A first assessment of the microbiota of Taurida Cave

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Abstract
Caves are considered as ecosystems isolated from the surface in varying degrees. Hypogean habitats are mostly oligotrophic, with discretely distributed nutrients, where chemolithoautotrophic species can be found among the producers. In this case, vital activity is provided due to the nutrients of endogenous genesis. Of particular interest are the cavities, which were completely isolated from the surface impact for a long time. As a consequence, unique landscapes and mineral environments were formed in such cavities. An example is given by Taurida Cave, located on the Crimean Peninsula (Piedmont Crimea) and discovered during the construction of Taurida Highway. Samples of sediments were taken right after opening the cave in July 2018. For the cultivation of bacteria and microfungi, standard media, extracts from substrates were used. The number and biomass of microorganisms were determined by luminescence microscopy. Chemical composition of the main and trace elements of the cave deposits samples was determined by XRF WDS spectrometer. As a result, a difference in the number and biomass of microorganisms in different parts of the cave was revealed. The main contribution to the biomass of microorganisms is made by actinomycetes and microfungi. The bacteria were dominated by gram-positive bacteria of the genera Bacillus, Arthrobacter, Micrococcus. Among actinomycetes, species of the genus Streptomyces predominated. The species of microfungi Penicillium chrysogenum, Trichoderma sp., Aspergillus sp. were identified, Penicillium janczewskii dominated. The high abundance and biomass of microorganisms in the substrates of the cave may be related to the summer sampling period.

Key words: karst caves, underground habitats, microfungi, cave sediments, speleothems, actinomycetes.

Introduction
Underground ecosystems, including karst caves, are considered as a variant of an extreme habitat, where the supply of organic matter from the surface is limited and where special trophic connections can be realized. Some cave systems actively communicate with the surface due to water and air flows, but despite this, the
underground biota differs significantly from the surface one. Even more unique are caves or their sections, which are isolated from surface effects, such as closed cavities that communicate with the environment only through cracks in the rock, isolated from water flows. In these habitats, given the special conditions of the caves, such as constant temperature, high humidity and gas composition, special mineral environments are formed in which specific microbiota develops, including chemolithoautotrophic species (Madigan et al., 2000).

The identification of underground microbiota is associated with both the standard problems of the presence of a large number of uncultivated microorganisms and the poorly developed methodological approaches for assessing the functional significance of species in communities (Engel, 2015). In particular, this concerns the identification of live, dead and viable but non-culturable species developing in the conditions of underground space.

The use of standard culture media for oligotrophic microorganisms does not give good results, for a number of reasons: from the inappropriateness of standard media, for example, causing osmotic stress in organisms, to the inability to isolate slowly growing microorganisms under conditions of rapid growth of heterotrophs and limited cultivation time (Koch 1997, 2001).

By analogy with surface habitats and based on the available data on the anthropogenic impact on the microbiota of underground cavities (Jurado et al., 2010) it can be assumed that the ecosystem must change with the beginning of the exploration of the caves. These changes are of a complex nature and can affect both the transformation of conditions (temperature, humidity, gas composition, mineral conditions, etc.), and the introduction of organic matter and microbiota into the cavity (Hoyos et al., 1998; Pusz et al., 2015). Therefore, it is important to study the composition of microorganisms in different habitats and its changes starting from the moment of opening the cavity.

Despite the fact that today, most of the cave microbial diversity studies start with the evaluation of 16S rRNA gene sequences, ecological research, in particular the identification of autochthonous microbiota, involves the use of classical methods using selective media.

One of the recently discovered caves is Taurida, found on the Crimean Peninsula during the construction of Taurida Highway (Lopatin et al., 2019).

The aim of the work was to carry out the microbiological analysis of various sediments of Taurida cave.

**Material and Methods**

Taurida Cave was opened in June 2018 in the Belogorsk region on the Crimean Peninsula. It is represent a large horizontal labyrinth with a length of more than 1200 m located on the 17th km of the Simferopol-Kerch road, at an elevation of 330 m above sea level. There are no reservoirs and water streams in the cave, minor infiltration water inflows are observed in the far part of the northeastern branch of the cave. The temperature in the cave is 12°C with 96-97% air humidity (Amelichev, 2019). As suggested by Amelichev with co-authors (Amelichev, 2019) the cave was formed in paleo-arterian conditions, with an ascending flow of pressure water from the deposits of the Mazan Formation to the overlying Eocene limestones (Amelichev et al. 2018).

