Tourism Management 78 (2020) 104037

Contents lists available at ScienceDirect

Tourism Management

journal homepage: http://www.elsevier.com/locate/tourman

Management of water bodies in show caves – A microbial approach

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ARTICLE INFO

Keywords:
Bacteria
Fungi
Human impact
Show caves
Management
Monitoring
Carpathians
Romania

ABSTRACT

The aim of this study was to monitor factors affecting the microorganisms of water bodies in caves and apply the findings to improve their management. Four show-caves in Romania were sampled monthly to obtain an overview of the changes occurring in the fungal and bacterial communities of cave waters in relation to surface and underground impacts. Test plates were used for microbiological monitoring, alongside the recording of environmental parameters - physicochemical, drip rates, and number of both bats and tourists. A higher number of microbes were obtained during springtime and the summer tourist peak-season. None of the identified strains were cave-specific, and the presence of human-associated taxa was observed from all of the sites. A lack of significant correlation between the abundances of the microorganisms and the physicochemical parameters of the water emphasise the reliability of microorganisms as significant proxies for impacts on underground environments and their use in management decisions.

1. Introduction

Caves were used for thousands of years by humans although only recently their recreational, aesthetic and scientific values were recognized (Gillieson, 2011). In the last decades, caves became exceptionally vulnerable to human impact, due to the development of tourism and expansion of land use. At present most countries have at least one cave opened for tourism (Cigna & Forti, 2013), with more and more caves being opened each year all over the world. Therefore, repercussions, in the form of the gradual degradation of cave resources and threats against cave life, need to be properly quantified. The IUCN Guidelines for Cave and Karst protection provide a list of possible impacts in caves (Watson, Hamilton-Smith, Gillieson, & Kiernan, 1997), with effects on physical structure, chemistry, hydrology, microclimate, floor, sediments, speleothems and fauna, including impacts on the surface and introduction of allochthonous organisms and materials.

Show caves, in particular, face the direct impact of their touristic arrangements (paths, lighting), and the indirect, cumulative effects on the cave microclimate and objects, such as chemical pollution, changes in humidity, increase in carbon dioxide concentration and temperature, corrosion of speleothems, high input of novel inorganic and organic matter from the surface (Baker & Genty, 1998; Cigna, 1993, 2002; Dragovich & Grose, 1990; Pulido-Bosch, Martin-Rosales, López-Chicano, Rodriguez-Navarro, & Vallejos, 1997; Russell & MacLean, 2008; Sánchez-Moral et al., 1999; Tomczyk-Żak & Zielenkiewicz, 2016). Human presence in caves leads to changes in biogeochemistry and the balance of organic matter, which in turn impacts on the autochthonous microbial communities, replacing them by fast-growing heterotrophic bacteria (Saiz-Jimenez, 2012). It has been demonstrated that the number of microorganisms occurring underground, even potential pathogens, increases with the growing number of tourists (Lavoie & Northup, 2006; Jurado et al., 2010; Shapiro & Pringle, 2010; Fernandez-Cortes et al., 2011; Northup, 2011; Porca et al., 2011; Saiz-Jimenez, 2012; Ogórek, Lejman, & Matkowski, 2013, 2014, 2016a, 2016b; Griffin, Gray, Lyles, & Northup, 2014; Mulec, 2014; Pusz, Ogórek, Knapik, Kozak, & Bujak, 2015; Bercea et al., 2018). Studies comparing visited and unvisited parts of a cave have shown higher diversities in tourist-accessible areas (Adetutu & Ball, 2014; Adetutu et al.,

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https://doi.org/10.1016/j.tourman.2019.104037
Received 31 August 2018; Received in revised form 20 September 2019; Accepted 6 November 2019
Available online 19 November 2019

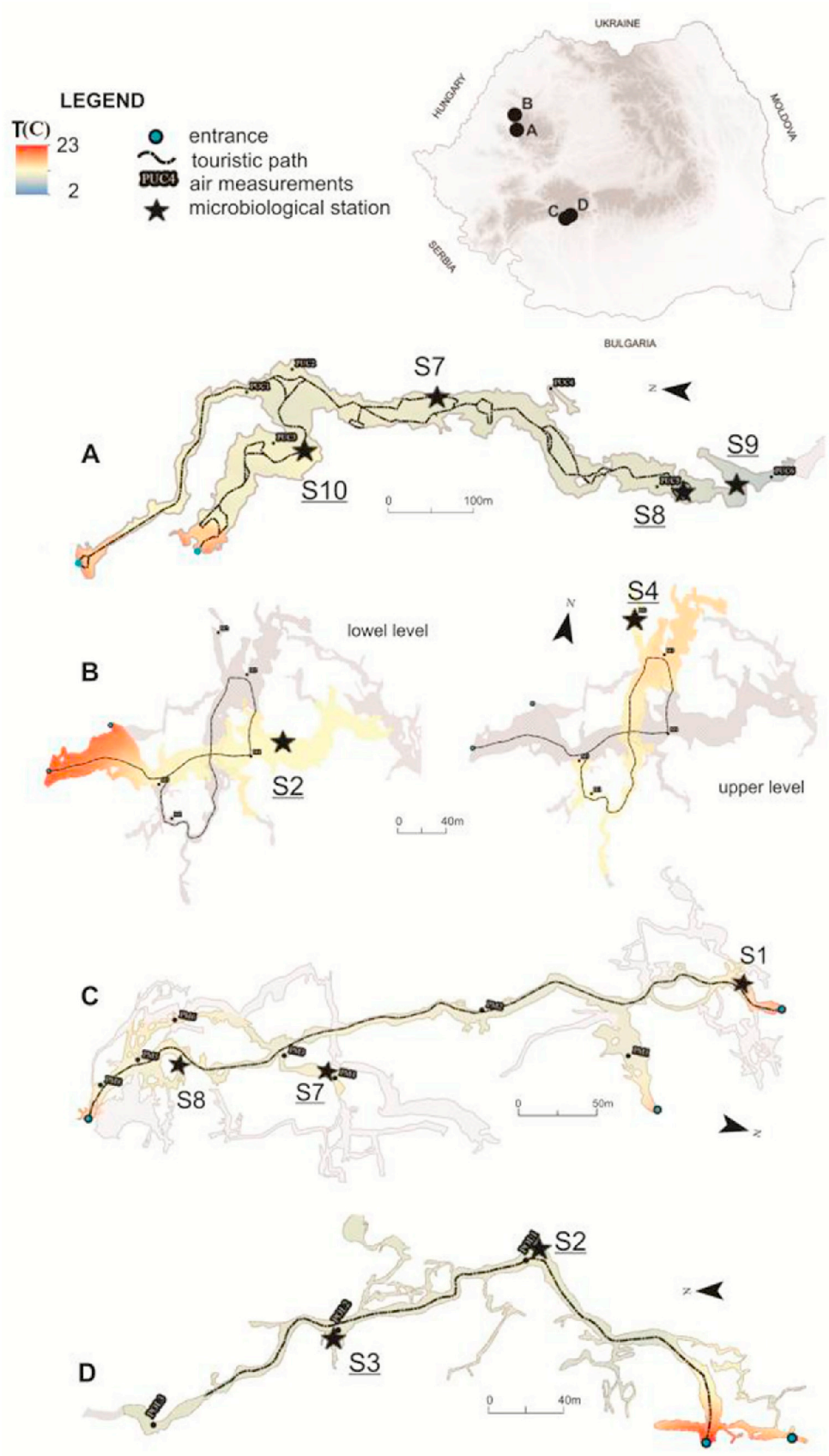

Fig. 1. Location of the studied caves in Romania, showing cave maps and sample site locations in Ursilor (A; modified after Rusu & Racoviță, 1981); Meziad (B; modified after Rusu, Racoviță, & Crăciun, 1974); Muierilor (B; modified after a map by Grigore, Fofirică, Dăscălescu and Iliescu, unpublished); Polovragi (D; modified after Ponta & Aldica, 1985); chemical analysis were done in the underlined stations. Cave maps present the distribution of the summer air temperatures (June–August means).

Table 1
Short descriptions of each of the microbiological monitoring sites (see also Fig. 1); physicochemical measurements were performed on-site, and samples for chemical analysis were taken when water was present in higher volume than a fine film.

Cave	Sampling site	Habitat type	Description	Presence of bats	Chemical analysis	Presence of water
Ursilor	S7	Pool on calcite	Next to the trail	No	No	Permanent
	S8	Pool on calcite	Next to the trail	No	Yes	Seasonal
	S9	Small pond on sediments	Restricted area	No	Yes	Permanent
	S10	Pool on calcite	Next to the trail	No	Yes	Permanent
Meziad	S2	Pool on calcite	~50 m from the trail	Yes	Yes	Seasonal
	S4	Dripping water	~50 m from the trail	Colony present nearby	Yes	Permanent
Muierilor	S1	Pool on calcite	Next to the trail	Yes	No	Seasonal
	S7	Pool on calcite	Restricted area	Yes	Yes	Seasonal
	S8	Dripping water	Next to the trail	Yes	No	Seasonal
Polovragi	S2	Pool on calcite	Next to the trail	Yes	Yes	Permanent
	S3	Pool on calcite	Next to the trail	Yes	Yes	Seasonal

2011; Barton & Jurado, 2007; Engel, 2010; Vanderwolf, Malloch, Mcalpine, & Forbes, 2013).

