

Article

Diversity of NC10 Bacteria and ANME-2d Archaea in Sediments of Fault Zones at Lake Baikal

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Abstract: Anaerobic oxidation of methane (AOM) reduces methane emissions from marine ecosystems, but we know little about AOM in freshwater reservoirs. Lake Baikal is the world's only freshwater reservoir where gas hydrates (GH) are found. Despite that AOM has been demonstrated in deep sediments of Lake Baikal did not reveal methane-oxidizing archaea ANME1, 2, or 3, which are responsible for AOM in marine sediments. A search for representatives *Candidatus Methyloirabilis oxyfera* (*M. oxyfera*)-like bacteria (NC10) and *Candidatus Methanoperedens nitroreducens* (*M. nitroreducens*)-like archaea (ANME-2d) has been carried out in the different types of Lake Baikal methane enriched sediments. We used different molecular biology methods including high-throughput sequencing and PCR analysis, using 16S rRNA genes as well as different functional genes of AOM (*mcrA* and *pmoA*). We found diverse *M. oxyfera*-like bacteria and *M. nitroreducens*-like archaea in various geological structures in Lake Baikal (methane seep and mud volcano), which were different from the composition of the discharged fluid. We also considered possible electron acceptors for this process in the sediments of Lake Baikal.

Keywords: anaerobic oxidation of methane; Lake Baikal; high-throughput sequencing of the 16S rRNA gene; *pmoA* gene; *mcrA* gene; *Candidatus Methanoperedens nitroreducens* (ANME-2d); *Candidatus Methyloirabilis oxyfera* (NC10)

1. Introduction

Lake sediments are globally important carbon storages [1,2], but they are also important contributors of methane (CH₄) to the atmosphere [3]. Approximately 70% of atmospheric methane is of microbial origin. CH₄ emissions from these environments are controlled by both methanogenesis and CH₄ oxidation. Methanotrophs oxidize the resulting methane under both aerobic and anaerobic conditions, decreasing significantly the emission of this greenhouse gas into the atmosphere. In the presence of oxygen, widespread methanotrophic species of *Alpha*- and *Gammaproteobacteria*, which have the key enzyme of a methylotrophic pathway (methane monooxygenase), oxidize methane [4–9]. Aerobic methanotrophs were also described within the phylum *Verrucomicrobia* [10].

The process of AOM was described long ago [11–14]. Currently, the most studied processes are sulfate-dependent methane oxidation via the reverse methanogenesis pathway performed by archaea of the ANME clusters in marine sediments [15–17]. Approximately 90% of CH₄ is assimilated through anaerobic oxidation by ANME clusters [16,18]. There are three known ANME archaea clusters (ANME1, 2 and 3), which were further divided into subclusters. ANME-1 and ANME-2 are very widespread in marine sediments, whereas members of ANME-3 are mainly found in deep mud

volcanoes. The highest density of methanotrophic archaeal populations is observed in cold methane seeps [16].

In recent years, there has been an active search for microbial agents that carry out AOM in freshwater ecosystems. Recent studies have shown that in oxygen-free soil layers and bottom sediments, anaerobic methanotrophic bacteria, and/or archaea, which are used as electron acceptors of nitrite or nitrate ions, consume methane [19–24]. Methane oxidation under anaerobic conditions, which is associated with nitrite reduction, was first demonstrated in the enrichment cultures derived from freshwater sediments [23]. Microorganisms responsible for this process were classified as bacteria that belong to the candidate phylum NC10 [21] and are named *M. oxyfera*. Usually, the 16S rRNA gene is applied as a biomarker for analyzing NC10 bacteria [21]. However, the *pmoA* gene catalyzes the aerobic conversion of methane to methanol, is highly conserved, and can be applied as a functional marker to identify NC10-like bacteria [25]. Furthermore, nitric oxide dismutase (*nod*) functional gene can be also as phylogenetic marker for oxygenic methanotrophs within the NC10 phylum [26]. Analyses of various environmental samples using primers on the *pmoA* and *nod* genes have indicated *M. oxyfera* various anoxic environments including lake sediments, wastewater treatment systems [27–31]. Members of the ANME-2d subcluster named *M. nitroreducens* perform AOM using nitrate as the terminal electron acceptor [19,22]. The resulting nitrite is reduced to gaseous nitrogen due to syntrophic interaction with ANAMMOX-bacteria (in the presence of ammonium) or with *M. oxyfera* (phylum NC10) [32]. Nitrate, being a product of anammox process can be reused by members of ANME-2d, which are widespread in environments and detected in freshwater ecosystems, soils, paddy fields, and treatment plants [22,23,33–35]. *M. nitroreducens* consist of all genes of the reverse methanogenic pathway (*mcrABCDG*) [22,36].

Although the 16S rRNA gene is most commonly used for phylogenetic analysis, the *mcrA* gene is an alternative and more specific biomarker for detecting not only methanogens but ANMEs clusters in the environments. Previously published *mcrA* primers were designed to mainly target all known methanogens and methanotrophic archaea, and most of them have a strong bias toward certain methanogens or specific groups of ANMEs [37–40]. Available general *mcrA* primers do not suit well to capture *mcrA* sequences of ANME-2d in the nature, potentially resulting in under representation in molecular investigation. Therefore, for detection of *mcrA* sequences in the environment, novel *mcrA* primers were developed, which specifically target *M. nitroreducens* for refined phylogenetic analysis [41].

More ANME sequences in different environments and possibly new archaeal phyla involved in AOM may yet have to be detected. For example, in genomes representatives *Bathyarchaeota* and *Verstraetearchaeota* the *mcrA* genes were found [42,43]. Sequences of phylum *Verstraetearchaeota* were detected in two habitats: a mesophilic methanogenic granular sludge reactor and deep sediment of the freshwater Lake Pavin (France). Fe^{3+} , Cr^{4+} , and Mn^{4+} may also act as electron acceptors in the course of anaerobic methane oxidation, but the mechanisms of these processes have not been determined yet [33,34,44]. Fe(III)-dependent AOM is likely a prevalent process both marine [20,45] and freshwater [46] ecosystems, where large amounts of iron oxides are detected in the sediments. Iron and manganese ions were present in freshwater lakes that lack SO_4^{2-} and NO_3^- ions and where AOM was detected [47,48]. Some representatives of ANME-2d have also been reported coupling CH_4 oxidation to the reduction of Mn^{4+} and/or Fe^{3+} [44,49]. One of the first study sites that provides information about AOM activity in freshwater sediments is Lake Ørn. In the sediments of this lake, which are characterized by low concentrations of SO_4^{2-} and Fe^{2+} ions [47,50], ANME-2d subcluster sequences were detected [24] in the absence of nitrate ions. This finding suggests that *M. nitroreducens*-like archaea perform AOM coupled to sulfate and/or iron reduction and may be capable of assimilation of CH_4 and dissolved inorganic carbon. Thus, ANME-2d archaea play an important role in controlling methane emissions from freshwater systems with low concentration NO_3^- and SO_4^{2-} ions [24].

