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First Report on a 5-Year Monitoring of Lampenflora in a Famous Show Cave in Serbia

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Abstract
Many show caves are vulnerable to various disturbances, meaning that conservation of such habitats, which would include monitoring of their ecological parameters and lampenflora (a growing problem worldwide), should be a priority. For the first time in Serbia, lampenflora was monitored continously for 5 years (2016–2020), three times per year during the tourist season, in the Lazar Cave. Artificial light created favorable conditions for the proliferation of phototrophic microorganisms that were developed not only as epiliths, but also endoliths, which poses a greater danger for cave substratum and structures. Although a higher diversity in general was found in Cyanobacteria (coccoid forms mostly), Chlorophyta were more widespread and abundant in samples, among which *Chlorella*, *Stichococcus bacillaris*, and *Klebsormidium flaccidum* stood out. *Chlorella* is one of the genera making lampenflora dangerous, as it can switch from an autotrophic to a mixotrophic, and finally to a heterotrophic lifestyle. The mosses protonema and mosses itself were also present. Even though the cave is closed for 6 months every year, lampenflora "legacy" always persisted on all sites from the previous year, spreading further over the years. Measured parameters (temperature, relative air humidity, light intensity, substrate pH, and substratum moisture), primary production, and biofilm parameters showed yearly, seasonal, or sampling site variations. Statistical analyses were used to examine the effect of the sampling year, the season, and sampling site on the selected measured parameters, while multivariate analyses were performed with taxa in relation to year, season, site, and main ecological parameters.

Keywords Cyanobacteria and algae · Lampenflora · Monitoring · Show cave · Ecological threat · Conservation

Introduction

Phototrophic microorganisms occupy various niches of the biosphere, including subterranean environments, such as caves. Most are transported into such environments by air, water, sediment, or animals (Northup and Lavoie 2001), where they inhabit a variety of different mineral surfaces in different microhabitats (Saiz-Jimenez 2012). The limited air circulation in caves can favor an increase of concentration of different particles, as well as their chances for settlement on cave surfaces (Albertano 2012). Due to the presence of light, phototrophs are always found at cave entrances extending to a penetration depth of light. Unless an artificial light is present, those organisms could not be found in deep cave zones (Saiz-Jimenez 2012). Artificial light in show caves serves to highlight their aesthetic value and ensure the safety of visitors. However, it can also inadvertently cause invasive growth of phototrophs (Meyer et al. 2017), especially considering that various surfaces along illuminated tour trails are moistened, thus representing ideal sites for colonization (Smith and Olson 2007). Once hypogean environments are illuminated by any light source, microbial community called lampenflora starts to develop. It is composed of different bacteria, Cyanobacteria, algae, sometimes also mosses and ferns and usually strongly adheres to the substratum surface (Mulec 2012). Its presence can lead to aesthetic problems and alterations of the delicate cave environment, as well as considerable input of organic matter in the cave ecosystem (excretion of different organic compounds and lysis of dead organisms), and damage to cave fauna (Cañaveras et al. 2001; Castello 2014). However, the most negative side of its presence is reflected in cave structure biodeterioration

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(Cañaveras et al. 2001). Microorganisms can alter rocks through various mechanisms: mechanical actions, secretion of exoenzymes, organic and mineral acids (Northup and Lavoie 2001). During respiration, they often produce carbonic acid, which is highly corrosive, especially for limestone (Smith and Olson 2007). Consequently, the dissolutional pits which can be observed on rock surfaces in caves are more frequent beneath biofilms (Cañaveras et al. 2001).

Caves represent very sensitive environments of unusual and exceptional beauty, natural and cultural importance, and many of them are enlisted on the United Nations Educational, Scientific, and Cultural Organization (UNESCO) World Heritage List. In general, they are very stable, but their environment can be easily altered. Transformation into important touristic attractions can significantly affect their natural environmental and microclimatic conditions and existing equilibrium (Cigna and Forti 2013; D'Agostino et al. 2015). Path construction, electricity, and water infrastructure can cause irreversible changes, and so can tourism, if not properly regulated (beside lampenflora, changes in air temperature, relative humidity, carbon dioxide, and radon concentrations, and higher concentration of fungal spores and bacteria are noticed). Consequently, conservation of such unique places should be a priority in every sense. Nevertheless, they are frequently poorly conserved and left vulnerable to various types of disturbances (Mulec 2014; Guirado et al. 2019; Piano et al. 2021). For conservation purposes, from the moment of the cave opening to the public, the monitoring in general (of environmental parameters, as well as lampenflora monitoring) should be the most important management aspect and performed regularly, as it is key for observing potential changes in cave environment and intervening accordingly (Parise 2011; D'Agostino et al. 2015). However, according to Day and Koenig (2002, p.131), "cave monitoring is poorly developed and is hampered by a general lack of awareness of its importance and utility." Some show cave managers choose economic interests over conservation, which can negatively affect the cave environment over longer periods of time and lead to various detrimental effects (Saiz-Jimenez 2012). Others ignore the presence and the consequence of lampenflora development until the problem becomes very obvious and difficult to solve (Mulec 2014) requiring different methods (Mulec and Kosi 2009; Borderie et al. 2014; Esteban Pérez 2017; Meyer et al. 2017; Pfendler et al. 2017). Scientific research requires space, time, and money, but can help the management of a show cave in preserving the cave equilibrium (Cigna and Forti 2013). Thus, scientific researchers together with cave management can ensure its sustainable exploitation in every possible manner (Parise 2011; Cigna and Forti 2013).

Long-term monitoring of lampenflora in show caves has never before been done in Serbia. Therefore, the goal of this work was to monitor the lampenflora in the Lazar Cave during a 5-year period. The monitoring program included sampling three times per year: at the beginning, in the middle, and at the end of the tourist season. Changes in lampenflora development, diversity of phototrophs, and environmental parameters during the 5 years of sampling were recorded and statistically processed.

Material and Methods

Sampling Location

Lazar Cave is situated in Eastern Serbia, 3 km northwest of the village Zlot, at the end of the left side of the Lazar River gorge. The entrance to the cave lies at 291.41 m a.s.l., 6.71 m above the Lazar River bed. The cave is built in layered limestones of lower Cretaceous period (Đurović 1998; Lazarević 1998).

Lazar Cave is a spring cave with two different types of cave canals that can be differentiated as (i) dry fossil and (ii) active ones, characterized by periodical or permanent water flow (Lazarević 1998).

Initially, the cave entrance was very small, cluttered with river material from its temporary water flow and it could only be entered by crawling. Then, during the first phase of reconstruction, a 15.2-m wide and 5.42-m high entrance was opened in 1953. However, the opening of such a relatively closed ecosystem, with a stable microclimate, resulted in a significant deterioration caused by external climate conditions. This was reflected in a lower amount of seeping water, the drying of cave structures, and halting of calcite material deposition. Second phase of the reconstruction was finished in 1978, when the entrance canal was partitioned with a massive wall made of stones and concrete. This had a positive influence on physical and chemical processes in the cave, and the cave microclimate was revitalized and restored. The cave was opened for tourists in 1978, with a 694.5-m-long trail (Lazarević 1998).

At the entrance to the cave, several canals diverge from the entrance hall: two shorter ones spread before the stone wall, and several of them after it. The main canal consists of a northwestern and a northern canal (Fig. 1). It can be defined as an erosive canal type, with a width varying from 7 to 15 m, and height from 2 to 10 m. With the exception of several stalagmites and stalactites, this part of the cave is not characterized by particular richness of the cave structures (Lazarević 1998).

