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Experimental equipment for Lake Baikal deep biosphere microorganism's exploration and some results obtained using this equipment

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ABSTRACT. The development of special autoclaves with automatic temperature control (Nikolaev Institute of Inorganic Chemistry) had enabled reproduction of Lake Baikal tectonically active zone typical conditions (80°C, 5 MPa). This article describes in details the equipment developed and demonstrates some results obtained using the equipment. In series of thermobaric experiments on the cultivation of bottom sediment microbial communities, we have determined the microbial community potential to transform organic matter via the formation of dibenzothiophenes, tri- and monoaromatic steroids as well as petroleum biomarkers (retene and gammacerene). The particular bottom sediment geochemical environment associated with hydrocarbon discharge imply the composition of microbial communities and, hence, the organic matter transformation degree as well as a compound spectrum resulting from the destruction of organic matter under thermobaric conditions. The presence of microorganisms with an unusual metabolism suggests the promising potential for such studies both in application to Lake Baikal and worldwide

Keywords: special autoclaves, thermobaric experiments, microbial communities, transformation of organic matter, bottom sediments, Lake Baikal

1. Introduction

One of the modern microbiology and ecology relevant areas is the investigation of individual species or entire communities capable to function in "extreme" environments under conditions far from the "standard" temperature 4°C to 40°C, pH in the range 5–8.5 and water salinity of over 37 g kg^{-1} (Kristjánsson and Hreggvidsson, 1995; Bartlett and Bidle, 1999; Mouser et al., 2016). These environments include bottom sediments of marine and freshwater bodies that are the Earth's largest organic carbon reservoir and unique ecological niches rich in uncultivated, just discovered or poorly studied yet microorganisms (Hedges and Keil, 1995; Tranvik et al., 2009; Teske at al., 2013). Investigation of composition, structure and metabolic capabilities of microbial communities inhabiting bottom sediments with different physicochemical conditions is an essential step towards understanding the biochemical processes and the biosphere evolution mechanisms (Frank et al., 2016; Hug et al., 2016; Drake et al., 2017; Kadnikov et al., 2017; Jones et al., 2018). The specific characteristics of deep bottom sediments as the habitat of microbial communities are: a lack of oxygen, low temperatures, high hydrostatic pressure, a deficiency of electron donors and acceptors as well as a shortage in easily accessible organic carbon sources due to active destruction of organic matter in the water column and surface sediment layer (Parkes et al., 2014). Recent studies have shown that these extreme conditions lead to a rapid decrease in number of microorganisms with sediment depth (Kallmeyer et al., 2012). However, in the areas with geological anomalies where bottom sediments have high concentrations of organic matter and/or inorganic electron donors and acceptors the density and activity of microbial populations are much higher (Parkes et al., 2014). These anomalies include

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fields of hydrothermal sources, discharge zones of oil- and gas-saturated fluids, mud volcanoes, gas-hydrate deposits, buried layers of sapropels, etc. (Parkes et al., 2000; Bonch-Osmolovskaya et al., 2003; Horsfield et al., 2006; Bennett et al., 2013; Anderson et al., 2014; Ruff et al., 2015). In these areas, additional sources of carbon and energy are available with gases and fluids inflow from the basalt layer of the Earth's crust or as products of buried organic matter thermal activation and degradation in the deep sediment layers (Cowen et al., 2003; Parkes et al., 2005; Horsfield et al., 2006; Parkes et al., 2007; Engelen et al., 2008; Boetius and Wenzhöfer, 2013). In addition to nutrition for growth, ascending fluid flows can carry representatives of deep thermophilic microbial communities to the surface layers of bottom sediments (Hubert et al., 2009). Metabolic capabilities of these microorganisms, as well as their role in biogeochemical cycles, are currently the objects of intensive research (Orcutt et al., 2011; Biddle et al., 2012; Edwards et al., 2012; Parkes et al., 2014).

Baikal, the world's deepest lake 25 – 30 million years old, is one of the promising sites for studying microbial communities associated with geological anomalies (Biddle et al., 2012). Location of Baikal in the tectonically active zone contributes to the formation of underwater mud volcanoes, methane seeps and gas-hydrate deposits in the lake (Kuzmin et al., 1998; Kontorovich et al., 2007; Khlystov et al., 2013). In some areas, oil and gas migrate to the surface of the sediment layer from depths of approximately 7 km (Khlystov et al., 2007; Kontorovich et al., 2007) with abnormally high concentrations of some ions in pore waters as well as thermogenic methane and ethane (Kalmychkov et al., 2006; Granina, 2008; Kalmychkov et al., 2017).

