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Yearly microbial cycle of human exposed surfaces in show caves

Silviu Bercea[1], Ruxandra Năstase-Bucur[1], Oana Teodora Moldovan[1], Marius Kenesz[1], Silviu Constantin[2]

1 *Emil Racoviță Institute of Speleology, Cluj Department, Cluj-Napoca, Romania* **2** *Emil Racoviță Institute of Speleology, Geospeology and Palaeontology Department, București, Romania*

Corresponding author: *Silviu Bercea* (silviu.bercea@gmail.com)

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Abstract

The human impact upon the subterranean microbiomes is not only a peril to the cave environment but might also affect future visitors. We focused on the changes that humans induced on the surfaces they came in direct or indirect contact with inside two intensely visited Romanian show caves, by means of commercially available microbial rapid test kits and molecular identification.

Overall culturable bacteria abundance in the caves maintained high levels year-round while *Enterobacteriaceae*, coliform bacteria and *Escherichia coli* levels peaked during the touristic season, reaching levels that could pose a threat to the health of the visitors. Culturable fungi abundance usually peaked in the spring, remained at a high level in the summer and started to slowly decrease towards the winter months. Differences were observed between the direct and indirect exposed surfaces, as the later had lower overall levels of bacteria and fungi, with increased *Enterobacteriaceae* loads. Most of the taxa identified are known biodeteriorants of subterranean surfaces and were previously associated with human altered caves. A *Dothideomycete* sp. previously unknown to the cave environments was detected.

This was the first study to analyse the dynamics of the microbial communities of delicate subterranean surfaces in show caves through the use of commercially available test kits. We revealed that exposed surfaces in show caves, in direct or indirect contact with tourists, are host to high concentrations of cultivable microbes. The touristic activity was shown to influence the abundance and dynamics of the microbial communities inhabiting surfaces of show caves.

Keywords

subterranean, bacteria, fungi, 16S 18S rRNA, Romania, Rida Count, tourists, *Enterobacteriaceae*, *E. coli*, coliform bacteria

Introduction

Humans willingly and unwillingly alter the biomes they visit or inhabit, modifying the microbiology of these sites, including caves (Saiz-Jimenez et al. 2011). The human induced changes in cave microbiomes are sometimes obvious as stains, colorations or microbial mats appear on speleothems, cave rocks and walls (Saiz-Jimenez et al. 2011). While some of the microbes carried by tourists into caves are affecting the natural environment through degradation of the host rock (Northup and Lavoie 2001), others, such as opportunistic pathogens, might affect future visitors, particularly immunodeficient persons (Jurado et al. 2010a).

Pristine subterranean systems self-regulate and adapt to what is usually an environment scarce in resources, lacks light and has limited exchange with the outside world (Hobbs III et al. 2017). Although limited interaction between caves and humans has little effect on the microbiology of caves (Fraschetti et al. 2001), once it becomes a true show cave widely open to the public the situation changes drastically (Fernandez-Cortes et al. 2011). The extensive human impact begins with the physical alterations that involve blasting, constructions of walkways and installing various utilities (Van Beynen and Townsend 2005). As soon as the cave starts to be prepared for mass visitation, biological and atmospheric changes occur and can range from migration of microscopic species to nontouristic parts of the cave or local extinctions, to foul odours emanating from pools or muds, stains on speleothems, relicts or cave art (Lavoie and Northup 2006; Fernandez-Cortes et al. 2011; Saiz-Jimenez et al. 2011; Hobbs III et al. 2017).