Lake Baikal is the world's only freshwater reservoir where gas hydrates (GH) are found, and it describes more than 50 geological structures that differ in the composition of the discharged

fluids [51,52]. In deep sediments near GH, the rate of AOM ranged from 35 to 273.2 $\mu\text{L CH}_4/(\text{dm}^3 \text{ day})$ [53], increasing at depths of 20–60 cm, which may indicate the functioning of the anaerobic community of microorganisms [54–56]. The process of AOM in deep sediments of Lake Baikal was detected by radioisotope techniques, with a maximum that was usually close to the GH layers, as well as by the shape of methane profile with depth typical for AOM [56,57]. Despite high methane concentrations in deep sediments of some sites of Lake Baikal (up to 15000 μM) that are sufficient to carry out of AOM [58], members of methane-oxidizing ANME clusters were not detected [57,59,60]. In this regard, it was suggested that the process could be carried out in methane-enriched freshwater lake sediments by other microorganisms, including ANME-2d archaea and NC10 bacteria. The latest study showed the simultaneous presence of these phyla in enrichment culture with nitrate ions as a nitrogen source in AOM [61] and in enrichment Baikal cultures obtained from the cultivation of the microbial community from the mud volcano Peschanka (Southern Baikal) [62]. Chemical analysis of pore water deep sediments from Lake Baikal generally indicated the absence or low concentrations of nitrate and nitrite ions (up to 9.6 μM) [56,63], which is insufficient for AOM. Therefore, the question about the presence of electron acceptors and microbial agents that carry out AOM remains open, and it is unclear which of the known mechanisms of this process dominate in the deep sediments of Lake Baikal.

The aim of this study was to detect representatives of the ANME-2d subcluster and NC10 bacteria in Baikal sediments with different types of geological structures, which discharge sulfate-calcium and sodium-calcium fluids with increased concentrations of Fe^{2+} and NH_4^+ , as well as to determine the presence of functional AOM genes in their genomes (*mcrA* and *pmoA*) using methods of high-throughput sequencing and PCR analysis.

2. Materials and Methods

2.1. Description of Sampling Sites, Sample Collection, and Pretreatment

Sediment samples were collected during the 2010 and 2012 expeditions on board the research vessel (RV) “G.Yu. Vereshchagin” using the gravity corer (GC) at two geomorphologically distinct sites: the mud volcano Malenky (M, ~1371 water depth) and the methane seep Posolsk Bank (PB, 400 m) (Figure 1). GH layers occurred in the sediments of the mud volcano M at depths from 130 to 139 cm and between 100 and 140 cm in those, the methane seep PB. After lifting the core onto the RV board and cutting it into two lengthwise halves, the sediment samples were collected for chemical analysis of pore waters and DNA extraction. Bottom sediments selected for DNA extraction were stored in liquid nitrogen until the laboratory analysis.

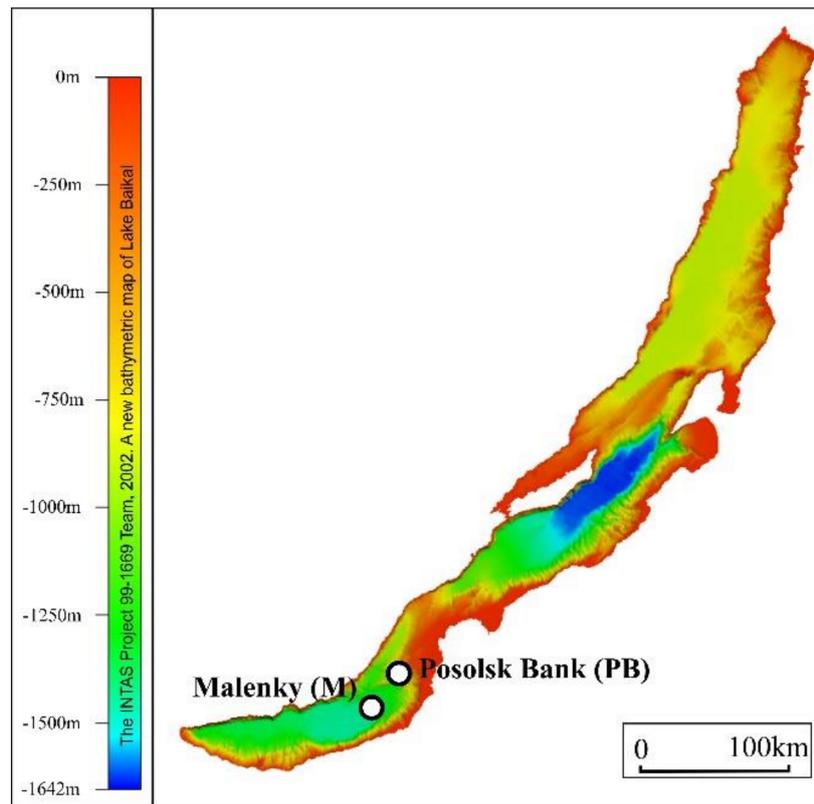


Figure 1. Sampling sites in Lake Baikal. Methane seep, Posolsk Bank (52.03 N, 105.84 E); a mud volcano, the Malenky (51.92 N, 105.63 E).

2.2. Measurements of Methane Concentrations and Analysis of the Chemical Composition of Pore Waters

Methane concentrations in the sediments were determined using headspace analysis [64] on an EKHOPIID chromatograph equipped with a flame ionization detector (2-m packed column, internal diameter 2 mm, Porapak as the sorbent, and isothermal mode at 100 °C). The measurement error for methane concentration was $\pm 5\%$.

Pore water extractions were obtained from 9 samples (100 g each), which were collected throughout the core. Pore waters were obtained on board the ship by sequential centrifuging the sample for 20 min at 5000 g, then for 10 min at 12,000 g. The pore water anion and cation contents were determined the methods described previously and are only briefly repeated here [57,65,66]. Pore water anion (SO_4^{2-} , NO_3^- , HCO_3^- , and CH_3COO^-) concentrations were measured onboard the ship by means of liquid chromatography immediately after preparation (5–10% relative error). The detection limits were 0.1 mg/L. The ions NO_2^- and NH_4^+ were determined onboard the ship by colorimetric methods (5% relative error). The detection limits were 0.003 mg/L for NO_2^- and 0.05 mg/L for NH_4^+ . The pore water samples for Fe^{2+} analysis were stored at 4 °C in polystyrene vials pre-acidified with 50 μL of ultra-pure concentrated HNO_3 . The Fe^{2+} concentrations were determined by atomic absorption method (3–5% relative error) in the laboratory LIN SB RAS. The detection limit was 0.01 mg/L.

2.3. Molecular Analyses

DNA was extracted according to phenol-chloroform method [67] in a modified version [68] from five samples of the PB site: the surface layer and the layers from depths of 10, 70, 100, and 140 cm; the M site—the surface layer and the layers of 60–65, 130–131, 132–139, and 140–146 cm. For PCR analyses were used primers for the *mcrA* gene of ANME-2d subcluster and methanogenic archaea were used, as well as for 16S rRNA and *pmoA* gene of NC10 bacteria (Table 1).

