Anaerobic oxidation of petroleum hydrocarbons in enrichment cultures from sediments of the Gorevoy Utes natural oil seep under methanogenic and sulfate-reducing conditions

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Abstract
This article presents the first experimental data on the ability of microbial communities from sediments of the Gorevoy Utes natural oil seep to degrade petroleum hydrocarbons under anaerobic conditions. Like in marine ecosystems associated with oil discharge, available electron acceptors, in particular sulfate ions, affect the composition of the microbial community and the degree of hydrocarbon conversion. The cultivation of the surface sediments under sulfate-reducing conditions led to the formation of a more diverse bacterial community and greater loss of n-alkanes (28%) in comparison to methanogenic conditions (6%). Microbial communities of both surface and deep sediments are more oriented to degrade polycyclic aromatic hydrocarbons (PAHs), to which the degree of the PAH conversion testifies (up to 46%) irrespective of the present electron acceptors. Microorganisms with the uncultured closest homologues from thermal habitats, sediments of mud volcanoes, and environments contaminated with hydrocarbons mainly represented microbial communities of enrichment cultures. The members of the phyla Firmicutes, Chloroflexi, and Caldiserica (OP5), as well as the class Deltaproteobacteria and Methanomicrobia, were mostly found in enrichment cultures. The influence of gas-saturated fluids may be responsible for the presence in the bacterial 16S rRNA gene libraries of the sequences of “rare taxa”: Planctomycetes, Ca. Atribacteria (OP9), Ca. Armatimonadetes (OP10), Ca. Latescibacteria (WS3), Ca. division (AC1), Ca. division (OP11), and Ca. Parcubacteria (OD1), which can be involved in hydrocarbon oxidation.

Keywords Lake Baikal · Anaerobic oxidation · Petroleum hydrocarbons · Microbial community

Introduction
Deep petroleum reservoirs and deep sediments associated with oil discharge have long been considered biotopes unsuitable for life [1]. In recent decades, the use of a set of methods has described microbial diversity in petroleum reservoirs, revealed dominant groups of microorganisms that carry out anaerobic oxidation of oil, determined metabolic pathways, and the resulting oxidation products [2–11]. Comparison of microbial diversity in petroleum reservoirs throughout the world indicated an obvious correlation of prokaryotic communities with temperature and depth of reservoir as well as no influence of the geographical distance between reservoirs. The members of Epsilonproteobacteria and Deltaproteobacteria were mostly detected in relatively shallow and low-temperature petroleum reservoirs, whereas Clostridiales and Thermotogales were more often found in deeper and higher-temperature

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petroleum reservoirs [11]. The study of the strategy of energy and carbon adsorption by microbial communities from three deep oil seepages (water column depth 3 km) in the eastern part of the Gulf of Mexico using metagenomic, geochemical, and metabolomic analyses revealed that deep sediments contain phylogenetically and functionally diverse microbial communities that carry out anaerobic metabolism of hydrocarbons where acetate and hydrogen are the central intermediates underpinning community interactions and biogeochemical cycling in these deep sediments. The microbial community was dominated by the members of the phylum Chloroflexi (mostly classes Dehalococcoidia and Anaerolineae), Ca. Atribacteria, Proteobacteria (mostly class Delaproteobacteria), and Ca. Bathyarchaeota, whose genomes contain genes of anaerobic oxidation of hydrocarbons through hydroxylation and addition of hydrocarbons to fumarate using fumarate-adding enzymes (FAE) as well as of degradation of anaerobic aromatic compounds through class I benzoyl-CoA reductase [12].

In the absence of oxygen, petroleum hydrocarbons can be biodegraded with NO$_3^-$, Fe$^{3+}$, SO$_4^{2-}$, and HCO$_3^-$ as alternative electron acceptors, which link to four typical reducing conditions [13]. In the past two decades, researchers have started to compare the performance of petroleum hydrocarbon degradation when different electron acceptors are employed, and it was indicated that the biodegradation behaviors of petroleum hydrocarbons may vary under various reducing conditions [14].

In contrast to marine ecosystems, natural oil seepages in freshwater lakes are rather a rare phenomenon known only for deep Lake Tanganyika (Central Africa), shallow Lake Chapala (Mexico), and deep oligotrophic Lake Baikal (Russia) [15–17]. The processes of anaerobic oxidation of oil in sediments of freshwater lakes have not been studied previously. Lake Baikal is one of the promising sites for studying ecology, taxonomic structure, and geochemical activity of anaerobic microbial communities in sediments associated with the discharge of hydrocarbons. Among the unique characteristics of Lake Baikal, there are not only its age (more than 25 million years), depths, and dimensions close to marine ones but also the presence of natural oil seepages. Oil in Lake Baikal, which was formed during the Oligocene and the Early Miocene, is the permanent component of the ecosystem characterized by a young age, specificity of the original organic matter, and the presence of a complex of unique biomarker molecules in its composition [17, 18].

There are two known sites of oil seepage in Lake Baikal: one is located at the estuary of the Bolshaya Zelenovskaya River, which was discovered at the end of the eighteenth century, and the second near Gorevoy Utes Cape (discovered in 2005) (Fig. 1a). The site of Gorevoy Utes Cape is an oil and methane seep with seepages of gas, oil, and deep waters migrating through reservoirs of the lower seismic complex (from a depth of ~ 4 km) to the permeable fault zone where via vertical migration they come to the bottom surface [18]. Oil is discharged through asphalt structures. In places where oil accumulated on a flat area and near the structures, hydrocarbon gases discharged, which contained 99% of methane and approximately 1% of its homologues [20]. There were dense populations of benthic animals on asphalt structures, whose density was an order of magnitude higher than that at the reference sites of the bottom [21] (Fig. 1b).

