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Original Research

Influence of the External Environment on Airborne Fungi Isolated from a Cave

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Abstract

Atmospheric air may contain a variety of biological components, e.g. microorganisms, which may be harmful to the internal environment of caves and the health of its visitors. Our study aimed at mycological evaluation of the air inside and outside of Niedźwiedzia Cave in Poland. Between 123 and 214 CFU fungi per 1m^3 of air were isolated from the air sampled in the cave, and ca. 600 CFU from the air sampled outside of it. *Cladosporium herbarum* and *Rhizopus stolonifer* are the species most frequently isolated from the air inside the cave and *Cladosporium herbarum* from the outside.

Keywords: airborne fungi, Niedźwiedzia Cave, environmental, Lower Silesia, Poland

Introduction

People inhale and exhale about 10 liters of air per minute [1]. Atmospheric air contains fixed amounts of oxygen, nitrogen, and carbon dioxide, as well as various biological contaminants [2]. These include viruses, protozoa, bacteria, cells, and tissue fragments of plants and animals, as well as fragments of fungal mycelia and spores [3]. Microbial air contaminants may emit secondary metabolites such as mycotoxins, endotoxins, enterotoxins, and enzymes that may adversely affect human health [4]. Air contaminated with microorganisms (and especially with fungi) has a direct impact on human health, which results in more frequent incidences of allergies and respiratory diseases [5].

The presence of microorganisms and visits by tourists can both result in changes to the microclimatic conditions of a cave. The fact of the cave being accessible to visitors can alter its microclimate because tourists can contribute *inter alia* to the increase of air temperatures and CO_2 concentration [6]. The visitors also can be a source of organic matter and new microorganisms [7]. Secondary metabolites of fungi and bacteria may cause chemical reactions with the substrate, contamination of the substrate by pigments [8, 9], and lithogenic processes, *inter alia* speleothem deposition or cavern enlargement [10, 11] as well as litholitic processes [12]. Evidence of the microbial activity in a cave includes dots on cave surfaces, unusual coloration of speleothems, precipitates, corrosion residues, structural changes, and the presence of biofilms [13]. However, fungi and bacteria play an important role in the feeding strategies of cave fauna, e.g. in the microbial decomposition of organic material [14]. Microbial presence is very well observed in bat guano, animal excrements, and dead animals [12].

One of the most universal techniques for studying microbial air pollution is the collision method. It uses an air sampler and Petri dishes containing solidified culture medium. The impact force causes the microorganisms and their spores to stick to the medium [15]. The type of culture medium is a very important factor in determining microbial air pollution for the collision method. The type of medium affects directly the number and species composition of

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fungi and bacteria isolated from the air. This effect is related to the variable availability of nutrients, such as sources of carbon, nitrogen, and microelements to the sampled and cultured microorganisms, in different types of media. It has been demonstrated by the study of Ogórek et al. [16, 17], who had used different culture media to determine the mycological quality of air. The results have shown that, depending on the culture medium, different numbers and species composition of fungi were obtained from air samples taken in the same room. The best medium for the isolation of dermatophytes were Czapek-Dox Agar medium.

The Study Area

Niedźwiedzia Cave is located in the village of Kletno, Stronie Śląskie commune, within Kleśnica Stream Valley in the Massif of Śnieżnik of Sudety range, Lower Silesia. The cave discovery was made in 1966 as a result of the exploitation of "Kletno III" mines. Until today about 3,300 m of corridors have been investigated on three levels (upper, middle, and lower, connected by chimneys). Corridors in a total length of 360 m have been opened as tourist tracks since 1983. The cave is protected as Bear Cave Reserve, with an area of about 89 hectares. The length of underground rivers is about 5 km and they carry about 30 m^3 of rock material per year out of the inner space of the cave [18]. The geographic coordinates of the Cave are 50º14,068' N and 16º50,558' E, and its entrance is 800 m a.s.l.

The middle level is partly developed for visitors and this level was tested in the presented study. The tourist trail starts and ends with a system of air locks. The Lions' Hall is a flat room of 10 m × 10 m size, filled with the internal, rubble-clayey sediments full of bones of Pleistocene fauna. An exhibition there demonstrates methods of palaeontological studies. In the walls of the Hall the potholes and the speleothems can be observed. The Palace Hall is the most spectacular part of the cave, with attractive speleothems on the rocks, e.g. stalactites and stalagmites. The Primitive Man Corridor is the final element of the tourist trail.