According to the recent data, the Lazar Cave has 16,041 m of explored canals and two entrances, with a great potential for further research and the discovery of new unexplored areas. In addition to it being the longest cave
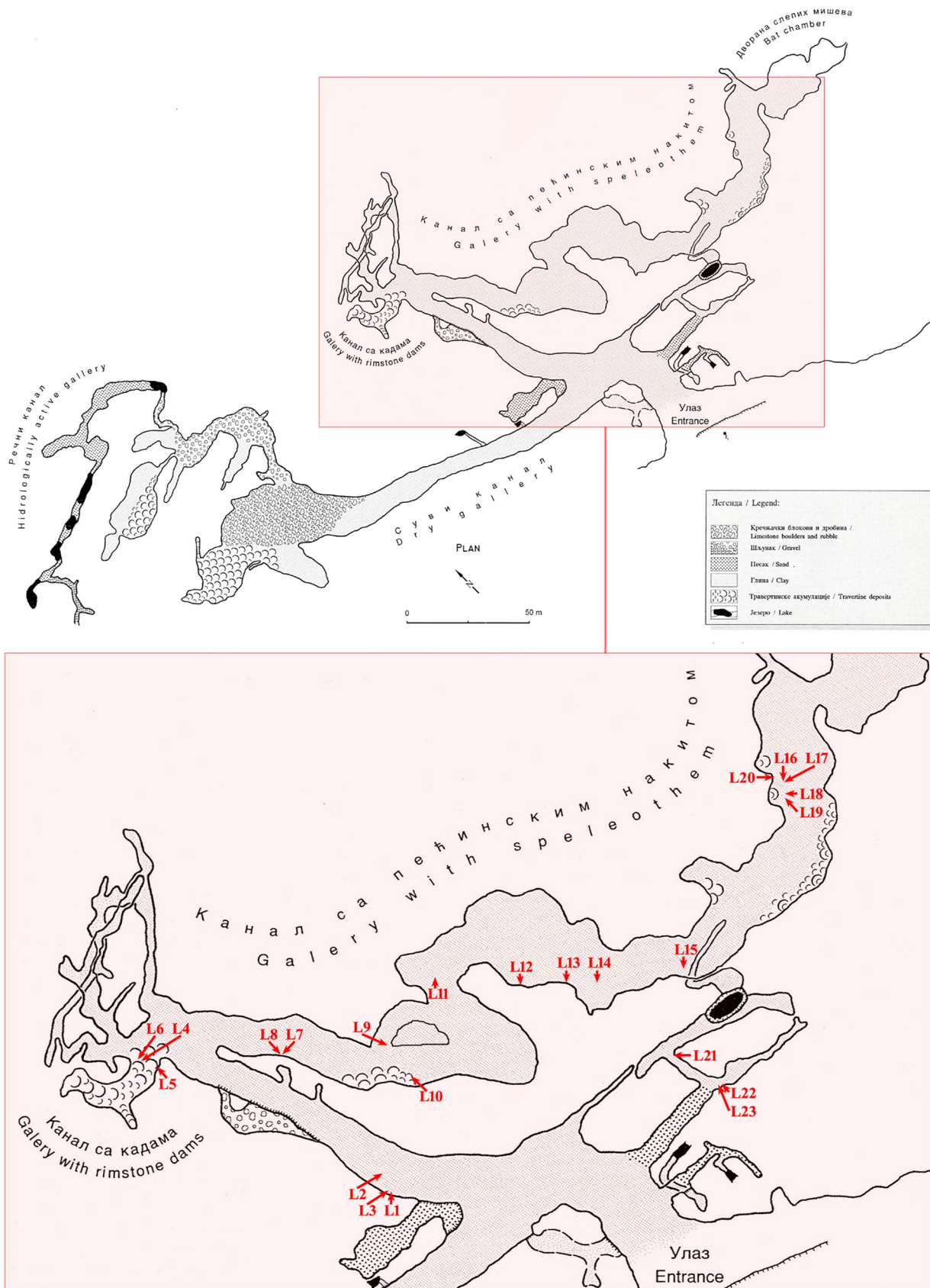
Fig. 1 Modified map of the main canal in the Lazar Cave (scanned page from Đurović et al. 1998), with designated sampling sites along the tourist trail in the northern canal

system in Serbia, it is also included in the list of the longest cave systems of the world (Mišić et al. 2019). Its attractiveness and uniqueness also reside in the fact that it is a well-known and significant archeological and paleontological site (Đurović 1998; Lazarević 1998).

The main canal, or more precisely the northern canal (marked red on Fig. 1), through which the main part of the tourist trail leads, stands out as the most attractive part of the cave for tourists. Artificial light, mainly originating from old reflectors which emit warm white light, is installed along the whole trail, but does not work continuously; it is active only during the cave tour, after which it is switched off until the entry of the next group of tourists. The number of cave tours during the day is not clearly defined and depends on the number of visitors. The sampling of lampenflora was performed in the northern canal, and positions of all sampling sites are shown in Fig. 1 and listed in Table 1. The cave is open for tourists every year from the May 1st until October 31st. The exception was the year 2020, when the cave was opened on June 21st and closed on November 16th, due to the coronavirus outbreak. From June 21st until September 21st, the cave was accessible to tourists only on weekends, and after that, until closing, for 4 days a week.

Lampenflora Sampling Sites and Sampling Methodology

Each time during the monitoring period, places near artificial light and in its surroundings were observed for the possible presence of phototrophic microorganisms. The aim was to record and perform sampling at as many as possible sites where the lampenflora, manifested mostly as green coloration of the stone substratum, was present. Samples were taken from vertical and horizontal substrates (cave walls, cave structures, sediment), or the cave ceiling (Table 1). Regretfully, many places overgrown with lampenflora were not accessible (high places of the cave) and thus could not be sampled.

As seen in Table 1, the research included localities from which sampling was done with different dynamics. There are nine constantly monitored sites: L1, L4, L5, L7, L16–L19, and L22; some of them are shown in Fig. 2 as they looked in 2016 and in 2020. L9 was sampled only in 2016 and 2017, but since it was hardly accessible and on muddy surface, it was excluded from further monitoring. The number of sites from which lampenflora was sampled increased over time, so the sampling site L13 was included from 2017 onwards, L10 and L11 were sampled from 2018, and L2 and L21 from 2019 onwards. However, there were also some sites which were sampled only for one year during this 5-year period, such as L23 that was sampled only in 2016. The sampling site L22 was near an artificial light source, but at the end of the tourist season, lighting was redirected, causing the development of lampenflora not far away at a new site, marked as L23. However, the following year, the artificial lightning was returned to its original place, L23

was excluded, and sampling was again performed only on L22. Lampenflora at L12 was spotted only in 2017. Lampenflora was present at L14 only in 2018 and after sampling, we blocked light near this sampling site with a pile of stones to see if that will show some effect the following year. The blocking of light had a positive effect, which causes lampenflora to recede and the following year it was not observed here. Several sites were sampled only in 2019: L3 (additional sampling site for L1), L6 (additional sampling site near L5), L8 (additional site near L7), and L20. L15 was sampled for the first time in 2020, since biofilm at this sampling site differed from others.

Due to the nature of biofilm (more endolithic than epilithic), lampenflora sampling was performed predominantly by using adhesive tape strips (Gaylarde and Gaylarde 1998; Urzi and Leo 2001) and in some cases additionally with a flame-sterilized scalpel (Popović et al. 2015, 2017). In addition to qualitative, biofilm was also sampled for quantitative analyses as described in the next section. This included applying round metal molds which cover a certain surface area, as described in Popović et al. (2017). Microscopic slides with adhesive tape strips were stored in microscope slide boxes and biofilm samples kept in sterile polyethylene bags were transferred to the laboratory for further processing and analysis.

Determination of Ecological Parameters, Chlorophyll A, and Biofilm Parameters

Prior to sampling, the distance from each sampling site to the light source was measured using a meter. Ecological parameters such as temperature (T (°C)) and relative humidity (RH (%)) were determined by Humidity Meter, Extech, USA. Light intensity (LI (lx)) was measured using DMV 1300 Luxmeter, Velleman, Belgium. pH meter was used to determine the pH value of the stone substratum (where possible), while the moisture content of the substratum (SM) was determined with the moisture meter.

Very often, samples could only be taken by using the adhesive tape method (Table 1), since there was not enough epilithic biofilm developed to be sampled for additional analyses. When possible, the biofilm was also sampled with a scalpel to estimate the Chl *a* concentration and biofilm parameters. Chlorophyll *a* content (µg Chl a/cm^2) was determined following the method described in Popović et al. (2015), so primary production could be assessed. Finally, biofilm parameters water content (WC), content of inorganic (IM), and organic matter (OM), expressed in mg/cm^2 and shown as a percentage share in biofilm samples, were determined as described in Popović et al. (2017).