The discovery of the new site of oil seepage in 2005 provided online monitoring of qualitative and quantitative changes in the oil composition as well as study of the diversity of the microbial community and its role in the processes of oil degradation. In 2005, the oil collected at the moment of its emergence on the water surface showed an extremely high n-alkane concentration and was identified as non-biodegraded paraffinic oil [17, 18]. At present, the oil composition shows a narrowing of the homologous series of n-alkanes and a decrease in the total concentration of normal hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) as well as partial degradation of oil coming to the water surface [19]. Previous studies have revealed that aerobic hydrocarbon-oxidizing microorganisms contribute significantly to the self-cleaning of the lake from oil “pollution” [22].

At the same time, studies of microorganisms involved in oil degradation in the anaerobic zone of the sedimentary strata of Lake Baikal are at an early stage [23]. The question of the possible electron acceptors, the composition of microbial community, and the role of anaerobic prokaryotes in these processes remain open. In this regard, we carried out the model experiments with the sediments from the Gorevoy Utes natural oil seep. They aimed to determine phylogenetic diversity of oil-degrading microorganisms in the cultures enriched with H$_2$:CO$_2$/sulfate ion in comparison with the conversion degree of n-alkanes and PAHs (two important petroleum hydrocarbon components) under methanogenic and sulfate-reducing conditions.

**Material and Methods**

**Sampling**

Two integrated samples of sediments were collected with a gravity corer in the zone of hydrocarbon discharge near Gorevoy Utes Cape (10 km off the coast, water column depth 890 m, Central Baikal; coordinates 53°30′45″N, 108°39′12″E) in 2018. The first core sample, St.5 GC. 3 (GUI), corresponded to a core depth of 10 to 30 cm and the second (GUI) corresponded 100 to 120 cm (Fig. 1c). The GUI sample contained oil; the GUII contained not only oil but also gas hydrates.
Enrichment Culture

To obtain enrichment cultures, samples of sediments were aseptically taken from the central part of the core and immediately placed into 116-mL vials containing 50 mL of sterile anaerobic modified Pfennig’s mineral solution with reduced salt content, taking into account low-mineralized conditions of Lake Baikal (0.25 g/L NaCl, 0.1 g/L KCl, 0.1 g/L NH₄Cl, 0.1 g/L KH₂PO₄, 0.2 g/L MgCl₂·6H₂O, 0.1 g/L CaCl₂·2H₂O, 1 g/L NaHCO₃, 1 mL vitamin solution, 1 mL trace element solution according to and Na₂S (0.5 mM) as a reducing agent). The vials were closed with rubber stoppers and aluminum caps, purged with oxygen-free nitrogen, and shaken for 15 min at 160–180 rpm in orbital shaking (OS-20, BioSan, Riga, Latvia). To obtain methanogenic enrichment cultures, the resulting suspension was transferred with a syringe into vials with a mineral solution (50 mL) of the above composition and gaseous mixture of H₂:CO₂ (80:10 v/v). Sulfate-reducing conditions were established by the addition of Na₂SO₄ (final concentration 20 mM) to the solution in the N₂:CO₂ atmosphere (90:10 v/v). Therefore, four enrichment cultures were obtained for the analysis: GUI_H₂:CO₂, GUI_SO₄, GUII_H₂:CO₂, and GUII_SO₄. Approximately 50 μL of non-biodegraded crude oil (Angarsk Petrochemical Company, Russia) was added as a carbon and energy source to triplicate enrichment culture. A sterile medium with oil without adding sediment samples was used as a negative control. All enrichment cultures were incubated at 10 °C in the dark without mixing.

Gaseous hydrocarbons in the experimental vials and sediments were determined by a modified phase-equilibrium degassing method where the error in determining the methane concentration was ± 5% [26].

Determination of Polycyclic Aromatic Hydrocarbons and n-Alkanes in Model Experiments

The samples taken in the course of model experiments were heterogeneous mixtures, including a mineral aqueous solution, sediments with a microbial community formed under conditions of oil seepage and crude oil additives. Before analysis, the samples were centrifuged; the aquatic fraction was separated from the sediment of the heterogeneous mixture. Before the determination of n-alkanes and PAHs, 20–100 μL of squalane (in dichloromethane, 0.60–30 μg/μL), 30–200 μL of a mixture of deuterated polycyclic aromatic hydrocarbons: naphthalene-d₈, acenaphthene-d₁₀, phenanthrene-d₁₀, chrysene-d₁₂, and perylene-d₁₂ (in a mixture of n-hexane:acetone (1:1); 5–600 ng/μL; Supelco, USA), were added to the aqueous fraction and the sediment of the heterogeneous mixture of samples as internal standards. The volume of the added standards was determined by the expected content of hydrocarbons in the sample.

During the determination of n-alkanes in the aqueous phase, hydrocarbons were twice extracted with dichloromethane; the extracts were combined; and the total extract (9 mL) was centrifuged for 3 min at 2000 rpm. Then, anhydrous Na₂SO₄ was added to ~ 1 mL aliquots of the extract, the
mixture was shaken and centrifuged, and the supernatant was transferred to the autosampler vial of the chromatograph. Hydrocarbons were extracted from the sediment of the heterogeneous mixture by ultrasonic (35 kHz) extraction with 5 mL of dichloromethane for 15 min. The extract was separated by centrifugation at 3000 rpm; anhydrous Na$_2$SO$_4$ was added to ~ 1 mL aliquots of the extract; the mixture was shaken and centrifuged; and the supernatant was transferred to the autosampler vial of the chromatograph.

