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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 *Desulforomonadales* 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 (ΔG) 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 54º31'15N, 10º01'28E) 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 CH_4 (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 4ºC 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_2) flow and transferred into stock bottles with a N_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_4 partial pressure on the initial activity. The effect of the CH_4 partial pressure on the CH_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_{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_2. After removing the N_2 gas with a syringe and needle, 3 ml $^{13}CH_4$ (purity 5.5) was added. The glass tubes were incubated statically at 20ºC 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_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)ºC 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

146 frozen at -20ºC for subsequent DNA extraction for molecular analysis. From the last sampling
147 point at 240 days, only one membrane capsule was taken.
148
149 **Geochemical analyses.** Total dissolved sulfide species (H_2S, HS^- and S^{2-}) were measured
150 photometrically using a standard kit (LCK 653) and a photo spectrometer (Xion 500) both
151 from Hach Lange (Dusseldorf, Germany).
152 Gas composition was measured on a gas chromatograph-mass spectrometer (GC-MS) from
153 Interscience (Breda, The Netherlands). The system was composed of a Trace GC equipped
154 with a GS-GasPro column (30m by 0.32 mm; J&W Scientific, Folsom, CA), and a Ion-Trap
155 MS. Helium was the carrier gas at a flow rate of 1.7mL min^{-1}. The column temperature was
156 30°C. The fractions of $^{13}CH_4$, $^{12}CH_4$, $^{13}CO_2$ and $^{12}CO_2$ were derived from the mass spectrum
157 as described (12), with a retention time for CH_4 at 1.6 min in the gas chromatogram and 1.8
158 min for CO_2.
159 The pressure in the bottles and tubes was determined using a portable membrane pressure
160 unit, WAL 0–0.4 MPa absolute (WalMess- und Regelsysteme, Oldenburg, Germany).
161 The pH was checked by means of pH paper (Macherey-Nagel, Dűren, Germany).
162
163 **Calculations.** For explanation on calculations of total $^{13}CO_2$, $^{12}CO_2$, $^{13}CH_4$ and $^{12}CH_4$, see
164 supplementary information and Table S1.
165
166 **DNA extraction.** DNA was extracted from the membrane capsules using the Fast DNA Kit
167 for Soil (MP Biomedicals, Ohio, USA) according to the manufacturer's protocol with two 45-
168 second beat beating steps using a Fastprep Instrument (MP Biomedicals, Ohio, USA). In
169 parallel, DNA was extracted from stored samples of reactor AP and from the original
170 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'-GYTACCTTGTTACGACTT-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 95ºC followed by 35 cycles of 95ºC for 30 s, 52ºC for 40 s and 72ºC for 1.5 min. Lastly, a post-elongation step of 5 min at 72ºC 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'-ACGGGGYGCAGCAGGCGCGA-3') and ARC519r (5'-GWATTACCGCGG 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 94ºC followed by 10 cycles of 94ºC for 10 s, 61ºC for 10 s (-0.5ºC/cycle), 72ºC for 40 s and 25 cycles of 94ºC for 10s, 56 ºC for 20s, 72ºC for 40 s and a post-elongation step of 30 min at 72ºC. 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'- CGGTGTGTACAAGACCC-3') (16). Bacterial amplicons were produced with a G-Storm cycler (G-storm, Essex, UK) using a pre-denaturing step of 30 s at 98ºC followed by 35 cycles of 98ºC for 10 s, 56ºC for 10 s, 72ºC for 30 s and a post-elongation step of 1 min at 72ºC. 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 60ºC 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'- TAATACGACTCACTATAGGG-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).

