New insight into stratification of anaerobic methanotrophs in cold seep sediments

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Abstract

Methane seepages typically harbor communities of anaerobic methane oxidizers (ANME); however, knowledge about fine-scale vertical variation of ANME in response to geochemical gradients is limited. We investigated microbial communities in sediments below a white microbial mat in the G11 pockmark at Nyegga by 16S rRNA gene tag pyrosequencing and real-time quantitative PCR. A vertical stratification of dominating ANME communities was observed at 4 cmbsf (cm below seafloor) and below in the following order: ANME-2a/b, ANME-1 and ANME-2c. The ANME-1 community was most numerous and comprised single or chains of cells with typical rectangular morphology, accounting up to 89.2% of the retrieved 16S rRNA gene sequences. Detection rates for sulfate-reducing Deltaproteobacteria possibly involved in anaerobic oxidation of methane were low throughout the core. However, a correlation in the abundance of Candidate division JS-1 with ANME-2 was observed, indicating involvement in metabolisms occurring in ANME-2-dominated horizons. The white microbial mat and shallow sediments were dominated by organisms affiliated with Sulfurovum (Epsilonproteobacteria) and Methylococcales (Gammaproteobacteria), suggesting that aerobic oxidation of sulfur and methane is taking place. In intermediate horizons, typical microbial groups associated with methane seeps were recovered. The data are discussed with respect to co-occurring microbial assemblages and interspecies interactions.

Introduction

At the continental margins large amounts of methane are stored in the subsurface sediments as crystalline methane hydrates, dissolved in pore water, and as free gas (Kvenvolden et al., 1993). Despite the large flux of methane in the sediment, as much as 90% of the biogenic methane is instantly consumed during anaerobic oxidation of methane (AOM) (Knittel & Boetius, 2009), hence restricting the diffusion of this potent greenhouse gas into the atmosphere. AOM is suggested to be mediated through syntrophic consortia of methanotrophic archaea [anaerobic methane oxidizers (ANME)] together with sulfate-reducing bacterial (SRB) partners. The process is catalyzed according to the following overall reaction (Nauhaus et al., 2002):

\[ \text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O} \]  

The ANME clades (ANME-1, -2 and -3) are phylogenetically affiliated with the methanogens, and form three distinct clusters related to the orders Methanosarcinales and Methanomicrobiales (Knittel & Boetius, 2009). ANME are often observed in consortia with SRB, and theories of ANME being dependent on a bacterial partner to compensate for its missing capacity for sulfate reduction were suggested early on (Hoehler et al., 1994). In ANME-1 and ANME-2, the partner is typically from the Desulfovarcina/Desulfococcus group (Knittel et al., 2005; Schreiber et al., 2010), while ANME-3 is affiliated with sulfate reducers within the Desulfobulbus group (Niemann et al., 2006; Lösekann et al., 2007). However, observations of ‘free-living ANME’ cells, in particular ANME-1 (Knittel et al., 2005), indicate that ANME mediate AOM with sulfate without a bacterial partner, challenging the universality of the syntrophic hypothesis. The major habitats of ANME clades are...
methane-enriched environments, and consequently these methanotrophs are found worldwide, in for example cold seeps and mud volcanoes (Lanoil et al., 2001; Orphan et al., 2001; Knittel et al., 2005; Løsekann et al., 2007). In cold seep ecosystems, where methane is present just below the seawater-sediment interface, the ANME populations can reach a level of $> 10^{10}$ cells m$^{-3}$, whereas in deeper subsurface sulfate methane transition zone the cell number decreases to as few as $< 10^6$ cells cm$^{-3}$ (Orphan et al., 2001). The upper layers of cold seep ecosystems are primarily dominated by ANME-2 and ANME-3 (Knittel & Boetius, 2009); however, two seep systems, in the Gulf of Mexico (Lloyd et al., 2006, 2010) and microbial mats from the Black Sea (Knittel et al., 2005), are known to be dominated by members of the ANME-1 clade. Aiming to provide insight into how geochemical gradients constrain the diversity, abundance and stratification of ANME clades in cold seep sediments, a sediment core sampled in a pingo-structure inside the G11 pockmark at Nyegga, the mid-Norwegian margin, was analyzed by 16S rRNA gene tag-encoded FLX pyrosequencing. The G11 pockmark is one of the most active and best described fluid seepage structures (Hovland et al., 2010) and microbial mats from the Black Sea (Knittel et al., 2005), are known to be dominated by members of the ANME-1 clade. Aiming to provide insight into how geochemical gradients constrain the diversity, abundance and stratification of ANME clades in cold seep sediments, a sediment core sampled in a pingo-structure inside the G11 pockmark at Nyegga, the mid-Norwegian margin, was analyzed by 16S rRNA gene tag-encoded FLX pyrosequencing and real-time quantitative PCR (qPCR). The G11 pockmark is one of the most active and best described pockmarks in Nyegga, located at a water depth of $\sim 740$ m and characterized by a large number of pockmarks and fluid seepage structures (Hovland et al., 2005; Hjelstuen et al., 2010; Ivanov et al., 2010; Reiche et al., 2011). Our approach provided fine scale taxonomic information, and in combination with extensive sampling, we revealed a dynamic change in community structure with depth, influenced by the geochemical gradients in the sediment. The data are discussed with respect to vertical variation in dominating ANME-clades and their co-occurring microbial assemblages.

