

EXPERIMENTAL ARTICLES

Microbiota of the Kinderlinskaya Cave (South Urals, Russia)

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Abstract—The mesophilic and psychrotolerant microbiota of the air, soil, water, and bottom sediments of the Kinderlinskaya cave (South Urals, Russia) and the factors affecting the structure of microbial communities were investigated. The pattern of microbial distribution in soils was shown to depend on both the configuration of the cave and the level of recreational load. The lowest numbers of bacteria and micromycetes were found in the poorly visited, difficult-to-access sites. Coliform bacteria were revealed in all soil and sediment samples and in some water samples. Micromycetes belonged to 19 genera, with *Geomyces pannorum* as the dominant species. Air movement was shown to be the main factor affecting the density of the aerial microbiota.

Keywords: microbiota, mycelial fungi, mesophilic and psychrotolerant microorganisms, cave.

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Caves are specific ecosystems with unique characteristics determined by the surrounding rock, subterranean water, and karst morphology. The microclimate and physicochemical conditions developing in deep caves, such as the absence of light, constant low temperature of 3 to 4°C, high humidity (close to 100%), and scarcity of organic matter, are responsible for formation of specific associations of organisms relatively isolated from the surface ecosystems. They are connected to terrestrial ecosystems by air flows and infiltration and flood water; cave tourism plays an important role in the penetration of allochthonous materials and microorganisms into these environments. Active introduction of organic matter into the caves and development of phototrophic organisms under lamp illumination promote the changes in the trophic state of the cave systems, which result in development and preservation of allochthonous microorganisms, including pathogenic ones [1–3].

The Kinderlinskaya cave is attractive to tourists; it is located in an accessible site and has been actively visited by tourists (over 1000 per year) since the early 1980s. Recently, the number of visitors increased sharply to 10000 per year [4]. Excursions and expeditions resulted in organization of the underground base camps and recreational areas, as well as formation of unorganized toilet facilities, garbage dumps, etc. Increased anthropogenic load calls for more detailed and task-oriented monitoring of all the elements of the fragile cave ecosystem.

The goal of the present work was to determine the structure and characteristics of microbial communi-

ties developing in the Kinderlinskaya cave and to assess the changes caused by abiotic factors and recreational load.

MATERIALS AND METHODS

The Kinderlinskaya cave (30th Anniversary of the Victory) is located in the southern part of the Ulutau ridge (South Urals, Republic of Bashkortostan), on the right slope of the valley of the Kinderly river (right tributary to River Zilim), near its estuary. It has the highest amplitude among the Urals caves (~250 m) and is the second longest among Bashkortostan caves (8600 m). It is located in the gray and dark-gray Upper Devonian limestone, with asphaltic interlayers on the eastern wing of the Tash-Asty sinclinal with the rock age of D3fm. The cave is an inclined horizontal system of galleries and passages in the northern, northeastern and western, northwestern directions, which were formed on four hypsometrical levels (Fig. 1). Modern watercourses in the cave follow the limestone dip, from east to west [5].

Microclimate parameters of the cave. In the near-entrance part, the seasonal temperature variations are significant. Beyond the entrance part, a glacier-filled gallery is located, where ice and hoarfrost are accumulated. The temperature reaches its minimum of –1°C 460 m from the entrance and further on remains practically constant at 4 to 8°C (Table 1). The cave contains underground lakes, siphons, and pools. Infiltration water seeping through the base rock is the main source of water.

For microbiological analysis, samples of soil (0–5 cm), water (0–10 cm), bottom sediments (from the

