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Characterization of methanogenic and prokaryotic assemblages based on *mcrA* and 16S rRNA gene diversity in sediments of the Kazan mud volcano (Mediterranean Sea)

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ABSTRACT

The diversity of the methyl-coenzyme reductase A (*mcrA*) and 16S rRNA genes was investigated in gas hydrate containing sediment from the Kazan mud volcano, eastern Mediterranean Sea. *mcrA* was detected only at 15 and 20 cm below seafloor (cmbsf) from a 40-cm long push core, while based on chemical profiles of methane, sulfate, and sulfide, possible anaerobic oxidation of methane (AOM) depth was inferred at 12–15 cmbsf. The phylogenetic relationships of the obtained *mcrA*, archaeal and bacterial 16S rRNA genes, showed that all the found sequences were found in both depths and at similar relative abundances. *mcrA* diversity was low. All sequences were related to the Methanosarcinales, with the most dominant (77.2%) sequences falling in group *mcrA*-e. The 16S rRNA-based archaeal diversity also revealed low diversity and clear dominance (72.8% of all archaeal phylotypes) of the Methanosarcinales and, in particular, ANME-2c. Bacteria showed higher diversity but 83.2% of the retrieved phylotypes from both sediment layers belonged to the δ-Proteobacteria. These phylotypes fell in the SEEP-SRB1 putative AOM group. In addition, the rest of the less abundant phylotypes were related to yet-uncultivated representatives of the Actinobacteria, Spirochaetales, and candidate divisions OP11 and WS3 from gas hydrate-bearing habitats. These phylotype patterns indicate that AOM is occurring in the 15 and 20 cmbsf sediment layers.

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INTRODUCTION

Marine mud volcanoes (MVs) are formed when fluids, gas, and sediment under tectonic compression escape from the subsurface to the seafloor, often through extensive layers of accumulated sediment. Gas hydrates (GH) are often associated with deep-sea MVs and are formed by conventional low-temperature gas expulsion processes around the central part of an MV and by metasomatic processes at its periphery. The vast amounts of methane included in GH are believed to play a potentially important role in climate regulation (Milkov, 2000; Dimitrov, 2002).

In the eastern Mediterranean Sea, MVs and seep areas were first discovered during the late 1970s (Cita *et al.*, 1981); continuing research has revealed several such formations on the accretionary prism of the Hellenic arc (Mediterranean Ridge) and within the Anaximander Mountains (Woodside *et al.*, 1998). The Anaximander Mountains comprise a group of three main mountains between the Cyprus and Hellenic arcs (Zitter *et al.*, 2005). Based on their geology, they are interpreted as southward rifted blocks of south-western Turkey (Woodside *et al.*, 1997, 1998). They are currently undergoing a neotectonic deformation phase characterized by strike slip faulting with subsidiary normal faulting and some minor thrusts (Zitter *et al.*, 2005) within a zone of accommodation between the westerly moving Anatolian Plate and the African Plate (McClusky *et al.*, 2002).

The first GH sampling in the Anaximander Mountains took place in 1996 at the Kula MV (Woodside *et al.*, 1997, 1998), while GH have also been sampled from four other MVs of the area. High seafloor methane fluxes are associated with the MVs as well as with the accompanying cold vents and seeps (Charlou *et al.*, 2003) and the available gas provides energy for rich benthic communities which include chemosynthetic symbiotic fauna (Olu-Le Roy *et al.*, 2004). Carbonate crusts may be formed in these environments (Aloisi *et al.*, 2002) as well as GH under certain conditions.

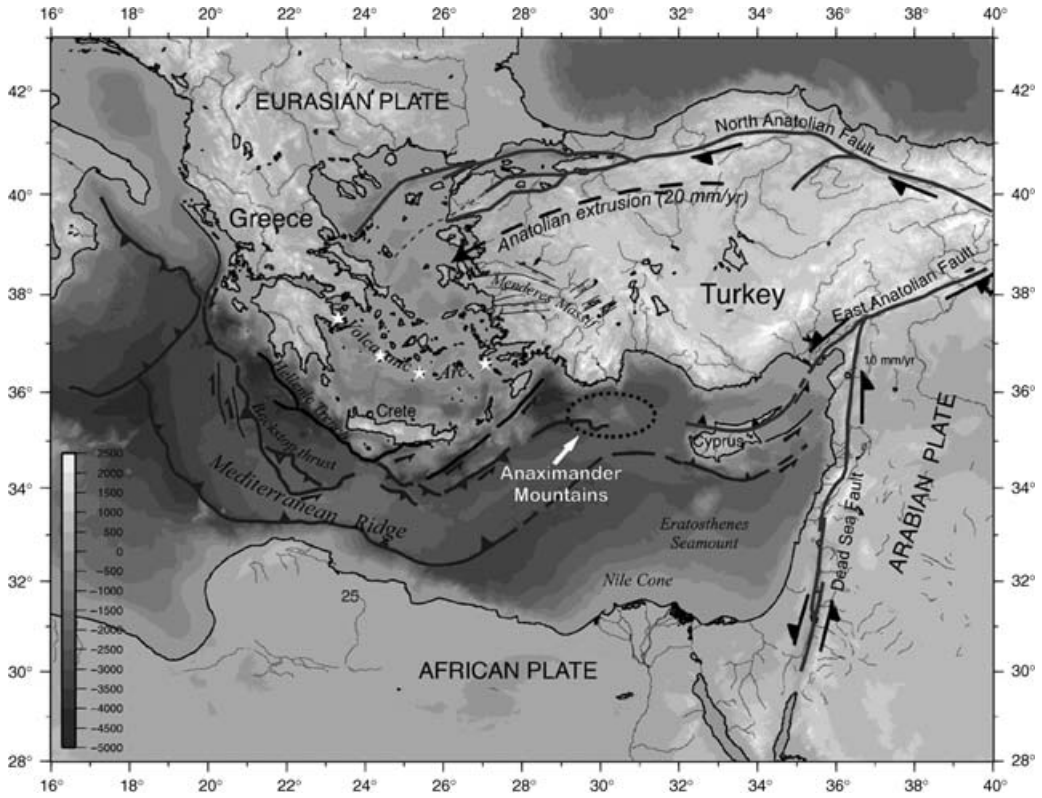

Fig. 1 Map of the study area and sampling site.

