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# Distribution of anaerobic methane-oxidizing and sulfate-reducing communities in the G11 Nyegga pockmark, Norwegian Sea

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**Abstract** Pockmarks are seabed geological structures sustaining methane seepage in cold seeps. Based on RNA-derived sequences the active fraction of the archaeal community was analysed in sediments associated with the G11 pockmark, in the Nyegga region of the Norwegian Sea. The anaerobic methanotrophic *Archaea* (ANME) and sulfate-reducing bacteria (SRB) communities were studied as well. The vertical distribution of the archaeal community assessed by PCR-DGGE highlighted the presence of ANME-2 in surface sediments, and ANME-1 in deeper sediments. Enrichments of methanogens showed the presence of hydrogenotrophic methanogens of the *Methanogenium* genus in surface sediment layers as well. The active fraction of the archaeal community was uniquely composed of ANME-2 in the shallow sulfate-rich sediments. Functional methyl coenzyme M reductase gene libraries showed that sequences affiliated with the ANME-1 and ANME-3 groups appeared in the deeper sediments but ANME-2 dominated both surface and deeper layers. Finally, dissimilatory sulfite reductase gene libraries revealed a high SRB diversity (i.e. *Desulfobacteraceae*, *Desulfobulbaceae*, *Syntrophobacteraceae* and *Firmicutes*) in the shallow sulfate-rich sediments. The SRB diversity was much lower in the deeper section. Overall, these results show that the microbial community in sediments associated with a pockmark harbour classical cold seep ANME and SRB communities.

**Keywords** Pockmark · Cold seeps · *Archaea* · ANME · SRB

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## Introduction

Pockmarks are marine depressions that may be formed by gas expulsion, or intense and sustained fluid seeping through the seabed. They can remain active, emitting gas over long periods of time by microseepage, or display periods of quiescence between eruptions (Dimitrov and Woodside 2003). The Norwegian continental margin contains large pockmark fields on its seafloor, connected to subsurface chimney structures. A smaller pockmark field Nyegga, is situated on the Northeastern flank of the Storegga Slide, known to host gas hydrates, in the mid-Norwegian margin at 600–800 m of water depth. Nyegga is surmised to be a area of large regional fluid flow associated with pipes and polygonal faults (Hovland et al. 2005). Seismic

investigations provided evidence for underlying gas chimneys associated with active or recent seeps and biological activity (Foucher et al. 2009). This region is comprised of morphologically complex pockmarks, containing chaotic heaps of large carbonate rocks and slabs (Hovland and Svensen 2006). The G11 pockmark is the deepest of the studied pockmarks in the Nyegga region, and includes small interior basins (Hovland et al. 2005). Mazzini et al. (2006) showed that methane-derived authigenic carbonates of the G11 pockmark were formed by anaerobic oxidation of methane (AOM) process, and that the precipitation took place in the shallow sediment depths of the sulfate-reducing zone. Recent observations reported that the rates of anaerobic methane oxidation in the sulfidic sediments of the innermost part of the pockmark reach up to 100 mmol $m^{-2}$ $d^{-1}$ (Foucher et al. 2009). Methane fuelling the carbonate precipitation seems to have mixed sources (thermogenic/biogenic) in G11 [$\delta^{13}C$ values between −29.4 and −58 ‰ V-PDB, (Hovland et al. 2005; Mazzini et al. 2006)].

In cold seep ecosystems, AOM is a major sink of the greenhouse gas methane. AOM is driven by anaerobic methanotrophic *Archaea* (ANME), and is sometimes coupled to sulfate-reduction (SR) driven by sulfate-reducing bacteria (SRB). Based on thermodynamic estimations, AOM could also be coupled to the reduction of alternative electron acceptors such as nitrite/nitrate, iron and manganese oxides with higher energy yields (Beal et al. 2009; Ettwig et al. 2010; Raghoebarsing et al. 2006). ANME *Archaea* are divided into three phylogenetic groups of the *Euryarchaeota*, ANME-1, ANME-2 and ANME-3. The ANME-1 *Archaea* are distantly affiliated with methanogenic clades of the *Methanosarcinales* and *Methanomicrobiales*, and the ANME-2 and ANME-3 *Archaea* with the *Methanosarcinales*. The presumably syntrophic ANME *Archaea* often form aggregates with SRB of the *Desulfosarcina-Desulfococcales* and the *Desulfobulbaceae* (Boetius et al. 2000; Knittel et al. 2005; Niemann et al. 2006). Even though an *Archaea-Bacteria* consortium mediating AOM coupled to SR has been obtained in stable enrichments (Nauhaus et al. 2007), the ANME *Archaea* have not yet been obtained in pure culture, and the biochemical pathways of AOM remain unknown. However, the initial step in methane oxidation is considered a reversal of the terminal reaction of methanogenesis (Thauer 1998), using a modified methyl coenyme M reductase (MCR) enzyme (Hallam et al. 2003; Hallam et al. 2006; Krüger et al. 2003). The SRB play a crucial role in the degradation of organic matter in many marine sulfate-rich shallow sediments (Joulian et al. 2001). They all share the ability to obtain energy from dissimilatory reduction of inorganic sulfate. They are widespread in anaerobic habitats and are composed of diverse physiological groups able to degrade a large range of organic substrates.

Recently, AOM and SR activities were detected in sediments of pockmarks in the Nile Deep Sea Fan (Omoregie et al. 2009), suggesting that AOM presumably coupled to SR could have occurred in these pockmark sediments. Also, ANME-1, 2 and 3 sequences (Cambon-Bonavita et al. 2009), as well as specific ANME and SRB lipid biomarkers (Boulouboassi et al. 2009), have been detected in the REGAB giant pockmark, in the southeast Atlantic. However, rates of fluid flow and related microbiological processes in pockmark structures are still not well understood (Hustoft et al. 2009). In this study, we sought to analyze the diversity of anaerobic methanotrophs and sulfate-reducing communities in sediments of the G11 pockmark in Nyegga. The depth stratification of the archaeal community with depth was assessed by Denaturing Gel Gradient Electrophoresis (DGGE) fingerprinting based on PCR-amplified 16S rRNA genes. The metabolically active fraction of the archaeal communities was examined. Total RNA was extracted from sediment depths 0–4 cmbsf, and subjected to reverse transcription-PCR, with specific archaeal primers. Then, microbial community structure of the ANME and SRB were monitored with sediment depth, by examining the methyl coenzyme M reductase subunit alpha (*mcrA*) and dissimilatory sulfite reductase subunit beta (*dsrB*) functional genes respectively. Finally, as methane was previously shown to mainly have a biogenic origin in the G11 pockmark, cultures for methanogens were carried out at all sediment depths.

## Materials and methods

### Sediment sampling and porewater analysis

The sediment samples were collected from the Nyegga G11 pockmark (N 64°39.9925, E 005°17.3457) located in the Norwegian continental

margin at the Storegga Slide off the western coast of Norway in May/June 2006 during the Ifremer Vicking cruise with the research vessel "Pourquoi Pas?". The sediment sample was recovered during dive PL 272-02 05 by the VICTOR 6000 (Ifremer)-operated push core CT22 from 732 m of water depth. Brown greenish bacterial mats were observed in the Nyegga pockmarks region. The sampled bacterial mat was surrounded by sediments populated by black tubeworms. Immediately after retrieval, the sediment core was sectioned horizontally and aseptically in 2-cm-thick layers in the cold room (4°C), and then frozen at −80°C for nucleic acid extractions.