To better understand the life of prokaryotes in the deep biosphere, the characteristics of the environment that microorganisms inhabit are registered and reproduced during the cultivation carried out in the special installations. For ex situ studies of microbial communities from deep bottom sediments of Lake Baikal, researchers from Nikolaev Institute of Inorganic Chemistry SB RAS have developed and assembled special autoclaves with automatic temperature control allowing to reproduce conditions typical for tectonically active zone of Lake Baikal.

2. Materials and methods

Bottom sediment samples from Lake Baikal were cultivated in special autoclaves (Fig. 1). A set of autoclaves and furnaces was designed to perform long-term experiments on the cultivation of microorganisms under conditions of protocatagenesis, i.e. at pressures up to 20 MPa and temperatures up to +150 ºC (Fig. 1, Fig. 2).

Sample (1) was placed into a glass beaker usually 50 mm in internal diameter and 70 mm in height with a Teflon lid (2). For free gas income and release conical cuts were made in the upper part of the jar. These cuts were covered from the outside with lid protrusion, reliably protecting sample from the ingress of any

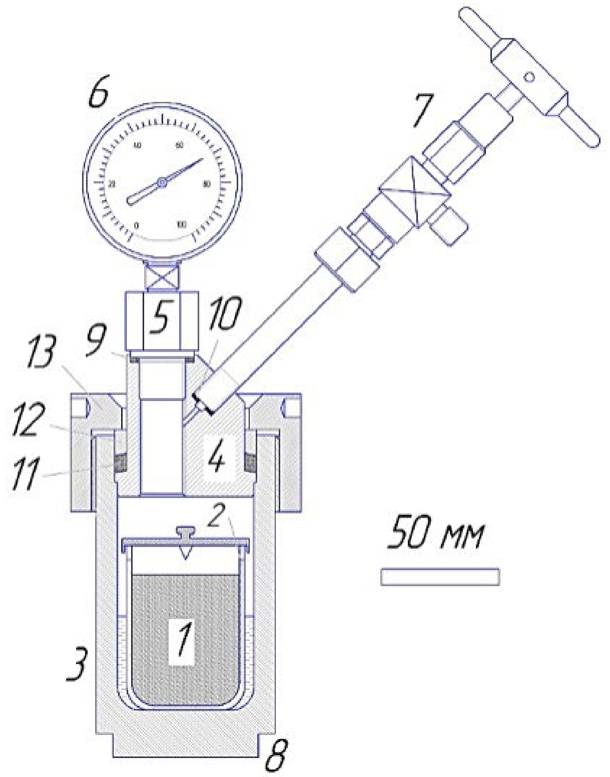

Fig.1. Scheme of an autoclave with a loaded sample

solid particles. A beaker with a sample was placed in the autoclave body (3). To prevent water evaporation from the sample during the experiment by producing saturated water vapour conditions, 10–5 ml of water sampled from Lake Baikal were poured between the outer walls of the beaker and the autoclave body. The autoclave body (3) is made of hardened 40 × 13 steel. It was shaped as a thick-walled cylindrical jar with the external M80 × 2 thread in the upper part. The autoclave internal space was comprised of two cylindrical parts. An obturator (4) with a Bourdon tube pressure gauge (6) connected with membrane separator (5) as well as shutoff valve (7) was inserted into the upper part of the body. This part polished cylindrical surface was used for sealing. The lower cylindrical part 58 mm in diameter and 90 mm in height provided a utility volume of the autoclave. The lower part of the autoclave had two notches (8) to fix it tight in a bench during assembly and disassembly of the appartatus. The obturator (4) was made of 40 ×13 steel as depicted in Fig. 1. Obturator had two sockets to accommodate a membrane separator (5) and a shutoff needle valve (7). A Bourdon tube pressure gauge (6) was screwed into the membrane separator (5). A membrane separator was installed to prevent water condensation in the internal volume of the pressure gauge (6). In our experiments we used RMM membrane separators and pressure gauges manufactured by YuMAS R&D production facility and shutoff valves manufactured by the SB RAS Experimental Plant (Novosibirsk) with a capacity of up to 40 MPa. All the structural elements of these units that faced internal workspace were made of stainless steel (12 × 18H10T, Russian analogue of steel 321), seals were made of Teflon. The socket for attaching the RMM membrane separator had a M20 ×1.5 thread; Teflon seal (9) was located in the upper part of the socket and was oppressed by the cylindrical part of the membrane separator. The socket for shutoff valve attachment had a M12 ×1.5 thread, and it's design was the same as of the socket for pressure gauges attachment with an appropriate fitting. The connection of the obturator (4) to the valve (7) was also tightened by a Teflon gasket (10). The outer part of the obturator (4) had a