Materials and methods

Site description and sampling

Push cores for detailed microbial diversity studies were retrieved from the Nyegga area (64°39.788′N; 05°17.317′E), located on the upper mid-Norwegian continental slope, during August of 2008. The core GS08-155-29ROV used in this study was taken from an active pingo-structure within pockmark G11 at 746 m of water depth (Supporting Information, Fig. S1a). Tabular methane hydrates are present in sediments below 0.75 m inside pockmark G11 (Chen et al., 2010). Upward-migrating methane fluids are spatially variable in the pockmark, with methane fluxes ranging from below detection limit outside the G11 to 0.30–0.54 mol m$^{-2}$ year$^{-1}$ inside the pockmark, with exception of the center area (Chen et al., 2010).

The video facilities on the ROV Bathysaurus were utilized to find and localize precisely the core site within a white microbial mat in the central ‘hot spot’ area of the pingo-structure. The Argus Bathysaurus ROV system applied on the research vessel G.O.Sars was used to retrieve the 22-cm-long push core, including a white microbial mat in the seawater-sediment interface (Fig. S1b). The ambient seawater temperature measured during sampling was $-0.6^\circ$C.

Immediately after retrieval, the push core was sectioned aseptically into 1-cm layers down to 10 cmbsf (cm below seafloor) and thereafter into 2-cm layers. From the center of each sediment layer, four sediment subsamples were taken for DNA extraction using sterile 1-mL tip cut plastic syringes, snap-frozen in liquid N$_2$ and stored at $-80^\circ$C. One half of the sediment sample was used for separation of sediment and pore water. The remaining bulk sediments were stored at $-80^\circ$C.

Geochemical characterization

Pore waters from five different horizons (3, 7, 11, 15 and 19 cmbsf) were obtained by centrifuging the sediment subsamples at 9000 $g$ for 20 min at $4^\circ$C, followed by a filtration of the supernatant through 0.2-μm membrane filters. In addition, bottom seawater was collected from 3 cm above the sediment surface. Sample aliquots were preserved in glass vials and kept cool until they were used in geochemical analyses.

The dissolved sulfate (SO$_4^{2-}$) concentrations were determined using a Dionex DX-120 ion chromatograph, as described by Chen et al. (2010). The total dissolved hydrogen sulfide (ΣH$_2$S) in pore water was quantitatively measured by precipitation as ZnS using 20% zinc acetate solution, and was determined by the methylene blue method of Cline (1969) using a Thermo GENESYS 10 UV spectrophotometer. The dissolved inorganic carbon (DIC) and its stable carbon isotopes ($\delta^{13}$C$\text{DIC}$) were determined at the stable isotope laboratory at Oregon State University, using continuous flow technology (Chen et al., 2010).

DNA extraction and 16S rRNA gene amplicon sequencing

Total genomic DNA was extracted from $\sim 0.5$ g of sediment from each subsample throughout the core, using FastDNA Spin kit for soil and the FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA) according to the protocol supplied with the kit. The DNA was eluted in 50–60 μL DNase/Pyrogen-Free Water supplied with the kit, and the concentration was determined by $A_{260\text{nm}}/A_{280\text{nm}}$ ratio measurements using a Cary 300 Bio UV–Vis Spectrophotometer (Varian Inc., Palo Alto, CA). Total genomic DNA from subsamples at 0–1, 2–3, 4–5, 6–7, 9–10, 14–16 and 20–22 cmbsf were applied in a two step PCR before the 454-amplicon sequencing. In the first PCR the V5–V8 region of the 16S rRNA gene was amplified using the universal primers.
Un787f (5'-ATTAGATACCCNGGTAG) (Roesch et al., 2007) and Un1392r (5'-AGCGGCCGTGWGTRC) modified from (Lane et al., 1985).