To determine PAHs, 1 mL of n-hexane was added to aliquots of the obtained extracts, ~ 0.1 mL; the mixture was shaken, left for ~ 24 h at a temperature of +4 °C, and then centrifuged. The upper layer of n-hexane was separated; anhydrous Na$_2$SO$_4$ was added; the mixture was shaken and centrifuged; and the supernatant was transferred into the autosampler vial of the chromatograph.

The prepared samples were analyzed by gas chromatography-mass spectrometry (GC-MS, Agilent, GC 6890, MSD 5973, USA) under the following conditions: OPTIMA® 17 MS Macherey-Nagel column (30 m × 0.25 mm 0.25 × μm) and He as a carrier gas. The GC operating parameters were as follows: from 50 (0.5 min initial time) to 300 °C at 10 min$^{-1}$ (isothermal for 25 min final time) for n-alkanes and from 95 (0.5 min initial time) to 310 °C at 10 min$^{-1}$ (isothermal for 5 min final time) for PAHs. The temperature of the injector was 290 °C; the temperature of the dispenser was 25 °C; the volume of the injected sample was 2 μL without flow splitting; and the electron impact ionization was 70 eV. For n-alkane quantification, the peaks were registered by ion monitoring with 57 and 71 m/z; for PAHs quantification, the peaks were registered by ion monitoring with 128, 142, 152, 154, 166, 178, 192, 202, 228, 252, 276, and 278 m/z. The quantification of n-alkanes and PAHs in the supernatant and the sediment were carried out according to the method of an internal standard. The results were summarized and presented as the concentration of n-alkanes and PAHs in the samples collected during the model experiment. Recovery averages were 85% for n-alkanes and PAHs. The precision measurements of absolute concentrations of n-alkanes and PAHs were less than 15%.

The chemical composition of pore waters from the sediments was determined as described previously [27].

**Molecular Identification Methods**

DNA was extracted from enrichment cultures according to the modified method of enzymatic lysis technique followed by phenol-chloroform extraction [28]. The fragments of the 16S rRNA gene were amplified using universal bacterial (27F and1350R) [29] and archaeal primers (21F and 958R) [30]. PCR was carried out using kits (Intifinica, Russia) according to the manufacturer’s instructions. The obtained fragments of the 16S rRNA gene were cloned and transformed using the pGEM-T Easy Vector Systems reagent kit (Promega, USA) according to the manufacturer’s protocol. For the analysis, 30 clones containing inserts were selected from each library. The Sanger sequencing was performed using the BigDye Terminator Kit v.3.1 reagents on an ABI 3130XL Genetic Analyzer (Applied Biosystems, USA). The primary analysis of the similarity in the nucleotide sequences of the 16S rRNA genes obtained from the enrichment cultures with known sequences from GenBank was carried out using the BLAST software package (www.ncbi.nlm.nih.gov/blast) and EzBioCloud (http://www.ezbiocloud.net/eztaxon) [31]. A total of 120 bacterial and 120 archaeal sequences were obtained, with the maximal length of 899 bp. The presence of chimeras was determined through analysis of sequences using the PINTAIL program (http://www.cardiff.ac.uk/biosi/research/biosoft). The phylogenetic tree was constructed using neighbor-joining clustering method and Kimura’s two-parameter model implemented in the MEGA X software [32]. Sequences used for phylogeny construction were deposited in the NCBI nucleotide database under accession numbers: MW595796–MW595818, MW599325–MW599327, MW599328, MW617235–MW617240, MW617242–MW617261, MW624372–MW624377, and MW633226–MW633230.

For alpha diversity measurement, the obtained sequences were combined into operative taxonomic units (OTUs) with cluster distances of 0.03 to 1.0. The rarefaction curves, the ACE and Chao indexes of species richness, as well as the Shannon diversity index, were calculated for each community based on the identified OTUs using the Mothur software v. 1.39.5 [33].

**Results and Discussion**

**Lithological Characteristics of Sediments**

In the upper interval (from the surface to 50 cm) of the St.5 GC. 3 core, sediments are represented by reduced diatomaceous alurite-pelitic ooze with oil inclusions; in the middle interval (from 50 to 65 cm), by a watered and oil-saturated alurite layer; and in the lower one (from 120 to 151 cm), gray clay with many oil inclusions and massive stratified gas hydrates. The sediments of the St.5, GC.3 core were highly saturated with gas; the methane concentration varied from 4 to 18 mM/L along the core depth (Fig. 2a). The highest methane concentrations were recorded at depths of 20 and 100 cm (13 and 18 mM/L, respectively). Methane homologues in the gas were mainly represented by ethane (from 1 to 10 μM/L). The content of $\Sigma_{\text{alk}}$ and $\Sigma_{\text{PAH}}$ in bottom sediments is presented in Supplementary Table 1S [34]. Pore waters of sediments in all sedimentary layers were of bicarbonate-calcium-sodium type. Salinity was higher than...
Degradation of Hydrocarbons Under Methanogenic and Sulfate-Reducing Conditions

