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Airborne microorganisms in gypsum maze caves of the western Ukraine: biodiversity and geoecological control

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Microbiological and microclimatic studies of two giant gypsum caves in Bukovina (Prypruttia) and Podillya, Ukraine, have characterized the microbial air quality and the microclimate within them, *via* stationary air sampling using the volumetric (impact) method. Dominant species of bacteria and fungi were identified by MALDI-TOF mass spectrometry by comparing the mass spectra of ribosomal proteins (molecular fingerprints) with the spectra in a database. Basic meteorological elements such as air temperature, air humidity, and airflow velocity were measured. Bacterial aerosol concentrations ranged from 37 to 232 CFU · m^{-3} in the indoor air of the caves, and for fungi from 10 to 365 CFU · m^{-3}. The range of bacterial aerosol concentrations in the outdoor environment ranged from 140 to 535 CFU · m^{-3}, being significantly higher than inside the caves. The most common microorganisms in the cave air were mesophilic Gram-positive cocci (*Staphylococcus*), non-spore-forming Gram-positive rods (*Arthrobacter* and *Rhodococcus*) as well as *Bacillus* and *Lactobacillus*, mesophilic actinobacteria (*Streptomyces*) and filamentous fungi (*Alternaria, Penicillium*). The microclimatic measurements carried out in both caves testify to the high stability of temperature and humidity. Measurements made using the katathermometric method showed that the speed of air movement in the static part of both caves ranges between 0.01 and 0.03 m · s^{-1}. The stability of the microclimatic conditions of the cave interior suggests that most microorganisms come from outside and enter the caves during an exchange of air with the external environment. In general, the concentration of microorganisms in the air of these caves is characterized by significant spatial variation within the cave fields but clearly tends to decrease as one moves away from the cave entrance. Our study shows that the content of airborne microorganisms and their spatial distribution in caves are determined by both external factors and the environment of the caves' interior, especially microclimatic, morphometric and morphological factors such as the cave volume, size of the chambers and corridors and maze structure.

Key words: biospeleology, aerobiology, bioaerosol, airborne microorganisms, cave microclimate.

INTRODUCTION

The gypsum maze caves of western Ukraine are among the largest cave systems in the world. The total length of their corridors is measured in tens of kilometres (Zoloushka – 91.0 km, Mlynky – 53.0 km, Kryshtaleva – 22.6 km) and even in hundreds (Optymistychna – 260.0 km, Ozerna – 142.2 km) (Klimchouk and Andreychouk, 2017).

These caves (including smaller ones, not listed above) form a cluster of genetically related karst objects, developed under hypogenic conditions, i.e. from bottom-up circulation of underground waters through a fractured "sieve" of a 15–25 m thick gypsum layer of the upper Badenian (Klimchouk and Andreychouk, 2017).

The caves are characterized by an environment with unusual ecological conditions. The specificity of this environment results from both general factors characteristic of caves (lack of light, high air humidity, oligotrophic), and specific ones, due to local conditions and the parameters of these objects. The specific conditions include that these caves have a large volume, measured in hundreds of thousands of m^3. They usually have only one, usually small, entrance, a factor that shapes the character of their microclimate. They are very stable over the course of the year; seasonal fluctuations of microclimatic parameters (mainly temperature and air humidity) occur only within the entrance zones, constituting a small part of their total area. Due to their deep (up to several tens of metres) location beneath the ground surface, the air temperature in the caves is usually higher than the average annual air temperature outside the cave, which makes them "warm" caves. The large volume, closed nature of the space, slower (due to one entrance hole and a large volume) air exchange with the surface and infiltra-

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tion water inflow explain the high (95–100%) moisture saturation of the air.

The microclimatic similarity of the majority of the caves results from not only their morphological, but also their environmental, geomorphological and climatic conditions, the caves occupying the same climatic zone and being separated by short distances, usually not exceeding several kilometres.

These characteristic environmental features of the maze caves determine the ecological conditions for the living organisms that inhabit them. In general, this environment has few living organisms, and almost no troglobionts. Sparse vegetation (algae, lichens, mosses) can be found only in the area near the cave entrance, which receives a small amount of reflected and diffuse solar radiation. Bats and insects, which treat the entrance area as their shelter, can also be found here. However, these organisms, with few exceptions, do not penetrate deep into the caves. The deeper parts of the caves, and in fact the vast majority of their surfaces, remain the habitat of microorganisms only: bacteria and fungi. Their development is facilitated by the stable microclimate, the relatively warm and humid air, the abundance of organic material dispersed in the clayey-silty bottom sediments, as well as by anthropogenic pollution, i.e. substances brought into a cave by speleologists exploring it.

Among geomicrobiological studies in caves, the greatest attention (and many publications) has been devoted to microorganisms related to the mineral substrate of the cave environment (walls and ceiling, speleothems) and deposits (silt), whereas little attention has been paid to microorganisms present in the waters of cave reservoirs and the condensate, and research into airborne microorganisms in caves is most limited. There are several reasons for this. 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, contains few interesting microorganisms. Secondly, sample collection for the determination of airborne microorganisms in caves presents more methodological and technical difficulties than sampling sediments or water (Wang et al., 2010; Porca et al., 2011; Ghosh et al., 2017), and so present knowledge of microorganisms in the cave air is superficial and scarce. Studies include those by Nakaew et al. (2009) exploring rare strains of Actinobacteria in Thai caves, by Wang et al. (2010) on mycological research in Chinese caves, by Bastian et al. (2009) exploring pathogenic bacteria and protozoa in the Lascaux Cave in France, and by Mulec et al. (2012) describing the airborne microflora of eutrophic caves in Slovenia and Slovakia. Among Polish studies, research has been carried out in the limestone caves of the Ojców National Park (ONP) (Wojkowski, 2013) and in the Bear Cave in Kletno (Ogórek and 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 as regards specific groups of microorganisms.

This study analyses the quantitative and qualitative diversity of microorganisms in the air of the maze caves. This area has been little studied. Studies of gypsum maze caves have so far focussed on their origin, morphology, sedimentology, mineralogy and geochemistry, with considerable success. Aspects of the organic, microbial life, however, remain poorly explored. The microorganisms that can be found in mineral cave deposits, above all in Zoloushka Cave, are fairly well-recognized, but almost exclusively in terms of the sediments and the geochemical processes occurring in them where microorganisms participate (bacteria: ferruginous, manganese, methane-producing and other) (Andreychouk and Klimchouk, 2001; Andreychouk, 2007; Andreychouk et al., 2009). By contrast, the microbiological content of the cave atmosphere has not been well studied. A partial exception is Zoloushka Cave where, a few years ago, the authors assessed the number of microorganisms (bacteria and fungi) present in the cave air (Wojkowski et al., 2019). These studies showed significant microbial diversity, in quantitative terms, but did not include the qualitative (regarding species) characteristics of the microorganisms: our present paper helps fill this gap. We also compare, quantitatively and qualitatively, the abundance of the microorganisms and their species composition in the Zoloushka Cave air (Bukovyna region) with those in another maze cave, Mlynky Cave (Podillya region). This is because, while all of the maze caves of Podillya and Bukovyna are basically similar, Zoloushka Cave differs somewhat from the others as it was exposed artificially, by a gypsum quarry, while still at the stage of waterlogging. Thus it is unlike the other caves, which are now at the dry stage of development, which is more advanced in evolutionary terms, and so is characterized by a slightly different environment. Its underground lakes make its environment more humid than in other Podillya caves, such as Mlynky, Kryshtaleva, Optymistychna and Slavka. There are microclimatic differences also as regards the speed of air exchange between the caves and the external atmosphere, and the caves also differ in the gaseous composition of the air. While in the dry and well-ventilated caves of Podillya, the gas composition of the air inside and outside the cave is similar, the inner air in Zoloushka Cave is clearly enriched in carbon dioxide, its content exceeding 5% in places (Andreychouk et al., 2011). At the same time, the oxygen content is low at 17–19%. The caves studied also differ significantly in the length of their corridors (92 km Zoloushka, 53 km Mlynky), and above all in their volume and shape: in Zoloushka, large galleries prevail, while Mlynky has narrow and high passages. These differences, and in the morphological and morphometric parameters of the caves studied (see below), indicate slightly different geoecological conditions as regards the occurrence and development of microorganisms within them.

STUDY CAVES

The study caves are located in the western region of Ukraine, in its southern part, where gypsum karst associated with Badenian evaporites is extensive. The area is adjacent to the contact zone of the East European Craton with the Carpathian Foredeep, stretching from the north-west (from the Polish border) to the south-east (to the Romanian border) along the outer periphery of the Eastern Carpathian Mountain arc (Fig. 1).

The caves are concentrated in the southern part of the gypsum area (see Peryt, 1996, 2001) – in Podillya (Optymistychna, Ozerna, Kryshtaleva, Mlynky, Slavka, Verteba and others) and the extreme southeastern part of Bukovyna, near the border with Romania (Zoloushka and Bukovynka).

