Title: Growth of anaerobic methane oxidizing archaea and sulfate reducing bacteria in a high pressure membrane-capsule bioreactor

Running title: High pressure activity and growth of ANME and SRB

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ABSTRACT

Anaerobic methane oxidizing communities of archaea (ANME) and sulfate reducing bacteria (SRB) grow slowly, which limits physiological studies. High methane partial pressure was previously successfully applied to stimulate growth, but it is not clear how different ANME subtypes and associated sulfate reducing bacteria (SRB) are affected by it. Here, we report growth of ANME/SRB in a membrane-capsule bioreactor inoculated with Eckernförde Bay sediment that combines high pressure incubation (10.1 MPa methane) and thorough mixing (100 rpm) with complete cell retention by a 0.2 µm membrane. Results were compared to previously obtained data from an ambient-pressure (0.101 MPa methane) bioreactor inoculated with the same sediment. Labelled-methane oxidation rates were not higher at 10.1 MPa, likely because measurements were done at ambient pressure. The subtype ANME-2a/b was abundant in both reactors, but subtype ANME-2c was only enriched at 10.1 MPa. SRB at 10.1 MPa mainly belonged to the SEEP-SRB2, Eel-1 group and Desulfomonadales and not to the typically found SEEP-SRB1. Increase of ANME-2a/b occurred in parallel with increase of SEEP-SRB2 which was previously only found associated with ANME-2c. Our results imply that the syntrophic association is flexible and that methane pressure and sulfide concentration influence growth of different ANME-SRB consortia.

We also studied the effect of elevated methane pressure on methane production and oxidation by a mixture of methanogenic and sulfate-reducing sludge. Here, methane oxidation rates decreased and were not coupled to sulfide production, indicating trace methane oxidation during net methanogenesis and not anaerobic methane oxidation, even at high methane partial pressure.

Keywords Anaerobic methane oxidation – methanotrophs - high pressure - AOM - TMO - ANME
INTRODUCTION

Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) is a process influenced by the CH$_4$ partial pressure. The SR rate of sediment from Hydrate Ridge was significantly higher at elevated CH$_4$ partial pressure (1, 2). Between 0 and 0.15 MPa, there is a positive linear correlation between the CH$_4$ partial pressure and the AOM and SR rates of an anaerobic methanotrophic enrichment obtained from Eckernförde Bay sediment (3). The methane-dependent sulfide production by microbial mats from the Black Sea increased 10 to 15-fold after increasing the methane partial pressure from 0.2 to 10.0 MPa (4). The affinity constant ($K_m$) for methane of anaerobic methanotrophs from Gulf of Cádiz sediment is around 37 mM which is equivalent to 3 MPa CH$_4$ (5). Because of the more negative Gibbs free energy change ($\Delta G$) at elevated CH$_4$ partial pressures, growth of the anaerobic methanotrophs might be faster when the CH$_4$ partial pressure is increased (Fig. S1). Bioreactor studies with high methane pressure have been performed (4, 5), but it is not clear how the different ANME subtypes and associated SRB are affected by the methane pressure. This information would contribute to the understanding of the process of AOM coupled to SR and would help in further attempts to cultivate the responsible organisms.

In this study, we investigated the effect of the CH$_4$ partial pressure on methane oxidation and methane production rates in Eckernförde Bay sediment from the Baltic Sea. We also studied the effect of long-term (240-days) incubation under a high methane pressure (10.1 MPa CH$_4$) on the activity of this sediment (‘reactor HP-1’). These results, together with the results of microbial community analysis, were compared with data from a bioreactor at ambient pressure (‘reactor AP’) (6, 7) inoculated with the same sediment as reactor HP-1 and with the original Eckernförde Bay sediment (EB). We also investigated the effect of the CH$_4$ partial pressure on methane oxidation and methane production rates in mixed methanogenic and sulfate-reducing granular sludge, both in short and long-term incubation (‘reactor HP-2’).
This was done to evaluate the capacity of methanogenic and sulfate reducing communities to perform methane oxidation under favorable conditions. A summary of the experimental set-up is given in Fig.1.

MATERIALS AND METHODS

Origin of the inocula. The samples of the Eckernförde Bay sediment used for the initial activity assays and to inoculate reactor HP-1 were taken at Eckernförde Bay (Baltic Sea) at station B (water depth 28 m; position 54°31’15N, 10°01’28E) during a cruise of the German research vessel Littorina in June 2005. This sampling site has been described by Treude et al. (8). Sediment samples were taken with a small multicore sampler based on the construction described previously (9). The cores had a length of 50 cm and reached 30-40 cm into the sediment bed. Immediately after sampling, the content of the cores was mixed in a large bottle, which was made anoxic by replacing the headspace by anoxic artificial seawater. Back in the laboratory, the sediment was homogenized and transferred into 1L bottles in an anoxic chamber. The 1-L bottles were closed with butyl rubber stoppers and the headspace was replaced by CH₄ (0.15 MPa).

The mixed sludge used for the initial activity assays and to inoculate reactor HP-2 was sampled at two full-scale mesophilic UASB reactors: a methanogenic reactor treating wastewater from paper mills (Industriewater Eerbeek, Eerbeek, the Netherlands, June 2005) and a sulfate-reducing reactor fed with ethanol (Emmtec, Emmen, the Netherlands, May 2006). The two sludge types were crushed by pressing them sequentially through needles with diameters of 1.2, 0.8 and 0.5 mm, mixed and transferred into anaerobic bottles.