At first, the microbial indicators of human impact in caves were considered *Bacillus* sp., *Escherichia coli*, and *Staphylococcus aureus*, and were regarded as 'Human Indicator Bacteria', based on studies by Lavoie and Northup (2006) in Lechuguilla Cave (USA). The degree of human impact is reflected in a decrease of diversity, as determined by Ikner et al. (2007) in a study of the Kartchner Caverns (USA). Investigations, performed due to the deterioration of the Palaeolithic paintings of Lascaux Cave (France), revealed that the cave was a reservoir of potential pathogenic protozoa and bacteria such as *Ralstonia*, *Pseudomonas*, *Legionella*, *Achromobacter*, *Bordetella*, *Shigella* or *E. coli*, previously linked to outbreaks related to air-conditioning systems and cooling towers in community hospitals and public buildings (Bastian et al. 2009). The use of designated 'indicator organisms' such as *Enterobacteriaceae* (Peters et al. 2018), *E. coli* (Anderson et al. 2005) or coliform bacteria (Shakoor et al. 2018) can detect human induced disturbances, as well as faecal associated contamination (Lavoie and Northup 2006). It was found that bacterial abundance varies greatly at different sampling times, as shown by Wang et al. (2010; 2012) in four different sites in the Mogao Grottoes (China). The increase in number of visitors lead to an increase of the bacterial abundance in all of the accessible sampled sites.

A number of studies have examined the diversity of surface-associated communities in bathrooms, as these are potential harbourers of pathogens. Skin-associated bacteria dominate on surfaces that are routinely touched by hands, although 19 bacterial phyla were identified with most sequences belonging to *Actinobacteria*, *Bacteroidetes*,

Firmicutes and *Proteobacteria* (Flores et al. 2011). Analyses of the contamination of high-touched surfaces in hospitals showed that average bacterial load was 1.32×10^4 bacteria per cm^2, almost half of the samples were culture-positive and some included multidrug resistant organisms (Costa et al. 2019). Confocal laser scanning microscopy demonstrated live bacteria on 76.7% of culture-negative samples while biofilm was present on all surfaces subjected to microscopy (Costa et al. 2019).

We focused on the microbial changes on surfaces humans came in direct or indirect contact with inside show caves. The surface microbiota was sampled on stalagmites that tourists come in direct contact with by touching and on cave bear (*Ursus spelaeus*) skeletons that are on display in the caves. The skeletons are indirectly exposed as they are behind a protective railing and cannot be touched. We monitored monthly changes in the culturable bacterial and fungal abundances focusing on constantly exposed surfaces from the visited sectors, as these are subject to high external allochthonous input. For the microbial monitoring itself we used commercially available Rida Count (R-Biopharm, Germany) rapid test kits. These test kits are based on the principle of using a specific chromogenic detection system imbedded in the standard nutrient media used for cultivating microorganisms. During growth, microbial colonies form and, due to the presence of specific enzymes, they will have distinctive colours. Second and third generation microbes were grown *ex-situ* on specific media for the purpose of ribosomal ribonucleic acid (rRNA) extraction and identification. Along the microbiological changes we monitored the cave climate. Such a monitoring program was not yet used for the subterranean surfaces, as previous studies used longer or unequal intervals between samplings, single samplings, or monitored few microbial groups and parameters. While environmental parameters influence the microbial communities, human presence is also known to alter such communities. We aimed to establish how this influence is identifiable upon the culturable bacterial and fungal abundances of surfaces exposed to tourists inside two Romanian show caves. We analysed the variability of these abundances over a yearly cycle and between surfaces that were directly and indirectly exposed to humans.

Materials and methods

Study sites and sampling locations

The microbiological monitoring of total aerobic bacteria, yeasts and moulds and indicator organisms (*Enterobacteriaceae*, *E. coli* and coliform bacteria) took place from March 2015 to March-April 2016, in two of the most visited caves in Romania, Peștera Urșilor de la Chișcău (Ursilor) and Peștera Muierilor (Muierilor) (Figure 1). The caves were sampled monthly to obtain an overview of the microbial changes that occur over a yearly cycle. All monitoring sites were exposed to tourists yearlong. Two of the monitoring sites were on stalagmites that were in direct contact with tourists (e.g. touched by hands). The other two monitoring sites were on the skeletons and were not in direct contact with tourists.