Despite the knowledge on the importance of cave microorganisms in subterranean processes (e.g. speleotheme formation or alteration), and the possible threats of allochthonous species to human health, cave managers all over the world are not primarily concerned with microbial pollution. Some exceptions are notable, following dangerous microbial outbreaks in painted caves, when the managers of show caves changed the visiting rules (i.e., Dupont et al., 2007; Jurado et al., 2010).

In a study on Romanian show caves, pursued between 2014 and 2017, that used multidisciplinary monitoring to develop protocols for a sustainable use of caves for tourism and an improved management, we tested whether the microbial load of air can be used in quantifying human impact. The air microorganisms, including pathogens, showed variations depending on the: number of tourists, CO_2 concentration, air relative humidity, number of bats, and radon level (Bercea et al., 2018).

In this study, we extended the monitoring to cave water microhabitats – dripping water and pools – and test their possible use in the management of show caves. For this purpose four of the most visited Romanian show-caves were chosen. Cave water microhabitats are important in the general energetic budget of a cave because drip water brings nutrients and organisms from the surface, and water accumulates inside caves in pools, providing a living space for groundwater organisms. Both, drip water and pools represent particular cave habitats in which microbial communities can be explored.

Until present less attention was given to changes in the caves water bodies. Šebela & Turk, (2014) demonstrated that the susceptibility of water temperature to anthropogenic influences is far higher than that of cave air and that the heat absorption by pools and the rock mass is relatively greater than into the cave air. Water bodies in caves also act as reservoirs for allochthonous microbiological input, and the microbiota is one of the first elements affected by human presence (Cigna, 2016; Engel, 2010; Pronk, Goldscheider, & Zopfi, 2007; Shabarova & Pernthaler, 2010). A direct human impact on cave waters was studied in Lechuguilla Cave (USA) by Hunter, Northup, and Dahm (2004). They found that various allochthonous materials present in pools led to an increase in biofilms, and so to an increase in coliform bacteria abundance. Humans are a source of nitrogen into these otherwise nitrogen-limited environments, as studied by Johnston and Muench (2012), who showed that organisms can take advantage of nitrogenous compounds. Their study revealed the presence of human-related bacterial groups in contaminated sites. In the Harmanecká show-cave (Slovakia), human-associated and pathogenic fungi from humans and animals were the most frequent types isolated from the cave water (Ogórek et al., 2016b).

2. Materials and methods

The study monitored over a period of 14 months changes in the microbiota (bacteria and fungi) in water bodies (pools and dripping water) in visited and non-visited sectors of four of the most visited Romanian show-caves. Along with the microbiological changes, the physicochemical parameters of the water and both numbers of bats and tourists were monitored. For the microbiological sampling, pools and dripping sites that were observed as being fed by a source with relatively constant drip-rate at the beginning of the study were chosen. They were situated in cave sectors with different human exposure, both along the trail that experienced a high touristic impact and in areas restricted to tourists that suffered low or no impact. Water flow and samples for water chemical analysis were sampled at the same sites.

Measurements in the same caves showed that wind speed was the most stable parameter measured inside the cave, with general small fluctuations of less than 0.1 m/s and only exceptionally more than 1 m/s. The average relative humidity fluctuated from around 90 to 100% inside the caves, and the increase of the number of tourists was synchronous with an increase in the levels of CO_2 and temperature inside the caves during the spring and summer months (Bercea et al., 2018).

2.1. Sample sites

Four show caves were chosen for this study. Show caves in Romania belong to the state and are managed by different entities.

Urşilor Cave in Chişcău (Ursilor) is located in the Apuseni Mountains (north-western Romania; 491 m.a.s.l.) (Fig. 1A). Monthly sampling was undertaken at four sites: S7, S8 and S10 at a distance of 1 m from the tourist trail, and S9 in a restricted area of the cave (Table 1). The cave has two artificially tunnels as entrances, each protected by airlock double doors. The manager of the cave is a private company, Transylvania Tour SRL, and the cave is part of the Apuseni Natural Park.

Meziad Cave (Meziad) is also located in the Apuseni Mountains (north-western Romania; 397 m.a.s.l.) (Fig. 1B). Both sampling sites, S2 and S4, were located at a distance of ~50 m from the tourist trail (Table 1). The show cave is developed on two levels and the entrance is semicircular (16 m high/10 m wide). Bats form summer colonies in the upper level and winter colonies within both levels. The manager of the cave is an NGO: Centrul pentru Arii Protejate şi Dezvoltare Durabilă Bihor.

Muierilor Cave (Muierilor) is located in the Southern Carpathians (735 m.a.s.l.; Fig. 1C). The three sampling sites were: S1 and S8 at a distance of 1 m from the trail, and S7 in a non-touristic passage (Table 1). The show cave has two entrances: 3 m height/2 m wide and 2 m height/4 m wide, the path is of concrete for most of its length, and bats are also present along the path. Bats form summer and winter colonies in two chambers but they are also present along the whole touristic path. The management of the cave belongs to the Baia de Fier town hall.

Polovragi Cave (Polovragi) is in the close vicinity of Muierilor Cave, within the Olteț river gorge (650 m.a.s.l.; Fig. 1D). Both sampling sites, S2 and S3, were next to the tourist path. This cave has no marked trails, and there is no set limit from the trail to the sampling sites (Table 1). The

cave entrance is 7 m high/11 m wide. Bats are found all along the touristic path during summer and winter. The Polovragi town hall manages this cave.

At the surface, above the caves, there are broad-leaved forests and brown soils at Ursilor, Meziad and Muierilor and mixed forests with redzinic soils at Polovragi. Only a few invertebrate representatives were found inside the caves and the only observed vertebrates were the bats.

In Ursilor, high airborne microbiological load was found for aerobic bacteria (TB) in some stations during the touristic period of May–August, while yeast and molds (YM) remained always at low abundances. Abundant airborne microorganisms in Muierilor were found for TB during August (only in two stations not included in our study) and for YM when bats became active after the winter hibernation. In Meziad, a high YM was recorded during summer, but the high number of TB in December and of YM in April could not be correlated to any other feature than the presence of the big bat colonies that seek shelter in this cave. In Polovragi, the number of TB and YM was very low (see also Table S1).

2.2. Microbiological sampling, environmental parameter measurement and laboratory protocols

A monthly sampling interval was used for Muierilor and Ursilor, due to the high number of visitors (>100,000/year), while for Polovragi and Meziad, a bimonthly sampling interval was chosen, considering the lower number of tourists.

Monitoring of underground water using rapid test kits, similar to those used in this study, was done by Mulec, Krištůfek, and Chroňáková (2012), with the method proving to be fit for regular monitoring of eutrophication and human influence in karst caves. Sampling of total viable counts was done using RIDA®COUNT (R-Biopharm AG, Germany) test sheets for TB, YM, and Enterobacteriaceae. At each sampling site, 1 mL of water was directly applied, with a sterile plastic pipette, onto the RIDA®COUNT growth medium surface, and this was then transported to the laboratory for incubation. The plates were transported at constant temperature in a cooler bag, and placed in incubators at 37 °C for TB and Enterobacteriaceae in an RB 360 incubator (Heraeus Habau, Germany), and at 25 °C for YM in a CulturaR Mini Inkubator (Almedica AG, Switzerland). Normal incubation was between 24 and 72 h (R-Biopharm), with additional periods suggested by both the manufacturer and previous studies, as samples from caves tend to have a slower rate of growth on selected media (Mulec et al., 2012). The plates were analyzed at 24-h intervals, the results being expressed in the total readings after five days. The counts are expressed as colony-forming units per mL of water (CFU), for ease of comparison with other studies.

Conductivity (μS/cm), temperature (°C) and pH were measured in situ with a Combo pH and EC HI 98129 Low Range pH/Conductivity/TDS Tester (Hanna Instruments). Drip water samples for chemical analysis were taken on a bimonthly basis and analyzed for total hardness (°dH) by titration with EDTA. Chlorine (mg/L), nitrate (mg/L), sulphate (mg/L), calcium (mg/L), magnesium (mg/L), potassium (mg/L), sodium (mg/L), barium (μg/L), iron (μg/L) and strontium (μg/L) were measured using a GENESIS ICP-OES Spectrometer (SPECTRO Analytical Instruments GmbH, Germany). Total dissolved oxygen (TDS; mg/L) was deduced from the conductivity values, and bicarbonate (mg/L) from the total hardness. Dripping rates were measured with Stalagmate Mark3 drip-loggers (Driptych, UK) (Collister & Mattey, 2008). The tourist traffic flow was continuously monitored with Long Range IR Beam Indoor People Counters (LIR8_D) with internal data loggers (Chamber Electronics, Scotland).