Material and Methods

The air samples were taken on 5 May 2012 from one location outside the cave (near the entrance pavilion) and from three locations inside of it (Lion's Hall, Palace Hall, and Primitive Man Corridor). The air temperature and relative humidity inside the cave amounted to 6ºC and almost 98%, respectively. The air temperature and relative humidity outside the cave were measured using an LB-522 thermo-hygrometer (LAB-EL) and during the study amounted to about 24.5ºC and 58.4%.

Mycological Evaluation of the Air

In order to examine the air acidified PDA medium (potato dextrose agar with 0.5% acetic acid, Biocorp) was used. The sampler (Air Ideal 3P) was programmed for air sample volumes of 50 L, 100 L, and 150 L. Measurements in particular locations were performed in six-plicate for each volume. The sampler was positioned 1.5 m above the cave floor level. The incubation of the cultures on Petri dishes of 90 mm diameter was carried out at room temperature (22ºC) for 2-7 days, in the darkness. After incubation the number of CFU/1.0 m^3 (colony-forming units per 1.0 m^3 of air) was calculated and the fungi were identified.

Fungal Identification

The fungal colonies grown on all the Petri dishes were counted and identified. The specific identification of the sampled fungi was performed using macro- and microscopic observations, namely the morphology of hyphae, conidia, and sporangia, of the colonies that had grown on the culture media according to the commonly accepted methods used in mycological laboratories. The fungi were identified using diagnostic keys [19-23].

Media

The following media were used: acidified potato dextrose agar (PDA with 0.5% acetic acid, Biocorp), potato dextrose agar (PDA, Biocorp), Czapek-Dox Agar (1.2% agar, Biocorp) and malt extract agar (MEA, Biocorp). Acidified PDA medium was used for the isolation of fungi from the air, whereas we used the standard PDA medium for identification of some fungi species. Czapek-Dox agar medium and MEA medium were used for identification of the *Penicillii* and *Aspergillii*.

Results

The number of filamentous fungi isolated from the air at the tested locations inside the cave ranged between 123 to 214 CFU in 1m^3 of air. In particular locations, between 5 to 9 species of filamentous fungi were isolated, whereas the total number of detected species was 12. The majority of filamentous fungi were isolated from Lion's Hall and the smallest number of species from this group was observed in Primitive Man Corridor (Table 1, Fig. 1). More than 600 CFU/1 m^3 and 9 fungi species were isolated from outside air (in front of the entrance pavilion; Table 2, Fig. 2).

Cladosporium herbarum and *Rhizopus stolonifer* were found as the most frequently isolated airborne fungi in this cave, where *C. herbarum* was the most isolated microfungal species from outside air. Conversely, the smallest group of the isolates obtained from the inside air was made by *Penicillium citrinum* and from the outside – by *Epicoccum nigrum* (Tables 1, 2 and Fig. 2).

Fungi such as *Acremonium strictum*, *Beauveria bassiana*, *Mucor mucedo*, *Penicillium citrinum*, *Sordaria fimicola*, and *Ulocladium alternariae* were isolated only from the air sampled inside the cave, whereas *Alternaria alternata*, *Botrytis cinerea*, and *Epicoccum nigrum* were exclusively isolated from outside air.

Table 1. Fungi isolated from the air inside Niedźwiedzia Cave.

Location of measurement	Fungi species	Acidified PDA medium		
		Air – CFU/m³		Species [%]
		Species	In total	
Lion's Hall	*Aspergillus niger* Tiegh.	1	214	0.5
	Beauveria bassiana (Bals.-Criv.) Vuill.	53		24.8
	Cladosporium herbarum (Pers.) Link	90		42.1
	Mucor mucedo de Bary & Woron	7		3.3
	Penicillium chrysogenum Thom	26		12.1
	Penicillium citrinum Thom	1		0.5
	Rhizopus stolonifer (Ehrenb.) Vuill.	29		13.6
	Sclerotinia sclerotiorum (Lib.) de Bary	3		1.4
	Sordaria fimicola Roberge ex Desm.	4		1.9
Palace Hall	*Aspergillus niger* Tiegh.	3	152	2.0
	Beauveria bassiana (Bals.-Criv.) Vuill.	10		6.6
	Cladosporium herbarum (Pers.) Link	39		25.7
	Penicillium chrysogenum Thom	15		9.9
	Penicillium expansum Link	10		6.6
	Rhizopus stolonifer (Ehrenb.) Vuill.	71		46.7
	Ulocladium alternariae (Cooke) E.G. Simmons	4		2.6
Primitive Man Corridor	*Acremonium strictum* W.Gams	5	123	4.1
	Aspergillus niger Tiegh.	15		12.2
	Beauveria bassiana (Bals.-Criv.) Vuill.	3		2.4
	Cladosporium herbarum (Pers.) Link	45		36.6
	Rhizopus stolonifer (Ehrenb.) Vuill.	55		44.7