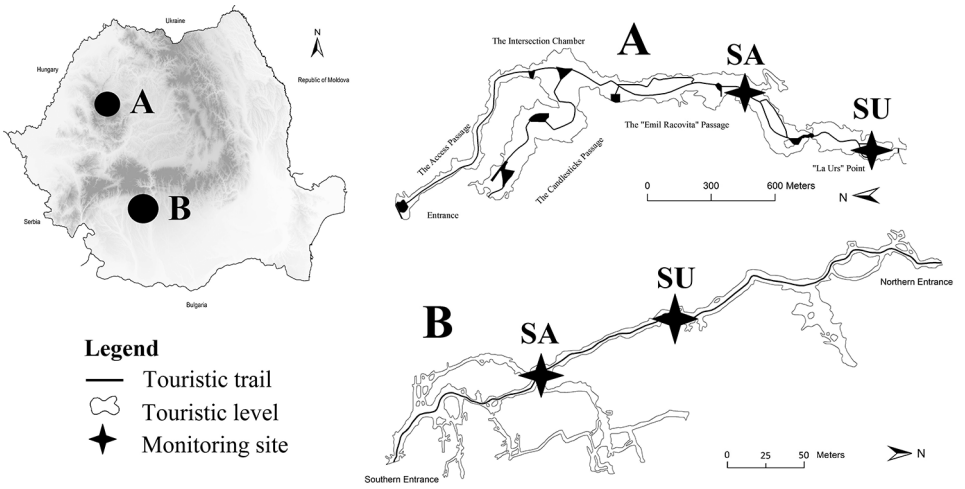

Figure 1. Monitoring sites; Location of the studied caves in Romania with the cave maps and monitoring sites in Ursilor (**A** modified after Rusu and Racoviţă 1981) and Muierilor (**B** modified after map by Grigore, Fofirică, Dăscălescu and Iliescu, unpublished).

Environmental parameters (temperature, airflow, relative humidity, CO_2 and particles per cubic centimetre of air) in the monitoring areas were also measured.

Ursilor is located in the Bihor Mountains (46°33'14"N, 22°34'10"E, Apuseni Mountains, Western Romania,) (Figure 1). The cave is formed in Late Jurassic (Tithonian) recrystallized limestones and had been sealed until it was discovered in 1975 during blasting in a limestone quarry. The cave entrance is housed in a large building constructed into the hill that hosts the cave. The trail is mostly made out of concrete, with one footbridge made of wood planks on a metal structure. The trail has a length of ~500m, with a metal handrail being present through most of the length of the trail. Some stalagmites are part of this handrail. One of these stalagmites (SA), along with the *U. spelaeus* skeleton that is on display (SU), were sampled monthly. The SA monitoring site was at a height of 1.2m from the trail (Figure 1A). A dark coloration was present on the touched part of the stalagmite. The SU monitoring site, was situated at the end of the tourist trail (Figure 1A), at a distance of 1.8m from the inner side of the trail and was displayed on the cave floor. The display area was littered with coins and presented lampenflora whose growth was promoted by the display lights.

Muierilor is located in the southern part of the Parâng Mountains (45°11'13"N, 23°45'9"E, Southern Carpathians) (Figure 1). The cave developed within a stripe of massive limestones of Late Jurassic on at least four levels, of which only the upper level is open to the public. The trail is made out of concrete, has sectors that are dug into the flour of the cave and has no handrail throughout most of its length. The monitoring site for the touched speleothem is around halfway through the touristic path (SA). The site was at a height of 1.2m, on a stalagmite where the pathway was 2m wide and the tourists used this speleothem as a handrail. A dark coloration was present on the touched part of the stalagmite. The monitoring site represented by the *U. spelaeus*

skeleton (SU), displayed upright on a metal stand, was at a height of 1.2m and at a distance of 1.8m from the tourist trail. The display area presented lampenflora whose growth was promoted by the display lights.

Microbial sampling and laboratory protocols

Sampling was performed using Rida Count (R-Biopharm, Germany) test sheets for total aerobic heterotroph bacteria, yeasts and moulds and indicator organisms (*Enterobacteriaceae*, *E. coli* and coliform bacteria). At each of the sampling sites, each test sheet was moistened and thus activated by adding 1 ml of sterile physiological saline solution (0.9% NaCl), just before contact with the sampled surface. After *in-situ* inoculation, the plates were transported at ambient cave temperature in a cooler bag. In the laboratory, the plates were placed in CulturaR Mini incubators (Almedica, Switzerland) at 37 °C for total bacteria and indicator organisms plates. Yeasts and moulds plates were incubated at 25 °C, according to the manufacturer's instructions. Incubation was between 24 and 72h with additional periods suggested by both the manufacturer and previous studies for cave microbes, as they have a slower rate of growth on selected media (Mulec et al. 2012b; 2012a; Mulec et al. 2012c; Bercea et al. 2018). The plates were analysed at 24 h intervals, the results being expressed in the total readings after five days. A five day incubation period was found to be better suited for cave studies as some colony forming units (CFU) can be identified only at the final analysis (Bercea et al. 2018). The counts were expressed as CFU/100cm^2.