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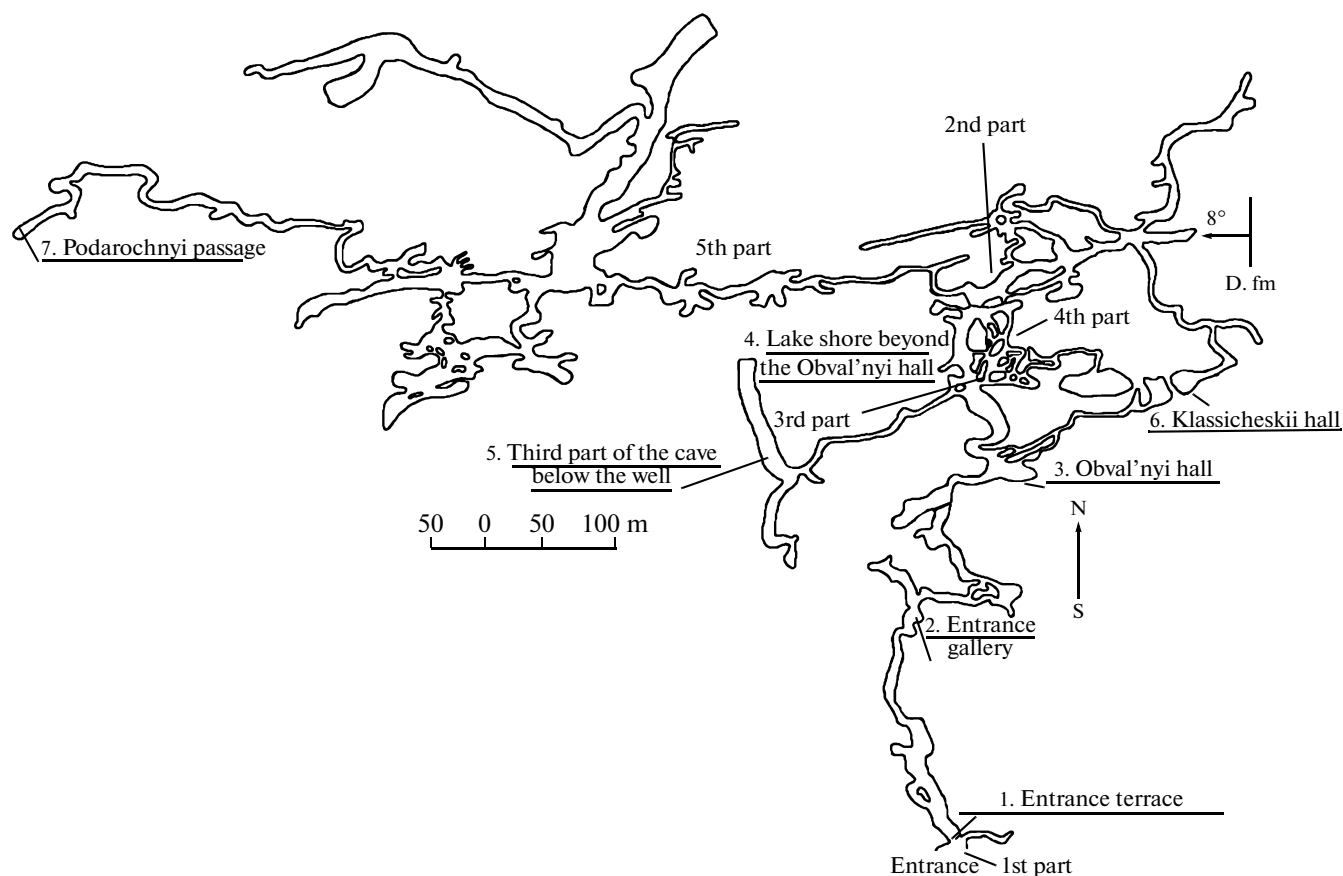


Fig. 1. Map of the Kinderlinskaya (30th Anniversary of the Victory) cave.

depth to 5 cm), and air (at 50-cm height) were collected (Table 1). The samples were placed in sterile bags or flasks and stored at 4°C. Analysis was carried out in the laboratory 1 to 3 days after sampling.

Air samples were collected by the Koch sedimentation method on nutrient agar (NA) and Czapek medium [6]. The following conventional criteria were used to characterize the ventilation of the different zones of the cave: I for the well-ventilated zones with constant exchange of air masses, with elevations and depressions, and II for stagnation of the air masses, with narrow passages in obstructed sites and dead ends.

Acidity was determined for water and for the water extracts [7] from soil and sediment samples.