The carbon-rich fluids in MVs often sustain highly diverse and productive microbial ecosystems based on chemosynthesis below and/or at the seafloor. Probably the most important biogeochemical process fuelling these communities at these locations is the anaerobic oxidation of methane (AOM) (Boetius & Suess, 2004). Earlier work has provided evidence for the occurrence of AOM in Kazan carbonate crusts, based on carbon isotopic signatures and porewater chemistry and general molecular fingerprints (Aloisi *et al.*, 2000; Werne *et al.*, 2002; Bouloubassi *et al.*, 2006; Heijs *et al.*, 2006), but also in the sediments of Kazan MV (Heijs *et al.*, 2007). The current study provides molecular evidence for the occurrence of AOM-related microorganisms within the Kazan MV sediments with 5-cm depth resolution. Here we report the composition of the methanogenic and methane-oxidizing microbial communities, based on the occurrence and diversity of the methyl-coenzyme reductase A (*mcrA*) gene within the top 40 cm of the Kazan MV sediments, integrated with geochemical parameters and with 16S rRNA analysis of the archaeal and bacterial assemblages.

METHODS

Sampling

Gas hydrate-containing sediment samples were collected from the Kazan MV (35°25′55″ N, 30°33′42″ E) of the Anaximander Mountains (Fig. 1), eastern Mediterranean Sea, with the R/V AEGEO in May 2003. The push cores were obtained from an Eckman box core (0.16 m^2), which collected approximately 40 cm of sediment. Upon retrieval of the box core on the deck, the fizzing out of GH at the size of rice grains or larger was observed at the bottom half of the box core. To achieve simultaneous sampling for geochemical and microbiological analyses of high spatial resolution, we used a predrilled plastic push core that contained, at intervals of 5 cm along the core, two adjacent holes (*c.* 2.5 cm diameter) that were 1–2 cm offset from each other horizontally (Fig. 2). All holes were originally covered with plastic tape during coring; the tape was removed after coring, stepwise opening up each pair of holes which were then used for syringe sampling. Each 5-cm interval was sampled by using two sterile 20-mL syringes with cut-off luer end. This handling minimized the loss of sediment gases during sampling. For porewater geochemical analyses, extra sediment samples were retrieved with finer depth resolution (every 2–2.5 cm), obtained by parallel sampling from this core and from an adjacent, second push core that was vertically offset by 2.5 cm.

For microbiological analyses of syringe samples, the outer sediment layer that was in contact with the plastic tape was removed by pushing off the top 1 mm of the syringe-sampled sediment; the rest was transferred to a 50-mL Falcon sterile tube and stored immediately at –20 °C on board. After returning to

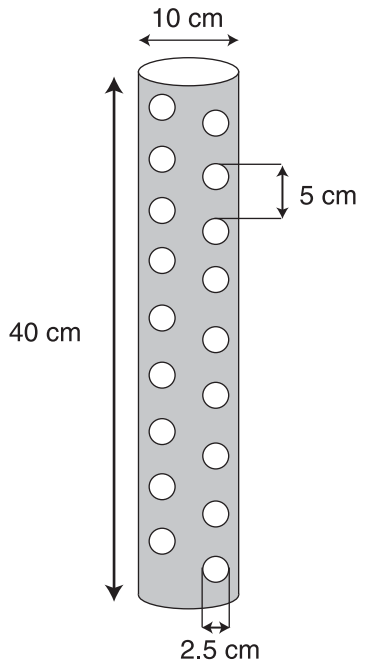

Fig. 2 Schematic drawing of the pre-drilled push core used for sediment sampling in Kazan mud volcano.

the laboratory (≤12 days), the samples were transferred at –80 °C until further analysis.

For methane analysis, 3 mL sediment samples (in most cases from the same core that yielded the microbiology syringe samples) were taken immediately after core retrieval with a cut syringe. At the sediment surface, an additional methane sample was taken from the second, vertically offset core. The sediment was put into a 30-mL glass bottle prefilled with saturated NaCl solution (*c.* 300 g L^{-1}) that was quickly sealed with a gas-tight butyl stopper, allowing no air to remain in the bottle. A headspace of 5 mL was made with nitrogen gas while simultaneously removing 5 mL of saltwater using a second needle. The bottles were shaken to make a homogeneous suspension and equilibrated up-side down for 24 h at room temperature prior to gas analysis. Methane concentrations were determined on board, injecting 1 mL gas sample into a Shimadzu gas chromatograph GC-14B with flame Ionization detector and equipped with a packed stainless steel Porapack Q (6 ft, 2 mm i.d., 80/100 mesh; Alltech, Deerfield, IL, USA). The oven was kept at 30 °C. Methane standards of 15, 100, and 1000 p.p.m.v. C_1 were measured three times prior to the analysis of a set of samples. The precision of the method is ~3%. The gas concentration in the headspace was calculated off-line from the peak area, from which the methane concentration in the liquid phase was calculated using the Bunsen solubility coefficient β (Wiesenburg & Guinasso, 1979; Werne *et al.*, 2004). Due to the large oversaturation in dissolved gases for most sediments, a substantial but unknown amount of gas may have escaped during core retrieval and on-deck handling. The measured concentrations for sediment samples are therefore minimum values. All concentrations are corrected for volume, salinity, and temperature, and are reported in µmol or mmol kg^{-1} of wet sediment.

For regular pore waters, 10–25 mL of sediment was extracted by centrifugation. The pore water was filtered over a 0.2-µm cellulose acetate, and subsampled inside an anoxic glovebox to avoid oxidation artefacts. To subsamples of 2 mL for H_2S, 10 µL of 0.1 N NaOH was added. Analyses for H_2S were carried out on board on a TRAACS800 continuous flow analyzer, applying colorimetric methods after (Grasshoff *et al.*, 1983). Sulfate concentrations were measured onshore as S, using inductively coupled plasma-atomic emission spectrometry. The standard deviation for all measurements is 3% or better.

