MIDDLE POMERANIAN SCIENTIFIC SOCIETY OF THE ENVIRONMENT PROTECTION

**Rocznik Ochrona Środowiska**

Volume 21. Year 2019 ISSN 1506-218X 1116-1135

# Airborne Microorganisms of Hypogenic Maze Caves Based on the Example of the Zoloushka Cave, Ukraine-Moldova

*Jakub Wojkowski[1*], Viacheslav Andreychouk[2], Krzysztof Frączek[1]*
*[1]University of Agriculture in Krakow, Poland*
*[2]University of Warsaw, Poland*
*[*]corresponding author's e-mail: rmwojkow@cyf-kr.edu.pl*

## 1. Introduction

The cave environment, due to its specificity (stable microclimate, high humidity, abundance of organic material, etc.), greatly facilitates the occurrence and growth of various types of microorganisms. They occur in all sub–environments of the underground: in the air, water, sediments, speleothems, at the bottom of the lakes, on the surface of the walls etc. Numerous studies conducted in recent decades by geo-microbiologists resulted in discovering extraordinary richness and diversity of the microbial life in caves, and led to a number of interesting discoveries referring to a geochemical role of microorganisms in the accumulation of mineral (biomineral) compounds in the caves (Wang et al. 2010, Muhammad 2018).

Among various geomicrobiological studies in caves, the greatest attention (and numerous publications) has been devoted to microorganisms related to the mineral substrate of the cave environment (walls and ceiling, speleothems) and deposits (silt), whereas very little attention has been paid to microorganisms present in the waters of cave reservoirs and the condensate. However, research on airborne microorganisms in caves is most limited. There are several reasons for that. Firstly, the cave air as an object of study does not arouse as much interest as sediments or water because it is believed that it is generally clean and in comparison with other elements of the cave environment does not contain any interesting microorganisms. Secondly, sample collection for the determination of airborne microorganisms in caves presents more severe methodical and technical difficulties than sampling sediments or water (Monte & Ferrari 2000, Wang et al. 2010, Porca et al. 2011, Ghosh et al. 2017).

In view of the above, the present knowledge of the microorganisms in the cave air is superficial and scarce. The literature on this subject comprises the works by Nakaew et al. (2009) exploring rare strains of *Actinobacteria* in Thai caves, the paper by Wang et al. (2010) presenting mycological research in Chinese caves, the works by Bastian et al. (2009) exploring pathogenic bacteria and protozoa in the Lascaux Cave in France, the works by Mulec et al. (2012) showing the airborne microflora of eutrophic caves in Slovenia and Slovakia and the works by Mulec & Oarga (2014) showing airborne bacteria, yeasts and moulds in Cuban cave the Great Cavern of Santo Tomás.

Among a few domestic works dealing directly with this subject, one can mention research carried out in the limestone caves of the Ojców National Park (ONP) (Wojkowski 2013) and in the Bear Cave in Kletno (Ogórek & Leyman 2013). Both of them showed that the airborne microflora of caves is diverse and may contain more microorganisms than the outer atmospheric air, especially in relation to specific groups of microorganisms. The studies also pointed to the relation of the abundance of microorganisms in the cave air with the intensity of the air exchange with the outside, which constitutes the type of the cave microclimate. Several studies were also carried out in the artificially created underground environment, i.e. mines (Pusz et al. 2018, Frączek et al. 2013).

This paper presents the results of studies conducted in a cave which is totally different from the aforementioned limestone caves, which are relatively small and generally characterised by a strong air exchange with the outside environment. The cave being the object of this study is the Zoloushka Cave (Eng. Cinderella Cave), which belongs to the group of caves in Western Ukraine making up the world's largest complex of gypsum caves. The major aim of this research was to determine the number of selected groups of microorganisms present in the air filling the halls and chambers of the Cave. These groups of microorganisms included: heterotrophic bacteria, *Actinobacteria* and fungi. The microbiological studies were accompanied by measurements of microclimate parameters to determine thermal conditions, humidity and circulation occurring in the Cave.

## 2. Study area, materials and methods

Zoloushka Cave is a large maze cave with a huge total length of corridors (over 90 km) located at a considerable depth below the surface (20-60 m). It is characterised by extremely poor (hindered) air exchange with the outside atmosphere (Andreychouk 2007). The cave is located in the southern part of Western Ukraine, in the district of Chernivtsy (historically – Northern Bukovina), at the place where the borders of three countries (Ukraine, Moldova and Romania) meet (Fig. 1).


**Fig. 1.** Location of the Zoloushka Cave (black badge) on the background of the Western Ukraine gypsum karst area: 1 – gypsum karst area

One of the borders – between Ukraine and Moldova runs directly above the cave thus dividing it into two parts: the western (Ukrainian) and eastern (Moldovan) (Fig. 2). The greater part of the cave labyrinth is located on the Ukrainian side (about 2/3). The borders of Ukraine and Moldova with Romania are situated within the distance of 0.8-1.3 km to the south of the cave and run along the border river of Prut. The entrance to the cave is located on the Moldovan side. The nearest settlements are: a Ukrainian village of Podvirne (800 m from the entrance) and a Moldovan village of Criva (1.5 km).

The cave was artificially exposed during the exploitation of the gypsum quarry founded in the late '40s of the last century. The discovery of the cave was made in 1977 by speleologists from Chernivtsy, who also carried out its first exploration. Due to the mining activity, a considerable part of the cave labyrinth was destroyed. As a result of the intervention of speleologists and scientific communities in the late '80s the cave was put under the protection by law (it was given the status of a natural monument) on both the Ukrainian and Moldovan sides, and mining operations were altered. The western part of the quarry with the cave holes in the gypsum wall was filled with loose material of dumps, while at the same time a 28-meter high concrete shaft with ladders was built into the ground, thus enabling entering the cave.

Until the cave labyrinth was opened by the quarry, the underground system of cavities had been almost completely filled with water and constituted a natural part of the rich in water karst aquifer. In order to facilitate the exploitation of gypsum, the quarry was being gradually deepened and the water present in gypsum was pumped out. This resulted in creation of a depression cone around the quarry and dehydration of the underground labyrinth, which has allowed its penetration by speleologists and conducting research in the cave.

Since the late '80s of the last century, the cave has been a "training ground" for a variety of research – geological, geochemical, hydrogeological, geotechnical, microclimatic, speleogenetic and others. The value of these studies arises from the fact that the cave became exposed while being at the stage of its active formation, and its artificial dehydration enabled researchers to observe the ('accelerated') course of various processes associated with the transition of the caves from the watered (freatic) stages to vadose and dry. These studies have led to the acquisition of a number of regularities in the development, and also some discoveries. The results have been published in numerous papers, including a synthesizing monograph of one of the discoverers of the cave (Andreychouk 2007).

