Algae and cyanobacteria in the aphytic habitats of Veternica Cave (Medvednica Mt., Croatia) and selected caves of the Dinaric karst (Southeast Europe)

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Abstract: Microphototrophs (algae and cyanobacteria) in karst environments have been intensively studied in aquatic epigean habitats. In recent decades knowledge about the communities inhabiting cave entrances and lampenflora has grown substantially, but the data about the communities in aphytic cave zone are scarce. This study aimed to investigate spatio-temporal presence of microphototrophs in the aphytic zone of Veternica Cave (Mt. Medvednica karst) and to present additional preliminary data from 22 caves of the Dinaric karst. The data were collected over ten years, in parallel with research on cave phagotrophic protists. In addition to the remains of microphototrophs, living algae and cyanobacteria were found in the cave aphytic zone. Diatoms (Bacillariophyta) were the most frequent group found, followed by green algae (Chlorophyta), golden-brown algae (Chrysophyta) and the filamentous cyanobacteria (Cyanobacteria). The presence of microphototrophs was detected throughout the year but showed spatio-temporal variations. Microphototrophs were absent in the parts of Veternica Cave with seeping and dripping water, while they were occasionally present in the hydrologically active parts of the cave. The presence of diatoms in the aphytic zone of Veternica Cave was related to hydrological conditions, and was not affected by the distance from the cave entrance. The presence of microphototrophs in caves of the Dinaric karst has been associated with caves subject to various types of flooding by endogenous and exogenous water. Despite the fact that microphototrophs are passively transported to the caves from the surface habitats, the presence of live individuals in the cave aphytic zone implies that they should not be neglected in discussions about cave food webs. Future research of microphototrophs should be focused on the species identification, their abundance, survival time, and detail description of conditions that determine their presence in caves.

Keywords: aphytic cave zone; microphototrophs; cave diatoms; flooding; karst hydrology

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INTRODUCTION

Cave ecosystems are characterized by a lack of light and therefore rely on connectivity to the surface or internal microbial production for energy supply (Simon, 2019). Howarth (1983) categorized the terrestrial environment of caves into five zones: (1) entrance, (2) twilight, (3) transition, (4) deep, and (5) stagnant. These zones are in fact a gradient from a surface along which light declines and phototrophs become rare and disappear. Algae and cyanobacteria (often called microphototrophs) in karst habitats have been the most extensively studied in surface aquatic habitats (e.g., Krivograd Klemenčič et al., 2004; Jasprica et al., 2006; Cunha Pereira et al., 2011). Special attention was drawn by the discovery of an important role of periphyton in tufa deposition processes (Scholl & Taft, 1964; Plenkovic-Moraj et al., 2002; Golubić et al., 2008).

In contrast to surface habitats, with abundance of light, freshwater cave habitats have been much less investigated regarding the microphototrophs. Still the data about the microphototrophs in cave entrances in
temperate zones have grown substantially in recent decades as the higher humidity and light gradation in cave entrances enable characteristic communities (Pouličková & Hasler, 2007; Martinez & Asencio, 2010; Czerwik-Marcinkowska & Mrziński, 2011; Czerwik-Marcinkowska, 2013; Popović et al., 2015, 2020a, b). Some new diatom species for science like Diploneis mausmairi (Bhatt & Karthick, 2020) and Diprora haenaensis (Main, 2003) have been discovered and described from the cave entrances. Mulec et al. (2007) demonstrated, on the example of Škocjanske Jame, that algae promote growth of stalagmites and stalactites near cave entrance. Many cave microorganisms are known to form complex biofilms and secrete various chemical compounds (Kosznik-Kwaśnicka et al., 2022). For example, the aerophytic alga Coccomyxa subglobosa, isolated from Głowniowa Nénya Cave (Poland), synthesizes fatty acids that could potentially be used for biodiesel production (Czerwik-Marcinkowska et al., 2020).