DNA extraction, amplification, and cloning

DNA was extracted using the UltraClean Soil DNA kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol with minor modifications: bead beating was reduced from 10 to 5 min, and this step was immediately followed by three cycles of freeze-and-thaw (–80 °C for 3 min and then immediately in 65 °C water bath for 5 min). The amplification of the *mcrA* gene was achieved by using the ME1 (5′-GCMATGCARATHGGWATGTC-3′) and ME2 (5′-TCATKGCRTAGTTDGGRTAGT-3′) primers (Hales *et al.*, 1996), which amplify ca. 750 bp of the subunit α of the methyl-coenzyme reductase, in a MyCyler (Bio-Rad Inc., Hercules, CA, USA) thermal cycler. The amplification included an initial denaturation step at 95 °C for 1 min, 30 cycles of (95 °C denaturation for 30 s; primer annealing at 55 °C for 1 min; and primer extension at 72 °C for 1 min) and one final cycle of (at 94 °C for 30 s; at 55 °C for 3 min; at 72 °C for 10 min). Bacterial 16S rDNA was amplified using the bacterial primers BAC-8F (5′-AGAGTTTGATCCTG-GCTCAG-3′) and BAC-1492R (5′-CGGCTACCTTGTTA-CGACTT-3′) (Lane, 1991) followed by a nested polymerase chain reaction (PCR) with BAC-8F and BAC-1390r (5′-TGTACACACCGCCCGTC-3′). The first PCR included an initial denaturation step at 94 °C for 1 min was followed by 30 cycles consisting of denaturation at 94 °C for 45 s, annealing at 52.5 °C for 45 s, and elongation at 72 °C for 2 min; a final 7-min elongation step at 72 °C was added. The second PCR included an initial denaturation step at 94 °C for 1 min was followed by 15 cycles consisting of denaturation at 94 °C for 45 s, annealing at 52.5 °C for 45 s, and elongation at 72 °C for 2 min; a final 7-min elongation step at 72 °C was added. Archaeal 16S rDNA was amplified using the primer combination ARC-8f (5′-TCCGGTTGATCCTGCC-3′) and ARC-1492r (5′-GGCTACCTTGTTACGACTT-3′) (Teske *et al.*, 2002). An initial denaturation step at 94 °C for 1 min was followed by 22 to 38 cycles consisting of denaturation at 94 °C for 45 s, annealing at 52.5 °C for 45 s, and elongation at 72 °C for 2 min; a final 7-min elongation step at 72 °C was added. PCRs

were repeated with different cycle numbers, and the lowest number of cycles that gave a positive signal (26 cycles for Archaea and *mcrA*, 30 cycles for bacteria) was then used for cloning and sequencing in order to avoid differential representation of 16S rDNA genes with low and high copy numbers (Spiegelman *et al.*, 2005).

Polymerase chain reaction products were visualized on a 1.2% agarose gel under ultraviolet light, bands were excised, and PCR products were extracted with the Wizard SV Gel and PCR Clean-up kit (Promega Inc., Madison, WI, USA) following the manufacturer's protocol. The purified products were A-tailed to improve cloning efficiency by mixing 2.5 μL 10× PCR buffer (200 mM Tris, pH 8.55), 2.5 μL deoxynucleoside triphosphate 2 mM, 1.5 μL $MgCl_2$ 25 mM, 5.9 μL PCR water, 0.1 μL Taq polymerase and 12.5 μL of the purified sample. The mixture was incubated at 72 °C for 10 min in a water bath or thermal cycler. The PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen Corporation, Hercules, CA, USA) using chemically competent cells according to the manufacturer's specifications. For each sample and each gene, 22–49 clones were analysed. These clones were screened for unique restriction fragment length polymorphism patterns after digestion at 37 °C for 18 h with the *RsaI*, *AluI*, and *HinfIII* (Fermentas UAB, Vilnius, Lithuania) enzymes (final concentrations of 3 U for each enzyme). Clones with unique restriction fragment length polymorphism patterns were grown in liquid LB medium with kanamycin and their plasmids were purified using the NucleoSpin Plasmid QuickPure kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) for DNA sequencing.

Sequencing and phylogenetic analysis

Sequence data were obtained by Macrogen Inc. (Seoul, South Korea) using capillary electrophoresis by the BigDye Terminator kit (Applied Biosystems Inc., Foster City, CA, USA) with the primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′). Every sequence read was approximately 850 bp. For each individual clone, forward and reverse reads were assembled, and then the assembled sequences were checked for chimeras using the CHIMERA_CHECK function of the Ribosomal Database Project II (Maidak *et al.*, 2001).

For the detection of closest relatives, all sequences were compared with the BLAST function (www.ncbi.nlm.nih.gov/BLAST/) for the detection of closest relatives. Sequence data were compiled using the ARB software (Ludwig *et al.*, 2004) and aligned with sequences obtained from the ARB and GenBank (www.ncbi.nlm.nih.gov) databases, using the ARB FastAligner utility, and followed by manual aligning according to secondary structure in ARB. Phylogenetic analyses were performed using minimum evolution and parsimony methods implemented in PAUP* version 4.08b (Swofford, 2000). Heuristic searches under minimum evolution criteria used 1000 random-addition replicates per data set, each followed by tree bisection–reconnection topological rearrangements. The topology of the tree was based on neighbour-joining according to Jukes-Cantor. Bootstrapping under minimum evolution and parsimony criteria was performed with 1000 replicates. Sequences of unique phylotypes found in this study have GenBank numbers DQ103596–DQ103602 for bacterial 16S rRNA genes, DQ084449 to DQ084452 for archaeal 16S rRNA genes, and AY883169 to AY883179 for *mcrA* genes.