Both caves investigated comprise extensive mazes (Fig. 2). Their basic morphological characteristics are given in Table 1. Zoloushka is much larger, though aspects regarding the cave length, reflecting the history of research into maze caves in this region, have a largely scientific and technical nature. The real dimensions of the caves, as shown by geological indications, may be much larger than the mapped underground fragments.

The only human presence in their interior is through rare visits by speleologists exploring them.

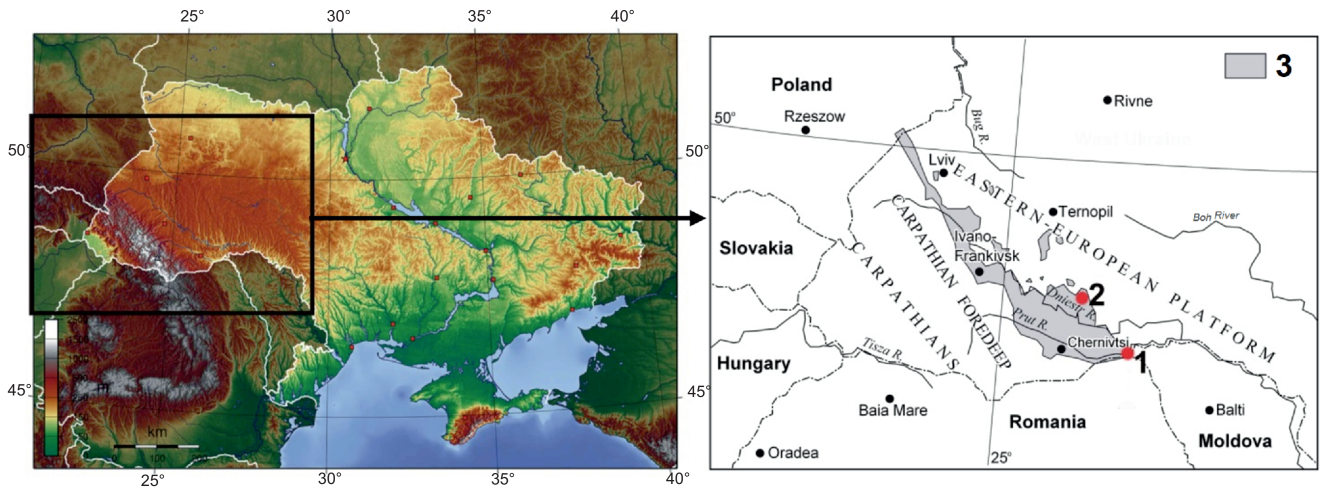

Fig. 1. The western Ukraine gypsum karst area (on the left) and location of the research objects (on the right): 1 – Zoloushka Cave (Bukovyna), 2 – Mlynky Cave (Podillya), 3 – gypsum karst area

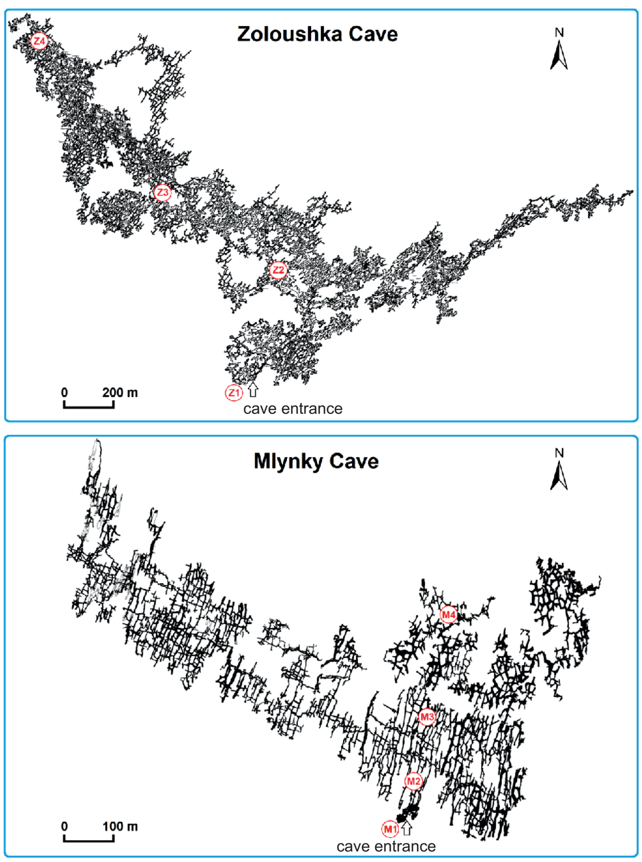

Fig. 2. The maps* of Zoloushka Cave and Mlynky Cave with distribution of measurement sites

Zoloushka Cave: Z1 – outside the cave, Z2 – Chamber of Chernivtsi Cavers, Z3 – Fakela, Z4 – Telephone; Mlynky Cave: M1 – outside the cave, M2 – Central, M3 – Star Skay, M4 – Eureka Grotto; * – maps of caves are property of the Speleological Clubs: Abis (Kishineu, Moldova), Troglodite (Chernivtsi, Ukraine) and Crystal (Chortkiv, Ukraine)

Table 1

Morphological parameters of the caves studied (after Andreychouk, 2016; Ukrainian Speleological Association, 2018, 2021)

Parameter	Zoloushka Cave	Mlynky Cave
Total length of the passages [km]	91.0	53.2
Total volume of the passages [m^3]	700 000	158 000
Territorial extent of the cave [km]	2.1	1.2
Average height of the passages [m]	3.2	2.4
Average width of the passages [m]	2.7	1.5
Total area of the passages [m^2]	185 027	63 000
Height difference [m]	35.0	20.0
Karstification coefficient [%]	47.1	12.0

ZOLOUSHKA CAVE

In terms of volume (700,000 m^3), it is the largest gypsum cave in the world. The cave is situated in the Prut River Valley, not far from where the borders of three countries meet: Ukraine, Moldova and Romania (Fig. 1).

The cave is embedded in a 20–25 m thick layer of Miocene gypsum exposed in the Criva gypsum quarry (Peryt, 1996). The cave became accessible for speleologists due to the pumping out of underground water and the drying of the gypsum unit, a basic condition for gypsum excavation that persists today. Speleological research, involving mainly the mapping of the dehydrated maze, started only in 1977. The part of the quarry with entrances to the maze was filled with overburden sediments within a few years, leaving a 28 metre vertical shaft enabling speleologists to descend into the cave. This shaft now functions as the only entrance to the cave maze and as the main ventilation hole. Currently, a square hole (with an area of ~0.75 m^2) in the ceiling of the shaft entrance is covered (permanently) with a lid made of thick sheet metal, opened only when speleologists visit the cave. The lid is not airtight but it significantly slows the flow and exchange of air in the cave.

The cave maze of Zoloushka was exposed at a stage when the cave was still almost completely (85–90%) filled with water. The currently dry maze caves of Podillya, including Mlynky Cave, underwent this developmental stage tens of thousands of years earlier (Andreychouk, 2007). This circumstance determines the main differences between the cave environments of Zoloushka Cave and the caves of Podillya. Due to the recent water saturation and artificial drainage, Zoloushka remains a rather moist cave. Thick layers of clayey deposits which fill its corridors, reaching 70–90% of their cross-section, are still water-soaked. In the lowest parts of the maze, there are several tens of lakes: dynamic water reservoirs which indicate the location of the water table of the drained karst aquifer (Andreychouk, 2007; Andreychouk et al., 2011, 2021; Andreychouk and Klimchouk, 2017; Wojkowski et al., 2019).

The basic features of the microclimate of Zoloushka Cave have been previously described in detail (Wojkowski et al., 2019), and only those microclimatic conditions present in the caves during air sampling will be described below (Table 2).

MLYNKY CAVE

Mlynky Cave is the fourth largest maze cave in the Podillya group of caves (after Optymistychna, Ozerna and Zoloushka). The total length of its corridors is >53 km, and its volume is ~158,000 m^3. The cave is situated on the right slope of Mlynochky Creek Valley, which is a tributary of the larger Seret River, a left tributary of the Dnister River. The entrance to the cave opens in an exposure of a 16 metre thick gypsum layer in the middle of the valley slope.

As at Zoloushka, the cave maze was exposed artificially (in 1960). The fissure in the gypsum massif was discovered by accident during gypsum mining by inhabitants of the nearby village of Zalissia (cf. Peryt, 1996). As a result of excavation, speleologists have managed to gain access to new parts of the maze several times.

Unlike Zoloushka, Mlynky is a dry cave, currently lacking any watercourses or lakes. Moisture condensation occurs on protruding walls; after heavy rainfall, water dripping from the ceiling gathers in places where it has fallen. The entrance to the cave is located high above the valley floor which, together with the opening and the relatively small volume of the cave, ensures a fairly active exchange of air with the external atmosphere.

Microclimatic measurements performed during air sampling for microorganisms gave very similar results for both caves, of air temperature and humidity, as well as air velocity (Table 2). A radical difference concerns only the content of carbon dioxide in the air: in Zoloushka it is very high (2–5%), while in Mlynky it is very low, similar to that in the air outside the cave (0.04%).