The communities of microphototrophs associated with electrical light in show caves called lampenflora have been studied intensively in recent years in an effort to reduce a possible negative impact on the natural and cultural heritage in caves (Mulec et al., 2008; Mulec & Kosi, 2009; Falasco et al., 2014; Baquedano Estévez et al., 2019). New diatom species Nupela troglophila (Falasco et al., 2015) was described from lampenflora in Bossea cave (Italy).

The presence of microphototrophs in habitats characterized by the constant absence of light was mentioned in Baković et al. (2019) as part of the interpretation of testate amoebae diversity in caves. Hajong et al. (2021) found 23 species of microphototrophs in the aphotic zone of six caves in Meghalaya (India). A recent study by Cahoon and VanGundy (2022) using environmental DNA metabarcoding in two caves in the Appalachian Mountains (USA) revealed dinoflagellates, diatoms, chrysophytes and green algae in samples of cave streams and pools. Some algae have been recorded in a suboxic chemosynthetic ecosystem of Moviele Cave, but it was attributed to contamination and diffusion of the Black Sea waters into the cave (Reboul et al., 2019).

The Dinaric karst, extending from northeast Italy to northwest Albania (Mihevc et al., 2010), is a global hotspot of both aquatic and terrestrial subterranean biodiversity (Culver & Sket, 2000). Within these overarching patterns, knowledge on subterranean algae and cyanobacteria is still deficient. With the presented results on microphototrophs from the selected caves, this gap will be partially filled.

The aim of this study is to present data on microphototrophs (algae and cyanobacteria) in aphotic zone of the karst caves collected in the period from 2011 to 2021. For this purpose, Veternica Cave (in an isolated karst area in Northern Croatia) was selected where the protists were researched during several years. During this study data on microphototrophs were recorded. In the same time period samples were collected and examined in search for microphototrophs from twenty two caves of the Dinaric karst from the territories of Croatia and Bosnia and Herzegovina. This study brings data on spatio-temporal presence of microphototrophs in relation to distance from the cave entrance and hydrological conditions in Veternica Cave (based on more frequent samplings), together with some preliminary data from other very diverse caves of the Dinaric karst (based on a small number of samples). Regardless, possibilities of the comparison from these data are limited, but they present general information on spatio-temporal presence of microphototrophs in case study of Veternica Cave and in addition of selected caves of the Dinaric karst.

The motivation of this study was to provide the starting point for future research on microphototrophs in the aphotic cave zone. First we wanted to provide data on the spatial presence of microphototrophs along cave channels in various seasons in order to test whether they are present only close to the cave entrance. Secondly we wanted to investigate what is the relation between the hydrological conditions and presence of microphototrophs. Some microphototrophs in deep parts of the cave (even one kilometer deep) could be possible mixotrophic or even heterotrophic species, thus they should be thoroughly researched in the future considering their ecology and possible biotechnological applications. The third topic we wanted to address is the presence of live microphototrophs inside caves as it opens an important topic on the role of the microphototrophs in cave food webs.

**RESEARCH LOCATION AND METHODS**

Data presented in this research were collected during the research projects of protozoa in freshwater karst caves of Croatia and Bosnia and Herzegovina in the period of 2011 - 2021. Veternica Cave (Figs. 1, 2; Table 1) is located in an isolated karst area in the south-western part of Medvednica Mountain (Croatia). It is a complex speleological object with a ground plan length of 5996 m, the 10th longest cave in Croatia (Croatian Speleological Server, 2022). The cave passages are formed mainly adjacent to the unconformable contact between the Triassic dolomites and the Miocene limestones and fractures within these deposits (Lacković et al., 2011). The first 900 m of the entrance channel and some higher cave floors in deeper parts of the cave are hydrologically inactive (only drip-water is present). After around 900 m, the hydrological activity is more pronounced consisting of 15 recorded endogenous streams located in the deeper parts of the cave (Ozimec & Šincek, 2011) together with water ponds, seeping and dripping water. The first 380 m of the channel of the cave are open to tourist on weekends from April to November and are electrically illuminated. Microphototrophs in the Veternica Cave aphotic zone have not been researched before, but the presence of algae and cyanobacteria has been reported near the cave electrical lamps in part of the cave opened for tourist visits (Baković, 2016). Additional studied caves are located in the Dinarides, 19 on the territory of Croatia and 3 in Bosnia and Herzegovina (Fig. 1; Table 2).
According to the biogeographical region defined in the Red book of Croatian cave-dwelling fauna (Ozimec et al., 2009), the majority (12 caves) belong to the North Dinarides (ND), eighth to the Middle Dinarides (MD) and two to the South Dinarides (SD). Each cave in Table 2 is presented with: Cave name, Cave location, Distance from the cave/tunnel entrance (m), Sampling date, Total number of samples, Samples with microphototrophs, Hydrological activity and Light presence. There are no published data on microphototrophs from the aphotic zone of the selected caves from the Dinaric karst (Table 2).