Isolation and enumeration of microorganisms in soil, water, and bottom sediments were carried out by plating water or suspensions on solid media [8]. Members of the family *Enterobacteriaceae*, heterotrophs, oligotrophs, and micromycetes were isolated on the Endo medium, NA, starvation agar (SA), and Czapek medium, respectively. The plates were incubated at 10 and 28°C for 7 to 14 days. Plates with Endo medium were incubated for one day at 37°C. For convenience, the psychrotolerance coefficient was used, which is the ratio of colonies formed at 10 and 28°C. This param-

eter was determined in the case of reliable differences in the number of colonies formed at these temperatures.

The safety level of soil and water depending on the abundance of the *E. coli* group bacteria (ECG) was determined according to the sanitary regulations [9–11]. The sanitary and hygienic assessment of airborne microflora was carried out according to the values accepted for closed environments in winter. For bacteria, pure and contaminated conditions were defined as $< 4.5 \times 10^3$ and $4.5\text{--}7.0 \times 10^3$ [12], respectively. For micromycetes, very low, low, medium, high, and very high spore content corresponded to < 50 , $50\text{--}2 \times 10^2$, $2 \times 10^2\text{--}10^3$, $10^3\text{--}10^4$, and $> 10^4$ CFU/m³, respectively [13].

Micromycetes were identified according to the identification guides [14–18]. Statistical treatment of the results was carried out using the Student criterion at 5% significance level [19].

RESULTS AND DISCUSSION

The Kinderlinskaya cave was formed in Devonian carbonate strata and the cave soils are carbonate eluvium [5], which has an alkaline reaction. In our work,

Table 1. Sampling sites in the Kinderlinskaya cave

No.	Sample	Sampling site (distance from the entrance, m)	pH, pH _{H₂O} /pH _B	T, °C	Anthropogenic load per year [4]
1	S, A	Entrance terrace (0)	8.1	−10...−15	M, <10 000
2	S, A	Entrance gallery before the Lagernyi passage, stairway (210)	8.1	−1	M, BC, ≈3000
3	S, A	Obval'nyi hall, camping site (460)	8.0	5	M, BC, ≈2000
4	S	Lake shore beyond the Obval'nyi hall, left wall (560)	7.6	8	T, <1000
	H ₂ O, B	Permanent lake (8 × 3.5 m ² , h = 2 m)	7.9/7.8	4	
5	S, A	Third part of the cave below the well, 2 m from the slope (730)	7.9	6	T, ≈1000
	H ₂ O, B	Pool in the course of an old stream, infiltration water	7.4/8.0	4	
6	S	Klassicheskii hall, wall along the entrance (930)	8.1	7	M, ≈2000
	H ₂ O, B	Drop pool (20 × 20 cm ²), infiltration water	7.2/8.0	6	
7	S, A	Podarochnyi passage, 2 m from the lake (1530)	8.1	4–6	C, <100
	H ₂ O, B	Permanent lake (1 × 1 m ²)	7.6/8.0		

Note: S, A, and B indicate soil, air, and bottom sediments, respectively. H₂O indicates water samples; pH_{H₂O}, pH, and pH_B indicate pH of the water, the water extract from soil, and bottom sediments, respectively. C, T, M, and BC indicate clean zone, temperate (~1000) and mass load (~2000), and the underground base camp, respectively.

the pH_{H₂O} value for cave soils and bottom sediments varied from 7.9 to 8.1. The pH of lake water and pools varied from 7.6 to 7.9 and from 7.2 to 7.4, respectively (Table 1). These results indicate acceptable conditions for a normophilic microbiota.

Analysis of microbial communities in the soils of the Kinderlinskaya cave revealed that the numbers of mesophilic heterotrophic bacteria, oligotrophs, and *E. coli* group bacteria (ECG) varied from 10⁵ to 10⁸, from 10⁴ to 10⁷, and from 1 to 10^{−2} CFU/g, respectively (Fig. 2). Psychrotolerant microorganisms predominated among bacteria, with the psychrotolerance coefficient of 2–35.

The highest numbers of heterotrophic bacteria were found in the areas with the highest recreational load (entrance gallery, Klassicheskii hall, and the 3rd part of the cave), indicating organic contamination. High ECG level (200 CFU/g) corresponding to dangerous contamination was revealed in the entrance gallery, possibly because this is the most visited part of the cave (3000 per year). The Klassicheskii hall with the load of 2000 per year and the 3rd part of the cave also had significant levels of ECG (133–28 CFU/g), indicating moderately dangerous contamination.