Cultivation of microbial communities in the cultures enriched with H₂:CO₂ and sulfate ions for 1 year revealed a different degree of conversion for n-alkanes and PAHs. The amount of n-alkanes and PAHs in bottom sediments was insignificant compared to the content of PAHs and n-alkanes in crude oil (Supplementary Table 1S). In the GUI sample, the greatest loss of n-alkanes (28%) was determined during the cultivation of the microbial community under sulfate-reducing conditions where the Σalk content in the sample decreased to 5000 μg in comparison with control enrichment cultures (7000 μg). Under methanogenic conditions, a decrease in the number of alkane fraction of oil was insignificant (6600 μg), accounting for 6%. The ΣPAH concentration in the presence of sulfate ion and H₂:CO₂ in the GUI_SO₄ and GUI_H₂:CO₂ enrichment cultures decreased by 20 to 37%, respectively (Fig. 3). Conversion of oil hydrocarbons in GUI_H₂:CO₂ was accompanied by the generation of methane. A native sample of sediments was initially saturated with gas; the methane concentration in the enrichment cultures at the beginning of the experiment was 13 mM/L. After 3 months of cultivation, the methane concentration increased to 27 mM/L. Its highest concentration (32 mM/L) was recorded after 6 months of cultivation in the enrichment cultures containing the surface sample of sediments. This level of methane concentration was maintained until the end of the experiment (31 mM/L).

In the cultures with deep sediments (GUII) in methanogenic microcosm, the conversion of n-alkanes was 20%, and in those enriched with sulfate ion less than 1.5% (Fig. 3). The degree of PAH conversion was 45–46% irrespective of the present electron acceptors. In the GUII enrichment cultures, the methane concentrations during the entire experiment remained almost the same (3.0 to 3.8 mM/L) because the values were comparable to those determined at the beginning of the experiment (3.2 mM/L). The loss of sulfate ions in the enrichment cultures containing both surface and deep samples was 25% of the initial concentration.

Diversity Assessment

At the level of cluster distances of 0.03, the rarefaction curves obtained (Supplementary Fig. 1S), as well as the values of the indices, indicate that the sequencing volume is sufficient to fully characterize the Ace and Chao diversity of archaea in the GUI_SO₄, GUII_H₂:CO₂, and GUII_SO₄ enrichment cultures. The bacterial diversity in all samples, as well as archaeal one in the GUII_H₂:CO₂ enrichment culture, was estimated at 75–86%. However, we can assume that most groups of significant abundance were identified (Supplementary Table 2S).

Bacterial and Archaeal Community Composition in Enrichment Culture Under Methanogenic Conditions

Analysis of the 16S rRNA gene clone libraries of bacterial communities revealed the members of 12 phyla in the background one [35]; total ions varied from 136.6 to 278.8 mg/L along the depth of the core. Pore waters were enriched with bicarbonate ions (up to 3.3 mM/L at a depth of 80 cm) (Fig. 2b). Nitrate (0.7–1.4 μM/L) and nitrite ions (0.7–4.5 μM/L) were present along the entire profile of the core. The concentration of sulfate ions was lower along the entire core depth than at the reference sites and in the Baikal water [27, 35], accounting for 2.9 to 8.3 μM/L (Fig. 2c).

Fig. 2 Concentration profile of the components in the chemical composition of pore waters from the St.5, GC.3 core: (a) methane, (b) bicarbonate ion, (c) nitrate ions (1), nitrite ions (2), and sulfate ions (3)
GUI_H2:CO2 and GUII_H2:CO2 enrichment cultures. Bacteria assigned to Firmicutes, Chloroflexi, Proteobacteria (δ), and Armatimonadetes (OP10) were common to two samples. The members of the phylum Bacteroidetes and Ca. Aminicenantes (OP8) were found only in the enrichment culture of the surface sample – Proteobacteria (α), Caldiserica (OP5), Ca. Atribacteria (OP9), Ca. division (AC1), Ca. division (OP11), and Ca. Parcubacteria (OD1) – only in the enrichment culture of the deep sample (Fig. 4).

In the GUI_H2:CO2 clone library, the bulk of the Firmicutes sequences (20 clones) belonged to uncultured bacteria from the sediments of freshwater lakes, Antarctic cold seeps, and oil sand tailings (Supplementary Table 3S). In the 16S rRNA gene library of the GUII_H2:CO2 sample, five sequences showed the highest similarity to the members of Peptococcaceae (Fig. 5).

The members of the phylum Chloroflexi were the second most common bacteria in gene libraries. In the clone libraries of the surface and the deep samples, representatives of the orders Anaerolinales and Dehalococcoidales were identified, respectively. Their largest number (10 clones) was identified in the GUII_H2:CO2 library. In the phylogenetic tree, they did not form a single cluster but separate branches with the sequences of uncultured bacteria retrieved in the sediments of the cold methane seep in the Sea of Okhotsk, mud volcanic sediments, and oil sand tailings during anaerobic biodegradation of longer-chain n-alkanes (Fig. 5). The sequences from the surface sample (three clones) were homologous to the sequences of uncultured bacteria from riverine sediments contaminated with nitrobenzene as well as to consortium of microorganisms involved in anaerobic digestion of sludge.

The members of the phylum Proteobacteria (δ) and Armatimonadetes (OP10) were minor (one clone in each library). The uncultured Syntrophaceae bacteria and bacterial sequences of the genera Syntrophus sp. and Smithella sp., whose cultured homologues were obtained from syntrophic associations of methanogenic archaea and propionate-, benzoate-, and alkane-oxidizing microorganisms, represented the phylum Proteobacteria (δ).