2.3. DNA extraction and amplification and sequencing

The most visible colonies were transferred from RIDA®COUNT plates to R2A agar PPM CS100 (VWR Chemicals) for TB growth and to Czapek Yeast Extract Agar (Atlas, 2010) for YM growth. After incubation, DNA was extracted from 23 TB to 8 YM water samples taken in

Table 2
Mean values and standard deviation (in parentheses) of water physicochemical parameters for the sampled sites during the entire study period; * = sites where laboratory chemical analysis were also done; - = insufficient water.

Cave	Site	Drip (L/h)	Conduct. (μS/cm)	Temp. (°C)	pH	Hardness (°dH)	HCO_3 (mg/L)	Cl (mg/L)	NO_3 (mg/L)	SO_4 (mg/L)	Ca (mg/L)	Mg (mg/L)	K (mg/L)	Na (mg/L)	Ba (ug/L)	Sr (ug/L)
Ursilor	S7		440 (10)	11.1 (0.4)	7.56 (0.2)	–	–	–	–	–	–	–	–	–	–	–
	S8*	1.23 (0.55)	411 (10)	10.9 (0.5)	7.4 (0.1)	13.5 (0.5)	293 (14)	1.03 (0.05)	1.05 (0.44)	7.68 (0.19)	86.34 (3.16)	0.31 (0.01)	1.14 (0.02)	0.56 (0.15)	6.18 (0.57)	30.58 (1.29)
	S9*		370 (10)	10.6 (0.4)	7.58 (0.2)	10.5 (0.3)	228 (7)	0.95 (0.06)	0.88 (0.19)	8.85 (0.13)	69.29 (1.34)	0.47 (0.01)	0.19 (0.03)	0.69 (0.13)	6.22 (0.77)	31.34 (0.44)
	S10*	0.20 (0.17)	367 (58)	10.9 (0.7)	7.63 (0.2)	11.1 (1.6)	242 (35)	1.14 (0.05)	1.68 (0.61)	11.4 (0.37)	67.22 (3.2)	0.39 (0.02)	0.27 (0.05)	0.81 (0.16)	8.45 (0.69)	33.92 (1.87)
Meziad	S2*	0.17 (0.12)	336 (52)	9.5 (0.3)	7.48 (0.2)	8.7 (1.0)	189 (23)	1.2 (0.12)	0.45 (0.06)	10.88 (0.74)	50.86 (0.67)	0.82 (0.06)	0.45 (0.28)	1.03 (0.14)	6 (0.25)	41.12 (1.85)
	S4*	0.06 (0.02)	316 (35)	9.5 (0.2)	7.45 (0.3)	11.7 (1.4)	255 (31)	1.08 (0.13)	5.02 (0.72)	11.4 (0.72)	77.98 (3.9)	0.7 (0.03)	0.43 (0.19)	0.89 (0.09)	5.9 (0.12)	40.4 (1.01)
Muierilor	S1		357 (43)	10.5 (0.3)	7.85 (0.2)	–	–	–	–	–	–	–	–	–	–	–
	S7*	0.30 (0.16)	363 (38)	9.9 (0.2)	7.64 (0.1)	11.5 (1.4)	24 (31)	0.82 (011)	7.3 (2.26)	7.66 (0.35)	73.76 (1.9)	0.64 (0.06)	0.43 (0.21)	0.61 (0.1)	4.84 (0.24)	38.74 (1.96)
	S8	0.11 (0.05)	366 (26)	10.0 (0.0)	8.0 (0.0)	–	–	–	–	–	–	–	–	–	–	–
Polovragi	S2*	0.77 (0.47)	494 (60)	10.3 (0.5)	7.78 (0.1)	14.6 (2.3)	317 (51)	1.06 (0.13)	5.33 (1.21)	2.55 (0.24)	75.6 (8.61)	3.2 (0.45)	0.53 (0.16)	0.79 (0.27)	14.98 (0.74)	70.47 (2.41)
	S3*		532 (50)	11.0 (0.5)	7.0 (0.2)	17.2 (1.3)	374 (28)	0.86 (0.13)	5.95 (1.39)	7.4 (0.17)	86.29 (18.01)	12.32 (0.68)	0.57 (0.36)	0.76 (0.23)	12.52 (1.17)	72.22 (2.62)

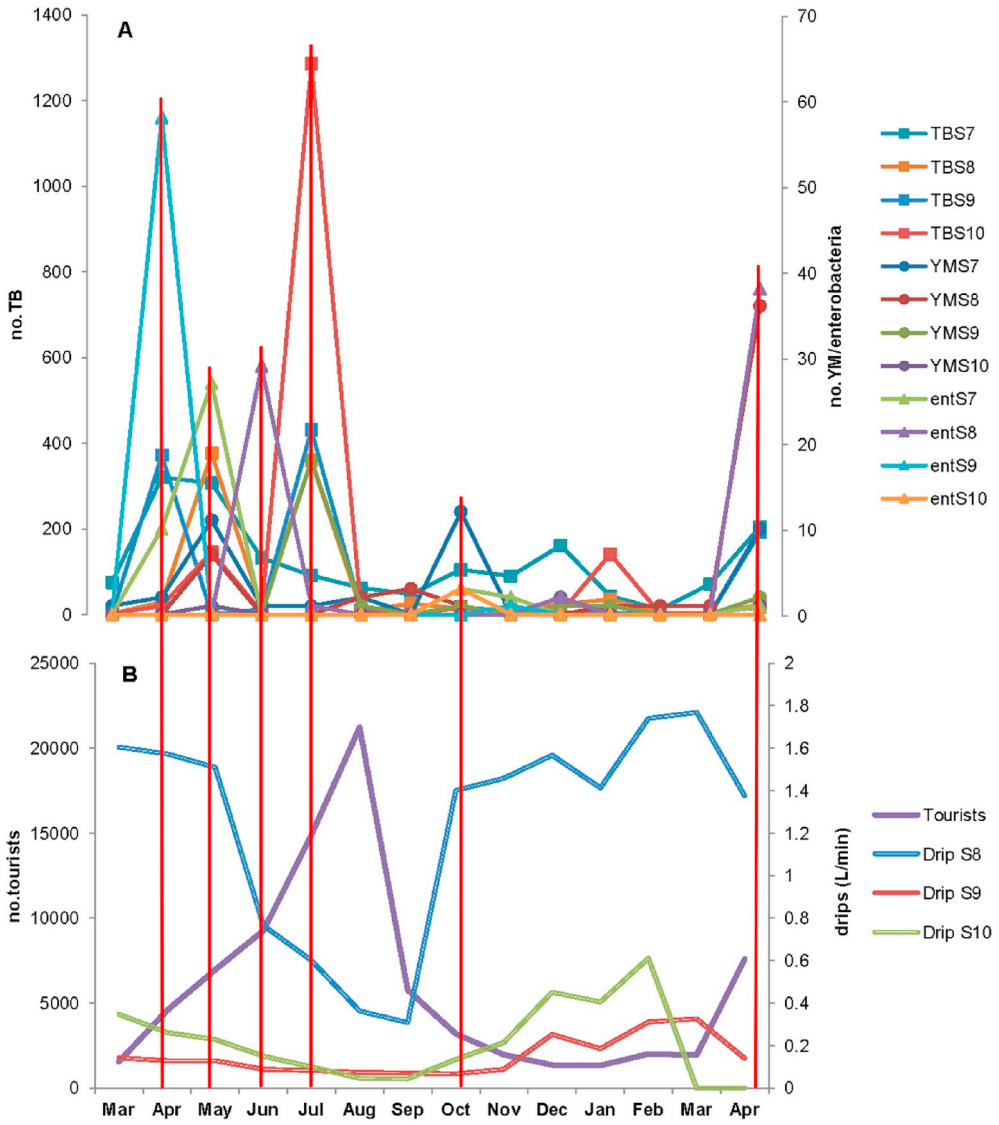

Fig. 2. Abundances of TB, YM and Enterobacteriaceae (A), and the number of tourists and drip flow (B) in the same period in Ursilor. Red lines indicate periods with increased microbiological load of water bodies; see also Tables 2 and 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.).

April, September and December 2016 from the four investigated caves. Bacterial DNA extraction was performed for 5 colonies in Ursilor, 7 in Meziad, 9 in Muierilor, and 2 in Polovragi. For YM, DNA was extracted from 2 colonies in Ursilor, 2 in Meziad, 3 in Muierilor, and 1 in Polovragi.

Extraction was performed with Chelex®100 (Bio-Rad) [5% solution in TE buffer, according to Walsh, Metzger, and Higuchi (1991)]. When this method was not successful we used DNA extraction with Isolate II Genomic DNA Kit (Bioline). Total DNA concentration was measured using a Nanodrop Spectrophotometer (BioDrop, U.K.) and expressed as ng/μL.