shape of an inverted mushroom. The "cap" of the mushroom was hooked on the protrusion (3) in the body channel; the part adjacent to the "cap" was polished. A cylindrical "stem" of the mushroom served for sealing. A five mm wide and 5-6 mm high silicone rubber ring (11) on the "stem" was used for sealing. The rubber ring was opressed by a bushing held tightened by enforced screw (11) matching the external M80 × 2 thread of the body (3). This screw was in fact a load-bearing element that fixed obturator (4) in the body (3). To reduce the probability of jamming the large diameter thread, the screw (13) was made of CuAl10Fe1 bronze. Obturator (4) and bushings (12) surfaces adjacent to the rubber ring were bevelled by 10° for better oppressing of the rubber. During the screw (13) tightening, the rubber ring is being squashed and flatten against the polished surfaces of the body (3) and obturator (4), resulting in primary compression in the autoclave internal volume. While the pressure builds inside the autoclave the squashing force applied to the rubber ring (11) is growing as well as the flattening of the rubber against the polished surfaces, i.e. the construction is presumably self-sealing. To reduce the rubber flowing into the gaps between the bushing (12), body (3) and obturator (4), a two mm thick tightly fitted to the polished surfaces of the body and obturator Teflon ring was designed to be inserted between the rubber ring (11) and bushing (12) (not shown in Fig. 1). The described autoclave proved to be able to withstand pressures of up to 20 MPa and temperatures of up to 150°C for a long time.

Furnaces shown in Fig. 2 were used for long-term heating of autoclaves. The main block of the construction (14) was made of aluminium alloy and had a socket to insert the assembled autoclave into. To improve heat transfer, the autoclave body (3) was inserted into block socket (14) with a gap of 0.2 – 0.3 mm, while the gap between the screw (13) and the block walls had to be at least 1 mm. The cover (15) and the bottom of the furnace (16) were made of textolite. They clamp together the main block (14) and the whole construction was assembled by a shell (17). The space between the shell (17) and the block (14) was filled by mineral wool (18) for thermal insulation. To reduce the heat transfer between the block (14) and parts (15) and (16), the contact surface between them had 4 mm wide circular grooves.

Four channels were drilled in the block (only one is shown). Three channels contained cartridge heaters 100 W of power each, and the fourth channel contains a copper resistance thermometer sensor (50 Ohms) (19). Power cables and wires from the sensor were placed in an annular groove (20) in the upper part of the block (14) and were brought out through the textolite cover (15). The wires from the temperature sensor were shielded. The autoclave was covered with a compound textolite cover (21) for an additional thermal insulation. The Termodat 10K4 temperature controller with thyristor power units (manufactured by Scientific and Production Enterprise Sistemy Kontrolya LLC) (22) controlled the furnace. Since a long-term (a year or more) continuous operation of the furnace was envisaged in the absence of continuous supervision, the additional safety measures were taken. The power of heaters (19) and the method of their connection (sequential) were selected in such a way that even in the event of a temperature controller failure and an unregulated voltage of 220 V application to heaters the temperature in the furnace would not rise above 110–120°C.