The bacterial sequences assigned to the phylum Bacteroidetes (two clones), which were detected only in the surface sample, were homologous to the uncultured bacteria from chemolithotrophic denitrification reactor and sediments of low-sulfate Lake Pavin. Three clones were assigned to Candidate division OP8. These clones were closely related to uncultured bacteria from water-flooded petroleum reservoirs, wastewater, and freshwater ecosystems.
The 16S rRNA gene library of bacteria from the deep sample was very diverse. There were six sequences assigned to the uncultured candidate division AC1 bacterium, poorly known taxa detected in the deeper layers of lakes [36], forming two subclusters in the phylogenetic tree. One sequence cluster was homologous to the uncultured bacteria from the PAH degrading bacterial community of contaminated soil and another to the sequences of the uncultured bacteria from the sediments of Lake Biwa and phreatic limestone sinkholes in Mexico. The phylum Caldiserica was the next most represented in the gene library (three clones). The sequences were 96 to 99% homologous to the sequences of the uncultured bacteria from boreal oligotrophic peat wetlands and subalpine stream sediments and 94% to the uncultured bacteria from thermal vents in Yellowstone Lake (Supplementary Table 3S).

Minor sequences (one clone each) were identified as the members of the phyla Proteobacteria (α) and Ca. Atribacteria (OP9). The Caldovatus sediminis and Crenalkalicoccus roseus thermophils isolated from hot springs were the closest homologues of the MW595807 (Proteobacteria α) sequence. The MW595808 clone was identified as uncultured bacteria closely related to microorganisms from the candidate phylum Atribacteria (OP9) found in the methanogenic reactor and boreal oligotrophic peat wetlands (Fig. 5). Two sequences from the gene library of the deep sample had low similarity (82 to 93%) with uncultured bacteria from the candidate phylum, OD1 (also referred to as Parcubacteria), and Ca. division (OP11) (not shown in the phylogenetic tree).

The members of the phylum Eurarchaeota and TACK group archaeon were detected in the 16S rRNA clone library of archaeal genes from the GUI_H2:CO2 enrichment culture. The phylum Eurarchaeota (20 clones) was represented by the orders Thermoplasmata and Methanomicrobia, whose closest homologues had been identified in Canadian oil sands reservoir, gas hydrate potential area, freshwater, and sea floor sediments (Supplementary Table 4S) (Fig. 6). TACK group archaeon consisted of 10 sequences, identified as Ca. Bathyarchaeota, MCG clade, according to EzBioCloud database. The closest homologues of the obtained sequences were detected in groundwater of the deep-well injection site, Tomsk-7 (Russia), and in sediments from different geographical locations. Bathyarchaeota, formerly known as the Miscellaneous Crenarchaeotal Group (MCG), is a phylum of microorganisms that are able to anaerobically utilize (i) detrital proteins, (ii) polymeric carbohydrates, (iii) fatty acids/aromatic compounds, (iv) methane (or short chain alkane)
and methylated compounds, and/or (v) potentially other organic matter [37]. The MW617261 clone (according to NCBI database) was related to uncultured “Aigarchaeota” archaeon from the microbial community of thermal vents in Yellowstone Lake. Currently, “Aigarchaeota” is a proposed archaeal phylum combining features of hyperthermophilic and mesophilic life during the evolution of its lineage [38]. Notably, the sequences from the 16S rRNA gene library of bacteria from the microbial community of thermal vents in Yellowstone Lake already appeared in this study during the analysis of the gene library of bacteria from the GUII H2:CO2 enrichment culture. Thus, the MW595804 clone showed a similarity of 94% to uncultured candidate division OP5 from Yellowstone Lake.

The 16S rRNA library of archaeal genes of the GUII H2:CO2 enrichment culture was less diverse in the composition than the GUI H2:CO2 enrichment culture. The gene library was 100% composed of the sequences, the closest homologues of which were identified in peatland ecosystems (Supplementary Table 4S). Of them, 87% were the sequences of the uncultured Methanomicrobiales archaeon, and 13% were the sequences of uncultured bacteria that formed a branch in TACK group archaeon (Ca. Bathyarchaeota, MCG clade) (Fig. 6).

**Bacterial and Archaeal Community Composition in Enrichment Culture Under Sulfate-Reducing Conditions**

In the gene libraries of both samples, more than 30% of the detected sequences belonged to microorganisms of the phylum Caldiserica, an anaerobic, thermophilic, and thiosulfate-reducing bacterium [39] (Fig. 4). Sequences of the uncultured bacteria detected in the hydrocarbon-contaminated aquifer and pristine subalpine stream sediments were the closest homologues (Fig. 4). The members of the phylum Firmicutes and the families Thermoactinomycesaceae, Gracilibacteraceae, and Peptococcaceae were the second most common microorganisms. The members of the phyla Proteobacteria (δ), Ca. Atrribacteria (OP9), and Chloroflexi were common for both samples.

The sequences assigned to the phylum Proteobacteria (δ) had high similarity with uncultured Deltaproteobacteria, assigned to order Desulfobacterales from Zacaton (volcanically controlled hypogenic karst, Tamaulipas, Mexico) and with Syntrophus sp. previously detected in the enrichment cultures of this study under methanogenic conditions (Supplementary Table 5S). The phylum Chloroflexi (orders Anaerolinaeales, Dehalococcoidales) was also represented by the sequences that were previously detected in the enrichment cultures enriched H2:CO2.