Sediment porewater was obtained by centrifuging approximately 10 g of crude sediment for 15 min at 3,000×*g* and 4°C; the supernatant porewater were removed, transferred to clean polypropylene tubes, and stored at −20°C. The depth distributions of dissolved sulfate and chloride concentrations were measured using ion exchange chromatography, with a isocratic DX120 ion chromatogtaphy system (DIONEX Corporation, Sunnyvale, CA) fitted with Ionpac AS9-SC columns and a supressor (ASRS-ultra II) unit in combination with a DS4-1 heated conductivity cell. Components were separated using a sodium carbonate gradient, with a flow of 1.5 mL $min^{-1}$.

## Culture media for enrichment of methanogens

Ten gram of wet sediment subsample was transferred into an anaerobic cabinet and then into 50 mL vials containing one volume (10 mL) of sterile and reduced Artificial Sea Water (ASW) medium immediately after sampling on board. ASW corresponded to medium 141 of DSMZ devoid of organic carbon substrates. The sediment slurries were stored at 4°C until processing. Enrichment cultures were performed anaerobically in 50 mL vials according to Balch and Wolfe (1976). In order to target methanogens, medium 141 from the DSMZ was used with slight modifications: organic substrates were omitted except yeast extract with a concentration adjusted to 0.2 g $L^{-1}$. The medium was prepared, sterilized under 80% $N_2$ and 20% $CO_2$ gas atmosphere and autoclaved. The medium was adjusted to pH 7.0. In order to enrich $CO_2$-reducing, aceticlastic and methylotrophic methanogens, three enrichment media supplemented with $H_2$ (200 kPa), acetate (10 mM), trimethylamine (TMA, 20 mM) were used as catabolic substrates. One mL of sediment suspension from the different sections of the push core CT22 was inoculated into 9 mL of medium. The suspension was mixed and serially diluted until $10^{-3}$. The cultures were incubated at 15°C to mimic in situ conditions. Cultures were periodically checked for methane production for one year. The methane detection was performed directly in the headspace of vial cultures by a micro MTI M200 Gas Chromatograph equipped with MS-5A capillary column and Poraplot U capillary column. Positive enrichment dilutions of methanogens were monitored by PCR-DGGE. For dilutions showing a single DGGE band on the fingerprint, 16S rRNA genes were amplified using the 8F and 1492R primers, cloned and sequenced, as subsequently described.

## Nucleic acids extraction and purification

Total genomic DNA was directly extracted and purified from 5 g of wet sediment from all sections in duplicates, by using the Zhou et al. (1996) method with modifications. Sediment samples were mixed with DNA extraction buffer as described by Zhou et al., frozen in liquid $N_2$, and then thawed at 65°C, 3 times. The pellet of crude nucleic acids obtained after centrifugation was washed with cold 80% ethanol, and resuspended in sterile deionized water, to give a final volume of 100 μL. Crude DNA extracts were then purified using the Wizard DNA clean-up kit (Promega, Madison, WI). DNA extracts were aliquoted and stored at −20°C until required for PCR amplification. Total RNA was directly extracted and purified from 2 g of wet sediment from pooled selected sediment sections 0–4 cmbsf, using the RNA PowerSoil Total RNA Isolation Kit (MO BIO Labs. Inc., Carlsbad, CA) according to manufacturer recommendations. Aliquots of RNA extracts were treated by Turbo™ DNAse (Applied Biosystems, Foster City, CA) and purified using the RNeasy® Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocols. The quality of RNA samples was examined by agarose-gel electrophoresis and concentrations were determined using spectrophotometry (Nanodrop ND-100, NanoDrop Technologies Wilmington, DE, USA).

## PCR of archaeal 16S rRNA genes

Archaeal 16S rRNA genes were amplified by PCR from purified DNA extracts using the primers pair 8F (5′-CGGTTGATCCTGCCGGA-3′) and 1492R (5′-GGCTACCTTGTTACGACTT-3′) (Lane 1991). All PCR reactions (total volume reaction 25 μL) contained 1 μL purified DNA template, 1× PCR buffer (Promega, Madison, WI), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer (Eurogentec) and 0.6 U Go*Taq* DNA polymerase (Promega, Madison, WI). Amplification was carried out using the GeneAmp PCR 9700 System (Applied Biosystems, Foster City, CA). The PCR conditions were as follows: denaturation at 94°C for 1 min, annealing at 49°C for 1 min 30 s, and extension at 72°C for 2 min for 30 cycles. All the archaeal 16S rRNA gene PCR products were then re-amplified in a nested PCR reaction with primers 340F (5′-CCCTACGGGGYGCASCAG-3′) (Vetriani et al. 1999) containing a GC clamp (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′) at the 5′ end and 519R (5′-TTACCGCGGCKGCTG-3′) (Ovreas et al. 1997). The PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 72–62°C (touchdown −0.5°C per cycle) for 30 s, and extension at 72°C for 1 min, for 20 cycles, then denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min, for 10 cycles, and a final extension at 72°C for 30 min (Janse et al. 2004).

To restrict contamination to a minimum, PCR experiments were carried out under aseptic conditions (Captair® bio, Erlab, Fisher Bioblock Scientific) using autoclaved and UV-treated plasticware and pipettes, and only sterile nuclease-free molecular grade water (MP Biomedicals, Solon, OH, USA). Positive (DNA extracted from pure cultures) and negative (molecular grade water) controls were used in all PCR amplifications.

## Denaturing gradient gel electrophoresis fingerprinting analysis, band excision, and sequencing

DGGE was carried out as described by Toffin et al. (2004) with some modifications. PCR products were separated by DGGE using the D-Gene™ System (Bio-Rad Laboratories, Hercules, CA) on 8% (w/v) polyacrylamide gels (40% acrylamide/bis solution 37.5:1 Bio-Rad) with a gradient of denaturant between 20 and 60% (100% denaturing conditions are defined as 7 M urea and 40% (v/v) formamide). Gels were poured with the aid of a 30 mL volume Gradient Mixer (Hoefer SG30, GE Healthcare, Buckinghamshire, UK) and prepared with 1 X TAE buffer (MP Biomedicals, Solon, OH, USA). Electrophoresis was carried out at 60°C, 200 V for 5 h (with an initial electrophoresis for 15 min at 80 V) in 1× TAE buffer. Polyacrylamide gels were stained with SYBRGold nucleic acid gel stain (Invitrogen, San Diega, CA) for 30 min, and viewed using the Typhoon 9400 Variable Mode Imager (GE Healthcare, Buckinghamshire, UK). Individual DGGE bands of interest were excised using a DarkReader® transilluminator DR-45 M (Labgene, Archamps, France) and washed in sterile nuclease-free molecular grade water for 10 min. Bands were then air-dried and crushed in 10–20 μL molecular grade water and incubated overnight at 4°C. The supernatant (1 μL) was used as template DNA in a nested PCR using primer set 340F and 519R. The PCR amplicons were sequenced with primer 519R, using an ABI PRISM 3100-Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Biogenouest platform of Roscoff Marine laboratory (France).

