BIOGEOCHEMICAL INVESTIGATIONS OF THE SPELEOTHEM MOONMILK IN THE KARST PROSCHALNAYA CAVE (FAR EAST, RUSSIA)

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Abstract

Results of investigations of natural waters (drip and fracture) and speleothem moonmilk from the karst Proschalnaya Cave (Russia, Far East) are reported. Concentrations of Fe and Mn in drip water were highest in spring, while the concentration of Mn was lowest in the fracture water, which may be due to the nature of infiltration of water through different channels after spring snowmelt and autumn rains. Molecular genetics investigation of the moonmilk mass revealed the presence of iron bacteria of the genera Rhodoferax and Geothrix. The visually plastic and homogeneous mass of moonmilk was shown to be highly heterogeneous, containing various microstructures. Tubular microstructures had a richer elemental composition (C, O, Ca, Fe, Mn, Si, Al, and S), in comparison with claviform formations (C, O, Ca, and Na). Binding matrix in the composition of moonmilk is represented by reticular structures similar to nanofibers. The results of this research conducted in a monsoon climate may be interesting for speleologists working with karst caves in other climatic conditions.

Introduction

Microbes present in the specific habitats of aquifers and pore space of rocks play an important role in the processes occurring in the water-rock contact zone (Perry et al., 2004). Various organic compounds and microorganisms that are capable of colonizing the surface of rocks enter karst caves from the ground biotopes with infiltration waters (Chelius et al., 2009). However, the cave microbial composition varies by the types and configuration of caves (Barton et al., 2004; Velikonja et al., 2014) and depends on the sampling location (Ghosh et al., 2017).

As the results of analysis of microbial communities sampled from the walls of caves located in Spain, Czech Republic, and Slovenia, Porca et al., (2012) proposed the hypothesis that the colonization of caves with microorganisms occurred through water infiltration from the overlying rock and soil. The heterogeneity and main mechanism of microbial diversity in caves are well-connected with surface environments (Wu et al., 2015). Microbial exopolysaccharides, alginate acids, siderophores, and other chelating compounds act as important factors determining the colonization and dissolution rate of mineral rocks (Perry et al., 2004; Ercole et al., 2007; Kuhn et al., 2014).

Alternatively, microbial cells have been shown to act as centers for precipitation and crystallization of many elements (Barton and Northup, 2007). Microorganisms are also capable of altering the mineral composition and solubility of carbonates, as well as crystal size and morphology, as demonstrated by the large, poorly soluble CaCO₃ crystals formed in the presence of Bacillus pasteurii (Mitchell and Ferris, 2006).

Dissolution of carbonate minerals and morphogenesis of karst cavities may be partially explained by bacterial activity (Hill and Forti, 2007). Infiltration, flood water, and airflow also introduce microbes into caves, where they can begin to influence the structure of the microbial community of caves. As weather parameters and water conditions change, the introduced microbial pool may also change strongly. As in surface environments, microorganisms act as active and passive promoters of redox reactions in the sedimentary processes in caves (dissolution, redeposition, secondary formation of minerals with participation of microorganisms) (Fornós et al., 2014).

The investigation of karst caves is presently carried out in several fields, including speleology, geology, and ecology. Research varies from large-scale analyses of landforms and processes involved in the formation of karst landscapes, to speleothems, including stalactites and stalagmites, to microscopic investigation of sinter formations. Among speleothems, moonmilk is a formation of high interest (Borsato et al., 2000; Cacchio et al., 2014). Moonmilk, one of the most common types of carbonate deposits (speleothems) formed in caves, has long been known as a habitat for microorganisms that are thought to be responsible for the origin of these commonly white and soft secondary calcite deposits (Reitschuler et al., 2016). Various forms of moonmilk deposition have been described, including encrustations, films, thick layers, deposits, and veins in clay. The metabolic activity of complex microbial communities can play an important role in the formation of moonmilk (Portillo and Gonzales, 2011).

The presence of microorganisms in moonmilk formations has been observed in caves around the world, from the tropics to high latitudes. It has been found that microbes participate in formation of the white and soft secondary calcite (calcium carbonate) deposits that can coat the walls, floors, and ceilings of caves. In this biologically-driven process,
upper surface layers are actively formed, while the deeper and older parts become progressively dehydrated, encrusted, and inactive (Canaveras et al., 2006).