The bottles with sediment and sludge were stored in the dark at 4°C until the experiments were started.
Medium preparation. The basal marine medium used for the incubations with Eckernförde sediment was made as described previously (10). The basal fresh water medium used for the incubations with mixed sludge was made according to Meulepas et al. (11). Both media were minimal media and did not contain any carbon source and no other electron acceptor than sulfate. The media were boiled, cooled down under a nitrogen (N\textsubscript{2}) flow and transferred into stock bottles with a N\textsubscript{2} headspace until use. The final pH of the media was 7.2. The phosphate provided buffering capacity to maintain a neutral pH value.

Effect of the CH\textsubscript{4} partial pressure on the initial activity. The effect of the CH\textsubscript{4} partial pressure on the CH\textsubscript{4} oxidation and methane production rate of both the Eckernförde Bay sediment and the mixed sludge was assessed in triplicate incubations with 0.02 gram volatile suspended solids (g\textsubscript{VSS}) at atmospheric (0.101 MPa) and elevated (10.1 MPa) methane pressure (Fig. 1, experiment 1). These tests were performed in glass tubes (18 ml), sealed with a butyl rubber stopper and capped at one side and equipped with a piston at the opposite side (De Glasinstrumentenmakerij, Wageningen, the Netherlands)(11). The glass tubes were filled with sediment or mixed sludge and filled with 9 ml marine medium or freshwater medium, respectively. Then, tubes were closed and flushed with N\textsubscript{2}. After removing the N\textsubscript{2} gas with a syringe and needle, 3 ml \textsuperscript{13}CH\textsubscript{4} (purity 5.5) was added. The glass tubes were incubated statically at 20°C in a non-pressurized incubator or in a 2.0 L pressure vessel (Parr, Moline, IL, USA) filled with 1.8 L water. The vessel was pressurized with N\textsubscript{2} gas. The pH, liquid volume, gas volume and gas composition in the tubes were measured weekly. To do so, the pressure vessel had to be depressurized. Both pressurization and depressurization were done gradually over a period of two hours.
Effect of long-term high-pressure incubation. Two high-pressure vessels (Parr, Moline, USA) were controlled at 20 (±1)°C and equipped with a stirrer controlled at 100 rpm (Fig. 1, experiment 2). One vessel was filled with 1.8 L marine medium and inoculated with 25 membrane capsules, each containing 0.038 (±0.003) g VSS Eckernförde Bay sediment (reactor HP-1). The other vessel was filled with 0.5 L fresh water medium and inoculated with 25 membrane capsules, each containing 0.072 (±0.006) g VSS mixed sludge (reactor HP-2). The membrane capsules were cylindrically shaped, 14 mm in diameter, 20 mm long and had a membrane surface of 840 mm$^2$. The polysulfone membranes (Triqua BV, Wageningen, the Netherlands) had a pore size of 0.2 µm to retain microorganisms. The filled capsules were slightly lighter than water, which made them float when the stirrer was turned off. During inoculation, the lid of the vessel was removed in an anaerobic glove box containing 90% N$_2$ and 10% H$_2$. Afterwards, the high-pressure vessel was connected to a bottle with pressurized CH$_4$ (purity 5.5). The vessel was flushed with approximately 10 L CH$_4$ (the gas entered the vessel at the bottom to remove any dissolved gas) and subsequently slowly pressurized to 10.1 MPa. At four time points (at 60, 110, 160 and 240 days), the pressure was gradually released and the vessel was opened in an anaerobic glove box to replace the medium and to sample two membrane capsules per reactor. Subsequently, the vessel was closed, flushed and pressurized again with CH$_4$ gas as described above. The high-pressure vessels were equipped with sampling ports for liquid phase sampling just before depressurization for sulfide determination. For activity determination, the sampled membrane capsules were incubated in 25-ml serum bottles at ambient pressure, closed with butyl rubber stoppers and filled with 20 ml medium. The 5 ml headspace was filled with pure $^{13}$C-labeled CH$_4$ (0.13 MPa). The serum bottles were incubated at 20°C in orbital shakers (100 rpm). For around 30 days, weekly the pH, liquid and gas volume, pressure, gas composition and sulfide concentration in the serum bottles was measured. After these assays, the two membrane capsules per sampling point were
frozen at -20°C for subsequent DNA extraction for molecular analysis. From the last sampling point at 240 days, only one membrane capsule was taken.

**Geochemical analyses.** Total dissolved sulfide species (H$_2$S, HS$^-$ and S$^{2-}$) were measured photometrically using a standard kit (LCK 653) and a photo spectrometer (Xion 500) both from Hach Lange (Dusseldorf, Germany).

Gas composition was measured on a gas chromatograph-mass spectrometer (GC-MS) from Interscience (Breda, The Netherlands). The system was composed of a Trace GC equipped with a GS-GasPro column (30m by 0.32 mm; J&W Scientific, Folsom, CA), and a Ion-Trap MS. Helium was the carrier gas at a flow rate of 1.7mL min$^{-1}$. The column temperature was 30°C. The fractions of $^{13}$CH$_4$, $^{12}$CH$_4$, $^{13}$CO$_2$ and $^{12}$CO$_2$ were derived from the mass spectrum as described (12), with a retention time for CH$_4$ at 1.6 min in the gas chromatogram and 1.8 min for CO$_2$.

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).

**Calculations.** For explanation on calculations of total $^{13}$CO$_2$, $^{12}$CO$_2$, $^{13}$CH$_4$ and $^{12}$CH$_4$, see supplementary information and Table S1.