RESULTS

Geochemistry

The porewater sulfate concentration decreases from bottom water values of near 30 mM to background values of ca. 1 mM at ~15 cm sediment depth. Sulfide is low in the topmost 3 cm (0.026 mmol L^{-1} wet sediment), but increases rapidly from thereon to a maximum of 13 mmol L^{-1} wet sediment at 13 cm depth. The methane content is low (3.4–134.3 mmol kg^{-1} sediment) in the topmost few centimetres, but rapidly increases with sediment depth from 11 to 16 cm, being above sea-level saturation value in higher depths (Fig. 3).

Microbial profiling

In the investigated core AN07-BC1, the *mcrA* gene was detected only at 15 and 20 cm below seafloor (cmbsf). At these depths, the diversity of the *mcrA* and archaeal and bacterial 16S rRNA genes was assessed. The clone library coverage for each depth and gene investigated, was satisfactory according to Good's C estimator (Kemp & Aller, 2004) as all curves reached a plateau above 0.80 with the number of clones analysed (Fig. 4).

A total of 62 and 65 of *mcrA* clones were sequenced from 15 and 20 cmbsf, respectively, and 11 representative phylotypes were detected based on a ≥98% similarity cut-off (Table 1). Nine out of 11 clones were detected in both sediment layers. Two of the most abundant sequences from 15 cmbsf (AN07-BC1-15cmbsf-31 and AN07-BC1-15cmbsf-34) and two less abundant sequences (AN07-BC1-15cmbsf-29 and AN07-BC1-20cmbsf-5) were >99% similar with each other, representing possibly the same phylotype (Table 1). All but one (AN07-BC1-15cmbsf-33) phylotypes fall in the ANME-2 group (Fig. 5A) with their closest relatives (91–98% similarity) originating from the Haakon Mosby MV, Monterey Canyon-seep sediment, Eel River Basin, and anoxic methane-seep sediments and mats (Table 1). In particular, the most abundant phylotypes (79.0% and 75.4% at 15 and 20 cmbsf, respectively) belonged to the ANME-2e group while the less abundant phylotypes (14.5% and 23.1% at 15 and 20 cmbsf, respectively) belonged to the ANME-2c and -2d groups. Phylotype AN07-BC1-15cmbsf-31 (6.4% abundance) fell in the ANME-1 group, showing very low similarity with any of the existing sequences in GenBank.

Table 1 16S rRNA and *mcrA* gene sequences retrieved from the Kazan mud volcano (cmbsf, cm below seafloor)

	No. of similar (≥98%) clones				
	15 cmbsf	20 cmbsf	Putative affiliation	Closest relative (GenBank accession no.), similarity	Reference
mcrA					
AN07-BC1-20-1	15	11	ANME-2e	Clone HMMVBeg-ME20, Haakon Mosby mud volcano (AM407727), 91%	Lösekann *et al*. (2007)
AN07-BC1-20-9	11	15	ANME-2e	Clone HMMVBeg-ME20, Haakon Mosby mud volcano (AM407727), 93%	Lösekann *et al*. (2007)
AN07-BC1-15-34	11	12	ANME-2e	Clone HMMVBeg-ME20, Haakon Mosby mud volcano (AM407727), 92%	Lösekann *et al*. (2007)
AN07-BC1-15-31	12	11	ANME-2e	Clone K8MV-C28mcrA-16, anoxic methane-seep sediments (AB362208), 98%	Miyazaki *et al*. (unpublished data)
AN07-BC1-20-7	2	4	ANME-2c & -2d	Clone K8MV-C22mcrA-11, anoxic methane-seep sediments (AB362199), 96%	Miyazaki *et al*. (unpublished data)
AN07-BC1-20-11	2	3	ANME-2c & -2d	Clone K8MV-C22mcrA-11, anoxic methane-seep sediments (AB362199), 97%	Miyazaki *et al*. (unpublished data)
AN07-BC1-20-4	0	5	ANME-2c & -2d	Clone GZfos26B2, Eel River Basin (AY714839), 96%	Hallam *et al*. (2004)
AN07-BC1-15-33	4	1	ANME-1	Clone 0418F12, methane-oxidizing microbial mat (BX649197) (70%)	Krüger *et al*. (2003)
AN07-BC1-20-5	1	2	ANME-2c & -2d	Clone GZfos26B2, Eel River Basin (AY714839), 98%	Hallam *et al*. (2004)
AN07-BC1-15-28	2	1	ANME-2c & -2d	Clone GZfos35D7, Eel River Basin (AY327049), 98%	Hallam *et al*. (2004)
AN07-BC1-15-29	2	0	ANME-2c & -2d	Clone GZfos26B2, Eel River Basin (AY714839), 98%	Hallam *et al*. (2004)
Archaea					
AN07-BC1-15cmbsf-157A	26	20	ANME-2c	Clone fos0626f11, Hydrate Ridge (AJ890142), 99%	Meyerdierks *et al*. (2005)
AN07-BC1-15cmbsf-162A	4	11	ANME-2a & -2d	Clone fos0642g6, Hydrate Ridge (CR937012), 99%	Meyerdierks *et al*. (2005)
AN07-BC1-20cmbsf-153A	10	11	ANME-2c	Clone fos0644c1, Hydrate Ridge (CR937009), 98%	Meyerdierks *et al*. (2005)
AN07-BC1-20cmbsf-154A	3	7	ANME-2a & -2d	Clone fos0642g6, Hydrate Ridge (CR937012), 99%	Meyerdierks *et al*. (2005)
Bacteria					
AN07-BC1-15cmbsf-104B	12	9	WS3	Clone Zplanct36, anaerobic, sulfide- and sulfur-rich Zodletone Spring (EF602497), 94%	Elshahed *et al*. (2007)
AN07-BC1-15cmbsf-105B	18	13	Desulfobacterales	Isolate Eel-36e1H1, Eel River Basin and Santa Barbara Basin (AF354164), 98%	Orphan *et al*. (2001)
AN07-BC1-15cmbsf-106B	4	16	WS3	Clone Zplanct36, anaerobic, sulfide- and sulfur-rich Zodletone Spring (EF602497), 94%	Elshahed *et al*. (2007)
AN07-BC1-15cmbsf-107B	3	4	Actinobacteria	Clone VHS-B3-77, Victoria Harbour sediments (DQ394962) 96%	Zhang and Qian (unpublished data)
AN07-BC1-15cmbsf-108B	5	2	Spirochaetales	Clone S11-50, Pacific arctic surface sediment (EU287233), 89%	Li *et al*. (unpublished data)
AN07-BC1-15cmbsf-109B	22	29	Desulfobacterales	Clone Hyd89-04, Cascadia margin (AJ535240), 99%	Knittel *et al*. (2003)
AN07-BC1-15cmbsf-110B	5	6	OP11	Clone Amsterdam-2B-13, Amsterdam mud volcano (AY592373), 98%	Heijs *et al*. (2006)