The environments of the two caves differ in their morphological features. Both caves represent the same morphological type, a maze system of underground corridors; nevertheless, the form of the corridors and their sizes in both cases are different. Zoloushka has predominantly large corridors and galleries, often isometric in cross-section, whereas Mlynky is characterized mostly by relatively narrow and high fissure passages (Figs. 2 and 3). The caves also differ considerably in the amount and character of bottom sediments: Zoloushka has widely developed, thicker – up to several metres thick – moist clayey sediments, while Mlynky is dominated by dry and thin clay-carbonate deposits.

The rock overburden above Zoloushka Cave ranges from 20 to 65 m, and above Mlynky Cave from 10 to 40 m. Its lithological nature (Miocene clays plus a series of Quaternary terraces of clayey, sandy and gravelly lithology) effectively isolates the underground space from the external atmosphere. The air temperature in the caves is constant and does not fluctuate seasonally, at ~11°C, slightly higher than the annual average at the surface (9.2°C near Zoloushka Cave and 7.7°C near Mlynky Cave). With similar air humidity, Zoloushka Cave is generally wetter due to the presence of lakes.

MATERIALS AND METHODS

As part of the study, 3 measurement sites were determined in the interior of each cave and, to define the "background", 1 measurement site outside of the cave (Z1, M1), in an open area (Fig. 2). Stationary air sampling was conducted using the volumetric (impact) method with a MAS impactor (model 100, Merck, Darmstadt, Germany; Fig. 4). Additionally, in front of the caves, outdoor air samples were collected to obtain data on the background (atmospheric) level of microbial contamination, at a distance of ~100 m from the caves investigated. At each measurement site, the impactor was placed at a height of ~1.0 m above the cave floor and air samples were collected in triplicate. Trypticase Soy Agar (TSA, bioMérieux) and Malt Extract Agar (MEA, Merck) were used to sample bacterial and fungal aero-

Table 2

The results of microclimatic measurements and amounts of CO_2 from air sampling in Zoloushka Cave (21.02.2020) and Mlynky Cave (23.02.2020)

Measurement sites		Air temperature [°C]	Relative humidity [%]	Vapour pressure [mb]	Water saturation deficit [mb]	Absolute humidity [$g \cdot m^{-3}$]	Air velocity [$m \cdot s^{-1}$]	Amounts of CO_2 [%]
Zoloushka Cave								
Z1	outside the cave	6.2	84	7.9	1.6	6.2	2.40	0.04
Z2	Chamber of Chernivtsi Cavers	11.2	99	13.1	0.2	10.0	0.01	1.9
Z3	Fakela	11.8	95	13.2	0.6	10.0	0.03	2.6
Z4	Telephone	12.2	98	13.9	0.3	10.6	0.01	5.3
Mlynky Cave								
M1	outside the cave	7.6	63	13.2	7.7	9.8	0.92	0.04
M2	Central	11.0	98	12.8	0.3	9.8	0.02	0.04
M3	Star Skay	10.4	98	12.3	0.3	9.4	0.01	0.05
M4	Eureka Grotto	10.5	99	12.5	0.3	9.6	0.01	0.05

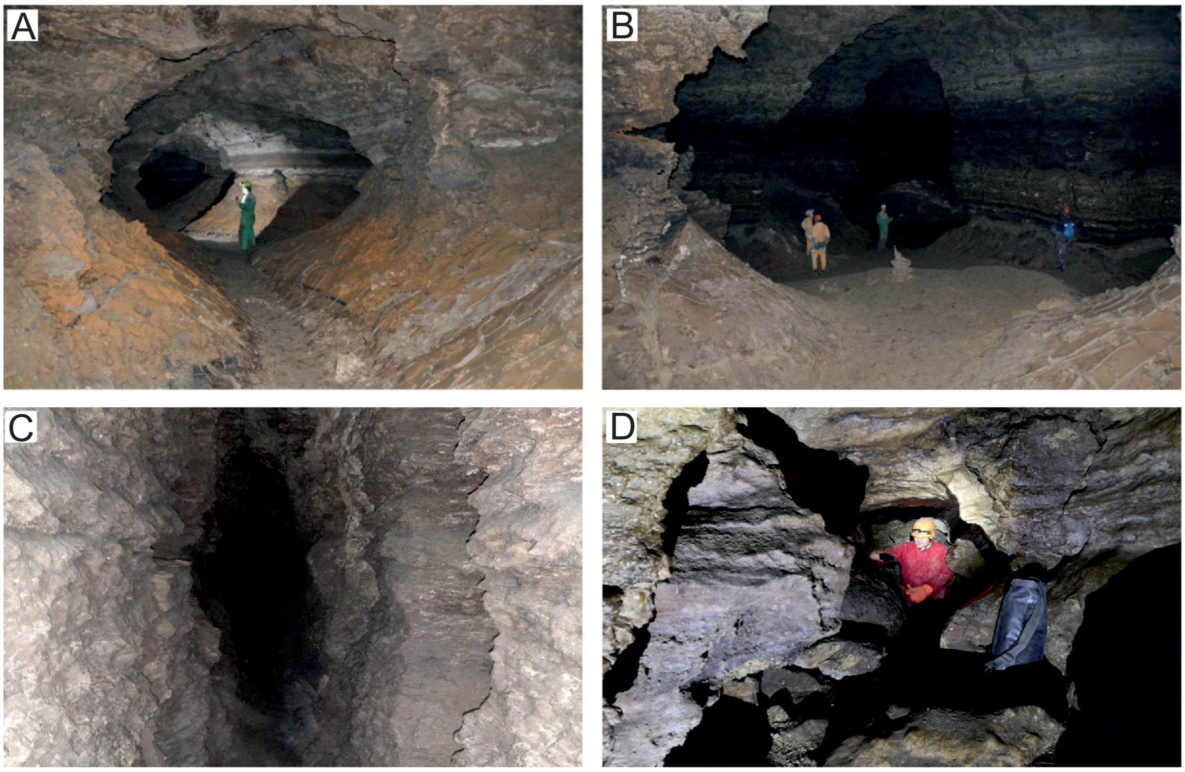

Fig. 3. Characteristic passages in Zoloushka Cave (at the top) and Mlynky Cave (at the bottom)

A – typical passage in the central part of Zoloushka Cave; **B** – Dinosaur's Hall in Zoloushka Cave; **C** – a typical corridor developed along a fracture in Mlynky Cave; **D** – small hall in the north part of Mlynky Cave; photos: B. Ridush (A, B), M. Więckowski (C, D)

sols, respectively. Petri dishes with microbiological media were transported to and from the laboratory to the sampling sites, and on the way back in a thermally insulated transport container, maintaining a constant temperature of ~4°C to prevent any possible damage. TSA plates were incubated for 1 day at 37°C followed by 3 days at 22°C and another 3 days at 4°C, and MEA plates were incubated for 4 days at 30°C followed by 4 days at 22°C. Following the incubation of the microbiological air samples, the concentration of microorganisms, expressed as the number of colony-forming units (CFU) present in 1 m^3 of sampled air (CFU · m^{-3}), was calculated. Dominant species of bacteria and fungi were identified by MALDI-TOF mass spectrometry to determine species/genus affiliation. The MALDI-TOF mass spectrometer identifies microorganisms by comparing the mass spectra of ribosomal proteins (as molecular fingerprints) with spectra collected in a database.

Measurements of basic meteorological elements such as air temperature, air humidity and air flow velocity were carried out in February 2020 (February 21 in Zoloushka Cave and February 23 in Mlynky Cave). Measurements were taken at a height of ~1.0 m above the cave floor. Air temperature and air humidity indices such as relative humidity, water vapour pressure, humidity deficit and absolute humidity were measured using an *Assmann* aspiration psychrometer (Fig. 4). Additionally, using HOBO® Temp/RH data logger sensors, air temperature and relative air humidity were also automatically recorded at 1.0 m above the cave floor. Due to the very slow air movement (<1.0 m · s^{-1}) in the caves studied, it was not possible to measure its velocity with a classical anemometer. Therefore, a *Hill* katathermometer was used to measure the airflow velocity (Fig. 4).

The content of carbon dioxide in the air of the caves studied was determined with the use of a SHI-11 gas interferometer.

The measurement data obtained were statistically analysed using the Kruskal-Wallis test and Spearman correlation analysis with the use of the *Statistica* data analysis software system package, version 12 (StatSoft, Inc., Tulsa, OK, USA), with p values <0.05 as statistically significant.

In the caves studied, these fluctuations occur only at a distance of up to several tens of metres from the entrance to the caves. Beyond the entrance zones, the interiors of the caves are characterized by stable environmental conditions in seasonal, annual, and multi-year courses. We have earlier monitored these conditions extensively, allowing recognition of the spatial distribution of microclimatic conditions in these caves (Andreychouk, 2007; Andreychouk et al., 2011; Wojkowski et al., 2019).