**DNA extraction.** DNA was extracted from the membrane capsules using the Fast DNA Kit for Soil (MP Biomedicals, Ohio, USA) according to the manufacturer’s protocol with two 45-second beat beating steps using a Fastprep Instrument (MP Biomedicals, Ohio, USA). In parallel, DNA was extracted from stored samples of reactor AP and from the original Eckernförde bay sediment (EB) (Fig. 1, experiment 0).
Clone library construction. Extracted DNA from the last sampling point at 240 days was used for clone library construction. To amplify almost full-length bacterial 16S rRNA genes for cloning, primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GYTACCTTGTACGACTT-3’) (13) were used. The archaeal 16S rRNA genes were amplified using primers A109f (ACKGCTCAGTAACACGT) (14) and universal reverse primer 1492R. PCR amplification was done with the GoTaq Polymerase kit (Promega, Madison, Wisconsin, USA) using a G-Storm cycler (G-storm, Essex, UK) with a pre-denaturing step of 2 min at 95°C followed by 35 cycles of 95°C for 30 s, 52°C for 40 s and 72°C for 1.5 min. Lastly, a post-elongation step of 5 min at 72°C was done. PCR products were pooled and purified using the PCR Clean & Concentrator kit (Zymo Research Corporation, Irvine, CA, USA) and were ligated into a pGEM-T Easy plasmid vector (pGEM-T Easy Vector System I, Promega, Madison, Wisconsin, USA) and transformed into E. coli XL1-Blue Competent Cells (Stratagene/Agilent Technologies Santa Clara, CA, USA). Both ligation and transformation were performed according to the manufacturer’s instructions.

DGGE analysis. Extracted DNA from the membrane capsules at every sampling point was used for DGGE analysis, as well as DNA from reactor AP and from EB. The V3 region of the archaeal 16S rRNA sequences was amplified with primers GC-ARC344f (5’-ACGGGGYGCAGCAGGGCG-3’) and ARC519r (5’-GWATTACGGCGG CKGCTG-3’) (15) using the GoTaq Polymerase kit (Promega, Madison, Wisconsin, USA). PCR reactions were performed in a G-Storm cycler (G-storm, Essex, UK) with a pre-denaturing step of 5 min at 94°C followed by 10 cycles of 94°C for 10 s, 61°C for 10 s (-0.5°C/cycle), 72°C for 40 s and 25 cycles of 94°C for 10s, 56 °C for 20s, 72°C for 40 s and a post-elongation step of 30 min at 72°C. Bacterial 16S rRNA V6-V8 regions were amplified using Phire Hot
start II Polymerase (Thermo Scientific, F-122L, Waltham, MA, USA) with the DGGE primer pair F-968-GC (5’-AACGCGAAGAACCT TAC-3’) and R-1401 (5’- CGGTGTGTACAGACTTACCTACGACCC-3’) (16). Bacterial amplicons were produced with a G-Storm cycler (G-storm, Essex, UK) using a pre-denaturing step of 30 s at 98°C followed by 35 cycles of 98°C for 10 s, 56°C for 10 s, 72°C for 30 s and a post-elongation step of 1 min at 72°C. Forward primers had a GC clamp of 40 bp attached to the 5’ end as used by Yu et al. (15). DGGE analysis was performed as previously described (17, 18) in a Dcode system (Biorad, Germany) at 60°C for 16 hours with a denaturing gradient of 30-60% for bacterial profiles and a 40-60% denaturing gradient for archaeal profiles, as recommended (15).

To clarify which of the most intense DGGE bands correspond to an OTU found in the clone library, clones were subjected to PCR-DGGE after cell lysis, using the same primer pairs that were used for previous DGGE profiling. One clone of every OTU was loaded on a DGGE gel parallel to the last sample (240 days) of reactor HP-1. Clones that corresponded to bands of the DGGE pattern of reactor HP-1 were annotated as such using the Bionumerics software V4.61 (Applied Maths NV, Belgium).

**Phylogenetic analysis.** For the archaeal and bacterial clone library, 75 and 82 picked white colonies were sent for sequencing respectively, with the primer pair SP6 (5’- ATTTAGGTGACACTATAGAA-3’) and T7 (5’- TAATACGACTCAGATAGG-3’) to GATC Biotech (Konstanz, Germany). All reverse and forward sequenced overlapping reads were trimmed of vector and bad quality sequences, and were assembled into contiguous reads using the DNA baser software (Heracle BioSoft S.R.L., Pitesti, Romania). After assembly, possible chimeras were removed using the Greengenes Bellerophon Chimera check (http://greengenes.lbl.gov) (18). Whole 16S rRNA sequences were checked with BlastN (20).
Sequences were aligned using the SINA online alignment tool version 1.2.11 (21). Phylogenetic trees were constructed after merging aligned sequences with the Silva SSU Ref database release 111 (22) using the ARB software package version 5.3-org-8209 (23). Phylogenetic trees were calculated by the ARB neighbor-joining algorithm.