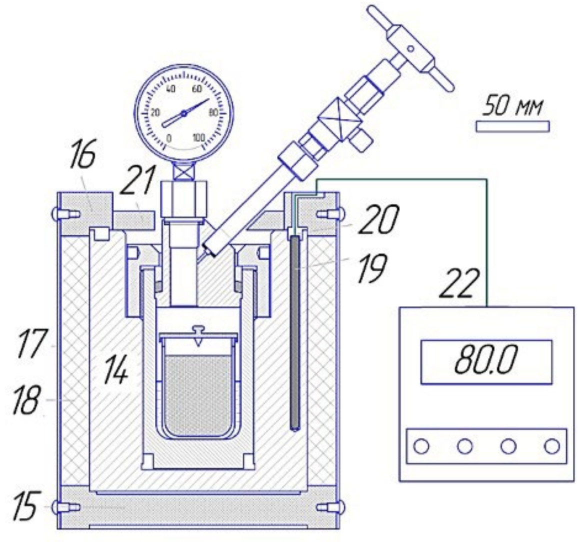

Fig.2. A heating furnace with the autoclave inside it. See text for the explanations

The standard experiment conducting procedure for the installation included loading of the sample into the autoclave, assembling the autoclave, flushing working fluid, setting the required working fluid pressure, heating the autoclave to the set designated temperature, and adjusting the pressure (if necessary). The working fluid was flushed by repetition of filling the autoclave with gas (~1 MPa) and discharging gas into the atmosphere. Depending on the permissible residual amount of air in the autoclave, the flushing is carried out three to six times.

Biomass of diatom *Synedra acus*, which is among the dominant species of phytoplankton in Lake Baikal (Grachev et al., 1998), was added as a complementary organic matter to the samples of bottom sediments from Lake Baikal taken for the experiments (methane seeps Goloustnoye and Posolsk Bank as well as mud volcano Khoboy). Department of Cell Ultrastructure at Limnological Institute SB RAS provided the axenic culture of this diatom (Shishlyannikov et al., 2011). The composition and distribution of hydrocarbons from the maltene part in the preliminarily obtained chloroform extract from samples of bottom sediments before (including the additionally introduced organic substrates) and at the end of the experiment were analysed by chromatography-mass spectrometry method according to (Kashirtsev et al., 2001).

To access changes in the structure and composition of microbial communities from samples of original sediments and sediments after cultivation, total DNA were extracted by enzymatic lysis followed by phenol-chloroform extraction (Sambrook et al., 1989). Loci subject to analysis were amplified by polymerase chain reaction (PCR) and subsequently used for massive parallel sequencing libraries preparation and analysis on Illumina MiSeq platform (SB RAS Genomics Core Facility, Novosibirsk). Massive parallel sequencing and phylogenetic analysis were performed as described previously (Bukin et al., 2016).

3. Results and discussion

We used autoclaves in several model experiments on cultivation of natural microbial communities from bottom sediments of Lake Baikal with different geochemical conditions: methane seeps Goloustnoye and Posolsk Bank as well as mud volcano Khoboy, which feature different temperatures, pressures and carbon and energy sources. We have shown the ability of some taxa from Lake Baikal deep bottom sediment layers to survive under conditions typical for protocatagenesis, as well as in typical involvement known for these taxa in organic matter transformation locations. The metagenomic analysis of DNA samples before and after the experiments allowed us to characterize the dominant and minor components of microbial communities and identify the putative main participants in the observed processes inhabiting deep bottom sediments of Lake Baikal (Bukin et al., 2016; Pavlova et al., 2016; Pavlova et al., 2019a). We have noticed that different geochemical conditions of bottom sediments associated with hydrocarbon discharge determine different compositions of microbial communities and, hence, the transformation degree of organic matter as well as spectrum of compounds resulting from the destruction of organic matter under thermobaric conditions. Samples of mud volcano after cultivation were dominated by representatives of thermophilic taxa (*Deinococcus-Thermus, Firmicutes*, etc.), which could be transferred to the surface sediments with gas-saturated material from depths of several kilometres (Pavlova et al., 2019a). In cultivated samples of bottom sediments from methane seeps characterized by presence of a fluid convection loop we detected typical mesophilic inhabitants of bottom sediments with a “flexible” metabolism (Bukin et al., 2016; Pavlova et al., 2016). This composition probably allows adapting to the changes in environmental conditions during the burial of individual bottom sediment layers and the circulation of fluid flows. The lack of changes in composition of organic matter, as well as in cells of microorganisms and 16S rRNA sequences of the *Bacteria* and *Archaea* members in control (sterile) sediment samples confirmed the destruction of organic matter under experimental conditions to be attributed to the activity of microorganisms.