Caves Vjetrenica (Ozimec et al., 2021) and Samograd are touristic caves with the installed electrical lights, but samples were collected in deeper parts of the caves, where the light was absent.

The designation “hydrologically active caves” was given to the caves that hold permanent or occasional endogenous or exogenous streams and/or lakes within the cave or resemble estavelles. The designation “hydrologically inactive caves” was given to the caves that are characterized only by the seeping and dripping water that does not form lakes or streams.

Two samples from the Markov Ponor (10/2016) were collected from the subterranean lake using plankton nets of 65 µm and 120 µm. The material collected by this method included debris (mostly wood) that was imported into the cave by flood waters and living organisms. Sample containing debris and meiofauna was then transferred into plastic containers. Other samples of cave freshwater sediments, terrestrial sediments and transitional habitats were collected by using plastic pipettes, brushes and spoons and transferred into plastic containers. Samples from caves were held in the total darkness at temperatures varying from 4 to 8°C, while the samples from the Vjetrenica Cave entrance were held away from the direct daylight at room temperature. Samples were examined within 48 h after the sampling. The exception were samples from caves Miljacka II (12/2014), Markov Ponor (10/2016), Špilja na Crvenom jezeru (09/2018), Stara jametina (12/2014) that were held at room temperature and examined within time period longer than 48 hours.

Detailed sampling methodology is presented in Baković et al. (2019). Total 0.6 ml (3 x 0.2 ml) of every sample was examined using Carl Zeiss Primostar and Carl Zeiss Axiostar light microscopes at magnifications of 100, 400, and 1000 times. The algae and cyanobacteria present were identified based on microscopical appearance of algal divisions presented in Bellinger & Siegee (2015). Species were determined by assessing the shape and ornamentation of the cells or colonies, presence of flagella, silica frustules, cell walls, motility type (non-motile, swimming, and gliding) and cell color. Cell viability was assessed by observing cell motility and signs of cell deterioration (e.g., uneven distribution of cytoplasm and organelles, cell damage).

For the data analysis only descriptive statistical methods were performed using MS Excel, while the maps were made in programs QGIS 3.4 Madeira and AutoCAD 10LT.
Table 1. Presence of microphototrophs (MPH) in Veternica Cave (Medvednica Mountain, Croatia) with respect to distance from the cave entrance and hydrological activity.