In the '80s of the last century geo–microbiological research was also carried out in the cave. It was focused on the prevalence of microorganisms in the cave sediments, their typological diversity and their role in the creation of iron–manganese formations (Andreychouk & Klimchouk 2001). These studies indicated a very wide distribution and high biochemical (metabolic) activity of microorganisms in the sediments and on the surface of the walls, which is facilitated by a unique microclimate of the cave and the abundance of organic matter in clay sediments. However, the study did not examine microorganisms in the cave air. Nevertheless this issue appeared to be very interesting due to the specific nature of the cave environment (dehydrated and dried from water but still moist, with very stable climatic conditions) and the presence of a large number of microorganisms in solid sub-environments (on rocks, in sediments, including the bottom sediments of underground reservoirs etc.) which may potentially be a source of airborne microorganisms.

To resolve the issue of the origin of microorganisms in the air of the cave ('exogenous' source – the outer atmosphere or 'endogenous' one – the cave environment), the stability of its microclimate in the course of the year is of considerable importance. Stable microclimate conditions may constitute a background reflecting both external and internal factors. Therefore it is vital to recognise the exact characteristics of the cave microclimate.

Microclimate and microbiological analyses were carried out in measurement series of a few days in two seasons: in winter (2-3.03.2017) and in summer (22-23.06.2017). Fixed measurement sites were established in selected locations of the cave (Fig. 2).

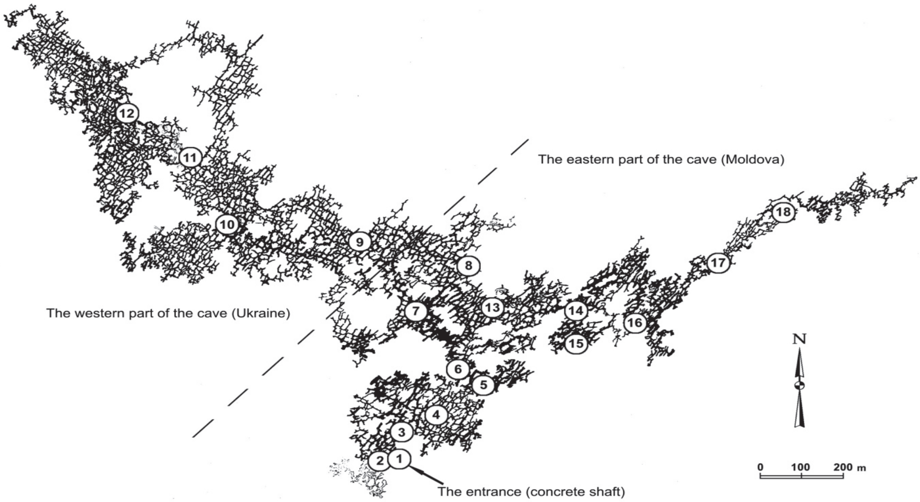


**Fig. 2.** The distribution of measurement sites within the field of the cave

Air samples for microbiological examination were collected by employing the method of collision detection with the MAS Merck air sampler. At each measurement site the device was placed at a height of about 1.0 m above the cave bottom and air samples were collected in triplicates. The bacterial aerosol was tested on tryptic–soy agar (TSA), *Actinobacteria* – on Gauze agar, and fungi on malt extract agar (MEA). TSA plates were incubated for 24 hours at 37°C, followed by 3 days at 22°C, and for the next 3 days at 4°C. Gauze plates were incubated at 28°C for 7 days, while MEA plates for 4 days at 30°C, then for 4 days at 22°C and for the next 3 days at 4°C. Prolonged incubation of bacterial cultures occurring in the air of caves has allowed the growth of strains growing slowly in the lower temperature range (Jensen & Schafer 1998, Ghosh et al. 2017). After the incubation of the plates, quantitative analysis of the grown microorganisms was performed – the number of grown colonies were counted and the bioaerosol concentration was expressed as the number colony forming units per one cubic meter of the air ($cfu \cdot m^{-3}$). Identification of isolated *Actinobacteria* cultures (Fig. 6) was prepared on the basis of macro- and microscopic morphological features and their biochemical properties based on Bergey's Manual of Determinative Bacteriology (1974).

Microclimate measurements were performed with Assman aspiration psychrometer at a height of 1.0 m above the bottom of cave corridors. Additionally, the temperature and relative humidity at a height of 1.0 m above the cave bottom were automatically recorded with StowAway miniature electronic sensors. The error of the temperature recorder was ±0.2°C, whereas of the relative humidity ±5%. The applicability of the recorders in environmental research, and the accuracy of their measurement were examined in the Department of Meteorology and Climatology of the University of Agriculture in Krakow (Wojkowski & Olechnowicz-Bobrowska 1997).

Microclimate measurements were also performed in the vertical profile of the shaft being the entrance to the cave at noon 23.06.2017 and at midnight 22/23.06.2017.

## 3. Results

### 3.1. The main features of the cave microclimate

The following brief characterisation of the cave microclimate is based on previous studies (Korzhyk & Andreychouk, 1981, Andreychouk 2007), and on research conducted by the authors of this article in 2012-2017. According to the nature of air exchange with the outer atmosphere, Zoloushka Cave belongs to static caves, characterised by a stable time course of basic meteorological elements such as pressure, temperature and humidity of the air. Owing to the characteristics of the cave such as a large area of the underground system, its location under impermeable (to water and air) overlying rocks (mostly clay deposits) of a considerable thickness, and connection with the surface through a vertical shaft of a small ventilation cross-section, stable microclimate conditions (static zone) occur in a larger part of the labyrinth (9/10), and these conditions undergo daily and seasonal changes (dynamic zone) only in the vicinity of the entrance (Fig. 3). The border between the zones runs, depending upon the season, at the distance of 30-100 m from the entrance opening (shaft).

The measurements of basic meteorological elements carried out in the cave in recent years show that the air temperature in the static zone is stable and fluctuates, regardless of the season, within the range of 10.8-11.2°C, whereas the relative humidity reaches almost always 100%. However, the latest research conducted by the authors in 2017 point to a minor difference (of 1.0°C) between the temperatures of the cold and warm period (Table 1). At this stage, we are not able to state clearly whether these observed differences really occur, or are the result of systematic measurement errors resulting from technical reasons. Nevertheless the microclimate stability within the major part of the cave is a clear and established fact, which is important for the air circulation in the cave, i.e. the velocity of the exchange of the cave air with the external atmosphere. The observations carried out in recent years have indicated that this exchange is weak. Due to a very slow movement of the air in the static part of the cave it is not possible to measure its velocity using conventional methods, for example with anemometers.