## Construction of RNA-derived 16S rRNA gene libraries

In order to obtain sufficient ribonucleic acids material, purified RNA were extracted from the 0 to 4 cmbsf depths and used for reverse transcription. RNA-derived cDNA was synthesised by reverse transcription using the archaeal 16S rRNA primer 915R (5′-GTGCTCCCCCGCCAATTCCT-3′) (Casamayor et al. 2002) and the Moloney Murine Leukaemia Virus reverse transcriptase (M-MuLV, MP Biomedicals, Irvine, CA) according to the manufacturer's protocol. Purified RNA (100–150 ng) was initially denatured by incubating at 65°C for 10 min, and 7.7 μM of 16S reverse primer 915R was added to the denatured RNA. The reaction mixture was incubated at 70°C for 10 min. The reverse transcription reaction mixture (total volume of 22 μL) consisted of 100–200 ng of denatured RNA and 16S rRNA reverse primer 915R, 1× M-MuLV buffer, 200 μM of deoxynucleoside triphosphate mix, and 10 mM DTT (dithiothreitol). The reverse

transcription reaction mix was incubated at 42°C for 2 min. A 200-unit aliquot of M-MuLV Reverse Transcriptase was added prior to a 80 min incubation at 42°C that resulted in the transcription of the RNA into complementary 16S ribosomal DNA (cDNA). The reverse transcriptase reaction was then stopped by heating the solution at 70°C during 15 min. The cDNA end product was used as a template for archaeal 16S based PCR using the following primers: 340F and 915R. The PCR amplification involved 20 cycles each consisting of 94°C for 1 min, 71–61°C (touchdown −1°C per cycle) for 1 min, and 72°C for 2 min. The purified RNA extracts that were not reverse transcribed were also amplified by PCR using the same primers, in order to monitor possible DNA contaminations of RNA templates. No contaminating DNA was detected in any of these reactions. PCR products were purified with the QIAquick® Gel Extraction kit (QIAGEN, Hilden, Germany) and analyzed on 1% (w/v) agarose gels run in 1 X TAE buffer stained with ethidium bromide and then UV-illuminated. Purified PCR products were cloned into TOPO® XL PCR Cloning Kit, and transformed into *Escherichia coli* TOP10 chemically competent cells (Invitrogen, San Diego, CA) according to the manufacturer's recommendations.

## Construction of *mcrA* and *dsrB* environmental gene libraries

Purified DNA extracted from the 0 to 2, and 8 to 10 cmbsf depths subsamples were used for the construction of *mcrA* and *dsrB* gene libraries. The *mcrA* genes were amplified by PCR using the ME1 (5′-GCMATG CARATHGGWATGTC-3′) and ME2 (5′-TCATKGC RTAGTTDGGRTAGT-3′) primers previously developed to specifically target methanogens and also anaerobic methanotrophs (Hales et al. 1996). The PCR conditions were as follows: denaturation at 94°C for 40 s, annealing at 50°C for 1 min 30 s, and extension at 72°C for 3 min, for 30 cycles. The *dsrB* genes were amplified by PCR using the dsr1F (5′-A CSCACTGGAAGCACG-3′) and dsr4R (5′-GTGTA GCAGTTACCGCA-3′) primers previously developed to specifically target sulfate-reducers (Wagner et al. 1998). The PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 2 min 15 s, for 30 cycles. PCR products were purified on a 1% agarose gel using the QIAquick® Gel Extraction kit (QIAGEN, Hilden, Germany) and cloned using the TOPO® XL PCR Cloning Kit (Invitrogen, San Diego, CA) according to the manufacturer's protocols. Sequencing was performed using an ABI PRISM 3100-Genetic Analyzer (Applied Biosystems, Foster City, CA). The M13R (5′-CAGGAAACAGCTAT-GAC-3′) universal primer was used for determining the RNA-derived 16S rRNA, *mcrA* and *dsrB* gene sequences.

## Phylogenetic analysis of DNA

The gene sequencing was performed by *Taq* cycle sequencing and determined on a ABI PRISM 3100-Genetic Analyzer (Applied Biosystems, Foster City, CA) using the M13R primer (5′-CAGGAAA-CAGCTATGAC-3′). Sequences were analyzed using the NCBI BLASTN search program within GeneBank (http://blast.ncbi.nlm.nih.gov/Blast) (Altschul et al. 1990). Chimeric sequences in the clone libraries were identified with the CHIMERA CHECK program of the Ribosomal Database Project II (Center for Microbial Ecology, Michigan State University, http://wdcm.nig.ac.jp/RDP/html/analyses.html). The RNA-derived 16S rRNA sequences and the enrichment culture-derived 16S rRNA gene sequences were then edited in the BioEdit v7.0.5 programm (Hall 1999) and aligned using the SINA webaligner (http://www.arb-silva.de/, Pruesse et al. 2007). The *mcrA* and *dsrB* sequences were translated into amino acid sequences using BioEdit and amino acid sequences were aligned using ClustalX (Larkin et al. 2007). Sequence data were analysed with the MEGA4.0.2 program (Tamura et al. 2007). The phylogenetic trees were calculated by the neighbour-joining analysis. The robustness of inferred topology was tested by bootstrap resampling (1,000). Sequence groups, using a 97% sequence similarity, were generated with the BlastClust program of the BLAST package (ftp://ftp.ncbi.nih.gov/blast/). Rarefaction curves were calculated for the RNA-derived 16S rRNA, *mcrA* and *dsrB* gene libraries using the RarFac program (http://www.icbm.de/pmbio/), and a 97% similarity cutoff value for sequence-based OTUs. The homologous coverage of the gene libraries were calculated using the following formula: $C=[1-(n_1/N)]*100$, where $n_1$ is the number of unique OTUs containing one 16S cDNA, *mcrA* or *dsrB* clone, and N is the number of

clones analyzed for respective genes (Singleton et al. 2001).

### Nucleotide sequence accession numbers

The sequence data reported here appear in GenBank nucleotide sequence databases under the accession no. GU989516 to GU989530 for DNA-derived 16S rRNA DGGE band sequences, GU989531 for the enrichment culture-derived 16S rRNA gene sequence, GU989532 to GU989593 for *mcrA* gene sequences, HM004722 to HM004784 for *dsrB* gene sequences, and GU989470 to GU989515 for RNA-derived 16S rRNA gene sequences.

## Results and discussion

### Geochemical characteristics

In the G11 pockmark sediments, porewater concentrations analysis of sulfate showed a decrease from 24 mM at the water–sediment interface down to 7 mM at 10 cmbsf (Fig. 1), indicating a possible sulfate-reduction zone. However, sulfate concentrations never reached complete depletion. Thus, the sulfate to methane transition zone was not reached in the CT-22 sediment push core. The chloride concentrations were low (Fig. 1) ranging from 497 to 527 mM. Low chloride concentrations were measured in porewater subsurface sediments of the Haakon Mosby mud volcano, also situated in the Norwegian Sea (Lein et al. 1999). These low chloride porewater concentrations characteristic of gas hydrate sediments were linked to subsurface clay dewatering. Methane was measured in the water column a few cms above the sampled sediment, and was below 0.45 μM (J-C Caprais, pers comm). This could indicate that methane is seeping through the G11 pockmark. However, no clear sulfate-methane transition zone was present in the sediment of the Nyegga pockmark.