<table>
<thead>
<tr>
<th>Location within cave / distance from cave entrance (m)</th>
<th>Sampling date</th>
<th>Total number of samples (with MPH)</th>
<th>Group of MPH</th>
<th>Hydrological activity</th>
<th>Light presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cave entrance/0 m</td>
<td>2011, 2014, 2021</td>
<td>3 (3)</td>
<td>Bacillariophyta, Chrysophyta, Cyanobacteria</td>
<td>Hydrologically inactive (seeping and dripping water)</td>
<td>Partially present daylight</td>
</tr>
<tr>
<td>Koncertna dvorana (KON)/90 m</td>
<td>2011-2013, 2015, 2016, 2019</td>
<td>22 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water, small water pools)</td>
<td>Diffuse electrical light during weekends between April and November; rest of the year the light is absent</td>
</tr>
<tr>
<td>Skupštinska dvorana (SKP)/200 m</td>
<td>2014</td>
<td>2 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water, condensed water)</td>
<td>Diffuse electrical light during weekends between April and November; rest of the year the light is absent</td>
</tr>
<tr>
<td>Separe (SEP)/250 m</td>
<td>2011-2015, 2019, 2021</td>
<td>35 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water, condensed water, small water pools)</td>
<td>Diffuse electrical light during weekends between April and November; rest of the year the light is absent</td>
</tr>
<tr>
<td>Kalvarija (KAL)/380 m</td>
<td>2011, 2012, 2014, 2015</td>
<td>9 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water, small water pools)</td>
<td>Diffuse electrical light during weekends between April and November; rest of the year the light is absent</td>
</tr>
<tr>
<td>Pakao (PAK)/800 m</td>
<td>2013</td>
<td>2 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water)</td>
<td>Absent</td>
</tr>
<tr>
<td>Limunova dvorana (LIM)/840 m</td>
<td>2013</td>
<td>2 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water)</td>
<td>Absent</td>
</tr>
<tr>
<td>Stream near Pakao (SIF)/920 m</td>
<td>2011</td>
<td>1 (1)</td>
<td>Bacillariophyta</td>
<td>Hydrologically active (running water, seeping, and dripping water)</td>
<td>Absent</td>
</tr>
<tr>
<td>Bijela dvorana (BIJ)/1220 m</td>
<td>2011, 2014, 2016, 2019, 2021</td>
<td>10 (7)</td>
<td>Bacillariophyta</td>
<td>Hydrologically active (running water, water pools, seeping, and dripping water)</td>
<td>Absent</td>
</tr>
<tr>
<td>Viktorija (VIK)/1230 m</td>
<td>2013, 2014</td>
<td>2 (0)</td>
<td>-</td>
<td>Hydrologically active (running water, water pools, seeping, and dripping water)</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Total: 103 (18)
Table 2. Microphototrophs (MPH) found in the selected investigated caves of the Dinaric karst. Light is absent at all locations.