When planning this survey, we had no equipment or logistical limitations regarding the number of measurement sites. Due to the static microclimate of the caves, we decided that 4 measurement sites in each would be sufficient, given the stable temperature and humidity and very slow air flow, constituting a continuum that can be approximated to the entire interior of the caves or, as we did, to "research routes" inside them.

RESULTS

The basic meteorological measurements made during air sampling are given in Table 2. They show the high stability of the microclimatic conditions, as in previous studies (Wojkowski

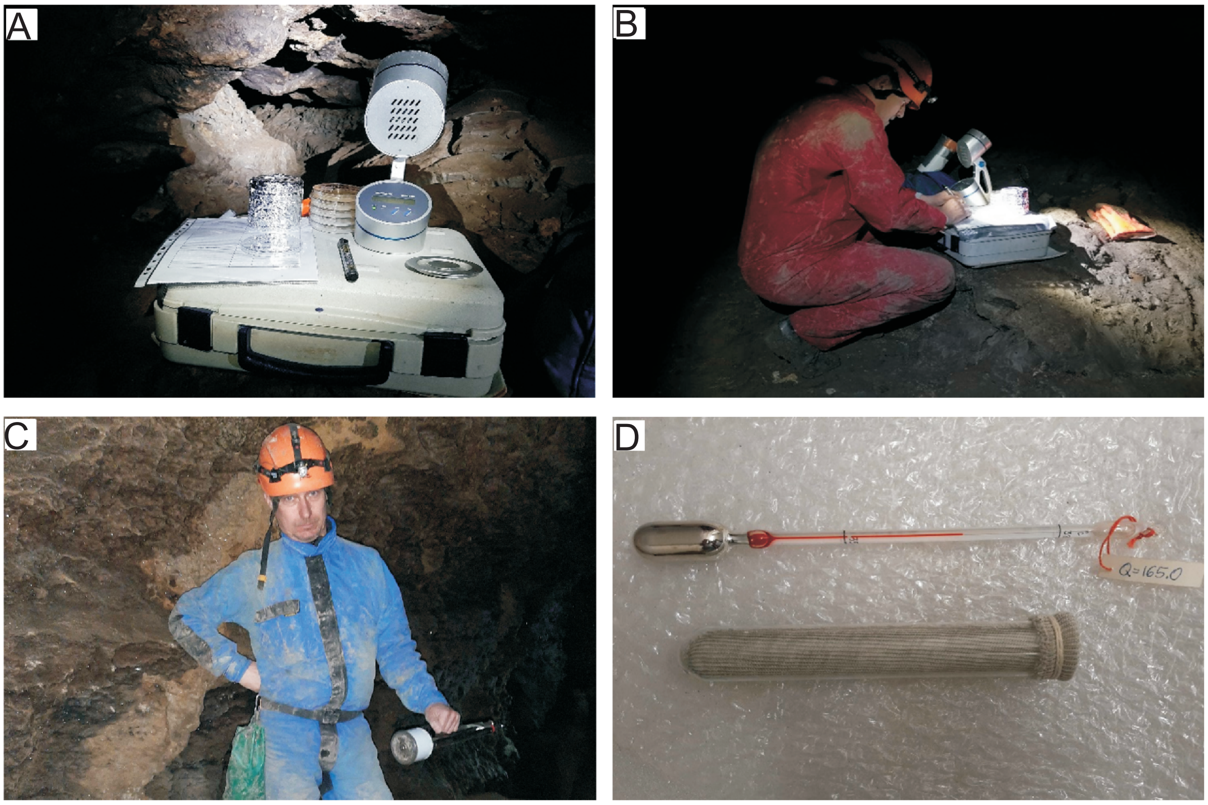

Fig. 4. Microbiological and microclimate measurements

A – air sampler; **B** – air sample collecting; **C** – humidity measurement with aspirated *Assmann* psychrometer; **D** – katathermometer; photos: M. Więckowski

et al., 2019). The air temperature in the static zone of these caves was stable and ranged between 10.4 and 12.2°C. The values of air humidity indices such as relative humidity, water vapour pressure, moisture deficit and absolute humidity reflect the high water vapour content inside the caves. As with the temperature, the air humidity was characterized by high stability, a condition favoured by very weak air circulation in both of the caves. The air between the interior of the caves and the external atmosphere is exchanged at a very slow rate, corresponding to the low air velocity. Measurements made using the katathermometric method showed that the speed of air movement in the static part of both caves ranges between 0.01 and 0.03 m · s^{-1} (Table 2).

Throughout the year, the direction of air circulation in the cave changes, primarily due to differences in the temperature of the air outside and inside the cave, which affect its density and flow direction. The studies in both caves were conducted in February. In winter, the air outside the cave is typically colder and denser than the air inside, which maintains a relatively constant temperature. Consequently, the direction of air flow is from the outside into the cave. This type of winter circulation was clearly observed during measurements in both Zoloushka and Mlynky caves.

QUANTITATIVE ANALYSIS OF MICROORGANISMS IN THE ENVIRONMENT OF THE ZOLOUSHKA AND MLYNKY CAVES

The concentration of microorganisms in the air of the caves studied was characterized by a clear spatial variation, reflected in the spatial distributions of bacterial and fungal aerosols along the measurement profiles in the Zoloushka (Fig. 5) and Mlynky caves (Fig. 6). These were created within a *GIS* environment using deterministic interpolation (natural neighbour) that used barriers (Sibson, 1981). This method helped constrain the interpolated values within the cave boundary. The data processing was carried out by *ArcGIS* software (version 10.4.1).

The values of mean bioaerosol concentrations and the range of its variability in the external environment and in the interiors of the caves investigated are shown in Figures 7 and 8. In the case of bacteria, concentrations ranged from 37 to 143 CFU · m^{-3} and 122 to 232 CFU · m^{-3} in the indoor air of the caves, respectively, for fungi from 10 to 365 CFU · m^{-3} and from 93 to 145 CFU · m^{-3}. The range of bacterial aerosol concentrations in the outdoor environment ranged from 140 to 535 CFU · m^{-3}, being significantly higher than inside the caves (Kruskal-Wallis test: $p < 0.05$). In the external background, no statistically significant differences were evident in the Zoloushka and Mlynky caves with respect to bacterial aerosol concentrations ($p > 0.05$). As regards the cave interiors, in both the Zoloushka and Mlynky caves, all of the studied interiors, regardless of their location, did not differ statistically significantly from each other in the degree of bacterial aerosol contamination (Kruskal-Wallis test: $p > 0.05$) and their concentrations were invariably lower than 232 CFU · m^{-3}. The highest concentration of bacteria in Zoloushka Cave was recorded at measurement site Z3 (Fakela), and the lowest at site Z2 (Chamber of Chernivtsi Cavers) (Fig. 7). In Mlynky Cave, their highest concentration was observed at measurement site M2 (central) and the lowest at M4 (Grotto of the Speleologist "Eureka") (Fig. 7), but these differences were not statistically significant (Kruskal-Wallis test: $p > 0.05$).