After extraction, DNA was stored at −20 °C until further use. Two bacterial specific primers, targeting the 16S rRNA gene, were used for screening the bacteria diversity: 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-ACG GHT ACC TTG TTA CGA CTT-3′) (McGenity, Gemmell, & Grant, 1998). For YM analysis primer sets FR1 (5′- AIC-CAT-TCA-ATC-GGT-AIT 3′) and FF390 (5′-CGA-TAA-CGA-ACG-AGA-CCT-3′) of 18S rDNA were used. For TB samples a standard amplification program with initial denaturation for 3 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s and final elongation 72 °C for 10 min, was applied, while for YM the program was: initial denaturation 97 °C for 3 min, 35 cycles 95 °C for 60 s, 49 °C for 25 s, 72 °C for 35 s, and final elongation 72 °C for 5 min. The PCR was performed with Techne TC-512 (Bibby Scientific, U.K.). After PCR the products were purified with FavorPrepTM GEL/PCR Purification Kit (Favorgen Biotech Corp., Taiwan) following the manufacturer's instructions.

PCR products were purified with FavorPrep™ GEL/PCR Purification Kit (Favorgen Biotech Corp., Taiwan) following the manufacturer's instructions.

DNA was sequenced by the Sanger method at a commercial company (Macrogen Europe, The Netherlands). The sequences obtained in this study were compared to bacterial 16S rRNA gene sequences deposited in GenBank using blastn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences have been deposited in the NCBI database.

2.4. Statistical analysis

Canonical correlation analysis (CCorA) was used to relate microorganism abundances to the physicochemical features, and were analyzed with XLSTAT v.2016 software. The correlation biplots with n/p coefficients are displayed with vectors and labels for each point. CCorA is a

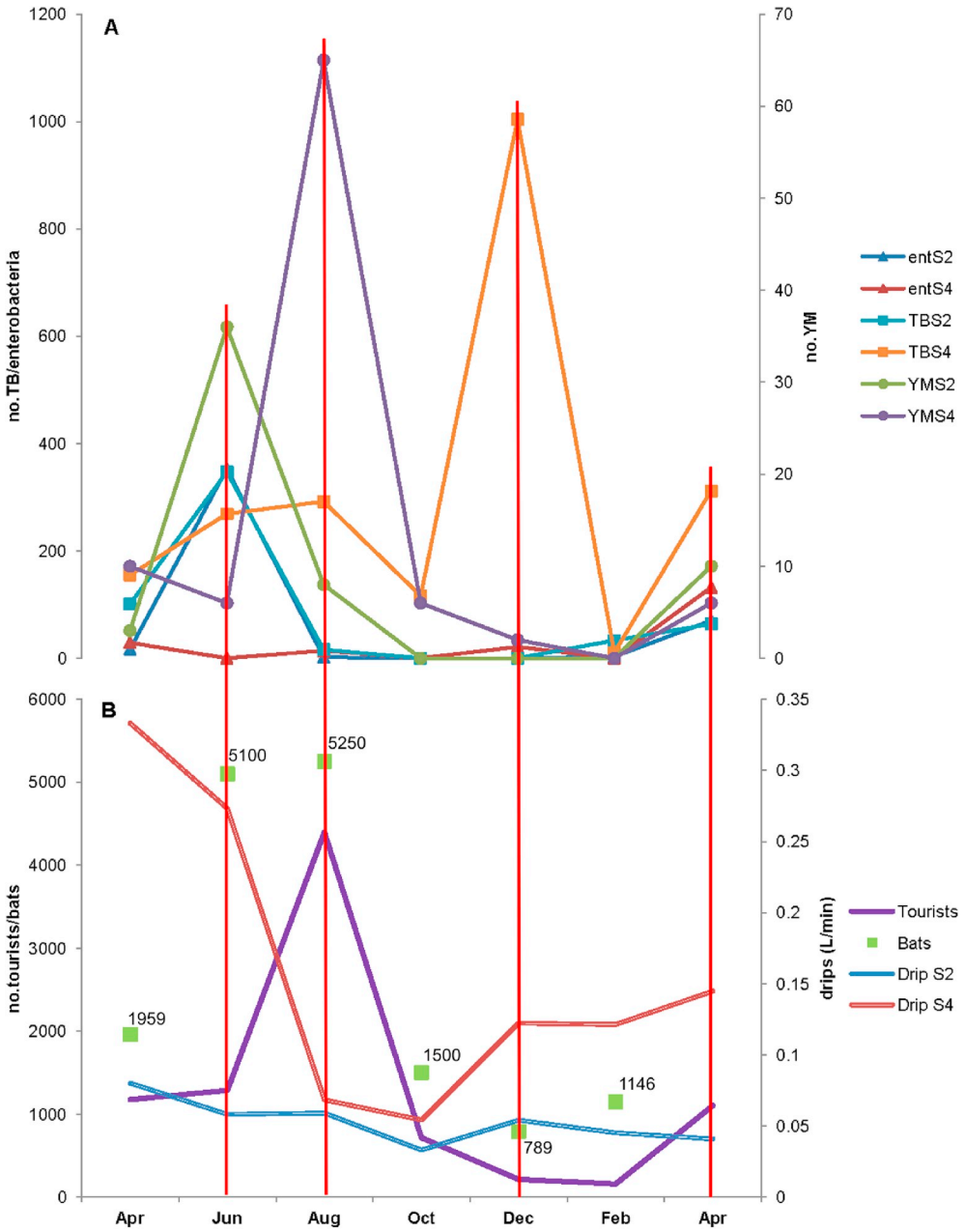

Fig. 3. Abundances of TB, YM and Enterobacteriaceae (A), and the number of tourists, bats (numbers next to green squares) and drip flow (B) in the same period in Meziad. Red lines indicate periods with increased microbiological load of water bodies; see also Tables 2 and 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.).

multivariate analysis that determines the relationships between groups of variables in a data set, even if some of the data are 0. The purpose of canonical analysis is then to find the linear combination of variables, which are most highly correlated.

3. Results

3.1. Environmental parameters

The physicochemical parameters showed relatively low variation for each sampling site (Table 2), and none of the values were higher than accepted drinking water standards (World Health Organization, 2018). The average conductivity values in the sampled waters were between 316 µS/cm and 532 µS/cm, with the lowest value belonging to the waters of Meziad, and the highest to those of Polovragi. The average water temperature varied within less than 1 °C in each of the studied caves, with the lowest value in Meziad (9.5 °C) and the highest in Ursilor (11.1 °C). The average pH of the cave water was between 7.4 (Ursilor) and 8.0 (Muierilor).

Compared to the rest of the parameters, the levels of K, Cl, Na, NO_3 and SO_4 fluctuated slightly more at some sites (e.g., Meziad S4, Polovragi S2, Ursilor S8), while others, such as pH or water temperature, were relatively constant across all caves. Most of the waters were classified as very hard (≥10.12 °dH) or hard (8.69 °dH).

Rainfall events occurred during the autumn–spring intervals, resulting in higher inputs of percolating water (Figs. 2–5), while the summer months were mostly dry. In Ursilor S8, extremely low flows were recorded during summer (less than 0.5 L/h), while higher inputs were observed at the beginning of winter, during periods of heavy rainfall or snow melt. There were lower quantities of dripping water in