<table>
<thead>
<tr>
<th>Cave name and location/ distance from the cave entrance (m)</th>
<th>Sampling date</th>
<th>Total number of samples (with MPH)</th>
<th>Group of MPH</th>
<th>Hydrological activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betina velika jama, Kokorići, Vrgorac, HRV/52 m</td>
<td>2020</td>
<td>5 (5)</td>
<td>Bacillariophyta, Chlorophyta, Cyanobacteria</td>
<td>Hydrologically active (estavelle, seeping and dripping water)</td>
</tr>
<tr>
<td>Buklina pecina, Mlakvena greda, Poljana, HRV/20 m</td>
<td>2016</td>
<td>1 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water)</td>
</tr>
<tr>
<td>Čakovac, Bobići, Kosinj, HRV/13-15 m</td>
<td>2016</td>
<td>4 (0)</td>
<td>-</td>
<td>Hydrologically active (running water, cave lake, seeping and dripping water)</td>
</tr>
<tr>
<td>Dankov ponor, Lipovo Polje, Kosinj, HRV/400 m</td>
<td>2016</td>
<td>2 (2)</td>
<td>Bacillariophyta, Chlorophyta, Cyanobacteria</td>
<td>Hydrologically active (sinkhole, seeping and dripping water)</td>
</tr>
<tr>
<td>Horvatova špilja, HE Sklope, Kosinj, HRV/75 m</td>
<td>2016</td>
<td>2 (1)</td>
<td>Bacillariophyta</td>
<td>Hydrologically active (flooding of cave by artificial lake, seeping and dripping water, water pools)</td>
</tr>
<tr>
<td>Hukavica, Donja Vidovska, Velika Kladuša, B&amp;H/18 m</td>
<td>2019</td>
<td>3 (2)</td>
<td>Bacillariophyta, Chlorophyta</td>
<td>Hydrologically active (occasional spring, endogenous river, seeping and dripping water)</td>
</tr>
<tr>
<td>Jama II kod Kolića, Kolići, Generalski Stol, HRV/25 m</td>
<td>2012</td>
<td>1 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water)</td>
</tr>
<tr>
<td>Kaverna km 12+188, Tunnel Debeli brije, Danćanje, Ston, HRV/1250 m</td>
<td>2021</td>
<td>2 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water, water pools)</td>
</tr>
<tr>
<td>Kotluša, Kotluša, Vrljka, HRV/35 m</td>
<td>2011</td>
<td>1 (0)</td>
<td>-</td>
<td>Hydrologically active (occasional spring, cave lakes, water pools, seeping and dripping water)</td>
</tr>
<tr>
<td>Marinovića betina, Kokorići, Vrgorac, HRV/86 m</td>
<td>2020</td>
<td>4 (1)</td>
<td>Bacillariophyta</td>
<td>Hydrologically active (estavelle, seeping and dripping water)</td>
</tr>
<tr>
<td>Markov ponor, Lipovo Polje, Kosinj, HRV/200 m</td>
<td>2016</td>
<td>5 (5)</td>
<td>Bacillariophyta, Chrysophyta, Cyanobacteria</td>
<td>Hydrologically active (sinkhole, cave lake, water pools, seeping and dripping water)</td>
</tr>
<tr>
<td>Miljacka II, Canyon of river Krka, Oklaj, HRV/80-85 m</td>
<td>2014, 2018</td>
<td>12 (4)</td>
<td>Bacillariophyta, Chlorophyta, Chrysophyta, Cyanobacteria</td>
<td>Hydrologically active (running water, cave lakes, water pools, seeping and dripping water)</td>
</tr>
<tr>
<td>Mračnjača, Plitvička Jezera, HRV/15 m</td>
<td>2021</td>
<td>1 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water)</td>
</tr>
<tr>
<td>Ponor Dražice, Lipovo Polje, Kosinj, HRV/70 m</td>
<td>2016</td>
<td>1 (1)</td>
<td>Bacillariophyta</td>
<td>Hydrologically active (sinkhole, seeping and dripping water)</td>
</tr>
<tr>
<td>Pražina pecina, Mlakvena greda, Kosinj, HRV/12 m</td>
<td>2016</td>
<td>2 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water)</td>
</tr>
<tr>
<td>Samograd, Grabovača, Perušić, HRV/300 m</td>
<td>2016</td>
<td>2 (0)</td>
<td>-</td>
<td>Hydrologically inactive (water pools, seeping and dripping water)</td>
</tr>
<tr>
<td>Špilja na Crvenom jezeru, Crveno jezero, Imotski, HRV/10 m</td>
<td>2018</td>
<td>1 (1)</td>
<td>Bacillariophyta</td>
<td>Hydrologically active (occasionally flooded with waters of surface lake Crveno jezero, seeping and dripping water)</td>
</tr>
<tr>
<td>Stara jametina, Koštan, Krići, HRV/20 m</td>
<td>2014</td>
<td>1 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water)</td>
</tr>
<tr>
<td>Vila jezerčica, Plitvička Jezera, HRV/40 m</td>
<td>2021</td>
<td>2 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water)</td>
</tr>
<tr>
<td>Vjetrenica, Popovo polje, Ravno, B&amp;H/700-750 m</td>
<td>2020</td>
<td>11 (0)</td>
<td>-</td>
<td>Hydrologically active (running water, cave lakes, occasional spring, water pools, seeping and dripping water)</td>
</tr>
<tr>
<td>Vranjevača, Grabovača, Tomislavgrad, B&amp;H/35 m</td>
<td>2019</td>
<td>1 (0)</td>
<td>-</td>
<td>Hydrologically inactive (seeping and dripping water)</td>
</tr>
<tr>
<td>Vukušića betina, Kokorići, Vrgorac, HRV/54 m</td>
<td>2020</td>
<td>3 (0)</td>
<td>-</td>
<td>Hydrologically active (estavelle, seeping and dripping water)</td>
</tr>
</tbody>
</table>

Total: 67 (22)

RESULTS

During the research in Veternica Cave (Table 1; Figs. 2, 3), microphototrophs were detected in all samples from the cave entrance that is partially illuminated by daylight. We detected the following groups: diatoms (Bacillariophyta), golden-brown algae (Chrysophyta) and cyanobacteria (Cyanobacteria).