### Diversity of the archaeal community

Archaeal 16S rRNA gene PCR products were obtained from all sediment samples by nested PCR. The DGGE pattern (Fig. 2) displayed a complex and diverse distribution of the archaeal communities, with 81 discernable bands from 0 to 10 cmbsf. Sequencing of excised DGGE bands confirmed that a majority of sequences were related to environmental clones retrieved from sediments or subseafloor sediments mainly associated with methane seeps (Table 1). These included sequences related to the ANME of the *Methanosarcinales* presumably involved in the anaerobic oxidation of methane. These ANME sequences dominate the DGGE band sequences data set throughout the entire core. For example, band sequences B8 and B9 could be related to the presumed methanotrophic ANME-1 group, with low sequence

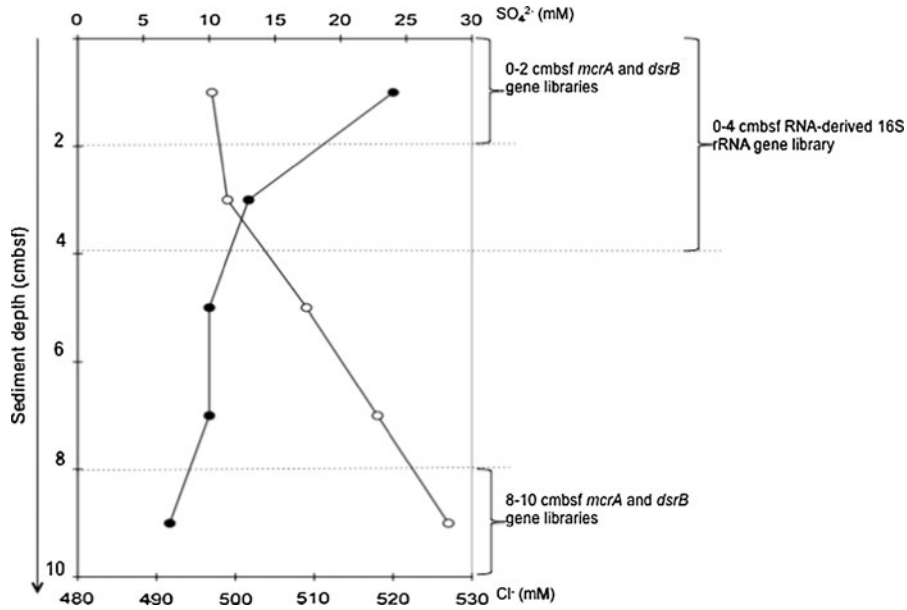



**Fig. 1** Sulfate (*filled circles*) and chloride (*open circles*) porewater concentrations with depth in push core CT22 of the G11 Nyegga pockmark sediments. The *scale* represents sediment depth below the seafloor

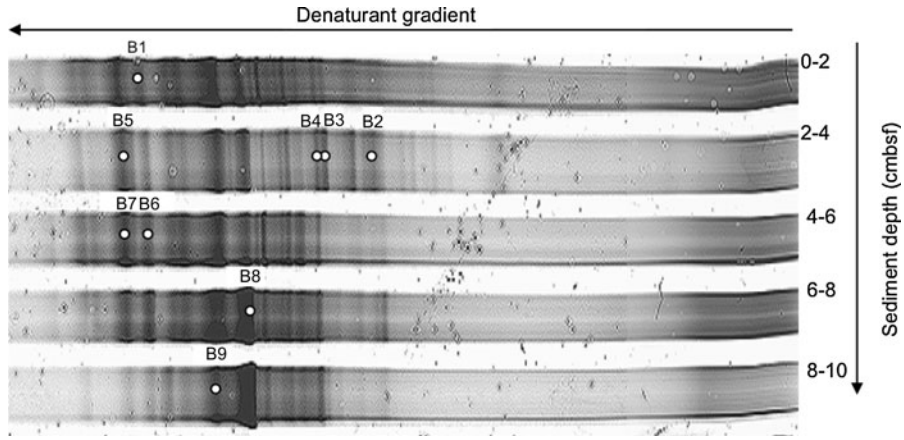



**Fig. 2** DGGE analysis of archaeal 16S rRNA gene sequences from various G11 Nyegga pockmark sediment depths by nested PCR. *White dots* show representative bands that were excised and sequenced. Numbers B1 to B9 are bands named Nye-dggeB1 to Nye-dggeB9. Depths in cmbsf was labelled at the *right* of individual lanes

similarity percentages. Band sequences Nye-dggeB1, B5, B6, and B7, could be related to the presumed methanotrophic ANME-2 clade. In addition, three band sequences were related to uncultured archaeal groups, *i.e.* Marine Benthic Group D (MBG-D) (Nye-dggeB3 and B4), and Marine Group I (MG-I) (Nye-dggeB2). The uncultured archeal groups MBG-D and MG-I are typically found in cold seeps around the world (Teske and Sorensen 2008).

Based on the DGGE fingerprinting observations, *mcrA* gene libraries were constructed from selected surface sulfate-rich sediments and deeper sediments (0–2, and 8–10 cmbsf) in order to compare methanogen/methanotroph population compositions in sulfate-rich and –poor sediments respectively. From the 0–2 and 8–10 cmbsf sections, a total of 31 and 30 *mcrA* clones were sequenced respectively. Calculation of the coverage values displayed 100% for the 0–2 cmbsf section clone library, and 90.3% for the 8–10 cmbsf clone libraries. Furthermore, rarefaction curves (Supplementary material. SM1) reached an asymptote indicating that sufficient clones were analysed to cover the major part of the *mcrA* diversity. The gene libraries were constituted solely by sequences related to ANME clades. A phylogenetic tree based on the *mcrA* genes (Fig. 3) showed a limited diversity and a majority of sequences most closely related to environmental clones rather than culture isolated representatives (Supplementary material. SM2). In the 0–2 cmbsf section, the major *mcrA* group was group e (ANME-2a), followed by *mcrA* group c/d (ANME-2c) as defined by Hallam et al. (2003). In the 8–10 cmbsf section, *mcrA* group e dominated, followed by the group c/d, the group f (ANME-3, as defined by Lösekann et al. (2007)), and the group a/b (ANME-1).