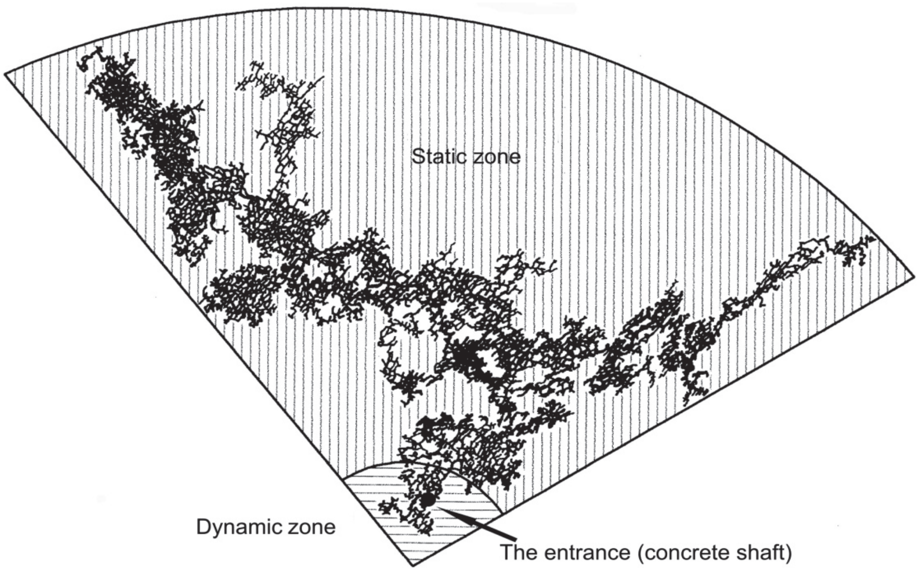


**Fig. 3.** Microclimatic zones of the Zoloushka Cave (Andreychouk 2007)

Initially, the velocity of the air flow was attempted to be estimated by employing the method of 'smoke' which consists in burning a smoky and fragrant substance, such as incenses flavoured with oils, and observing the spread of smoke in the cave (measurement of how quickly the smoke moves between the specified sites). Repeated observations and measurements performed with the aforesaid method showed that the air in the static part of the cave moves within a distance of approximately 30-100 m per hour, which is equivalent to a velocity of 0.01-0.03 $m \cdot s^{-1}$.

The velocity of the air flow estimated with the method of "smoke", however, contains a serious error. A much more accurate method for measuring the velocity of the air flow turned out to be a katathermometric method. A silver-plated Hill katathermometer was used to measure the cooling quantity of the air, and then the velocity of the air movement was calculated on the basis of empirical formulas (Bradtke and Liese, 1958). The measurements carried out with the katathermometric method showed that in the static part of the cave the velocity of the air movement ranges between 0.05 and 0.08 $m \cdot s^{-1}$ (Table 2). The air movement slightly accelerates on its approaching the dynamic area of the cave.

The general direction of the air movement is always towards the zone around the entrance hole, and in particular towards the area of the cave D-System, which probably has a connection through the rock fissures with the quarry.

**Table 1.** The results of microclimatic measurements in the Zoloushka Cave during winter and summer periods (italics – measurement sites situated in a dynamic zone, near the entrance)

| Measurement sites | | Air temperature (°C) | | Relative humidity (%) | |
|---|---|---|---|---|---|
| | | Winter period | Summer period | Winter period | Summer period |
| 1 | Outside | 4.2 | 27.5 | 94 | 63 |
| *2* | *Lower part of entrance shaft* | *9.1* | *14.0* | *97* | *97* |
| *3* | *The Hall of Kobylanska Prospect* | *11.0* | *13.1* | *98* | *99* |
| 4 | The Window | 11.0 | 12.4 | 100 | 100 |
| 5 | The Hall of Perspectives | 11.0 | 12.4 | 100 | 100 |
| 6 | The Overture | – | 12.2 | – | 100 |
| 7 | The Hall of the Speleologists of Chernivtsy | 10.8 | 12.3 | 100 | 100 |
| 8 | The Stalactitic Couloir | 11.2 | – | 100 | – |
| 9 | The Western Crossing | 11.0 | 12.4 | 100 | 100 |
| 10 | The Torchs | 10.8 | 12.1 | 100 | 99 |
| 11 | The Cellars | 11.0 | 12.3 | 100 | 100 |
| 12 | The Transition Geochim-Bukovina | 11.2 | – | 100 | – |
| 13 | The Metro | 10.9 | 12.3 | 100 | 100 |
| 14 | The Dinosaur Hall | 10.9 | 12.3 | 100 | 99 |
| 15 | The Colorado Labyrinth | 10.9 | 12.4 | 99 | 99 |
| 16 | Wet Well | 10.8 | 12.3 | 99 | 100 |
| 17 | The Far Eastern region (beginning) | 10.5 | – | 99 | – |
| 18 | The Far Eastern region (end) | 11.3 | – | 99 | – |

**Table 2.** The results of measurements of air velocity ($m \cdot s^{-1}$)

| Measurement sites | | Air velocity ($m \cdot s^{-1}$) |
|---|---|---|
| 1 | Outside | 0.96 |
| 4 | The Window | 0.05 |
| 5 | The Hall of Perspectives | 0.06 |
| 7 | The Hall of the Speleologists of Chernivtsy | 0.07 |
| 9 | The Western Crossing | 0.05 |
| 10 | The Torchs | 0.06 |
| 14 | The Dinosaur Hall | 0.08 |

Weak intensity of the exchange of the cave air with the outside atmosphere generally leads to a stagnating regime of the air circulation within a greater part of the cave. This results, among others, in the accumulation of significant amounts of carbon dioxide ($CO_2$) (1-5%) in the air (Fig. 4). An increase of its content towards deeper parts of the cave reflects weakening of the circulation activity in areas situated further away from the entrance and from the area of D-System ventilating the cave.

In the dynamic zone around the entrance hole, the exchange of the air is much more active. When approaching the bottom of the shaft, a gentle current of air is felt on the face. It is also indicated by a clear seasonal variation of temperature and humidity of the air in this part of the cave (Table 1).

A concrete shaft itself nowadays constitutes an important element of the microclimatic system of the cave enabling its ventilation. The role of the slot ventilation through the quarry still has to be explained. Figures 4 and 5 show that the microclimatic conditions in the shaft, particularly temperature, undergo stratification, especially within the first 10 metres of the shaft, pointing to its buffer (compensating) role in relation to the interior of the cave.