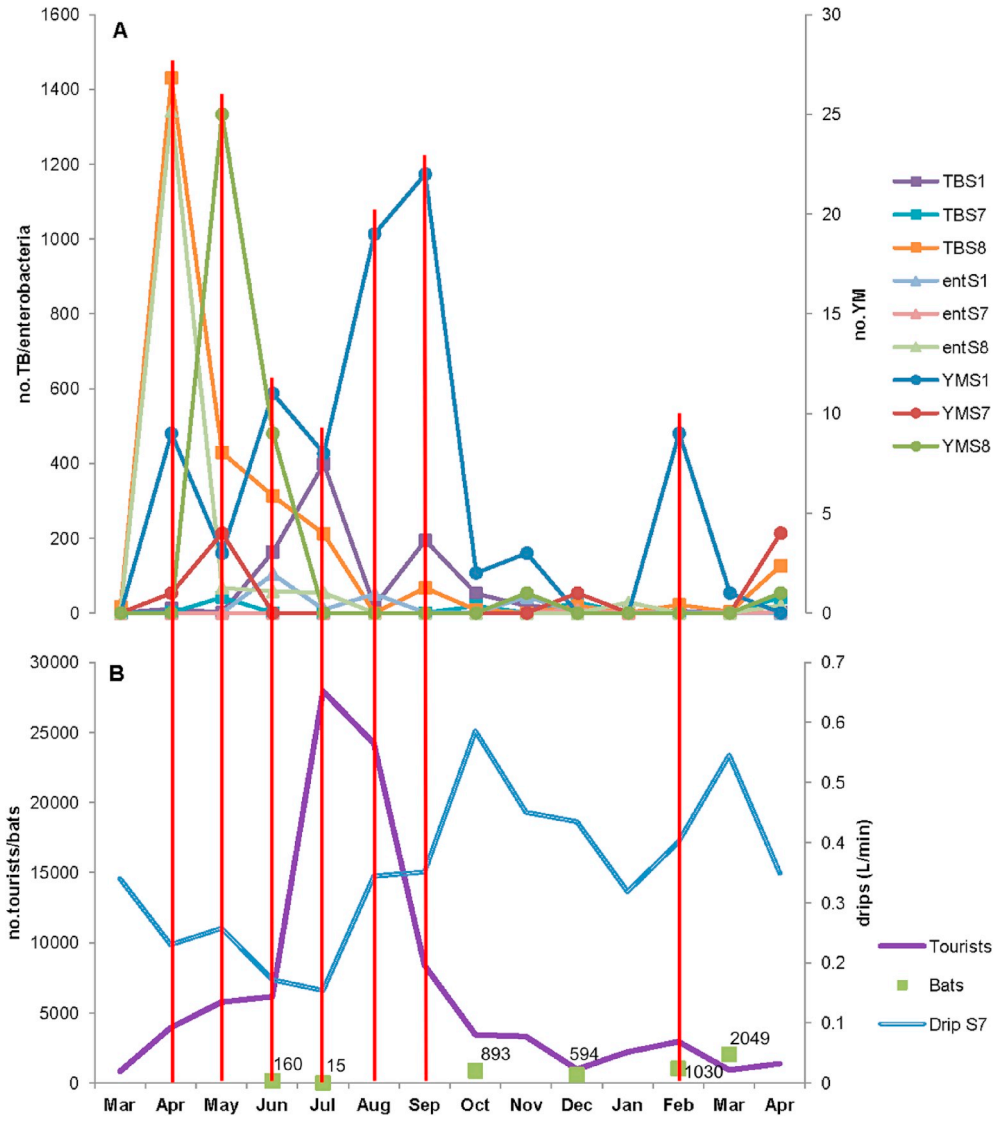

Fig. 4. Abundances of TB, YM and Enterobacteriaceae (A), and the number of tourists, bats (numbers next to green squares) and drip flow (B) in the same period in Muierilor. Red lines indicate periods with increased microbiological load of water bodies; see also Tables 2 and 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

summer, with the lowest at the end of autumn. Similar increases in water input in Ursilor S10 were observed for the winter–spring period, with the same reduction in the warm season. In quantitative terms, the flow rate for the dripping point in Meziad was much lower than that recorded in Ursilor, through the same time-interval. Flow peaks were recorded in spring and autumn, but they were not related to major rainfall episodes. In Muierilor, lower input values were recorded in the summer, with occasional spikes and a general increase in the water input starting in September. In this respect, the drip point S7 was the fastest to react to torrential events. In Polovragi, the input followed the same pattern, but with different inflow values. Quantitatively, the recorded flows reached up to 3.5 L/h at the Polovragi S2 station.

The tourist traffic showed a similar pattern in all of the studied caves: a steady grow starting in April, followed by a steep increase from June to September (Figs. 2–5). The months with the highest numbers of tourists were, in descending order, August and July for all caves. The rest of the touristic visits were spread across the spring–autumn part of the year, while the numbers for the winter months were very low for Meziad and Polovragi, when these caves are usually closed.

3.2. Distribution and abundance of microorganisms

In Ursilor, a total of 5,358 TB CFU were found in the water bodies during the monitoring program, with peaks in April, May and July (Table 3, Fig. 2). The total YM count in this cave was 122 CFU, predominantly in May, July and October. Enterobacteriaceae had a total number of 175 with the highest numbers in S9, in the protected part of the cave. The restricted area of Ursilor (S9) showed higher abundances of the monitored groups, including Enterobacteriaceae.

In Meziad, the overall number of TB was 2,719 CFU, with peaks in April, July, August and December (Table 3, Fig. 3). YM had a similar pattern of presence, in April, July and August, in lower number (152 CFU). Of the total 641 Enterobacteriaceae, most were from April and June. The two different habitats (drips and pools) showed considerable differences between them: the dripping site in Meziad (S4) contained significant amounts of TB and YM, while S2 pool had higher levels of enterobacteria (Fig. S1).

The total TB number for Muierilor was 2,195 CFU, most of which were present in the April–July period, with a smaller peak in September (Table 3, Fig. 4). Although in much lower numbers (133 CFU), YM had a similar peak presence from April until close to the end of the tourist

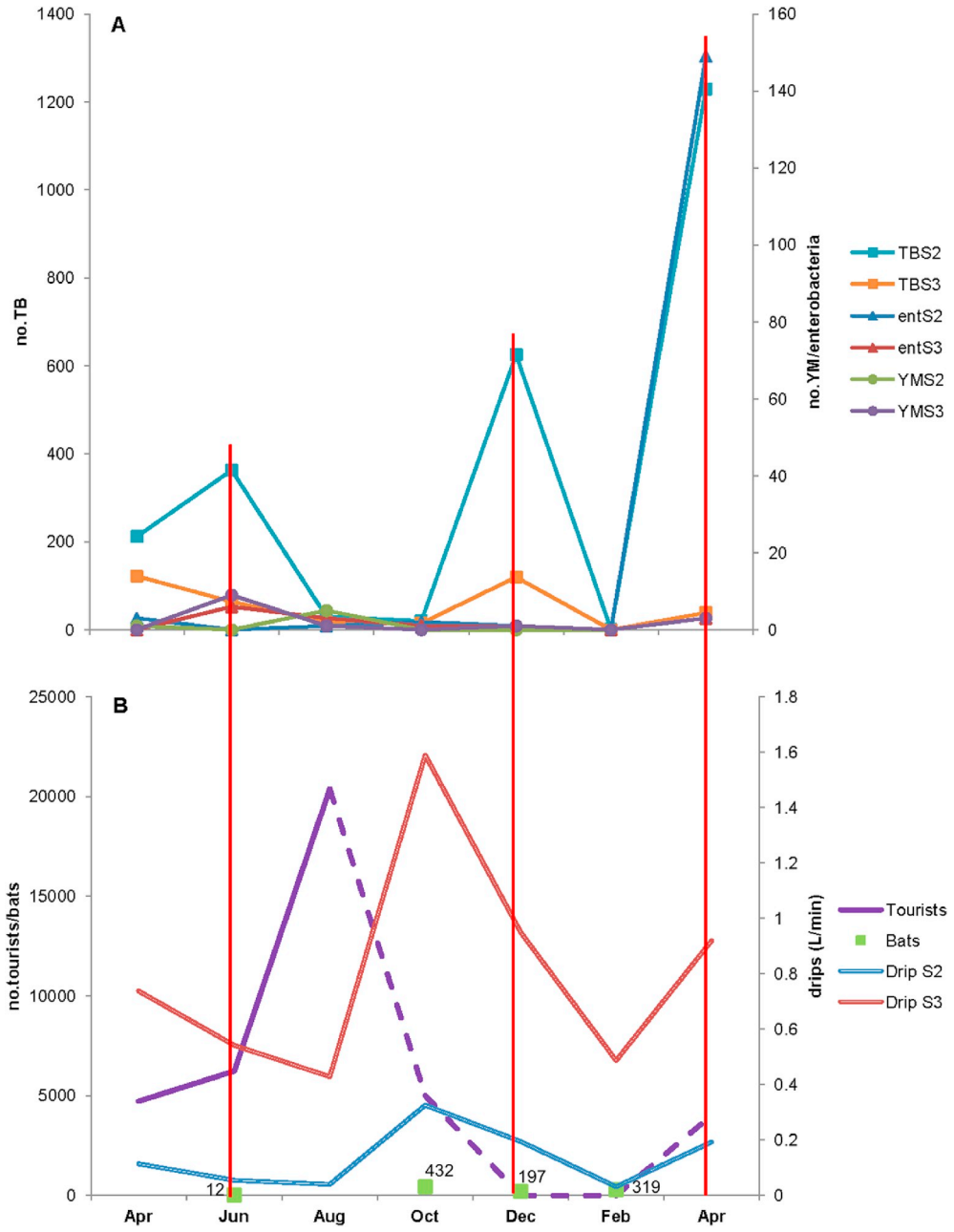

Fig. 5. Abundances of TB, YM and Enterobacteriaceae (A), and the number of tourists, bats (numbers next to green squares) and drip flow (B) in the same period in Polovragi. Red lines indicate periods with increased microbiological load of water bodies; see also Tables 2 and 3 Note that the dotted purple line represents the approximation of the tourist number. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

season in September. The highest number of Enterobacteriaceae was 1,790 CFU, with a major peak in April. Values for the unvisited part of the cave (S7) were the lowest for total numbers of TB and YM. Out of the total CFU measured during the study, only a small fraction of YM, TB and Enterobacteriaceae were from the restricted area (S7). The dripping site in Muierilor (S8) had higher levels of TB, YM and Enterobacteriaceae compared to the pools in the same cave, except for YM in S1 (Fig. S1).