The template DNA concentrations were titrated in order to achieve optimum PCR conditions (1–10 ng DNA ml⁻¹). Each reaction contained 1 × HotStar Taq® Master Mix kit (Qiagen, Hilden, Germany), 1 µM of each primer and 1 µL template. The thermal cycling program was 95 °C for 15 min and then 25–30 cycles of 45 s at 95 °C, 45 s at 53 °C and 1 min at 72 °C, followed by 72 °C for 7 min and cooling to 4 °C. Each sample was amplified in triplicate, and PCR products were pooled before purification with MinElute® PCR purification kit (Qiagen). The resulting DNA concentrations ranged from 0.98 to 5.3 ng µL⁻¹, measured by SYBR-Green quantification (modified from Zipper et al., 2003). Briefly, duplicate samples were measured in 10-fold dilution, each reaction contained 5 µL sample and 20 µL 5 × SYBR-Green 1 nucleic acid gel stain (Invitrogen, Eugene, OR). The samples were incubated in Step One Plus real-time PCR system (Applied Biosystems Inc., Foster City, CA) instrument for 15 min at 22 °C, followed by a plate read. A duplicate dilution series of genomic Escherichia coli DNA (DNA sodium salt from E. coli strain B, Sigma-Aldrich Inc., St. Louis, MO) was used to generate the standard curve.

In the second PCR, the primers were modified according to specifications in Lib-L chemistry corresponding to the unidirectional 454-pyrosequencing applied in this study. In the forward primer Un787f the GS FLX Titanium Primer A sequence and a specific MID sequence of 10 bp for unidirectional 454-pyrosequencing applied in this study. In the reverse primer Un1392r had the GS FLX Titanium Primer B sequence included. The second PCR contained 1 × HotStar Taq® Master Mix kit, 0.8 µM of each primer and 10 µL template from the first PCR, in 25 µL reactions. The PCR was performed as described above, using five cycles.

Excess primers were removed using Amicon Ultra 50K centrifugation filter units (Millipore, Carrigtwohill, Ireland). Briefly, two centrifugation steps (14 000 g, for 10 and 25 min, respectively) and Tris-HCl EDTA (TE) buffer (pH = 8) (500 µL in total) were applied. The concentrations of the PCR products were determined to range from 5.5 to 11.9 ng µL⁻¹ by SYBR-Green quantification. All samples were pooled in a 1 : 1 ratio, comprising 35 ng DNA each in the final suspension. To ensure complete removal of excess primers and other impurities, a final purification was performed using Agencourt AMPure Beads (Beckman Coulter Genomics) according to the protocol supplied with the kit.

Sequencing of the 16S rRNA gene amplicons were performed on a Roche/454 GS-FLX system following the guidelines for Titanium amplicon sequencing. The sequencing service was provided by the Norwegian High-Throughput Sequencing Centre.

Filtering and removal of noise and chimeras from 16S rRNA gene amplicon sequences

Quality filtering and noise removal of pyrosequencing reads of 16S rRNA gene amplicons was carried out using AMPLICONIOSE (version 1.1) (Quince et al., 2011). In summary, this method includes four steps: filtering, flowgram clustering, sequence-clustering and chimera removal. In the filtering step, reads with fewer flows than 360 or with a noisy signal (flow intensity 0.5–0.7) before this position were removed, in addition to all reads not matching the barcode and primer sequences. In the sequence-clustering step, reads were truncated at 400 bp. Barcode and primer sequences were removed before further analysis such as taxonomic classification and linkage clustering.

Taxonomic classification

All filtered, denoised and chimera-filtered sequences were aligned to a reference database prepared from Silva SSURef release 100 (Lanzén et al., 2011) using BLASTN (default parameters) as implemented in the NCBI standalone BLAST suite. All sequences with a bit-score above 150 to any database reference sequence were classified as SSU rRNA. The BLAST result was analyzed using MEGAN version 3.7 (Huson et al., 2007) in order to assign each sequence to a taxon in the modified Silva Taxonomy described above. To this end, MEGAN uses a lowest common ancestor (LCA) algorithm for which we used a minimum absolute cut-off of 150 bits and the default settings for relative cut-off. Thus, all sequences matching a reference sequence above the threshold were assigned to the node that represents their LCA of the 10% range of the best BLASTN bitscore in the taxonomy. MEGAN assignments were then exported and weighed according to its cluster’s copy number. Abundance counts of each taxon at different ranks were calculated using these weighed assignments.