220 Sequences were aligned using the SINA online alignment tool version 1.2.11 (21).
221 Phylogenetic trees were constructed after merging aligned sequences with the Silva SSU Ref
222 database release 111 (22) using the ARB software package version 5.3-org-8209 (23).
223 Phylogenetic trees were calculated by the ARB neighbor-joining algorithm.
224
225 **Quantitative real-time PCR.** Extracted DNA from the membrane capsules at every sampling
226 point was used for qPCR analysis, as well as DNA from reactor AP and from EB. The DNA
227 concentration was determined with the Qubit 2.0 fluorometer (Thermo Fisher Scientific, MA,
228 USA). Amplifications were done in triplicate in a BioRad CFX96™ system (Bio-Rad
229 Laboratories, Hercules, CA, USA) in a final volume of 25 µl using iTaq Universal SYBR
230 Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 5 ng of template DNA and
231 primers with optimal concentrations and annealing temperatures for highest efficiency and
232 specificity (Table S2), all according to the manufacturer's recommendations. New primer sets
233 were designed using the ARB software package version 5.3-org-8209 (23) . Triplicate
234 standard curves were obtained with 10- fold serial dilutions ranged from $2x10^{5}$ to $2x10^{-2}$
235 copies per µl of plasmids containing 16S rRNA archaeal inserts of ANME-2a/b and ANME-
236 2c and bacterial inserts of SEEP-SRB2 and Eel-1 group. The efficiency of the reactions was
237 up to 100% and the R^2 of the standard curves were up to 0.999. All used primers were
238 extensively tested for specificity with cloned archaeal inserts of ANME-1, ANME-2a/b,
239 ANME-2c, *Methanococcoides* and *Methanosarcinales* and bacterial inserts of SEEP-SRB1,
240 SEEP-SRB-2, Eel-1 group, *Desulforomonadales*, *Desulfosarcina* and *Myxococcales* and with
241 genomic DNA of *Methanosarcina mazei* TMA (DSM-9195) and *Desulfovibrio sp.* G11
242 (DSM-7057). PCR conditions consisted of a pre-denaturing step for 5 min at 95ºC, followed
243 by 5 touch-down cycles of 95ºC for 30s, annealing at 60ºC for 30s with a decrement per cycle
244 to reach the optimized annealing temperature (temperatures are shown in Table S2), and

extension at 72ºC (times are shown in Table S2). This was followed by 40 cycles of denaturing at 95ºC for 15s, 30s of annealing and extension at 72ºC. PCR products were checked for specificity by a melting curve analysis (72-95ºC) 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 CH_4 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 $^{13}CH_4$ partial pressure on the oxidation of $^{13}CH_4$ to $^{13}CO_2$ and the $^{12}CH_4$ production of Eckernförde Bay sediment and mixed sludge. In both incubations with Eckernförde Bay sediment and mixed sludge, we observed $^{12}CH_4$ production and $^{13}CO_2$ production. Since no other carbon source than $^{13}CH_4$ was added, the $^{12}CH_4$ must have been produced from endogenous organic matter. At 0.101 MPa CH_4, both Eckernförde Bay sediment and mixed sludge showed $^{13}CO_2$ production during net methanogenesis. At 10.1 MPa, the Eckernförde Bay sediment showed no methane production and 4 times higher oxidation rates of $^{13}CH_4$ to $^{13}CO_2$ than at 0.101 MPa. The oxidation of $^{13}CH_4$ to $^{13}CO_2$ by the mixed sludge was approximately 2 times higher at 10.1 MPa CH_4 than at 0.1 MPa CH_4 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 *Desulforomonadales* (7.4%). Members of the *Desulfobacteriaceae* 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 phyloptypes 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_{VSS}^{-1} d^{-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_{VSS}^{-1} day^{-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^{-2} d^{-1} (= 0.025 mmol g_{VSS}^{-1} d^{-1} * 0.038 g_{VSS} / 8.8 cm^2). At this flux the $\Delta[CH_4]/\Delta x$ is 16 mM cm^{-1}, according to Fick's first law of diffusion (CH_4 flux = - $\O D_{methane}$ $\Delta[CH_4]/\Delta x$). At 10.1 MPa CH_4 and 20ºC, the CH_4 concentration in the bulk liquid was approximately 152 mM. The average CH_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_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