Polovragi had an overall TB count of 2,857 CFU, mostly present in April and July, with high levels also observed in December (Table 3, Fig. 5). The low YM number in this cave (23 CFU) was mostly from April, July and August. The Enterobacteriaceae peak was in April, consisting of 167 CFU.

3.3. Relationships between microbiological abundances and environmental parameters

There was no significant correlation between the abundances of the three monitored groups of microorganisms and the physicochemical parameters measured on site and in laboratory (Fig. 6, Table S2). The CCorA for the on-site physicochemical parameters showed a 51.92% correlation on the x-axis, defined by TDS, while on the y-axis, defined by Cl, NO_3, Na and SO_4, a lower correlation of 32.14% was observed.

The August peak of tourism was not followed by an increase in the microbiological load of water bodies, except for S4 in Meziad. However, this station is also next to the summer bats colony which also shows an important increase during summer.

The spring and autumn high dripping is correlated with high and, respectively, low microbial load in the water bodies in Ursilor,

Table 3
Monthly values of CFU of the monitored microbiological groups in each of the sampling sites in the studied caves; * – drip water sites; in bold – surpassed limits of contamination (limits indicating human, faecal or environmental contamination in CFU: TB > 1,000, YM > 1,000, Enterobacteriaceae >10; Organisation for Economic Cooperation and Development, 2011).

Cave	Ursilor				Meziad		Muierilor			Polovragi		Date
Site/Group	S7	S8	S9	S10	S2	S4*	S1	S7	S8*	S2	S3	
TB	75	2	0	2	–	–	1	0	16	–	–	Mar-15
	320	29	372	20	101	155	11	1	**1430**	212	122	Apr-15
	307	376	0	146	–	–	1	42	429	–	–	May-15
	132	16	12	6	347	269	163	0	313	363	64	Jun-15
	91	359	431	**1285**	–	–	399	0	211	–	–	Jul-15
	62	9	10	5	16	292	18	0	0	30	17	Aug-15
	48	25	2	0	–	–	194	0	67	–	–	Sep-15
	105	14	18	16	0	116	52	19	8	20	15	Oct-15
	90	11	8	5	–	–	19	0	0	–	–	Nov-15
	161	24	5	4	0	**1004**	13	28	19	625	120	Dec-15
	43	35	2	141	–	–	1	0	0	–	–	Jan-16
	12	4	3	2	33	10	5	0	22	1	0	Feb-16
	71	2	0	3	–	–	0	0	3	–	–	Mar-16
	204	23	192	18	64	312	1	42	126	**1229**	39	Apr-16
Total	**1721**	**929**	**1055**	**1653**	**561**	**2158**	**878**	**132**	**2644**	**2480**	**377**	
Mean/station	**123**	**66**	**75**	**118**	**80**	**308**	**63**	**9**	**189**	**354**	**54**	
YM	1	0	0	0	–	–	0	0	0	–	–	Mar-15
	2	0	0	0	3	10	9	1	0	1	0	Apr-15
	11	7	0	1	–	–	3	4	25	–	–	May-15
	1	0	0	0	36	6	11	0	9	0	9	Jun-15
	1	0	18	0	–	–	8	0	0	–	–	Jul-15
	2	2	1	0	8	65	19	0	0	5	1	Aug-15
	0	3	0	0	–	–	22	0	0	–	–	Sep-15
	12	1	1	0	0	6	2	0	0	0	0	Oct-15
	0	0	0	0	–	–	3	0	1	–	–	Nov-15
	1	0	1	2	0	2	0	1	0	0	1	Dec-15
	1	1	1	0	–	–	0	0	0	–	–	Jan-16
	0	1	0	0	0	0	9	0	0	0	0	Feb-16
	0	1	0	0	–	–	1	0	0	–	–	Mar-16
	10	36	2	1	10	6	0	4	1	3	3	Apr-16
Total	**42**	**52**	**24**	**4**	**57**	**95**	**87**	**10**	**36**	**9**	**14**	
Mean/station	**3**	**4**	**2**	**0**	**8**	**14**	**6**	**1**	**3**	**1**	**2**	
ENTEROBACTERIACEAE	0	0	0	0	–	–	0	0	1	–	–	Mar-15
	10	0	**58**	0	**17**	**29**	0	0	**1342**	3	0	Apr-15
	27	0	0	0	–	–	0	0	**67**	–	–	May-15
	0	**29**	0	0	**353**	0	**104**	0	**58**	0	6	Jun-15
	0	1	0	0	–	–	9	0	**54**	–	–	Jul-15
	0	0	0	0	2	**14**	**51**	0	0	1	3	Aug-15
	0	0	0	0	–	–	1	0	0	–	–	Sep-15
	3	0	0	3	0	0	0	0	0	2	1	Oct-15
	2	0	1	0	–	–	**43**	0	0	–	–	Nov-15
	0	2	0	0	0	**21**	0	8	0	1	1	Dec-15
	0	0	0	0	–	–	0	0	**28**	–	–	Jan-16
	0	0	0	0	3	0	1	0	0	0	0	Feb-16
	0	0	0	0	–	–	0	0	0	–	–	Mar-16
	1	**38**	0	0	**70**	**132**	0	3	**20**	**149**	3	Apr-16
Total	**43**	**70**	**59**	**3**	**445**	**196**	**209**	**11**	**1570**	**156**	**14**	
Mean/station	**3**	**5**	**4**	**0**	**64**	**28**	**15**	**1**	**112**	**22**	**2**	

Muierilor, Polovragi and Meziad S2 (Figs. 2–5). The exceptions is S4 in Meziad with increased dripping that was synchronous with a TB abundance increase.

Different microbial load between April 2015 and April 2016 observed in all the four studied caves, with lower or higher abundances in the different stations, can be related only to slightly different dripping rates with various microbial input inside the cave.

Bats present in the three of the studied caves (Muierilor, Meziad and Polovragi) were in especially high numbers in Meziad during spring–summer and Muierilor during spring (Figs. 3–5). The high number of bats was associated in Muierilor and Meziad with an increase in YM number in S1 and S4, respectively.

Comparisons of abundances between airborne and water microorganisms are not showing significant correlations for TB (Table 4, Fig. 7) and water abundances were higher for both TB and enterobacteria, except for TB in Ursilor. However, in some months, an increase of airborne microorganisms also showed an increase of water microbes in the same stations. For example in Polovragi TB and enterobacteria were at high values at S2, during April (2016 and 2015, respectively). For YM, there is a strong correlation between air and water microbial load, with a higher load in the air.

3.4. Identified taxa

The composition of the water TB community was determined by 16S rRNA gene sequencing (Table 4). A total of 23 sequences were assigned to genus and, in some cases, to species level, according to their similarities to sequences from GenBank (identity values 99–100%). The most

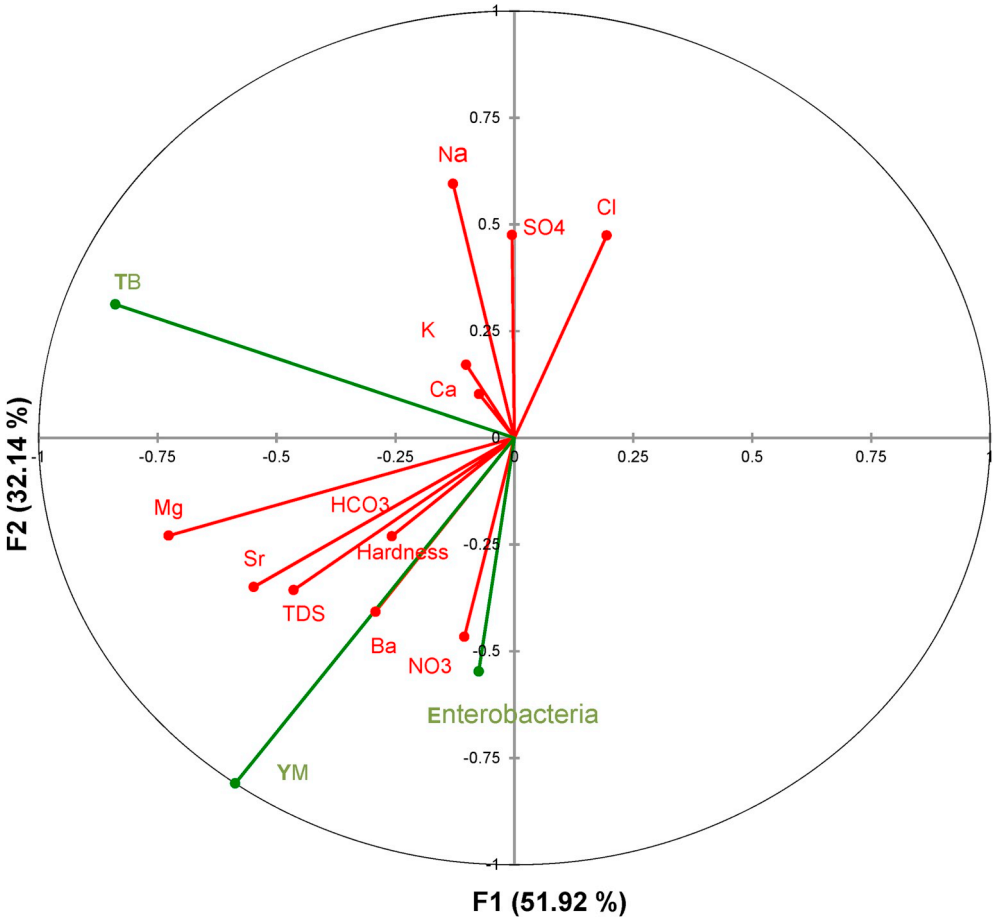

Fig. 6. CCorAs for the microbiological abundance of each sampling occasion, and the physicochemical content of the water (see also Tables 2 and S2). Note that only months with all measured parameters were considered here.