Molecular analyses, statistical analyses and environmental parameters

The most visible and well-grown CFU were transferred from Rida Count plates to R2A agar PPM CS100 (VWR Chemicals, USA) for bacteria growth and to Czapek Yeast Extract Agar (Atlas 2010) for yeasts and moulds growth. After incubation, rRNA was extracted from the resulted colonies. Extraction was performed with Chelex 100 (Bio-Rad, USA) 5% solution in TE buffer (Walsh et al. 1991). When this method was not successful, we performed rRNA extraction with Isolate II Genomic DNA Kit (Bioline, UK) and followed the manufacturer's instructions. Total rRNA concentration was measured using a BioDrop DUO Spectrophotometer (BioDrop, UK). After extraction the rRNA was stored at -20 °C until further use. Two bacterial specific primers, targeting the 16S rRNA gene, were used for screening the bacterial diversity: 27F (5'-AGA GTT TGA TCC TGG CTCAG-3') and 1492R (5'-ACG GHT ACC TTG TTA CGA CTT-3') (McGenity et al. 1998). For yeasts and moulds analysis primer set FR1 (5'-AIC-CAT-TCA-ATC-GGT-AIT-3') and FF390 (5'-CGA-TAA-CGA-ACG-AGA-CCT-3') of 18S rRNA were used. For bacterial samples, an amplification program with initial denaturation for 3min at 95 °C, followed by 35 cycles of 95 °C for 30s, 55 °C for 30s, 72 °C for 90min and final elongation at 72 °C for 10min, was

used. For yeasts and moulds the program was initial denaturation at 97 °C for 3min, 35 cycles 95 °C for 60s, 49 °C for 25s, 72 °C for 35s and final elongation at 72 °C for 5min. The polymerase chain reaction (PCR) was performed with a Techne TC-512 gradient thermal cycler (Bibby Scientific, UK). PCR products were purified with FavorPrep™GEL/PCR Purification Kit (Favorgen Biotech Corp., Taiwan) following the manufacturer's instructions. rRNA was sequenced by Sanger method (Sanger and Coulson 1975; Sanger et al. 1977) at a commercial company (Macrogen Europe, The Netherlands). The sequences obtained were compared to 16S and 18S rRNA gene sequences deposited in NCBI BioSystems Database (GenBank) using BLAST algorithm (Geer et al. 2010; Sayers et al. 2019a; Sayers et al. 2019b). Sequences have been deposited in GenBank. Statistical analyses were performed with SSS 2019 One-Way Repeated Measures ANOVA Calculator (Stangroom 2019).

The following parameters were measured *in-situ* at each sampling site: temperature and airflow with a PCE-423 Hot Wire Anemometer (PCE Instruments, UK); relative humidity with a Hygropalm 3 Digital Humidity Meter (RoTronic, Switzerland); CO_2 values with a Vaisala Measurement Indicator MI70 equipped with a Vaisala Carbon Dioxide Probe GMP70 (Vaisala, Finland); particles per cubic centimetre of air with a Condensation Particle Counter 3007 (TSI, USA). The tourist traffic flow was continuously monitored with a Long-Range IR Beam Indoor People Counters with internal data logger (Chamber Electronics, Scotland). Over the course of the study, the average temperature and CO_2 in Ursilor was overall slightly higher than in Muierilor, while particles per cubic centimetre of air was higher in Muierilor. Relative humidity and airflow were stable during the study period for all the sites. The average number of visitors in the years preceding the study were estimated by the cave managers at around 130,000 and 120,000 for Ursilor and Muierilor, respectively. The pattern of visitation during the study period was similar for the two caves, with numbers rising from spring up to the beginning of autumn, followed by a steep drop for the winter season (Bercea et al. 2018). Muierilor had bat populations in both the touristic and non-touristic sectors that congregated in the winter and also summer colonies. This could have contributed to an increase in the CFU of Muierilor as bats introduce low, but not negligible, concentrations of microbes on their fur (Borda et al. 2014).