Moonmilk is primarily water by mass (60–90 %). In this geochemical environment, microbial cells can act as centers of precipitation and crystallization for many elements (Barton and Northup, 2007). As 90 % of Earth’s biomass resides in the subsurface, and many of those environments are exposed to constantly cold conditions (below 5 °C), basic research on exotic habitats such as moonmilk through cultivation of microorganisms and geochemical analyses is important for understanding potentially widespread processes (Rodrigues and Tiedje, 2008). Low-temperature biotopes are successfully colonized by cold-adapted organisms, which include a large range of representatives from all three domains: Bacteria, Archaea, and Eukarya. As a result, psychrophiles are the most abundant in terms of biomass, diversity, and distribution (Struvay and Feller, 2012). The ability of psychrophilic microorganisms to grow at temperatures below 5 °C can be associated with their successful adaptation to the natural habitat. It is known that the microbial activity of psychrophiles and growth yield at low temperatures is higher than the growth rate at what is normally considered the optimal growth temperature (Margesin, 2009).

Microorganisms utilize several metabolic strategies to survive in the cave environment, such as synthesis of new organic matter from inorganic carbon (chemolithoautotrophy) and decomposition of organic matter (heterotrophy) (Chen et al., 2009). These processes, or its byproducts, can play a role in the transformation of rock through dissolution or formation of minerals (Lefevre et al., 2016).

Based on past studies conducted in the Snezhnaya Cave (Abkhazia) (Kondratyeva et al., 2016), it was hypothesized that the elemental composition of groundwater and the structure of microbial communities play a key role in determining the elemental composition of the moonmilk. Our research is devoted to the study of the elemental composition of groundwater and moonmilk, as well as the activity of microorganisms in the Proschalnaya Cave (Far East, Russia).

Investigation of moonmilk from Proschalnaya Cave was conducted in two stages: (1) microbiological research (molecular genetic techniques, isolation of cultured bacterial strains, determination of their physiological and biochemical activity) and (2) the analysis of nanostructures in the moonmilk mass by scanning electron microscopy with determination of their elemental composition.

The main objective of our research was to determine environmental factors that characterized peculiarities of biofilm from moonmilk in a large karst cave on the Far East of Russia. For the first time interdisciplinary studies including physicochemical, microbiological, molecular genetic methods, and scanning electron microscopy of moonmilk from the Proschalnaya Cave were conducted. The results of research on biofilm and rock interactions in a monsoonal climate can be interesting for speleologists working with karst caves in other climatic conditions.

Materials and Methods

Sampling site

Proschalnaya Cave is on the eastern slope of the Sagdi-Selanka River valley (Amur River Basin) in the Khabarovsk region, Far East, Russia (47°18’32.7ʺ N; 136°29’56.3ʺ E) (Fig. 1). The climate of this region is moderately continental with signs of monsoonal: humid summers with frequent rains and winter with little snow. A monsoonal climate is characterized by a sharp contrast in the amount of precipitation over the year seasons and stability of the wind direction for one season with a sharp wind variation in the opposite direction during changing seasons. The cave is remote from settlements, not visited by tourists, and accessible for speleologists only. The cave does not have access for animals due to the complex labyrinths and deep depth.

The cave is a labyrinth with a total length of approximately 6 km, multiple levels, and a large number of halls, galleries, and grottos. There is a watercourse and many sources of drip and fracture water inside the cave; the walls and ceilings are covered with various speleothems, including moonmilk (Fig. 2). The chemical composition of the karst waters of the Russian Far East are primarily hydrocarbonate-calcium, and more rarely, chloride-hydrocarbonate-calcium with an average degree of mineralization (5–15 g/L) (Bersenyov, 1989). Surface waters of the Sagdi-Selanka River and groundwaters of Proschalnaya Cave are characterized by an increased content of Ca ions (82–86 % mg-Eq) and a very low concentration of Mg ions (9–12 % mg-Eq) (Shesterkin, 1983). Hydrochemical studies of natural waters in the Proschalnaya Cave have not been carried out in recent years.