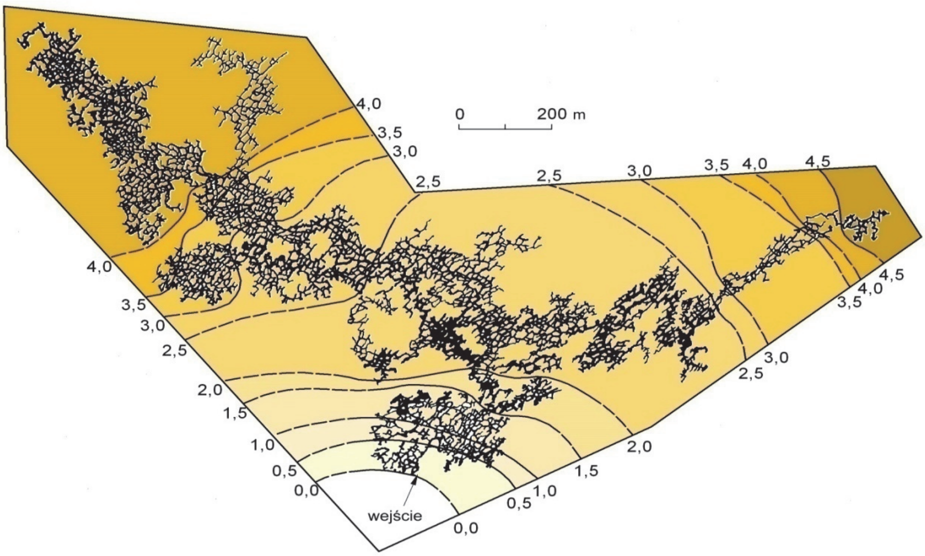


**Fig. 4.** The content of carbon dioxide ($CO_2$) in the air of the Zoloushka Cave (Andreychouk et al. 2011)

In the context of the evaluation of air exchange in the cave, extremely important are measurements of the velocity of its movement in the shaft entrance. The performed measurements (Fig. 5) show that it changes within the range of 0.1-1.3 $m \cdot s^{-1}$. Maximum values of the velocity appear on the bottom of the shaft, which results from a distinct narrowing of the transitional opening (< 0.5 $m^2$) – from the bottom of the shaft to the interior of the cave (the cross section of the shaft is approximately 4 $m^2$). These data are important for determining the velocity of the exchange of the cave air. Unfortunately, a proper quantitative balancing of the air circulation between the cave and the external environment requires data from a continuous annual monitoring, because the velocity of the exchange is highly variable over time, both in a seasonal course and daily.

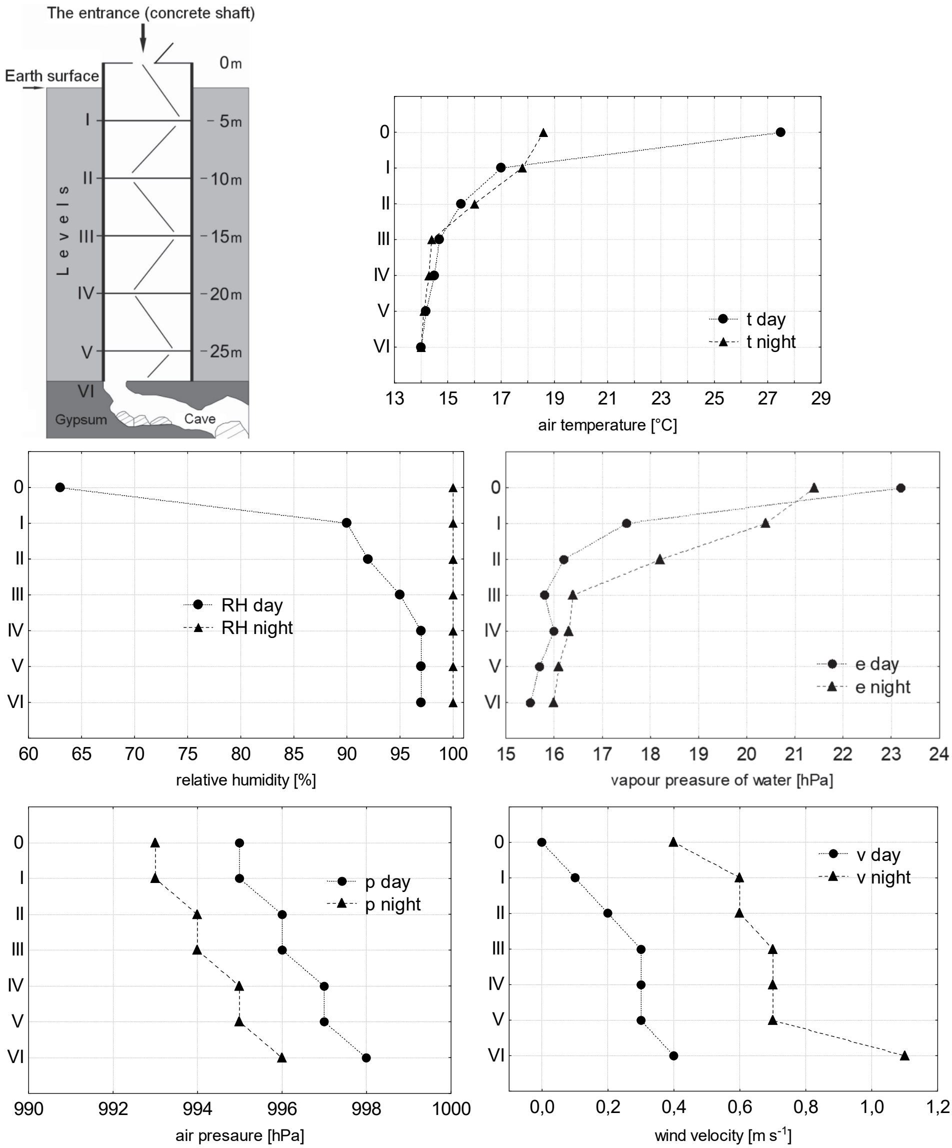


**Fig. 5.** Selected meteorological elements in the vertical profile of the shaft entrance of the Zoloushka Cave during the daytime and night-time

Periodic observations show, however, that this velocity generally ranges from 0.0 to 1.5 m · $s^{-1}$. Basing on these values, an attempt may be made to determine the time necessary for a complete exchange of the air in the cave. Given the capacity of the cave – 650 000 $m^3$, the ventilation opening of 0.5 $m^2$, and assuming the average value of the velocity of the air flow of 0.7 m · $s^{-1}$, a complete air exchange in the cave labyrinth may take 21-22 days (about 30 000 $m^3$ per day).

Nevertheless these calculations should be considered very carefully due to the lack of data from regular year-long measurements. Most likely, however, the total time of the air exchange is much longer because of the peculiarities of the internal circulation of the air in the cave – a stagnating circulation regime in areas distant from the entrance, and more active movement of the air in the area in the vicinity of the opening.