The raw sff-files of 16S tag-encoded amplicons from all subsamples in core 29ROV from Nyegga have been submitted to the Sequence Read Archive under the accession number SRA026733.1.

Real-time qPCR

Total genomic DNA from all subsamples was used as templates in real-time q-PCR to enumerate 16S rRNA genes from both Archaea and Bacteria. The DNA template concentration used in the PCR was 1 ng per 20 µL reaction, in duplicates. Each reaction contained 1 × Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 1 mM of each primer and 1 µL template. Genomic DNA from Archaeoglobus fulgidus and E. coli were used as negative controls during quantification of Bacteria and Archaea, respectively.
For quantification of 16S rRNA genes of bacterial origin, the primers B338f (5'-ACTTCTACGGGAGGCAGC) (Amann et al., 1995) and B518r (5'-TTACCGCGGTCTGCTG) (Muyzer et al., 1993) were used. The PCR was performed using a Step One Plus real-time PCR system (Applied Biosystems Inc., Foster City, CA) with 40 cycles of the thermal program as described by Einen et al. (2008).

A purified PCR product obtained by amplification of the 16S rRNA gene from E. coli was used in a 10-fold dilution series to generate the quantification standard curve. Briefly, the PCR product was obtained using the 16S rRNA gene-specific primers B8f (5'-AGAGTTTGATCCTGGCTCAG) (Edwards et al., 1989) and Un1492r (5'-GGTTACCTTGT TAGCAGT) (Lane, 1991) in a conventional PCR with genomic E. coli DNA as template. The PCR product was quantified by A260 nm/A280 nm ratio measurement, and the concentration was used to calculate 16S rRNA gene copies µL⁻¹ as described by Whelan et al. (2003). The standard curve was applied as duplicates, ranging from 1.38 x 10⁷ to 1.38 x 10² 16S rRNA gene copies µL⁻¹. The R² value for the standard curve was 0.99 and the slope value was −3.49, estimating a PCR amplification efficiency of 93.43%.

Archaeal 16S rRNA genes were quantified using the primers Un519r (5’-TTACGCGGCGKGGCTG) (Ovreas et al., 1997) and A907r (5’-CCGTCAATTCCTTTRAGTTT) modified from (Muyzer et al., 1995; S.L. Jorgensen, unpublished data). Quantification standard curve consisted of a dilution series of linearized fosmid 54d9 (Treusch et al., 2005) with a copy number of archaeal 16S rRNA genes between 14.8 and 1.48 x 10⁷ copies µL⁻¹. The R² value for the standard curve was 0.99 and the slope value was −3.34, giving an estimated PCR amplification efficiency of 99.25%.

### Total cell number [4',6'-diamidino-2-phenylindole (DAPI)] and FISH

Samples of approximately 1 g in 15 mL sterile seawater were homogenized in dispersing tubes (DT-20, IKA-Werke, Staufen, Germany) for 30 s at 3000 r.p.m. The homogenate was diluted to 50 mL in sterile seawater, centrifuged at 1000 g, 15 min, 4 °C. Cells in the supernatant were fixed by adding formaldehyde to a final concentration of 1% and cells were stained for quantification using DAPI staining. When prepared for FISH, the cells were fixed with a 2% final concentration of paraformaldehyde, transferred to a polycarbonate membrane filter (0.2 µm pore size, Whatman, Schleider & Schuell, UK) and stored at −20 °C until used.

FISH was performed on filters with fluorescently labeled oligonucleotides (Glöckner et al., 1996; Ronimus et al., 1997) and DAPI (Morikawa & Yanagida, 1981). Alexa-488-labeled EUB338 I–III (Daime et al., 1999), NON338 (Christensen et al., 1999) and Atto-590 labeled ANME1-350 (Boetius et al., 2000) were used in addition to Cy-3 double-labeled (Stoecker et al., 2010) ARC917 (Loy et al., 2002). Hybridizations were performed in 35% formamide for the probes ARCH917 and EUB338, while 40% formamide was used for the ANME1-350 and EUB338 probes. Positive control for the bacterial probe was fixed cells of E. coli, and for the archaeal probe fixed cells of A. sulfatilis (Steinbuer et al., 2010). Stained slides were immersed in Immersol 518F (Zeiss) and evaluated in Zeiss Axios Imager Z1 microscope (Carl Zeiss), equipped with illumination system HXP-120 and filters for Cy3 and GFP, and Colibri with filterset 62HE (BFP+GFP+HcRed).