Table 1. PCR primers used for *mcrA*, *pmoA*, and 16S rRNA genes amplification. PCR reaction conditions are shown in references.

Primer	Detected Microbial Groups	Sequence (5'–3')	References
159F 345R	ANME-2d <i>mcrA</i> gene	AAAGTGC GGAGCAGCAATCACC TCGTCCCATTCTGCTGCATTGC	[41]
NC10-202Fdeg NC10-1043Rdeg	NC10 16S rRNA gene	RACCAAAGGRGGCGAGCG TCTCCRCGYTCCCTTGCG	[27]
A189F NA720R	NC10 <i>pmoA</i> gene	GGNGACTGGGACTTCTGG TCCCCATCCACACCCACCAG	[27,69]
pUC/M13F pUC/M13R	Universal group	GTTTCCCAGTCACGAC CAGGAAACAGCTATGAC	Promega Promega
A2Fa A519R	Archaea 16S rRNA gene	TTCCGGTTGATCCYGCCRG GGDTTACCGCGGCKGCKGCTG	[70]

PCR fragments were cloned in the competent cells of *E. coli* DH5 α . The purified amplification products 16S rRNA, *pmoA*, *mcrA* genes of bacteria, and archaea were cloned into the pGEM_T Easy Vectors plasmid vector (Promega, USA) according to the manufacturer's specifications. Transformation of the competent cells was carried out according to the standard procedure [67]. The clones with the PCR fragment inserts (15 to 25 clones per each of the four libraries) were selected using Xgal/IPTG. Plasmid DNA was isolated from 12 h cultures of *E. coli* DH5 α grown in liquid LB medium with ampicillin (50 U/mL). The plasmid DNA samples were stored at -18 °C. For confirmation of the existence of inserts of the necessary size, plasmid primers were used (Table 1).

The amino acid and nucleotide sequences of independent clones were determined via Sanger method using BigDye Terminator Kit v.3.1 (Applied Biosystems) and genetic analyzer ABI 3130x1 at SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia).

Initial analysis of the similarity of the 16S rRNA gene sequences to the known GenBank sequences (<http://www.ncbi.nlm.nih.gov/genbank/>) was carried out using the BLAST software package. Amino acid sequences of the *mcrA* and *pmoA* genes were obtained online at <http://web.expasy.org/translate/>. Chimeral sequences of the 16S rRNA genes were excluded using the Pintail online system (<http://www.bioinformaticstoolkit.org/WebPintail/>). The operational taxonomic units (OTUs), comprising the sequences with at least 98% similarity, were accepted as the phylotypes that represent the members of the community at the species level. These OTUs were used for further analysis. Sequences were edited using BioEdit. Nucleotide (gene 16S rRNA of NC10 bacteria) and amino acid (*mcrA*, *pmoA*) sequences were aligned using the CLUSTALW algorithm. Corresponding phylogenetic trees were constructed by the NJ and Kimura two-parameter methods implemented in MEGA version 6.0 [71].

The sequences determined in this study for NC10 phylum specific 16S rRNA, *pmoA*, and *mcrA* genes for anaerobic methanotroph archaeon have been deposited in the GenBank database under accession numbers MN595984–MN596011, MN603447–MN603470, and MN595903–MN595983 respectively.

2.4. 16S rRNA Gene High-Throughput Sequencing of Archaeal Communities

For amplification of archaeal 16S rRNA gene fragments, the primers for the V1–V3 region (Table 1) were used. Pyrosequencing of the amplicons was carried out on a GS FLX 454 genome analyzer (Roche, United States). Diversity and taxonomic composition of archaeal communities were analyzed using the Mothur v. 1.31.2 software package. For this purpose, the sequences were aligned, and cluster analysis was carried out. The sequences were grouped into operational taxonomic units (OTUs) at genetic distances of 0.03. The taxonomic position of the representative sequences in a cluster with a genetic distance of 0.03 was determined by comparison to the following 16S rRNA databases: SILVA and NCBI. For analysis of molecular genetic diversity of the community, the following values were calculated: number of observed OTUs. The obtained sequences were processed by the PyroNoise algorithm [72] to remove sequencing errors. Chimeric sequences were detected using the UCHIME algorithm [54] with standard parameters. Pyrosequencing of the 16S rRNA gene V1–V3 region yielded 27,231 sequences

for deep sediments M and 12,154 sequences for deep sediments PB after initial filtration, alignment of reads, and the removal of chimeric sequences. Sequences shorter than 180 bp were excluded from analysis. Comparison with the known 16S rRNA gene sequences was carried out using BLASTN. The sequence arrays were deposited to GenBank, SRA section, under accession nos. SRR2912890 (Archaea, PB) and SRP052288 (Archaea, M).

3. Results

3.1. Chemical Composition of Pore Waters

Sediments of the investigated core from the methane seep PB were highly saturated with gas. Methane concentration increased with depth (up to 10 μM in surface sediments), reaching maximum values (up to 9000 μM) in the sedimentary strata where GHs were detected (Figure 2). Throughout the core, there were increased concentrations of CH_3COO^- ions up to 379 μM and Fe^{2+} up to 134 μM . The concentration of NH_4^+ ions in pore waters from the investigated station varied from 16.7 μM to 205.5 μM , and the highest NH_4^+ concentrations were in deep layers of the bottom sediments at the boundary with GHs (70 cm). The concentrations of SO_4^{2-} ions in pore waters from the investigated core had maximum values in the surface sediments (62 μM). Pore water NO_3^- content was 20 μM in surface sediments, and it was not detected in other pore waters.

In addition to GHs, bottom sediments of the mud volcano M contained carbonate (oligonite - $\text{Fe}(\text{Mn},\text{Zn})(\text{CO}_3)_2$) at a depth of 145 cm, whose structure was determined on a JCSA-733 wave X-ray microanalyzer at the Institute of Geochemistry SB RAS. Unlike the methane seep PB, methane concentrations in sediments of the mud volcano M exceeded 90 μM almost from the surface and showed uneven distribution with the maximum value at a depth of 140 cm (up to 1200 μM) (Figure 2). The concentrations of SO_4^{2-} in pore waters of the investigated core reached 3781 μM in sediments that contained carbonate (140–146 cm); the minimum concentrations were recorded in GH layers (130–131 cm and 132–139 cm). Higher concentrations of HCO_3^- ions in comparison with the reference values (up to 1098 μM) are typical of all studied sediments with the maximum in GH layer of 130–131 cm (up to 4049 μM). We did not detect NO_3^- and CH_3COO^- ions there, which were present in sediments of the methane seep PB. Notably, unlike the methane seep PB, all layers of the investigated sediments had lower concentrations of Fe^{2+} ions with the maximum values of 19.64 μM in GH layer (130–131 cm).