In order to assess the active fraction of the microbial communities, RNA was extracted from two different sediment depths (0–2 and 2–4 cmbsf) of the G11 Nyegga pockmark. RNA extractions from deeper sediment sections were unsuccessful even after several attempts. RNA extracts were not of sufficient quality and quantity (36.4 and 5.5 ng $\mu L^{-1}$ respectively) to be reverse transcribed, hence we pooled RNA extracted from both sections for retro-transcription. A total of 46 archaeal cDNA sequences from sediment depths 0–4 cmbsf were sequenced. Calculation of the coverage values displayed 97.8%, and the rarefaction curve (Supplementary material. SM1) indicated that sufficient clones were analysed to cover the major part of the diversity. All analyzed sequences were related to clones retrieved from similar marine environments (Hydrate Ridge, Santa Barbara Basin, Milano Mud Volcano, and North Sea, Supplementary material. SM4). This active fraction of the archaeal communities (Fig. 4) was related to the ANME-2 clade, with sequences affiliated with the ANME-2a/b (43.5% of total sequences), and with the ANME-2c (56.5% of total sequences). Hence, the 16S PCR-DGGE bands, *mcrA* gene libraries and the 16S rRNA gene library are almost all composed

**Table 1** Closest 16S rRNA gene sequences matches to the excised dominant DGGE bands using the NCBI BLASTN search

| Phylogenetic affiliation | DGGE band | Closest uncultured relative (accession number) and origin | Sequence identity (%) |
|---|---|---|---|
| *Crenarchaeota* MG-I | Nye-dggeB2 (2–4 cmbsf) | SAGMA-8 (AB050238) South African gold mine | 88 |
| *Euryarchaeota* MBG-D | Nye-dggeB3 (2–4 cmbsf) | A163E07 (FJ455958) Santa Barbara Basin sediments | 87 |
| | Nye-dggeB4 (2–4 cmbsf) | NapMat-6_10-rtD02 (HQ443471) Napoli mud volcano sediments | 80 |
| ANME-1 | Nye-dggeB8 (6–8 cmbsf) | Hyd24-Arch03 (AJ578110) Hydrate Ridge, and Black Sea mats | 87 |
| | Nye-dggeB9 (8–10 cmbsf) | BA1b1 (AF134382) Methane seep, Eel river basin | 82 |
| ANME-2 | Nye-dggeB1 (0–2 cmbsf) | AMSMV-30-A24 (HQ588685) Amsterdam mud volcano sediments | 98 |
| | Nye-dggeB5 (2–4 cmbsf) | LT-SA-15 (FJ755742) Lake Taihu sediments, China | 84 |
| | Nye-dggeB6 (4–6 cmbsf) | AMSMV-30-A24 (HQ588685) Amsterdam mud volcano sediments | 91 |
| | Nye-dggeB7 (4–6 cmbsf) | Milano-WF1A-23 (AY592819) Milano mud volcano sediments | 86 |

*MG-I* marine group I, *MBG-D* marine benthic group D

uniquely of ANME affiliated sequences, and mainly the ANME-2 clade.

The fingerprint pattern obtained by DGGE analysis suggests a vertically structured community with an archaeal community mainly composed of anaerobic methanotroph sequences. ANME-1 sequences were detected from 6 to 10 cmbsf, whereas ANME-2 were detected down until 6 cmbsf. In addition, metabolic genes indicative of methanogens/methanotrophs (*mcrA*) showed a heterogeneous community structure composed mainly of ANME-2a (*mcrA* group e) and ANME-2c (*mcrA* group c/d) from the surface and deeper sediment layers. Furthermore analysis of the RNA-derived 16S rRNA genes in shallow sediment layers (from the surface to 4 cmbsf) were solely affiliated with the ANME-2. Despite no clear SMTZ is present, ANME probably involved in AOM were distributed in the entire sediment depths. These anaerobic methanotrophs were mainly dominated by ANME-2. The environmental conditions at Nyegga certainly determined specific niches for establishment of ANME-2 communities. It is assumed that AOM is influenced by environmental factors such as methane and sulfate fluxes from subsurface reservoirs (Nauhaus et al. 2005). The ANME-2a and 2c subgroups were mostly retrieved from environments with moderate advective fluid flows, such as gas hydrate mounds and *Beggiatoa* mats covered sediments in the Gulf of Mexico, or *Bathymodiolus* and *Calyptogena* populated sediments in the Kuroshima Knoll and the Sagami Bay (Fang et al. 2006; Inagaki et al. 2004; Mills et al. 2004; Mills et al. 2003). As a working hypothesis, ANME-1 could outcompete ANME-2 for metabolites in so-called "high regimes" simulating in situ conditions (Girguis et al. 2005). Fluids regimes in the G11 Nyegga pockmark sampled sediments could be considered moderate as hinted by the presence of tubeworms close to the sampled sediments, as showed for sediments of the Haakon Mosby mud volcano (Niemann et al. 2006). Hence, these probable low fluid flows could have favored the development of ANME-2 *Archaea* explaining the dominance of ANME-2 sequences in the G11 Nyegga pockmark sediments. Also Krüger et al. (2008) suggested that ANME-2 *Archaea* prefer environments with high sulfate concentrations and effective sulfide removal, whereas ANME-1 could thrive in environments with lower sulfate concentrations. The observed vertical ANME distribution with depth in the Nyegga sediments seems to follow this pattern.