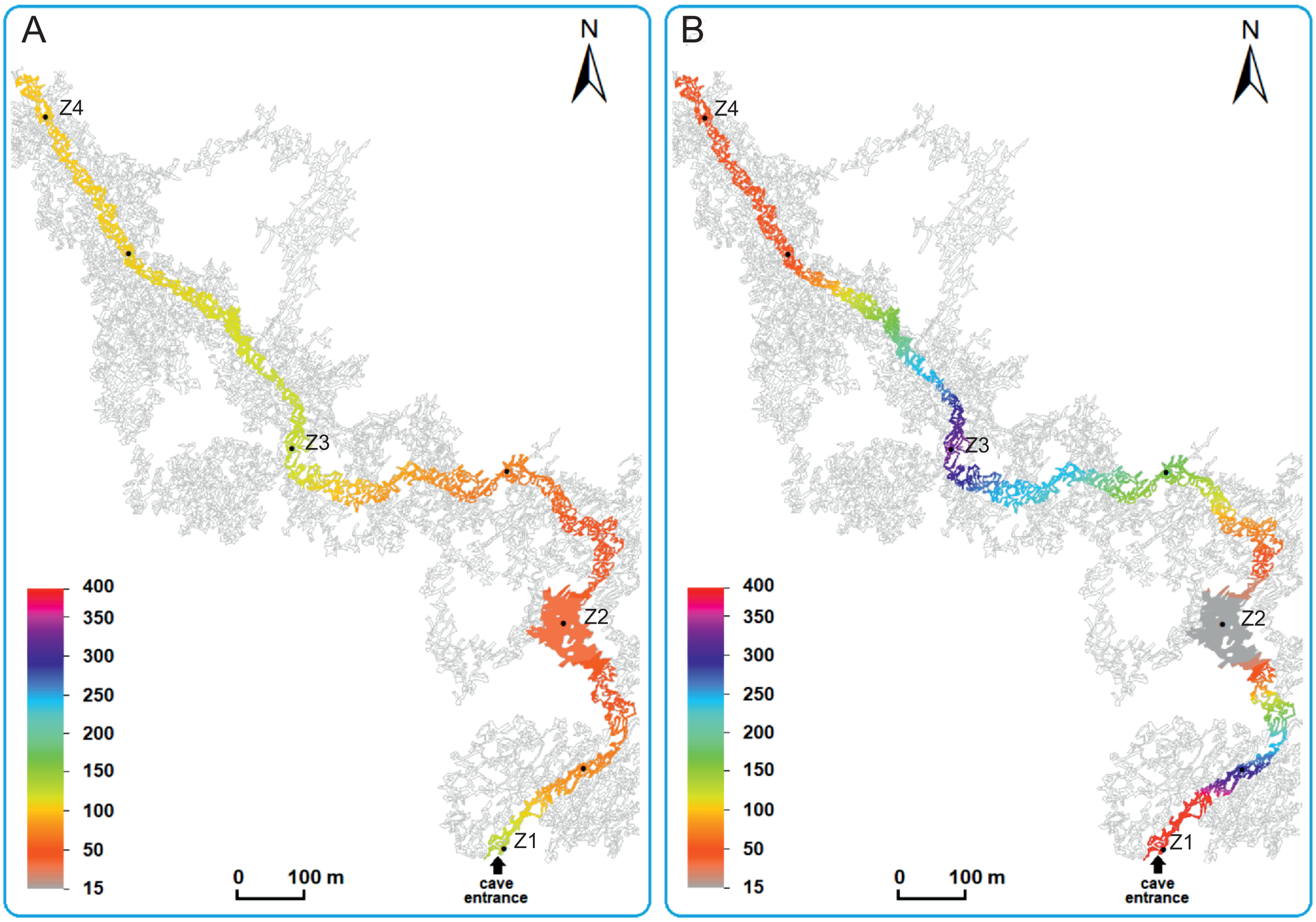

Fig. 5. Spatial distribution of the bacterial (A) and fungal (B) aerosol concentrations (CFU · m^{-3}) along the measurement profile in Zoloushka Cave

While analysing the fungal aerosol in Zoloushka Cave quantitatively, as with bacteria, the highest concentration was observed at measurement site Z3 (Fakela), and the lowest at site Z2 (Chamber of Chernivtsi Cavers; Fig. 8). These differences were not statistically significant (Kruskal-Wallis test: $p > 0.05$). In Mlynky Cave, the highest fungal aerosol concentrations were observed at measurement site M2 (central) (Kruskal-Wallis test: $p < 0.05$) (Fig. 8).

Fungal concentrations were significantly higher in the external background air (range 396 to 750 CFU · m^{-3}) than in the caves (Kruskal-Wallis test: $p < 0.05$). With regard to bacterial and fungal aerosol measurements inside the caves, the mean value of bacterial concentration in the air of Mlynky Cave was significantly higher ($p < 0.01$) than the mean value of the concentration inside Zoloushka Cave.

There was no statistically significant difference in the level of concentration of the fungal aerosol between the two caves (Kruskal-Wallis test: $p > 0.05$). There were also no statistically significant differences in the levels of concentrations of microorganisms between the individual measurement sites located inside the caves ($p > 0.05$). In all interiors studied, irrespective of location, there were no statistically significant differences in the degree of bacterial and fungal aerosol contamination (Kruskal-Wallis test: $p > 0.05$).

When comparing the mean values of bacterial concentrations between the two cave interiors, the highest differences were observed between measurement site Z2 (Chamber of Chernivtsi cavers) located in Zoloushka Cave (38 CFU · m^{-3}) and measurement site M2 (Central) in Mlynky Cave (211 CFU · m^{-3}). When comparing the mean values of fungal concentrations, the greatest differences were found between site Z2 (Chamber of Chernivtsi cavers) (15 CFU · m^{-3}) and Z3 (Fakela) (344 CFU · m^{-3}) located in Zoloushka Cave. Nevertheless, the highest external bacterial and fungal aerosol concentrations (535 and 750 CFU · m^{-3}, respectively) were >2 times higher than those observed inside.

As regards the influence of the microclimatic conditions in Mlynky and Zoloushka on the bioaerosol concentrations, air velocity and moisture deficit had a significant effect on the observed levels of microorganisms. Correlation analysis showed that any increase in these meteorological elements resulted in a significant increase in the levels of both bacteria and fungi in the air (Spearman correlation coefficient for airflow velocity: $R = 0.84$ at $p < 0.05$ and $R = 0.93$ at $p < 0.05$, respectively, and for moisture deficit: $R = 0.76$ at $p < 0.05$ and $R = 0.73$ at $p < 0.05$, respectively). However, the levels of bacteria and fungi in the cave interiors were statistically significantly ($p < 0.05$) negatively correlated with relative air humidity ($R = -0.76$ $p < 0.05$ and $R = -0.73$ $p < 0.05$, respectively). However, as shown by the analyses, no significant correlation ($p < 0.05$) was observed between the temperature and absolute air humidity, water vapour pressure and CO_2 concentration, and the presence of the microorganisms studied in the cave air.

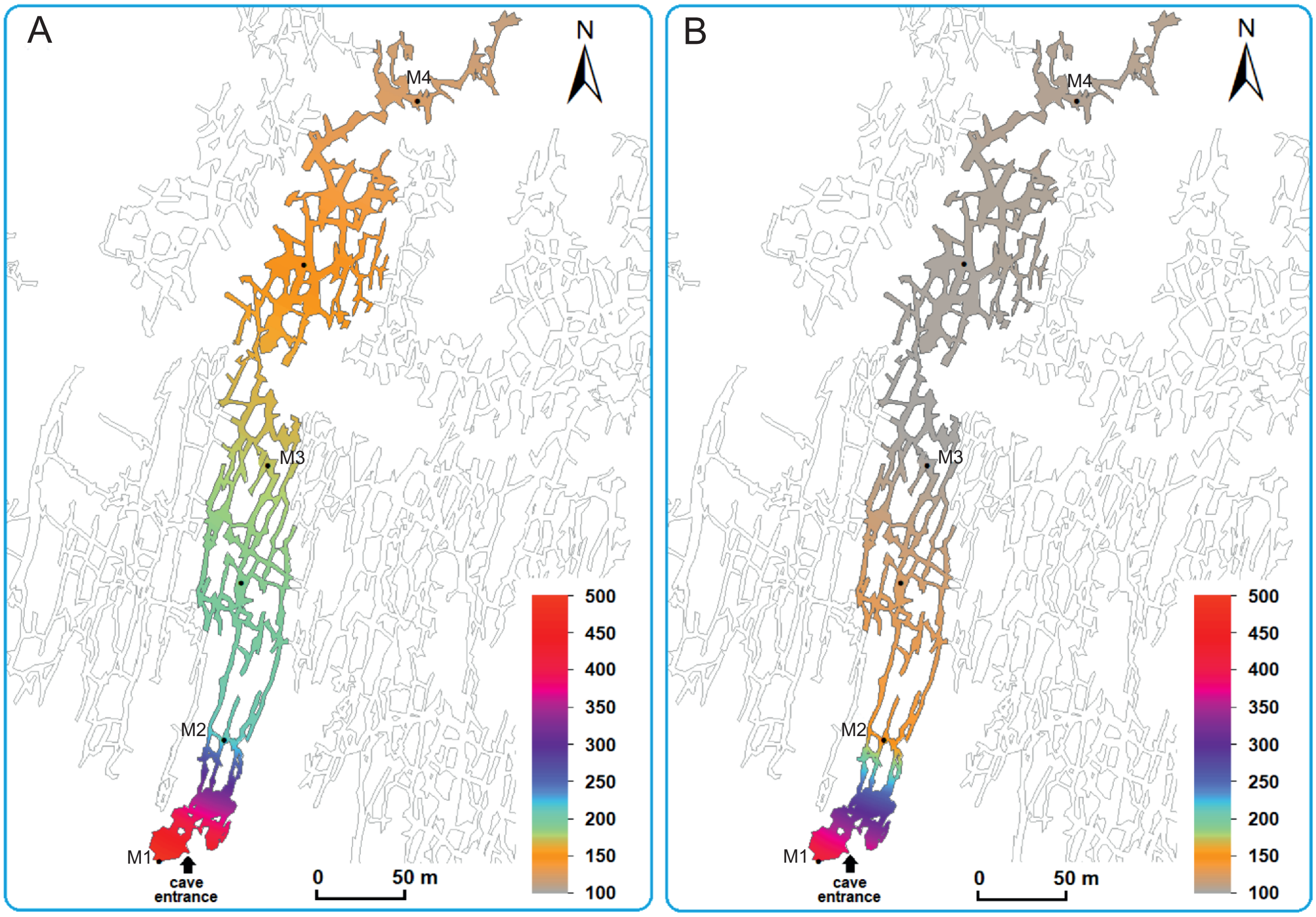

Fig. 6. Spatial distribution of the bacterial (A) and fungal (B) aerosol concentrations (CFU · m^{-3}) along the measurement profile in Mlynky Cave