The cave is laid in nummulite limestones of the Ypresky-Lutetian stages (Simferopol regional stage) of the Lower-Middle Eocene (Simferopol suite) (Kopachev et al., 2008).

Samples were taken in July 2018, right after the opening of the cave and stored at the cave temperature until the analysis. Sampling points are shown at Fig 1.

The concentration of the main and trace elements in the samples was determined by X-ray fluorescence analysis (XRF) with WDS spectrometer Axios, PANalytical (4 kW x-ray tube, Rh-anode). Spectrometer calibrated with using of standard samples, samples of geological rocks and pure minerals. The reference material samples registered by United States Geological Survey (USGS) were used for quality control of analytical measurements. Pre-dried at 110°C samples were pulverised in a vibration disk mill (Herzog) with a tungsten carbide grinding set, which does not contain any analyzed chemical elements. The main oxides of samples were analysed using sample preparation as beads fused with lithium borates (technique code: NSAM VIMS 439-RS 2010, Russia). To perform the analysis of trace elements, samples were prepared in form of tablets by cold pressing of the dry sample powder substance mixed with the addition of a wax.
The absolute moisture content of the soil was determined as the difference between the mass of the initial and dried samples in a drying oven during the day at a temperature of 105 °C. The acidity of the soils was determined in an aqueous extract. The method of scanning electron microscopy was used, a CamScan microscope was used.

The number of bacteria in the soils was determined by a direct microscopic method. A 10 g sample was mixed with 10 ml of sterile water and sonicated for 3 minutes at a current of 0.4 A and a frequency of 15 kHz in order to disperse. A suspension with a volume of 0.02 ml was applied to degreased glass with an area of 4 cm$^2$, dried and fixed in a burner flame, and then stained with a solution of acridine orange (dilution 1: 10000) for 4 minutes. The excess dye was washed off with water for 10 minutes, after which the slides were placed on a glass slide and the preparation was observed by a fluorescent microscope.

The length of the mycelium of actinomycetes and the number of cells was determined by recalculating them per gram of air-dry substrate (Zvyagintsev, 1991). To determine the number of spores and mycelium of fungi, the samples were stained with white calcofluor (dilution 1: 10000) for 10 minutes. For each sample, 6 preparations were prepared, and 50 visual fields per preparation were evaluated. The number of cells and biomass was counted out by calculating the area of the mycelium and the number of cells in the photo using the Image Pro program, the results were calculated for 1 g of air-dry substrate.

The calculation of the fungal biomass was carried out assuming that the density of spores is 0.837 g/cm$^3$, and the mycelium of micromycetes is 0.628 g/cm$^3$; the density of the mycelium of actinomycetes was calculated similarly (Polyanskaya & Zvyagintsev, 2003). Calculations of bacterial biomass were carried out taking into account that the mass of one cell with of 0.1 μm$^3$ is $2 \times 10^{-14}$ g (Polyanskaya & Zvyagintsev, 2003). The microbial biomass content was calculated per gram of absolutely dry soil or mineral deposits (speleothems).

Figure 1. Taurida cave, location of sampling sites. The plan was drawn up by G.V. Samokhin according to the expeditions of the Crimean Federal University, 2018.
### Table 1. Characteristics of cave sediments at sampling points.

| Type of cave deposits                                                                 | pH of the aqueous extract | Moisture | Total number of prokaryotes in billion cells per g of substrate |
|---------------------------------------------------------------------------------------|---------------------------|----------|---------------------------------------------------------------|
| Dark viscous coating on limestone and in limestone cracks on the cave walls            | 7.23                      | 19       | 1.81±0.02                                                    |
| Clay sediments on the cave floor with carbonate white, light yellow and cream inclusions and veins. | 7.26                      | 28       | 0.62±0.02                                                    |
| Gray pasty moist deposits on the ceiling and walls of the cave with dark point inclusions and small light carbonate inclusions. | 6.91                      | 44       | 0.57±0.02                                                    |
| Soft, pasty, cream-colored thixotropic deposits in ceiling domes with crystalline structure, identified as moonmilk. | 6.86                      | 48       | 0.21±0.02                                                    |
| Loose clay deposits on the cave floor                                                | 7.12                      | 23       | 0.69±0.02                                                    |
A total of 7 samples were analyzed (Table 1), 20 samples from each were prepared and analyzed with a count of at least 200 microscope fields of view. Microbiological studies were carried out using agar culture media for the isolation of microorganisms by the method of serial dilutions.