367 pressure had a positive effect on the AOM mediating microorganisms which was not reflected
368 in AOM activity measurements.
369 A less likely explanation could be that reactor HP-1 was operated in fed-batch mode. Here,
370 sulfide and bicarbonate accumulated until the medium was replaced. Sulfide levels during the
371 first (days 0-60) and the last (days 160-240) incubation periods reached 2.7 mM (Table 2).
372 This could have been limiting the overall activity of the AOM mediating microorganisms as
373 2.4 (±0.1) mM sulfide was found to completely inhibit AOM and SR in reactor AP (7). In
374 reactor AP, sulfide levels were below 1.5 mM in the first 800 days of the reactor run, reaching
375 only 1.9 mM in the last 7 day period.
376
377 **Microbial community of Eckernförde Bay sediment reactor.** Increase in 16S rRNA gene
378 copies of ANME-2c archaea was only observed in the high pressure reactor HP-1. In the
379 ambient pressure reactor AP, only ANME2a/2b was present (6), which was verified by DGGE
380 and qPCR (Fig. 4). ANME-2a/b also showed growth at high pressure, indicating that both
381 phylotypes could grow at high methane partial pressure. Previous studies showed
382 predominance of ANME-2c archaea at high methane partial pressure (27), in interior of
383 hydrates (28), and showed a transition of ANME-2a/b to ANME-2c sequence abundance with
384 increasing sediment depth and sulfide concentration (29). Also, ANME-2a/b archaea seem to
385 exist in sediments with little or no free sulfide (30). Because ANME-2c archaea were not
386 present in reactor AP at atmospheric pressure and lower sulfide concentration, it is likely that
387 these methanotrophs do not grow at low methane pressure and that they have higher sulfide
388 tolerance. This could have resulted in higher growth rates than for ANME-2a/b. Indeed,
389 ANME-2c showed faster growth at the end of the run of reactor HP-1 as compared to
390 ANME2a/2b (Fig 4) and a shorter doubling time of 0.75 months vs. 0.97 months in the
391 exponential phase. An eventual predominance of ANME-2c in reactor HP-1 after prolonged

392 incubation time is therefore plausible. ANME-1b archaea were the least abundant
393 methanotrophs in both AOM-SR reactors, which could be explained by the continuous high
394 sulfate and low sulfide concentrations that seems to preferentially select for ANME-2 archaea.
395 Several studies showed a dominance of ANME-1 archaea in sulfate-depleted environments
396 (31) together with elevated sulfide levels (30) and it was suggested that ANME-1 could
397 perform AOM independent of sulfate reducing bacteria (32-34) or even perform
398 methanogenesis (35).
399 Archaeal DGGE bands that were intense throughout incubation of reactor HP-1 belong to the
400 MCG-15 and MBG-D (Fig. S2). The MBG-D represent 8% of our clone library sequences
401 and have been found in many cold marine (deep sea) sediments (36, 37, 38), and were
402 consistently found in bioreactors (7, 39). These archaea are related to the sulfur reducing
403 order *Thermoplasmatales,* and appear to include methanogens named "*Methanoplasmatales"*
404 (40). The MCG that were present until the end of the reactor run are abundant in marine deep
405 subsurface sediments (41). One hypothesis is that MCG archaea are heterotrophic anaerobes
406 (42) and carbon-isotopic signatures and polar lipid analysis also indicated an organic carbon
407 metabolism in sediments dominated by MCG sequences (43). Recently, it was found with
408 single cell genomic sequencing that the MCG and MBG-D archaea could play a role in
409 protein degradation (44). The batch mode of operation of our reactor implies long retention
410 time of products of endogenous activity that could function as potential new substrates. This
411 may have led to less selective enrichment and could explain the richness in archaeal diversity
412 in our reactor.
413 *Deltaproteobacteria* of the Eel-1 and the SEEP-SRB2 clade were present during run of
414 reactor HP-1 as qPCR and clone library results showed. Eel-1 members are closely related to
415 the marine sulfate reducer *Desulfobacterium anilini* (45). Most members of the SEEP-SRB2
416 are related to *Dissulfuribacter thermophilus* (92% similarity) and *Desulfobulbus propionicus*