## Cyanobacterial and Algological Analyses

Light microscope Zeiss Axio-ImagerM.1 with AxioVision 4.8 software was used for the observation and identification

Table 1 Sampling sites in the Lazar Cave during the 5-year monitoring period (2016–2020). Sampling times during the tourist season (B – beginning, M – middle, E – end of the tourist season), sampling method/type of analyses for which the samples were taken, distance between the artificial light source and the sampling site, and place of every sampling site are specified. *At* adhesive tape method, *S* use of scalpel for additional biofilm sampling for qualitative or other analyses (determination of water, inorganic and organic matter content), *Chl* biofilm sampling for Chl *a* analysis. Gray cells filled with dash designate seasons in which neither biofilm sampling for qualitative and quantitative analysis, nor measurement of other parameters were performed

Sampling sites	Time of touristic season	2016	2017	2018	2019	2020	Artificial light distance	Place of sampling site
L1	B	-	At	-	At	At, S	2.48	Cave wall vertical
	M	At	At, S, Chl	At, S, Chl	At, S, Chl	At, S		
	E	At, S, Chl	At, S, Chl	At, S, Chl	At, S, Chl	At, S		
L2	B				At, S, Chl	At, S	5.10	Cave wall vertical
	M				At, S, Chl	At, S		
	E				At, S, Chl	At, S		
L3	B				-		1.70	Cave wall vertical
	M				-			
	E				At, S, Chl			
L4	B	-	At	-	At	At	2.55	Cave structure horizontal
	M	At	At	At	At	At		
	E	At	At	At, S, Chl	At	At		
L5	B	-	At	-	At	At	4.60	Cave wall vertical
	M	-	At	At, S, Chl	At	At		
	E	At, S, Chl	At	At, S, Chl	At	At		
L6	B				-		4.80	Cave structure horizontal
	M				-			
	E				At, S, Chl			
L7	B	-	At	-	At	At	3.50	Cave structure vertical
	M	At	At	At	At	At		
	E	At, S, Chl	At	At, S	At	At		
L8	B				-		3.30	Cave structure vertical
	M				-			
	E				At			
L9	B	-	At				1.00	Cave structure with sediment nearly vertical
	M	At	At					
	E	At, S, Chl	At					
L10	B			-	At	At	1.00	Cave structure horizontal
	M			At	At	At		
	E			At	At, Chl	At		
L11	B			-	At	At	2.30	Cave structure vertical
	M			-	At	At		
	E			At	At	At		
L12	B		-				1.95	Cave structure vertical
	M		-					
	E		At					
L13	B		-	-	At	At	2.31	Cave structure vertical
	M		At	At	At	At		
	E		At, S, Chl,	At, S, Chl	At	At		
L14	B			-			7.50	Cave structure vertical
	M			-				
	E			At				
L15	B					-	0.50	Cave ceiling
	M					At, S		
	E					At, S		
L16	B	At	At	-	At	At	6.00	Cave structure vertical
	M	At	At	At	At	At		
	E	At	At	At, S, Chl	At	At		
L17	B	At	At	-	At	At	6.00	Cave structure vertical
	M	At	At	At	At	At		
	E	At, S, Chl	At	At, S, Chl	At	At		
L18	B	-	At, S, Chl	-	At, S, Chl	At	1.50	Cave sediment horizontal
	M	At, S, Chl	At, S, Chl	At, S, Chl	At, S, Chl	At		
	E	At, S, Chl	At, S, Chl	At, S, Chl	At, S, Chl	At		
L19	B	-	At, S, Chl	-	At, S, Chl	At	1.50	Cave sediment horizontal
	M	At, S, Chl	At, S, Chl	At, S, Chl	At, S, Chl	At		
	E	At, S, Chl	At, S, Chl	At, S, Chl	At, S, Chl	At		
L20	B				-		7.50	Cave wall vertical
	M				-			
	E				At, S, Chl			
L21	B				-	At	1.20	Cave wall vertical
	M				At	At		
	E				At	At		
L22	B	At	At	-	At	At	1.00	Cave structure vertical to horizontal
	M	At	At	At	At	At		
	E	-	At	At	At	At		
L23	B	-					1.50	Cave structure vertical to horizontal
	M	-						
	E	At						

Fig. 2 Selected constantly monitored sampling sites in the Lazar Cave as they looked in 2016 and in 2020
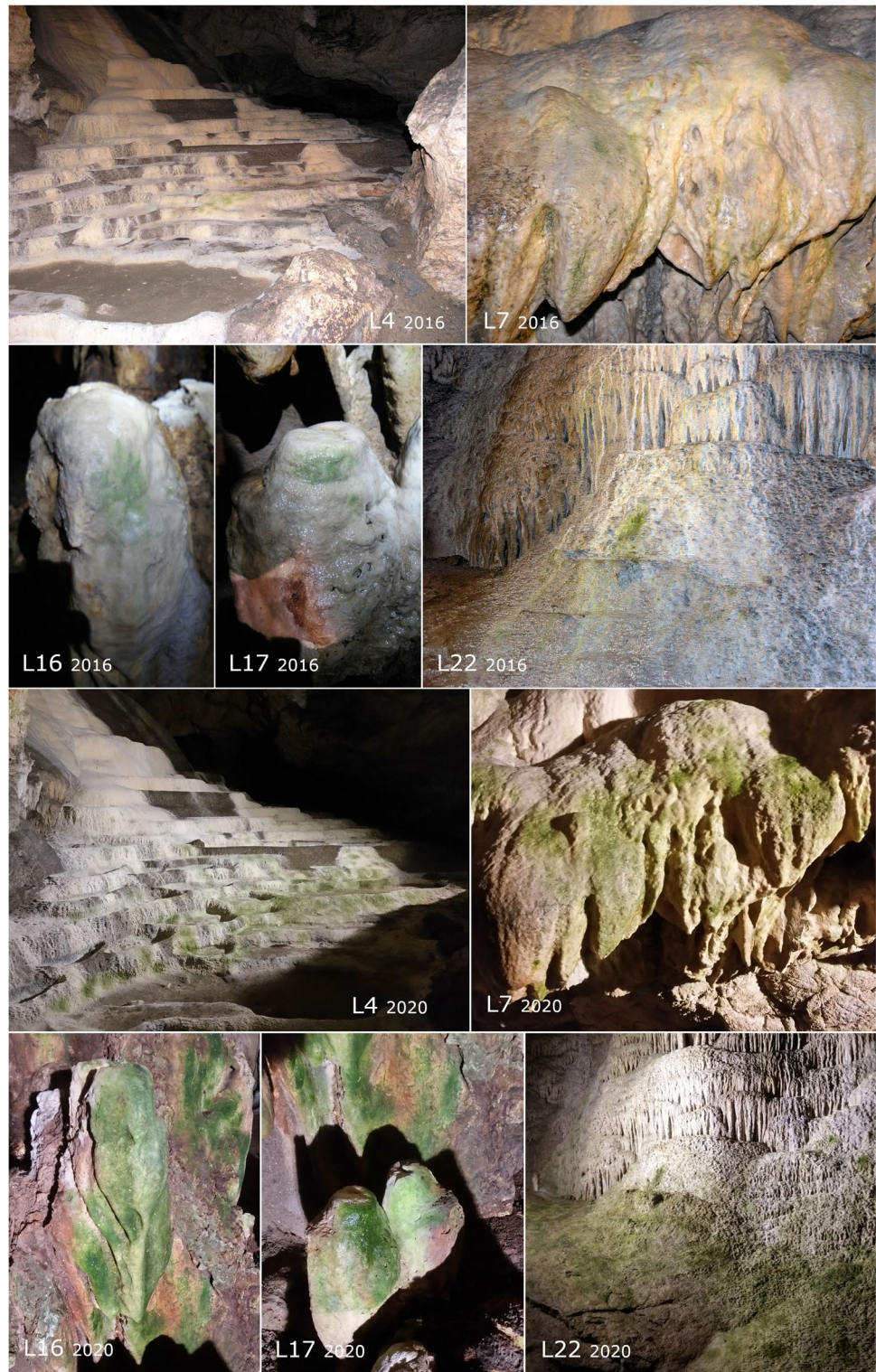
of phototrophic representatives present in the lampenflora samples. For that purpose, adhesive tape strips and temporary microscopic slides containing a small amount of sampled biofilm mixed with a drop of glycerine were analyzed using different magnifications ($400\times$, $640\times$, and $1000\times$). Observed cyanobacterial and algal representatives were identified to the species or genus level, using standard literature: Komárek and Anagnostidis (1998, 2005, 2013), John

et al. ([2003](#)), Hofmann et al. ([2013](#)), and Ettl and Gärtner ([2014](#)).

Statistical Analyses

Effects of the sampling year (when every season is considered separately and overall), the season and sampling site were tested on the selected measured parameters. Prior to these analyses, normal distribution (Shapiro-Wilks test) and homogeneity of variance (Levene's test) were assessed. If normal distribution and homogeneity of variance were met, one-way analysis of variance (ANOVA) was performed. If non-homogeneity was detected and data were not normally distributed, non-parametric test Kruskal–Wallis one-way analysis of variance was used. A threshold of $p < 0.05$ was applied for all tests. All of the aforementioned analyses were performed using Microsoft Excel and the statistical package XLSTAT (Addinsoft [2020](#)). Microsoft Excel was also used to determine correlations between certain parameters.

