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EXPERIMENTAL ARTICLES

Diversity of Novel Uncultured Prokaryotes in Microbial Communities of the Yessentukskoye Underground Mineral Water Deposit

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Abstract—Caucasian Mineral Waters is a unique territory, where various types of mineral waters with overall daily flow over 16000 m^3 are concentrated in a relatively small area. The Yessentukskoye deposit is characterized by high diversity of water types, of which Yessentuki nos. 17 and 4 are produced in the greatest amounts. Biogeochemical activity of microorganisms inhabiting the subsurface hydrosphere is one of the proposed mechanisms responsible for the genesis of these waters. The influence of microbial communities on the quality of balneological water resources is presently quite poorly studied. This is the first report on characterization of two communities inhabiting the water-bearing rocks and mineral waters of the Yessentukskoye deposit. The 16S rRNA gene profiling of these communities revealed predominance of uncultured archaea of the phylum *Hadarchaeota* (36.6%) in the Yessentuki no. 17 water retrieved from the well 46 and of several new classes of uncultured actinobacteria (29.4%) in the Yessentuki no. 4 water retrieved from the well 49-E. Significant differences were revealed in the structure of microbial communities inhabiting the water-bearing horizons of these two wells having different hydrochemical characteristics. Enrichment and pure cultures of the microorganisms belonging to the less abundant taxa were obtained. Analysis of metadata on genomic properties of prokaryotes of the dominant taxa, revealed in this work, indicates their ability to grow chemoautotrophically and thus, their potential involvement in redox transformations of the water-bearing rocks and the gas component of mineral waters.

Keywords: mineral waters, subsurface ecosystems, microbial communities, uncultured microorganisms, rare biosphere, chemolithotrophy

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Natural mineral waters have been widely used for medicinal purposes since ancient times. The ancient Greek healing center Asklepion in Epidaurus considered the "cradle" of modern spa medicine was formed over 3000 years ago around thermal mineral springs (Christopoulou-Aletra et al., 2010). The genesis of mineral waters has been studied for more than a century, and assumptions about the significant role of microorganisms in this process have arisen for a long time, although microbiological studies of mineral waters have been fragmentary so far. Initially, the methods for studying the microbial communities of mineral waters were chosen mainly in accordance with sanitary and epidemiological requirements and provided little information about the functional diversity of microorganisms and their influence on formation of the composition of the waters themselves. However, since the late 1970s, there has been significant progress in the study of subsurface mineral waters, and cultured microorganisms have been detected even in very deep aquifers. The works of that time concentrated on determination of the number of aerobic heterotrophic microorganisms in mineral waters using the standard methods of plating on solid nutrient media. Most of the bacteria revealed by this method were identified as members of the genus *Pseudomonas,* which led to conclusions about the probable dominance of this genus in the microbial communities of mineral waters (see the review by Leclerc and da Costa, 2005 and references therein). Later, culture-based methods revealed in mineral waters a significant number of representatives of other heterotrophic bacterial genera: *Cytophaga, Flavobacterium,* and *Flexibacter* (Quevedo-Sarmiento et al., 1986). With the rise of molecular ecology, microbial communities of several types of European bottled mineral waters have been characterized. All analyzed communities were dominated by *Gammaproteobacteria*; however, at the level of orders

and families, the profiles of communities of different waters differed significantly from each other (Lesaulnier et al., 2017; Sala-Comorera et al., 2020). Pathogenic microorganisms were not detected in significant quantities either in the original samples of mineral waters or in bottled water during its storage (see the work of Sala-Comorera et al., 2020 and references therein). Moreover, antagonistic activity of the pseudomonads detected in mineral waters against some pathogenic test strains was shown even earlier (Leclerc and da Costa, 2005). Thus, while the issue of safety of the natural microflora of mineral waters is currently resolved, the issue of participation of this microflora in the formation of the composition of waters still requires a detailed study. One of the few such works characterized the microbial communities of natural discharges of acidic carbonaceous mineral waters of the Cheb basin in the Czech Republic (Krauze et al., 2017). Based on isotopic studies, the authors suggested that terrigenous CO_2 was a significant carbon source for these microbial communities. Molecular studies revealed the predominance of anaerobic and microaerophilic microorganisms, presumably involved in the biogeochemical cycles of sulfur and iron, including sulfur-oxidizing bacteria of the genera *Sulfuricurvum* and *Sulfurimonas* and Fe(II)-oxidizing bacteria of the genera *Gallionella* and *Sideroxydans* (Krauze et al., 2017). Moreover, the researchers of Cheb mineral springs found that the microbial communities of carbonaceous waters contained various low-abundant phylotypes—methanogenic archaea of the genus *Methanoregula* and representatives of archaeal deep phylogenetic lineages of uncultured *Hadesarchaea* and *Bathyarchaeota*, typical for marine and deep subsurface ecosystems (Parks et al., 2020). It is suggested that scarce prokaryotic populations, whose relative abundance in a microbial community comprise 1% or less, may significantly influence the activity of such a community. According to modern concepts, such phylotypes constitute a part of the global phenomenon of the "rare prokaryotic biosphere" (Skopina et al., 2016; Jousset et al., 2017). It is suggested that these organisms may be responsible for a number of important ecological functions in various ecosystems: acting as a reserve of genetic material that is activated upon sharp changes of environmental conditions (Jousset et al., 2017); being the first to populate novel ecological niches; using rare substrates, for example, xenobiotics (Skopina et al., 2016); and ensuring consistent production of important growth factors at a constant (low but critically required) level (Sohm et al., 2011; Zhang et al., 2019). With regard to subsurface water ecosystems, the ecological role of the representatives of the rare biosphere, as well as the functions of the dominant phylotypes, have not yet been the subjects of detailed studies.

Assumptions about a significant role of microorganisms in the formation of the water composition of the Caucasian Mineral Waters (CMW) region arose quite a long time ago (Shinkarenko, 1941; Kudlaenko, 1976; Muravleva et al., 1989). Early studies mainly assessed the significance of sulfate-reducing bacteria as sulfide producers and oxidizers of organic matter buried in the water-bearing rocks. In the last decade, studies of the microbial communities of subsurface mineral waters of productive aquifers at the Yessentukskoye, Nagutskoye, and Pyatigorskoye deposits, for which the role of cultured sulfate reducers in the formation of "sulfate-free" soda-type carbonaceous waters has been assessed, were started (Potapov et al., 2014). The presence of carbon dioxide with an admixture of isotopically light biogenic carbon, originating from microbial oxidation of organic matter, which was revealed during the analysis of gases from the Yessentukskoye deposit, indicated high activity of sulfate reducers, completely oxidizing dispersed organic matter to CO_2 (Potapov et al., 2017; Potapov, 2019). The effect of activity of other physiological groups of prokaryotes and entire microbial communities on formation of the composition of mineral waters was not determined.

The most widely used strategy for determining the structure of prokaryotic communities is currently the sequencing and analysis of the 16S rRNA genes (Soriano-Lerma et al., 2020). This technique has already made it possible to characterize a wide variety of ecosystems.

In the present work, we describe the phylogenetic diversity of microbial communities in the CMW region based on analysis of the libraries of 16S rRNA gene amplicons obtained from total DNA isolated directly from the water of two wells of the Novoblagodarnensky area of the Yessentukskoye deposit. This area produces balneologically valuable carbonaceous mineral waters of the Yessentuki no. 17 and Yessentuki no. 4 types circulating in the fractured zone of Upper Cretaceous limestones at a depth of 500 to 1000 m, where, according to preliminary geological and hydrochemical studies (Potapov et al., 2017; Potapov, 2019), a high geochemical activity of microorganisms can be expected. The results of our analysis represent one of the first molecular ecological characteristics of such ecosystems.