For inoculation 10g samples were placed in sterile 100 mL volumetric flask. The samples were rinsed with sterile deionized water on a shaking incubator at 10°C, 150 rpm within 6 hours. The following dilutions were used: 100, 10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\) with cave sediment washings volume of 1 ml per Petri plate.

A glucose-peptone-yeast medium with the antibiotic nystatin was used to isolate saprotrophs, a Czapek-Dox medium for isolating fungi, and a Gause1 medium for isolating actinomycetes (Netrusov, 2005).

For inoculation of all samples, an agarose medium based on a soil extract was used, a soil sample was taken about 200 meters from entering the cave, the preparation of the extract was carried out according to the standard method (Netrusov, 2005). Additionally, for each sample, an extract from the soil was prepared using the collected soil, while the sample of soil for extraction was 20 g, the rest of the medium components were proportionally reduced.

The plates were incubated at 12° and 24 ºС till 7-8 weeks/until the visible colonies were observed. Morphologically distinguishable colonies were selected and re-streaked on the same media plates to obtain pure cultures.

The primary identification of bacteria and actinomycetes was carried out according to Bergey’s manyal (2000) for bacteria, Domsch (1993) for microfungi.

**Results**

Sediments samples are typical for caves and represent clay deposits with a predominance of silicates (samples TT2, TT5, TT7) or carbonates (samples TT3, TT6, TT4, TT1) (Table 2). Sample TT4 is identified as a moonmilk (Fig. 2).

The maximum value of the number of prokaryotes was found in a sample TT1, and the lowest in TT4, TT6, and TT7, while the biomass of prokaryotes was the highest in TT1 and TT5, where the length of the mycelium of actinomycetes and fungi was similar (Fig. 3). The least development of microbiota was noted in a sample of moonmilk TT4.

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**TABLE 1**

| Sample | Description | pH | Count | Biomass |
|--------|-------------|----|-------|---------|
| TT6    | Brown crumbly deposits of heterogeneous structure with ferruginous and carbonate inclusions on the walls and ceiling of the cave | 7.24 | 32    | 0.16±0.01 |
| TT7    | Loose clayey deposits with rare light carbonate inclusions | 7.11 | 25    | 0.14±0.01 |
In all of the samples, the biomass of fungal spores prevailed over the biomass of the mycelium, spores were represented as large (d = 2-3 µm) and small (d = 5-7 µm) sizes, small spores dominated, especially in the wet area TT5. The main contribution to the biomass of microorganisms is made by actinomycetes and microfungi (Fig. 4-5).

![Figure 2. Sample TT4 taken with a SEM.](image)

![Figure 3. The total number of prokaryotes in samples.](image)
The use of cultural methods showed that the number of cultivated forms of saprotrophs in the substrate samples is lower, on average, by 2 orders of magnitude, compared with direct counting of bacteria and ranged from $1.6 \times 10^7$ in 1 sample to $1.4 \times 10^6$ in 7 sample. Fungi were mainly represented by sterile mycelium, the numbers ranged from 103 to 105 CFU/g.