Results

Microbiota

On the surfaces of Ursilor and Muierilor, the total bacterial communities presented high numbers year-round. *Enterobacteriaceae*, coliform bacteria and *E. coli* peaked during the touristic season. An exception was represented by the *U. spelaeus* skeleton on display in Muierilor, were *Enterobacteriaceae* were in high numbers year-round. Yeasts and moulds usually peaked in the spring, remained at a high level in the summer, and slowly decreased towards the winter months. The overall total bacterial counts (~87000 CFU) and yeasts and moulds (~83000 CFU) were at close levels to each other. Overall

Enterobacteriaceae (~12000 CFU) were far behind, whilst coliform bacteria (~3600 CFU) and *E. coli* (~1100 CFU) were even lower.

On the monitored stalagmite in Ursilor, yeasts and moulds values peaked in the spring (>6500 CFU) and slowly declined towards the winter period (~200 CFU) (Figure 2A). Total bacterial levels were relatively constant from spring to autumn (~1500 CFU), dropping only for the winter (~200 CFU). *Enterobacteriaceae*, coliform bacteria and *E. coli* all peaked in the summer season, reaching an all high in July (~3500 CFU), with smaller peaks in May and June. The highest levels of yeasts and moulds, recorded during the study period in Ursilor, were registered in May (>6500 CFU) (Figure 2A). On the similar surface in Muierilor, yeasts and moulds values peaked in the spring (~5800 CFU) and autumn periods (~6500 CFU), with slightly lower values for the summer season. Total bacteria followed an almost identical pattern, although the summer period values did not drop as much as the fungal ones. *Enterobacteriaceae*, coliform bacteria and *E. coli* all peaked in the summer season, reaching an all high in June (~1800 CFU) and a smaller peak in August (~900 CFU). Coliform bacteria also peaked during the winter season (~500 CFU). The highest level of yeasts and moulds CFU, recorded during the study period in all sites, was registered in September in Muierilor (6559 CFU), while that of total bacteria peaked in April in Muierilor (7155 CFU) (Figure 2B).

On the exposed and not touched *U. spelaeus* skeleton on display in Ursilor, yeasts and moulds followed a similar trend as on the touched surface, only with lower levels, peaking in the spring period (~2400 CFU) and then slowly declining. As in the case of yeasts and moulds, total bacteria followed a similar trend, with lower and relatively constant levels from spring to autumn, dropping for the winter (Figure 3A). *Enterobacteriaceae*, coliform bacteria and *E. coli* all peaked in the summer season, reaching an all high in July (~600 CFU), with a smaller peak June. On the *U. spelaeus* skeleton on display in Muierilor, the yeasts and moulds followed a similar trend as on the touched surface, only with lower levels, peaking in the spring (~3400 CFU) and registering lower values for the summer and autumn seasons. Total bacteria and *Enterobacteriaceae* started to rise in the spring, peaking in the autumn (~4300 and ~1100 CFU, respectively) and then declining in the winter. Coliform bacteria and *E. coli* peaked in the spring season, with smaller peaks in the summer (Figure 3B).

Differences were observed between the overall yeasts and moulds loads of the sampling sites on touched stalagmites and the *U. spelaeus* skeletons on display, as the overall counts for the first reached ~55,000 CFU, whilst for the later it was an order of magnitude lower, at only ~4,300 CFU. A smaller difference was observed for total bacteria, as the overall load reached ~50,000 CFU on stalagmites and ~37,000 CFU on skeletons. There were no bacteria cultivated in Ursilor cave from March to April 2015. *Enterobacteriaceae* had a higher count on the *U. spelaeus* skeletons on display, especially on the one in Muierilor. Coliform bacteria and *E. coli* followed a similar trend as yeasts and moulds and total bacteria. As the differences between the caves go, yeasts and moulds had slightly higher numbers in Ursilor than in Muierilor, while for total bacteria it was the opposite. An almost double number of *Enterobacteriaceae* were present in Muierilor compared to Ursilor, while coliform bacteria and *E. coli* were only slightly higher than in Ursilor (Figures 2, 3).