The archaeal 16S rRNA gene clone library included only four phylotypes resulting from the sequencing of 43 and 49 clones from 15 and 20 cmbsf, respectively, and occurred in both sediment layers (Table 1, Fig. 4B). Based on practically identical (98–99% similarity) environmental sequences from other methane-related cold seeps and hydrothermal fields, the two most abundant clones (AN07-BC1-15cmbsf-157A and AN07-BC1-15cmbsf-153; A83.7% and 63.3% at 15 and 20 cmbsf, respectively) fell in the ANME-2c group while the remaining two fell in the ANME-2a and -2b groups, of the Methanosarcinales order.

The bacterial 16S rRNA gene clone library was more diverse than the archaeal one (Table 1, Fig. 6). In total, 69 and 79 clones were sequenced from the 15 and 20 cmbsf sediment layers, respectively, and seven phylotypes were revealed which occurred in both layers. Phylotypes AN07-BC1-15cmbsf-104B and -106B were >99% similar, suggesting that they represent the same phylotype. This phylotype was related to the candidate division WS3. Phylotypes AN07-BC1-15cmbsf-109B and -105B (31.9% and 36.8% at 15 and 20 cmbsf, respectively) were related to phylotypes from similar environments of the order Desulfobacterales (δ-Proteobacteria). The rest of the phylotypes, representing 18.8% and 15.2% at 15 and 20 cmbsf, respectively, were related to uncultivated members from similar environments of the Actinobacteria, Spirochaetes phyla, and candidate division OP11.

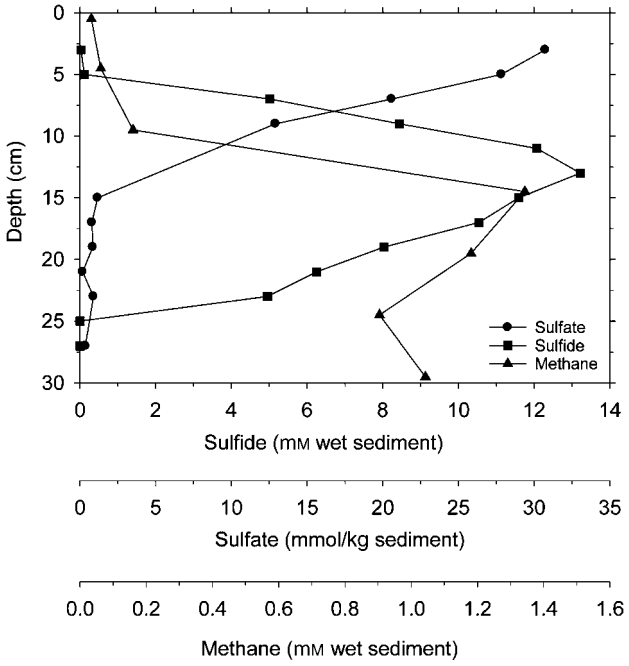

Fig. 3 Chemical profiles of site AN07-BC1 in Kazan MV.

DISCUSSION

In this study, the prokaryotic molecular diversity was studied in the sediment depth horizons of the Kazan MV, where methanogens occur, based on the occurrence of the *mcrA* gene. Although the full extent of the prokaryotic diversity is difficult to unravel in natural samples due to technical innate limitations (e.g. Wintzingerode *et al.*, 1997), our clone library coverage (Fig. 4) is satisfactory based on the Good's C estimator (Kemp & Aller, 2004), suggesting that the major part of the existing prokaryotic diversity in the studied sediment layers has been revealed.

Geochemistry

Sulfide formation is taking place at ~13 cmbsf, where the maximum of sulfide concentration occurs (Fig. 3). The peak shape of the concentration profile suggests that this formation is rapid compared to any potential removal processes. Accordingly, there is a rapid downward decrease in sulfate and an increase in methane in the upper half of the core. These data give a clear indication for a sulfate reduction/methane oxidation transition zone (Iversen & Jørgensen, 1985) at about 13 cmbsf. In studies