Principal component analysis (PCA) was used to demonstrate the relationship of water content (WC), organic matter (OM), and inorganic matter (IM) and sampling sites. Two diagrams were given, one representing relation of sampling sites and WC, OM, and IM expressed as a percentage share in a biofilm, and another representing sampling sites related to WC, OM, and IM expressed per surface area. These parameters were not determined for every site, but only for those where it was possible to sample the needed amount of biofilm.

Four canonical correspondence analyses (CCA) were also performed. In all analyses, recorded taxa (presence/absence) were used as response variables and were shown in relation to certain explanatory variable/s (separately): 1. sampling time (years of sampling), 2. seasons, 3. sampling sites, 4. main ecological parameters (T, RH, and LI). All CCAs showed significance, except for one which included seasons as an explanatory variable. Ordination diagrams were shown only for the first and fourth analyses.

Multivariate analyses were done in Canoco for Windows (Ter Braak and Šmilauer [2012](#)).

Results and Discussion

Lampenflora in the Lazar Cave

Prior to the tourist season of 2016, the cave was cleaned from previously developed lampenflora. Consequently, at the beginning of the tourist season of 2016, the remains of old lampenflora (epilithic/endolithic) were observed only on a few sampling sites as a form of very pale green biofilm developed on the cave stone substrate (see Fig. [2](#)). From this year onwards, lampenflora just spread on the existing sampling sites and throughout the cave over the years. At the beginning of each tourist season from 2017 onwards, lampenflora always persisted on all previously recorded sites, even if the cave was closed for tourists out of season. According to Meyer et al. ([2017](#)) lampenflora "legacy" remains even after the removal of lighting. In general, the spread was also obvious during a single year, from the beginning to the end of the tourist season. Over time, the number of places affected by the development of lampenflora increased, as well as the surface areas with green coloration, as a result of cyanobacterial and algal growth. This was especially true for inaccessible areas, such as high parts of the cave and its ceilings. Only in the case of 2020 lampenflora looked almost the same at the end as at the beginning of the season. This was probably due to shorter working hours of the cave due to the pandemic, lack of tourists, and bigger pauses between the periods when the cave was open to the public.

Lampenflora/biofilm in the Lazar Cave had one specific characteristic: it was mainly thin and rather developed in the surface part of the substrate at a majority of sampling sites. This is why biofilm was mainly sampled using adhesive tape. Using the scalpel caused the surface layer of the substrate to fall off in some places (i.e., L16 in 2017). However, in cases when biofilm became thicker, it could be sampled by scalpel. This usually happened at certain sites, mostly at the end of the tourist season or in later years, which allowed us to perform some additional analyses (indicated in Table [1](#)). On the other hand, a pure epilithic biofilm was observed at L15. Color of the biofilm in the whole cave was bright green, except on L15 where a darker shade of green was observed.

As already mentioned, lampenflora can cause a lot of problems in cave environments. But lampenflora developing predominantly in the substrate instead of on its surface can have an extremely detrimental effect. According to Golubić et al. ([1981](#)), endoliths are divided into chasmoendoliths, cryptoendoliths, and euendoliths. Chasmoendoliths inhabit surface rock cracks and fissures, cryptoendoliths grow inside the porous rocks, and euendoliths actively deteriorate the rock (Albertano [2012](#); Keshari and Adhikary [2014](#)). Based on the description provided by Golubić et al. ([1981](#)), we believe that the type of endoliths present in the Lazar Cave belongs to chasmoendoliths. Some believe that endoliths in general play the main role in the process of rock deterioration, weathering, and mineral dissolution, since they provide a wider range of microhabitats that could be colonized by other organisms that can be even more dangerous to rock substrate (Keshari and Adhikary [2014](#)).

Ecological and Biofilm Parameters

Ecological factors in caves are considered to be relatively stable. Nevertheless, they may be easily influenced by natural

and anthropogenic factors such as climate conditions outside the cave, geothermal influences, condensation-evaporation processes, microenvironmental conditions, and human activities (Ravbar and Kosutnik 2013).

Average T in the Lazar Cave varied from 10.7 °C (L3) to 13.43 °C (L1) during all 5 years (all seasons included). The average T was higher at a number of sampling sites positioned closer to the cave entrance (i.e., L1, L4 near the beginning of the tourist trail and L23 at the end of the tourist trail). This was expected since the external temperature during tourist season is higher than the temperature inside the cave. Higher values compared to other sites were also observed at sampling sites L16–L19. At these four sampling sites, higher variations in the values of this parameter were also recorded, as well as on L1, L4, L7, L9, and L13 (Fig. 3a). The air temperature is relatively stable in the isolated part of the cave, but is observed to be very variable in the parts that are closer to the cave entrance (heat is conducted through the entrance) and to the surface (T is controlled to a lesser extent through the rock walls) (Ravbar and Kosutnik 2013; D'Agostino et al. 2015). Also, T can be strongly influenced by cave morphology, ventilation (poorly ventilated parts of the cave are characterized by higher T), and position inside the cave. Furthermore, temperature is also dependent on the presence of tourists and artificial lightning (Ravbar and Kosutnik 2013; D'Agostino et al. 2015). As reported by Constantin et al. (2021), a group of only 5–6 people can cause a temperature increase by 0.5 °C in a short time span (1–2 days) in a medium-sized gallery (~6×1.5 m), after a working time of 6–8 h per day; with temperature returning to its initial state after 1–3 days. Nevertheless, the same authors emphasized that in the other studied cave, no direct correlation between the variation of air temperature and the number of tourists was observed. While conducting this study, we noticed that lamps which are installed in the Lazar Cave warm up the air in their immediate proximity, which certainly influences T and RH, as concluded by Mulec and Kosi (2009). Local air currents caused by warming in the proximity of strong lamps are an important factor which contributes to the spread of lampenflora (Mulec and Kosi 2009). Constantin et al. (2021) reported that they documented an increase in air temperature by 0.3–0.4 °C at a distance of 1 m from the reflector about 20 min after switching it on, with this effect being felt throughout the day. Nevertheless, they state that an increase in temperature in the cave is the cumulative effect of the tourist presence and operation of the lighting installation. According to Mulec and Kosi (2009), the frequency of switching lights on and off also affects the relative humidity and temperature. We already mentioned that lighting in the Lazar Cave did not work continuously and was turned on only during cave tours.

Average RH varied from 63 to 85% (Fig. 3b). The lowest average values were recorded near the entrance (L1,

L2, L23) but inside the cave, they varied depending on the sampling site. Great variations of this parameter were recorded at many sampling sites. The lowest value of RH was found on L1 (48.7%), and the highest on L16–L19, where it reached 90%. We can presume that differences in this parameter between the sites derive from differences in cave morphology, microclimatic conditions, or the influence of artificial light and its distance from the sampling site. According to Wigley and Brown (1976) (taken from Perry 2013), during the warm period of the year (the author refers to summer), relative humidity near the cave entrance has lower values because the warmer air meets the cold cave walls creating condensation on the cave walls, thereby removing moisture from the air. Since tourist season mainly includes the warmer part of the year, this could be a valid explanation for lower RH near the cave entrance. Meanwhile, deeper in the cave, humidity rebounds due to lower temperatures (Perry 2013).