417 DSM 2032 (89% similarity), both sulfur disproportionating bacteria (46, 47).
418 *Desulforomonadales* related sequences were equally abundant in the clone library as the Eel-1
419 and clustered closely to the *Pelobacter* genus. *Pelobacter* is distinguished from
420 *Desulforomonas* species by being able to ferment specific hydrocarbons and being unable to
421 reduce Fe(III) and/or elemental sulfur (48). Both the SEEP-SRB2 and the Eel-1 group had
422 increased in 16S rRNA gene copies at 160 days but Eel-1 decreased in abundance with reactor
423 time, in parallel with total *Bacteria* (Fig 6). The Eel-1 group was previously hypothesized to
424 be *in situ* directly or indirectly involved in AOM (24). We however found that only growth of
425 SEEP-SRB2 coincided with growth of ANME-2a/b with a stable ratio of around 1:2 (Fig.
426 6C), excluding at least the direct involvement of Eel-1 members in AOM. This finding,
427 together with the observed similar doubling times, could indicate that ANME2a/2b is growing
428 in consortia with SEEP-SRB2, which to our knowledge has not been shown before. ANME-
429 2c archaea could have been paired with the other most abundant *Desulforomonadales.* This
430 SRB group was previously found in AOM mediating enrichments (27) and in cold seep
431 sediment (29, 49). However, as with the Eel-1 group, abundance is not an indication for the
432 involvement in AOM-SR. It could be that ANME-2c is actually forming consortia with
433 SEEP-SRB2 as well, but a strong correlation was not found because ANME-2c copies were
434 very low at the start of the reactor run and increased most between 160 and 240 days. A
435 stronger correlation between ANME-2c and SEEP-SRB2 may have been found if the reactor
436 would have been monitored longer.
437 Only 2.5% of the sequences in the clone library of reactor HP-1 belong to the SEEP-SRB1
438 branch. In previous research on different AOM sediments, cloning results show a co-
439 occurrence of ANME-2 archaea and SEEP-SRB1. In contrast, when there is presence of
440 ANME-1 archaea, the Eel-1 and SEEP-SRB2 group seem to be more abundant (Table 3).
441 With microscopy techniques, other researchers recently found ANME-2c to be associated

442 with SEEP-SRB2 (50), or other ANME-2 partners such as *Desulfobulbus* spp. related SRB
443 (51, 52), and unidentified bacteria (32). Other ANME types besides ANME-2 were also found
444 to aggregate with SEEP-SRB1 (53, 54). Recently, a novel bacterial partner named 'HotSeep-
445 1' was found in thermophilic AOM (55) and ANME-1a was even found at 90ºC in absence of
446 SRB (56).
447 Our findings clearly indicate that the syntrophic relationship between different types of
448 ANME and SRB is flexible and dependent on environmental factors. It was suggested before
449 that syntrophy in AOM depends on the metabolism or ecological niche of the SRB (50, 52)
450 and nitrate was suggested as the basis for niche differentiation between some groups of SRB
451 (57). Uncultivated SRB belonging to SEEP-SRB2 are dominating seep habitats and are
452 believed to be able to use non-methane hydrocarbons (50, 57). We observed growth of SEEP-
453 SRB2 in reactor HP-1, indicating that this clade is indeed involved in AOM and does not need
454 other non-methane hydrocarbons for growth. More likely, environmental parameters such as
455 methane partial pressure and sulfide concentration play a key role in growth of SEEP-SRB2
456 and ANME-2c. This could explain the lack of ANME-2c and SEEP-SRB2 in reactor AP at
457 ambient methane pressure and low sulfide levels and the lack of SEEP-SRB1 at high pressure
458 and increased sulfide levels in reactor HP-1. Further studies are however needed to clarify
459 which environmental parameters are crucial and which mechanism underlies the syntrophic
460 interaction between ANME and SRB. A continuous flow bioreactor which mimics *in situ*
461 conditions with little disturbance, already showed differential growth dynamics between
462 ANME-1 and ANME-2 populations dependent on altering pore water flow rates (58). Similar
463 studies where only the methane partial pressure or sulfide concentration is the varying factor
464 could also give more insight into the differential growth and activity of ANME-2a/b and
465 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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731 **Fig. 1** Schematic representation of the different experiments performed in this study.
732 Experiment 0 represents the study of reactor AP published previously (6,7) and the original
733 Eckernförde Bay sediment (EB) of which samples were stored and analyzed in this study.
734 Experiment 1 and 2 were fully conducted in this study. The piston picture was modified with
735 permission from ref. 11.

736 **Fig. 2** The $^{13}CO_2$ (○), $^{12}CO_2$ (Δ),$^{12}CH_4$ (x) and sulfide (□) production rates derived from the
737 ambient pressure activity measurements with $^{13}CH_4$ of sampled capsules of reactor HP-1 (A)
738 and reactor HP-2 (B) after different periods of incubation at 10.1 MPa $^{12}CH_4$ and 20ºC. Error
739 bars represent standard deviations from independent measurements.