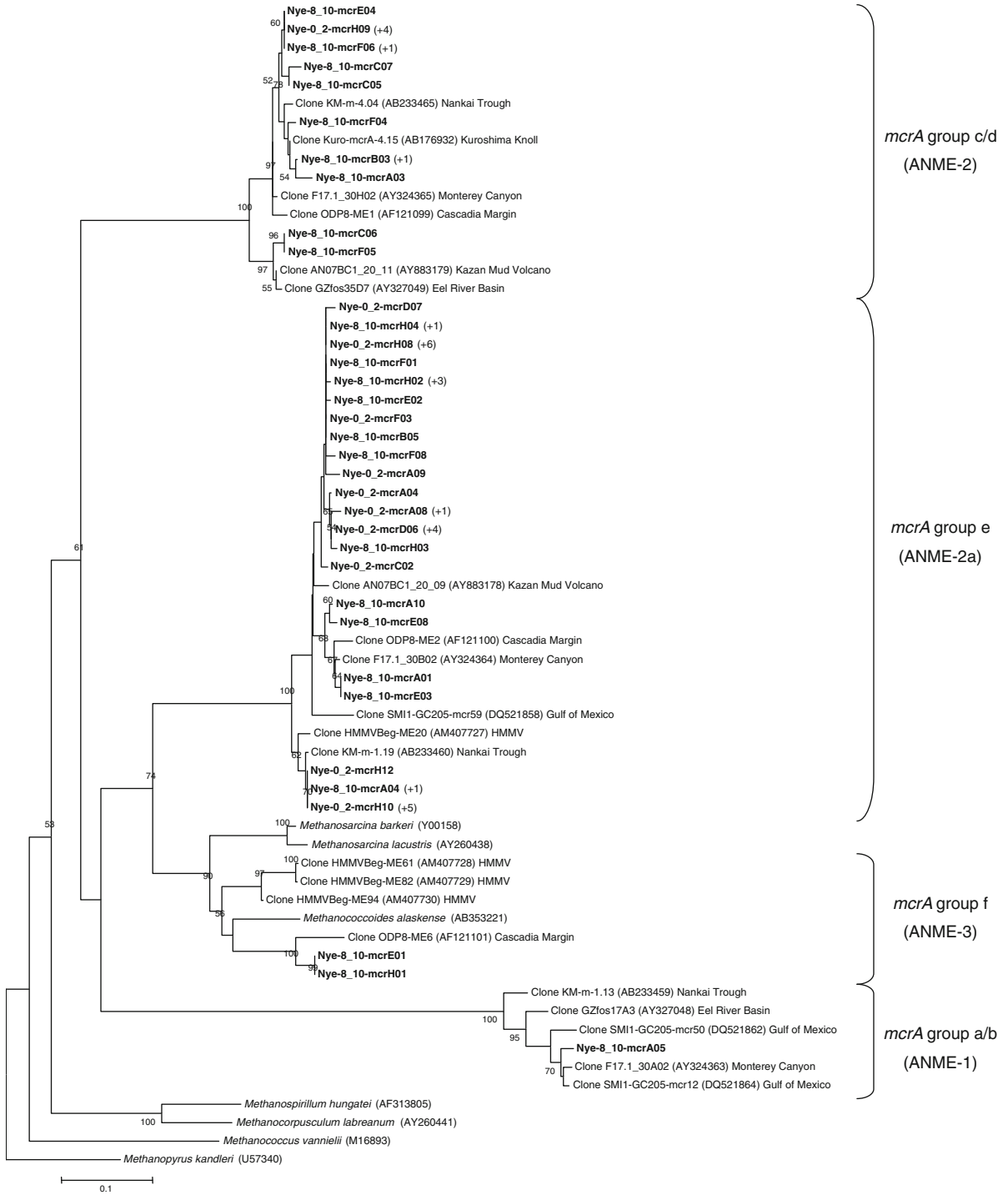



**Fig. 3** Phylogenetic analysis of MCR amino acid sequences from the G11 Nyegga pockmark sediments based on the neighbour-joining method with approximately 258 amino acid positions. Boostrap values (in percent) are based on 1,000 replicates and are indicated at nodes for branches values ≥50% boostrap support. Gene sequences from the G11 Nyegga pockmark sediments are in *boldface*. Clones with designation beginning Nye-0_2 are from sections 0 to 2 cmbsf, and clones with designation Nye-8_10 are from sections 8 to 10 cmbsf. Numbers in *brackets* indicate the number of analyzed clones that have more than 97% sequence identity

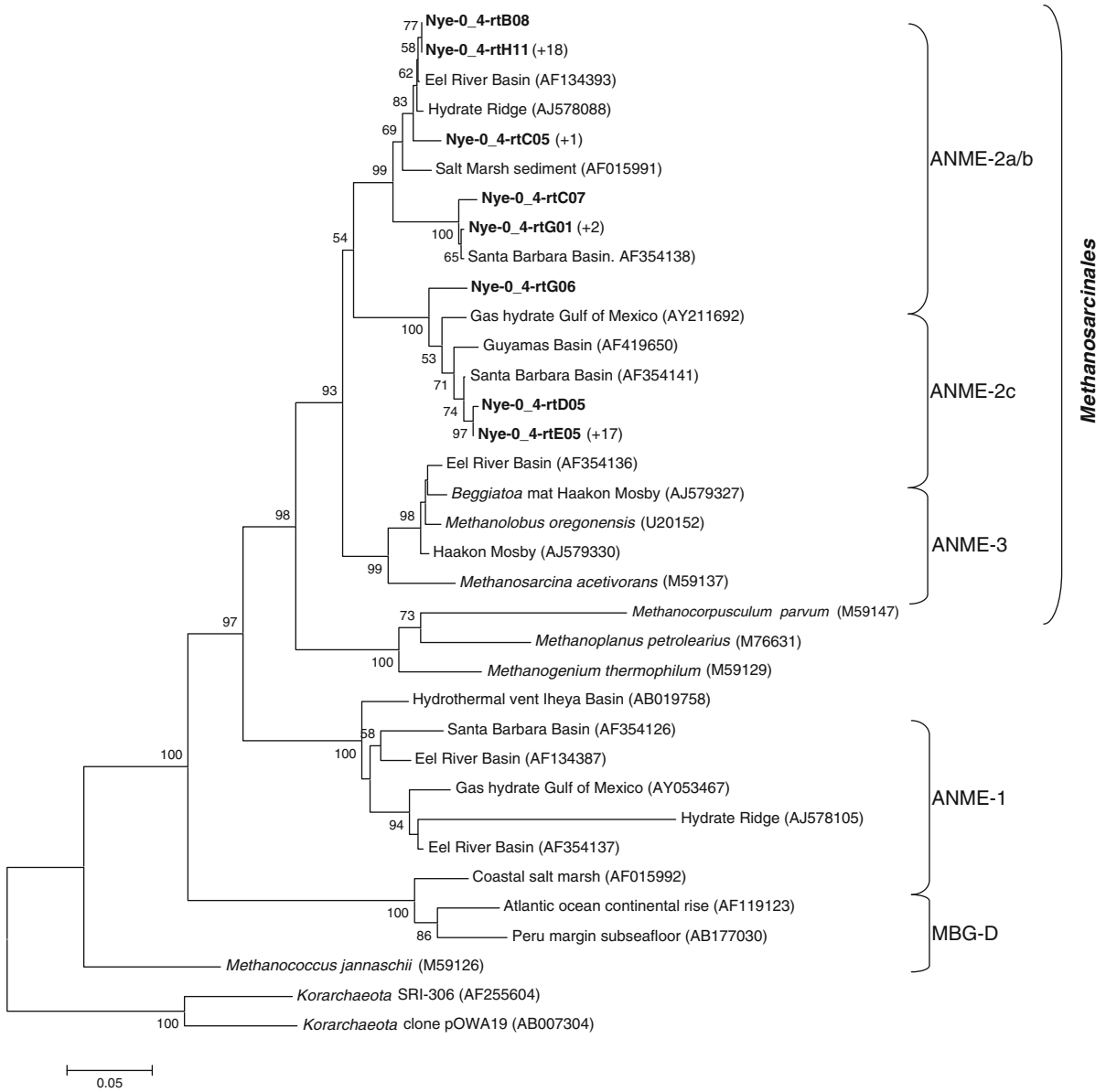



**Fig. 4** Phylogenetic tree of the archaeal RNA-derived 16S rRNA genes of the G11 Nyegga pockmark sediments based on the neighbour-joining method with 575 homologous positions. Boostrap values (in percent) are based on 1,000 replicates and are indicated at nodes for branches values ≥50% boostrap support. Gene sequences from the G11 Nyegga pockmark sediments are in *boldface*. Clones with designation beginning Nye-0_4 are from sections 0 to 4 cmbsf. Numbers in *brackets* indicate the number of analyzed clones that have more than 97% sequence identity

## Culturable methanogenic diversity

Methane production was detected in media designed to enrich hydrogenotrophic methanogens ($H_2/CO_2$) in the shallow sulfate-rich 0–2 cmbsf sediment section. Microscopic observations of positive enrichments from the $H_2$ medium suggest that these methanogens were regular cocci. Under UV light, autofluorescent methanogens were detected as free cells. Total DNA was extracted from the $H_2$ enriched medium of the 0–2 cmbsf section. Phylogenetic affiliation of clones Nye-0_2-enr40 showed 99% sequence similarity with

cultured methanogen *Methanogenium marinum* isolated from sediments of Skan Bay in Alaska (NR_028225, Chong et al. 2002).