In the Obval'nyi hall and the entrance terrace, ECG numbers corresponded to moderately dangerous contamination (Fig. 2). It is interesting to note that the numbers of heterotrophic and oligotrophic bacteria in these sites did not differ significantly. Evidently, the soils of these parts of the cave contained significant amounts of allochthonous organic matter, and the processes of mineralization had occurred.

The Podarochnyi passage and the lake shore beyond the Obval'nyi hall, which are less frequently

visited parts of the cave, contained less heterotrophic and oligotrophic bacteria (Fig. 2). The Podarochnyi passage contained also the lowest number of ECG, corresponding to uncontaminated soil.

Enumeration of mesophilic and psychrotolerant micromycetes yielded the values of 10²–10⁴ and 10²–10⁵ CFU/g, respectively (Fig. 3). The micromycetes growing at 10°C predominated in the samples collected at the entrance terrace, entrance gallery, Obval'nyi hall, lake shore beyond the Obval'nyi hall, and Podarochnyi passage. The psychrotolerance coefficient varied from 2.6 to 6.6. The lowest number of micromycetes was detected in the Podarochnyi passage, the most difficult-to-reach site. The candle remnants in the Klassicheskii hall were found to carry visible micromycete colonies. Thus, analysis of the abundance of microscopic fungi revealed predominance of the psychrotolerant species. The number of micromycetes decreased with distance from the entrance. The psychrotolerant nature of the cave microfungi resulted from the microclimate conditions, while their distribution was probably to a greater degree associated with anthropogenic load.

Mycological analysis revealed 93 strains of micromycetes belonging to 19 genera (Table 2). Most of the samples contained complexes of one dominant species and several rare ones. Importantly, the rare species have been isolated from different, sometimes quite remote sites. A similar phenomenon was reported for the mycobiota of karst caves in the Arkhangelsk oblast [20]. It may probably result from the random character of the distribution of available substrates.

The cave mycobiota contained both the eurybiontic species characteristic also of the soils of the day surface (*Aspergillus restrictus*, *A. versicolor*, *Clonostachys*