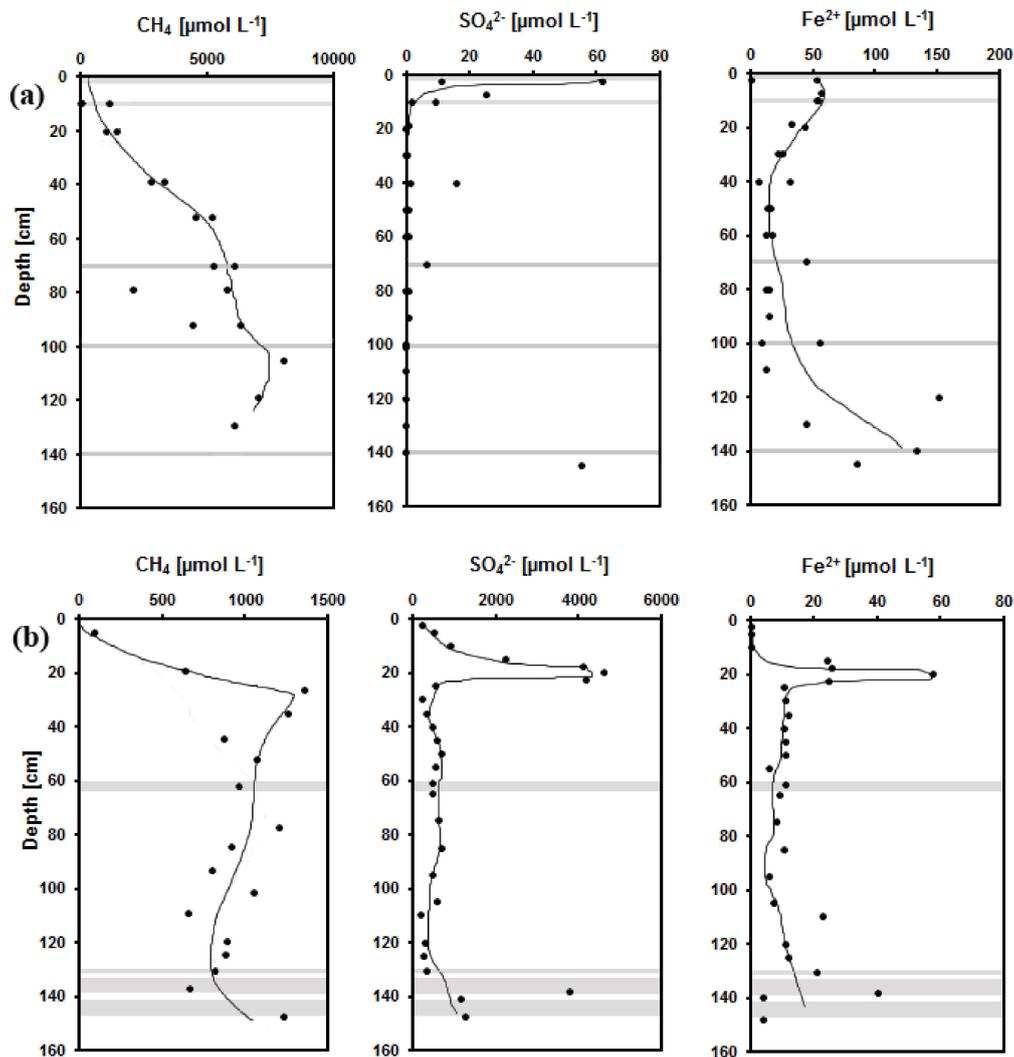


Figure 2. Concentration depth profiles of methane (CH_4), sulfate ions (SO_4^{2-}), and dissolved iron (Fe^{2+}), (a) the methane seep Posolsk Bank; (b) the mud volcano Malenky. Samples of bottom sediments taken for molecular biological studies are highlighted.

3.2. Diversity of the ANME-2d 16S rRNA Genes in Libraries

Near the mud volcano M, we detected the representatives of the ANME-2d subcluster in all obtained DNA samples. Whereas in site of the methane seep PB we not detected sequences of the ANME-2d in sediments 100 cm and 140 cm. The number of OTUs varied from one to five per one analyzed sample. In the phylogenetic tree, the recovered nucleotide 16S rRNA gene sequences formed six clusters, and one cluster was common for DNA from the two investigated sites.

Analysis of 16S rRNA gene libraries of the ANME-2d subcluster has shown that near the mud volcano M, the bulk of the genes (22% and 20% of all analyzed sequences) was in sediments with GHs (130–131 cm) and oligonite (140–146 cm), respectively. At a depth of 60–65 cm, we detected approximately 15% of the ANME-2d 16S rRNA gene sequences, and in sediments adjacent to GHs (132–139 cm) their quantity was approximately 6% (Figure 3).

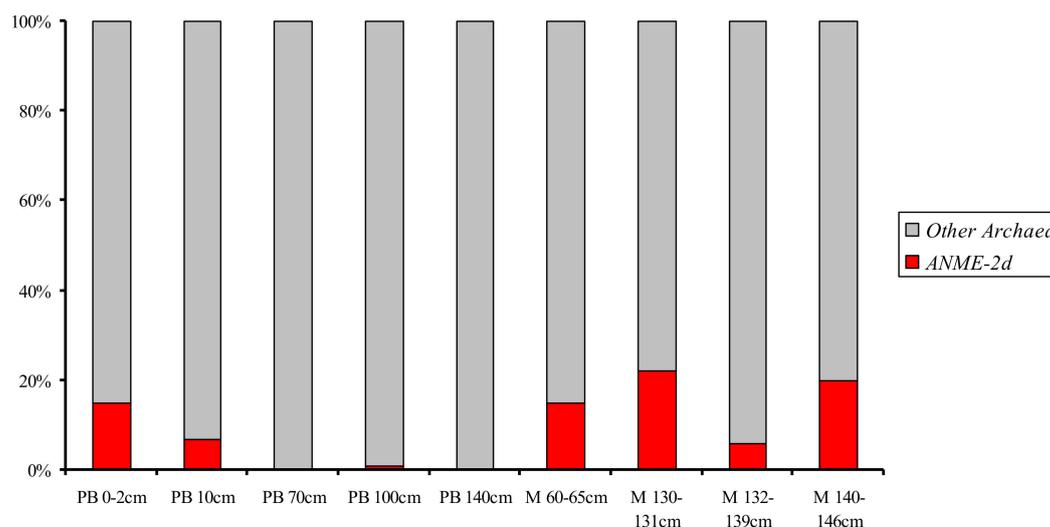


Figure 3. Share of archaea of the ANME-2d subcluster in archaeal communities from bottom sediments.

Nucleotide sequences of 16S rRNA gene fragments of the ANME-2d representatives in the sediments of the methane seep PB predominated in the surface sediment (0–2 cm), decreasing with depth to 6.8% at 10 cm, and to 1% at 100 cm. Notably, we did not detect the ANME-2d sequences in the DNA samples taken from depths of 70 and 140 cm (Figure 3).