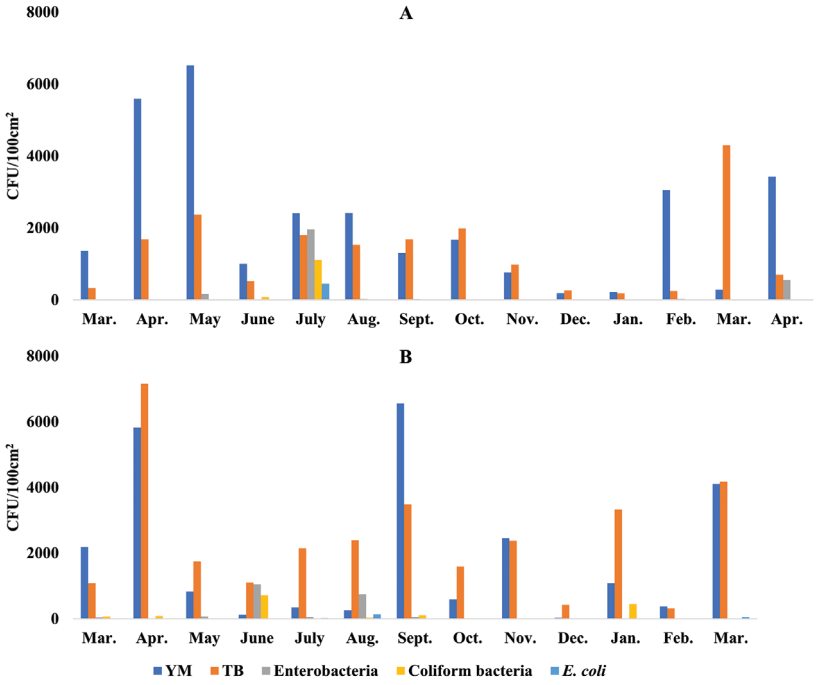

Figure 2. Distribution of culturable bacterial and fungal abundances on touched stalagmites from 2015 to 2016 in Ursilor (**A**) and Muierilor (**B**). TB, total aerobic bacteria; YM, yeasts and moulds; *E. coli*, *Escherichia coli*.

The one-way analysis of variance performed for each site resulted in p-values < .00001 for all but one site (the skeleton site from Muierilor, p .000236) for a significance value of p .01. The F-ratio value for the stalagmite site in Ursilor was F = 12.46052 (4/65 numerator/denominator degrees of freedom) and F = 21.52592 (4/65) for the skeleton site, while for the stalagmite site in Muierilor it was F = 11.84504 (4/60) and F = 6.56377 (4/65) for the skeleton site.

Sequence diversity

Identification of bacteria by 16S rRNA gene sequencing revealed the presence of *Mortierella* sp. (closest GenBank match, KT964847.1; identity, 100%; length of matched segment, 272 base pairs [bp]) in Ursilor SU site and of *Staphylococcus* sp. (closest GenBank match, KX262673.1; identity, 100%; length of matched segment, 899 bp) in Muierilor SU site. The yeasts and moulds group identified by 18S rRNA gene sequencing was represented in the SU site of Muierilor by *Dothideomycete* sp. (closest GenBank match, AY275186.1; identity, 100%; length of matched segment, 309 bp) and *Penicillium* sp. (closest GenBank match, KU350746.1; identity, 99%; length of matched segment, 277 bp) while *Apiotrichum* (*Trichosporon*) sp. (closest GenBank match, KF036718.1; identity, 99%; length of matched segment, 263 bp) was found in the SA site.