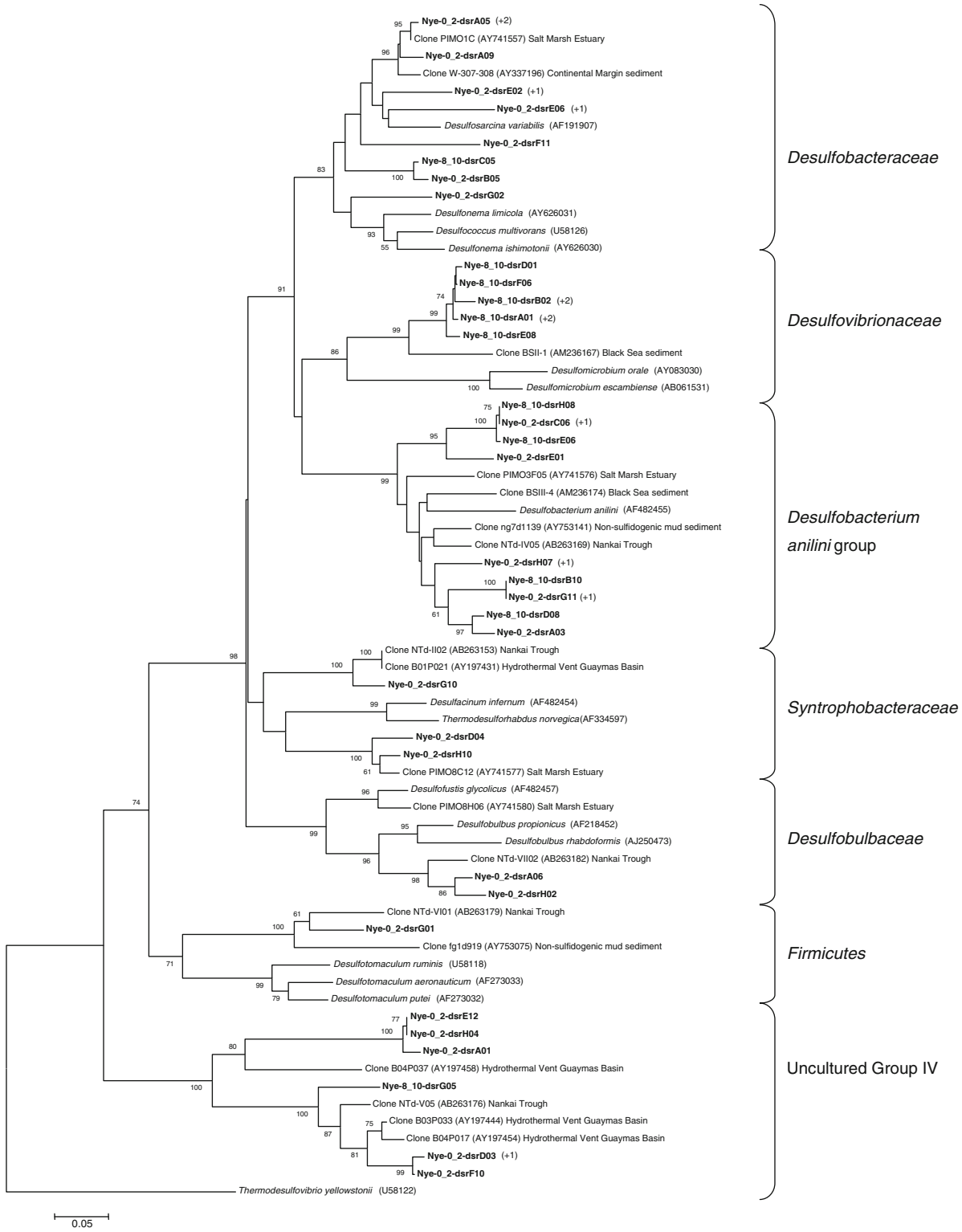
*M. marinum* is able to use $H_2/CO_2$ as catabolic substrates, but is also able to grow by reducing $CO_2$ with formate (Chong et al. 2002). Growth with formate is slower than with $H_2$, however formate could be an alternative electron donnor in the sulfate-rich sediment layers probably containing SRB that outcompete hydrogenotrophic methanogens for $H_2$. The absence of molecular signatures of methanogens in the gene libraries suggests that methanogenic populations probably represented a small fraction of the archaeal community (Kendall et al. 2007). Also, the specific primers used to amplify *mcrA* genes do not seem to successfully amplify all *Methanobacteriales* or *Methanosaetaceae mrtA* genes (Banning et al. 2005). However no methanogens, whether they belong to these orders or the other orders, have been detected.

ANME-2 communities were present and probably active in the surface sediments, whereas culturable methanogens of the *Methanomicrobiales* were enriched from the same sediment layers. These results could point out that AOM and methanogenesis processes took place simultaneously in the sulfate-rich surface sediment layers of the G11 Nyegga pockmark, as was previously described in a brine seep site and a gas hydrate site of the Gulf of Mexico (Orcutt et al. 2005), in microbial mats of the Black Sea (Seifert et al. 2006), or in non-seep sediments of Skagerak (Parkes et al. 2007).

## Diversity of sulfate-reducers

*dsrB* gene libraries were constructed from 0 to 2, and 8 to 10 cmbsf sediment sections of the CT22 push core. A total of 38 and 26 *dsrB* clones were sequenced from the 0–2 cmbsf and 8–10 cmbsf depths respectively, corresponding to 43.6% for the 0–2 cmbsf gene library, and 80.8% for the 8–10 cmbsf gene library. Furthermore, rarefaction curves (*Supplementary material*. SM1) indicated that sufficient clones were analysed to cover the major part of the *dsrB* diversity. The surface sulfate-rich sediment layers (0–2 cmbsf) displayed a high SRB diversity (Fig. 5), and contained sequences affiliated to the *Desulfobacteraceae* which dominated the clone library. Other sequences were affiliated with the *Desulfobacterium anilini*-related group, the uncultured Group IV as defined by Dhillon et al. (2003), the *Syntrophobacteracea*, the *Desulfobulbaceae*, and the non-*Deltaproteobacteria Firmicutes* (Supplementary material. SM3). The deeper sediment layers (8–10 cmbsf) displayed a much lower diversity than the surface sediments, and included three different clades (Fig. 5). Indeed, a majority of the clones were affiliated with the *Desulfovibrionaceae*, and a minority with the *Desulfobacterium anilini*-related group, the *Desulfobacteraceae,* and the uncultured Group IV (Supplementary material. SM3).