Sequences of the phyla Ca. Latescibacteria, Bacteroidetes, Actinobacteria, and Planctomycetes were detected only in the surface sample, and the phyla Acidobacteria and Ca. division (AC1) are only in the deep sample (Fig. 7). The sequences assigned to the phylum Planctomycetes (class Ca. Brocadiaceae) had a low similarity (90 to 92%) with the closest homologues from methane hydrate-bearing deep marine sediments in the Pacific Ocean and deep-sea mud volcanoes in Eastern Mediterranean. One of the sequences was assigned to Ca. division WS3 (Latescibacteria). Metabolic reconstruction suggests a prevalent saprophytic lifestyle in all “Latescibacteria” orders, with marked capacities for the degradation of proteins, lipids, and polysaccharides predominant in the plant, bacterial, fungal/crustacean, and eukaryotic algal cell walls [40]. Uncultured eubacterium clone from industrial and mining acid sulfate wastewaters was the only closest homologue (98%) of this sequence.

Two clones related to uncultured Actinobacteria from boreal oligotrophic peat wetlands and an ammonium-rich aquifer-aquitard system in the Pearl River Delta (China) had a low similarity (Supplementary Table 5S). In the phylogenetic tree, four clones from the gene library of the deep sample (MW617251, MW617252) formed a branch in a separate cluster and were similar to uncultured candidate division AC1 bacterium from soil contaminated of PAH, deepest phreatic sinkhole, and sediment of a freshwater Lake Biwa (Fig. 7).

The clone library of the 16S rRNA archaeal genes from the GUI SO4 enrichment culture consisted of 100% members of Euryarchaeota. In the phylogenetic tree, sequences of the order Methanomicrobia formed three branches (Fig. 8). The group with the greatest number of sequences (22 clones) clustered with uncultured euryarchaeota from sinkhole ecosystems, which had been previously identified in the archaeal gene library of the cultures enriched with H2:CO2 and of Methanoregula formicica, methane-producing archaeon isolated from methanogenic sludge. Five sequences showed the highest similarity with the members of Methanoseta sp. from the microbial community of anaerobic methanotrophic archaea of the ANME-2d cluster in freshwater sediments of Lake Ørn. The sequences homologous to the archaeal sequences from the microbial community of freshwater sediments of Lake Ørn were already identified in the gene library of archaea from the surface sample, which had been cultivated under methanogenic conditions. Three clones were related (98%) to uncultured euryarchaeote from the microbial
community of thermal vents in Yellowstone Lake (Supplementary Table 6S).

In the 16S rRNA library of archaeal genes from the GUII_SO₄ enrichment culture, as in GUII_SO₄, sequences of the order Methanomicrobia from sinkhole ecosystems dominated (87%). Five sequences showed the highest similarity to uncultured Methanomicrobiales archaeon from the Canadian oil sands reservoir. TACK group archaeon was represented by...
Anaerobic oxidation of petroleum hydrocarbons in enrichment cultures from sediments of the Gorevoy Utes...
two sequences with 97–96% identity with the sequences of uncultured archaea from sediments of various ecosystems (Supplementary Table 6).

Discussion

In subsurface and deep sediments of the Gorevoy Utes natural oil seep, under methanogenic and sulfate-reducing conditions, we recorded the loss of \( n \)-alkanes and PAHs accompanied by the methane formation. In the enrichment cultures containing surface sediments, the \( n \)-alkane conversion was the most intense in the presence of sulfate ions and in those containing deep ones – of bicarbonate ions, which can be due to the composition of microbial communities developing under various conditions. In deep sediments, the microbial community is more oriented to the anaerobic oxidation of PAHs, to which a high degree of their biodegradation (up to 46%) testifies, regardless of the present electron acceptors.

Cultivation of the surface sediment under methanogenic conditions led to the dominance of the phylum Firmicutes members, family Gracilibacteraceae. The type species of the genus Gracilibacter is described as chemoorganotrophs; its metabolism is not associated with anaerobic oxidation of hydrocarbons [41]. The members of the phylum Chloroflexi and Ca. Aminicinantes (OP8) can provide the loss of \( n \)-alkanes in the methanogenic enrichment culture. The members of the phylum Chloroflexi are regarded as microorganisms with a high level of hydrolytic enzymes indicating their involvement in the decomposition of complex organic matters [42]. The reconstructed central metabolic pathways suggested that Aminicenantes bacterium is an anaerobic organotroph capable of fermenting carbohydrates and proteinaceous substrates and of performing anaerobic respiration with nitrite [43]. At the same time, the members of the phylum Chloroflexi and Ca. Aminicinantes (OP8) are increasingly found in ecosystems associated with hydrocarbons. Ca. Aminicinantes are often found associated with fossil fuels and hydrocarbon-impacted environments; Chloroflexi harboring genes for anaerobic hydrocarbon degradation have been found in hydrothermal vent sediments [12, 44, 45]. Perhaps, archaea assigned to the Ca. Bathyarchaeota and the order Thermoplasmata, comprising 33 and 43%, respectively, of the archaenal gene library of the GUL_H2CO2 sample, participate in anaerobic alkane oxidation. Phylogenetic reconstructions, protein homologue modelling, and functional profiling of metagenomes and genomes revealed that among Archaea, in addition to Archaeoglobi previously shown to have this capability, genomes of Ca. Bathyarchaeota, Heimdallarchaeota, Lokiarchaeota, Thorarchaeota, and Thermoplasmata also suggest fermentative hydrocarbon degradation using archaea-type FAE [12, 46].