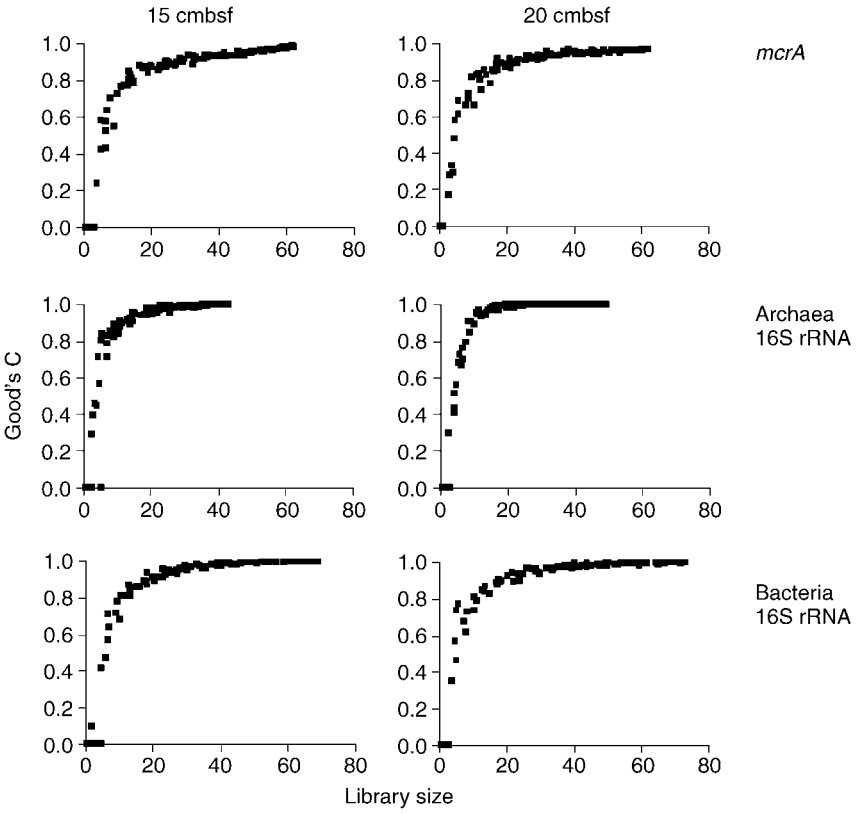

Fig. 4 Clone library coverage based on Good's C estimator of the prokaryotic clone libraries from the Kazan mud volcano.

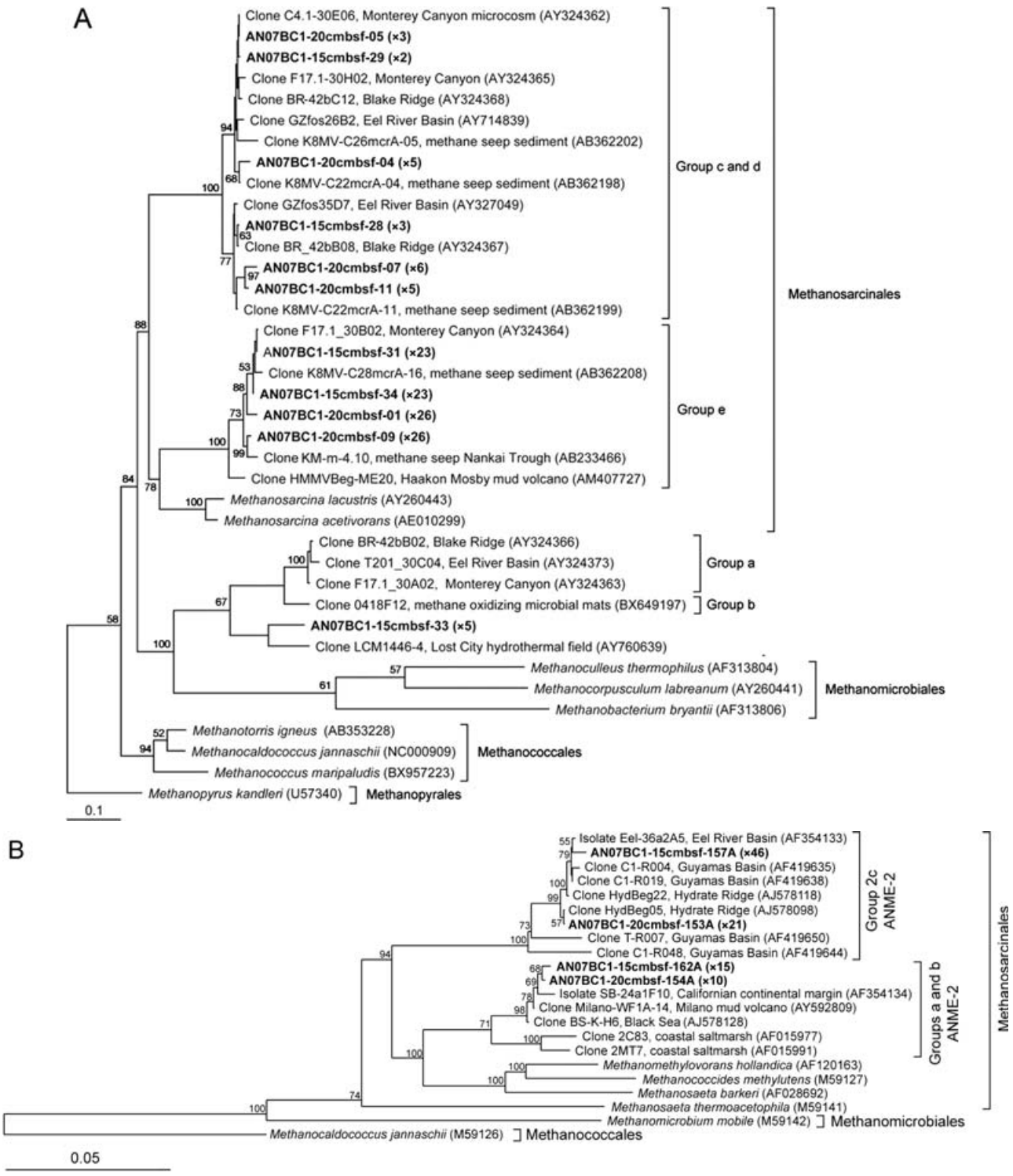

Fig. 5 Phylogenetic trees of amino acid sequences of PCR-amplified *mcrA* genes (A) (ca. 770 bp) and archaeal 16S rDNA (B) (ca. 1490 bp) from sediment core AN07BC1, Kazan mud volcano, Eastern Mediterranean Sea, based on the neighbour-joining method as determined by distance Jukes-Cantor analysis. Each clone is named after its sediment depth origin (cm below sea floor, cmbsf). One thousand bootstrap analyses (distance) were conducted, and percentages greater than 50% are indicated at the nodes. Group names are based on those of Hallam *et al*. (2003).