### Results and discussion

**Geochemistry**

Bottom seawater had a sulfate concentration of 27.5 mM, which is lower than the typical seawater value. The DIC concentration of 2.5 mM was similar to the regional value of 2.3 mM, although the δ¹³C value of −3.9% PDB (Pee Dee Belemnite) was much depleted in ¹³C compared with the regional value of 0 ± 0.5‰ PDB (Chen et al., 2010). Just below the seafloor surface, the pore water profiles showed a distinct pattern, where the concentration of SO₄²⁻ decreased from 16 mM at 3 cmbsf to 7.1 mM at 19 cmbsf, while the ΣH₂S concentration increased accordingly from 2.7 to 5.8 mM. Decrease of sulfate showed a strong correlation (R² = 0.92) with increase in hydrogen sulfide, indicating that hydrogen sulfide is produced during sulfate reduction (Fig. 1a). However, gas loss during sampling due to decreasing pressure apparently influenced the sulfide measurements, and sulfide concentrations should therefore be considered as minimum values.

The DIC concentration increased with depth, from 2.5 mM at the surface to 20.4 mM at 19 cmbsf. This was consistent with increasing concentrations of sulfide, possibly generated during AOM in deeper horizons. In addition, the δ¹³C values of pore waters were distinctly depleted in ¹³C, ranging from −47.1‰ to −49.0‰ PDB, which shows that DIC is derived from anaerobic oxidation of isotopically light methane by microorganisms (Reeburgh, 2007).

**Abundance and taxonomy of bacterial and archaeal groups**

454-pyrosequencing of 16S rRNA gene tag-encoded PCR amplicons was used to study the distribution of taxa within seven depth horizons in core 29ROV. Amplicon libraries comprising 922–18652 reads with an average read length of 230 bp (excluding primer sequence and barcode) were obtained after removal of poor-quality and chimeric sequences, excluding 14.7–22.6% of the reads from the...
original dataset (Table S1). The majority of the reads were successfully taxonomically classified, with 99% classified to phylum level, 87% to class level and 77% to order level or better. However, only 46% of reads could be classified to family level and a meager 1.4% to genus level (Appendix S1). This is mainly caused by the samples being dominated by taxa that are not well described below phylum and class level, and uncultivated groups within Thaumarchaeota, Candidate divisions and Planctomycetes among others. In addition, the use of primer based PCR in the preparation of the amplicon libraries introduces a potential bias that could lead to incomplete detection of specific taxa and likewise distort their relative abundance. This should be taken into consideration when evaluating the data.

The top 8 cm of the sediment were dominated by Bacteria, while Archaea gradually increased in abundance at greater depths as revealed by taxonomic classification of the noise-filtered amplicon sequences (Fig. 2). This observation was supported by real-time qPCR of Archaea and Bacteria, separately (Fig. 1b).

The most dominating taxon in shallow horizons between sediment surface and 3 cmbsf were Gammaproteobacteria, constituting between 35.1% and 32.7% of the reads, of which the majority was affiliated with the order Methylocoales. The Methylocoales was also found in deeper horizons, but the abundance decreased significantly below 2–3 cmbsf (Fig. 3). In a study by Chen et al. (2010), the methane fluxes of 0.3–0.54 mol m$^{-2}$ year$^{-1}$ in G11 pockmark were measured (Chen et al., 2010). Due to the high methane flux within the active pingo-structures, sufficient methane apparently reaches the oxic surface sediments to support the population of aerobic methanotrophs. The high abundance of Methylocoales (Fig. 2b) may also influence the observed $\delta^{13}$C$_{\text{DIC}}$ values of pore waters (Fig. 1a) as the metabolically produced CO$_2$, in addition to the degradation of dead methanotrophs, may be isotopically light. Unique sequences within the Methylocoales were extracted from MEGAN and used in a BLASTN search on the NCBI server. This revealed that the sequences were between 97% and 100% similar to published sequences found in similar environments, such as mud volcanoes in the East Mediterranean Sea or on the Barents Sea margin (Lösekann et al., 2007; Pachiadaki et al., 2010), cold seeps in Sagami Bay and Northern North Sea (Fang et al., 2006; Wegener et al., 2008) and hydrothermal vent systems on the Mid-Atlantic Ridge (Brazelton et al., 2006; Hugler et al., 2010).