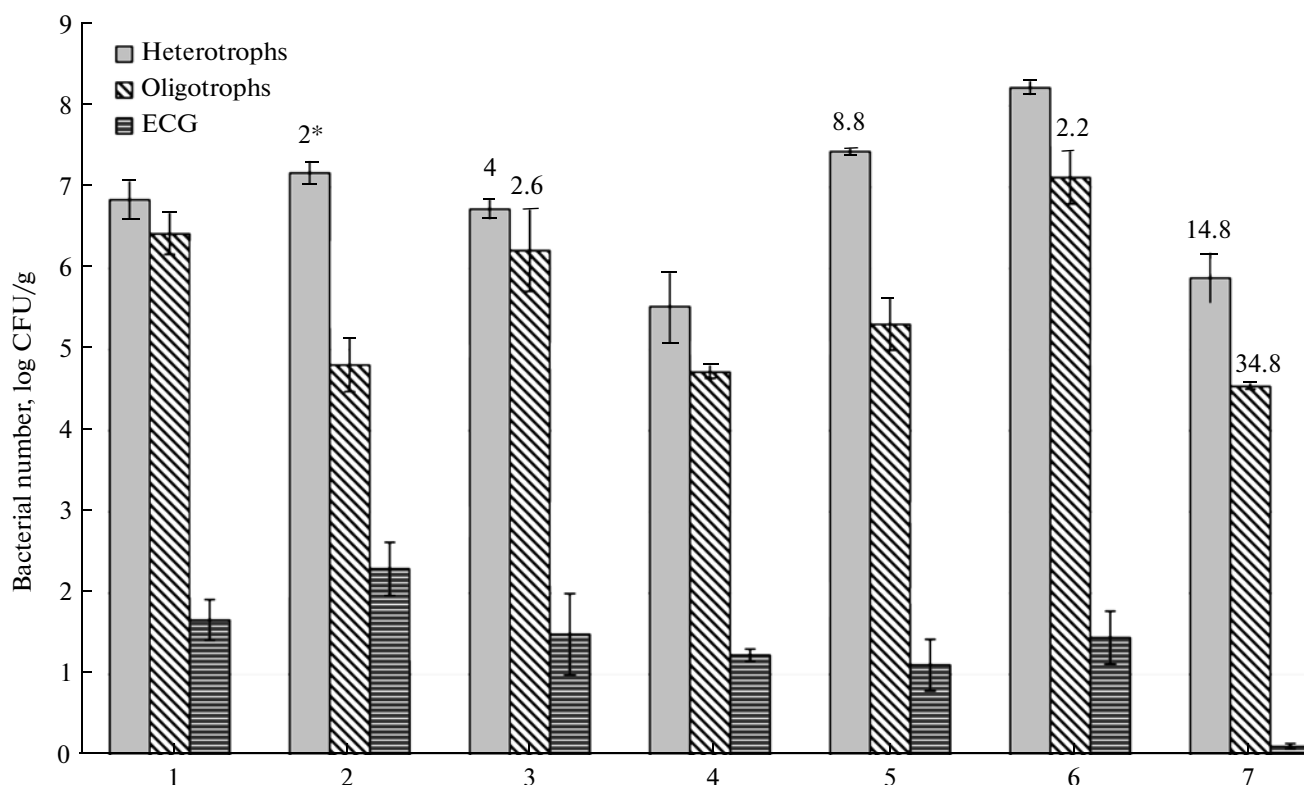


Fig. 2. Number of mesophilic bacteria in the soils of the Kinderlinskaya cave: entrance terrace (1), entrance gallery (2), Obval'nyi hall (3), Lake shore beyond the Obval'nyi hall (4), third part of the cave (5), Klassicheskii hall (6), and Podarochnyi passage (7). * indicates the psychrotolerance coefficient. The confidence interval at $p = 0.95$ is marked by vertical lines.

rosea f. rose, *Acremonium charticola*, *Cladosporium cladosporioides*, and *Penicillium* species) and mycomycetes not usual in Bashkortostan soils. Analysis of the literature data suggests that these species may be specifically associated with the underground (cave) habitats. Such micromycetes as *Cephalotrichum stemmitis*, *Geomyces pannorum*, *Trichoderma polysporum*, as well as some *Oidiodendron*, *Stachybotrys*, and *Talaromyces* species, have been revealed in the soils of Slovakian karst caves [21] and in the underground mine tunnels of the Kola peninsula and northern Norway [22]. *Aspergillus aureolatus*, which we found to predominate in the samples from 930-m depth, also predominated in Virginian karst caves [23]. *Geomyces pannorum* was the dominant species in the other analyzed samples, probably due to its broad adaptive capacity [24]. It should be noted that this species is dangerous to humans, with some data confirming its ability to cause onychomycosis [25].

Different cultivation temperatures made it possible to reveal certain patterns in the frequency of occurrence of some species. Mesophilic species prevailed at the sites closely connected with the surface, while the ratio of psychrotolerant micromycetes among the isolates increased with depth.

According to four-level scale by Nesterenko [26], which is based on the numbers of micromycetes in

soil, the condition of the Kinderlinskaya cave (3 points) is characterized as bad, suggesting limited construction of underground base camps, regular sanitary cleaning of the cave, and monitoring of the major environmental parameters.

Investigation of the microbiological state of the cave water bodies (Table 3) demonstrated that the number of enterobacteria was zero in lake water and 1–3 CFU/mL in other sites (pools), which does not exceed the safe level for lakes and other water sources (Sanitary Norms and Regulations 2.1.4.1175-02 and 4630-88).

The numbers of ECG revealed in the bottom sediments (Table 3) were higher than in nearby soils. This may be an indication of bottom sediments acting as a reservoir for potentially dangerous microorganisms.

Lake sediments also contained higher numbers of heterotrophic and oligotrophic bacteria, while their numbers in small water bodies (pools) were comparable to those in soils (Table 4). The shares of mesophilic and psychrotolerant bacteria in the bottom sediments were equal; only in one sample (lake in the Podarochnyi passage) the psychrotolerance coefficient was 1.6.