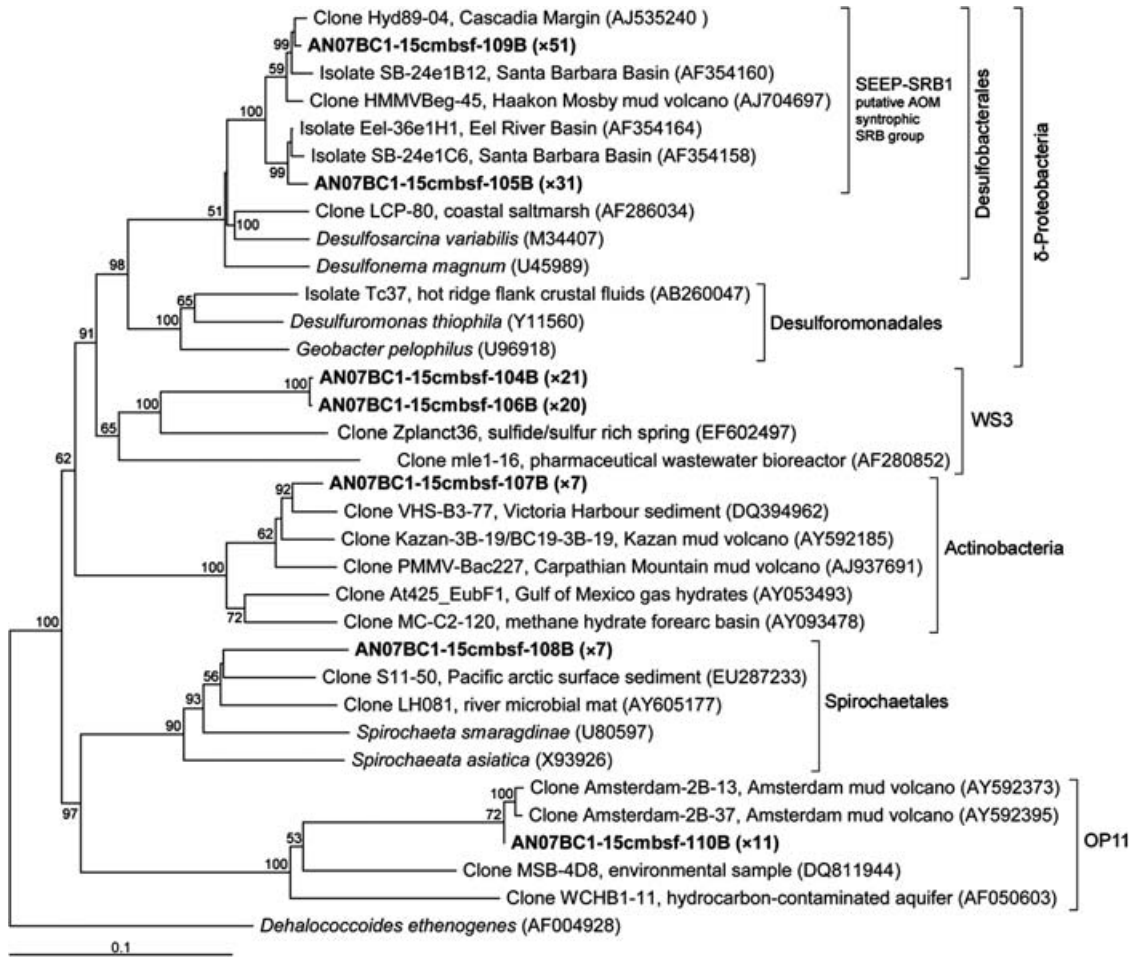

Fig. 6 Phylogenetic tree of relationships of 16S rDNA of the representative bacterial clones found in Kazan MV sediments, based on near-complete sequences (ca. 1390 bp), based on the neighbour-joining method as determined by distance Jukes-Cantor analysis. Each clone is named after its sediment depth origin (cm below sea floor, cmbsf). One thousand bootstrap analyses (distance) were conducted, and percentages greater than 50% are indicated at the nodes. The tree was rooted by using *Dehalococcoides ethenogens* as an outgroup. Groups (bold letters) that have ≥98% similar nucleic acid sequences within each sediment depth are represented by a single sequence, with the number of clones out of the total in parentheses. The numbers in brackets are GenBank accession numbers. Scale bar represents 10% estimated distance.

based on lipid biomarkers, it has been suggested that AOM occurs in Kazan MV sediment layers of *c.* 7–20 cmbsf (Werne *et al.*, 2002, 2004; Haese *et al.*, 2003). This finding corroborates with the geochemical profile we acquired for methane, sulfate, and sulfide concentrations and with our findings of AOM-related prokaryotic assemblages at 15 and 20 cmbsf (see below).

mcrA and archaeal 16S rRNA diversity

The vast majority (96.1%) of the Kazan *mcrA* clones were affiliated to the *mcrA* e, c, and d groups and the remaining to the *mcrA* a and b groups. All but one of the 11 sequences appeared both in sediment layers and in similar relative abundances, thus, pointing to no spatial heterogeneity. The predominant clones (77.2%) fell in the *mcrA* e group while the predominant 16S rRNA phylotype fell in the ANME-2c group. A very similar occurrence pattern for both genes was observed in methane-seep sediments of the Nankai Trough (Nunoura *et al.*, 2006).

In contrast to most GH sediments, where archaeal diversity is rather medium to high and ANME-1, ANME-2 and ANME-3 often co-occur (Biddle *et al.*, 1999; Hinrichs *et al.*, 1999; Orphan *et al.*, 2001; Aloisi *et al.*, 2002; Teske *et al.*, 2002; Mills *et al.*, 2003; Knittel *et al.*, 2005; Mills *et al.*, 2005; Nauhaus *et al.*, 2005; Lloyd *et al.*, 2006; Lösekann *et al.*, 2007), in Kazan the archaeal community showed very low diversity. The archaeal communities at 15 and 20 cmbsf consisted of two phylotypes belonging to the ANME-2c and two at the ANME-2a and -2b groups. Similarly, low archaeal diversity was reported in Hydrate Ridge (Knittel *et al.*, 2005) and in the mat-covered surficial sediment of the near-by Milano MV (Heijs *et al.*, 2005). Furthermore, ANME-2 and Methanosarcinales phylotypes outnumbered ANME-1 in sediments