MATERIALS AND METHODS

Sample collection. Samples for DNA isolation were taken directly through the wellheads 46 and 49-E using FM02-1000 membrane filtration units with a volume of 1 L (Institute for Analytical Instrumentation of RAS, St. Petersburg, Russia) with 0.2-μm pore size track membrane filters (JINR, Dubna, Russia). Pre-sterilized filtration units were assembled on-site, connected to the wellhead fittings via sterile connectors and hoses, and 100 L of water per each sample were passed through the filters under natural excess pressure of the wells (4–6 atm at the wellhead). Filtration began immediately after filling the next tank

truck, supplying water to the bottling plant with a volume of at least 19 m^3, which ensured obtaining the samples directly from the water-bearing sediments without admixture of the water, settling in the wellbores between the fillings of the tank trucks. At the end of the filtration, the residual water was completely pushed through the filters by the gases released from the wells. A quarter of each of the obtained wet filters with microbial biomass was cut off with sterile scissors, transferred to a sterile 120-mL glass vial, completely filled with water from the well, and sealed with a rubber stopper and a screw cap. The resulting material was transported to the laboratory, stored in a refrigerator, and used as an inoculum. The rest of the filter was packed with sterile tweezers into sterile 15-mL Falcon tubes and completely covered with 2 mL of sterile buffer A (100 mM Tris-HCl, 100 mM EDTA, 150 mM NaCl; pH 8.0) for DNA fixation. Tightly closed tubes were immediately placed in a cooler bag on ice, transported in this form to the laboratory, and then stored at −20°C until DNA isolation.

DNA isolation, preparation and sequencing of 16S rRNA gene amplicon libraries. DNA was isolated directly from fixed filters using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, United States) according to the manufacturer's instructions.

The preparation of amplicon libraries of the V4 region of the 16S rRNA gene was carried out as described previously (Gohl et al., 2016) using a pair of primers 515F (5'-GTGBCAGCMGCCGCGGTAA-3'; Hugerth et al., 2014)—Pro-mod-805R (5'-GGAC-TACHVGGGTWTCTAAT-3'; Merkel et al., 2019). The libraries were sequenced on a MiSeq system (Illumina, California, United States) using a 150-nucleotide length paired-end read cartridge. Bioinformatic analysis was performed as described by Merkel et al. (2021). All sequencing data were deposited into the NCBI BioProject PRJNA database under accession number 760784.

Preparation of enrichment and pure cultures. Water from the vials containing 1/4 part of the filter, obtained in process of DNA isolation, served as inoculum (10% by volume). The vial was shaken vigorously before taking an aliquot. To obtain enrichment cultures of microorganisms, a modified method of selective media was used. Bottled Yessentuki water nos. 4 and 17 obtained from the same wells as the inoculum was used as a mineral base for the preparation of culture media. The use of water from the wells as the mineral base of the media increased the likelihood of accumulation of the target groups of microorganisms and at the same time, ensured their development under optimal physicochemical conditions.

The media were prepared using the routine technique of anaerobic cultivation by boiling bottled Yessentuki no. 4 water (Aqua Holding LLC, GOST R (Russian National Standard) 54316-2011), pH 7.0, for enrichment cultures from well 49-E, or bottled Yessentuki no. 17 water (Aqua Holding, GOST R 54316-2011), pH 6.8, for enrichment cultures from well 46, followed by cooling under CO_2 flux (100%). Vitamins (1 mL/L) (Wolin et al., 1963) and microelements (1 mL/L) (Kevbrin and Zavarzin, 1992) were added to the cooled medium. If necessary, pH was adjusted to the required values with 1 N HCl solution. The media were dispensed into 10-mL Hungate tubes, containing one of the following iron minerals as an electron donor or acceptor (20 g/L): natural glauconite $K_{0.8}(Mg_{0.4}Fe^{2+}_{0.1})(Fe^{3+}_{1.1}Al_{0.4})[(Si_{3.7}Al_{0.3})O_{10}](OH)_2$ (Maasdu deposit, Estonia), biotite $(K_{0.89}Na_{0.03})(Mg_{0.9}Fe^{2+}_{1.1}Al_{0.43}Fe^{3+}_{0.22}Ti_{0.09}Mn_{0.03})[Al_{1.2-6}Si_{2.8}O_{10}](OH,F)_2$ (Karelia, Russia), siderite ($FeCO_3$) (Bakal'skoye deposit, Russia), or synthesized magnetite (Fe_3O_4, calculated final total concentration of ferrous and ferric iron in the medium—50 mM). The synthesized magnetite (SM), a mixed Fe(II/III) oxide, was prepared by titration of equimolar amounts of $FeSO_4 \cdot 7H_2O$ and $Fe_2(SO_4)_3 \cdot 6H_2O$ solutions with 10% NaOH solution to pH 8.5 under constant stirring. The resulting magnetic precipitate was washed three times with distilled water to remove NaCl, centrifuged for 3 min at 1000 *g*, and added to the tube before dispensing the medium. CH_4 (2 mL) was added to the gas phase (to its final volume concentration of 30%); after that, the tubes were autoclaved at 121°C for 30 min. Primary enrichments were incubated for 7 days at 37 and 47°C. The growth of enrichment cultures was monitored by direct counting of acridine orange-stained cells under an Axio Lab.A1 fluorescence microscope (Zeiss, Germany), by the change in the composition of the gas phase, or in the color of the added iron minerals. If the growth of microorganisms in primary enrichments was confirmed, they were transferred (5% by volume) into tubes with fresh sterile medium under the same conditions that were used for the corresponding primary enrichment cultures. Three to five sequential transfers ensured obtaining of stable enrichments, from which further attempts were made to isolate pure cultures of dominating microorganisms by the method of limiting tenfold dilutions.

Aerobic cultivation of pure cultures was carried out on bottled Yessentuki no. 17 water dispensed under air (5 mL per Hungate tube) with thiosulfate (10 mM) as an electron donor.

RESULTS AND DISCUSSION

Geological and hydrochemical characteristics of the research subjects. The Novoblagodarnensky area located 5–10 km north of the city of Yessentuki was explored in 1949–1956. At present, the reserves of mineral waters in this area comprise 65 m^3/day of Yessentuki no. 17 water and 80 m^3/day of Yessentuki no. 4 water. Wells 46 and 49-E, from which subsurface carbonaceous mineral waters of the Novoblagodarnensky

area are extracted, have depths of 685.8 (intake interval at a depth of 552.0–685.8 m) and 770 m (intake interval at a depth of 508.0–770.0 m), respectively, and are located at a distance of 3 km from each other. The wells operate in a regulated self-dispensing tap mode and are used for industrial bottling. The water temperature at the wellheads reaches 45°C. In terms of composition, the waters are chloride-hydrocarbonate sodium with a mineralization of 8.0–14.0 g/L; the content of dissolved carbon dioxide is 0.6–1.4 g/L. The gas phase of these waters, apart from CO_2 (40–70%), contains CH_4 (10–40%) and N_2 (1–5%). The chemical composition of the sampled waters of the two production wells of the Novoblagodarnensky area according to the Kurlov formula is presented below:

Yessentuki type no. 17, well 46:

$$CO_2 0.7\ M 10.5 \frac{HCO_3 63\ Cl 37}{(Na + K) 93 Mg 4\ Ca 3} H_3BO_3 0.051\ H_2SiO_3 0.029\ pH 6.9\ T 36°C;$$

Yessentuki type no. 4, well 49-E:

$$CO_2 0.7\ M 8.6 \frac{HCO_3 60\ Cl 40}{(Na + K) 96\ (Mg 2\ Ca 2)} H_3BO_3 0.053\ H_2SiO_3 0.031\ pH 7.0\ T 41°C.$$

Phylogenetic composition of the microbial community of Yessentuki no. 17 water from well 46. According to the results of microscopy of the sampled water, the number of cells varied from 10^5 to 10^6 cells/mL. For water samples from this well, two phylogenetic profiles were recovered—for samples taken in August and in October, 2019. For each time replicate, several experimental replicates of the 16S rRNA gene libraries were obtained. In total, for well 46 libraries, over 28 thousand sequences of V4 fragments 16S rRNA gene amplicons were obtained. Phylogenetic profiles of the microbial community in different replicates were, on the whole, similar to each other, which allowed us to further analyze the average values of taxa representation. The average Shannon index value was 2.7, that of the Simpson index, 0.1, which indicate a relatively low diversity of the studied microbial community.