Discussion

The most important factors affecting the survival of fungi in the environment are temperature and humidity [24]. During the presented study the air temperature inside the cave was approximately 6ºC and the relative humidity approached 100%. However, outside the cave (near the entrance pavilion) the temperature was higher and reached 24.5ºC, but the humidity was lower (58.4%). In particular, the air temperature affected a number of the isolated fungi, for it was three times as high outside the cave as in its interior.

The location of Niedźwiedzia Cave and its surroundings also contributed to a larger number of spores sampled outside of it. The cave is located in Śnieżnik Mt. Massif in lower subalpine zone at 800 m a.s.l. Matuszkiewicz [25] reports that the vegetation layer of the lower subalpine zone is mainly covered by spruce monocultures and grasses. Numerous vegetation provides good conditions for fungi development. Furthermore, studies were carried out in the middle of spring (5 May), in a period of intensive plant growth. This period is particularly conducive for fungal development.

Viable microbial propagules have their specific low gravities and are therefore disseminated by air currents in the air both inside and outside caves. Passive microbes

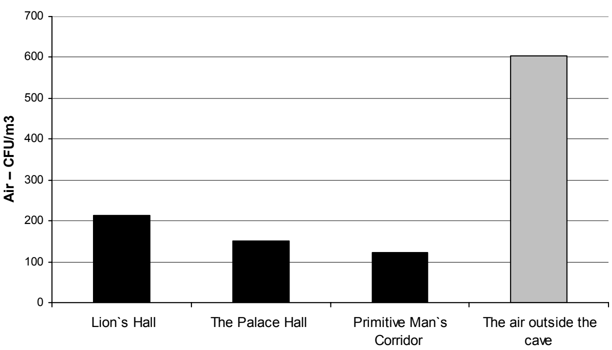

Fig. 1. Total number of fungi isolated from the outside and inside air of Niedźwiedzia Cave.

Table 2. Fungi isolated from the outside air of Niedźwiedzia Cave (near the pavilion entrance to the cave) – height about 800 m a.s.l.

Fungi species	Acidified PDA medium		
	Air – CFU/m³		Species [%]
	Species	In total	
Alternaria alternata Keissl.	10	605	1.7
Aspergillus niger Tiegh.	9		1.5
Botrytis cinerea Pers.	15		2.5
Cladosporium herbarum (Pers.) Link	490		81.0
Epicoccum nigrum Link	5		0.8
Penicillium chrysogenum Thom	18		3.0
Penicillium expansum Link	13		2.1
Rhizopus stolonifer (Ehrenb.) Vuill.	9		1.5
Sclerotinia sclerotiorum (Lib.) de Bary	36		6.0

transport by air currents are an important mode for spreading their inoculum but, at the same time, it is dependent on the seasonal air circulation [12]. In the air outside the cave more fungi were obtained than in its interior. This was probably related to the date of sampling (spring) and stronger air currents outside the cave than inside, as the entrance and exit of the cave are closed by a system of air locks. This system isolates the cave environment from external factors. Apart from the air currents, another carrier of microbes into caves is dripping and seeping water. The introduction of microbes by animals and humans also represents an important mode of transport deep into the karst underground.

Most of the filamentous fungi on the acidified PDA medium were isolated from Lion's Hall and the least of them were sampled from Primitive Man's Corridor. Lion's Hall is the first location of research and is located closest to the entrance to the cave, while Primitive Man Corridor is the last location of research and is located closest to the cave exit. The abundance of fungi decreases from the entrance toward the cave exit. Similar results were obtained by Ogórek et al. [7] on the same day of research by using the PDA medium. They report that this situation is probably caused by the airflow that occurs in the cave during summer, or by the tourist traffic. Behind the entrance to the cave, different processes, anthropogenic and natural, may alter air circulation. During the summer season, a permanent stream of the warm external air flows next to the cave entrance area, and air from the outside is conveyed to the inside of the cave [26].