740 **Fig. 3** Phylogenetic tree of 16S rRNA gene sequences from an archaeal clone library
741 constructed of a sample taken at 240 days of incubation of reactor HP-1. The tree was
742 constructed with the ARB neighbor-joining method with terminal filtering and jukes-cantor
743 correction using almost full length 16S rRNA sequences. Clones detected in this study are
744 indicated in bold. The numbers in parenthesis indicate the number of sequences found of each
745 phylotype. Closed circles represent bootstrap values >70% (1000 replicates). The scale bar
746 represents the percentage of changes per nucleotide position.

747 **Fig. 4** Absolute 16S rRNA gene abundance of ANME-2a/b and total *Archaea* (A) and
748 ANME-2c (B) in reactor HP-1 sampled in duplicate (A and B) at 60, 110 and 160 days,
749 except at 240 days. Results are compared to the ambient pressure reactor (AP) and the
750 Eckernförde bay sediment inoculum (EB). Standard deviations represent triplicate analysis.

751 **Fig. 5** Phylogenetic tree of 16S rRNA gene sequences from a bacterial clone library
752 constructed of a sample taken at 240 days of incubation of reactor HP-1. The tree shows only
753 the canonical sulfate-reducing bacterial phylotypes found. The tree was constructed with the
754 ARB neighbor-joining method with terminal filtering and jukes-cantor correction using
755 almost full length 16S rRNA sequences. Clones detected in this study are indicated in bold.
756 The numbers in parenthesis indicate the number of sequences found of each phylotype.
757 Closed circles represent bootstrap values >70% (1000 replicates). The tree outgroup
758 *Clostridium* was removed after tree construction. The scale bar represents the percentage of
759 changes per nucleotide position.

760 **Fig. 6** Absolute 16S rRNA gene abundance of SEEP-SRB2 and Eel-1 group (A) and total
761 *Bacteria* (B) with standard deviations representing triplicate analysis and the ratio of ANME-
762 2a/b and SEEP-SRB2 (C) with combined standard deviations calculated as described (64).
763 Reactor HP-1 was sampled in duplicate (A and B) at 60, 110, 160 days, except at 240 days
764 and was compared to the ambient pressure reactor (AP) and the Eckernförde bay sediment
765 inoculum (EB).

766 TABLE 1 Initial activity experiment with the $^{13}CO_2$, and $^{12}CH_4$ production rates by

767 Eckernförde Bay sediment and mixed sludge at 0.101 and 10.1 MPa $^{13}CH_4$[a]

768
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	Eckernförde Bay sediment		Mixed sludge	
Production rates	0.101 MPa $^{13}CH_4$	10.1 MPa $^{13}CH_4$	0.101 MPa $^{13}CH_4$	10.1 MPa $^{13}CH_4$
	(μmol g_{VSS}^{-1} day^{-1})		(μmol g_{VSS}^{-1} day^{-1})	
$^{13}CO_2$	5.8 (±0.3)	20.9 (±4.5)	8.6 (±0.9)	16.3 (±6.2)
$^{12}CH_4$	8.5 (±1.4)	0.0 (±0.1)	47.1 (±1.9)	36.6 (±7.3)

770

771 [a] Standard deviations represent biological triplicates of 0.02 g VSS inoculum per glass tube

772 TABLE 2 Sulfide concentration in reactor HP-1 inoculated with Eckernförde bay sediment

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774

Time (Days)	Sulfide concentration (mM)
0	-
60	2.7
110	1.5
160	2.1
240	2.7

775

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

Sampling site	Eel River Basin			Hydrate Ridge	Santa Barbara	Gulf of Mexico	Guaymas Basin		Santa Barbara			Eckernförde Bay	
	0-4 cm	20-22 cm	4-7 cm	*Beggiatoa* Mat	139 cm	15-18 cm	Core A	Core C	13-16 cm	HR	Isis	Reactor AP	
Reference	(24)			(65, 53)	(66)	(67)	(68)		(24)	(27)		(6)	This research
Archaea													
ANME-1	++	++	+	+	+	+	+	-	-	-	-	-	+
ANME-2	+	+	++	++			-	+	+	+	+	+	++
Bacteria													
SEEP-SRB1	+	+	+	++	+		-	+	+	++	++	++	+
SEEP-SRB2	+	+	-	+	-	++	+	-	-	-	-	+	++
SEEP-SRB3	+	-	+	+			+		-				-
SEEP-SRB4				+				+					-
Eel-1	+	+	-	-	++	+	-	-	-	-	-	+	+
Eel-3	+	-	-						+				-

777

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

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