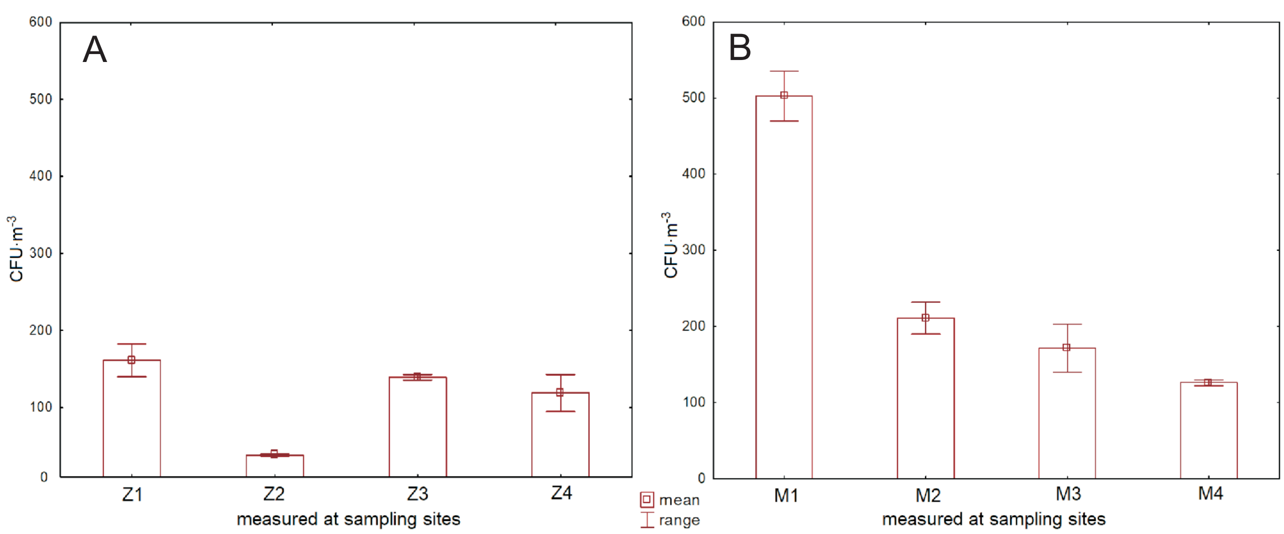

Fig. 7. Mean bacterial aerosol concentrations (CFU · m^{-3}) at sampling sites in Zoloushka Cave (A) and Mlynky Cave (B)

QUALITATIVE ANALYSIS OF MICROORGANISMS IN THE ENVIRONMENT OF THE ZOLOUSHKA AND MLYNKY CAVES

Detailed qualitative data of microorganisms isolated from the air samples at the measurement sites studied are given in Table 3, which lists taxa isolated from the air of the cave interiors, as well as from the outdoor air. In the atmosphere, Gram-positive rods (*Bacillus*), Gram-positive cocci (mainly *Staphylococcus*) and fungi (*Cladosporium, Alternaria*) were the dominant organisms, a typical composition of outdoor microbiota. In the cave interiors, airborne Gram-positive rods (mainly *Bacillus, Lacto-*

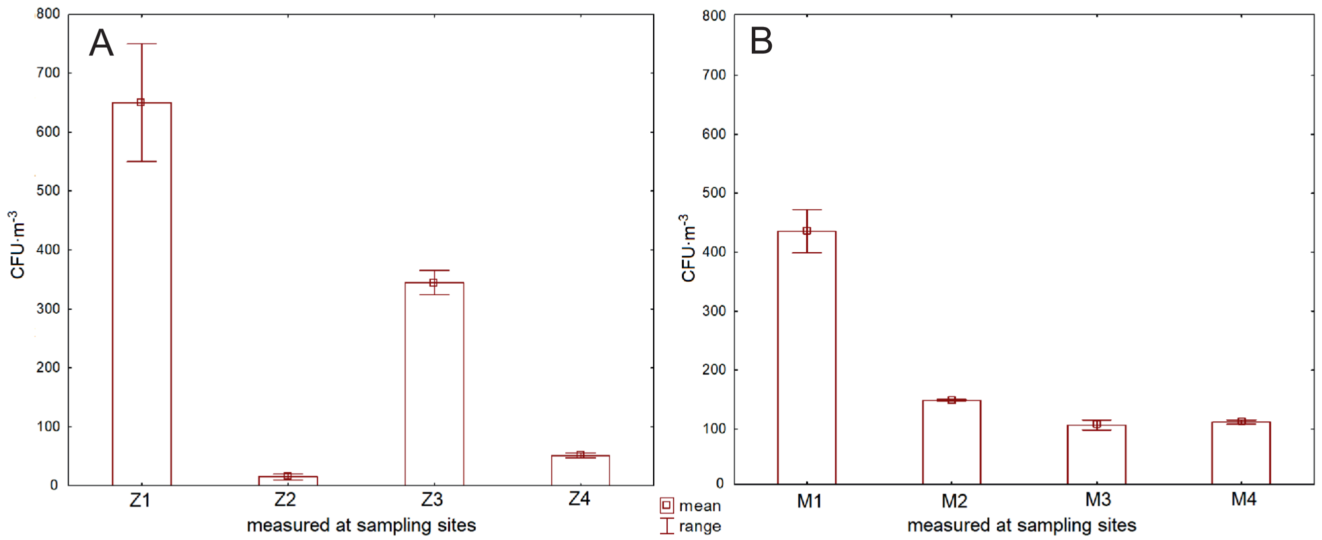

Fig. 8. Mean fungal aerosol concentrations (CFU · m^{-3}) at sampling sites in Zoloushka Cave (A) and Mlynky Cave (B)

bacillus and *Solibacillus*), non-spore-forming Gram-positive rods (*Arthrobacter*, *Rhodococcus*), non-spore-forming Gram-positive rods (mainly *Staphylococcus*) and mesophilic actinobacteria (*Streptomyces*) and fungi (*Alternaria* and *Penicillium*) were the most common organisms. In total, the isolated and identified strains of microorganisms can be classified into 27 bacterial species from 15 genera and 3 fungal genera. The number of isolated microorganism species in the caves investigated ranged from 12 to 18. The highest number of bacterial species was identified in the inner air of Mlynky Cave. The most common species were bacteria from the genera *Bacillus* (8 species), *Lactobacillus* (4 species), *Arthrobacter* and *Staphylococcus* (2 species each). Some species of microorganisms isolated from the air of the caves were not present in the outdoor air. Such bacterial species include *Arthrobacter gandavensis, A. koreensis, Staphylococcus simulans, Rhodococcus erythropolis, Bacillus indicus, B. simplex, B. muralis, Clostridium innocuum, Lactobacillus coryniformis, L. gasseri, L. paracasei, L. perolens, Solibacillus silvestris, Brevundimonas diminuta and Myroides odoratus.*

DISCUSSION

BACTERIAL AND FUNGAL AEROSOL CONCENTRATIONS IN THE CAVES

Cave air has been little studied given the technical difficulties in sampling for the determination of microorganisms in it.

Table 4 summarises results of work published to date in the field of cave aerobiology. It includes average values of bioaerosol concentrations (CFU · m^{-3}) and the range of their variability in the air of the caves listed. Table 4 shows that most microbiological studies have concerned tourist caves, frequently visited by people. Significantly fewer studies have examined natural caves, whose environment has been less exposed to anthropogenic microbiological contamination. Reliable comparison of the results of these studies is difficult, due to the different methods of air sampling, as well as the different periods of the measurements performed. Hence, the current knowledge of microorganisms in cave air is still superficial, and the results obtained in the present study may provide a new point of reference for subsequent researchers addressing this issue.

Our research characterized the cave air of Zoloushka and Mlynky qualitatively and quantitatively under different environmental conditions. Bacterial and fungal aerosol concentrations measured at the designated measurement sites varied depending on the sampling location in the caves selected for the study, which may be attributed to changes in environmental conditions and the spread of microorganisms at the time of sampling (Wang et al., 2010; Wojkowski, 2013).

Airborne fungi usually occur as spores, whereas most airborne bacteria bind to dust particles. The analysis of the bioaerosol concentration values shows that none of the samples exceeded 232 CFU · m^{-3} for bacteria and 365 CFU · m^{-3} for fungi, being within the low range of concentrations generally observed in this type of underground cave (Wojkowski, 2013; Ogórek and Lejman, 2013; Ogórek, 2016; Wojkowski et al., 2019). Similar results were obtained by Wang et al. (2010) in Mogao Grottoes (China), where the average abundance of fungi was 187 CFU · m^{-3}, while in Bear Cave in Kletno (Poland), Ogórek and Lejman (2013) determined the abundance of fungi in the air as 58 CFU · m^{-3}. Fungi in underground facilities very rarely show active growth in the form of overgrowth or efflorescence on rocks or sediments, yet such situations do occur (Bastian et al., 2010; Jurado et al., 2010; Garcia-Anton et al., 2013; Martin-Sanchez et al., 2015). Unfavourable factors for fungal growth in caves are most likely the nutrient-poor environment and moisture deficit, as well as the relatively low temperature of the cave air and high carbon dioxide content (Nováková, 2009).