In the present study, by using the acidified PDA medium 12 species of filamentous fungi were obtained from the air inside the cave. The most frequently isolated among them were *C. herbarum* (from the internal and external air samples) and *R. stolonifer* (only internal air). The incidence level of the fungi isolated from the cave`s interior (*C. herbarum* and *R. stolonifer*) may be considered similar, as the small observed difference may be caused by imprecision of the testing methods used or by the difference between the samples collected from the same site. Ogórek et al. [7] reported that in the same period of study in 2012 only 9 species of filamentous fungi and one yeast species were isolated using PDA medium, and that the most abundantly isolated fungi was *R. stolonifer*. Most probably, this situation was due to the precision of the method and type of the medium used, because medium type directly affects the number and species composition of fungi and bacteria isolated from the air [16, 17].

In the current work *Cladosporium herbarum* was the fungus most frequently isolated from Niedźwiedzia Cave. Species of the genus *Cladosporium* are common in many

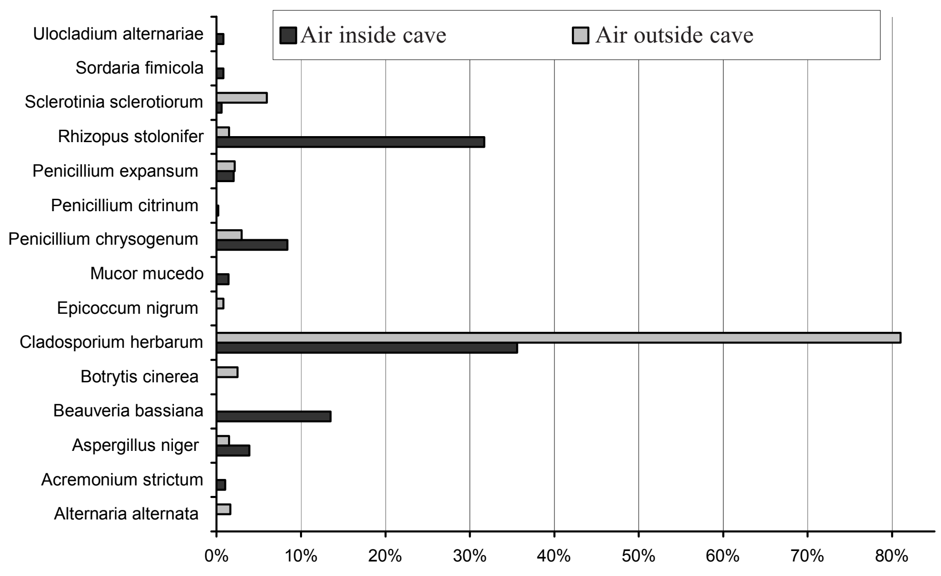

Fig. 2. The percentage proportion of fungi species isolated from the outside and inside air of Niedźwiedzia Cave.

parts of the world, as they are cosmopolitan organisms. Their conidia can be found in air, soil, and water [27, 28]. They also are commonly isolated from residential areas and public use terrains as well as from food products [29, 30]. These fungi are active at low temperatures and in high humidity [31]. Studies of atmospheric air of various regions of Europe show that the spores of *Cladosporium* spp. dominate in 80% of all the caught spores and the peak season is in the summer [32, 33]. The number of *Cladosporium* spores necessary to induce symptoms of allergic respiratory system disease in most patients with hypersensitivity to these allergens in the Polish population was estimated at 2,800 spores in 1 m^3 of air [34]. Some species are pathogenic to various crops, causing economically important plant diseases, while others are important only endemically. Fungi commonly occur as saprotrophs on crop remnants and plant remnants [35]. These fungi can cause diseases of plants, e.g. the black point of cereals, scabs of cucurbits in *Cucurbita,* and brown spots on tomato leaves [28, 36, 37]. *Cladosporium* spp. can cause allergic reaction in humans, which sometimes leads to asthma. Rarely, they can cause opportunistic infections, and that is mainly related to patients with their immune system compromised, such as patients with haematological diseases or those showing AIDS [38].

Some of the fungi isolated from the air inside Niedźwiedzia Cave may be harmful to the rocks, e.g. fermentative microorganisms that excrete many organic acids into the environment, including acids which may cause biodeterioration of natural stones, as well as brick, concrete, or mortars [39, 40]. Fungi of the genus *Penicillium* and *Aspergillus* belong to this group of microorganisms.

Conclusions

1. Internal microclimate of the cave directly contributes to the decrease in the number of filamentous fungi as compared to the external air.
2. The fungus most frequently isolated from the air inside Niedźwiedzia Cave and outside of it is *Cladosporium herbarum*.
3. The environment around the cave and time of the research directly influence the high content of *Cladosporium herbarum* in the air.
4. The incidence level of the fungi isolated from the cave's internal air constitutes no threat to the health of tourists visiting Niedźwiedzia Cave.

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