from the Cascadia margin (Lanoil *et al.*, 2001), Monterey Canyon, and Blake Ridge (Hallam *et al.*, 2003). The dominance of ANME-2 has been related to low methane fluxes (Girguis *et al.*, 2005; but see Lloyd *et al.*, 2006) and it has also been suggested that ANME-1 prefers more highly reductive habitats than ANME-2 (Knittel *et al.*, 2005). In Kazan MV, methane fluxes are not known yet but the sediment core we studied (AN07BC1) was characterized by the absence of overlaying pelagic sediments, indicating that the sampling site was located on a relatively recent mud flow (e.g. Stadnitskaia *et al.*, 2005), suggesting possible oxygen diffusion down the sediment. Also, the presence of small (<1 cm) rice-like GH at around 20–25 cmbsf could suggest inherent low gas diffusion from the subsurface. At the bottom of the core (35–40 cmbsf), we found larger hydrates that could be subject to outcropping causing resuspension and sediment mixing (e.g. Lloyd *et al.*, 2006) and enhancing thus oxygen diffusion from the sediment surface. Finally, fluorescence *in situ* hybridization-based studies in Kazan MV, have shown no ANME-1 (Kallionaki & Kormas, unpublished data). ANME-2 (groups a, b, and c) were the dominant Euryarchaeota in a number of phylotypes and relative abundances at the 22–34 cmbsf sediment layer in Kazan MV in a previous study (Heijs *et al.*, 2007). It seems that the occurrence of ANME-2 is important in the Kazan MV sediments. What remains to be investigated is whether this population is metabolically active.

Bacterial 16S rRNA diversity

Bacterial diversity at 15 and 20 cmbsf in the Kazan MV was higher compared to the archaeal and comparable with that of other methane-rich environments like the Cascadia margin (Knittel *et al.*, 2003) and the Nankai Trough (Reed *et al.*, 2002). No depth heterogeneity was observed as all phylotypes occurred in both sediment layers. Most of the bacterial phylotypes found (58.0% and 53.2% at 15 and 20 cmbsf, respectively) were related to uncultured members of the δ-Proteobacteria subphylum found in similar environments. In particular, the two most abundant phylotypes (AN07-BC1-15cmbsf-109B and -105B) were affiliated with the SEEP-SRB1 group. This lineage consists of environmental sequences from similar environments such as methane seeps from the Eel River Basin, Santa Barbara Basin, the Guaymas Basin, Gulf of Mexico (Knittel *et al.*, 2003), and the Haakon Mosby MV (Lösekann *et al.*, 2007), and are often involved in AOM.

Phylotypes AN07-BC1-15cmbsf-104B and -106B, which were practically identical, comprised 27.70% of the total bacterial phylotypes and were the second most dominant ones. No known relatives were found for these sequences, and in particular, they were affiliated to the candidate division WS3 for which no cultivated representatives are known. The first sequences of the WS3 group originate from the methanogenic zone of soil contaminated with hydrocarbons and chlorinated solvents (Dojka *et al.*, 1998). Other phylotypes related to the WS3 group occur in H_2S-containing sediments of the Florida Escarpment, Gulf of Mexico (Reed *et al.*, 2006), in hydrocarbon-seep sediment from Coal Oil Point Seep Field at a depth of 22 m in the Santa Barbara Channel, California (LaMontagne *et al.*, 2004), and in anoxic marine sediments of Loch Duich, Scotland (Freitag & Prosser, 2003).

The three remaining unique clones, which comprised only 16.90% of all phylotypes, belonged to different phylogenetic groups. Phylotype AN07-BC1-15cmbsf-107B was related to other sequences from methane-bearing sediments and MVs, including Kazan. Although Actinobacteria dominated the top 6 cm of the Kazan MV, such phylotypes were also found in the 6–22 cmbsf sediment layer in a previous study (Heijs *et al.*, 2007). These actinobacterial sequences were not closely related to cultured actinobacterial species; therefore, a specific role for these phylotypes could not be inferred. The fact, however, that such representatives seem to appear more and more often in similar habitats, suggests that this group must be studied in more detail.

Phylotype AN07-BC1-15cmbsf-108B was distantly related to the Spirochaetales. Its closest relative, *Spirochaeta smaragdinae*, is an obligate anaerobe and the first characterized member of the Spirochaetaceae that reduces thiosulfate and sulfur to hydrogen sulfide (Magot *et al.*, 1997). Thiosulfate was not measured in this study but a Spirochaetales-related phylotype has been recently reported which is associated with the gills of the cold-seep clam *Lucinoma* aff. *kazani* from Kazan MV (Duperron *et al.*, 2007).

Phylotype AN07-BC1-15cmbsf-110B was very closely related with two previously found sequences from the 6–22 cmbsf sediment layer of the Kazan MV (Heijs *et al.*, 2007). These sequences were attributed to the Chloroflexi. However, we place phylotype AN07-BC1-15cmbsf-110B and the two Kazan MV sequences in the candidate division OP11. This is supported not only by our high bootstrap values but also by the putative affiliation given by the authors of other related sequences (Fig. 6), like clone WCHB1-11 which originated from the methanogenic zone of soil contaminated with hydrocarbons and chlorinated solvents (Dojka *et al.*, 1998). Thus, it is possible that this phylotype takes part in methane cycling at the Kazan MV.

The occurrence of the *mcrA* gene in depths 15 and 20 cmbsf could indicate possible biogenic methane production. However, the isotopic composition of C in methane (–51 ± 2‰, data not shown here) depicts towards a mixture of methane from thermocatalytic and biogenic source (Welhan, 1988; Mastalerz *et al.*, 2006). In addition, the ^{13}C-isotopic signature could also show the imprint of anaerobic methane oxidation, which selectively removes and oxidizes lighter methane and generates a heavier methane profile towards the upper part of the sediment surface (e.g. Lloyd *et al.*, 2006). The occurring phylotypes indicate AOM at 15 and 20 cmbsf depths, and in particular, the clear dominance of ANME-2 and sulfate-reducing bacteria of the δ-Proteobacteria representatives, which have been observed only in sediments that exhibit AOM (Boetius *et al.*, 2000; Orphan *et al.*, 2001; Knittel *et al.*, 2005).

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