The studies conducted by Wojkowski (2013) in the interiors of Jama Ani, Okopy Górna, Sąspowska and Złodziejska caves, located in the Ojców National Park (Poland), illustrate the influence of air circulation on changes in the air concentrations of microorganisms. It has been observed that very poorly ventilated caves tend to be more microbiologically polluted. As regards the authors' studies, it is also likely that the low concentrations of bacteria and fungi observed in the cave interiors were also influenced by air exchange, masking environmental factors.

The results have also shown that the content of microorganisms was shaped by the distance from the entrance hole to the cave. The greatest differences can be seen between measure-

Table 3

The microbial taxa isolated from the air sampled in the Zoloushka and Mlynky caves and in outdoor air

Microorganisms		Mlynky Cave	Zoloushka Cave	Outdoor air
BACTERIA				
Gram-positive cocci	*Kocuria rosea*			*
	Staphylococcus equorum		*	*
	Staphylococcus simulans		*	
Non-sporing Gram-positive rods	*Arthrobacter gandavensis*	*	*	
	Arthrobacter koreensis	*		
	Methylobacterium mesophilicum			*
	Glutamicibacter uratoxydans		*	*
	Rhodococcus erythropolis	*	*	
Endospore forming Gram-positive rods	*Bacillus clausii*			*
	Bacillus licheniformis	*	*	*
	Bacillus megaterium	*	*	*
	Bacillus cereus	*	*	*
	Bacillus subtilis	*	*	*
	Bacillus indicus	*		
	Bacillus simplex	*		
	Bacillus muralis	*		
	Clostridium innocuum	*		
	Lactobacillus coryniformis	*	*	
	Lactobacillus gasseri	*		
	Lactobacillus paracasei		*	
	Lactobacillus perolens	*		
	Solibacillus silvestris	*	*	
	Viridibacillus neidei	*		*
Gram-negative rods	*Pseudomonas putida*			*
	Brevundimonas diminuta	*		
	Myroides odoratus	*		
Mesophilic actinobacteria	*Streptomyces badius*	*	*	*
FUNGI				
	Alternaria spp.	*	*	*
	Aspergillus spp.	*	*	
	Penicillium spp.	*	*	
	Cladosporium spp.			*

ment site Z2 (Chamber of Chernivtsi cavers) (38 CFU · m^{-3}) located at a considerable distance from the Zoloushka entrance, and site M2 (central) in Mlynky (211 CFU · m^{-3}), situated relatively close to the cave entrance. In the caves studied, lower bacterial and fungal aerosol concentrations were observed in chambers and passages farther from their entrance.

In both caves, bacterial and fungal aerosol concentrations differed significantly from those in the outdoor air. Their concentrations in the studied interiors were significantly lower than those measured externally ($p <0.05$). This pattern is consistent with the current state of knowledge on the sources of origin of the bioaerosols studied. Docampo et al. (2011), Ogórek and Lejman (2015) and Dominguez-Moñino et al. (2021) emphasised that, in the case of fungal aerosols, the most important sources are located in the external environment (e.g., soil, water, plants, etc.), and the constant – while of different intensities – air migration into a cave is the main process causing their biological contamination. For this reason, the greatest number of fungi in the underground air can be found in those parts of the caves that have the greatest contact with the external environment. Typically this is the cave entrance zone, but may also include deeper parts of a cave. For example, in Zoloushka Cave, the higher fungal bioaerosol concentrations observed at measurement site Z3 (Fakela) may result from a direct connection of the atmosphere to this part of the cave.

Being the main source of microbial contamination, the outdoor air may mask potential internal sources (Dominguez-Moñino et al., 2021).

MAIN FEATURES OF CAVE MICROCLIMATE

Measurements of microclimatic parameters were conducted simultaneously with the bioaerosol measurements. Microclimates can have varying effects on biological aerosol concentrations (Douwes et al., 2003). Measurements of relative air

Table 4

Sample values of average and range concentrations of airborne microorganisms (CFU · m^{-3}) in cave air

Cave	BACTERIA (CFU · m^{-3})		FUNGI (CFU · m^{-3})		Remarks	Reference
	average	range	average	range		
Altamira (Spain)	718	150–1800	133	10–390	SC/SM	Garcia-Anton et al. (2013)
Ardales (Spain)	–	–	510	10–1010	SC/SM	Porca et al. (2011)
Castanar de Ibor (Spain)	–	–	833	20–2200	SC/SM	Porca et al. (2011)
Demänovská (Slovakia)	–	–	104	87–127	SC/SM	Ogórek (2018)
La Garma (Spain)	–	–	733	10–5420	SC/SM	Sanchez-Moral et al. (2021)
Jama Ani (Poland)	2245	270–3439	206	137–318	WC/SM	Wojkowski (2013)
Jarkowicka (Poland)	–	–	134	76–205	WC/UM	Pusz et al. (2015)
Lapa Nova (Brazil)	–	–	51	18–86	SC/SM	Taylor et al. (2013)
Lascaux (France)	2381	70–15520	281	10–950	SC/SM	Martin-Sanchez et al. (2014)
Maijishan Grottoes (China)	–	–	645	216–1389	SC/UM	Duan et al. (2021)
Mlynky (Ukraine)	170	122–232	117	93–145	WC/SM	this study
Mogao Grottoes (China)	–	–	187	110–245	SC/UM	Wang et al. (2010)
Nerja (Spain)	–	–	346	93–754	SC/SM	Nováková et al. (2014)
Niedźwiedzia (Poland)	–	–	52	39–61	SC/SM	Ogórek and Lejman (2013)
Okopy Górna (Poland)	1283	383–2608	260	208–367	WC/UM	Wojkowski (2013)
Postojna (Slovenia)	–	10–100	–	10–100	SC/SM	Mulec et al. (2012)
Sąspowska (Poland)	2721	458–4075	269	100–442	WC/SM	Wojkowski (2013)
Škocjan (Slovenia)	81	2–382	–	–	SC/SM	Mulec et al. (2017)
Złodziejska (Poland)	1781	192–3067	256	175–350	WC/UM	Wojkowski (2013)
Zoloushka (Ukraine-Moldova)	100	37–143	137	10–365	WC/SM	this study

SC – show cave, WD – wild cave, SM – stable microclimate, UM – unstable microclimate

humidity and temperature are used to identify conditions that favour the growth of microorganisms. A temperature range between 18 and 24°C is optimal for the growth and development of many environmental microorganisms, which are mostly mesophilic (Macher, 1999; Flannigan et al., 2001). Although both temperature and air humidity (or water availability as its equivalent) control the growth of microorganisms, humidity is of critical importance. The development of microbial populations is closely proportional to the value of this limiting factor, e.g. it can be slow (or even stop) when relative humidity is low and pronounced when it suddenly increases (Institute of Medicine: Damp Indoor Spaces and Health). The National Academies Press, Washington 2004, WHO Guidelines for Indoor Air Quality: Dampness and Mould, Copenhagen 2009). In the present study, only air velocity, moisture deficit and relative air humidity had a significant effect on the abundance of observed microorganisms.

QUALITATIVE ANALYSIS OF BACTERIAL AND FUNGAL AEROSOLS

Airborne microorganisms are an integral element of the cave ecosystem, but cave aerobiology is still relatively poorly studied. Microbiological studies of caves have paid significant attention to microorganisms (mainly fungi) associated with the mineral substrate of cave walls, ceilings and infiltrates, as well as in sediments and guano (Vaughan et al., 2000, 2011; Northup and Lavoie, 2001; Gottstein Matocec, 2002; Sugita et al., 2005; Nakaew et al., 2009; Nováková, 2009; Bastian et al., 2010; Jurado et al., 2010; Mulec et al., 2012; Taylor et al., 2013; Mulec and Oarga, 2014; Martin-Sanchez et al., 2015; Epure et al., 2017; Lepinay et al., 2018; Muhammad, 2018; Nováková et al., 2018; Zhu et al., 2021). By contrast, few studies so far have considered microorganisms (especially rare bacteria) found in cave air (Wang et al., 2010; Docampo et al., 2011; Martin-Sanchez et al., 2014; Pusz et al., 2015; Mulec et al., 2017; Ogórek, 2018; Zhang et al., 2020; Dominguez-Mońino et al., 2021; Sanchez-Moral et al., 2021). In the present study, the air microbiota of the caves studied were composed of many species of microorganisms characteristic of this type of environment; Dominguez-Mońino et al., 2021). The analysis of the microbial composition of the ambient external air has shown that Gram-positive cocci (*Staphylococcus, Kocuria*), Gram-positive rods (*Bacillus* genus), mesophilic actinobacteria (*Streptomyces*) and fungi (*Alternaria, Cladosporium*) are the dominant organisms, illustrating the typical composition of the outdoor microbiota. In the cave interiors studied, mesophilic Gram-positive cocci (*Staphylococcus*), non-spore-forming Gram-positive rods (*Arthrobacter* and *Rhodococcus*) and bacilli of the genus *Bacillus* and *Lactobacillus* were the most common airborne organisms, and filamentous fungi (*Alternaria, Penicillium*) and mesophilic actinobacteria (*Streptomyces*) were isolated equally frequently. The microorganisms isolated include species of environmental origin (*Bacillus*), which survive in the external environment more easily than Gram-negative bacteria, and include those of human origin, naturally colonising the skin on a commensal basis (*Staphylococcus*) (Lis et al., 2004; Jones and Harrison, 2004). Species from the genera *Staphylococcus* and *Bacillus* were likely to find favourable living conditions in this environment, which is a factor that, with the presence of potential sources, increased their share in the composition of the airborne microbiota present here (Mulec et al., 2017).