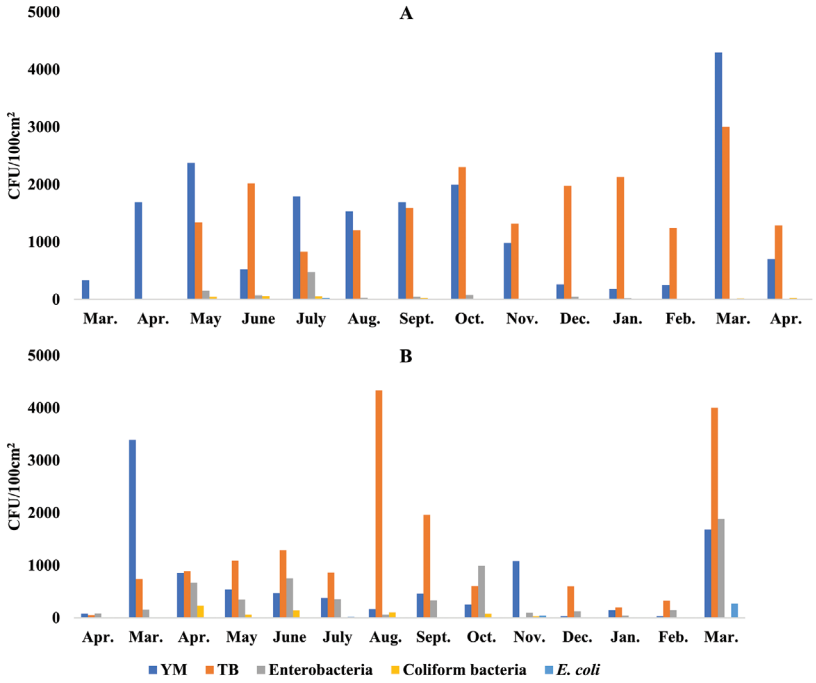

Figure 3. Distribution of culturable bacterial and fungal abundances on the *Ursus spelaeus* skeletons on display from 2015 to 2016 in Ursilor (**A**) and Muierilor (**B**). TB, total aerobic bacteria; YM, yeasts and moulds; *E. coli*, *Escherichia coli*.

Environmental parameters

As previously described for these caves, the airflow was the most stable environmental parameter measured, relative humidity was above 94% inside the caves and the increase in the number of tourists was synchronous with an increase in the levels of CO_2 and temperature inside the caves during spring and especially during summer months (Bercea et al. 2018). Average particulates levels were much lower in Ursilor compared to Muierilor (Table 1).

Table 1. Mean values of environmental parameters over the course of the study period. SD, Standard deviation; PT/CC, particles per cubic centimetre of air.

Site	Mean value ± SD				
	Temperature (°C)	Relative humidity (%)	Airflow (m/s)	CO_2 (ppm)	PT/CC
Ursilor					
Overall	11.33 ± 0.63	94.69 ± 2.44	1.40 ± 0.67	3057.14 ± 1834.94	469.86 ± 543.98
Skeleton	11.29 ± 0.68	94.66 ± 2.42	1.28 ± 0.07	3050.71 ± 1811.37	425.50 ± 493.01
Stalagmite	11.37 ± 0.56	94.71 ± 2.46	1.52 ± 0.93	3063.57 ± 1858.19	514.21 ± 587.24
Muierilor					
Overall	10.04 ± 1.33	95.19 ± 2.47	1.22 ± 0.11	620.37 ± 293.6	5175.63 ± 6176.29
Skeleton	10.18 ± 1.08	95.40 ± 2.58	1.24 ± 0.12	632.86 ± 299.94	5508.57 ± 6732.27
Stalagmite	9.89 ± 1.54	94.96 ± 2.33	1.20 ± 0.09	606.92 ± 286.02	4817.08 ± 5492.69

Discussion

The increase in total bacteria and yeasts and moulds CFU on the touched surfaces of Ursilor and Muierilor mirrors the visitation pattern of these caves and of other Romanian show caves. The number of CFU start to rise in the early spring, maintaining elevated levels up to the end of touristic season. Peaks of indicator organisms were recorded mainly during the summer, concomitant with high tourist influx in the caves. Despite the reduced number of caves that this study involved and based on the coinciding visitation pattern observed for Romanian show caves (Bercea et al. 2018), there is a probability that the surfaces of most Romanian show caves could harbour similar evolving microbiomes, subject to human influence. The lower level of particulates in Ursilor compared to Muierilor could be attributed to the fact that Ursilor has air lock style entrances.