Sampling characterization

In May 2015, 2016, and 2017 and in November 2015, water samples of different origins were taken from the cave (watercourse, drip, and fracture water) and from the Sagdi-Selanka River (surface water) according to the standards of sampling in hydrochemistry and microbiology (Gerhardt, 1983; Kuznetsov and Dubinina, 1989). In the study area, the average amount of precipitation during the month was: 105 mm in May 2015; 136 mm in May 2016, and 48 mm in May 2017. In November 2015, precipitation was minimum, 9.9 mm. In the cave air temperature was 1–4 °C.
In the Marble room, samples of moonmilk deposits of different consistencies were aseptically collected in sterile tubes: M1—thin slimy white mass and M2—thick curdy ivory-white mass were taken from the walls; M3—dry white mass was taken from the surface of broken rocks. Samples were transported to the laboratory in coolers at 4 °C.

Determination of the elemental composition in samples of natural waters and moonmilk was carried out with use of the Total Quant ICP-MS method, PerkinElmer (USA), in accordance with standard methods (Federation Regulation, 2011).

**Microbial studies**

For the inoculum, 100 mg of wet mass of moonmilk was dispersed in 10 mL of sterile physiological saline; dilution was performed in 100-fold and 0.1 mL of suspension was used for spread-plating on the agar culture media. The abundance of heterotrophic and Fe-metabolizing cultivated bacteria (CFU/mL) in natural waters and in moonmilk was determined on the 7th day after cultivation on solid nutrient media with use of spread plates at 23 °C: SAA (starch ammonium agar) (Gerhardt, 1983); FPA (fish peptone agar) and FPA diluted 10 times (Kuznetsov and Dubinina, 1989); and Vinogradsky medium (Egorov, 1995). For the cultivation of Fe-metabolizing bacteria, Bromfield agar medium was used (Namsaraev et al., 2006).

Diagnostic system (SPA Microgen, Moscow, Russia) with color indicators and various carbon sources (carbohydrates, polyhydric alcohols, and amino acids) was used to determine the nutritional range of strains isolated from different types of water. The growth activity of the strains was evaluated by the color change of the dissolved substrate on the 7th day after cultivation at 23 °C. Amylase activity was determined on SAA after treating the colonies with Lugol’s solution according to the diameter of the starch hydrolysis zones.

**Figure. 1.** Geographical location of the Proschalnaya Cave in the Amur River Basin, Khabarovsk region (Far East, Russia).

**Figure. 2.** Proschalnaya Cave (Russia): (1) Entrance to the cave, (2) Gallery in the Albatross system, (3) moonmilk on the wall (M2 — thick curdy ivory-white mass).
qPCR analysis

Microbial investigation of moonmilk samples (M1 and M2) were carried out with use of quantitative PCR (qPCR) analysis according to standard procedures (Kubista et al., 2006). DNA was extracted using a GeneMATRIX Soil DNA Purification Kit (Roboklon, Berlin, Germany). The total number of eubacterial DNA copies and the DNA copies of bacteria of the genera *Rhodoferax* and *Geothrix* were determined with use of specialized primers which were offered by Prof. U. Szewzyk (Technische Universität Berlin): Eubacteria (Uni338F_RC ACT CCT ACG GGA GGC AGC, Uni907R CCG TCA ATT CMT TTG AGT TT), *Rhodoferax ferrireducens* group (RdoR_RC GAC CTG CAT TTG TGA CTG YA, Uni907R CCG TCA ATT CMT TTG AGT TT), *Geothrix* (Gx. 193F_GAC CTT CGG CTG GGA TGC TG, Gx. 448R_AGT CGT GCC ACC TTC GT) (Braun et al., 2016). Quantitative PCR was performed with an RG-6000-5 Plex real-time DNA cycler (Rotor-Gene 6000). Non-DNA-containing samples were used as negative controls to ensure the accuracy of the qPCR. Cycles of qPCR characterized: 40 cycles for Eubacteria (Initial denaturation 95 °C, 2 min; Denaturation 95 °C, 20 s; Annealing 60.4 °C 30 s; Extension 72 °C, 1 min); 40 cycles for *Rhodoferax ferrireducens* (Initial denaturation 95 °C, 2 min; Denaturation 95 °C, 20 s; Annealing 58 °C, 30 s; Extension 72 °C, 1 min); 45 cycles for *Geothrix fermentans* (Initial denaturation 95 °C, 3 min; Denaturation 95 °C 20 s; Annealing 58 °C, 20 s; Extension 72 °C, 30 s). Quantification was performed using standard curves obtained from the amplification profiles of known concentrations of the respective standard. A melt curve analysis (55–99 °C) was performed at the end of PCR cycles to confirm specificity of primer annealing. The parameters for the calibration curves were $R^2 > 0.99$, efficiency from 92 % to 98 %.