**Quantitative real-time PCR.** Extracted DNA from the membrane capsules at every sampling point was used for qPCR analysis, as well as DNA from reactor AP and from EB. The DNA concentration was determined with the Qubit 2.0 fluorometer (Thermo Fisher Scientific, MA, USA). Amplifications were done in triplicate in a BioRad CFX96™ system (Bio-Rad Laboratories, Hercules, CA, USA) in a final volume of 25 μl using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 5 ng of template DNA and primers with optimal concentrations and annealing temperatures for highest efficiency and specificity (Table S2), all according to the manufacturer’s recommendations. New primer sets were designed using the ARB software package version 5.3-org-8209 (23). Triplicate standard curves were obtained with 10-fold serial dilutions ranged from $2 \times 10^5$ to $2 \times 10^{-2}$ copies per μl of plasmids containing 16S rRNA archaeal inserts of ANME-2a/b and ANME-2c and bacterial inserts of SEEP-SRB2 and Eel-1 group. The efficiency of the reactions was up to 100% and the $R^2$ of the standard curves were up to 0.999. All used primers were extensively tested for specificity with cloned archaeal inserts of ANME-1, ANME-2a/b, ANME-2c, *Methanococcoides* and *Methanosarcinales* and bacterial inserts of SEEP-SRB1, SEEP-SRB-2, Eel-1 group, *Desulfomonadales*, *Desulfoarcina* and *Myxococcales* and with genomic DNA of *Methanosarcina mazei* TMA (DSM-9195) and *Desulfovibrio sp.* G11 (DSM-7057). PCR conditions consisted of a pre-denaturing step for 5 min at 95°C, followed by 5 touch-down cycles of 95°C for 30s, annealing at 60°C for 30s with a decrement per cycle to reach the optimized annealing temperature (temperatures are shown in Table S2), and
extension at 72°C (times are shown in Table S2). This was followed by 40 cycles of
denaturing at 95°C for 15s, 30s of annealing and extension at 72°C. PCR products were
checked for specificity by a melting curve analysis (72-95°C) after each amplification step
and gel electrophoresis. Quantification of specific archaeal and bacterial groups was
expressed as total 16S rRNA gene copies per ng DNA extracted from the capsules per g_vss.

Nucleotide sequences. Nucleotide sequence data reported are available in the
DDJB/EMBL/GenBank databases under the accession numbers HF922229 to HF922386.

RESULTS

Effect of the CH₄ partial pressure on the initial activity. The results of the initial activity
experiment (Fig.1, experiment 1) are shown in Table 1 which presents the effect of an
elevated ¹³CH₄ partial pressure on the oxidation of ¹³CH₄ to ¹³CO₂ and the ¹₂CH₄ production
of Eckernförde Bay sediment and mixed sludge. In both incubations with Eckernförde Bay
sediment and mixed sludge, we observed ¹₂CH₄ production and ¹³CO₂ production. Since no
other carbon source than ¹³CH₄ was added, the ¹₂CH₄ must have been produced from
endogenous organic matter. At 0.101 MPa CH₄ both Eckernförde Bay sediment and mixed
sludge showed ¹³CO₂ production during net methanogenesis. At 10.1 MPa, the Eckernförde
Bay sediment showed no methane production and 4 times higher oxidation rates of ¹³CH₄ to
¹³CO₂ than at 0.101 MPa. The oxidation of ¹³CH₄ to ¹³CO₂ by the mixed sludge was
approximately 2 times higher at 10.1 MPa CH₄ than at 0.1 MPa CH₄ but still showed net
methane production.
Effect of long-term high-pressure incubation. The long-term effects of an elevated methane partial pressure were tested in reactors with either Eckernförde Bay sediment or mixed sludge (Fig. 1, experiment 2). At 10.1 MPa CH$_4$, the methane oxidation rate in reactor HP-1 increased from 0.006 mmol g$_{VSS}^{-1}$ day$^{-1}$ to 0.024 mmol g$_{VSS}^{-1}$ day$^{-1}$ during the 240-day incubation (Fig. 2A and Table S3). The $^{12}$CO$_2$ production rate on the other hand decreased, likely because the available endogenous organic matter was depleted. After 240 days, $^{13}$CO$_2$ production was faster than the endogenous $^{12}$CO$_2$ production. Initially the SR rate by reactor HP-1 also decreased, but from day 110 onwards the SR rate was correlated to the methane oxidation rate. During long-term incubation of the mixed sludge, methane oxidation and sulfide production in reactor HP-2 did not increase, nor were they coupled during the 160-day incubation at 10.1 MPa CH$_4$. The total CO$_2$ and sulfide production rates decreased during the reactor run (Fig 2B and Table S3).

Microbial community of Eckernförde Bay sediment reactor. An archaeal clone library of a sample taken from reactor HP-1 at 240 days of incubation shows that the total of 75 sequences are dominated by different clades of ANME archaea (Fig. 3 and Table S4). The highest percentage of ANME clones belonged to the ANME-2a/b group (56% of all sequences), followed by ANME-2c (18.6%) and ANME-1b (4%). Other clones with relatively high frequency in the clone library cluster with the Miscellaneous Crenarchaeotal Group 15 (MCG-15) (9.3%) and the Marine benthic group D (MBG-D) (8%). Archaeal DGGE profiling of membrane capsule DNA from reactor HP-1 at all sampling points was done to see initial community changes. Afterwards, PCR-DGGE of cloned inserts with known composition revealed that bands belonging to ANME-2a/b and ANME-2c were increasing in intensity (Fig. S2). qPCR analysis of the same samples with specific 16S rRNA primers for ANME-2a/b, ANME-2c and total Archaea are shown in Fig 4. A significant increase (2-tailed t-test
with unequal variance p<0.05) of both ANME-2a/b and ANME-2c 16S rRNA gene copies at 110 days of incubation is observed, confirming initial DGGE results. The increase of ANME continued throughout reactor run and coincided with an increase of AOM and SR rates (Fig. 2A and Table S3). The ANME-2a/b clade comprised a major fraction of total Archaea whereas ANME-2c abundance was much lower during reactor operation (Fig. 4). However, ANME-2c 16S rRNA gene copies showed a faster increase than ANME-2a/b between 160 and 240 days.