In cold seep habitats, sulfur-oxidizing bacteria are often present at the seawater-sediment interface, being fueled by
the hydrogen sulfide produced during AOM in deeper horizons (Lösekann et al., 2007; Lloyd et al., 2010). In sediment horizons from 0 to 3 cmbsf, the Epsilonproteobacteria were one of the most abundant taxa, comprising 26.8% and 14.1% of the total reads, respectively (Fig. 2b). More than 99% of these reads were assigned to members of the sulfur-oxidizing groups *Sulfurovum* and *Sulfurimonas*, of which *Sulfurovum* was the most dominant genus. Only minor fractions of *Epsilonproteobacteria* were observed in deeper horizons. Cultivated representatives of *Sulfurovum* and *Sulfurimonas* are described as chemolithoautotrophs oxidizing elemental sulfur, sulfide or thiosulfate coupled to reduction of oxygen or nitrate, and are abundant in hydrothermal vent systems (Inagaki et al., 2003, 2004; Campbell et al., 2006; Takai et al., 2006; Glaubitz et al., 2010). Furthermore, *Sulfurimonas* has been identified in the pelagic redoxcline of the Black Sea (Glaubitz et al., 2010). However, in marine methane-enriched sediments, such as in the Gulf of Mexico, Håkon Mosby Mud Volcano and Hydrate Ridge, the sulfur-oxidizing bacteria *Beggiatoa* affiliated with Gammaproteobacteria form bacterial mats in the seawater-sediment interface (Sassen et al., 1993; Knittel et al., 2005; Lösekann et al., 2007). The high abundance of *Epsilonproteobacteria* in the microbial mat in the G11 pockmark could indicate that the Nyegga area has a different geochemical composition or involves different environmental factors compared to other cold seep areas, and that these could influence the microbial colonization of the sediment surface located directly above seepage structures.

The dominating archaeal phylum in the two uppermost horizons was *Thaumarchaeota*, constituting 4.9% of the total community (Fig. 2). Within this phylum all sequences were affiliated with Marine Group 1.1a, of which cultivated representatives are described as aerobic autotrophic ammonia oxidizers (Könneke et al., 2005; Walker et al., 2010). In conclusion, the sediment horizons above 3 cmbsf were dominated by microorganisms involved in aerobic oxidative processes.

From 3 to 10 cmbsf, there was a gradual increase in abundance of other bacterial taxa within *Chloroflexi*, *Planctomycetes* and *Bacteroidetes*, contributing between 1.9% and
7.5% to the microbial communities (Fig. 2b). These taxa are often detected in marine sediments (Heijs et al., 2007; Harrison et al., 2009; Liao et al., 2009); however, their ecological and metabolic role in these environments are not well studied. Remaining bacterial taxa constituting < 1% of the total reads are not described in detail (Appendix S1), although they contribute to the higher overall diversity of Bacteria compared with diversity of Archaea, especially in horizons above 10 cmbsf (Fig. S3).

The archaeal taxa Thermoplasmata within Euryarchaeota and Marine Benthic group B within Crenarchaeota were present mainly between 0 and 7 cmbsf, comprising 1.3–4.4%. Within Thermoplasmata, a majority of the reads were affiliated to Marine Benthic group D and the terrestrial miscellaneous euryarcheotal group, which are among the less abundant groups in marine sediments (Knittel et al., 2005; Webster et al., 2006). Marine Benthic group B, also known as deep-sea archaeal group (DSAG), might be involved in biochemical processes, including sulfate reduction or methane oxidation, based on increased presence of DSAG in the sulfate-reduction zone in hydrate-bearing sediments (Inagaki et al., 2006).

**Depth profile of ANME**

Anaerobic methanotrophs (ANME), previously described as AOM-performing Archaea (Hinrichs et al., 1999; Knittel & Boetius, 2009), dominated the Nyegga cold seep sediments below 4 cm (Fig. 3). In the oxic sediments surface horizons, dominated by sulfur-oxidizing bacteria and aerobic methanotrophs, only a few reads were assigned to ANME clades. The overall abundance of ANME increased with depth, and two of the major groups of ANME, ANME-1 and ANME-2, were identified. Transitions of ANME-2a/b-, ANME-1- and ANME-2c-dominated communities with increasing depth were observed, indicating distinct niche-specific stratification in this habitat (Fig. 3). Sulfide concentrations above 2.4 mM has been shown to inhibit AOM coupled to sulfate reduction (Meulepas et al., 2009) in a mixed-culture with ANME-2a as the sole ANME (Jagersma et al., 2009). The observed stratification of ANME may thus be caused by their ability to tolerate elevated sulfide concentrations and explain why ANME-2a/b was less abundant below 10 cmbsf. At 4–5 and 6–7 cmbsf, the ANME-2a/b subgroup comprised 38.1% and 29.8% of the total reads, respectively, whereas ANME-1 constituted < 15% in these layers. In the deepest layers (20–22 cmbsf), closest to the gas hydrates, the ANME-2c subgroup dominated the microbial community with 60% of the reads. The number of ANME-1 assigned reads increased with increasing sediment depth, and the highest abundance was observed at horizons 9–10 and 14–16 cmbsf, where 64.1% and 89.2% of the total reads were classified as ANME-1, respectively.