Similar results were obtained for micromycetes: less fungi was found in pool sediments than in surrounding soils (Table 4). Importantly, the silt micro-

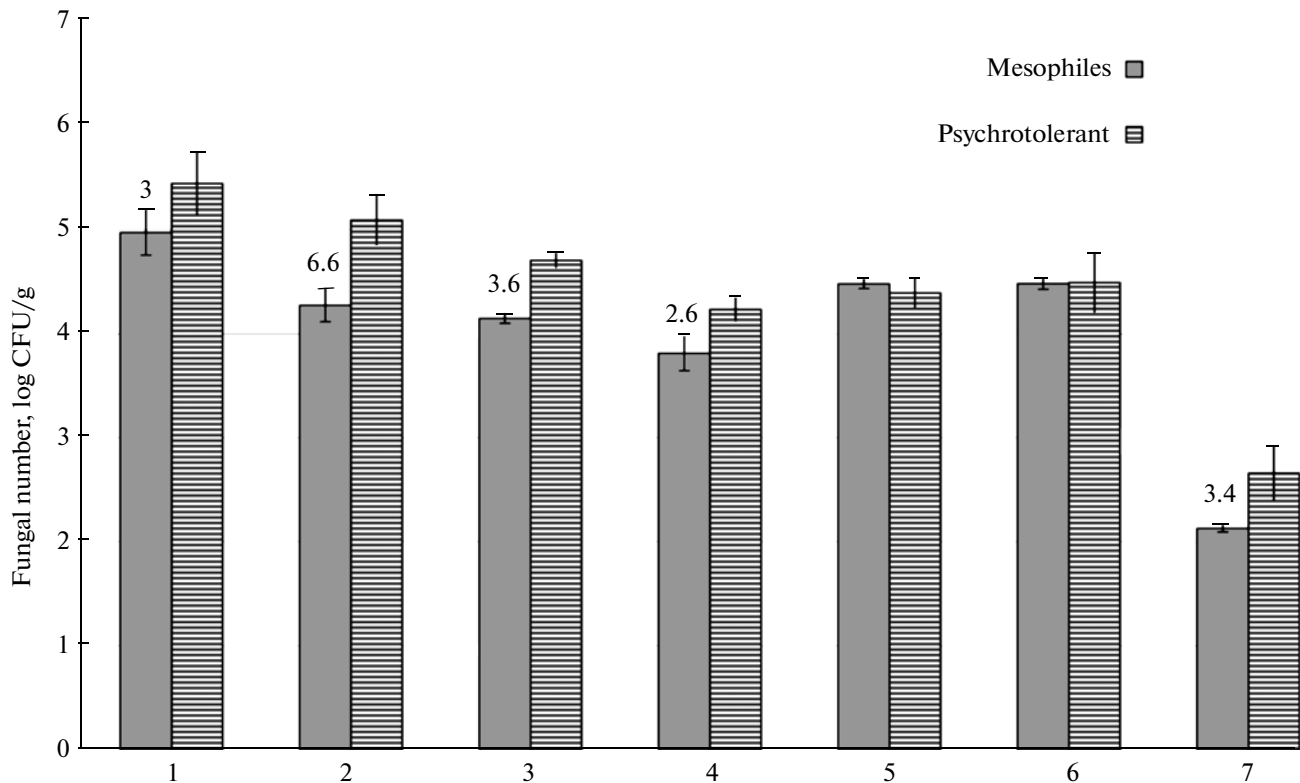


Fig. 3. Number of mesophilic and psychrotolerant fungi in the soils of the Kinderlinskaya cave. The designations are as on Fig. 2.

mycetes were more tolerant to low temperatures (the psychrotolerance coefficient varied from 3 to 5). Some fungal species isolated from water were not found in other substrates. Individual colonies of *Botrytis*, *Mortierella*, *Stachylidium*, and *Verticillium* species were isolated from the water of the Obval'nyi hall lake. Incubation of pool samples at 10°C revealed *Rhinochla-diella* and *Chaetomium* species. These data may indicate that the trophic regime developing in lakes formed by infiltration and flood water is more favorable for various groups of microorganism, including the potentially dangerous ones.