Analysis of the 16S rRNA gene clone libraries revealed predominance of uncultured archaea in well 46 water. Archaea accounted for more than a half (59.3%) of the total microbial diversity of the ecosystem (Table 1), with the highest abundance (36.6% of all reads) of the phylotypes of the order *Hadarchaeales*. Its first representatives were discovered by molecular methods in the technical and fracture water of South African deep subsurface gold mines and were originally designated as SAGMEG (South-African Gold Mine Miscellaneous Euryarchaeal Group; Takai et al., 2001). Based on the analysis of complete genomic data, this group was identified as a separate phylum *Hadarchaea* whose representatives were predicted to be capable of autotrophy using CO_2 as the only carbon source and electron acceptor during acetogenesis, as well as of hydrogenogenic oxidation of CO to CO_2 (Baker et al., 2016). Representatives of *Hadarchaea* have been detected in a variety of anaerobic ecosystems with a wide range of temperatures (from 4 to 80°C): in the subsurface continental biosphere, in terrestrial hot springs, in subsurface ecosystems of the ocean floor, in deep-sea, shallow-water, and freshwater sediments (Parkes et al., 2005; Biddle et al., 2006). However, in none of these ecosystems were such archaea the dominant phylotype. Thus, subsurface mineral waters and water-bearing rocks exposed by well 46 of the Yessentukskoye deposit are the first-described ecotope, the physicochemical conditions of which favor the dominance of this uncultured and still poorly studied group of archaea. It is likely that these organisms are the key primary producers of organic matter in the microbial community of water-bearing rocks and water of well 46, where their abundance and metabolic activity may have a significant effect on the CO_2 content in the gas phase of mineral waters.

Phylotypes belonging to a new order of the class *Thermoplasmata* of the phylum *Thermoplasmatota* were the second most abundant group of archaea (10.8% of all reads) in the Yessentuki no. 17 water from well 46. All the described *Thermoplasmata* species have been isolated from acidic mine drainages or acidic hot springs of volcanic origin. Most of them are facultatively aerobic organotrophic acidophiles, including hyperacidophiles with a growth pH optimum of about 0 (Rosenberg et al., 2014). Some members of the class are capable of autotrophic growth coupled with aerobic oxidation of Fe(II). The closest cultured organism related to the *Thermoplasmata* phylotypes from well 46 belonged to the species *Methanomassiliicoccus luminiensis* of the order *Methanomassiliicoccales* (Table 1), a neutrophilic obligate methylotroph that forms methane from methanol or methylamines and hydrogen (Iino et al., 2013). However, the very low identity between this organism and the Yessentuki phylotypes of *Thermoplasmata* (80.7%) makes it difficult to predict the physiological properties of the latter and, in particular, their capacity for methylotrophic methanogenesis.

Table 1. Representation of the main phylotypes (% of the total composition) in the microbial communities of mineral waters from wells 46 and 49-E of the Yessentukskoye deposit according to the results of 16S rRNA profiling. Phylogenetic groups with representation in the communities >1%, are highlighted in bold

Taxonomic (position(phylum, class, order, family, genus)	Samples from wells		Closest valid species (>80% similarity)	Closest clones
	46	49-E		
ARCHAEA				
Crenarchaeota *Bathyarchaeia*	0.5	**2.5**	*Archaeoglobus lithotrophicus* (82.4%)	KP341421.1 (98.1%), Filwoha hot spring, Ethiopia
Euryarchaeota *Methanobacteria Methanobacteriales Methanobacteriaceae Methanobacterium*	**4.0**	0.0	*Methanobacterium subterraneum* (100%) *Methanobacterium formicicum* (100%)	MK472013.1 (100%), anaerobic bioreactor; MG001723.1 (100%), methane reservoir in a coal layer (depth 1.2 km, 62°C), India
Euryarchaeota *Methanobacteria Methanobacteriales Methanothermobacteriaceae Methanothermobacter*	0.0	**11.5**	*Methanothermobacter thermautotrophicus* Delta H (100%)	MT013481.1 (100%), sludge of a mesophilic digester
Hadarchaeota *Hadarchaeia Hadarchaeales*	**36.6**	0.0		AB476720.1 (100%), sulfur drainage water of the Gotard Tunnel, Switzerland; KC926748.1 (100%), Ouzan River sediment, China; AB802437.1 (98.6%), ocean bottom core, Honshu Island shelf JQ315350.1 (98.1%), AM883015.1 (97.2%), coal layer core, depth to 2.3 km, New Zealand
	0.0	**2.4**		FJ936679.1 (97.6%), Avachinsky Volcano mud, Russia
Halobacterota ANME-1 ANME-1a	**7.3**	0.4	*Methanosaeta pelagica* (85.4%)	GU120524.1 (99.5%), Pitch Lake coastal natural bitumen lake, Trinidad, Carribean; JN605063.1 (99.1%), bottom sediment of White Oak River estuary, USA western coast
Halobacterota others (2 phylotypes)	0.1	0.1		
Thermoplasmatota *Thermoplasmata*	**10.8**	0.0	*Methanomassiliicoccus luminyensis* (80.7%)	AB665421.1 (93.2%), water of an undergonud hot spring (depth 0.25–1 km), Japan; FJ900727.1 (91.8%), oil well production water, China

Table 1. (Contd.)