In the studied locations KON, SKP, SEP, KAL, PAK, and LIM located 90 to 840 m away from the Veternica Cave entrance (FVCE), microphototrophs were absent in all 75 samples that were collected in various times of the year (Table 1; Figs. 2, 3). Diatoms (Bacillariophyta) were found occasionally in locations SIF, PLA, and BIJ in Veternica Cave (950–1220 m FVCE) where the light is constantly absent (complete darkness). Diatoms were detected in 15 out of 25 samples (60%). They were confirmed during all four seasons (winter, spring, summer and autumn). The locations where diatoms were found are characterized...
by subterranean streams, water pools and seeping water (Table 1). Microphototrophs were absent from two samples collected in location VIK in Veternica Cave (1230 m FVCE) that was characterized by running water, water pools and seeping water.

During the research of other caves of the Dinaric karst (Table 2), microphototrophs were detected in 9 caves (41\%) in complete darkness, including the following groups of algae: the most frequent diatoms (Bacillariophyta), followed by green algae (Chlorophyta) and golden-brown algae (Chrysophyta). Additionally, the filamentous cyanobacteria (Cyanobacteria) have been detected.

A connection between hydrological activity and the presence of microphototrophs in caves of the Dinaric karst was noticed. Out of the 13 hydrologically active caves, microphototrophs were detected in nine caves (69\%) (Table 2). Microphototrophs have not been detected in nine hydrologically inactive caves.

In the caves of the Dinaric karst microphototrophs were more frequent in parts of the caves that are far from the cave entrance (52-400 m), compared to entrance part, however completely absent in deep cave sites (700-1250 m) (Fig. 4 A, B).

Microphototrophs that were found in cave habitats characterized by constant darkness (Table 1 and 2) showed variable cell viability. Some of the found individuals of algae were actively moving within the samples and showing healthy-looking cell morphology. Some individuals of algae showed signs of cell deterioration such as uneven distribution of the cell organelles (e.g. plastids) and the cytoplasm. Together with the live individuals, empty diatom frustules have been found. The cells of some cyanobacteria were also detected just as recognizable empty cell walls. Usually, both living and dead individuals were detected.

Photographs of researched cave habitats and selected microphotographs of species are showed on Figs 5 and 6.

DISCUSSION

The research of microphototrophs in Veternica Cave (Table 1) is the first study of spatio-temporal presence of microphototrophs in the cave aphotic zone that was conducted during the period of eight years (2011-2016, 2019, 2021) based on 103 samples. This article also presents the first data on the presence/absence of microphototrophs in another 22 caves of the Dinaric karst based on 67 samples also collected during a period of eight years (2011-2012, 2014, 2016, 2018-2021) (Table 2). Despite the low degree of determination, our research is, to our knowledge, the most comprehensive study of the presence of microphototrophs in the aphotic zone of the caves, having total 170 samples (Table 1 and 2).

The partially illuminated entrance to Veternica Cave is a compatible habitat for microphototrophs, so their presence was expected. After the entrance, this cave very quickly narrows into a channel where the...
light was absent. The absence of microphototrophs from locations KON, SKP, SEP, KAL, PAK, and LIM in Veternica Cave (located 90 to 840 m distance FVCE) could be due to the specific hydrological conditions that do not favor the input of the microphototrophs from the surface. The conditions found in the first 840 m of the main channel of Veternica Cave reflect the conditions present in hydrologically inactive caves presented in Table 2, where hydrological activity is limited to seeping and dripping water and small water pools. The samples from nine hydrologically inactive caves of the Dinaric karst (Table 2) were also negative to microphototrophs. The locations KON, SKP, SEP, and KAL in Veternica Cave (Table 1) are in a part of the cave where diffuse electrical light was occasionally present, as this section of the cave is open for tourist visits. The presence of electrical lights in caves can induce colonization of lighted habitats by lampenflora – assemblages of cyanobacteria, algae and/or mosses (Mulec et al., 2008). Lampenflora has already been noted in Veternica Cave in the vicinity of some electrical lamps (Baković, 2016). Nevertheless, the electrical lamps were not directly pointed at the researched habitats, so the present diffuse electrical light probably did not induce the development of microphototrophs in these sites.