A bacterial clone library of reactor HP-1 at 240 days of incubation of 82 sequences shows a high bacterial diversity (Fig. 5 and Table S4). All but two sequences within the clone library showed 97% or less similarity to known cultivated members. From the Deltaproteobacteria, the most common phylotypes recovered belonged to the methane-seep associated ‘Eel-1’ (6.2% of all sequences) and ‘Eel-2’ (13.6%) clades as described by Orphan et al. (24) of which the Eel-2 clade clusters within the SEEP-SRB2 group. We also found sequences that are affiliated with the order Desulfomonadales (7.4%). Members of the Desulfo bacteriaceae were least abundant and only 2.5% belonged to the Desulfosarcinales/Desulfococcus cluster SEEP-SRB1. Some sequences found belonged to the Myxococcales group. The remaining bacterial phylootypes were very diverse and many groups are also found previously in AOM sediments and reactor systems. Some are only represented by one phylotype derived from the clone library (Table S4).

qPCR analysis results of membrane capsule DNA from reactor HP-1 at all sampling points with 16S rRNA primers for total Bacteria, specific primers for SEEP-SRB2 and the newly designed specific primers for Eel-1 are shown in Fig. 6. An 8-fold increase of SEEP-SRB2 16S rRNA gene copies was observed at 160 days of incubation and Eel-1 16S rRNA gene copies increased 4-fold. The abundance of Eel-1 decreased slightly in parallel with total Bacteria after 160 days of incubation whereas SEEP-SRB2 continued to slightly increase.
This results in a relative increase of SEEP-SRB2 throughout the reactor run whereas Eel-1 remained at a constant 2.5% of total Bacteria. From the qPCR results, we also calculated the ratios of ANME-2a/b and ANME-2c over Eel-1 and SEEP-SRB2 copy numbers. We observed that only ANME-2a/b and SEEP-SRB2 were detected in a constant ratio of around 1:2 throughout reactor operation and in EB (Fig. 6C). In reactor AP, much more ANME-2a/b copies were detected as compared to SEEP-SRB2. The Eel-1 copies did not show a constant ratio with any ANME subtype. We could not analyze Desulforomonadales within the reactor as we were not able to design specific primers for this clade.

**Microbial community of mixed sludge reactor.** Microbial community analysis of the mixed sludge reactor HP-2 was restricted to archaeal and bacterial DGGE analysis (Fig. S2 and S3) as no increase in methane oxidation was observed. On both the archaeal and bacterial DGGE profile, we did not see any community changes during reactor run.

**DISCUSSION**

**Activity of Eckernförde Bay sediment.** Our initial activity experiments showed that the Eckernförde Bay sediment performed trace methane oxidation (TMO) during net methanogenesis at 0.101 MPa CH$_4$ and net anaerobic oxidation of methane (AOM) at 10.1 MPa CH$_4$ without methane production (Table 1). Because the $^{13}$CO$_2$ production rate was also 4 times higher at 10.1 MPa CH$_4$ as compared to 0.101 MPa CH$_4$, we expect that the AOM activity of Eckernförde Bay sediment is stimulated by the higher methane partial pressure, although the sediment originates from relative shallow waters of 28 m depth (8). The AOM activity in reactor HP-1 did however not increase faster than the reported AOM activity of the same Eckernförde Bay sediment in reactor AP at 0.101 MPa CH$_4$. In reactor HP-1, the AOM
rate increased from 0.006 to 0.025 mmol g\textsubscript{VSS}\textsuperscript{-1} d\textsuperscript{-1} over 240 days (Fig 2A and Table S3) and in reactor AP, the AOM rate increased from 0.003 to 0.55 mmol g\textsubscript{VSS}\textsuperscript{-1} day\textsuperscript{-1} in 842 days (7). Despite the good mixing of reactor HP-1, the increase of the AOM rate could have been limited by the larger diffusion distances. In reactor HP-1 the biomass was present in membrane capsules with a diameter of 14 mm, whereas reactor AP was a membrane bioreactor (MBR) where the biomass was present as 0.1-mm flocks that were directly in contact with the bioreactor medium (7). In reactor HP-1 at day 240, the average methane flux though the membranes was 0.11 µmol cm\textsuperscript{-2} d\textsuperscript{-1} (= 0.025 mmol g\textsubscript{VSS}\textsuperscript{-1} d\textsuperscript{-1} * 0.038 g\textsubscript{VSS}/ 8.8 cm\textsuperscript{2}). At this flux the $\Delta [\text{CH}_4]/\Delta x$ is 16 mM cm\textsuperscript{-1}, according to Fick’s first law of diffusion ($\text{CH}_4$ flux = - $\nabla D_{\text{methane}} \Delta [\text{CH}_4]/\Delta x$). At 10.1 MPa CH\textsubscript{4} and 20ºC, the CH\textsubscript{4} concentration in the bulk liquid was approximately 152 mM. The average CH\textsubscript{4} concentration near the microorganisms was therefore only marginally lower than in the bulk liquid and cannot explain the slow activity increase. A more plausible explanation for the slow activity increase could be related to the method of measuring activity of the high pressure reactor samples. Sampled membrane capsules were incubated in 25-ml serum bottles at ambient pressure, using 0.13 MPa of pure $^{13}$C-labeled CH\textsubscript{4} (Fig.1, experiment 2). Activity measurement at ambient pressure previously showed decreased AOM activity as compared to high pressure measurements (25) but also the microorganisms could have adapted to the higher pressure and will be less active when incubated at ambient pressure as shown for true piezophiles (26). Indeed, the doubling times calculated from the exponential increase in AOM rate in both reactors was 3.8 months ($R^2$=0.98, N=12) for reactor AP and 3.9 months ($R^2$=0.90, N=15) for reactor HP-1. The doubling time calculated from qPCR analysis was 0.97 months for ANME-2a/b, 0.75 months for ANME-2c and 0.96 months for SEEP-SRB2. This indicates that high methane partial
pressure had a positive effect on the AOM mediating microorganisms which was not reflected in AOM activity measurements.