Analysis of 16S rRNA gene libraries of the ANME-2d subcluster has indicated the most diverse nucleotide sequences from the DNA sample from the surface layer of the methane seep PB, which were combined into five OTUs (Figure 4). Approximately 13% of all ANME-2d sequences (OTU16) from this sediment layer formed a cluster in the phylogenetic tree with sequences from marine sediments of the western cold sea, the Weddell Sea, Antarctica (Acc. no. FN429785); 4% of sequences (OTU74) formed a cluster with denitrifying anaerobic methane oxidation archaea [19]; the remaining sequences (approximately 2% of all analyzed ANME-2d sequences, OTU101) grouped with sequences of archaea from sediments of Honghu Lake (HM244209).

Sequences of ANME-2d 16S rRNA gene fragments from DNA of the surface layer of the methane seep PB (8% of all analyzed ANME-2d sequences), layer with oligonite (140–146 cm) of the mud volcano M and 82% of the sequences from 10 cm sediment layer of the methane seep PB (OTUs 14 and 32) formed a cluster, having a 100% similarity whether with the sequences from the coal-rich sediments (KJ424521), or freshwater iron-enriched microbial mat (AB722188), as well as Lake Ørn sediments [24].

In the phylogenetic tree, the ANME-2d 16S rRNA gene sequences from all analyzed DNA samples of the mud volcano M formed a cluster (OTU12) and had a 99–100% similarity with the sequences that were previously found in the sediments of Chaohu Lake [19] and those of Lake Baikal [63,73].

The molecular analysis did not reveal of typical for marine environment methane-oxidizing archaea ANME1, 2, or 3 in samples of investigated sediments (Figure 4).

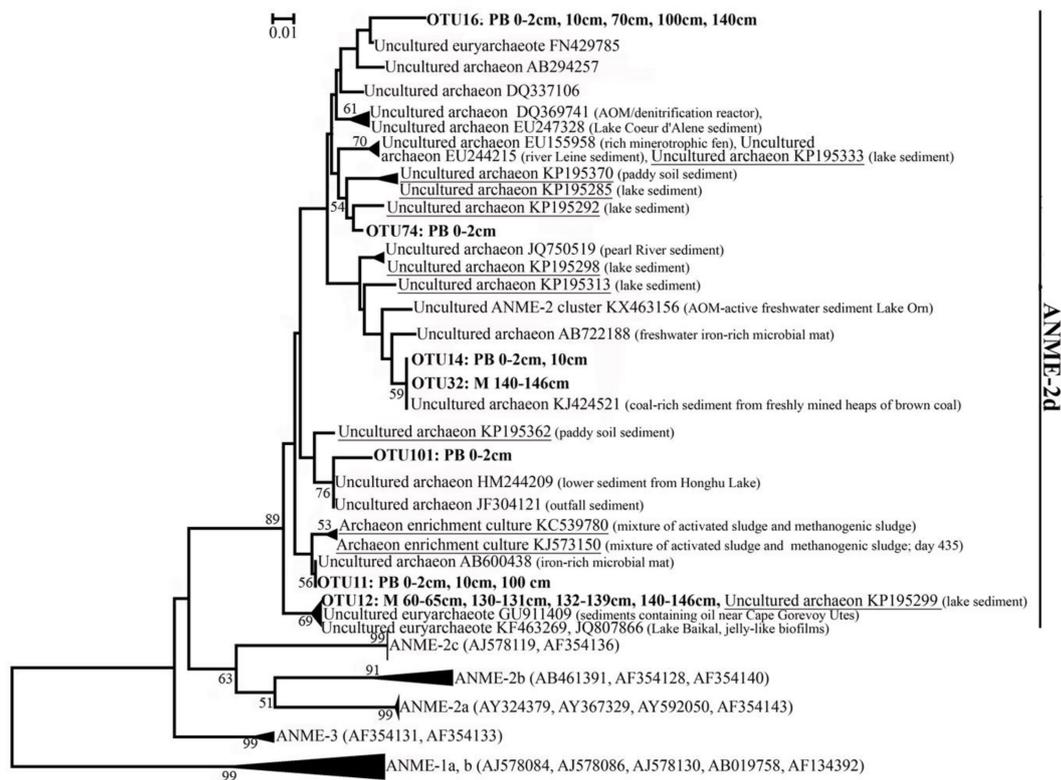


Figure 4. Maximum likelihood phylogenetic tree with the affiliations of the 16S rRNA genes of *M. nitroreducens*-like archaea recovered from deep sediments of the mud volcano Malenky (M) and the methane seep Posolsk Bank (PB). Bootstrap values > 50% (out of 100 replicates) are shown in front of respective nodes, and the scale bar represents 1% sequence divergence.

3.3. Diversity of *mcrA* Genes of ANME-2d Sequences in Clone Libraries

We tested all recovered DNA samples for *mcrA* genes of the ANME-2d subcluster for each of the recovered *mcrA* gene libraries. The number of OTUs was from 1 to 2 per sample. The analysis of the obtained translated amino acid sequences has confirmed the presence of *mcrA* genes of the ANME-2d in the communities. The analyzed amino acid sequences of the bottom sediments from the two investigated areas were similar to *mcrA* gene sequences of *M. nitroreducens*. The similarity of the recovered nucleotide sequences with those available in the NCBI database was 93–98% and amino acid sequences (97–100%). In the phylogenetic tree, the obtained sequences of *mcrA* gene fragments formed two clusters (Figure 5). The phylotype ANME-2d.*mcrA*-OTU1 dominated in the number of the recovered sequences. This phylotype included amino acid sequences from different sediments (Figure 5) of the methane seep PB and the mud volcano M and clustered with the *M. nitroreducens* sequences from the bioreactor (AQM52588) and rice soils (AQM52582) [41]. The phylotype ANME-2d.*mcrA*-OTU2 formed a cluster only with sequences from deep sediments of M and from the bioreactor, in which AOM processes occurred together with iron reduction (PWB49621) [49]. The phylotype ANME-2d.*mcrA*-OTU3 consisted of sequences from the surface sediments of the methane seep PB and the closest relatives from the microbial mat of Hydrate Ridge North, Pacific Ocean (PPA79495).