In opposition to the first 840 m of Veternica Cave, the cave hydrology is rapidly changing in the deeper parts of the cave. The presence of larger quantities of water in SIF, PLA, BJ, and VIK in Veternica Cave implies fast inflow of water to the subterranean environment that could be attributed to the wider hydrological surrounding of the cave. The dye tracing performed by Božičević in 1979 (in Lacković et al., 2011) showed that Veternica Cave is part of the karst channel system that drains water from sinkholes in karst polje Ponikve (located NNE from the end cave passages) and surrounding karst terrain, to the Dubravica spring (located SSW from the entrance to the cave). There are nine small temporary surface streams that drain into Veternica Cave by sinkholes that are small or completely filled with sediment thus inaccessible to humans (Ozimec & Šincek, 2011). In other words, conditions found in locations SIF, PLA, BJ, and VIK in Veternica Cave (Table 1) resemble the conditions present in the hydrologically active caves listed in Table 2.

Both findings from the hydrologically active parts of Veternica Cave (Table 1) and findings in the hydrologically active caves of the Dinaric karst (Table 2) show that microphototrophs were not present in all prospected samples. This implies that microphototrophs assemblages that are transported into caves are possibly not always detectable. Considering that only 0.6 ml of each sample was examined in this study, it cannot not be excluded that microphototrophs are much more abundant in caves, but below the detection limit of the methodology used in this study. Their presence could also be a consequence of the natural variation of their transport (e.g., presence of intensive flood events, retention capacity of bedrock). These phenomena should be studied further.

Findings from Veternica Cave (Table 1) and the caves of the Dinaric karst (Table 2) from this research are in accordance with the results of Baković et al. (2019) that found microphototrophs in aphytic habitats of five caves that were exposed to the occasional flooding, and one cave that was not flooded. Hajong et al. (2021) confirmed the presence of microphototrophs in aphytic habitats of five caves with subterranean streams and one cave with no distinct hydrological features. The results of Baković et al. (2019), Hajong et al. (2021), Cahoon and VanGundy (2022), along with those presented in this paper imply that the presence of microphototrophs in caves could be much more common than previously thought.

The more frequent presence of microphototrophs in deeper parts of the caves (Figs. 3, 4A, B) implies that the thickness of the bedrock above the cave, that is usually thinner near the cave entrance, is not the key element determining the presence of microphototrophs. The findings of microphototrophs in Veternica Cave and other researched caves from the Dinaric karst in a variable state of deterioration together with dead individuals (registered as empty shells/cell walls) confirm that microphototrophs are destined to diminish in cave aphytic conditions. Samples of

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**Fig. 5. A:** Small water pools formed on speleothem (Cave Miljacka II) and **B:** subterranean stream (Veternica Cave).
microphototrophs were collected during different seasons, confirming that the introduction of these organisms into caves is possible throughout the year. Regardless, it can be assumed that they are constantly introduced into subterranean habitats. Thus, the possible presence of live algae and cyanobacteria should not be ignored when discussing the subterranean food webs in caves and shallow subterranean habitats (Culver & Pipan, 2009, 2014). In order to identify the role of these organisms in subterranean food webs, additional research needs to be conducted. Algae associated with caves were noted also by some other authors presented in a review of Gittleson and Hoover (1969), but it is not clear if these findings are associated with cave entrances or areas characterized by constant darkness. The main reason why algae and cyanobacteria were ignored when discussing the true subterranean environment is the fact that they are primary phototrophic organisms – gaining energy by the process of photosynthesis. Thus, the presence of light is considered necessary for their survival. Still, it is often forgotten that these organisms have adaptations for surviving some time in the absence of light due to the ability to utilize their stored energy reserves (e.g., lipid compounds) and organic compounds from their environment (Tuchman, 1996; Tuchman et al., 2006; Kamp et al., 2011; Noth et al., 2013). For this reason, it is expected that microphototrophs could survive some limited time within subterranean habitats.