prevalent genus was *Staphylococcus*. The majority of the phylotypes present in the studied caves were *Staphylococcus* species (31%) and *Bacillus* (13%).

The composition of the YM community was assessed through retrieval and analysis of 18S rRNA gene fragments (Table 4). Based on the similarity with sequences deposited in GenBank, the 8 identified operational taxonomic units (OTUs) belonged to 6 different taxa (identity between 99 and 100% of entries in GenBank). The dominant groups were Agaricomycetes and Dothideomycetes, each with 25%.

4. Discussion

The microbiological communities of the studied cave water sites were dominated by TB, with lower abundances of enterobacteria and YM. The low number of YM compared to TB in the water bodies has been previously documented; this cannot be attributed to scarcity of nutrients in water bodies and is not exclusively characteristic of cave environments (Ainsworth & Sussman, 2013; Gessner & Van Ryckegem, 2003; Mugnier & Jung, 1985).

Among the identified strains, no cave-specific species were found throughout the monitoring program, although groups that were not previously known from cave waters were found; for example, members of the bacterial genus *Olivibacter* were isolated from bats in cave systems and surface locations in New Mexico and Arizona, USA (Hamm et al., 2017).

From the identified strains in our study, Bacillus spp. and *Staphylococcus aureus* are considered indicators of human impact in caves (Lavoie & Northup, 2006), and Pseudomonas was recovered from high-impact sites (Ikner et al., 2007). *Aeromonas* spp., *Enterobacter* spp., *Staphylococcus pasteuri*, *Klebsiella oxytoca*, *Rahnella* spp. and *Serratia fonticola* can be pathogenic for humans, while *Penicillium chrysogenum* and *Cladosporium* spp. spores produce human allergens. *Staphylococcus hominis*, *S. epidermidis* and *Streptococcus sanguinis* are commensal on humans, *Massilia suwonensis* was isolated from air samples, *Phoma* spp. are soil fungi, and *Lysinibacillus fusiformis, Olivibacter soli*, *Erwinia* spp., *Stenotrophomonas rhizophila*, Agaricomycetes and Dothideomycetes representatives were found on various organic substrates such as plants, wood, decaying leaves etc.

The physical characteristics and chemical contents of the studied water bodies showed low variability throughout the period of the study, while the microorganisms registered significant variations, even from one month to another. The high number of TB (>1,000 CFU), in some of the monitored stations and in some periods, far exceeded the levels found in other investigated water sources in Slovenian and Slovakian caves, which capped out at 434 CFU (Mulec et al., 2012), or those from Altamira Cave, which had only up to 310 CFU (Laiz, Groth, Gonzalez, & Saiz-Jimenez, 1999). The highest values of CFU in the caves monitored in this study were registered during spring-summer. August, the month with the highest tourist influx, had high but not highest number of YM and enterobacteria, with the exception of S1 in Muierilor. Dripping water had in general higher microorganism abundances than water in pools in Muierilor and Meziad, where these types of habitats were sampled in parallel. This indicates a high input of microorganisms from surface or subsurface habitats above the caves, an observation also supported by the increase in the number of TB and YM at most of the stations during spring - especially in Muierilor and Polovragi -, a period of high input of water from the surface. Nevertheless, high levels of CFU were maintained through the summer and autumn months, during the

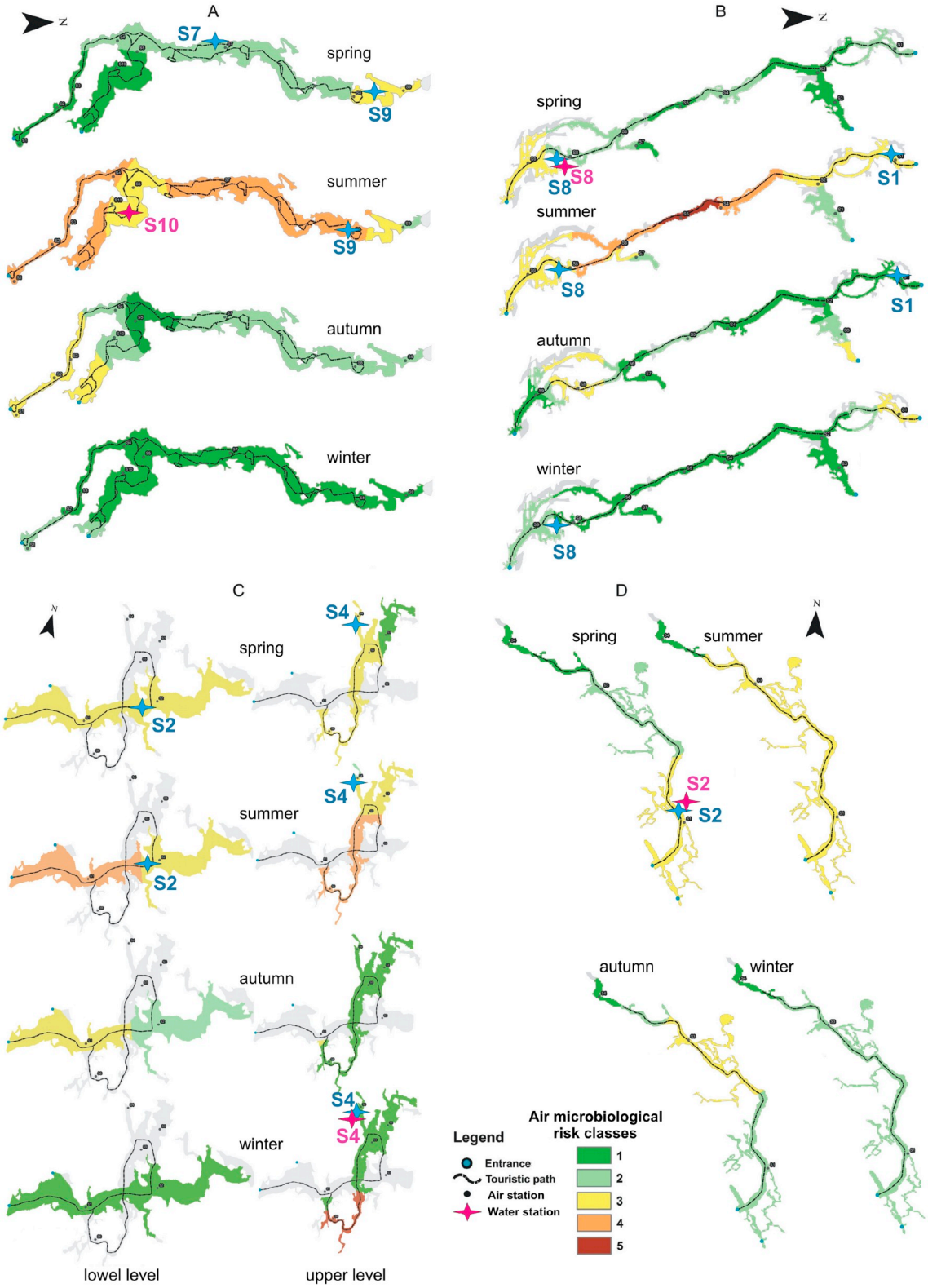

Fig. 7. Maps with classes of airborne microbial risks (modeled using abundances of airborne TB and YM; from 1 = low risk to 5 = high risk classes were defined and they were added within an ArcGIS 10.3.1. ESRI environment; modified after Bercea et al., 2018) and the critical values for TB (pink stars) and Enterobacteriaceae (blue stars) in water (see also Fig. 1 and Table 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
List of identified TB and YM in the studied caves.