Considering different environmental parameters that influence the development and growth of lampenflora, light intensity and duration of illumination are expected to play a key role (Piano et al. 2021). Light intensity, wavelength, and duration of illumination have been considered limiting factors (that (fortunately) can be controlled) for the development of cave phototrophs (Baquedano Estévez et al. 2019). Thus, LI was measured every time when lampenflora was sampled during this 5-year monitoring. LI varied between the sampling sites, and as expected, was negatively correlated with the distance from the light source (−0.5048). The highest average values of this parameter were measured at L15, L18, and L19 (Fig. 3c), sites located very close to the light source, which was directly pointed on the sampling sites. The range of measured values of this parameter at many sampling sites was also high (L18, L19, L1, L4, etc.). This could be explained by the fact that the measuring was not performed at exactly the same sampling point as previously done, so the exposition was different and caused changes in LI values. However, in some cases, light source was occasionally shifted or partially blocked (as mentioned in the "Material and Methods" section) over the years, which resulted in different LI values at the same sampling site over 5 years. Higher diversity of phototrophs, as well as an increase in biofilm thickness, is observed when light intensity increases (Roldán and Hernández Mariné 2009, *in* Baquedano Estévez et al. 2019). However, with very high light intensity, it is possible for epilithic algae to start to grow as endolithic, to protect themselves from excess light (Asencio and Aboal 2001, *in* Baquedano Estévez et al. 2019). This could be a possible mechanism which is present in the Lazar Cave and that can explain the occurrence of an endolithic community. A minimal level of light needed for the development of phototrophic microorganisms is 10 to 50 lx, according to Johnson (1979) but, as stated by Cigna

Fig. 3 Minimum, average, and maximum values of **a** temperature, **b** relative air humidity, and **c** light intensity at each sampling site in the Lazar Cave for the period of 5 years (2016–2020, all sampling seasons included)
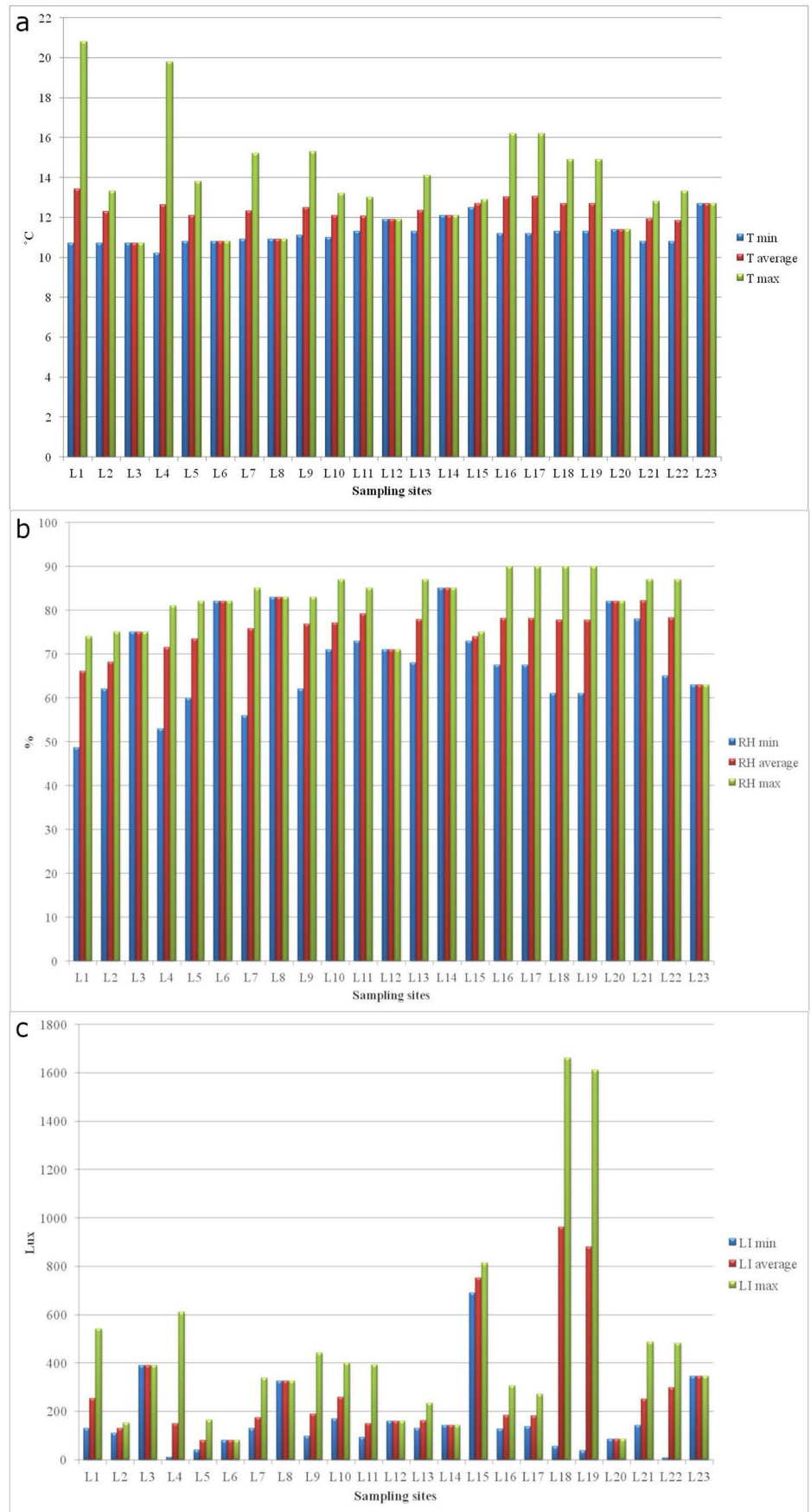
(2011a), 85% of the lampenflora develops at a minimum value of about 40 lx (Baquedano Estévez et al. 2019). However, it seems that some algae and cyanobacteria can survive at light intensities considerably below the "photosynthetic compensation point" (Mulec 2005 *in* Baquedano Estévez et al. 2019) and, as depicted, lampenflora can even survive in total darkness. According to Johnson (1979), as cited in Baquedano Estévez et al. (2019), lampenflora did not change or deteriorate after five full months in the dark. Similarly, the Lazar Cave is in completely darkness for 6 months between the tourist seasons, and at the beginning of the new tourist season, it seems that lampenflora almost did not change at all. According to Roldán and Hernández-Mariné (2009), *Chlorella* sp. which is very widespread and, in few samples, very abundant in the Lazar Cave (see later in Table 3) can

switch from an autotrophic to a mixotrophic, and finally to a heterotrophic lifestyle. This surely influences lampenflora to be persistent and to survive longer periods of time without light. Unfortunately, this also lowers the possibility of its reduction and its elimination in general.

The average pH ranged between 5.1 and 6.6 (Fig. 4a). The highest average value of this parameter was recorded on L15, where the sampled biofilm was rich in Cyanobacteria (see Supplementary material). It is known that aerophytic cyanobacteria more frequently inhabit circumneutral to alkaline substrata (Pentecost and Whitton 2012). At one point during the monitoring, the highest pH value was measured on L1. Changes of pH in different microhabitats depend mainly on the lighting regime, but fluctuation in H^+ concentration should also be expected during the light/dark