Taxonomic (position(phylum, class, order, family, genus)	Samples from wells		Closest valid species (>80% similarity)	Closest clones
	46	49-E		
BACTERIA				
Acidobacteriota *Aminicenantia Aminicenantales*	0.0	**2.3**	*Moorella glycerini* (83.2%)	MK067121.1 (100%), oil-contaminated soil, Nigeria
Actinobacteriota *Coriobacteriia* FS118-23B-02	0.0	**10.9**	*Egibacter rhizosphaerae* (85.8%)	GU982852.1 (89.2%), Western Pacific bottom sediment
Actinobacteriota *Coriobacteriia* OPB41	**1.4**	**5.2**	*Olegusella massiliensis* (87.7%)	MK035808.1 (100%), electrotrophic methanogenic microbial community from a water-bearing horizon of a gas deposit, Japan
Actinobacteriota RBG-16-55-12	0.1	**11.0**	*Caldalkalibacillus thermarum* (88.9%)	MF895411.1 (100%), coal layer, Australia
Caldisericota *Caldisericia Caldisericales* TTA-B15	0.0	**4.6**	*Calditerricola yamamurae* (84.4%)	KP109901.1 (100%), asociated water from an oil deposit, China; KM373089.1 (100%), terephthalate-converting thermophilic digester
Chloroflexi *Dehalococcoidia* Sh765B-AG-111	0.1	**1.6**	*Dehalogenimonas formicexedens* (90.6%)	EU385911.1 (100%), South China Sea bottom core
Firmicutes D8A-2	**3.2**	**2.3**	*Moorella humiferrea* (88.7%)	KX576599.1 (99.5%), subterranean gas reservoir (0.8 km), France
Firmicutes *Desulfitobacteriia Desulfitobacteriales Desulfitobacteriaceae Desulfitobacterium*	0.1	**10.4**	*Desulfitobacterium metallireducens* (93.9%)	MF895721.1 (99.1%), coal layer, Australia
Firmicutes *Desulfotomaculia Ammonifexales Ammonificaceae*	**4.0**	0.2	*Thermodesulfitimonas autotrophica* (88.7%)	MF628593.1 (99.5%), microcosm from a coal layer
Firmicutes *Desulfotomaculia Desulfotomaculales Desulfurisporaceae* SCADC1-2-3	0.0	**1.4**	*Desulfofundulus kuznetsovii* (97.2%)	MF897964.1 (100%), coal layer, Australia
Firmicutes Incertae Sedis DTU014	0.1	**3.3**	*Koleobacter methoxysyntrophicus* (90.7%)	MF950564.1 (92.3%), rice field soil, China
Firmicutes others (18 phylotypes)	1.0	**5.0**		

Table 1. (Contd.)

Taxonomic (position(phylum, class, order, family, genus)	Samples from wells		Closest valid species (>80% similarity)	Closest clones
	46	49-E		
Nitrospirota *Thermodesulfovibrionia Thermodesulfovibrionales Thermodesulfovibrionaceae Thermodesulfovibrio*	0.0	**2.3**	*Thermodesulfovibrio yellowstonii* (100%)	LC480817.1 (100%), sludge of a thermophilic digester; MF894795.1 (100%), coal layer, Australia
Nitrospirota *Thermodesulfovibrionia*	**23.5**	0.2	*Dissulfurispira thermophila* (88.2%); *Thermodesulfovibrio aggregans* (85.3%)	GQ921458 (99.5%), fracture water of the deep Northam mine (1.7 km), South Africa; KX974515.1 (96.2%), urban water intake, Singapore
Patescibacteria *Parcubacteria Candidatus* Yanofskybacteria	0.1	**1.1**		FR727651.1 (93.4%), hot spring, France
Patescibacteria *Parcubacteria Paceibacterales Paceibacteraceae Candidatus* Paceibacter	0.0	**4.0**		AB645175.1 (93.9%), subseafloor core sample, Honshu Island shelf; JN123513.1 (93.4%), ocean bottom core, Tainan Ridge, Taiwan shelf
Proteobacteria *Alphaproteobacteria Rhizobiales Rhizobiaceae Ciceribacter*	0.0	**1.3**	*Ciceribacter azotifigens* (100%); *Ciceribacter thiooxidans* (100%)	KX714809.1 (100%), hot spring, Iran
Proteobacteria *Gammaproteobacteria Pseudomonadales Moraxellaceae*	**3.0**	0.0	*Cavicella subterranea* (97.6%)	KT014926.1 (100%), Cr(VI)-contaminated soil, China
Proteobacteria *Gammaproteobacteria Halothiobacillales Halothiobacillaceae Halothiobacillus*	0.3	0.0	*Halothiobacillus neapolitanus* (100%)	KX714819.1 (100%), gas-processing plant, Iran
Other bacteria (a total of 44 phylotypes)	**3.1**	**10.5**		
Uncertain taxonomic position (No relative)	0.6	**5.4**	–	–

The third most abundant group of uncultured archaea (7.3% of the community) in the water from well 46 was represented by ANME-1a phylotypes in the phylum *Halobacterota*. Comparison of the obtained ANME-1a 16S rRNA gene sequences with the NCBI Nucleotide Collection database (version of September 14, 2021) revealed their strong homology with the ANME-1 phylotype detected in marine coastal sediments. It was this group for that a number of evidences was first obtained indicating the functioning of the process of anaerobic oxidation of methane to carbon dioxide (Hinrichs et al., 1999; Lloyd et al., 2011). The high (10–40%) content of methane in the gas phase of water from well 46, which correlates with a significant representation of ANME-1a, suggests that this substrate is a significant resource for biomass formation in the studied ecosystem. It is important to note that anaerobic methanotrophy is thermodynamically beneficial only in consortia with sulfate- or iron-reducing bacteria, which act as acceptors of reduced equivalents released during methane oxidation (Timmers et al., 2017). Mineral substances with electron conductivity, including the iron minerals magnetite and maghemite, can serve as alternative electron acceptors for ANME-1a methanotrophs. Such an opportunity for ANME archaea is provided by the electron transport protein genes present in their genomes (Wang et al., 2014). The processes of using insoluble electron acceptors are widespread in the subsurface biosphere and may be active in the aquifers of well 46. In turn, the particles of iron minerals can serve as a significant link in the interspecies electron transfer between organisms with different metabolism in the processes of syntrophic methanogenesis (Kato et al., 2012). These processes are especially important for aquifers, where the abundance of microniches in water-bearing rocks creates selective conditions for the development of microbial associations attached to minerals. Apart from uncultured archaea, known autotrophic hydrogenotrophic methanogenic archaea of the genus *Methanobacterium* were fairly widespread (4.0% of all reads) in the water community of well 46 (Whitman, 2015). Hypothetically, these archaea can serve as a source of methane for ANME-1a.

Bacteria accounted for 40.1% of the microbial community in the well 46 water. Among them, the phylotypes of the uncultured group of the new order-level in the class *Thermodesulfovibrionia* prevailed (23.5% of the community). According to GTDB taxonomy database, this class of nitrospira includes several clusters of uncultured bacteria and two cultured genera, sulfate reducers *Thermodesulfovibrio* (Whitman, 2015) and autotrophic sulfur and thiosulfate disproportionators *Dissulfurispira* (Umezawa et al., 2021). The phylotypes identified in the present work had a low level of identity with the closest relative, *D. thermophila* (the level of identity of the V4 region of the 16S rRNA gene was 88.2%; Table 1), which hinders unambiguous conclusions about their physiology and, accordingly, their ecological role in the community. The phylotypes of known sulfate reducers, related to the genus *Desulfovibrio*, were present in minor amounts in the water community of well 46 (≤1% of the 16S rRNA gene reads). However, together with nitrospira, they can be considered as possible syntrophic partners of ANME-1a archaea.

The phylotypes of the family "*Ammonificaceae*" (4.0%), which include three cultured species of chemolithotrophs that reduce nitrates, sulfur, or sulfur compounds with hydrogen or formate as electron donors (https://gtdb.ecogenomic.org/searches? s=al&q=ammonificaceae) accounted for a significant proportion of the microbial community of water in well 46, although substantially smaller than nitrospira. Similar shares of this microbial community (3.2 and 3.0%, respectively) belonged to the phylotypes of the uncultured D8A-2 group of the new class-level in the phylum *Firmicutes* and the new uncultured group of gammaproteobacteria of the family *Moraxellaceae*. The metabolic properties of the D8A-2 group are currently not described. Of interest is the work in which a sharp increase in the abundance of D8A-2 in microbial consortia was recorded when their methane production from complex organic substrates was stimulated, e.g. by introducing magnetite particles activating interspecies electron transfer (Lee et al., 2019). *Cavicella subterranea,* an organism closely related to the Yessentuki's *Moraxellaceae* phylotype (97.6% identity of the 16S rRNA gene), is an organotroph isolated from a well extracting subsurface mineral water in Portugal (Franca et al., 2015).