High heterogeneity was revealed in the number of bacteria within one sample, which may be associated with the discrete distribution of microorganisms on mineral aggregates and the formation of biofilms.
| Sample | L.O.I. | Na₂O | MgO | Al₂O₃ | SiO₂ | K₂O | CaO | TiO₂ | MnO | Fe₂O₃ | P₂O₅ | SO₃ | SrO | ZnO | Cl  | BaO |
|--------|-------|------|-----|-------|------|-----|-----|-----|-----|-------|------|-----|-----|-----|-----|-----|
|        | %     | %    | %   | %     | %    | %   | %   | %   | %   | %     | %    | %   | %   | %   | %   | %   |
| TT-1   | 40,45 | 0,04 | 0,25| 0,80 | 1,98 | 0,15| 51,12| <0,01| 3,16| 0,60  | 1,03 | 0,17| 0,03| 0,04| NI  | 0,101|
| TT-2   | 31,10 | 0,07 | 0,87| 5,84 | 19,67| 1,36| 35,52| 0,24 | 0,63| 4,00  | 0,27 | 0,11| 0,01| <0,01| NI  | NI  |
| TT-3   | 37,61 | 0,06 | 0,50| 3,28 | 9,43 | 0,80| 45,31| 0,15 | 0,28| 2,30  | 0,19 | 0,10| 0,02| <0,01| 0,01| NI  |
| TT-4   | 43,32 | 0,05 | 0,37| 0,20 | 0,43 | 0,03| 54,69| <0,01| 0,03| 0,10  | 0,03 | 0,65| 0,07| <0,01| NI  | NI  |
| TT-5   | 11,05 | 0,08 | 1,55| 13,88| 59,59| 2,26| 2,55 | 0,92 | 0,06| 6,23  | 1,22 | 0,30| 0,03| 0,03| NI  | NI  |
| TT-6   | 33,92 | 0,09 | 0,70| 4,65 | 13,90| 1,26| 39,65| 0,19 | 0,88| 3,26  | 1,10 | 0,10| 0,03| 0,04| 0,02| NI  |
| TT-7   | 9,61  | 0,09 | 1,25| 9,22 | 32,40| 1,44| 21,72| 0,44 | 0,68| 4,60  | 17,56| 0,33| 0,23| 0,20| 0,08| NI  |

L.O.I. – loss on ignition
NI – not identified
The following genera and species were identified on the basis of cultural and morphological characters: *Nocardia, Micromonospora, Micrococcus, Arthrobacter, Corynebacterium, Brevibacillus, Paenibacillus, Pseudomonas, Caulobacter, Hyphomicrobiurn*. Mainly common saprotrophic bacteria characteristic of soils dominated in gram-positive bacteria of the genera *Bacillus, Arthrobacter, Micrococcus* was isolated. Among actinomycetes, species of the genus *Streptomyces* predominated, and a wide variety of *Bacillus* strains was found among bacteria.

The species of microfungi *Penicillium chrysogenum, Trichoderma sp., Aspergillus sp.* were identified with *Penicillium janczewskii* predominated in all substrates.

**Discussion**

As a rule, microorganisms are excreted from the surface of rocks in which caves are laid, from mineral formations, drip deposits (speleothems) or soils, therefore, substrate samples were used to assess the biodiversity of microorganisms in Taurida Cave (Banerjee et al., 2012).

Bacteria are considered an important part of the underground ecosystem, as producers of organic matter and participants in the transformation of minerals in caves (Turchinskaia et al., 2019). The role of biota in the formation of soloids, as well as the contribution of bacteria to the emission of carbon dioxide into the atmosphere of a cave is estimated (Mazina et al., 2020). The species *Penicillium janczewskii*, which dominated the cave, is characterized by habitation in dry substrates depleted in organic matter, which is partially consistent with its presence in the cave (Marfenina, 2005).

The number of bacteria in the sediments of the cave was lower than in the soil, especially in the upper horizons (Polyansky et al., 2014), but rather high in comparison with other caves (Epure et al., 2014). This can be regarded as a result of the proximity of the cave to the surface or as evidence of active biological processes taking place in the cave; in general, the composition of the fungal biomass corresponds to the characteristics of the mineral horizons of soils (Lysak & Dobrovolskaya, 1982; Marfenina et al., 2016).

Considering the fact that the samples were taken in July, and for the majority of the soils, the maximum development of both mycobiota and prokaryotes was described in summer (Polyanskaya et al., 1995; Polyanskaya & Zvyagintsev, 2005), it is obvious that the summer period may be optimal for the development of cave biota. Further identification of the microbiota of Taurida cave using molecular genetic methods is planned.

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