Scanning electron microscopy

Textural and microstructural characterization of moonmilk was performed using a VEGA 3 LMH TESCAN scanning electron microscope (Czech Republic). The samples were prepared by air drying, and Pt coating. Then the moonmilk samples were placed on a conductive carbon tape, mounted on 12 mm diameter aluminum stubs that were then placed in the microscope chamber; magnification was up to 15,000×. Energy dispersive spectrometer X-max 80 with Aztec™ microanalysis system (Oxford Instruments, UK) was used for the elemental composition analysis of moonmilk. X-Max 80 provides a range of detected elements from boron to uranium with elements detection interval from 0.1–100 wt. %. The general process for sample preparation and scanning electron microscopy were carried out at the Khabarovsk Innovation and Analytical Center for Collective Use at the Institute of Tectonics and Geophysics, Far East Branch, Russian Academy of Science.

Results and Discussion

Elemental composition of water of different origins

The content of Ca in drip and fracture water in the Proschalnaya Cave is dependent on the amount of precipitation. The maximum amount of Ca in drip and fracture water was recorded in May 2016 (Fig. 3) at maximum amount of precipitation (136 mm/month). High Ca content is associated with dissolution of calcium carbonates contained in rocks in interaction with natural waters, especially when the acidity of the water increases in the presence of organic matter (OM) and microbial metabolic activities in the overlying soils.

We have shown (Kondratyeva et al., 2016) that in vitro the process of dissolution of CaCO$_3$ crystals was accelerated in the presence of nitrogen-containing OM. Microorganisms capable of synthesizing a polymer matrix played a determinative role. The formation of abundant slimy biofilms that formed on the surface of CaCO$_3$ crystals contributed to their dissolution (Fig. 4). During cultivation of moonmilk suspensions on agar nutrient media, we often observed growth of slimy colonies of heterotrophic bacteria capable of consuming different sources of carbon. The polymer matrix produced by these bacteria may be an active accumulator of other elements forming the moonmilk mass. It is known that over 99 % of microorganisms on Earth live within matrix consisting of a mixture of polymeric compounds (extracellular polymeric substance: EPS), which makes up the intercellular space of microbial aggregates and forms the structure and architecture of the biofilm matrix (Flemming, 2016).

Fe and Mn measurements for drip water were highest in the spring period. Enrichment of water with iron occurs as a result of leaching and dissolution of ferruginous minerals and rocks. Among the geochemical factors, ferric oxide was correlated with increased microbial diversity in the cave sediments (De Mandal et al., 2017). It should be noted seasonal asynchrony in the content of manganese in the drip and fracture water in the Proschalnaya Cave. In November 2015, the content of Mn in the fracture water was much higher than in the drip water and watercourses. In the spring of 2016, the concentration of manganese in the fracture water was lower than in other water samples, which may be due to the infiltration of water through different channels after spring snowmelt and autumn rains. Organic substances play an important role in determining the intensity of microbiological processes at the biogeochemical barrier of water-rock, which would also affect the content of dissolved forms of iron and manganese (Ferris, 2005).
Microbiological studies of water

In samples of water from the watercourse in the Proschalnaya Cave, regardless of the season, the predominant microorganisms preferred low concentrations of OM. Nitrifying and ferromanganese bacteria were also present in these samples. The most abundant microorganisms (6.28 × 10^3 CFU/mL) were in the cave watercourse in the spring, likely due to the increased transport of easily oxidized OM from the soil during snowmelt. During this time, the number of microorganisms present in the samples of surface water from Sagdi-Selanka River was lower due to increased flow velocity and volume. Autumn sampling from the cave watercourse and river surface water revealed decreased numbers of all physiological groups of cultivated microorganisms.