### 3.2. Microorganisms content in the cave air

The study of airborne microorganisms in the cave atmosphere was conducted in 16 sites under the ground, more or less evenly distributed within the cave field, on the surface of the earth above the cave, and in the entrance shaft to the cave (Fig. 2). The measurements have shown that the total number of the studied groups of microorganisms in the air of the cave varies in the course of year within the following ranges: heterotrophic bacteria 48-2,630 cfu·$m^{-3}$, fungi 80-3,395 cfu·$m^{-3}$, and *Actinobacteria* 5-51 cfu·$m^{-3}$ (Table 3). Mean values of the microbial aerosol concentrations with respect to the entire cave are: heterotrophic bacteria – 353 cfu·$m^{-3}$, fungi – 974 cfu·$m^{-3}$, and *Actinobacteria* – 17 cfu·$m^{-3}$.

Based on the analysis, it has been found that the microbial aerosol levels vary greatly between the examined seasons. In general, there is a regularity of an increase in their concentration during the warm period: 3-5 times higher values for bacteria (48-764 cfu·$m^{-3}$ in winter and 175-2630 cfu·$m^{-3}$ in summer), 4-5 times for fungi (80-990 cfu·$m^{-3}$ in winter and 390-3395 cfu·$m^{-3}$ in summer), and 0-1 times for *Actinobacteria* (0-51 cfu·$m^{-3}$ in winter and 5-55 cfu·$m^{-3}$ in summer) (Table 3). In comparison to the external environment, in winter the concentration of microbial aerosol the air of the cave is generally similar in the case of all groups of microorganisms. For heterotrophic bacteria the value recorded outside was – 133 cfu·$m^{-3}$, while the mean for the cave was 199 cfu·$m^{-3}$, for fungi the concentration for the outdoor air was 559 cfu·$m^{-3}$ compared with the mean value for the case of 497 cfu·$m^{-3}$, and for *Actinobacteria* – it was 13 cfu·$m^{-3}$ (outside) and 16 cfu·$m^{-3}$ (mean for the cave). In summer, the picture becomes more diverse, especially in relation to fungi and *Actinobacteria*. The content of fungi in the cave air shows a 4-fold increase from 390 cfu·$m^{-3}$ outside to 1668 cfu·$m^{-3}$ in the cave, whereas the content of *Actinobacteria* decreases from 55 cfu·$m^{-3}$ outside to 15 cfu·$m^{-3}$ inside the cave.

As regards the difference in the concentration of individual groups of microorganisms within the cave field, it should be noted that there are no clear patterns. Similarly low and high levels of airborne microorganisms may occur in the parts of the cave located both closer to the entrance and away from it. Only in the case of *Actinobacteria*, an increase in their concentration can be observed (both in summer and winter) in the part of the cave labyrinth near the entrance hole (measurement sites 2-5 in accordance with the Tables 1-2). As to other groups of microorganisms, what is characteristic for them is their high variability within the cave and their 'abnormally' high amount at certain sites. For example, the content of the heterotrophic bacteria at the measurement site at Wet Well (measurement site No. 16) reaches 764-2630 cfu·m$^{-3}$, which significantly exceeds the mean value for the cave, both in winter (199 cfu·m$^{-3}$) and summer (497 cfu·m$^{-3}$). In the case of fungi, a similar anomaly is characteristic for measurement sites 14-16 in the eastern part of the cave, especially in summer. During a cold period, an increased amount of fungi was noted in the part of the cave located closer to the entrance at the measurement sites No. 3-5 (Table 3).

## 4. Discussion

Seasonal variability is a very important factor affecting the microbiological quality of cave air (Wang et al. 2010). The aforementioned regularities in the occurrence of microorganisms indicate a rather complex nature of the phenomenon. Seasonal variability of the number of microorganisms in the cave air (and external air), namely an increase in their number in summer, clearly points to a close relationship with the external environment, characterised by a similar trend. This leads to a general conclusion that a majority of population (if not all) of airborne microorganisms in the cave originates from an exogenous (external) source. It seems that this is a natural phenomenon and results from the infiltration of external air carrying an additional load of microorganisms (Kummer & Thiel 2008, Ghosh et al. 2017). However, fungi are an exception as their numbers in summer substantially exceed the external values. Similar results were observed in studies conducted in caves in another part of the world (Wang et al. 2010). The reason for this phenomenon is not yet understood, given the stability of the cave microclimate throughout the year (which is somewhat contradicted by the data from the recent measurements of the microclimate) and relatively low values of the amount of fungi in the external atmospheric air. It is possible that the 'enrichment' of the cave atmosphere with fungi in summer time is not only related to the variability or stability of ecological conditions conducive to the existence of fungi in the cave. Perhaps during the summer period other factors grow in importance: physical (weaker or more active air circulation, drying out or moistening of clay substrate etc.) or biological (the peculiarities of the development cycle and metabolism of fungi). It seems that the widespread presence and dominance of fungi in the air of caves may be conditioned by the fungal production of very numerous spores, as well as their extremely modest nutritional and environmental requirements. Optimal growth conditions for these microorganisms are high air and substrate humidity, although many species are characterised by the ability to survive in very dry conditions (Wang et al. 2010, Porca et al. 2011, Ghosh et al. 2017). This topic, interesting for ecologists concerned with caves, requires more extensive research.

**Table 3.** Concentration of microorganisms (cfu·m$^{-3}$) in the air of Zoloushka Cave during winter and summer periods (*italics* – measurement sites situated in a dynamic zone, near the entrance)

| Measurement sites | | Bacteria (except *Actinobacteria*) (cfu·m$^{-3}$) | | Fungi (cfu·m$^{-3}$) | | *Actinobacteria* (cfu·m$^{-3}$) | |
|---|---|---|---|---|---|---|---|
| | | Winter period | Summer period | Winter period | Summer period | Winter period | Summer period |
| 1 | **Outside** | **133** | **710** | **559** | **390** | **13** | **55** |
| *2* | *Lower part of entrance shaft* | 171 | 360 | 404 | 1120 | 9 | 42 |
| *3* | *The Hall of Kobylanska Prospect* | 100 | 262 | 695 | 1355 | 29 | 20 |
| 4 | The Window | 221 | 230 | 668 | 1610 | 32 | 20 |
| 5 | The Hall of Perspectives | 116 | 370 | 990 | 1865 | 51 | 25 |
| 6 | The Overture | – | 318 | – | 725 | – | 12 |
| 7 | The Hall of the Speleologists of Chernivtsy | 131 | 775 | 403 | 1305 | 13 | 15 |
| 8 | The Stalactitic Couloir | 118 | – | 100 | – | 14 | – |
| 9 | The Western Crossing | 167 | 535 | 158 | 765 | 16 | 12 |
| 10 | The Torchs | 225 | 175 | 100 | 1175 | 8 | 12 |
| 11 | The Cellars | 48 | 232 | 98 | 1270 | 0 | 10 |
| 12 | The Transition Geochim-Bukovina | 106 | – | 80 | – | 0 | – |
| 13 | The Metro | 87 | 187 | 80 | 2125 | 17 | 5 |
| 14 | The Dinosaur Hall | 232 | 197 | 88 | 3395 | 20 | 5 |
| 15 | The Colorado Labyrinth | 169 | 185 | 384 | 2570 | 11 | 12 |
| 16 | Wet Well | 209 | 2630 | 285 | 2410 | 10 | 5 |
| 17 | The Far Eastern region (beginning) | 317 | – | 729 | – | 10 | – |
| 18 | The Far Eastern region (end) | 764 | – | 488 | – | 12 | – |
| **Mean values:** | | **199** | **497** | **359** | **1668** | **16** | **15** |