A less likely explanation could be that reactor HP-1 was operated in fed-batch mode. Here, sulfide and bicarbonate accumulated until the medium was replaced. Sulfide levels during the first (days 0-60) and the last (days 160-240) incubation periods reached 2.7 mM (Table 2).

This could have been limiting the overall activity of the AOM mediating microorganisms as 2.4 (±0.1) mM sulfide was found to completely inhibit AOM and SR in reactor AP (7). In reactor AP, sulfide levels were below 1.5 mM in the first 800 days of the reactor run, reaching only 1.9 mM in the last 7 day period.

**Microbial community of Eckernförde Bay sediment reactor.** Increase in 16S rRNA gene copies of ANME-2c archaea was only observed in the high pressure reactor HP-1. In the ambient pressure reactor AP, only ANME2a/2b was present (6), which was verified by DGGE and qPCR (Fig. 4). ANME-2a/b also showed growth at high pressure, indicating that both phylotypes could grow at high methane partial pressure. Previous studies showed predominance of ANME-2c archaea at high methane partial pressure (27), in interior of hydrates (28), and showed a transition of ANME-2a/b to ANME-2c sequence abundance with increasing sediment depth and sulfide concentration (29). Also, ANME-2a/b archaea seem to exist in sediments with little or no free sulfide (30). Because ANME-2c archaea were not present in reactor AP at atmospheric pressure and lower sulfide concentration, it is likely that these methanotrophs do not grow at low methane pressure and that they have higher sulfide tolerance. This could have resulted in higher growth rates than for ANME-2a/b. Indeed, ANME-2c showed faster growth at the end of the run of reactor HP-1 as compared to ANME2a/2b (Fig 4) and a shorter doubling time of 0.75 months vs. 0.97 months in the exponential phase. An eventual predominance of ANME-2c in reactor HP-1 after prolonged...
incubation time is therefore plausible. ANME-1b archaea were the least abundant methanotrophs in both AOM-SR reactors, which could be explained by the continuous high sulfate and low sulfide concentrations that seems to preferentially select for ANME-2 archaea. Several studies showed a dominance of ANME-1 archaea in sulfate-depleted environments (31) together with elevated sulfide levels (30) and it was suggested that ANME-1 could perform AOM independent of sulfate reducing bacteria (32-34) or even perform methanogenesis (35).

Archaeal DGGE bands that were intense throughout incubation of reactor HP-1 belong to the MCG-15 and MBG-D (Fig. S2). The MBG-D represent 8% of our clone library sequences and have been found in many cold marine (deep sea) sediments (36, 37, 38), and were consistently found in bioreactors (7, 39). These archaea are related to the sulfur reducing order Thermoplasmatales, and appear to include methanogens named “Methanoplasmatales“ (40). The MCG that were present until the end of the reactor run are abundant in marine deep subsurface sediments (41). One hypothesis is that MCG archaea are heterotrophic anaerobes (42) and carbon-isotopic signatures and polar lipid analysis also indicated an organic carbon metabolism in sediments dominated by MCG sequences (43). Recently, it was found with single cell genomic sequencing that the MCG and MBG-D archaea could play a role in protein degradation (44). The batch mode of operation of our reactor implies long retention time of products of endogenous activity that could function as potential new substrates. This may have led to less selective enrichment and could explain the richness in archaean diversity in our reactor. 

Delta proteobacteria of the Eel-1 and the SEEP-SRB2 clade were present during run of reactor HP-1 as qPCR and clone library results showed. Eel-1 members are closely related to the marine sulfate reducer Desulfobacterium anilini (45). Most members of the SEEP-SRB2 are related to Dissulfuribacter thermophilus (92% similarity) and Desulfobulbus propionicus.
DSM 2032 (89% similarity), both sulfur disproportionating bacteria (46, 47).

*Desulforomonadales* related sequences were equally abundant in the clone library as the Eel-1 and clustered closely to the *Pelobacter* genus. *Pelobacter* is distinguished from *Desulforomonas* species by being able to ferment specific hydrocarbons and being unable to reduce Fe(III) and/or elemental sulfur (48). Both the SEEP-SRB2 and the Eel-1 group had increased in 16S rRNA gene copies at 160 days but Eel-1 decreased in abundance with reactor time, in parallel with total *Bacteria* (Fig 6). The Eel-1 group was previously hypothesized to be *in situ* directly or indirectly involved in AOM (24). We however found that only growth of SEEP-SRB2 coincided with growth of ANME-2a/b with a stable ratio of around 1:2 (Fig. 6C), excluding at least the direct involvement of Eel-1 members in AOM. This finding, together with the observed similar doubling times, could indicate that ANME2a/2b is growing in consortia with SEEP-SRB2, which to our knowledge has not been shown before. ANME-2c archaea could have been paired with the other most abundant *Desulforomonadales*. This SRB group was previously found in AOM mediating enrichments (27) and in cold seep sediment (29, 49). However, as with the Eel-1 group, abundance is not an indication for the involvement in AOM-SR. It could be that ANME-2c is actually forming consortia with SEEP-SRB2 as well, but a strong correlation was not found because ANME-2c copies were very low at the start of the reactor run and increased most between 160 and 240 days. A stronger correlation between ANME-2c and SEEP-SRB2 may have been found if the reactor would have been monitored longer.