The qPCR results confirmed the predominance of Archaea in the horizons below 7–8 cmbsf (Fig. 1b), whereas the geochemical data implied AOM as a dominating process. The abundance of Archaea increased with increasing depth from 3.7 × 10^8 16S rRNA gene copies g^{-1} sediment at 7–8 cmbsf to a distinct peak in the ANME-1 dominated horizon at 14–16 cmbsf with 4.5 × 10^8 16S rRNA gene copies g^{-1} sediment. Overall, the total 16S rRNA gene copies enumerated by qPCR were in accordance with the relative numbers of Archaea and Bacteria in the amplicon dataset (Fig. 2 and Fig. S2), and also with other methane-enriched marine habitats such as the Black Sea (Leloup et al., 2007) and the Haakon Mosby Mud Volcano (Lösekann et al., 2007).

Between 33% and 98% of all ANME-1 affiliated reads throughout the sediment-core were not assigned to either ANME-1a or ANME-1b by the LCA algorithm in MEGAN. The ANME-1 sequences were thus extracted from MEGAN and subdivided into operational taxonomic units (OTUs) using the AMPLECONNOISE software and an in-house PERL script. The ANME-1 affiliated sequences were distributed in seven OTUs when a cut-off value of 0.03 was used (Table S2). The OTU-2 was present in all horizons and included 88.3% off all ANME-1 affiliated reads. A phylogenetic analysis based on one representative ANME-1 sequence from each OTU, and previously recovered ANME-1a/b sequences comprising a wide geographical distribution revealed that OTUs 1, 3, 4 and 5 were affiliated with ANME-1a whereas OTUs 2, 6 and 7 were classified as ANME-1b (Fig. S4). Hence, ANME-1b appears to dominate in the sediments within the pingo-structure in the G11 pockmark at Nyegga.

**ANME and interspecies relations**

ANME-1 and ANME-2 are most often associated with sulfate-reducing genera within Desulfosarcina and Desulfococcus (DSS) (Deaproteobacteria), defined as SEEP-SRB-1 (Knittel & Boetius, 2009). Overall, reads classified as Deaproteobacteria comprised 1.08–9.18% of the reads, of which the majority was assigned to known orders of sulfate reducers, such as Desulfobacterales and Desulfuromonadales. The abundance of the different families varied with increasing depth. Above the horizons enriched in ANME, Desulfobulbasaceae dominated, of which Desulfobulbus and Desulfoacapsa were the main genera. In the ANME-2a/b-dominated horizons, a predominance of Desulfoacteraceae was observed, and within this family the most abundant groups were Desulfococcus, described as a possible SRB partner of ANME during AOM (Boetius et al., 2000; Knittel et al., 2005; Schreiber et al., 2010), and Desulfobacterium.

The ANME-1 dominated horizons had the lowest abundance of Deaproteobacteria, contributing < 2% to the total community. All the bacterial reads were assigned to...
Desulfobacterales, and the majorities were affiliated with Desulfobacteraceae. ANME-1 accounts for up to 33% of total single cells in sediments from Hydrate Ridge (Knittel et al., 2005) and at least 70% of the microbial mat from the Black Sea (Michaelis et al., 2002), and can form dense colonies of monospecific aggregates (Treude et al., 2007). In order to provide further insight into the ANME-1 interspecies relationships, cells were extracted from the sediment at 14–16 cmbsf, and analyzed by FISH (Fig. 1c and d). Clearly, this horizon was dominated by ANME-1, occurring characteristically as single cells and in chains of two or more, where no direct contact with a bacterial partner was observed (Fig. 1d). The number of ANME-1 cells were approximately 2.54 × 10^9 g^-1 sediment whereas Bacteria only constituted 9.93 × 10^7 g^-1 sediment, in agreement with the qPCR results (Fig. 1b). Consequently, a highly abundant, naturally enriched ANME-1 community was present within the sediments at Nyegga.