The spatial diversity of the microbial concentration within the cave also reflects the influence of certain environmental 'endogenous' (internal) factors which causes both the variation in their abundance and is responsible for the occurrence of local (or regional) anomalies. Analyses of this type carried out by other researchers also confirm the large diversity of microorganisms in this type of environment, mainly depending on the specific characteristics of each of the studied caves (the size of caves, availability of nutrients) (Wang et al. 2010, Porca et al. 2011, Ghosh et al. 2017, Muhammad 2018).

In the case of fungi, a potential relation with the character of the clay substratum of the cave corridors is revealed. In areas where the ground is drier (especially measurement sites No. 2-6 located closer to the entrance hole, and sites No. 17 and 18), the values are higher, whereas in more humid areas they are relatively lower.

As regards several other anomalies, they may be related to local conditions. A very high (relatively) number of fungi in the Hall of Perspectives (measurement site No. 5) may (hypothetically) be the effect of storing up various kinds of tools used by cavers during the exploration work, which comprise wooden elements. Increased levels of both fungi and bacteria are also noted in places where speleologists stay longer (especially in summer), for example, in the Hall of the Speleologists of Chernivtsy (measurement site No. 7) or in the Dinosaur Hall (measurement site No. 14).

An ecological relationship with the external environment may be indicated by elevated values of *Actinobacteria* in the part of the cave located closer to the entrance, although in the winter their high concentration was also observed in the Perspective Room (measuring site No. 5), where the previously mentioned tools are stored. It cannot be ruled out that the source of *Actinobacteria* may also be present in a cave environment, especially in the winter, when the concentration of *Actinobacteria* outside the cave is 2-4 times smaller than within the cave. In the near-hole area of the cave, where they are observed in the greatest abundance, these microorganisms easily colonize the roof projections (Fig. 6).

It should be emphasized that this is a group of bacteria occurring relatively often in the environment of caves, which probably results from the fact that they occur always where there is a large amount of minerals and organic matter. In addition, these bacteria are able to form spores characterised by high resistance to stress caused by dehydration and remarkable metabolic activity (Lacey 1997, Górny 2004).

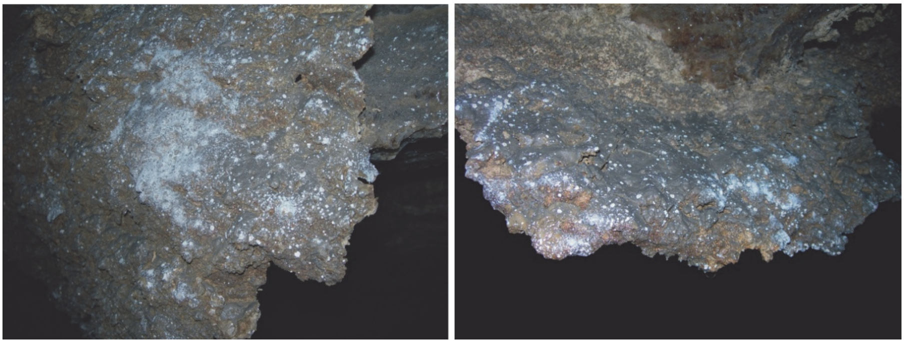

**Fig. 6.** Colonies of *Actinobacteria* covering the ceiling of the Hall of Kobylanska Prospect (measurement site No. 3), located near the entrance well (photo V. Andreychouk)

The results of the study show that the air of Zoloushka maze cave is characterised by a fairly high content of microorganisms, mainly bacteria and fungi, and lower of *Actinobacteria*. This is consistent with the data presented in the literature, which shows that *Actinobacteria* constitute usually about 5% of bacteria isolated from the air (Libudzisz et al. 2009). Nevertheless, when compared with the results of studies carried out in caves of a more dynamic exchange of air (Wojkowski 2013), the number of microorganisms in Zoloushka Cave is much lower (Table 4).

**Table 4.** Comparison of the mean concentrations of airborne microorganisms (cfu·m$^{-3}$) in the air of caves of the Ojców National Park and Zoloushka Cave

| Cave | Bacteria (except *Actinobacteria*) (cfu·m$^{-3}$) | Fungi (cfu·m$^{-3}$) | *Actinobacteria* (cfu·m$^{-3}$) |
|---|---|---|---|
| Jama Ani | 2245 | 206 | 251 |
| Okopy Górna | 1287 | 260 | 86 |
| Sąspowska | 2720 | 268 | 146 |
| **Zoloushka** | **348** | **1013** | **16** |

Fungi, whose amount in the air of Zoloushka Cave is almost 4 times higher, are an exception. In the case of the Ojców National Park caves, there was an average of 82% of bacteria (except *Actinobacteria*), 11% of fungi and 7% of *Actinobacteria* in the total number of microorganisms. In Zoloushka Cave the proportions were 25%, 74% and 1%, respectively. This may imply that the stable microclimate and humid environment of Zoloushka Cave is more favourable for the existence and growth of fungi (taking into account their 'explosion' in summer) than the dynamic climate and less humid environment of the Ojców National Park caves. The relative humidity and temperature measurements can be used to identify conditions that promote the growth of microorganisms in the cave environment. (Porca et al. 2011).

## 5. Conclusions

The study shows that there is a significant number of airborne microorganisms: bacteria, *Actinobacteria* in particular, and fungi in the large labyrinth caves, such as the studied Zoloushka Cave. The number of microorganisms is, in varying degrees, subject to seasonal fluctuations, and is characterised by a distinct spatial variability (within the cave field) culminating in the occurrence of specific 'anomalies' (high number of microorganisms significantly different from the mean).