Uncultured actinobacteria of the OPB41 group of the class *Coriobacteriia* (1.4% of the community) can also be assigned to prevailing bacterial groups of the Yessentuki no. 17 water community. Originally detected in hot springs, these organisms have since been detected in a wide variety of ecosystems. Based on genomic analysis, they are supposed to possess hydrolytic activity (Bird et al., 2019).

Among the minor components of the microbial community of well 46 water, each accounting for ≤1% of the 16S rRNA gene reads, noteworthy are the phylotypes attributed to the genera of organotrophic bacteria *Paracoccus, Halothiobacillus,* and *Cellulomonas,* which cultured representatives are capable of denitrification, oxidation of various sulfur compounds, and hydrolysis of polysaccharides, respectively (Rosenberg et al., 2014).

Phylogenetic composition of the microbial community of Yessentuki no. 4 water from well 49-E. According to the results of microscopy of the water samples of this type, the number of cells in them was less than 10^5/mL. For water samples from this well, phylogenetic profiling was only performed for one replicate, taken in August, 2019, for which two experimental replicates of the analysis of 16S rRNA gene libraries were made. In total, for the well 49-E libraries, more

than six thousand sequences of V4 fragment of the 16S rRNA gene amplicons were recovered. The average Shannon index value was 2.9, and the Simpson index value was 0.08, which also indicated a relatively low diversity of the studied microbial community, which, however, was higher than that of the well 46 water.

Profiling of the microbial community of water from well 49-E revealed predominance of uncultured actinobacteria, whose phylotypes made up 27.1% of reads in total (Table 1). Unlike water of the Yessentuki no. 17 type, the microbial community of Yessentuki no. 4 water contained significantly smaller share of archaea (only 16.9%). At the same time, its bacterial part was more diverse—about 80% of the community was represented by twenty bacterial phylotypes, while in the water of Yessentuki no. 17 type, 90% of the community belonged to only eight phylotypes, the majority of which were archaeal (Table 1). One of the reasons for the greater phylogenetic diversity of the microbial community in the well 49-E water, compared to that in the well 46, was its lower CO_2 content, as compared to well 46, which in its turn, was apparently caused by the admixture of fresh sodium bicarbonate water, circulating in the Upper Cretaceous limestones, to the mineral water in the horizons exposed by the well 49-E (Potapov, 2017).

The uncultured actinobacteria, which predominated in the microbial community of well 49-E water, belonged to the class *Coriobacteriia* and the new class-level phylogenetic group RBG-16-55-12. Cultured *Coriobacteriia* members, all concentrated in the orders *Coriobacteriales* and *Eggerthellales,* are animal and human epibionts (Rosenberg et al., 2014); however, the phylotypes found in the well 49-E water belonged to new orders, which representatives were detected in various ecosystems not related to animal organisms. For instance, the clone most closely related to the Yessentuki's phylotype of the FS118-23B-02 group (10.9% in the studied community) was detected in the bottom sediments of the Pacific Ocean (Table 1). Microorganisms of this group have been suggested to be free-living organotrophs (Huber et al., 2006), although their physiology remains unexplored. The putative hydrolytic coriobacteria of the OPB41 group constituted 5.2% of the water community in well 49-E, although their main substrate in this organic-depleted ecosystem remains unclear. There is little information about the metabolism of uncultured bacteria of the class-level group RBG-16-55-12—this is the most abundant form of actinomycetes in the water of the Yessentuki no. 4 type (11.0% of all reads). Bacteria of this group have previously been found in a variety of extreme ecosystems. In particular, a clone closely related to Yessentuki RBG-16-55-12 was found in the Australian coal seam. The taxon "*Candidatus* Hakubanella thermoalkaliphilus" was proposed for the representatives of RBG-16-55-12 isolated from a Japanese thermal alkaline spring. Single cell genomic analysis of these organisms revealed their ability to fix CO_2 during mixotrophic growth via the Wood–Ljungdahl pathway, which has not been previously described in actinobacteria. One of such phylotypes was also found to be capable of nitrate reduction (Merino et al., 2020). Actinobacteria are able to degrade complex organic substances as part of microbial associations, the formation of which could be provided by the ability of actinobacteria to form complex colonies on solid substrates (Rosenberg et al., 2014). Besides that, the role of primary producers could be equiprobably suggested for *Actinobacteriia* representatives detected in the well 49-E water, considering the detection of the Wood-Ljungdahl pathway genes in this phylum.

Archaea of the species *Methanothermobacter thermautotrophicus,* which constituted the second largest group of prokaryotes in the water community of well 49-E (11.5% of the community, Table 1), could also play the role of organic matter producers. These archaea are strict anaerobes, moderate thermophiles, typical hydrogenotrophic methanogens reducing CO_2 to methane with hydrogen or formate as electron donors (Wasserfallen et al., 2000). Apart from them, the microbial community of well 49-E water contained a significant proportion of two groups of uncultured archaea with the putative capacity for autotrophic acetogenic growth. These are archaea of the abovementioned order *Hadarchaeales* of the phylum *Hadarchaeia* (2.4% of the community) and the class *Bathyarchaeia* of the phylum *Crenarchaeota* (2.5%). Based on genomic analysis, the latter are supposed to have the capacity for hydrogenotrophic methanogenesis from CO, CO_2, and methanol, for acetogenesis, as well as for dissimilatory reduction of nitrogen and sulfur compounds (Evans et al., 2015; He et al., 2016; Thomas et al., 2020). Interestingly, the well 49-E water contained the *Hadarchaeales* phylotype, which was not detected in the well 46 water, where archaea of this class predominated; its most closely related clone was detected in a thermal volcanic habitat, while *Hadarchaeales*-related clones from Yessentuki no. 17 water originated from cold subsurface ecosystems (Table 1). Such a difference may reflect inequality of hydrochemical characteristics of these waters, which favors the accumulation of organisms with different metabolic properties.

Most likely, the dissimilarity of physicochemical conditions also explains the significant difference in the representation of *Firmicutes* of the genus *Desulfitobacterium* assigned to the separate class *Desulfitobacteriia* (Parks et al., 2020), in the waters of Yessentuki no. 4 (over 10% of the community diversity) and Yessentuki no. 17 (less than 0.3% of the community). Bacteria of this genus are organotrophs capable of various types of anaerobic respiration, of which iron reduction can be considered the most geochemically significant. In particular, *Desulfitobacterium metallireducens,* a cultured organism most closely related to

the Yessentuki phylotype of this genus (94% identity in the V4 region of the 16S rRNA gene; Table 1), is capable of reducing Fe(III), Mn(IV), and of reductive dehalogenation of chlorinated hydrocarbons (Finneran et al., 2002). In sediments of marine origin, constituting the aquifer exposed by well 49-E, chlorinated hydrocarbons can locally be present as products of pyrolysis or thermolysis of halogenated terpenes, amino acids, flavonoids, and other halogenated components of algal biomass (Paul and Pohnert, 2011).