Fifty strains were isolated from water samples on different media (FPA; FPA diluted 10 times; Vinogradsky media). Using cultural and morphological characteristics, ten strains with active growth on agar media were selected for the study of biochemical activity. Strains isolated from the surface water of Sagdi-Selanka River and fracture water were most active and capable of utilizing the monosaccharides β-galactose, glucose, mannose, arabinose, arginine, ornithine, and mannitol as a source of carbon (Table 1). Strains of bacteria isolated from the river surface water and watercourse in the cave recycled the disaccharides lactose and sucrose, associated with the enzyme carbohydrase, which is responsible for the hydrolysis of di-, tri-, and polysaccharides. This enzyme also plays a role in regulating equilibrium between different forms of inorganic carbon, including bicarbonate, which is involved in the precipitation of calcium in nature (Müller et al., 2014).

Some strains isolated from fracture water and one representative of drip water utilized various amino acids (arginine, lysine, and ornithine) as a carbon source. Strains from the cave watercourse and fracture water actively utilized alco-
## Table 1. Carbon utilization by the strains isolated from surface water of Sagdi-Selanka River and different water sources from the Proschalnaya Cave (May, 2017).

| Source of Carbon | B 44 | B 45 | B 46 | B 19 | B 21 | B 25 | B 26 | B 32 | B 38 | B 42 |
|-----------------|------|------|------|------|------|------|------|------|------|------|
| Glucose         | +    | +    | +    | +    | -    | +    | +    | +    | +    | +    |
| Mannose         | +    | +    | +    | +    | +    | -    | -    | -    | +    | +    |
| Arabinose       | +    | +    | +    | +    | -    | +    | +    | +    | +    | +    |
| Lactose         | +    | -    | -    | -    | -    | +    | +    | +    | -    | +    |
| Sucrose         | +    | +    | +    | +    | +    | -    | -    | -    | +    | +    |
| Arginine        | +    | +    | +    | +    | -    | +    | +    | +    | +    | +    |
| Lysine          | -    | -    | -    | -    | +    | -    | -    | -    | +    | +    |
| Ornithine       | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Inositol        | +    | +    | +    | +    | +    | +    | -    | -    | +    | +    |
| Mannitol        | +    | +    | +    | -    | +    | +    | +    | -    | +    | +    |
| Sorbitol        | -    | -    | +    | +    | +    | -    | -    | -    | +    | +    |
| β-galactose     | +    | +    | +    | -    | -    | -    | -    | -    | +    | +    |
| Sodium malonate | +    | +    | +    | +    | +    | -    | -    | -    | +    | +    |
| Sodium citrate  | +    | -    | +    | +    | +    | -    | -    | -    | +    | +    |
| Urea            | -    | -    | +    | +    | -    | -    | +    | -    | +    | +    |

Note: "+" is a positive reaction, "−" is a negative reaction.

hols (inositol, sorbitol, and mannitol). Most strains were also capable of using citrates as a source of carbon. Overall, strains isolated from fracture water had the most flexible carbon requirements.

### Microbiological studies of the moonmilk

The abundance of cultivated bacteria within moonmilk varies strongly depending on its consistency (Table 2). In all samples, heterotrophic microorganisms dominated, consuming high concentrations of nitrogen-containing organic

## Table 2. Structures of the microbial communities of moonmilk of different consistency from the Proschalnaya Cave.

| Media | Colony Morphotype | Sample No. M1 (thin slimy white mass) | Sample No. M2 (thick curdy ivory-white mass) | Sample No. M3 (dry white mass) |
|-------|-------------------|--------------------------------------|---------------------------------------------|-------------------------------|
| FPA   | PR                | 111 ± 10.5                           | 142 ± 16.8                                  | 15 ± 3.9                      |
|       | PO                | 41 ± 6.4                             | 5 ± 2.2                                     | 3 ± 0.7                       |
|       | Y                 | 74 ± 8.6                             | 3 ± 0.7                                     | ...                           |
|       | Total             | 226 ± 25.5                           | 150 ± 12.7                                  | 18 ± 4.6                      |
|       | FPA:10            |                                      |                                              |                               |
|       | ST                | 74 ± 8.6                             | ...                                         | ...                           |
|       | OA                | 32 ± 5.6                             | ...                                         | 5 ± 2.2                       |
|       | B                 | 2 ± 0.4                              | ...                                         | ...                           |
|       | G                 | ...                                  | 6 ± 1.4                                     | 22 ± 4.7                      |
|       | MM                | ...                                  | 86 ± 9.3                                    | ...                           |
|       | Total             | 108 ± 14.6                           | 92 ± 10.7                                   | 27 ± 6.9                      |
| SAA   | OS                | 70 ± 8.4                             | ...                                         | n/a                           |
|       | OSp               | 22 ± 4.7                             | 52 ± 5.2                                    | n/a                           |
|       | Total             | 92 ± 13.1                            | 52 ± 5.2                                    | n/a                           |