The seasonal variability of microorganisms in the cave air points to the impact of external factors. The stability of the microclimate (ecological) conditions in the cave in the course of the year allows the conclusion that most of the microorganisms come from the outside and enters the cave during the exchange of the air with the external environment. Nevertheless, the environment of the cave does not remain passive – it makes the air contents spatially (within the cave field) diverse and, in some cases, it determines them (in places of significant anthropogenic pollution of the cave, which probably facilitates the growth of fungi). It should be emphasised, however, that the issue of the role of the external and internal environments in the migration and infiltration of microorganisms into the cave air may be settled more or less unambiguously only after having examined the qualitative (species) composition of microorganisms, and having understood their ecology.

The study does not indicate that the cave is a clear 'trap' for microorganisms, as might be inferred from microclimatic assumptions (stagnating regime of the air circulation). Only a high content of fungi in the air during the summer period still remains unclear. The fact that the numbers of microorganisms in the cave air and in the outside are generally comparable interferes with the established fact for ONP caves that there is a relative 'accumulation' of microorganisms in the environment of the cave (Wojkowski 2013). It is possible that ecological factors prevailing in the Zoloushka Cave are unfavourable for the existence of microorganisms in the air, for example, a particularly high concentration of carbon dioxide, poor circulation or rather low air temperature.

In comparison with the caves of a dynamic air exchange, Zoloushka maze cave of 'stagnant' microclimate is characterised by lower concentrations of microorganisms and its atmosphere is generally more pure. Both cases, however, point to a critical role of the external environment in supplying microorganisms into the cave atmosphere.

The research carried out in the Zoloushka Cave, as well as the authors' studies in other caves, still do not allow drawing generalizing conclusions on the presence of microorganisms in the air of caves. It should be emphasized that in addition to microbiological tests, the microclimate study turns out to be equally important which may be helpful in interpreting the results and explaining the observed regularities. For now, it has been concluded that the microbial aerosol concentration of the cave air is individual and different, depending on specific characteristics of the cave. Conclusions of a generalizing character will be possible only after a considerably larger number of cases will have been analysed and tests in caves of various genetic, morphological and microclimate types will have been conducted.

## References


Andreychouk, V. (2007). *Peshchera Zoloushka*. Sosnoviec-Simferopol. 407.

Andreychouk, V., Klimchouk, A. (2001). Geomicrobiology and Redox Geochemistry of the Karstified Miocene Gypsum Aquifer, Western Ukraine. *The study from Zoloushka Cave. Geomicrobiology*. *18*(3), 275-295.

Andreychouk, V., Teleshman, I., Kuprich, P. (2011). Prostranstvenno-dinamiczeskiye osobennosti raspredeleniya $CO_2$ v vozduche peshchery Zoloushka. *Speleologia I Karstologia*. *7*, 15-25.

Bastian, F., Alabouvette, C. & Saiz-Jimenez, C. (2009). Bacteria and free-living amoeba in the Lascaux Cave. *Research in Microbiology*. *160*, 38-40.

Bergey's Manual of Determinative Bacteriology (1974). The Williams AT Wilkins Company. Baltimore.

Bradtke, F., Liese, W. (1958). Pomiary klimatyczne wewnątrz i na zewnątrz budynków w zakresie higieny, techniki sanitarnej, ochrony pracy i medycyny. Warszawa: Arkady.

Frączek, K., Górny, R. L., Ropek, D. (2013). Bioaerosols of subterraneotherapy chambers at salt mine health resort. Aerobiologia, *29*, 481-493.

Górny, R.L. (2004). *Cząstki grzybów i bakterii jako składniki pomieszczeń: właściwości, mechanizmy emisji, detekcja*. Sosnowiec: IMPiZŚ.

Ghosh, S., Kuisiene, N., Cheeptham, N. (2017). The cave microbiome as a source for drug discovery: Reality or pipe dream? *Biochemical Pharmacology*. *134*, 18-34.

Jensen, P.A., Schafer, M.P. (1998). Sampling and characterization of bioaerosols. *Manual of Analytical Methods*. *1*(15), 82-112.

Korzhyk, V., Andreychouk, V. (1981). Osobennosti mikroklimata peszczery Zoloushka i vozmozhnosti jego prakticheskogo ispolzovaniya. *Akkumulaciya zimnogo choloda v gornych porodach i yego ispolzovaniye w narodnom choziaystvie*. 92-95.

Kummer, V., Thiel, W.R. (2008). Bioaerosols – sources and control measures. *Int J Hyg Environ Health, 211*, 299-307.

Lacey, J. (1997). Actinomycetes in composts. *Ann. Agric. Environ. Med*. *4*, 113-121.

Libudzisz, Z., Kowal, K., Żakowska, Z. (2009). *Mikrobiologia techniczna. Mikroorganizmy i środowiska ich występowania*. Warszawa: Wydawnictwo Naukowe PWN.

Monte, M., Ferrari, R. (2000). Airborne microorganisms in a subterranean archaeological area of the basilica of San Lorenzo in Lucina (Rome). *Aerobiologia*. *16*, 435-439.

Muhammad, Y. (2018). Analysis of bacterial communities and characterization of antimicrobial strains from cave microbiota. *Brazilian Journal of Microbiology*. *49*, 248-257.
Mulec, J., V. Krištůfek and A. Chroňáková. (2012). Comparative microbial sampling from eutrophic caves in Slovenia and Slovakia using RIDA®COUNT test kits. *International Journal of Speleology*. *41*, 1-8.
Mulec, J., Oarga A. (2014). Ecological evaluation of air and water habitats in the Great Cavern of Santo Tomás, Cuba. *Revista Mexicana de Biodiversidad*. *85*, 910-917.
Nakaew, N., Pathomaree, W., Lumyong, S. (2009). Generic diversity of rare actinomycetes from thai cave soils and their possible use as new bioactive compounds. *Actinomycetologica*. *23*, 21-26.
Ogórek, R., Lejman, A. (2013). Analiza mikologiczna powietrza w Jaskini Niedźwiedziej w Kletnie. Doniesienie wstępne. *Episteme*. *18*(3), 121-130.
Porca, E., Jurado, V., Martin-Sanchez, P.M., Hermosin, B., Bastianb, F., Alabouvette, C., Saiz-Jimenez, C. (2011). Aerobiology: An ecological indicator for early detection and control of fungal outbreaks in caves. *Ecological Indicators*. *11*, 1594-1598.
Pusz, W., Grzeszczuk, J., Zagożdżon, P., Kita, W. (2018). Aeromycological Monitoring of Disused Mines in Poland. *Pol. J. Environ. Stud*. *27*(1), 257-266. DOI: https://doi.org/–10.15244/pjoes/75201.
Wang, W., Ma, X., Ma, Y., Mao, L., Wu, F., Ma, X., An, L., Feng, H. (2010). Seasonal dynamice of airborne fungi in different caves of the Mogao Grottoes. Dunhuang, China. *International Biodeterioration & Biodegradation*. *64*, 461-466.
Wojkowski, J. (2013). Microclimate and microflora of caves of the Ojcow National Park. Prądnik. *Prace Muz. Szafera*. *23*, 75-90.
Wojkowski, J., Olechnowicz-Bobrowska B. (1997). The usefulness of miniature temperature and humidity recorders for environmental measurements. Zeszyty Naukowe AR w Krakowie. *17*, 185-189.