In the cultures that do not contain sulfate ions, methane generation accompanied degradation of hydrocarbons. Methane generation rates in Lake Baikal vary significantly depending on the geological structure of the lake sites [47, 48]. The methane concentration (32.54 mM/L) identified during the cultivation of the surface sample after 6 months of the experiment significantly exceeded the values that had been
Anaerobic oxidation of petroleum hydrocarbons in enrichment cultures from sediments of the Gorevoy Utes...  

previously determined (up to 11.2 mM/L) under conditions of laboratory modelling during the cultivation of microbial communities from the methane seep and mud volcanoes [49, 50]. In the deep sample, under methanogenic conditions, bacteria from the phylum Chloroflexi (proportion in the gene library – 33%) and Firmicutes (17%) represented by the order Peptococcaceae can play the main role in the alkane degradation. Microorganisms from the order Peptococcaceae are most often detected in anoxic environments associated with the anaerobic degradation of aromatic hydrocarbons [51] and in methanogenic short-chain alkane-degrading culture together with methanogenic Archaea (Methanosetaeaceae and Methanomicrobiaceae) [52]. In the GUI\_H2:CO2 enrichment cultures, the bulk of the archaeal sequences (83%) was the members of the class Methanomicrobia. Despite the presence of sequences of syntrophic bacteria and methanogenic archaea in the gene libraries of the deep sample, there was no significant methane generation. In some cases, the absence of methane generation in deep sedimentary strata was previously shown both in native natural sediments and in the experimental conditions [48, 50], despite the presence of methanogenic archaea in the composition of microbial communities.

In marine sediments, sulfate ion is the most preferable electron acceptor, and the degradation rate of petroleum hydrocarbons gradually decreases under sulfate-reducing - methanogenic - nitrate-reducing conditions [53]. The content of sulfate ions in the freshwaters of Lake Baikal is not high (55 μM/L) [54]. In Baikal areas associated with hydrocarbon seepages, the concentrations of some ions in pore waters from sediments were abnormally high [27, 35]. No elevated concentrations of sulfate and nitrate ions were in the investigated core. The increased salinity was mainly due to the concentration of H2:CO2 that do not prevent the development of microorganisms with different types of metabolism. The addition of sulfate ions into the experimental vials containing surface sedimentary layer led to the formation of a more diverse bacterial community and greater loss of n-alkanes in comparison with methanogenic conditions. The members of the phyla Caldiserica, Firmicutes, and Chloroflexi, as well as of the class Deltaproteobacteria, occupied the dominant position there. Notably, in the 16S rRNA gene libraries of bacteria from GUI\_H2:CO2 and GUI\_SO4, we identified the same closest homologues assigned to Deltaproteobacteria, Chloroflexi, and Methanomicrobia, despite the difference in cultivation conditions. The ability to adapt to sulfate stress was shown for bacteria of the genus Smithella and archaea of the genus Methanoculleus, the key alkane degraders and methane producers. In conditions of mixed electron acceptors, in the medium, depending on the sulfate concentration, there is a competition and coexistence of sulfate-reducing and methanogenic populations during the anaerobic decomposition of hexadecane [55], which, probably, also takes place in the Baikal sediments where the same microorganisms participate in the hydrocarbon degradation irrespective of the present electron acceptors.

Microorganisms present in enrichment cultures can be involved not only in the degradation of n-alkanes but also PAHs. Dong X. et al. [12] revealed that aromatic compounds can be anaerobically degraded by bacteria related to Dehalococcoidia, Anaerolineae, Deltaproteobacteria, Aminicenantes, and TA06, as well as by archaea (Thermoplasmata and Ca. Batharchaeota), via channelling into the central benzoyl-CoA degradation pathway. A decrease in the PAH concentration in the sediments of the oil seepage site near Gorevoy Utes Cape has been observed over the past 10 years since the discovery of natural oil seepage in 2005. Thus, in 2006, Σ\_PAH (24 compounds) in the sediments varied from 0.9 to 70 ng/g and in 2016 from 1.6 to 16 ng/g [19, 56]. In contrast to PAHs in oil sampled from the lake and the water surface, the proportion of PAHs in oil from the sediments was relatively low [19]. The low PAH concentration is likely due to the impact of the microbial communities in both surface and deep sediments under anaerobic conditions regardless of the present electron acceptors where PAHs primarily undergo oxidation, and n-alkanes are mainly oxidized in the water column under aerobic conditions and subsurface sediments in case of their enrichment with sulfates. This experiment indicates that the presence of sulfate ion affects the n-alkane degradation that occurs only in enrichment cultures containing surface sediments and less significant for the processes occurring in enrichment cultures containing deep sediments, which corresponds to the results of determining the activity of the sulfate reduction in the sediments of Lake Baikal. The activity of sulfate reduction (from 0.3 to 1200 nM/(dm\(^3\)day)) is reliably recorded in the upper 15 to 20 cm [48] of sediments and up to 60 cm near the Posolsk Bank methane seep [57]. In deep sediments, the maximum rates of sulfate reduction did not exceed 7 nM/(dm\(^3\)day) [48].