Note: Colony Morphotype: PR: Pale-yellow, rugose; PO: Pale-yellow, oily; Y: yellow; ST: slimy, translucent; OA: opaline, asterial; B: brown; G: grey; OS: opaline, slimy; OSp: opaline, oily; OSp: opaline, spot, "−−" no colonies of this morphotype, "n/a" not available.
substances (NOS) and differing slightly in the dominant morphotype of the colonies. Moreover, in a thin layer of curdy mass the abundance of different groups was higher than in a thicker layer of moonmilk. During cultivation on SAA containing starch as a carbon source, the abundance of bacteria was low in three samples. Periodically, violet-colored colonies, growing on Bromfield media containing Fe(OH)₃, were isolated from moonmilk. Such differences can be associated with different stages of the formation of biofilms from moonmilk and physico-chemical conditions at the sampling sites.

Minimal diversity of colony morphotypes and low abundance were recorded in the sample of dense slimy moonmilk. There is evidence that the structure of the microbial community strongly affects the intensity of CaCO₃ deposition and the composition of moonmilk (Cirigliano et al., 2018). High concentrations of calcium carbonate are able to precipitate in the slimy matrix and inhibit the development of bacteria. The physiological adaptation of bacteria to toxic Ca²⁺ ions occurs by calcification in Ca²⁺-rich cave environments. Such activity creates the initial crystal nucleation sites that contribute to the formation of secondary CaCO₃ deposits within caves (Banks et al., 2010).

On the basis of cultural-morphological characteristics and a proposed scheme for identification of bacteria of the genus Bacillus (Vasiliev, 2013) with use of a series of tests (growth on citrate, arabinose, xylose, mannitol, urea, raffinose; catalase activity, and H₂S secretion). Among the twenty strains isolated from moonmilk in the Proschalnaya Cave, two strains were identified as Bacillus. It can be assumed the surface waters that drain the soil and karst rocks can act as the main source determining the composition of moonmilk. Bacillus are capable of producing polymeric slime and act as catalysts for the biogenic mineralization and weathering of rocks (Ercole et al., 2007). Bacillus can act as typical soil chemo-organotrophic bacteria that occur in freshwater, participate in the nitrogen cycle, and can reduce iron (Garcia et al., 2016).

These bacteria are the centers of crystal formation, affect the morphology of crystals, the solubility of carbonates (Mitchel and Ferris, 2006), and take an active part in induced calcium carbonate precipitation (Achal and Pan, 2014).

Molecular investigations of moonmilk sampled from the Proschalnaya Cave revealed the presence of iron bacteria of the genera Rhodoferax and Geothrix (Table 3) that are commonly found in iron-containing groundwater. Members of
the genus *Rhodoferax* are psychrotolerant facultative anaerobes that often use Fe(OH)$_3$ as an electron acceptor (Fin-
erant et al., 2003). *Geothrix fermentans* is found within the Fe (III) reduction zone of subsurface environments. Such
iron bacteria have been shown to attach to the surface of mineral particles by the production of adhesive biopolymer
(Nevin and Lovley, 2002). We assume that *Rhodoferax* and *Geothrix* acting as primary colonizers, initiate the first stage
of biofilm formation and create conditions favorable for the growth of other heterotrophic bacteria in moonmilk.

Bacteria capable of oxidizing iron and manganese have been repeatedly found in cave sediments. The presence of
*Flavobacterium* spp. in the Iron Curtain Cave indicates that it might potentially participate in iron oxidation (Ghosh et al.,
2017). *Flavobacterium* spp. was previously reported in abundance in ferromanganese deposits from the caves of the
Upper Tennessee River Basin, along with other bacteria indicating that this bacterium contributed to Mn (II) oxidation
(Carmichael et al., 2013).