Concentration of bacteria and micromycetes in cave air was assessed (Table 5). High number of airborne bacteria and fungi found at the entrance terrace was determined by the contact of the sampling site with exterior atmosphere.

At 210–460 m beyond the entrance, in the entrance gallery and Obval'nyi hall, content of bacteria and fungi made it possible to classify the air as clean and medium, respectively. In a poorly ventilated site on the lake shore beyond the Obval'nyi hall, 730 m from the entrance, the air was more saturated with microorganisms (contaminated condition and high spore content). The number of microorganisms in the air increased with distance from the entrance. In the Klassicheskii hall and Podarochnyi passage, the number of airborne heterotrophic bacteria corresponded to

high contamination, and the content of fungal spores was high. These zones of the cave are characterized by air stagnation. The psychrotolerance coefficient calculated for the aerial microbiota showed predominance of psychrotolerant bacterial populations in the areas contacting with atmospheric air, while psychrotolerant micromycetes played the main role in the fungal component of the airborne microbiota. The microbiota of cave air is mostly formed by the microorganisms arriving with soil dust, moisture, or carried by macroorganisms. The main factors responsible for air self-purification in caves are gravity (resulting in precipitation of microbial aerosols) and natural ventilation. Some publications, however, demonstrated the leading role of the anthropogenic factor in formation of the aerial microbiota in some caves characterized by numerous narrow curved passages [22].

Our investigation demonstrated that the microbial community developing in cave ecosystems is formed under the influence of both the factors of adaptation to the specific conditions of cave geology and microclimate and the anthropogenic pressure. The lowest number of microorganisms in soils was found in remote and rarely visited parts of the cave (Podarochnyi passage and lake shore beyond the Obval'nyi hall). The zones with high recreational load exhibited the highest numbers of microorganisms. Bacteria of the *E. coli* group were found in all analyzed samples of soil and silt, as well as in half of the water

Table 2. Species composition of fungi in the soils of the Kinderlinskaya cave (occurrence, %)

Species composition of fungi		Entrance terrace (0)		Entrance gallery		Obval'nyi hall		Lake shore beyond the Obval'nyi hall		Klas-sicheskii hall		Poda-rochnyi passage	
		10000, 0 m*		3000, 210 m		2000, 460 m		1000, 560 m		2000, 930 m		100, 1530 m	
		28°C	10°C	28°C	10°C	28°C	10°C	28°C	10°C	28°C	10°C	28°C	10°C
1	<i>Acremonium butyri</i> (J.F.H. Beyma)							26.2	12.0		1.1		
2	<i>A. charticola</i> (Lindau) W. Gams									1.3			
3	<i>Aspergillus aureolatus</i> Ment.-Cvetk. & Bata									93.5	92.4		
4	<i>A. restrictus</i> G. Sm.			2.8	3.5	7.5	3.3		6.0				
5	<i>A. versicolor</i> (Vuill.) Tirab.						4.3						
6	<i>Aureobasidium pullulans</i> (de Bary) G. Arnaud		7.6			3.2							
7	<i>Cephalotrichum stemonitis</i> (Pers.) Nees	1.3	1.4	22.0	5.6						1.1		
8	<i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries		6.4				7.0					20.0	
9	<i>Clonostachys rosea</i> (Link) Schoers, Samuels, Seifert & W. Gams f. <i>rosea</i>	1.0					6.0	7.8	6.0				
10	<i>Fusarium</i> sp.	1.0							15.0				
11	<i>Geomyces pannorum</i> (Link) Sigles & J.W. Carmich.	27.3	69.6	66.6	90.9	48.9	75.0	8.8	16.0	1.3	2.1	100	50.0
12	<i>Gliocephalotrichum simplex</i> (J.A. Mey.) B.J. Wiley & E.G. Simmons			3.8		1.7			5.0				
13	<i>Humicola nigrescens</i> Omvik.					1.7							
14	<i>Mucor</i> sp.						2.7				1.1		
15	<i>Oidiodendron cereale</i> (Thüm.) G.L. Barron	9.4	4.4			1.4							
16	<i>O. truncatum</i> G.L. Barron					35.6					1.1		
17	<i>Penicillium</i> sp.	1.0							5.0	1.3		30.0	
18	<i>Phialophora</i> sp.	1.0		4.8					5.0				
19	<i>Scolecobasidium constrictum</i> E.V. Abbott						1.7						
20	<i>Stachybotrys</i> sp.	1.0							10.0				
21	<i>Talaromyces luteus</i> (Zukal) C.R. Benj.	1.0							5.0		1.1		
22	<i>Trichoderma koningii</i> Oudem.							24.2	5.0				
23	<i>T. polysporum</i> (Link) Rifai	54.6						8.8	10.0				
24	<i>Trichosporiella cerebriiformis</i> (G.A. de Vries & Kleine-Natrop) W. Gams		8.8										
25	<i>Mycelia sterilia</i> (white)	1.4	1.8					24.2		1.3			
26	<i>Mycelia sterilia</i> (dark)									1.3			
Total number of species		11	7	5	3	7	7	7	12	6	7	1	3