The share of other *Firmicutes* in the water community of Yessentuki no. 4 comprised 13.2% and included 21 phylotypes. Of these, the phylotypes of the uncultured groups D8A-2 (2.3%) and DTU014 Incertae sedis (3.3%), as well as the phylotypes belonging to the classes *Clostridia* (2.3% in total) and *Desulfotomaculia* (2.0% in total), were the most abundant. The latter group of phylotypes includes thermophilic anaerobic autotrophs of the genera *Ammonifex* and *Thermodesulfitimonas,* which reduce nitrates, sulfur or its compounds with hydrogen or formate as electron donors, as well as syntrophic organotrophs of the genus *Pelotomaculum* and the SCADC1-2-3 group of uncultured bacteria within *Desulfurisporaceae* family of thermophilic sulfur reducers. The closest cultured organism related to the SCADC1-2-3 phylotype from well 49-E belongs to the species *Desulfofundulus kuznetsovii* (97.0% identity, Table 1) isolated from subsurface mineral thermal water and being a thermophilic organotrophic sulfate reducer (Nazina et al., 1988; Watanabe et al., 2018).

Apart from actinobacteria, methanogenic archaea, and *Firmicutes*, a significant share of the microbial community of water of the Yessentuki no. 4 type was represented by the phylotypes of uncultured class-level group *Parcubacteria* in the phylum *Patescibacteria* (5.1%). The class *Parcubacteria* includes several different groups of uncultured organisms, whose metabolism remains poorly characterized, despite the publication of several incomplete genomes of their representatives. Uncultured *Parcubacteria* were previously detected in a variety of anaerobic ecosystems with high concentration of organic matter, for example, in bottom sediments and the rumen of ruminants, but their greatest abundance was recorded in cold freshwater ecosystems of Svalbard (Sułowicz et al., 2020).

The family-level TTA-B15 phylotype in the order *Caldisericales*, detected in the well 49-E water (4.6% of the community), belongs to the phylum *Caldisericota*. This phylotype was previously detected in various thermal ecosystems (Chen et al., 2004). The clones most closely related to the Yessentuki TTA-B15 were also detected in anaerobic thermal habitats – in a deep oil field and a thermophilic digester (Table 1). Data on the physiology and genomics of these organisms are not yet available. The only cultured representative of the phylum, the species *Caldisericum exile,* is not closely related to the Yessentuki TTA-B15 phylotypes, whose 16S rRNA genes are more similar (84.0%) to that of the hyperthermophilic organotrophic firmicute *Calditerricola yamamurae* (Table 1).

An order-level uncultured bacterial group Sh765B-AG-111 within the class *Dehalococcoidia* (phylum *Chloroflexi*, 1.6% of the community) was first detected in soil samples from the tailing dump of a uranium mine in the Colorado Basin (https://www.ncbi.nlm.nih.gov/nuccore/26005692). Genomic data and, accordingly, information on the possible type of metabolism of representatives of this group are currently absent. A clone phylogenetically identical (100% identity) to the Yessentuki sequences of the Sh765B-AG-111 group was detected in a core of marine sedimentary rock in an anaerobic environment (Table 1). The closest cultured relative has a rather low similarity of the 16S rRNA gene sequences to the Yessentuki Sh765B-AG-111 members (90.6%; Table 1) and belongs to the species *Dehalogenimonas formicexedens* (Table 1), whose catabolism is based on reductive dehalogenation of chlorinated hydrocarbons with formate or hydrogen as electron donors (Key et al., 2017).

Uncultured acidobacteria of the class *Aminicenantales* (2.3% of the community) were first detected in the Obsidian Pool of Yellowstone National Park as the OP8 phylotype and have been subsequently identified in a variety of terrestrial and marine ecosystems. Based on genomic reconstruction, a representative of this taxon from a deep oil exploration well in the Tomsk region was predicted to play the ecological role of anaerobic degrader of polysaccharides by fermentation or via nitrite respiration (Kadnikov et al., 2019).

The bacteria of the class *Thermodesulfovibrionia,* which were predominant in the Yessentuki no. 17 water, accounted for a significantly smaller (2.3%) share of the microbial community in the well 49-E water and were represented by the species *Thermodesulfovibrio yellowstonii,* a thermophilic organotrophic sulfate reducer (Table 1). Phylotypes closely related to sulfate-reducing bacteria of the genus *Desulfobacca* of the phylum *Desulfobacterota* (1.1% of the community) were also detected.

Clostridia were represented in the water community of well 49-E by a wide variety of rare phylotypes, of which thermophilic anaerobes of the genus *Thermincola* (0.8%) were the most abundant. Representatives of this genus are capable of reducing Fe(III) minerals, as well as carrying out electrogenesis—electron transfer to chemically inert anodes or to solid compounds possessing electronic conductivity (Wrighton et al., 2008). Minerals of transition elements in water-bearing horizons of well 49-E could act as such compounds.

Minor part of the microbial communities in the waters of the studied wells 46 and 49-E was represented by a wide variety of phylotypes, different in metabolic properties of their cultured representatives.

Table 2. Phylogenetic composition of enrichment cultures obtained from water samples of Yessentuki nos. 17 and 4 from wells 46 and 49-E with iron minerals as electron donors or acceptors. Phylogenetic groups with relative abundance of ≥1% are indicated

Substrates	Well 46 water	Well 49-E water
Siderite + CO_2	*Halothiobacillus* (82.1%) *Desulfovibrio* (3.4%) *Aerosphaera* (3.0%) *Comamonas* (1.7%) *Pseudomonas* (1.0%)	*Actinotalea* (93.5%) *Pseudomonas* (1.2%)
Biotite + CH_4	*Halothiobacillus* (99.9%)	*Halothiobacillus* (95.7%) *Actinotalea* (3.8%)
Glauconite + CH_4	*Halothiobacillus* (99.8%)	*Cellulomonas* (95.7%) *Peptostreptococcaceae* uncultured (0.6%)
Magnetite + CH_4	*Halothiobacillus* (99.7%)	*Halothiobacillus* (92.7%) *Actinotalea* (6.6%)

Most of them have one or another type of organotrophic metabolism and can also function as degraders of organic matter in the community. Their geochemical role is uncertain, especially considering their low abundance. Determination of the specific ecological role of scanty representatives of rare biosphere in a particular microbial community is impossible without studying them by cultural methods, which is often hampered by the low concentration of cells of such microorganisms.

Enrichment and pure cultures from waters of well 46 and well 49-E. Using water samples from wells 46 and 49-E as inocula for selective media (see Methods), primary enrichment cultures with iron minerals (glauconite, biotite, siderite, and synthesized magnetite) were obtained. These minerals were selected as the most common electron donors or acceptors that can be used by microorganisms inhabiting deep water-bearing rocks. Siderite, a mineral containing only Fe(II), can only serve as an energy source (electron donor) for microorganisms. All other minerals contain both oxidized (Fe(III)) and reduced (Fe(II)) forms of iron and can be used by microorganisms as high-potential electron acceptors or donors. Taking into account the data obtained on the prevalence of CO_2 and methane in the gas phase of Yessentuki nos. 4 and 17 waters, as well as the data on a significant proportion of potential autotrophs and methanotrophs in the microbial communities of these waters, gaseous CO_2 and CH_4 were selected as an electron acceptor or donor, respectively, and the main source of carbon for the enrichment cultures. The growth of each of the cultures with siderite was accompanied by a change in the color of the mineral from red to light gray; the color of other minerals in the cultures did not change. Methane consumption was not recorded in any of the variants. All cultures were dominated by morphotypes of medium-sized rods associated with mineral particles and often forming dense aggregates (Figs. 1b, 1d). Due to dense colonization of the surface of minerals, it was difficult to determine the number of microorganisms. Approximate number of cells in the cultures with methane was at least 5×10^6 cells/mL, and in cultures with siderite and CO_2, it was an order of magnitude lower, 5×10^5 cells/mL. Addition of vitamins stimulated the growth of all enrichment cultures; therefore, their further transfers were carried out on the media supplemented with vitamin solution. The second transfer of primary enrichments under the same conditions did not lead to a decrease in the number of microbial cells and their morphotypes. For the second transfers of enrichments, phylogenetic composition was determined by high-throughput sequencing of V4 regions of 16S rRNA genes. The results of this analysis are presented in Table 2. The majority of enrichments were predominated by gammaproteobacteria of the genus *Halothiobacillus* (Whitman, 2015). Actinobacteria of the genus *Cellulomonas* prevailed in the culture from well 49-E grown on glauconite with methane (Figs. 1c, 1d), and actinobacteria of the