## Abstract

The article presents the results of microbiological and microclimatic research carried out in a large maze Zoloushka Cave (Ukraine-Moldova). The cave was artificially exposed during the exploitation of the gypsum quarry founded in the late '40s of the last century. Until the cave labyrinth was opened by the quarry, the underground system of cavities had been almost completely filled with water and constituted a natural part of the rich in water karst aquifer. The cave became exposed while being at the stage of its active formation, and its artificial dehydration enabled researchers to observe the (‘accelerated’) course of various processes associated with the transition of the caves from the watered (freatic) stages to vadose and dry.

Microbiological analyses aimed to determine the number of microorganisms (heterotrophic bacteria, *Actinobacteria*, and fungi) present in the air of the cave in two seasons – summer and winter. Microclimatic study aimed to determine the thermal, humidity and circulation characteristics of the cave microclimate The rules of occurrence of microorganisms in temporal and spatial (within a cave) cross-sections and the relative role of external and internal (cave) factors in shaping of the microbiological "image" of cave air were established.

The stability of the microclimate (ecological) conditions in the cave in the course of the year allows the conclusion that most of the microorganisms come from the outside and enters the cave during the exchange of the air with the external environment. Nevertheless, the environment of the cave does not remain passive – it makes the air contents spatially (within the cave field) diverse and, in some cases, it determines them (in places of significant anthropogenic pollution of the cave, which probably facilitates the growth of fungi). The measurements have shown that the total number of the studied groups of microorganisms in the air of the cave varies in the course of year within the following ranges: heterotrophic bacteria 48-2,630 cfu·m$^{-3}$, fungi 80-3,395 cfu·m$^{-3}$, and *Actinobacteria* 5-51 cfu·m$^{-3}$. Mean values of the microbial aerosol concentrations with respect to the entire cave are: heterotrophic bacteria – 353 cfu·m$^{-3}$, fungi – 974 cfu·m$^{-3}$, and *Actinobacteria* – 17 cfu·m$^{-3}$. In general, there is a regularity of an increase in their concentration during the warm period: 3-5 times higher values for bacteria (48-764 cfu·m$^{-3}$ in winter and 175-2630 cfu·m$^{-3}$ in summer), 4-5 times for fungi (80-990 cfu·m$^{-3}$ in winter and 390-3395 cfu·m$^{-3}$ in summer), and 0-1 times for *Actinobacteria* (0-51 cfu·m$^{-3}$ in winter and 5-55 cfu·m$^{-3}$ in summer).

**Keywords:**
airborne microorganisms, cave microclimate, hypogenic cave,
Zoloushka Cave (Ukraine-Moldova)

## Mikroorganizmy w powietrzu labiryntowych jaskiń hypogenicznych na przykładzie Jaskini Zołuszka, Ukraina-Mołdowa

### Streszczenie

Artykuł prezentuje wyniki badań mikrobiologicznych i mikroklimatycznych przeprowadzonych w dużej labiryntowej Jaskini Zołuszka (Ukraina-Moldowa). Jaskinia została odkryta podczas eksploatacji gipsowego kamieniołomu założonego pod koniec lat 40-tych ubiegłego wieku. Przed otwarciem labiryntu jaskiniowego system próżni podziemnych był prawie całkowicie wypełniony wodą i stanowił naturalną część zasobnego w wodę wodonośca krasowego. Otwarcie jaskini na etapie jej aktywnego formowania się oraz sztuczne jej odwodnienie stworzyło okazję do obserwacji (w trybie „przyśpieszonym”) przebiegu różnorakich procesów, towarzyszących przejściom jaskiń ze stadiów zawodnionych (freatycznych) do wadycznych i suchych.

Badania mikrobiologiczne zmierzały do określenia liczebności mikroorganizmów (bakterii, grzybów i promieniowców) występujących w powietrzu jaskini w dwóch kontrastowych porach roku, latem i zimą. Badaniom mikrobiologicznym towarzyszyły pomiary mikroklimatyczne, które miały na celu ustalenie termicznych, wilgotnościowych i cyrkulacyjnych charakterystyk mikroklimatu jaskini. Ustalono prawidłowości występowania mikroorganizmów w przebiegu czasowym oraz przestrzennym (w obrębie jaskini), a także względną role czynników zewnętrznych i wewnętrznych (jaskiniowych) w kształtowaniu „obrazu” mikrobiologicznego powietrza jaskiniowego.

Stabilność warunków mikroklimatycznych (ekologicznych) w jaskini w przebiegu rocznym pozwala wnioskować, że większość mikroorganizmów pochodzi z zewnętrz i trafia do jaskini w trakcie wymiany jej powietrza ze środowiskiem zewnętrznym. Nie mniej jednak, środowisko jaskiniowe nie pozostaje bierne, lecz różnicuje przestrzennie (w polu jaskiniowym) te zawartości, a w niektórych przypadkach również je warunkuje (w miejsca o znacznym antropogenicznym zanieczyszczeniu jaskini, sprzyjającym prawdopodobnie rozwojowi grzybów). Pomiary wykazały, że ogólna liczba badanych grup mikroorganizmów w powietrzu jaskini waha się w przebiegu rocznym w następujących przedziałach: bakterie 48-2630 jtk·m$^{-3}$ (rozrzut ponad 50-krotny), grzyby 80-3395 jtk·m$^{-3}$ (rozrzut ponad 40-krotny), promieniowce 5-51 jtk·m$^{-3}$ (rozrzut ponad 10-krotny). Średnie liczby zawartości mikroorganizmów w odniesieniu do całej jaskini wynoszą: bakterie 353 jtk·m$^{-3}$, grzyby 974 jtk·m$^{-3}$ i promieniowce 17 jtk·m$^{-3}$. Na ogół występuje prawidłowość wzrastania ich ilości w okresie ciepłym: dla bakterii 3-5 razy (48-764 jtk·m$^{-3}$ zimą i 175-2630 jtk·m$^{-3}$ latem), dla grzybów 4-5 razy (80-990 jtk·m$^{-3}$ zimą i 390-3395 jtk·m$^{-3}$ latem) i promieniowców 0-1 razy (0-51 jtk·m$^{-3}$ zimą i 5-55 jtk·m$^{-3}$ latem).

**Słowa kluczowe:**
mikroorganizmy, mikroklimat jaskiń, jaskinia hypogeniczna,
Jaskinia Zołuszka (Ukraina-Mołdowa)