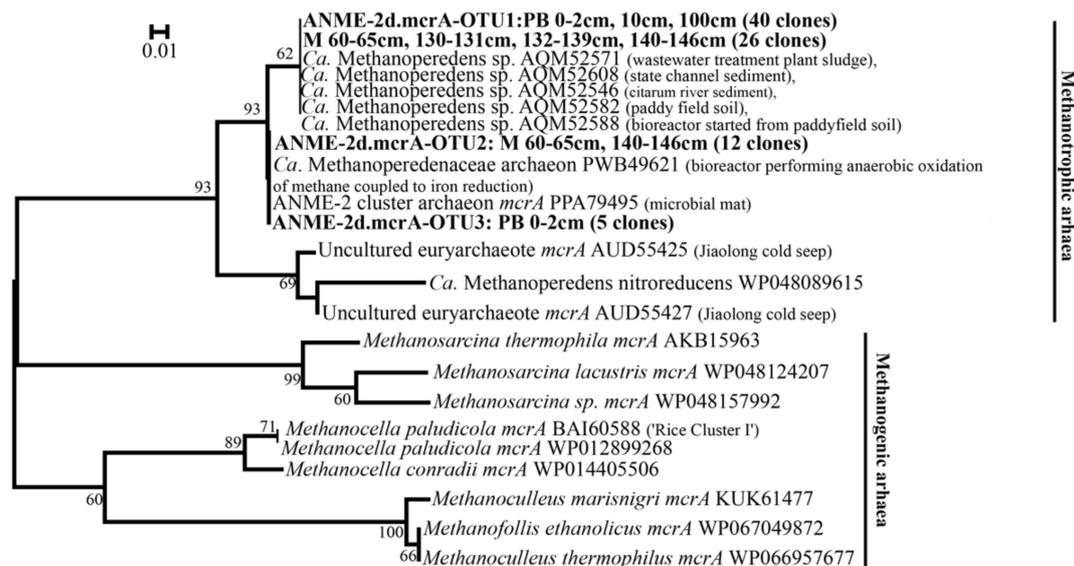


Figure 5. Maximum likelihood phylogenetic tree with the affiliations of the *mcrA* genes of *M. nitroreducens*-like archaea recovered from deep sediments the mud volcano Malenky (M) and the methane seep Posolsk Bank (PB). Bootstrap values > 50% (out of 100 replicates) are shown in front of respective nodes, and the scale bar represents 1% sequence divergence.

To solidify the absence of representatives typical of marine sediments with methane-oxidizing archaea ANME1, 2, or 3, the DNA samples were tested for *mcrA* gene of methanogenic and methanotropic archaea. Specific primers (Table 1) of *mcrA* gene did not detect members of the above mentioned ANME clusters in investigated DNA samples from deep sediments of Lake Baikal. Phylogenetic analysis indicated that all Baikal sequences of *mcrA* genes were similar to sequences of the order *Methanomicrobiales* and *Methanosarcinales* [60].

3.4. Diversity of NC10 Bacteria in 16S rRNA Gene Clone Libraries

We also tested all DNA samples for anaerobic methanotrophs of the phylum NC10. Since the metagenomic analysis with universal bacterial primers [63] did not confirm the presence of the members of this phylum at the investigated sites, we performed an additional PCR analysis with the specific primers for this bacterial phylum (Table 1). These primers detected the NC10 bacteria relative to *M. oxyfera* in all investigated sediments of the mud volcano M and methane seep PB, in which archaea of the ANME-2d subcluster were previously detected. Phylogenetic analysis showed that the recovered nucleotide sequences were 97–99% identical to the 16S rRNA gene of *M. oxyfera*. All recovered nucleotide sequences of 16S rRNA gene sequences of bacteria from the NC10 phylum grouped into four OTUs.

Phylogenetic analysis indicated that dominant phylotypes in the investigated 16S rRNA gene libraries were NC10-1-OTU1, having a 99% similarity with the uncultured candidate division of the NC10 bacteria (HQ906512, HQ906514) from the sediments of Lake Constance [27], and NC10-2-OTU2 (20 clones), having a 99% similarity with the uncultured bacteria from the denitrification zone of Lake Biwa (AB661499) [74]. Other phylotypes were minor in the 16S rRNA gene libraries, and they were represented by 5 (NC10-4-OTU4) and 10 (NC10-3-OTU3) clones. The phylotype NC10-3-OTU3 was close, with various similarity (97–99%), to the uncultured bacteria from the basalt underground ecosystem. The phylotype (NC10-4-OTU4) was close (99% similarity) to the uncultured *Methylomirabilis* sp. (JX252156, JX262159) from the enrichment cultures that carry out nitrite-dependent AOM (Figure 6).

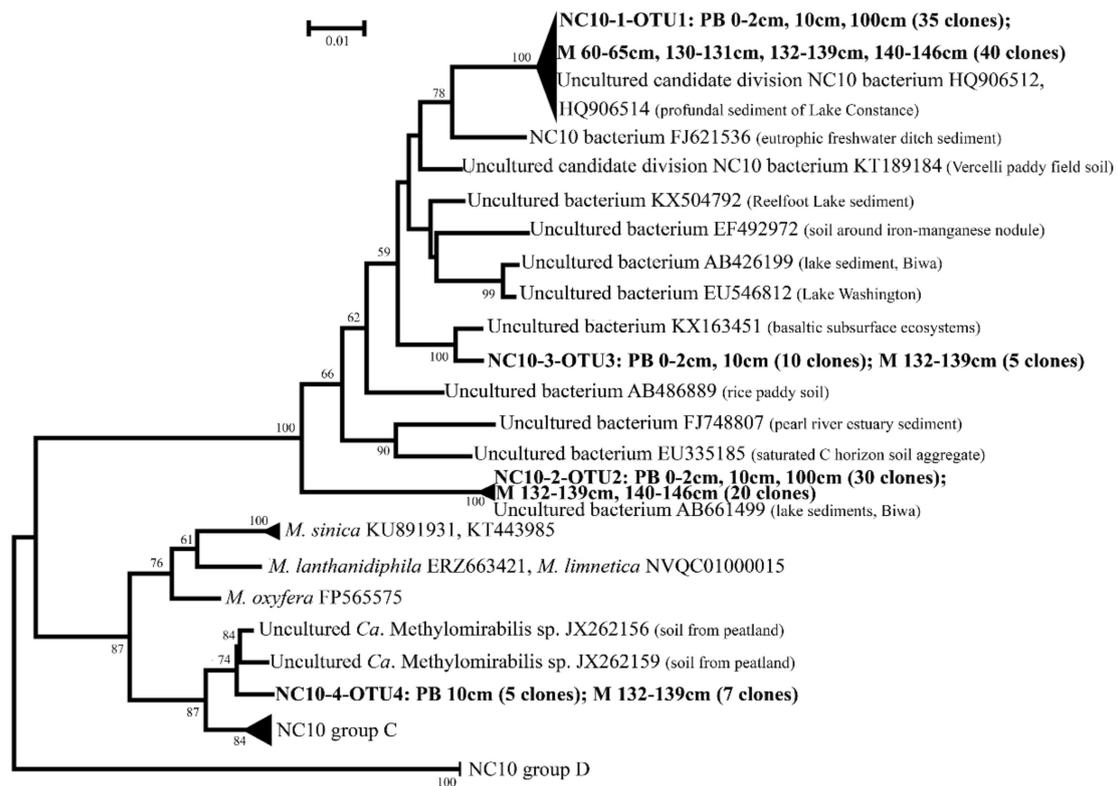


Figure 6. Maximum likelihood phylogenetic tree with the affiliations of the 16S rRNA genes of *M. oxyfera*-like bacteria recovered from deep sediments the mud volcano Malenky (M) and the methane seep Posolsk Bank (PB). Bootstrap values > 50% (out of 100 replicates) are shown in front of respective nodes, and the scale bar represents 1% sequence divergence.