Fig. 4 Minimum, average and maximum values of **a** pH and **b** substrate moisture at each sampling site in the Lazar Cave for the period of 5 years (2016–2020, all sampling seasons included)
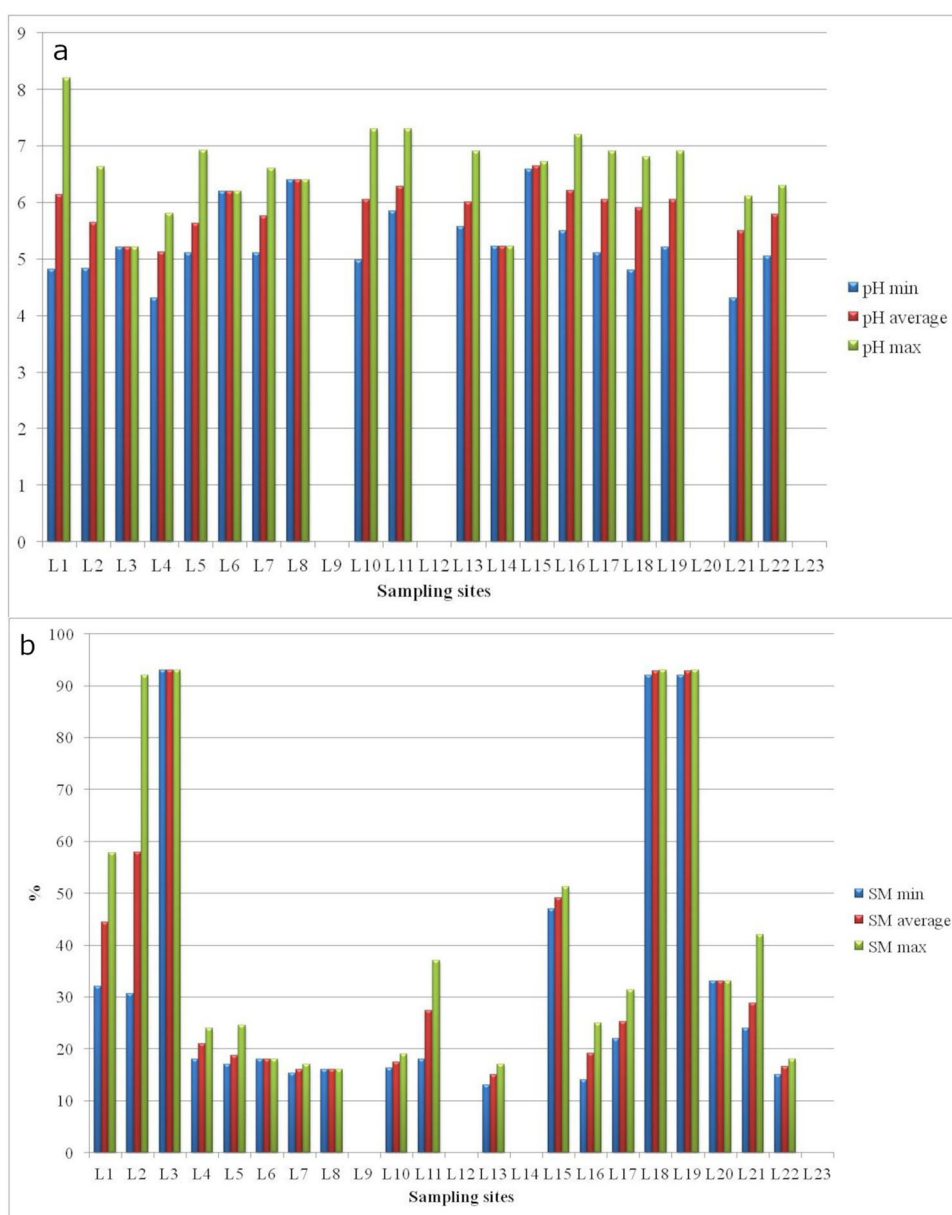
cycles, due to changes in CO_2 emission and consumption in respiration and photosynthesis processes. Consequently, higher values of pH occur during photosynthesis and lower ones during respiration or fermentation (Albertano and Urzi 1999; Albertano et al. 2000). Respiration of bacteria and fungi increases the concentrations of local CO_2, forming H_2CO_3 which decreases the pH of the rock surface in the contact zone (Gorbushina 2007). Additionally, biofilms and EPSs in general can slowly change the substrate, including pH of the surface. This process is referred to as the chemical vulnerability (Gorbushina 2007). According to de Paula et al. (2020), the pH values in subterranean environments exhibit a greater degree of variation. As reported in literature, a wider pH range, from near neutral to slightly alkaline, is most frequently reported. In alkaline areas water, which is usually present, dissolves the carbonate rock, thus raising the substrate pH. However, acidic cave substrates may result from "different intermixing ratios of the weathering residue of carbonate rock, sandy rock, or shale components and decomposed organic matter" (de Paula et al. 2020, p. 6).

As it is already known, the key factors which affect the growth of lampenflora are light and moisture (Mulec and Kosi 2009). The highest substratum moisture was found at L18 and L19 (sediment), but also at L3, even though this site was sampled only once (Fig. 4b). Higher average values (above 30%) were also recorded at L1, L2, L15, and L20, while at the remaining sampling sites, the average substratum moisture values were below 30%. Higher variations in moisture values were observed at L1, L2, L11, and L21. These sites were characterized by a rough surface substrate and a lot of cracks which certainly improved water retention during the period of the year when the cave was more hydrologically active. Overall, variation in substrate moisture depended on the season or hydrological activity of the cave. In line with de Paula et al. (2020), the substrate moisture was higher during the wet season, when compared to the dry season at all sampling sites.

The effects of the sampling year (when every season separate is considered and overall), the effect of the season, and sampling site were tested on the selected measured parameters (Table 2). The difference between the study years when only the beginning of the season was considered was significant only for RH. Meanwhile, when data from the middle or end of the tourist season were considered, differences for both T and RH were significant (Table 2). Significance was also observed only for T and RH when years were compared in general (with all data included). Significant differences for T and RH between the sampling years were expected, as these factors are prone to changes over the years due to outdoor climate differences. This is especially true near the entrance and in parts of the cave closer to the surface (Ravbar and Kosutnik 2013). The temperature inside the caves correlates with mean annual temperatures on the surface because the outside conditions are transferred through the bedrock by conduction (Domínguez-Villar et al. 2013). Meanwhile, changes in RH relate to the changes in T (Perry 2013). Additionally, tourist path in the Lazar Cave is not long in general, so these factors can be influenced to a higher extent when compared to some deeper caves (the changes in T and RH are reflected on Fig. 3). No significant differences were observed for any of the measured parameters when seasons were compared, while between the sampling sites, the difference was significant only for LI and SM. It was expected that the sampling sites would show significant differences when data for LI were compared, since LI values were specific for each site. The same can be applied to SM, as the sites differed in their position inside the cave, proximity to water, presence of seeping water, microclimatic parameters, etc.

As mentioned before, due to the nature of biofilm in the Lazar Cave, biofilm for additional analyses could be sampled only on special occasions and on certain sampling sites. The sampling sites from which biofilm was occasionally taken for analysis of biofilm parameters are shown on ordination diagrams below. The relationship of water content (WC), organic matter (OM), and inorganic matter (IM) and sampling sites is shown on two PCA diagrams (Fig. 5). Considering these parameters expressed as percentages in biofilm, WC% was highest in biofilms from L18 and L19, lowest on L5; OM% was highest on L3 and IM% was highest on L5 and L13, followed by L1 and L2 (Fig. 5a). When these parameters were considered per surface area, all were the highest on L18 and L19, while OM and IM were additionally high at L2 (Fig. 5b).

Table 2 Effects of the sampling year (when every season is considered separately and overall), effect of the season and sampling site were tested on the selected measured parameters; *NS* not significant, *all data included

		T	RH	LI	pH	SM
Difference between years	Beginning	NS	**0.02**	NS	NS	NS
	Middle	**< 0.0001**	**< 0.0001**	NS	NS	NS
	End	**< 0.0001**	**< 0.0001**	NS	NS	NS
Difference between years *		**< 0.0001**	**< 0.0001**	NS	NS	NS
Difference between seasons *		NS	NS	NS	NS	NS
Difference between sampling sites *		NS	NS	**< 0.0001**	NS	**< 0.0001**

Fig. 5 PCA showing the relationships between the water content (WC), organic matter (OM), and inorganic matter (IM) and sampling sites. **a** WC, OM, and IM are expressed as percentage in biofilm; **b** WC, OM, and IM are expressed per surface area
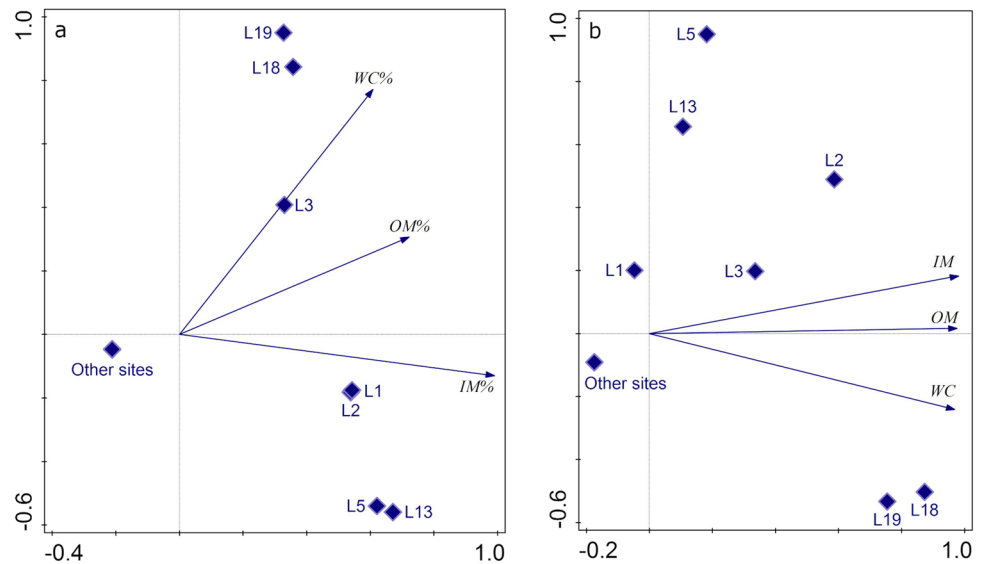
Average values of Chl *a* ranged from 0.10012 at T7 to 34.5943 μg Chl a/cm^2 at T19. A high value of Chl *a* was also observed on T18. Bearing in mind that T18 and T19 are horizontal surfaces with additional sediment, such results were expected. It also should be mentioned that high values of Chl *a* coincide with the observation that parameters WC, OM, and IM when considered per surface area were the highest on L18 and L19 (Fig. 5b).