Calcium salts promoting aggregation of bacterial cells and formation of slimy polymers can accelerate the formation
of biofilms and their interaction with rocks (Das et al., 2014). In many cases, microorganisms and their extracellular
polymeric substances act as effective centers for the formation of new structures that can lead to passive incrustation of
biofilms (Flemming, 2016) and affect the structure of the speleothem (Sallstedt et al., 2014). The production of carbonic
anhydrase, the enzyme regulating the equilibrium of inorganic carbon forms such as bicarbonate, can play the key role
in the mechanism of biomineralization (Smith and Ferry, 2000; Müller et al., 2014).

**Microstructure and elemental composition of the moonmilk from the Proschalnaya Cave**

While speleologists, geologists, and microbiologists have different views on moonmilk genesis, modern research
techniques have revealed an important role of biogenic factors in development of a number of sinter formations. Scanning
electron microscopy (SEM) of moonmilk from the Grotta Nera Cave (Italy) revealed fibrous formations with calcites
identified by X-ray refractometry (Cacchio et al., 2014). An array of elements were detected in the moonmilk, including
Ca, Mg, Al, P, Si, S, Mn, K, and Fe. The proportion of CaO was as high as 60.87 % in some samples, while the portion
of oxides such as MgO and Al$_2$O$_3$ never exceeded 1 %.

SEM imaging of moonmilk from the Proschalnaya Cave showed the presence of morphologically distinct micro-
structures with different elemental composition. Tubular structures in the composition of moonmilk (Fig. 5) distinguished
themselves by a rich chemical content. Except for the basic elements indicative of their carbonate genesis (C, O, and
Ca), in tubular structures Al, Si, and Fe were also present. In one of the loci, impurities of magnesium and sulfur were
observed. Al and Si oxides are often found as impurities in dolomite (CaMg(CO$_3$)$_2$), which is represented as inclusions
in calcite and as part of fine-grained sediments, including in moonmilk (Hill and Forti, 2007). Similar tubular structures
called nanofibres were found in caves and relate to secondary calcites (Bindschedler et al., 2014).

Calcium content in different samples of moonmilk from Snezhnaya Cave (Russia, Western Caucasus) indicated
differences in nanostructures (Kondratyeva et al., 2016). The highest calcium content (up to 61.54 % by weight) was ob-
served in the cubic crystal microstructures. In this locus, the contents of carbon and magnesium oxides were 33.79 %

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**Table 3. Molecular genetics (qPCR) analysis of moonmilk, sampled from the Proschalnaya cave.**

| Sample Description                | Total number of eubacterial DNA gene copies/g | Number of the *Rhodoferax* DNA gene copies/g | Number of the *Geothrix* DNA gene copies/g |
|-----------------------------------|-----------------------------------------------|---------------------------------------------|-------------------------------------------|
| M1: thin slimy white mass         | 1.17 × 10$^9$                                | 5.64 × 10$^6$                               | 3.25 × 10$^5$                             |
| M2: thick curdy ivory-white mass  | 1.08 × 10$^9$                                | 1.44 × 10$^6$                               | 3.76 × 10$^5$                             |

**Table 4. Elemental composition of nanostructures included in the composition of moonmilk in the Proschalnaya Cave.**

| Elements       | Weight Percent | Tubular Nanostructure | Claviform Nanostructure | Nanofibres |
|----------------|----------------|-----------------------|-------------------------|------------|
| C              | 19 ± 1         | 22 ± 2                | 41.5 ± 1.5              |            |
| O              | 59 ± 1         | 65 ± 3                | 64 ± 2                  |            |
| Ca             | 17.5 ± 2.5     | 9.5 ± 3.5             | 12.5 ± 2.5              |            |
| Na             | ...            | 0.325 ± 0.175         | 0.91 ± 0.41             |            |
| Fe             | 0.22 ± 0.04    | ...                   | ...                     |            |
| Mn             | 0.105 ± 0.005  | ...                   | ...                     |            |
| Si             | 0.87 ± 0.17    | ...                   | 0.135 ± 0.055           |            |
| Al             | 0.63 ± 0.1     | ...                   | ...                     |            |
| S              | 0.06 ± 0.01    | ...                   | 0.505 ± 0.245           |            |
and 3.26 % by weight, respectively. The highest level of carbon oxides (58.62 % to 82.73 % by weight) characterized the biofilm microstructures. These microstructures also had elevated levels of magnesium oxides (up to 16 % by weight). Detailed scanning of the images of moonmilk from Snezhnaya Cave moonmilk revealed specific microstructures resembling stacks of thin lamellars. Elemental composition of these plates was characterized by relatively low calcium content (0.1–0.14 % by weight) and considerably high magnesium content (14.65–22.6 % by weight).