3.5. Diversity of *pmoA* Genes of the NC10 Bacteria in Clone Libraries

The genome of the NC10 bacteria encodes the complete aerobic methane oxidation pathway [21], and the *pmoA* gene encodes a subunit of particulate methane monooxygenase (pMMO). The *pmoA* gene can be used as a biological marker to identify the NC10 bacteria [25,75]. PCR analysis of the investigated samples of bottom sediments successfully detected specific *pmoA* gene sequences of the NC10 bacteria using primers targeting *pmoA* gene (Table 1). All reserved amino acid sequences of *pmoA* bacterial genes of the phylum NC10 grouped into three OTUs. The bulk of the *pmoA* gene sequences (NC10.pmoA-OTU1), which were recovered from the samples, were the most similar to *Methylomirabilis* sp. (94–98%) from the soil and the uncultured NC10 bacteria of the Lake Biwa sediments (BAL70515) and water in dam reservoir (BAP16645). Whereas one-third of the *pmoA* gene sequences (NC10.pmoA-OTU2) were similar to NC10 bacteria (90–95%) from wastewater treatment plant Lieshout (AEJ33959) (Figure 7), *pmoA* sequences (NC10.pmoA-OTU3) from both the methane seep and the mud volcano again clustered together with *pmoA* sequences from the Lake Constance sediments (ADY76950) and *M. limnetica* (WP107560874).

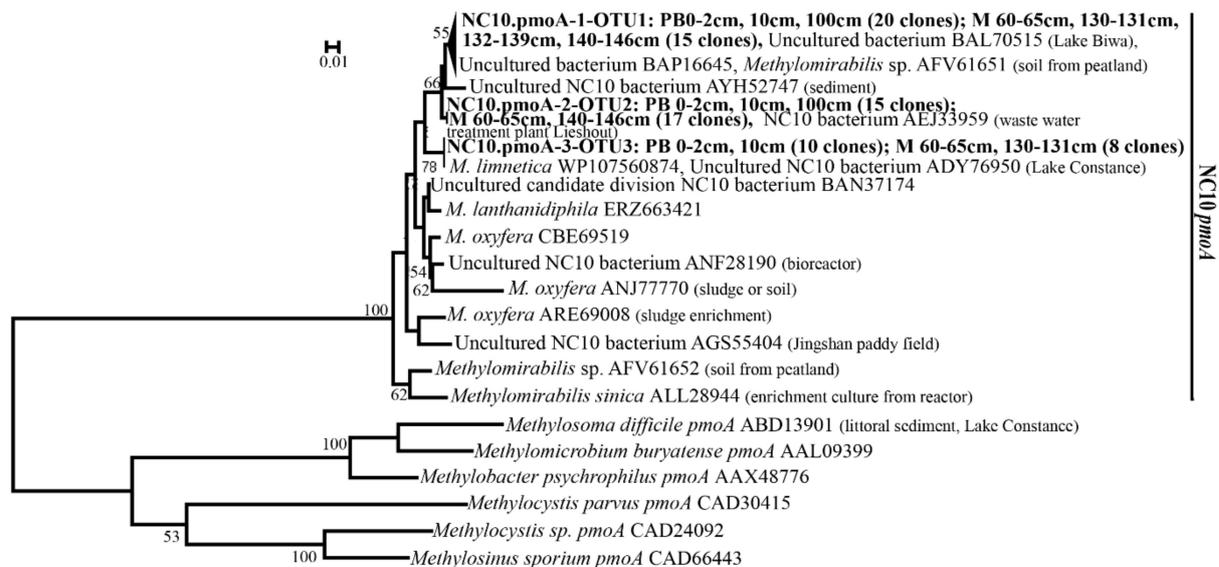


Figure 7. Maximum likelihood phylogenetic tree with the affiliations of the *pmoA* genes of *M. oxyfera*-like bacteria recovered from deep sediments the mud volcano Malenky (M) and the methane seep Posolsk Bank (PB). Bootstrap values > 50% (out of 100 replicates) are shown in front of respective nodes, and the scale bar represents 1% sequence divergence.

4. Discussion

Analysis of different genes has confirmed the presence of archaea and bacteria that most likely carry out AOM processes in sediments of various geological structures in Lake Baikal (methane seep and mud volcano), which are different in the composition of the discharged fluid and contained CHs. In sediment layers that contained GHs concentrations of all analyzed ions were two to three times higher compared to reference values [66]. The abnormally high values of some ions in such zones are due to the displacement of salts from the water during GH formation, which was repeatedly indicated at other sites with the near-surface occurrence of GHs [63]. In addition to GHs, bottom sediments of the mud volcano M contained carbonate, presence of which in GH-bearing sediments of mud volcanoes was previously indicated [76]. Methane concentrations in the sediments at the investigated sites increase with depth, reaching the maximum values, and the obtained profiles of methane distribution curves are similar to those in the AOM zones of other freshwater ecosystems [24,58].

The structure of microbial communities was previously studied in deep sediments of methane seeps and mud volcanoes in Lake Baikal. However, no marine uncultivated anaerobic methanotrophic archaea of the ANME clusters were found either in surface sediments or zones of GH occurrence [57,59,60,68,73]. The absence of ANME clusters that are typical for marine ecosystems is understandable since in deep sediments of some gas-saturated areas (oil seeps and methane seeps), the concentration of sulfate ions is comparable with the reference values (up to 82 μM) [55,56], which is insufficient for the occurrence of geochemically significant sulfate-dependent AOM. This is also consistent with the studies which indicate sulfate-dependent AOM as the main process for methane removal in marine environments where the concentrations of sulfate ions reach 28,000 μM [16]. In the investigated sediments of the mud volcano M, we observed relatively high concentrations of sulfate ions (up to 3781 μM) compared to other freshwater lakes (from 20 to 300 μM) [27,58,77]. Similar concentrations of sulfate ions in Baikal (up to 2000 μM) were observed in the sediments of freshwater Lake Cadagno, though, like in Lake Baikal, the members of the marine ANME clusters were also not detected there [58]. To carry out AOM, the marine ANME clusters require the presence of sulfate-reducing bacteria. According to the high-throughput sequencing data, their proportion does not exceed 2% of all analyzed 16S rRNA bacterial sequences in investigation deep sediments of the mud volcano M and the methane seep PB [63]. Rates of sulfate reduction in the sediments of site Lake Baikal varied from 0.001 to

0.07 nM/day [57,78]. There was a greater variety of sulfate-reducing bacteria and a high rate of sulfate reduction in the bottom sediments of the methane seep PB. Sulfate-reducing bacteria from this site accounted for 9.2–9.5% of all analyzed 16S rRNA gene sequences [59], among which members of the genus *Desulfosporosinus* were detected by molecular methods [55]. The range of sulfate-reduction rates was 1.2 nM in the sediments below 60 cm and 1000 nM in the surface layer (0–2 cm). The obvious activity of sulfate-reducing bacteria remained up to a depth of 40–50 cm where there was a high methane concentration [55]. Constant fluid flow from deep sediments enriched, in particular, with sulfate ions ensure the presence of sulfate-reducing microorganisms in the studied communities [59]. As far as sulfate content is mostly very low and no ANME1, 2, 3 known for coupling AOM to sulfate reduction sequences were detected, it is possible that other mechanisms of AOM are functioning.