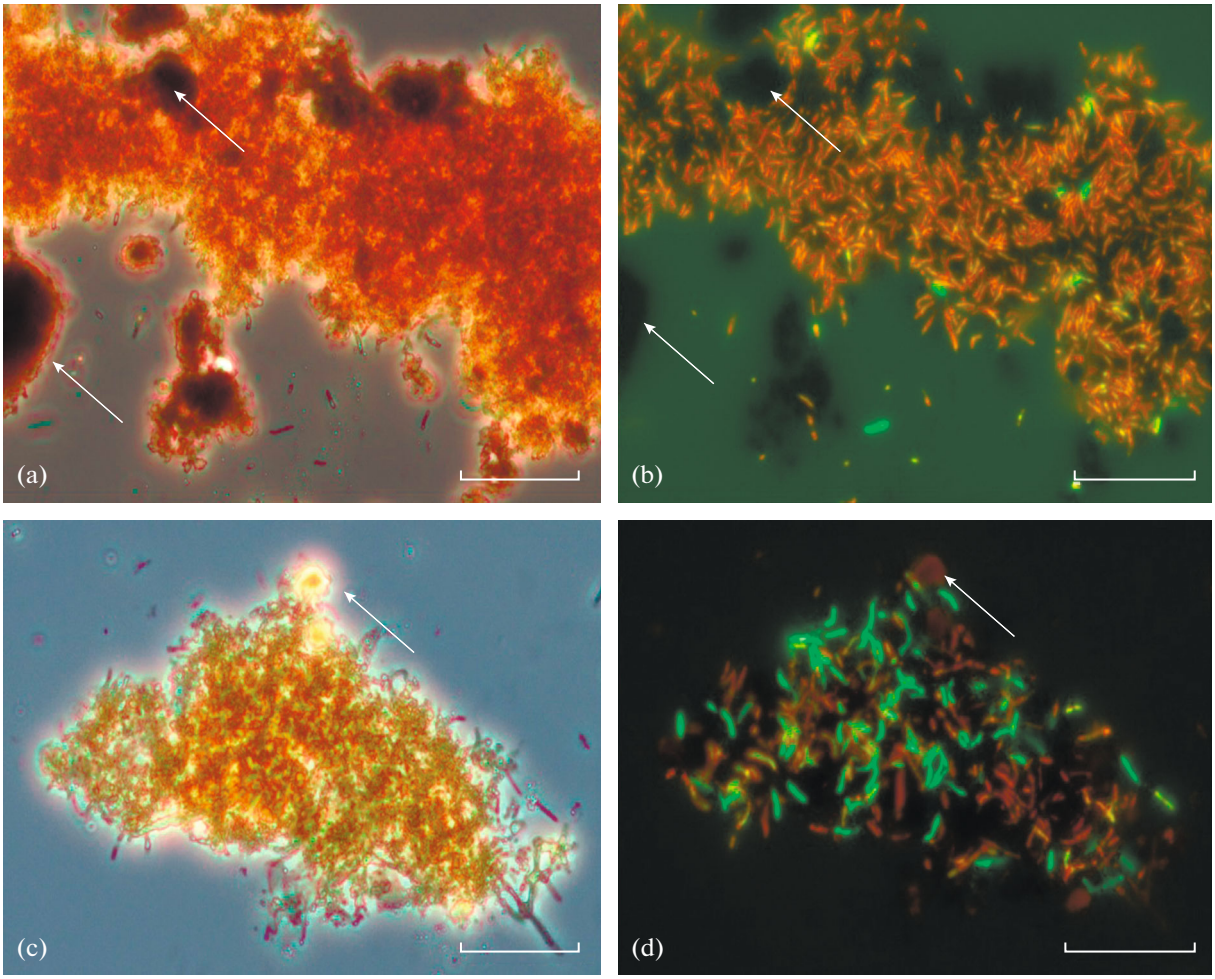

Fig. 1. Photographs of enrichment cultures from well 49-E water (second transfers) grown on magnetite (a, b) and glauconite (c, d) in the presence of methane in the gas phase. On the left (a, c), preparations under a phase contrast light microscope; on the right (b, d), the same preparations stained with acridine orange dye under a fluorescence microscope. The cells form dense aggregates around the mineral particles indicated by arrows. Scale bars: 10 μm.

genus *Actinotalea* prevailed in the culture grown on siderite with CO_2. The second transfers of enrichments from the well 46 water with methane and mixed Fe(II/III) minerals resulted in almost pure cultures containing up to 99.9% of *Halothiobacillus* according to 16S RNA gene profiling data.

Using tenfold dilutions, a representative of *Halothiobacillus* from well 46 water of the Yessentuki no. 17 type was isolated into a pure culture and designated as strain Es46-Z0520. Analysis of complete 16S rRNA gene sequence revealed 99.6% identity of the novel strain to the type strain of the species and genus *Halothiobacillus neapolitanus* (Whitman, 2015). Further experiments revealed the capacity of the isolated strain for aerobic growth with thiosulfate as an electron donor, which makes it possible to assign it to facultative anaerobes.

Relative abundance of gammaproteobacteria of this genus in the water community of well 46 did not exceed 0.3%. The type strain of the species *H. neapolitanus* is a chemolithoautotroph that oxidizes sulfur under aerobic conditions and fixes CO_2 through the Calvin cycle. This strain serves as a model organism for studying the structure and functions of carboxysomes containing the key enzyme of the Calvin cycle, ribulose bisphosphate carboxylase (Menon et al., 2008). The ecological role of this minor component of microbial communities in the waters of the Yessentuki no. 17 and no. 4 types requires further study. It is possible that the number of bacteria of this strain and its importance in the community as a producer of organic matter increases sharply in the case of occasional aerated fresh water admixing to anoxic mineral waters.

Thus, the analysis of microbial communities of mineral waters from wells 46 and 49-E revealed in both of them the predominance of uncultured microorganisms of deep phylogenetic lineages (levels of phyla, classes, or orders), which apparently evolved separately from the surface biosphere and may represent

relicts of the Upper Cretaceous microflora. Interestingly, the most closely related clones of most of the phylotypes identified in our study were detected in thermal aquatic ecosystems or in anaerobic ecotopes confined to deposits of various hydrocarbons (Table 1). In both studied microbial communities, numerous potential chemosynthetic microorganisms capable of fixing CO_2 into biomass, methane and/or acetate were revealed, most of which have not yet been isolated into pure cultures. Such organisms form secluded communities that are almost completely isolated from surface ecosystems of the Earth having no direct trophic links with their photosynthetic biomass production. Analysis of the microbial community of well 46 water revealed a structure typical of the classical food pyramid. It was dominated by potentially autotrophic microorganisms (such as *Hadarchaeota*), while organotrophic organisms that depend on the primary production of biomass and specialize in the consumption of its various components (for example, archaea of the class *Thermoplasmata,* nitrospira of the class *Thermodesulfovibrionia,* or *Firmicutes* of the D8A-2 group) were less represented, albeit more diverse (Table 1, Fig. 2a). The microbial community of well 49-E water was characterized by a different structure, namely, a significantly greater phylogenetic diversity; a significant share of prokaryotes with an unidentified phylogenetic position (5.4% of the community, Table 1); lower share of potential autotrophs; a wide variety of organotrophs potentially capable of degrading complex organic substances, including buried organic matter (polysaccharides, proteins, hydrocarbons); and prevalence of cultured hydrogenotrophic methanogens among gas-utilizing organisms (Fig. 2b). One of the most probable reasons for these differences is the instability of the temperature regime of well 49-E (Potapov, 2017), which, in turn, may be associated with the mixing of fresh sodium hydrocarbonate waters with mineral waters at the intake interval of well 49-E (at depths of 580–865 m). This results in the absence of the classical ratio of auto- and heterotrophic microorganisms in the well 49-E water community, which is characteristic of the balanced microbial community of the well 46 water.