In a Belgian Cave (Collembola Cave), abundant, randomly-oriented, single-crystal rods, and polycrystalline calcite fibers were present in the structure of moonmilk (Maciejewska et al., 2017). The tubular microstructures in moonmilk from the Proschalnaya Cave had high similarity with these microstructures. Also SEM images of the microstructures in moonmilk from the Proschalnaya Cave were similar to calcitic nanofibres, needle fibre calcite, tubular- and filament-like structures in other scientific literature (Shankar and Achyuthan, 2007; Maciejewska et al., 2015). However, the tubular nanostructures found by us are not similar to the reticulated filaments described earlier (Melim et al., 2008).

Various mechanisms for the formation of nanostructures are proposed: physicochemical processes, such as the deposition of salts on the cell surface or the deposition of calcite crystals on organic matrices; and calcination of fungal mycelium or actinobacteria (Bindschedler et al., 2014; Maciejewska et al., 2015). Proteobacteria, Acidobacteria, and Actinobacteria were the most common phyla in strong association with the needle calcite in moonmilk (Cirigliano et al., 2018).

Needle calcite is a common secondary speleothem (Cailleau et al., 2009). The presence of calcitic nanofibers and needle calcite in secondary CaCO₃ sediments can be used to characterize the paleoclimate and assess the ecological situation (Shankar and Achyuthan, 2007). Their ratio can indicate the alternation of arid and semi-arid climatic conditions, although both forms of calcite can also occur in a humid climate (Bindschedler et al., 2012).

For the first time in the mass of moonmilk in the Proschalnaya Cave we discovered claviform nanostructures (Fig. 6). In comparison with tubular structures, they have limited elemental composition (Table 4). The dominant components in these structures are carbon, oxygen, and calcium, but the calcium content in claviform nanostructure is lower than in tubular structures and nanofibers.

In some crystals of ancient calcites, needle structures composed of aragonite (CaCO₃) are found. It is assumed that, depending on the environmental conditions, sequential precipitation of calcite-aragonite-calcite can occur. The formation of aragonite in speleothems is associated with a high Mg/Ca ratio in the drip water, as Mg is an inhibitor of calcite growth (Wassenburg et al., 2012). In the nanotubes and claviform microstructures we observed that Mg was extremely rare. We assumed that during the formation of the moonmilk mass against the background of a decrease in the amount of rainfall, precipitation of calcites without magnesium occurred, in spite of its presence in groundwater.

Conclusions

The nature of the interaction of groundwater and surface water varies greater under the influence of the biochemical activity of microorganisms. Due to the movement of waters and the biochemical activity of microorganisms, the most intensive dissolution of the bedrock occurs in the spring, resulting in an increase in the calcium content where fracture and drip water interact with rock. Based on our studies, we assume that the formation of moonmilk in Proschalnaya Cave largely depends on the rate of entry of organic substances and the ratio of elements accumulating in bacterial polymers. Consequently, the movement of surface water and groundwater in the area of Proschalnaya Cave drive the biogeochemical processes important for the formation of moonmilk, and the origin of the water can dictate the mineral composition of the speleothems.

Climatic conditions are an important factor affecting the speed and stages of the formation of the biomass of moonmilk. Microorganisms are producers of polymeric compounds; they act as first settlers in the initial stages of the formation of the biofilm from moonmilk, and then accumulate other elements, forming biominerals. The biospheric approach to the moonmilk speleothem study is based on interdisciplinary research that relies on macro processes (geological and geochemical) and micro processes occurring at the scale of microbial cells and biofilms. Calcite transformation and geomorphology of karst caves are changed due to the specific formation of biofilms. Moonmilk provides clear evidence of the role of biofilms in transformation of rocks in underground ecosystems.

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