Only 2.5% of the sequences in the clone library of reactor HP-1 belong to the SEEP-SRB1 branch. In previous research on different AOM sediments, cloning results show a co-occurrence of ANME-2 archaea and SEEP-SRB1. In contrast, when there is presence of ANME-1 archaea, the Eel-1 and SEEP-SRB2 group seem to be more abundant (Table 3). With microscopy techniques, other researchers recently found ANME-2c to be associated...
with SEEP-SRB2 (50), or other ANME-2 partners such as *Desulfobulbus* spp. related SRB (51, 52), and unidentified bacteria (32). Other ANME types besides ANME-2 were also found to aggregate with SEEP-SRB1 (53, 54). Recently, a novel bacterial partner named ‘HotSeep-1’ was found in thermophilic AOM (55) and ANME-1a was even found at 90°C in absence of SRB (56).

Our findings clearly indicate that the syntrophic relationship between different types of ANME and SRB is flexible and dependent on environmental factors. It was suggested before that syntrophy in AOM depends on the metabolism or ecological niche of the SRB (50, 52) and nitrate was suggested as the basis for niche differentiation between some groups of SRB (57). Uncultivated SRB belonging to SEEP-SRB2 are dominating seep habitats and are believed to be able to use non-methane hydrocarbons (50, 57). We observed growth of SEEP-SRB2 in reactor HP-1, indicating that this clade is indeed involved in AOM and does not need other non-methane hydrocarbons for growth. More likely, environmental parameters such as methane partial pressure and sulfide concentration play a key role in growth of SEEP-SRB2 and ANME-2c. This could explain the lack of ANME-2c and SEEP-SRB2 in reactor AP at ambient methane pressure and low sulfide levels and the lack of SEEP-SRB1 at high pressure and increased sulfide levels in reactor HP-1. Further studies are however needed to clarify which environmental parameters are crucial and which mechanism underlies the syntrophic interaction between ANME and SRB. A continuous flow bioreactor which mimics *in situ* conditions with little disturbance, already showed differential growth dynamics between ANME-1 and ANME-2 populations dependent on altering pore water flow rates (58). Similar studies where only the methane partial pressure or sulfide concentration is the varying factor could also give more insight into the differential growth and activity of ANME-2a/b and ANME-2c phylotypes and the associated SRB.
Activity and microbial community of mixed sludge. Our initial activity experiments showed that mixed sludge performs TMO during net methanogenesis at both 0.101 MPa CH$_4$ and 10.1 MPa CH$_4$ (Table 1). Where reactor HP-1 showed increasing AOM activity during long term incubation, reactor HP-2 did not. The total CO$_2$ and sulfide production decreased during the reactor run as endogenous substrates became depleted. Microbial analysis was restricted to DGGE profiling which did not show major community changes as observed in the HP reactor performing net AOM (Fig S2 and S3). This demonstrates that even at 10.1 MPa CH$_4$, the anaerobic community in granular sludge was not able to utilize the available energy for AOM coupled to SR during 160 days of incubation or that it does not have the metabolic flexibility to do so. This is in agreement with previous findings that granular sludge mediates TMO during net methanogenesis (10, 59), which results in much higher $^{13}$CO$_2$ production rates from $^{13}$CH$_4$ than the reported carbon back flux (60). In contrast, Eckernförde Bay sediment showed a clear uncoupling between the methane oxidation and the endogenous methanogenic activity and a coupling of $^{13}$CO$_2$ and sulfide production after 120 days of incubation. The production of $^{12}$CO$_2$ dropped to around 37 μmol g$^{-1}$ d$^{-1}$ when AOM started to occur and kept on decreasing whereas the sludge reactor never reached less than 90 μmol g$^{-1}$ d$^{-1}$ $^{12}$CO$_2$ production during the 160 days of reactor run. According to Hoehler et al., 1994, the hydrogen concentration must be low enough for AOM to occur (61). Assuming that $^{12}$CO$_2$ production coincides with hydrogen production from organic matter degradation in anoxic sludge (62), then the hydrogen concentration was probably low enough in the Eckernförde Bay sediment reactor at 110 days, but too high in the mixed sludge reactor. If we would have allowed $^{12}$CO$_2$ production to drop as low as 37 μmol g$^{-1}$ d$^{-1}$ in the sludge reactor, it maybe could have allowed AOM to occur. It was shown recently that in anaerobic digestion of a diverse mixture of samples, the chemical oxygen demand also drastically drops in the first 150 days of reactor incubation and reaches steady state at around 160 days (63). Long term
incubation is therefore indispensable to distinguish between labeled-methane oxidation during net methanogenesis (TMO) or net anaerobic methane oxidation (AOM).

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the dominant sulfate-reducing bacterial partner of anaerobic methanotrophs of the 

community structure in Gulf of Mexico gas hydrates: Comparative analysis of DNA-


Fig. 1 Schematic representation of the different experiments performed in this study.

Experiment 0 represents the study of reactor AP published previously (6,7) and the original Eckernförde Bay sediment (EB) of which samples were stored and analyzed in this study.