Qualitative Analysis of Lampenflora

General Analysis

During the 5-year survey, 25 taxa belonging to Cyanobacteria, Chlorophyta, and Bacillariophyta were identified to the species or genus level from lampenflora community in the Lazar Cave (Table 3). In general, the highest diversity was found within Cyanobacteria, with exclusive domination of coccoid forms. Representatives of *Aphanocapsa* were documented each year since the beginning of the monitoring, while *Eucapsis* and *Leptolyngbya* were observed during 3 and 4 years, respectively. Lower diversity was found within Chlorophyta, but similar to Cyanobacteria, coccal forms were also dominant. Genus *Chlorella* and *Stichococcus bacillaris* were recorded each year in almost all seasons, while *Klebsormidium flaccidum* was observed during 4 years. Bacillariophyta were observed only in fresh material and recorded if spotted in the sample; thus, their diversity was seemingly low. It is highly concerning that mosses protonema and mosses itself were very widespread and present all the time during this 5-year monitoring. Regardless of their diversity, Cyanobacteria were sporadically found in samples (except in a few cases

that will be mentioned later), while Chlorophyta and/or mosses protonema were more abundant.

Seasonal Analysis

Cyanobacteria, Chlorophyta, and Bacillariophyta were documented in every season of each year, except at the beginning of the tourist season of 2016. That time, only the remnants of the old lampenflora were recorded; closer identification was not possible due to damaged cells, but all were representatives of Chlorococcales, i.e., coccal green algae. During 2017, even though three main groups of phototrophs were identified, it was still not possible to identify all representatives in samples due to lampenflora recovering, and many were also assigned to coccal green algae. The lowest diversity was recorded in 2016 and the highest in 2020. When years were analyzed separately and seasons in each year were compared, the highest diversity was observed in E 2016, M 2017, E 2018, M 2019, and M and E 2020, and the lowest in B 2016, E 2017, M 2018, E 2019, and B 2020. By analyzing every season during the 5-year period, Cyanobacteria were the most diverse only in M and E 2020 (mostly due to the sampling site L15), Cyanobacteria were equal to Chlorophyta in B 2017 and M 2019, while in the rest of the sampling periods, the highest diversity was recorded within Chlorophyta (M, E 2016, M, E 2017, M, E 2018, B, E 2019, B 2020). *Chlorella* sp. was the most widespread algae (found on most sampling sites), followed by *Stichococcus bacillaris* in all seasons during the study years, except B 2016, E 2017 when *Stichococcus bacillaris* took its place. *Klebsormidium flaccidum* was interesting since it was very abundant in samples from L19 in 2018. Table showing detailed qualitative analysis per year, season, and sampling sites is given in Supplementary material.

Table 3 Cyanobacterial and algal taxa identified from lampenflora in the Lazar Cave for the period of 5 years (2016–2020)

	2016			2017			2018			2019			2020		
	B	M	E	B	M	E	B	M	E	B	M	E	B	M	E
Cyanobacteria															
Aphanocapsa fuscolutea Hansgirg								+	+		+	+			
Aphanocapsa muscicola (Meneghini) Wille			+					+	+		+	+	+	+	+
Aphanocapsa rivularis (Carmichael) Rabenhorst			+												
Aphanocapsa C.Nägeli spp.		+			+			+	+	+				+	+
Chroococcus cohaerens (Brébisson) Nägeli								+	+					+	+
Chroococcus Nägeli sp.														+	
Eucapsis F.E.Clements & H.L.Shantz sp.								+	+		+	+	+	+	+
Gloeocapsa atrata Kützing														+	+
Gloeocapsopsis Geitler ex Komárek sp.													+	+	
Leptolyngbya foveolarum (Gomont) Anagnostidis & Komárek				+	+			+							
Leptolyngbya Anagnostidis & Komárek spp.					+									+	+
Phormidium Kützing ex Gomont sp.				+											
Pleurocapsa Thuret sp.														+	+
Pseudocapsa Ercegovic sp.													+		
Synechococcus C.Nägeli sp.						+						+		+	+
Chroococcales				+						+	+	+		+	+
Chlorophyta															
Chlorella Beyerinck [Beijerinck] sp.		+	+	+	+	+		+	+	+	+	+	+	+	+
Coccomyxa Schmidle sp.								+	+			+			
Desmococcus olivaceus (Persoon ex Acharius) J.R.Laundon				+	+	+		+	+	+	+	+	+	+	+
Klebsormidium flaccidum (Kützing) P.C.Silva, K.R.Mattox & W.H.Blackwell		+	+	+	+	+		+	+		+	+			+
Klebsormidium P.C.Silva, Mattox & W.H.Blackwell sp.															
Stichococcus bacillaris Nägeli		+	+	+	+			+	+	+	+	+	+	+	+
Trebouxia Puymaly sp.					+										
Chlorococcales	+	+	+	+	+	+				+	+	+	+	+	+
Bacillariophyta															
Humidophila (Lange-Bertalot & Werum) R.L.Lowe & al. spp.		+	+	+	+	+		+		+	+	+	+		
Nitzschia Hassall sp.			+			+			+						
Orthoseira roeseana (Rabenhorst) Pfitzer									+						
Unknown		+	+	+	+	+		+	+	+	+	+	+	+	+
Other															
Moss protonema		+	+	+	+	+		+	+	+	+	+	+	+	+
Mosses		+	+	+	+	+		+	+	+	+	+	+	+	+

As shown, according to the general analysis (Table 3), the highest diversity occurred in Cyanobacteria. But when the details are analyzed, Cyanobacteria were dominant only in two seasons in 2020, where only the L15 sampling site contributed to their general diversity.

The lampenflora consisted of Cyanobacteria and algae, which have been documented in many caves worldwide including the ones famous for their prehistoric rock paintings, i.e., Lascaux Cave, Tito Bustillo, Mammoth Cave (Saiz-Jimenez 2012), or Altamira Cave (Cañaveras et al. 2001). Saiz-Jimenez (2012) reported that the examination of lampenflora in Mammoth Cave by Smith and Olson (2007) resulted in discovering representatives of *Chroococcus*, *Gloeocapsa*, *Leptolyngbya*, *Chlorella*, and *Diadesmis* (*Humidophila*) (all among dominant taxa). Dayner and Johansen (1991) in Seneca Cave recorded *Chlorella* and *Humidophila* (*Diadesmis*) as the most abundant, while in the Kateřínská Cave, Faimon et al. (2003) also found *Chlorella*, *Leptolyngbya*, *Nitzschia*, and *Stichococcus bacillaris* among other genera. Albertano and Urzí (1999) explored the lampenflora in catacombs, characterized by the presence of *Eucapsis*, *Leptolyngbya*, and *Humidophila* (*Diadesmis*). Mazina and Kozlova (2018) conducted a study in the Lipska Cave in Montenegro where they recorded many genera similar to those found in our study: *Chroococcus*, *Gloeocapsa*, *Leptolyngbya*, *Nitzschia*, *Humidophila*, *Klebsormidium*, *Chlorella*, and *Stichococcus*, as well as mosses. Pentecost (2010) revealed that Cyanobacteria (mostly coccoid forms) dominated in lampenflora in three tourist caves in northern England (Ingleborough Cave, Stump Cross Cavern, and White Scar Cave), while considering the green algae, *Coccomyxa* was recorded. Mosses and ferns were also observed.