* Recreation load per year, depth, m.

Table 3. Bacteria of the *E. coli* group in the water, bottom sediments, and soil of the Kinderlinskaya cave

Sampling points	Water	Bottom sediments	Soil
	CFU/mL	CFU/g	CFU/g
Lake, Podarochnyi passage	0	ND	1.3 ± 1.1
Lake beyond the Obval'nyi hall	0	15.1 ± 1.7	17.2 ± 1.2
Drop pool, hall Klassicheskii	2.5 ± 1.2	35.1 ± 2.9	28.3 ± 2.1
Pool in the course of an old stream, Third part of the cave	1.1 ± 1.0	27.7 ± 2.9	13.1 ± 2.1

Note: ND stands for no data.

Table 4. Proportion of mesophilic fungi and heterotrophic and oligotrophic bacteria in the bottom sediments and soil of the Kinderlinskaya cave

Sampling points	Fungi		Bacteria			
			heterotrophic		oligotrophic	
	bottom sediments	soil	bottom sediments	soil	bottom sediments	soil
Lake, hall Obval'nyi	9	91	2	98	2	98
Lake, passage Podarochnyi	7	93	13	87	3	97
Pool, Third part of the cave	76	24	53	47	4	96
Drop pool, hall Klassicheskii	65	35	80	20	78	22

Table 5. Numbers of the mesophilic microflora in cave air

No.	Distance from the entrance, m	T, °C	Ventilation	Anthropogenic load	Bacteria, CFU/m ³ *	Fungi, CFU/m ³ *
1	0	−10...−15	atm. air	M	1.4 × 10 ⁴ ; 1.8*	4.2 × 10 ³
2	210	−1	I	M	3.4 × 10 ³ ; 2.9	2.6 × 10 ² ; 4.0
3	460	+5	I	M	2.6 × 10 ³	2.6 × 10 ²
4	730	+6	II	T	5.2 × 10 ³	1.6 × 10 ³ ; 1.3
7	1530	+6	II	C	2.2 × 10 ⁴	1.0 × 10 ³ ; 1.5
6	930	+7	II	M	4.8 × 10 ⁴	1.4 × 10 ³ ; 2.7

Note: Entrance terrace (1), entrance gallery before the Lagernyi passage (2), Obval'nyi hall (3), Lake shore beyond the Obval'nyi hall (4), Klassicheskii hall (6), and Podarochnyi passage (7). Atm. air indicates atmospheric air. Other designations are as in Table 1.

* Indicates the psychrotolerance coefficient.

samples, which may indicate danger for tourists and researchers. High occurrence of the microscopic fungi capable of causing onychomycosis was found in cave soils. Assessment of the content of bacteria and fungi in the air revealed their increase deeper in the cave due to air stagnation in poorly ventilated zones. Psychrotolerant fungal species were found to prevail in the air. No clear dependence was found between the number of airborne microorganisms and the recreational load. Our research demonstrated that the composition and structure of the microbial population of caves both reflects the specificity of cave ecosystems and characterizes the intensity of the anthropogenic load, suggesting microbiological assessment in the course of cave monitoring.

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