An important common feature of the microbial communities of the studied subsurface mineral waters was the predominance of gas-utilizing microorganisms, primarily methanotrophs and methanogens, which produce and consume CO_2, respectively. This observation is in good agreement with the predominance of carbon dioxide in the gas phase of mineral waters, as well as with their significant (tens of percent) methane content. Thus, the characterized microbial communities can influence the gas content of exposed mineral waters and, consequently, their balneological value. The metabolic activity of the dominant groups of microorganisms in these communities may be one of the reasons for the observed fluctuations in the content of dissolved and free carbon dioxide, as well as the hydrocarbonate ion concentration, in the mineral waters from wells 46 and 49-E. The high abundance of anaerobic methanotrophs in the waters of well 46 and a wide variety of anaerobically respiring organotrophs in the waters of well 49-E imply the formation of various syntrophic associations in the microbial communities of these waters, which increase the thermodynamic efficiency of the oxidation of methane or organic matter in an anaerobic reductive environment. As a rule, such syntrophic associations appear in biofilms overgrowing solid surfaces. Under the conditions of water-bearing rocks, biofilms may form on the surface of minerals of transition elements, primarily sulfur and iron, which can be used as donors or acceptors for interspecies electron transfer. With a high metabolic activity, syntrophic associations will have a significant effect on the composition and mobility of iron and sulfur compounds in water-bearing sediments exposed by wells 46 and 49-E of the Novoblagodarnensky area of the Yessentukskoye deposit. This effect can indirectly be confirmed by the increased content of iron in the waters discharged by wells 46 and 49-E, which, in turn, may reflect the increased content of this element in water-bearing rocks and its active involvement in biogeochemical cycles. This hypothesis correlates with the detection of various taxa of microorganisms, shown to be capable of dissimilatory reduction of Fe(III) or sulfur compounds, in the waters exposed by wells 46 and 49-E

Predominance of uncultured microorganisms of novel deep phylogenetic lineages in both microbial communities and the probable independence of these communities from the surface biosphere, afforded by the chemosynthetic biomass production, indicate the need for further detailed study of the functioning of subsurface ecosystems of water-bearing rocks of the Yessentuki mineral water deposit and the CMW region as a whole.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. This article does not contain any studies involving animals or human participants performed by any of the authors.

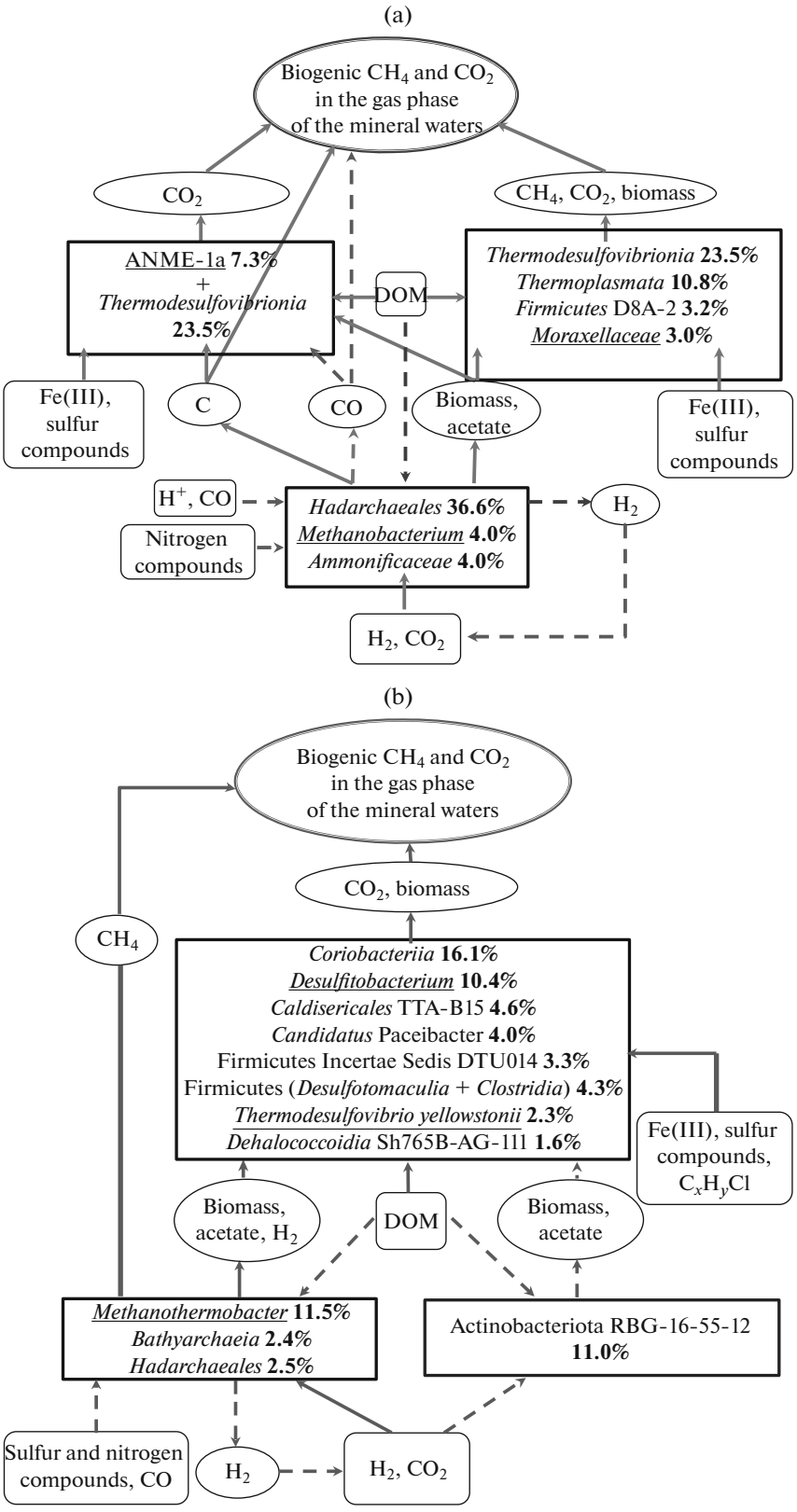

Fig. 2. Putative trophic relationships in microbial communities of waters and water-bearing rocks exposed by wells 46 (a) and 49-E (b). The dotted line indicates the relationships predicted with low confidence due to the lack of genomic data on the microorganisms involved. Underlined are the names of the taxa with available information on physiological properties or their genomic determinants. DOM stands for dissolved organic matter; C_xH_yCl, for chlorinated hydrocarbons, which may be present in the investigated waters.

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