Therefore, the conducted experiments have revealed a wide range of microorganisms that are potential participants in oil biodegradation under anaerobic conditions of Lake Baikal. The detailed analysis of phylogenetic diversity in petroleum reservoirs on the global scale determined the core of the microbiome that includes three classes of bacteria (γ- Proteobacteria, Clostridia, and Bacteroidia) and one class of archaea (Methanomicrobia), which are widespread in petroleum reservoirs and underlie the functioning of the ecosystem in petroleum reservoirs [11]. Gray and co-authors [58] presented similar results that the members of four phyla (Firmicutes, Proteobacteria, Bacteroidetes, and Methanomicrobia) are mostly found in petroleum reservoirs and environments contaminated with hydrocarbons (aquifers, sediments, and soils). The results of comparing the structure of microbial communities from sediments associated with oil and from petroleum reservoirs confirm the general idea that the identified main composition of microbial communities
participates in complex syntrophic interactions responsible for the complete degradation of alkanes and other hydrocarbon components [59]. Syntrophy is a key mechanism of anaerobic biodegradation of hydrocarbons not only under methanogenic conditions but also in the presence of sulfate ion, ferric iron, or nitrate ion [60]. We also do not exclude syntrophic interactions for microbial communities in the Baikal sediments because, in all investigated samples, there were microbial communities that carry out interdependent sequential reactions in the general metabolic process that one member of the community cannot carry out [8].

The phylogenetic diversity revealed in methanogenic and sulfate-reducing microcosms that were obtained in this experiment mostly coincides with the composition of microorganisms included in the “microbiome core” of petroleum reservoirs. The members of the phyla Firmicutes, Chloroflexi, and Caldiserica (OP5), as well as of the class Deltaproteobacteria, predominated in the bacterial 16S rRNA gene libraries. Archaea in the clone libraries of the 16S rRNA genes were mainly represented by the sequences of the class Methanomicrobia. In the experiment, there were no members of the class γ-Proteobacteria; the class Bacteroidia was present only in the surface sediments. At the same time, γ-Proteobacteria and Betaproteobacteria ranged from 0.3 to 9 and 0.5 to 15%, respectively, in the surface sediments of the oil seeps according to the analysis of the structure of microbial communities in sediments from the Gorevoy Utes oil seep areas using high-throughput sequencing [61]. In general, from 46 to 80% of microbial communities from the different sedimentary layers of oil seeps consisted of unique OTUs, and only 1 to 2% were shared [61, 62]. Sequences of Actinobacteria, Cyanobacteria, Proteobacteria, Thaumarchaeota, and Euryarchaeota dominated the communities in sediments [61, 62]. The members of the phyla Chlorobi, Gemmatimonadetes, Nitrospirae, Planctomycetes, Armatimonadetes, Ca. Saccharibacteria, Ca. Aminicenantes, Ca. Parcubacteria, and TM6 were minor in the 16S rRNA gene libraries [61].

The dominant taxa detected in the experimental enrichment cultures are more typical of microbial communities from deep methane hydrate-bearing sediments of the St. Petersburg methane seep (Lake Baikal) where Chloroflexi (38%), Armatimonadetes (previously OP10/JS1 group) (19%), and Caldiserica (OP5) (8%) were the major components [63]. The investigated sediments, in particular the deep sample, included not only oil but also gas hydrates. Therefore, the influence of gas-saturated fluids may be responsible for the composition of microbial communities close to hydrate-bearing sediments and for the presence in sediments and enrichment cultures of the members of “rare taxa”: Planctomycetes, Ca. Atribacteria (OP9), Ca. Armatimonadetes (OP10), Ca. division (OP11), Ca. Latescibacteria (WS3), Ca. division (AC1), and Ca. Parcubacteria (OD1), which can be involved in hydrocarbon oxidation.

Microbial communities of enrichment cultures are mainly represented by uncultured prokaryotes from thermal springs, sediments of mud volcanoes, and environments contaminated with hydrocarbons, which are rather distant geographically from Lake Baikal. The detection of the same phylotypes of anaerobic bacteria phylogenetically similar to microorganisms from marine oil strata and high-temperature sediments in cold sediments of distant geographical locations is owing to their distribution by ocean currents [64]. Ocean currents play a key role in the passive spread of spores of thermophiles to distant locations from their origins. The transfer of cells from underground habitats to the overlying ocean also contributes to the marine microbial biodiversity, including representatives of the “rare biosphere” [65].

Lake Baikal located in the central part of the Baikal Rift Zone is not connected with the World Ocean by ocean currents. The identification of sequences of microorganisms having the closest homologues from mud volcanoes, oil, and gas basin of the Sea of Okhotsk and Canada, as well as from thermal vents of Yellowstone Park, in the sediments of Lake Baikal associated with the discharge of hydrocarbons may be due to the activity of hydrothermal vents located at a depth of 5–6 km, the generation of which was the most intense at the beginning of the Neogene [66]. In the same period, a modern system of mid-ocean ridges was formed in the World Ocean at the boundary between the Miocene and Pliocene. The entry of thermophiles from terrestrial hot springs located in the area of the Baikal rift may be another probable source of their occurrence in surface sediments. Thanks to the complex system of gradient and convection currents that determine the general circulation of water masses covering all three basins of Lake Baikal [67], thermophilic prokaryotes from terrestrial thermal vents could be brought in and buried in the surface sediments. All these hypotheses require study and will be the subject of further research.

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**Author Contribution** Pavlova O.N. performed data analysis and wrote the manuscript; Izosimova O.N. and Gorskhov A.G. determined PAHs and n-alkanes in model experiments; Chernistsyna S.M. analyzed molecular biological data; Ivanov V.G. measured methane in bottom sediments.
Anaerobic oxidation of petroleum hydrocarbons in enrichment cultures from sediments of the Gorevoy Utes...

and experimental samples; Pogodaeva T.V. conducted a chemical analysis of the composition of pore waters from sediments and storage cultures; Khabuev A.V. sampled sediments and described lithological core; Zemskaya T.I. coordinated the project and revised the manuscript.

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**Declarations**

**Conflict of Interest** The authors declare no competing interests.

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