Experiment 1 and 2 were fully conducted in this study. The piston picture was modified with permission from ref. 11.
Fig. 2 The $^{13}$CO$_2$ (○), $^{12}$CO$_2$ (Δ), $^{12}$CH$_4$ (x) and sulfide (□) production rates derived from the ambient pressure activity measurements with $^{13}$CH$_4$ of sampled capsules of reactor HP-1 (A) and reactor HP-2 (B) after different periods of incubation at 10.1 MPa $^{12}$CH$_4$ and 20°C. Error bars represent standard deviations from independent measurements.
Fig. 3 Phylogenetic tree of 16S rRNA gene sequences from an archaeal clone library constructed of a sample taken at 240 days of incubation of reactor HP-1. The tree was constructed with the ARB neighbor-joining method with terminal filtering and jukes-cantor correction using almost full length 16S rRNA sequences. Clones detected in this study are indicated in bold. The numbers in parenthesis indicate the number of sequences found of each phylotype. Closed circles represent bootstrap values >70% (1000 replicates). The scale bar represents the percentage of changes per nucleotide position.
Fig. 4 Absolute 16S rRNA gene abundance of ANME-2a/b and total Archaea (A) and ANME-2c (B) in reactor HP-1 sampled in duplicate (A and B) at 60, 110 and 160 days, except at 240 days. Results are compared to the ambient pressure reactor (AP) and the Eckernförde bay sediment inoculum (EB). Standard deviations represent triplicate analysis.
Fig. 5 Phylogenetic tree of 16S rRNA gene sequences from a bacterial clone library constructed of a sample taken at 240 days of incubation of reactor HP-1. The tree shows only the canonical sulfate-reducing bacterial phylotypes found. The tree was constructed with the ARB neighbor-joining method with terminal filtering and jukes-cantor correction using almost full length 16S rRNA sequences. Clones detected in this study are indicated in bold. The numbers in parenthesis indicate the number of sequences found of each phylotype. Closed circles represent bootstrap values >70% (1000 replicates). The tree outgroup *Clostridium* was removed after tree construction. The scale bar represents the percentage of changes per nucleotide position.
**Fig. 6** Absolute 16S rRNA gene abundance of SEEP-SRB2 and Eel-1 group (A) and total *Bacteria* (B) with standard deviations representing triplicate analysis and the ratio of ANME-2a/b and SEEP-SRB2 (C) with combined standard deviations calculated as described (64).

Reactor HP-1 was sampled in duplicate (A and B) at 60, 110, 160 days, except at 240 days and was compared to the ambient pressure reactor (AP) and the Eckernförde bay sediment inoculum (EB).
TABLE 1 Initial activity experiment with the $^{13}$CO$_2$, and $^{12}$CH$_4$ production rates by Eckernförde Bay sediment and mixed sludge at 0.101 and 10.1 MPa $^{15}$CH$_4^a$

<table>
<thead>
<tr>
<th>Production rates</th>
<th>Eckernförde Bay sediment</th>
<th>Mixed sludge</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.101 MPa $^{13}$CH$_4$</td>
<td>10.1 MPa $^{13}$CH$_4$</td>
</tr>
<tr>
<td></td>
<td>($\mu$mol g$_{VSS}$$^{-1}$ day$^{-1}$)</td>
<td>($\mu$mol g$_{VSS}$$^{-1}$ day$^{-1}$)</td>
</tr>
<tr>
<td>$^{13}$CO$_2$</td>
<td>5.8 (±0.3)</td>
<td>20.9 (±4.5)</td>
</tr>
<tr>
<td>$^{12}$CH$_4$</td>
<td>8.5 (±1.4)</td>
<td>0.0 (±0.1)</td>
</tr>
</tbody>
</table>

$^a$ Standard deviations represent biological triplicates of 0.02 g VSS inoculum per glass tube
TABLE 2 Sulfide concentration in reactor HP-1 inoculated with Eckernförde bay sediment

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>Sulfide concentration (mM)</th>
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<tbody>
<tr>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>2.7</td>
</tr>
<tr>
<td>110</td>
<td>1.5</td>
</tr>
<tr>
<td>160</td>
<td>2.1</td>
</tr>
<tr>
<td>240</td>
<td>2.7</td>
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TABLE 3 An overview of archaeal and bacterial 16S rRNA genes detected in different studies on AOM mediating marine sediments

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Eel River Basin</th>
<th>Hydrate Ridge</th>
<th>Santa Barbara</th>
<th>Gulf of Mexico</th>
<th>Guaymas Basin</th>
<th>Santa Barbara</th>
<th>Eckernförde Bay</th>
<th>Reactor AP</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-4 cm</td>
<td>20-22 cm</td>
<td>4-7 cm</td>
<td>139 cm</td>
<td>15-18 cm</td>
<td>13-16 cm</td>
<td>HR</td>
<td>Isis</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>(24)</td>
<td>(65, 53)</td>
<td>(66)</td>
<td>(67)</td>
<td>(68)</td>
<td>(24)</td>
<td>(27)</td>
<td>(6)</td>
<td></td>
</tr>
</tbody>
</table>

**Archaea**

ANME-1

ANME-2

**Bacteria**

SEEP-SRB1

SEEP-SRB2

SEEP-SRB3

SEEP-SRB4

Eel-1

Eel-3

**Symbols** represent presence (+), dominance (++) or absence (-) of 16S rRNA gene sequences. HR = Hydrate ridge, Isis = Isis mud volcano.

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776 TABLE 3 An overview of archaeal and bacterial 16S rRNA genes detected in different studies on AOM mediating marine sediments

777 **Symbols** represent presence (+), dominance (++) or absence (-) of 16S rRNA gene sequences. HR = Hydrate ridge, Isis = Isis mud volcano.
1. Initial activity

- Eckernförde bay sediment
- Mixed granular sludge

2. Long-term high pressure reactor (10.1 MPa $^{12}$CH$_4$)

- Reactor HP-1: Eckernförde bay sediment
- Reactor HP-2: Mixed granular sludge

Ambient pressure activity measurements (2x)

- $^{13}$CH$_4$
- Membrane-capsule sampling points
- Storage for DNA extraction
- Cloning, DGGE, qPCR