CCA ($F = 2.6$, $P = 0.002$) representing the recorded taxa in relation to the sampling time (sampling years) is shown on Fig. 6a. Certain taxa were characteristic for only 1 year: *Aphanocapsa rivularis* and *Nitzschia* sp. for Y2016, *Phormidium* sp. and *Trebouxia* sp. for Y2017, *Coccomyxa* sp., *Desmococcus olivaceus*, and *Orthoseira roeseana* for Y2018, and *Chroococcus cohaerens*, *Chroococcus* sp., *Gloeocapsa atrata*, *Gloeocapsopsis* sp., *Pleurocapsa* sp., *Pseudocapsa* sp., and *Synechococcus* sp. for Y2020. There were no recorded taxa which were specific only for Y2019. Others were recorded in more than 1 year, or more than two. A variation is noticed between communities when years are compared and introducing new sampling sites over the years due to the spread of lampenflora could contribute to this. Also, even though the development of microorganisms on a certain site is influenced by the above-mentioned environmental parameters, microhabitat (surface roughness, porosity, hygroscopicity, chemical composition) and microclimatic conditions at sampling sites (Cuzman et al. 2010; Macedo et al. 2009), as well as the presence/absence of seeping water, play a very important role. The seeping water in the cave can bring different inorganic/organic particles, but other microbes as well (Ogorek et al. 2014). They can originate from other parts of the cave or enter the cave through the crack system from places above the cave (epikarst; Pipan and Culver 2013). These microorganisms are temporarily or permanently retained in the biofilm
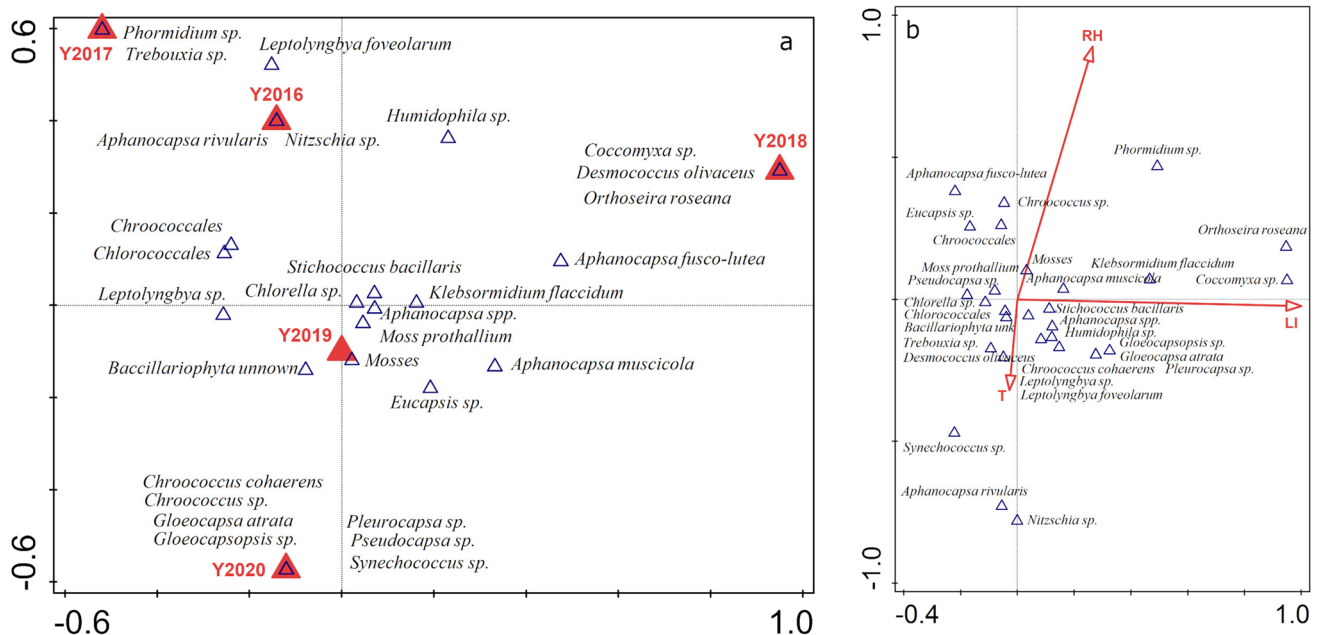

Fig. 6 CCA diagrams representing **a** recorded taxa in relation to sampling time (sampling year) and **b** recorded taxa in relation to the main ecological parameters (T, RH, LI) based on the 5-year data

which can influence changes in the algal composition of the lampenflora. Recorded taxa were observed also in relation to seasons and sampling sites (no ordination diagrams included). Seasons did not show any significance and only few taxa were specific for a certain season B: *Pseudocapsa* sp. and *Phormidium* sp., M: *Chroococcus* sp., *Gloeocapsopsis* sp., *Desmococcus olivaceus*, and *Trebouxia* sp., E: *Aphanocapsa rivularis*, *Nitzschia* sp., *Orthoseira roeseana*, and *Synechococcus* sp. Sampling sites as explanatory variable were significant ($F = 2.6$, $P = 0.002$) and some taxa were found only on one sampling site: *Nitzschia* sp. and *Trebouxia* sp. on L1, *Synechococcus* sp. on L2, *Desmococcus olivaceus* on L7, *Pseudocapsa* sp. on L10, *Gloeocapsa atrata*, *Gloeocapsopsis* sp., and *Pleurocapsa* sp. on L15, *Eucapsis* sp. on L16 and L17, *Orthoseira roeseana* on L19, *Chroococcus* sp. on L22, *Aphanocapsa rivularis* on L23, *Phormidium* sp. at L19. We noticed that in some cases, certain taxa were site specific, like the ones reported by Saiz-Jimenez (2012). For example, through seasons and years (see Supplementary material), *Klebsormidium flaccidum* was recorded mostly on L18 and L19, and only during 2020 on L15; *Stichococcus bacillaris* on L1, L2, L18, and L19 and *Eucapsis* sp. on L16 and L17. *Klebsormidium flaccidum* and *Eucapsis* sp. always were very abundant in samples, as well as *Stichococcus bacillaris* in some cases. Thus, *Eucapsis* sp. was the only Cyanobacteria not recorded sporadically in the Lazar Cave.

CCA representing the recorded taxa in relation to the main ecological parameters (T, RH, and LI) was significant ($F = 3.3$, $P = 0.002$) and shown on Fig. 6b. Taxa in the lower part of the ordination diagram such as *Aphanocapsa rivularis*, *Nitzschia* sp., and *Synechococcus* sp., are connected with higher T; those in the upper part of the ordination diagram, i.e., *Aphanocapsa fuscolutea*, *Chroococcus* sp., and *Phormidium* sp., are related to higher values of RH and taxa on the right side (i.e., *Coccomyxa* sp., *Klebsormidium flaccidum*, *Orthoseira roeseana*) with higher values of LI. It is believed that higher diversity of phototrophs relates to higher temperatures (Smith and Olson 2007). This assumption is partially supported by our study where slightly higher number of species correlates with higher temperatures (Fig. 6b). However, when Smith and Olson (2007) performed the regression analysis, they found no correlation between the temperature and species diversity and explained that the air flow fluctuations and wind at or near the cave entrances influenced this relationship.

The cave energy (Heaton 1986 *in* Baquedano Estévez et al. 2019) should be maintained at an optimal level, striving not to introduce a lot of additional energy which comes with various anthropogenic activities that are an inevitable part of the new role caves are given, especially in the case of show caves (i.e., input from the outside, number of visitors). Additional energy input from outside in the Lazar Cave is partially prevented with the previously mentioned wall that

has been built. In support of this, to maintain the needed balance of microclimatic parameters, Merdenisianos (2005) and Cigna (2011b) also suggested the installation of double doors and air curtains at cave entrances (Baquedano Estévez et al. 2019). Regarding the number of visitors, it should be limited at a level that would not cause permanent negative influence on the environment (carrying capacity); if the number would still remain high, the time they spend inside the show cave should be shortened (Baquedano Estévez et al. 2019). The number of visitors in the Lazar Cave is directly related to the number of tourist tours which dictate the time during which the artificial light is on.

Conclusion

The transformation of caves into tourist attractions is inevitable nowadays and can significantly affect their inherent equilibrium. Once stable and untouched cave environment is now made vulnerable to disturbances, leading it to suffer changes (some irreversible) due to the construction of paths, electricity, and water infrastructure, introduction of light and presence of tourists. The conservation of these sensitive environments should be a priority from the moment of opening and all parameters, including the potential changes around artificial lights, should be monitored. One of the most undesirable changes is the appearance of lampenflora. Its monitoring is of particular importance to prevent excessive development that could deteriorate cave substrate and cave structures that would require the use of certain methods for its removal. For the first time, lampenflora was long-term (for a period of 5 years) monitored in the Lazar Cave in Serbia. The sampling was done three times per year during the tourist season which lasts for 6 months. Even though the cave is closed for 6 months, the lampenflora "legacy" persisted from the previous year on all sites, continuously spreading over the years, also reaching parts of the cave that were not accesible for sampling. Furthermore, lampenflora in this cave is characterized by the presence of epilithic and endolithic community of phototrophs, of which endoliths pose a bigger threat. One of the most widespread and abundant genera was *Chlorella* which is capable of switching from an autotrophic to a mixotrophic, and finally to a heterotrophic lifestyle (Roldán and Hernández-Mariné 2009), thus lowering the possibility of lampenflora reduction and its elimination. We hope that monitoring will be performed more regularly in the future and that it will include a larger number of show caves, which would allow us to define the degree of damage done and accordingly define proper categorization.

Declarations

Conflict of Interest The authors declare no competing interests.

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