	Cave	Sampling Site	Closest GenBank match		% Identity	Length (bp)
			Taxa	Identified number		
TOTAL BACTERIA	Ursilor	S7	*Lysinibacillus fusiformis*	KY038703.1	100	999
		S8	*Olivibacter soli*	NR_041503.1	100	999
		S9	*Pseudomonas fluorescens*	LN651257.1	100	989
		S9	*Erwinia* sp.	KU991850.1	100	999
		S10	*Staphylococcus hominis*	KY623285.1	100	869
	Meziad	S2	*Aeromonas* sp.	KY124163.1	100	999
		S2	*Enterobacter* sp.	LT978460.1	100	849
		S2	*Massilia suwonensis*	LN774642.1	99	585
		S2	*Staphylococcus pasteuri*	KP261076.1	99	676
		S4	*Klebsiella oxytoca*	CP026715.1	100	929
		S4	*Staphylococcus aureus*	CP012978.1	99	760
		S4	*Staphylococcus aureus*	KX348313.1	99	714
	Muierilor	S1	*Staphylococcus hominis*	MF327693.1	99	729
		S1	*Staphylococcus epidermidis*	MF327691.1	99	721
		S1	*Hymenobacter* sp.	NR_108903.1	99	986
		S7	*Bacillus* sp. 1	KY622338.1	99	644
		S8	*Stenotrophomonas rhizophila*	KY800458.1	100	898
		S8	*Rahnella* sp.	KJ939700.1	100	999
		S8	*Bacillus* sp. 2	MG210674.1	100	759
		S8	*Bacillus* sp. 3	KY849439.1	99	860
		S8	*Streptococcus sanguinis*	LC145554.1	100	989
	Polovragi	S2	*Serratia fonticola*	CP013913.1	100	999
		S2	*Staphylococcus epidermidis*	LT686077.1	99	704
YEAST & MOLDS	Ursilor	S8	*Penicillium chrysogenum*	KU559908.1	100	250
		S10	*Phoma* sp.	KM387394.1	99	260
	Meziad	S1	Dothideomycetes	KC510276.1	100	284
		S7	Dothideomycetes	AY275186.1	100	284
		S8	*Cladosporium* sp.	KJ909958.1	100	279
	Muierilor	S2	Agaricomycetes	KU141330.1	100	321
		S4	Agaricomycetes	KU141330.1	100	277
	Polovragi	S8	*Penicillium chrysogenum*	KU559908.1	100	250

tourist season, despite the low water input into the cave. A special situation was in Meziad, where the highest concentration of TB was in December, possibly determined by the bats colony hibernating inside this cave, considering that the bacterial reservoir and vector capabilities of bats is well documented (Altizer, Bartel, & Han, 2011). Comparisons of abundances between airborne and water microorganisms can explain an increase of YM in Polovragi, with high values of TB and enterobacteria at S2 during April (2016 and 2015, respectively).

The results obtained by comparing the CFU in visited and non-visited parts of the same caves in Ursilor and Muierilor, are somewhat contradictory. In Ursilor, the TB peak was 1,285 colonies in the tourist sector at one station (S10), while for the restricted area they only got up to 431 CFU (S9), but this was still a larger value than the highest values at the other two stations from the touristic sector. The unusual abundance in TB at S9, represented by a pool supplied by water with occasional high flow rates, could indicate a relatively direct inflow from the surface. In this case, the higher levels of enterobacteria could have originated from the soil/land above and around the cave. The fecal bacteria loads of agriculture-influenced water from caves in Ozark (USA), at levels of up to 831 CFU (Graening & Brown, 2003), are lower than those in the caves studied herein, and much higher than the ones in dripping water from other caves (Mulec & Oarga, 2014; Mulec & Oarga-Mulec, 2016). The negative influence of organic pollution and agricultural practices at the surface on cave water quality has previously been demonstrated (i.e., Neill, Gutierrez, & Aley, 2004; Moldovan, Iepure, & Perşoiu, 2005; Pacioglu & Moldovan, 2016). In Muierilor, the difference was of two orders of magnitude between the maximum values in the visited and restricted sectors (1,430 versus 42 CFU, respectively, at S7) – a good example of the strong impact of tourism.

The microbiological loads of the waters in the four studied show caves were subject to variability caused by microhabitat type (pool or dripping water), seasonal water input from the surface (dripping), and the presence of both tourists and bats (Table 5).

We propose the following measures to be taken for a better management of the studied show caves (and of show caves, in general) and we communicated our proposals to their managers. Such measures cannot buffer tourism impacts which are inevitable but their effects can be minimized:

Table 5
Summary of the factors that are susceptible to impact water bodies microbiology in the studied caves.

	CAVE			
	URSILOR	MEZIAD	MUIERILOR	POLOVRAGI
Physico-chemistry	NO	NO	NO	NO
Tourism	NO	YES (S4)	YES (S1)	NO
Dripping rate	NO	YES (December)	NO	YES
Bats	N/A	YES	YES (S1)	NO
Air microorganisms	YES for YM	YES for YM	YES for YM	NO

(1) Microbiological monitoring. It should be assumed as an important instrument for the show caves management in general. It is an instrument that proved to be a better indicator of human impact, with seasonal or even monthly variations, than the relatively constant water physicochemical characteristics. The physicochemical water parameters are the least sensitive indicator of pollution/human impact on cave environments, and microbial load may be a much better proxy for the processes that can alter groundwater quality.

A simple method, such as the test-plates used in this study, can yield results that are efficient, in terms of time and money, for the rapid estimation of possible human impacts at the surface and underground, by sampling in cave water bodies. Differences between the microorganisms abundances in the same months of the two different years of the

study showed that monitoring should be a continuous process of surface and underground changes for a sustainable use of caves for tourism.

(2) Paths. In the cases of Ursilor and Muierilor the concrete path act as a the barrier that limits the tourists impact on the pools with water on the cave floor. Ursilor had the lowest mean value of all three groups of microorganisms in the water bodies. Concrete paths can be carefully cleaned and might be the best solution for show caves, where possible. The highest TB mean value was found in Polovragi, a cave where there is no marked touristic path. Therefore, the simplest measure to avoid water pollution would be to mark a narrow path that avoids contact with the pools on the floor.

(3) Bats. Meziad had high TB and the highest mean number of enterobacteria but the situation was different than in Polovragi. Meziad has a traced natural path which crosses several points where bats and guano are present. High abundance of enterobacteria were also found in Muierilor where bats are found all along the touristic path. For Meziad and Muierilor the management measures should concentrate on redesigning the paths, where possible, to avoid the direct contact of visitors with bats and guano.

(4) Cave water consumption. In Polovragi, people are encouraged to drink water from the pool on the cave floor (S2), recommended by the guides as having very good quality. It is from the physicochemical point of view, but the presence of Enterobacteriaceae forbids its use.

(5) Surface management. Protecting caves by limiting direct human impact is not sufficient, and a more extensive approach, which could potentially protect entire water basins and the areas at the surface that might feed into caves, should be implemented for their conservation (Watson et al., 1997; Moldovan, Pipan, Iepure, Mihevc, & Mulec, 2007, 2012). Managers of the show caves should advocate for the preservation of the primary vegetation on the surface and the avoidance of the land use as pastures where the direct or diffuse inlet(s) are identified. Therefore, hydrological tracing should be another management measure that can be undertaken in order to find the precise input of water pollutants in caves.

In conclusion, there are two main aspects regarding the importance of including water bodies in the management of show caves. The first aspect stems in the danger of an microbial outbreak, very hard to control in a confined underground environment. Such an outbreak can impact both, the objects inside the cave and the visitors. The second aspect is the scientific importance of cave microorganisms that are involved in nutrient cycling beneficial for other cave organisms, may produce biochemical substances of use for humans, and serve as analog for studying life forms on other planets (Northup, 2011).

Authors contribution

OTM designed the research, SC secured funding and analyzed chemical data, SB, RNB, MK, ICM, AP, MR, RAA, SC and OTM performed the field work, RNB and SB performed the laboratory work, OTM, SB and RNB wrote the paper. All coauthors contributed to improve the manuscript.

Acknowledgements

We are grateful to Stelian Grigore, Cristinel Fofirică, Arthur Dăscălescu, Marius Iliescu (Hades Speleological Club, Romania) for providing the map of Muierilor Cave, to Andreea Baricz, Adrian-Ștefan Andrei and Horia Leonard Banciu for the help with the molecular analyses, and to Stein-Erik Lauritzen for the chemical analyzes. We are also thanking Transylvania Tour SRL, Apuseni Natural Park, CAPDD Bihor, the town hall of Baia de Fier, the Gorj County Museum, and all the caves guides for allowing the access and the studies in the caves. This study was financially supported by the EEA Financial Mechanism 2009–2014 under the project contract no. 17SEE/2014, EEA Grants 2014–2021 under Projects EEA126 and EEA138, contracts no. 3/2019 and 4/2019, and by a grant of the Ministry of Research and Innovation, CNCS - UEFISCDI, project number PN-III-P4-ID-PCCF-2016-0016, within PNCDI III.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tourman.2019.104037.

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