Although *Dothideomycete* have high numbers of known inhabitants of caves, including known biodeteriorants that slowly erode the surfaces they inhabit (Mammola et al. 2017), the one identified via GenBank was not previously found in caves, marking a step forward in mapping the environments that this class inhabits and possibly a novel species for the cave environment. *Staphylococcus* sp. are also known biodeteriorants of the surfaces they inhabit in some caves (De Leo et al. 2012) while also being classified as human pathogens, correlated with speleological exploration and visitation (Boga et al. 2007). The genus is known to inhabit frequently touched subterranean objects and surfaces and was previously found in the studied show caves (Borda et al. 2014; Bercea et al. 2018). *Mortierella* is a known inhabitor of altered mycobiotas of show caves (Jurado et al. 2010b) and was previously found in Romanian caves (Epure et al. 2014). *Apiotrichum* (*Trichosporon*) have previously been correlated with fungal outbreaks in show caves and can lead to the establishment of biofilms on subterranean surfaces (Jurado et al. 2010a; Jurado et al. 2010b; Urzì et al. 2010). *Penicillium* are a common occurrence on various surfaces from the altered mycobiotas of show caves as a result of tourism (Urzì et al. 2010; Mammola et al. 2017).

Preventing the pollution of caves and cleaning them of what has already been inflicted upon these fragile habitats are easy-to-do actions with the higher purpose of quality management, whilst also implementing a proper land management for the surrounding areas, thus limit the allochthonous input from several sources (Watson 1997; Rechtschaffen 1998). A remediation program for a subterranean environment cannot be perceived only from the mindset of cleaning frequently touched surfaces, it has to be an overall policy that ameliorates the accumulated human disturbance.

Traditionally, microbial sampling is a burdensome task in caves as it involves tools that are fragile and need delicate handling for proper operation, like agar-based media in Petri dishes fabricated out of glass or rigid polymeric materials. Recent studies analysed and proved the potential of commercially available, ready to use test sheets (Mulec et al. 2012b; 2012a; Mulec et al. 2012c; Borda et al. 2014; Moldovan et al. 2015; Bercea et al. 2018). The advantages of such media compared to a Petri dish are, among others: the smaller size that allows scientists to carry a significantly larger number of media; toughness due to elasticity, as it can withstand repeated impacts that would shatter any

glass, or for that matter, rigid polymeric material Petri dishes; and the easy-to-handle characteristics. The robustness and universality of such test sheets for quantification of total aerobic bacteria, yeasts and moulds and indicator organisms (*Enterobacteriaceae*, *E. coli* and coliform bacteria) was previously tested for surfaces, waters and the air of caves, thus proving the viability of such methods (Mulec et al. 2012b; 2012a; Mulec et al. 2012c; Borda et al. 2014; Moldovan et al. 2015; Bercea et al. 2018). As selective plate media were used to assess the bacterial and fungal community, we must also mention this as a shortcoming, as these media were not specifically designed for use in caves.

Although some test sheets might be selective to some taxa, including the ones used in this study, their use, never the less, should be involved in future monitoring programs of show caves, and, if faced with high CFU levels, the deployment of molecular methods should be a top priority. When faced with dangerously high microbial loads, the first action by cave management should be to limit or even deny the access of visitors to the afflicted areas or to the whole cave. As proper cave management goes, the issue of surveying a site before opening it to the public should imply, among many others aspects, the microbial monitoring over at least a yearly cycle on a monthly basis and maintaining the said monitoring program through the operational life cycle of the show cave, albeit at lower frequencies, such as bimonthly or seasonally. As far as the type of selective media that should be used for sampling, aspects such as robustness, ease of use in the cave environments and affordability must be taken into consideration.

This was the first study to analyse the dynamics of the microbial communities of delicate subterranean surfaces and those of *U. spelaeus* skeletons displayed in show caves through the use of commercially available test kits. We revealed that exposed surfaces in show caves, in direct or indirect contact with tourists, host high concentrations of cultivable microbes. The presence of indicator organisms was confirmed through most of the year and in higher numbers on the surfaces with which tourist come in direct contact constantly. The touristic activity was shown to influence the abundance and dynamics of the microbial communities inhabiting surfaces within show caves.

Acknowledgements

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