Where ANME-2c was dominating, most of the reads within Deltaproteobacteria were assigned to Desulfobacteraceae at family level. ANME-2 has previously been observed with SRB in spherical shell-type or mixed type consortia (Boetius et al., 2000; Orphan et al., 2002; Knittel et al., 2005). The ANME:SRB ratio in shell-type consortia has been found to be 1 : 3 in sediment samples from Hydrate Ridge (Orcutt & Meile, 2008). At Hydrate Ridge ANME-2/DSS aggregates accounted for > 90% of the total cells, while single ANME-2 Archaea accounted for 1% (Knittel et al., 2005). In the Nyegga sample, the Deltaproteobacteria were less abundant and calculated ratios of ANME/Deltaproteobacteria were as follows for the different horizons: 5.9 : 1 (4–5 cmbsf), 4.5 : 1 (6–7 cmbsf), 50 : 1 (9–10 cmbsf), 86 : 1 (14–16 cmbsf) and 16.1 : 1 (20–22 cmbsf). Monospecific aggregates of ANME-2 have previously been detected by FISH analyses of ANME communities in sediments from Eel River and Eckernförde Bay (Orphan et al., 2002; Treude et al., 2005), and microbial mats from the Black Sea (Treude et al., 2007). The higher ratio of ANME/Deltaproteobacteria in the sediments at Nyegga could indicate that not all ANME-cells are aggregating with SRB, but may occur as single cells or in monospecific aggregates. Another interesting possibility is that ANME interspecies associations may extend beyond sulfate-reducing Deltaproteobacteria in the sediments at Nyegga, as observed for ANME-2c in Eel River sediments (Pernthaler et al., 2008). In the Nyegga sediments, the bacterial group Candidate division Japan Sea 1 (JS-1) accounted for 1.4–10.8% of the reads. No cultivated representatives of JS-1 group exist, making physiological inference impossible. However, the JS-1 group is widespread in deep marine subsurface sediments, and is associated with anaerobic organic-rich environments, methane hydrate containing sediments and anaerobic methanotrophic communities in sediments with high sulfate-reduction rates (Inagaki et al., 2006; Webster et al., 2007). To statistically analyze a possible correlation in abundance variations between ANME groups and JS-1, a Spearman rank order correlation test was used. The test revealed a correlation between ANME-2 and JS-1 with a P-value of 0.006 (R^2 = 0.86), indicating that JS-1 could be a potential syntrophic partner of ANME-2. In contrast, there was no correlation between ANME-1 and JS-1 (P-value = 0.6, R^2 = 0.21), showing that the abundances of these groups change with depth independent of each other.

**Conclusion**

There is a gradual transition of dominating taxa in the sediment below a white microbial mat sampled within an active pingo-structure in the G11 pockmark at Nyegga. In the microbial mat and shallow sediment horizons, aerobic methanotrophs within Gammaproteobacteria dominate, and are sustained by convection of methane to the sediment surface. Interestingly, another dominating taxon in these horizons is sulfur-oxidizing representatives within Sulfurovum (Epsilonproteobacteria), which is not frequently observed in cold seeps. Our geochemical data indicate AOM in the deepest horizons of the core, where sulfate is consumed and δ^{13}C_DIC is depleted. In horizons below 4 cmbsf, a stratification of ANME was observed in the order ANME-2a/b, ANME-1 and ANME-2c, which has previously not been described for cold seep sediments. As the concentrations of sulfide increases with depth, the observed stratification may reflect the sulfide tolerance of the different ANME subgroups, with ANME-2a/b being the most sensitive. The abundance of described sulfate-reducing partners within Deltaproteobacteria was low in ANME-dominated horizons. However, we hypothesize that Candidate division JS-1 could benefit from AOM or ANME-related metabolites as there was a clear correlation in abundance between JS-1 and ANME-2. Sediment horizons highly enriched in and dominated by free-living ANME-1 assemblages further challenge the theory of syntrophic AOM with sulfate.

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References


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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Reads from 454 pyrosequencing filtered by PyroNoise.

Table S2. Percent wise distribution of ANME-1 reads in OTUs.

Figure S1. Photos of the sampling site inside the G11 pockmark at Nyegga (a), and the core 29ROV used in this study (b).

Figure S2. Variation in abundance of Archaea quantified by both qPCR and amplicons in horizons throughout the core, shown as percent of total reads or archaeal and bacterial cells summarized, respectively.

Figure S3. Rarefaction curves of Archaea (a) and Bacteria (b).

Figure S4. Phylogenetic tree showing the affiliations of OTU representatives of ANME-1 16S rRNA gene sequences from Nyegga sediments and selected archaeal reference sequences.

Appendix S1. An overview of the taxonomic binning of all reads from each subsample.

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