The presence of staphylococci in the air of the cave interiors also demonstrates their ability to colonise the new environment in which they survive, an adaptability resulting from the plasticity of biochemical traits, which is determined by genetic metabolic regulation systems, such as the Sigma normal metabolism systems, as well as agr (accessory gene regulator) and sar (staphylococcal accessory regulator) regulation (Chmiel and Mickowska, 2002; Stańkowska and Kaca, 2005; Mulec et al., 2017). As regards *Bacillus bacilli*, their main environment is the external environment (soil, plants, etc.), from which they may have been transferred with atmospheric air. These bacteria are capable of forming endospores that are highly resistant to external physicochemical factors; in this state they can exist in the environment for a long time, being more resistant to adverse environmental conditions than other bacteria (Libudzisz et al., 2009). Some types of Gram-positive rods identified in the study, i.e. *Arthrobacter* and *Rhodococcus*, are ubiquitous in the natural environment, being present in soil and on plants, from where these microorganisms can penetrate the air inside caves (Mulec et al., 2017). Bacteria belonging to the genus *Arthrobacter*, capable of fixing atmospheric nitrogen, have already been found in caves and are associated with the precipitation of calcium carbonate (Gradziński et al., 2012; Ren et al., 2024). Gram-negative bacteria can be also identified in the air of the caves.

In the present study, mesophilic actinobacteria (*Streptomyces*) represented a relatively large proportion of the airborne microorganisms identified. These bacteria have the unique ability to colonise a variety of solid surfaces from rocks to ceramic materials, to construction and finishing materials. Moreover, they are able to form spores, highly resistant to dehydration stress and possessing remarkable metabolic activity (Reponen et al., 1998; Marcinowska, 2002; Górny, 2003).

The analysis of the fungal composition of the bioaerosol in the interiors reveals that moulds such as *Alternaria, Aspergillus* and *Penicillium* are frequently present in the cave interiors. The fungal genera isolated from the air of the study caves are considered to be typical of this type of interior (Nováková, 2009; Ogórek and Lejman, 2013; Dominguez-Moñino et al., 2021). Wang et al. (2010), while studying the air in Mogao Grottoes (China), also found that the most commonly isolated fungal genus was *Cladosporium*. In contrast, the data presented by Docampo et al. (2011) indicated *Cladosporium* as the most abundant genus of fungi isolated from the atmospheric air.

The widespread presence of fungi in cave air is primarily determined by their production of very abundant spores, as well as their extremely modest nutritional and environmental requirements. The optimal growth conditions for these microorganisms are high humidity of air and substrate, although many species are characterized by their ability to survive in very dry conditions (these include xerophilic species of the genus *Penicillium*) (Docampo et al., 2011; Ogórek and Lejman, 2013). Since many species from the fungal genera isolated are common in soil, their high frequency in the outdoor air may also naturally translate (e.g., by air penetration) into their high frequency in cave interiors. The relationships presented in this study are consistent with those previously observed in this type of interior environment by Nováková (2009), Docampo et al. (2011), Zhang et al. (2017, 2020).

The stable environmental conditions of caves may favour the growth of microorganisms (Taylor et al., 2013). The quantitative and qualitative differences discussed in the occurrence of microorganisms in the environment of the caves studied at this stage of the research cannot be unambiguously explained. Nevertheless, based on knowledge of the environments of both caves, we can propose some explanations of the facts found.

Our microclimatic measurements show that the values of such meteorological elements as temperature and humidity were very similar in both caves (Table 1) and, in ecological terms, should not cause such a large spatial variation of microorganisms as was found. The air velocity was also similar in both caves, but the conditions of movement and exchange of the cave air with the external environment were different. These differences concern the volume of the investigated caves (air capacity), labyrinth permeability, rate of exchange with the external atmosphere and gaseous composition of the air.

The morphometric factor, i.e. the much larger volume of Zoloushka Cave than of Mlynky Cave (Table 1), its large corridors, much larger than the fissure passages in Mlynky Cave, can cause, with the same air flow velocity and similar concentration of microorganisms in the entrance, a significant "dilution" of their content in the air of Zoloushka Cave. The morphological factor, in turn, such as the structure of the maze, as in Mlynky Cave with its dense network of narrow fissure passages giving a large total surface area of walls in contact with underground air, can cause a higher deposition of microorganisms on its walls than in Zoloushka Cave. Unlike Zoloushka Cave, which is a large air reservoir, Mlynky Cave is a filter for the air flowing through it, effectively retaining airborne microorganisms.

The morphological factor (the "filter" effect) in Mlynky Cave may be the reason for a clear tendency of its decreasing number of microorganisms on moving away from the cave entrance. The air flowing through the stone "sieve" of narrow fissures gradually cleanses itself of microorganisms that deposit on its walls. In contrast, the gigantic corridors of Zoloushka Cave are not able to perform the function of a filter as effectively, so the concentrations of microorganisms deep inside the cave, both in its air and on the surface of its walls, do not show a similar pattern. Moreover, measurement site Z2 (Chamber of Chernivtsi Cavers), representing the largest space (hall) in the cave (with a volume of 45,600 m^3), has the lowest amount of microorganisms (Figs. 5, 7 and 8).

Obviously, the quantitative variation of microorganisms in the caves may also be influenced by circulation factors, including the nature of the air circulation inside the cave itself, but the large size of both caves significantly reduces its potential role in this respect.

LIMITATIONS OF THE STUDY

The major limitation of this study seems to be the number of samples evaluated; however, the premises analysed were selected as reasonably representative of the study environments. The quantitative and qualitative results obtained are additionally biased by the sampling time and many environmental, spatial and temporal changes. In this study, the microbial aerosol sampling was limited in time (3 min) and space (stationary sampling only).

Anthropogenic factors may also play a role in the measurements, not least contamination by microorganisms brought into caves by speleologists exploring them. However, this issue needs further research. In this study, we tried to eliminate or minimise potential anthropogenic impact by choosing methodologically appropriate sites for air sampling, staying away from the impactor during air sampling and limiting acivities during the measurements. In addition, the cave surveys were planned so that there would be long periods of at least one month before the measurements, during which the caves would not be disturbed by speleologists.

Nevertheless, all these limitations should be kept in mind as they may, to some extent, underestimate the real environmental exposure.

CONCLUSIONS

Our study is one of the very few examples of aerobiological research in natural caves. The results obtained may provide a new point of reference for further research into cave aerobiology, especially for studies performed in caves with a natural environment, previously unpolluted by the presence of humans. Bioaerosol concentrations in the caves we studied (Zoloushka and Mlynky caves) were generally lower than in tourist caves (Table 4).

The study shows that the content of airborne microorganisms in gypsum maze caves of large sizes is determined by both external factors and by the environment of the caves' interiors, especially microclimatic and morphological factors. The results indicate a decisive role of the external environment in the penetration of microorganisms into the cave atmosphere. This results from the very weak internal air circulation and slower air exchange between the cave interiors and the external atmosphere. The stability of the microclimatic conditions of the cave interior shows that most microorganisms come from outside and enter the caves during the exchange of its air with the external environment.

The analysis of the relationship between microclimate elements and bioaerosol concentrations indicates that only air velocity and moisture deficit had a significant effect on the level and composition of microbial aerosols. However, no significant effect of temperature and absolute air humidity or CO_2 concentration on the number of microorganisms was observed.

The results of our study have demonstrated that morphometric and morphological factors such as the cave volume, the size of the chambers and corridors, and the maze structure can play an important role in shaping the level of microorganism concentrations in the cave air. The lower concentrations of microorganisms in the interior of Zoloushka Cave than in Mlynky Cave may result – given similar concentrations of microorganisms at the entrances to both caves – from the "dilution" of bioaerosols in the volumetrically much larger space of this cave. As for the decreasing numbers of microorganisms as one moves away from the cave entrance, a tendency observed particularly clearly in Mlynky Cave, may be a result of a "filtration" effect, i.e. the gradual settling and absorption of bioaerosol on the walls of narrow fissure corridors during the slow flow of air masses from the entrance into the cave.

In addition to microbiological studies, microclimatic studies are of equal importance, as they can help interpret the results and explain the patterns observed. We have ascertained that there are individual variations in the microbiological composition of cave air, depending on the specific features of the cave in question. More general conclusions on the occurrence of microorganisms in cave air will need analysis of a considerably larger number of samples, a task that we plan to carry out: we have already initiated new microbiological measurements and have selected caves of different genetic, morphological and microclimatic types for this planned study.

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