The experiments have shown that long-term cultivation of microbial communities from bottom sediments of methane seeps Goloustnoye and Posolsk Bank, which were enriched with detritus of the Baikal diatom *Synedra acus* and CH_4:H_2:CO_2 gas mixture, leads to the destruction of algal biomass and formation of petroleum biomarkers, such as retene or gammacerene (Bukin et al., 2016; Pavlova et al., 2016). These data indicate the involvement of microorganisms in the formation of retene during the destruction of diatoms under milder than pyrolysis conditions. Previously the involvement of anaerobic microorganisms in the formation of retene was suggested (Martin et al., 1999), but there was no experimental evidence. Considering that retene is used as a biomarker of conifers for the interpretation of the recent past geochemical changes, the obtained data could be applied for more correct interpretations of a more distant past geochemical processes.

The recorded changes in organic matter composition for bottom sediments of mud volcano Khoboy sample were not as significant (16%) as changes registered for methane seep Posolsk Bank bottom sediments sample (41%) (Bukin et al., 2016). However, even after small cultivation time (seven months) sediment sample from the mud volcano Khoboy displayed a decrease in concentration of phenanthrenes relative to methyl-substituted homologues, including retene, and an increase in concentration of dibenzothiophenes relative to normal alkanes. We identified also tri- and monoaromatic steroids, including 17-dismethyl-23-methylmonoaromatic steroids C_{27} (Pavlova et al., 2019a). An increase in concentrations of tri- and monoaromatic steroids in autoclaved sediments may indicate that biomass of Baikal diatom *S. acus* was in fact destructed, which led to an increase in concentration of steroids known to be produced by diatoms and associated with them (Volkman, 1986; Ponomarenko et al., 2004; Kodner et al., 2008; Kalinovsky et al., 2010). The dominance of the S isomers over R isomers in homohopanes, as well as the presence of biohopanes in trace concentrations, also indicate an increased organic matter transformation level after cultivation.

In the process of microbial communities from Lake Baikal fault zones cultivation, we have isolated several pure cultures of thermophilic bacteria with unusual for their species metabolism. Unlike cold marine sediments where endospores of thermophiles belong to obligate anaerobes, mantaining their metabolism via fermentation of organic substrates or sulphate reduction (Hubert et al., 2010; Müller et al., 2014; Chakraborty et al., 2018), in Baikal sediments associated with seepages of gas-saturated fluids we found facultatively anaerobic thermophilic prokaryotes of the genera *Paracoccus*, *Geobacillus* and *Thermaerobacter* (Pavlova et al., 2016; Khanaeva et al., 2017; Pavlova et al., 2019b). Genomic studies confirmed the potential of *Thermaerobacter* PB12/4term pure culture for mixotrophic type of nutrition, which is uncharacteristic for the members of genus *Thermaerobacter* as obligate aerobes (Baturina et al., 2018). The *Thermaerobacter* PB12/4term contains genes that encode [NiFe]hydrogenase, formate dehydrogenase, enzymes catalysing assimilatory nitrate and sulphate reduction, as well as an incomplete set of the denitrification enzymes, by which microorganism can sequentially reduce nitrate to nitrogen dioxide. The final pure cultures attributed to the genera *Geobacillus* and *Thermaerobacter* were deposited in All-Russian Collection of Microorganisms (VKM B-3150; VKM B-3151).

The presence of thermophiles in the surface Lake Baikal psychrophilic sediments is consistent with the data (Klerkx et al., 2003) on the role of gas-saturated fluids, which bring from deep sediment layers to the surface layers not only diatoms but also viable microorganisms that can develop and function at a temperature of +70°C or more.

Conclusions

Unique characteristics of Lake Baikal open for researchers new perspectives into the not yet fully explored world of the deep biosphere. Our study has demonstrated that the developed experimental equipment is a simple and effective tool to recreate the deep biosphere conditions in model experiments. Using this equipment we have shown that thermophiles from the deep biosphere enter the surface sediments together with gas-saturated fluids and mud-volcanic breccia. The thermobaric experiments have indicated that hydrocarbon molecular markers (n-alkanes, isoprenoids, terpanes, and polycyclic aromatic hydrocarbons) presence in the bottom sediments (Morgunova et al., 2018) can be a result of organic matter transformation with the participation of thermophilic microbial communities. The presence of microorganisms with an unusual type of metabolism suggests that these studies will be in demand for a long time both for Lake Baikal and worldwide.

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