns marked as circles and triangles.
Fig. 5. Effect of different sulfate concentrations on rates of methane oxidation (open symbols) and sulfide production (filled symbols). Data are shown for two replicate columns marked as circles and triangles.