In contrast to the ANME clusters, members NC10 bacteria and ANME-2d archaea do not need a partner to carry out AOM and are widespread in freshwater ecosystems [22,23,33–35]. However, in the investigated sediments of Lake Baikal, there were no nitrate and nitrite ions in case of the mud volcano M, or they were present in the surface sediments of the methane seep PB. Lack of these ions, which are necessary for AOM, in the Baikal sediments can be due to their rapid involvement in methane oxidation, owing to the members of the ANME-2d subcluster and bacteria of the phylum NC10. Perhaps lack of nitrate and nitrite ions, being electron acceptors for AOM, in the sediments of Lake Baikal can be compensated, owing to the activity of archaea of the phylum *Thaumarchaeota* [79,80]. Ammonium-oxidizing members of this phylum at the investigated sites of Lake Baikal comprised a significant part of all analyzed archaea: up to 40% near the methane seep PB [59] and 20% near the mud volcano M [60].

Our study has shown that sequences of the ANME-2d have different numbers of OTUs and diverse composition near the methane seep and the mud volcano. The most diverse sequences, which were included into five OTUs, we have recovered for the surface layer of the methane seep PB. At the same time, we have not detected the representatives of the nitrate-dependent AOM in two deep GH-containing sediments from this site (70 and 140 cm). The absence of the ANME-2d representatives in deep sediments and their wide variety in the surface sediments most likely result from the influence of convective mineralized fluids, which circulate in the GH stability zone [81,82]. Their constant flow may also be responsible for development of methanotrophic archaea in the surface sediments, ensuring reducing conditions of the environment and influx of the carbon sources and energy as well as their lack in deep anaerobic zones [59]. If the fluid circulation indeed takes place in the sedimentary stratum up to a depth of 400 m in the communities of surface sedimentary layers, there may be representatives of deep microorganisms together with cosmopolitan prokaryotes. Sediments of the methane seep M have 1–2 OTU sequences of the ANME-2d 16S rRNA gene fragments. According to the phylogenetic analysis, the recovered sequences of the ANME-2d representatives are similar to those from the river, lake and freshwater sediments as well as soils of rice fields and enrichment cultures of denitrifying methanotrophs [19,23,24,83]. In the investigated sediments, we have indicated the presence of the archaeal *mcrA* genes of the ANME-2d subcluster using the additional PCR analysis and recently developed primers [41]. Since the general *mcrA* primer pair ME1F/ME2R [37] did not detect *mcrA* sequences of ANME-2d [60]; further still, the presence of these microorganisms and their diversity in environmental studies may be underestimated, as presently used primers simply do not capture them. Phylogenetic analysis has revealed that *mcrA* gene sequences from the areas of the methane seep PB and mud volcano M clustered together with available *mcrA* sequences of the known *M. nitroreducens*-like archaea from freshwater habitats and bioreactor, starting from paddy field soil, the Citarum River sediments and microbial mat [41].

In bioreactors, the representatives of ANME-2d, NC10, and ANAMMOX bacteria can coexist and compete with each other for the availability of nitrate and nitrite ions, which can lead to the simultaneous conversion of ammonium, methane, and nitrogen oxides to harmless N_2 and CO_2 [44]. Both ANAMMOX bacteria and NC10 bacteria grow in anoxic conditions and use NO_2^- ions as terminal electron acceptor. Ions of NO_3^- , NO_2^- , NH_4^- , and methane often coexist in the anoxic conditions of

different freshwater ecosystems. It is assumed that wetlands may be a suitable environment for the coexistence of these two taxonomic groups of bacteria, despite their competition for NO_2^- as an electron acceptor for ANAMMOX or AOM processes, respectively [80]. Using molecular methods, showed that ANAMMOX bacteria and NC10 bacteria coexist in paddy soil and saline lake sediments [84,85]. For this reason, in this study, we have searched for representatives of candidate phylum of the NC10 bacteria in the sediments of the methane seep PB and the mud volcano M. In all investigated sediments with ANME-2d archaea, we detected the representatives of the phylum NC10. We did not find them at depths of 70 and 140 cm near the methane seep PB, which also confirms a possible coexistence of these microorganisms. The NC10 bacteria carry out AOM in the presence of NO_2^- ions in the environment, which were not found in pore waters of the investigated sites, or their concentrations were lower than the values of nitrate ions in the sediments. Lower nitrite values compared to nitrate ions can be due to the likely toxicity of nitrite in a concentration of $1.5 \mu\text{M}$ for the NC10 bacteria [79]. From the investigated sites, we have recovered four OTUs that belong to the candidate phylum NC10. The sequences of the NC10 phylum close to the Baikal ones were previously found in the sediments of Lake Constance [27], rice soils [86], and eutrophic lakes [87]. Moreover, in the investigated samples, we have confirmed the presence of the functional *pmoA* genes, which can serve as a biological marker for the identification of the NC10 bacteria. The *pmoA* gene sequences, which were recovered from the sediments of Lake Baikal, are similar in amino acid and nucleotide sequences with those from Lake Biwa [74], Lake Constance [27], as well as with *Methylomirabilis* sp. and *M. limnetica*.

We also hypothesize that iron ions can serve as electron acceptors during AOM in the sediments of Lake Baikal. Regardless of the location of the investigated sites, the chemical composition of pore waters and discharged fluid, all sediments had high concentrations of iron ions (Fe^{2+}): up to $134 \mu\text{M}$ in deep sediments and up to $52.5 \mu\text{M}$ in the surface sediments compared to the reference sites at Lake Baikal. Iron is present in freshwater bodies that are depleted of sulfate and nitrate and where methane is oxidized in anaerobic zone [20,46–48,88]. It is also proposed that Fe(III)-dependent AOM was largely responsible for oxidizing the entirety of methane produced on early Earth prior to the advent of oxygen [45]. This process can potentially occur by *M. nitroreducens* [22,44]. They are also universal anaerobic methanotrophs that can use various oxidizers as electron acceptors (iron or nitrate ions), like pore waters of Lake Baikal, which vary in chemical composition, and, thus, may play an important role in controlling methane freshwater systems [89].

Our study confirms the presence of the NC10 bacteria and ANME-2d archaea based on the analysis of 16S rRNA gene sequences and functional genes (*pmoA* and *mcrA*) of AOM with concentrations of nitrite and nitrate ions below $20 \mu\text{M}$ in freshwater sediments. We also show that recently discovered AOM process together with denitrification can occur in Lake Baikal in the absence of representatives of the ANME clusters, which are responsible for AOM in marine sediments. However, we think it could be plausible that the microorganisms that have been obtained in this study can use Fe^{3+} and SO_4^{2-} as electron acceptors to oxidize methane in the sediments of Lake Baikal.

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