Analysis of the dissimilatory sulfite reductase subunit beta, metabolic genes indicative of sulfate-reducers (*dsrB*), reflected a change in SRB diversity with depth. A correlation between phylogenetic affiliation of the dominant SRB and specific environmental parameters is likely. Indeed, sulfate pore-water concentrations rapidly decreased to values of 7 mM, and probably had a major influence on the SRB community structure. Organic substrates availability was also shown to be a factor impacting SRB diversity (Leloup et al. 2007). And, the shallow Nyegga sediment layers underlying a microbial mat may be replenished with compounds such as fatty acids and amino acids, released from dead cells of the mat. The sulfate-rich surface layers were dominated by sequences related to the *Desulfobacteraceae* and *Desulfobacterium anilini* members, both considered complete-oxidizing SRB (Bahr et al. 2005; Rooney-Varga et al. 1997), whereas very few sequences of incomplete-oxidizing SRB were found, such as members of the *Desulfobulbaceae*. Cultured members of the *D. anilini* group couple dissimilatory reduction of sulfate to the degradation of various aromatic hydrocarbons (Leloup et al. 2007). These SRB have been found in sulfate-rich sediments (Kaneko et al. 2007), but mostly in SMTZ where AOM rates were the highest (Lloyd et al. 2006). Three minor SRB clusters also found in the surface sediment layers were affiliated to the *Syntrophobacteraceae*, *Desulfobulbaceae*, and the *Desulfomaculum*. These are nutrionally versatile SRB that can use acetate, lactate, alcohols, pyruvate, and fatty acids as energy sources, and some can reduce toxic heavy metals (Bahr et al. 2005; Chang et al. 2001; Cook et al. 2008; Dhillon et al. 2003). Hence members of these clusters would have a competitive advantage in environments where a broad range of organic substrates are available (Cook et al. 2008; Liu et al. 2003).


Nye-0_2-dsrA05 (+2)
Clone PIMO1C (AY741557) Salt Marsh Estuary
Nye-0_2-dsrA09
Clone W-307-308 (AY337196) Continental Margin sediment
Nye-0_2-dsrE02 (+1)
Nye-0_2-dsrE06 (+1)
Desulfosarcina variabilis (AF191907)
Nye-0_2-dsrF11
Nye-8_10-dsrC05
Nye-0_2-dsrB05
Nye-0_2-dsrG02
Desulfonema limicola (AY626031)
Desulfococcus multivorans (U58126)
Desulfonema ishimotonii (AY626030)
Desulfobacteraceae
Nye-8_10-dsrD01
Nye-8_10-dsrF06
Nye-8_10-dsrB02 (+2)
Nye-8_10-dsrA01 (+2)
Nye-8_10-dsrE08
Clone BSII-1 (AM236167) Black Sea sediment
Desulfomicrobium orale (AY083030)
Desulfomicrobium escambiense (AB061531)
Desulfovibrionaceae
Nye-8_10-dsrH08
Nye-0_2-dsrC06 (+1)
Nye-8_10-dsrE06
Nye-0_2-dsrE01
Clone PIMO3F05 (AY741576) Salt Marsh Estuary
Clone BSIII-4 (AM236174) Black Sea sediment
Desulfobacterium anilini (AF482455)
Clone ng7d1139 (AY753141) Non-sulfidogenic mud sediment
Clone NTd-IV05 (AB263169) Nankai Trough
Nye-0_2-dsrH07 (+1)
Nye-8_10-dsrB10
Nye-0_2-dsrG11 (+1)
Nye-8_10-dsrD08
Nye-0_2-dsrA03
Desulfobacterium anilini group
Clone NTd-II02 (AB263153) Nankai Trough
Clone B01P021 (AY197431) Hydrothermal Vent Guaymas Basin
Nye-0_2-dsrG10
Desulfacinum infernum (AF482454)
Thermodesulforhabdus norvegica(AF334597)
Nye-0_2-dsrD04
Nye-0_2-dsrH10
Clone PIMO8C12 (AY741577) Salt Marsh Estuary
Syntrophobacteraceae
Desulfofustis glycolicus (AF482457)
Clone PIMO8H06 (AY741580) Salt Marsh Estuary
Desulfobulbus propionicus (AF218452)
Desulfobulbus rhabdoformis (AJ250473)
Clone NTd-VII02 (AB263182) Nankai Trough
Nye-0_2-dsrA06
Nye-0_2-dsrH02
Desulfobulbaceae
Clone NTd-VI01 (AB263179) Nankai Trough
Nye-0_2-dsrG01
Clone fg1d919 (AY753075) Non-sulfidogenic mud sediment
Desulfotomaculum ruminis (U58118)
Desulfotomaculum aeronauticum (AF273033)
Desulfotomaculum putei (AF273032)
Firmicutes
Nye-0_2-dsrE12
Nye-0_2-dsrH04
Nye-0_2-dsrA01
Clone B04P037 (AY197458) Hydrothermal Vent Guaymas Basin
Nye-8_10-dsrG05
Clone NTd-V05 (AB263176) Nankai Trough
Clone B03P033 (AY197444) Hydrothermal Vent Guaymas Basin
Clone B04P017 (AY197454) Hydrothermal Vent Guaymas Basin
Nye-0_2-dsrD03 (+1)
Nye-0_2-dsrF10
Uncultured Group IV
Thermodesulfovibrio yellowstonii (U58122)
0.05

◀**Fig. 5** Phylogenetic analysis of DSR amino acid sequences from the G11 Nyegga pockmark sediments based on the neighbour-joining method with approximately 265 amino acid positions. Boostrap values (in percent) are based on 1,000 replicates and are indicated at nodes for branches values ≥50% boostrap support. Gene sequences from the G11 Nyegga pockmark sediments are in *boldface*. Clones with designation beginning Nye-0_2 are from sections 0 to 2 cmbsf, and clones with designation Nye-8_10 are from sections 8 to 10 cmbsf. Numbers in *brackets* indicate the number of analyzed clones that have more than 97% sequence identity

Sequences affiliated with the uncultured Group IV were found in both surface sulfate-rich and deeper sediment layers. Group IV sequences were previously retrieved in the Guaymas Basin, in sediments devoid of visible microbial mats, and characterized by liquid petroleum mud (Dhillon et al. 2003). The Group IV sequences were phylogenetically distinct from *dsrAB* genes found in the AOM zone, suggesting that they were not ANME bacterial partners. Group IV sequences were also detected in sediments with rich complex organic compounds (Bahr et al. 2005).

In the Nyegga sediments, archaeal RNA-derived 16S rRNA, *mcrA* and bacterial *dsrB* gene libraries were dominated by sequences previously associated with AOM. Indeed, in the surface sediment layers, the ANME-2 dominated archaeal gene libraries, and SRB community was mainly represented by sequences belonging to organisms of the *Desulfosarcina* and *Desulfococcus*. This observation could suggest the occurence of a syntrophic association between the ANME-2 and the SRB in the Nyegga sediments, as previously described (Schreiber et al. 2010). However there exists no phylogenetic difference between the *dsrB* sequences of free living SRB and sequences of the syntrophic SRB (Lloyd et al. 2006). Further analysis, such as FISH would be necessary to acertain this hypothesis.

In conclusion, the results of this study show that the *mcrA* and RNA-derived 16S rRNA gene libraries, as well as the DGGE band sequence analysis, were dominated by sequences related to the ANME. The ANME communities occuring in the G11 Nyegga pockmark sediments are comparable to those found in other marine ecosystems. Vertical distribution of ANME and SRB seems to indicate that AOM could occur in the sediments of the Nyegga Pockmark but needs to be confirmed. Further studies such as FISH or activity measurements on sediments associated with other pockmarks would help confirm that AOM processes are ubiquitous and certainly an important control of methane sink in various geological structures at cold seeps.

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