One of the interesting results regarding the microphototrophs in caves is domination of diatoms within detected groups in this research that is also in accord with the results of Hajong et al. (2021) and Baković et al. (2019). Diatom frustules are made of silica, thus they are very resilient and can be preserved in the environment for a long time. This characteristic of diatoms is extensively used in paleoenvironmental studies (e.g., LeBlanc et al., 2004, Carballeira & Pontevedra-Pombal, 2020). Considering the constant inflow of water into caves from the surface and durability of the diatom frustules, it would be expected that they are found in all prospected samples, which is not the case as showed in our results. Thus, their transport to subterranean habitats is not that simple to explain.

The presence of microphototrophs in cave darkness poses a question about how they enter caves. It is widely accepted that the source of primary colonization of caves are surface habitats (Golemansky & Bonnet, 1994). In surface waters (rivers and lakes) microphototrophs are very easily transported due to the water turbulence that could carry organisms to large distances (including into the caves). Microphototrophs can enter caves by sinking rivers, but also by means of infiltration through karstified lake bottoms and river beds (Bonacci, 1987) typical for karst areas. In contrast, microphototrophs in surface non-aquatic habitats are constantly fighting desiccation and other unfavorable conditions. They adapted to these conditions by secreting protective polymers which also enables them to strongly adhere to the surfaces (Nayaka et al., 2017). Thus, their transport to the caves is less likely. The most emphasized media for the transport of the microorganisms to the caves is water, and to a lesser extent the air transport and transport by animals entering the caves (e.g., Gittleson & Hoover, 1969; Golemansky & Bonnet, 1994). The results of our research confirmed that microphototrophs were dominantly found in samples from the hydrologically active parts of Veternica Cave (Table 1) and the hydrologically active caves of the Dinaric karst (Table 2), indicating transport by water as the dominant mechanism of their introduction.

Hydrological activity implies fast water infiltration into the caves and higher probability of turbulent water movement (Ford & Williams, 2007). Higher water energy and quicker infiltration could induce more successful transport of microphototrophs from their original habitat to the cave. Thus, it was not surprising that microphototrophs were recorded in caves (Table 2) where concentrated infiltration of surface water by sinking rivers (in case of Dankov Ponor, Markov Ponor, Ponor Dražice), flooding by natural surface lake (in case of Red Lake Cave) or by artificial water accumulation (in case of Horvatova špilja) is present. In areas where concentrated infiltration (e.g., by sinking rivers) is not present, the water comes from the rainfall in the wider cave area (Ford & Williams, 2007). It means that the only source

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Fig. 6. Diatoms showing signs of cell deterioration. A: Dankov Ponor, B: Markov Ponor, and C: Empty diatom frustule (Markov Ponor).
of the microphototrophs are non-aquatic species that strongly adhere to the surfaces (Nayaka et al., 2017). Therefore, it is not surprising that microphototrophs are absent in so many locations. Seeping water is characterized by slow and laminar transport through karst intergranular spaces and smaller bedrock fractures, so there is an increased possibility that microphototrophs will be retained, predated or will die before entering the cave.

CONCLUSION

The results presented in this research bring basic data on the presence/absence of microphototrophs in cave darkness from 23 researched caves collected in the total period of ten years. Microphototrophs were detected in ten caves in different seasons. Considering the large number of prospected samples in this research, findings of microphototrophs in cave darkness could not be regarded as an exception. Their presence was associated with the hydrologically active caves where the fast and turbulent water transport was assumed. Because both hydrologically active and inactive conditions can exist in the same cave, it is important to consider spatio-temporal variations of cave features when planning sampling campaigns. The results of this research suggest that microphototrophs should be considered as potentially important members of subterranean food webs in caves and shallow subterranean habitats along with other microorganisms. The research in Veternica Cave and other caves of the Dinaric karst provides preliminary data on general groups of detected microphototrophs. In order to better perceive the role of microphototrophs in aphotic habitats, further research needs to be focused on the identification of species, their abundance, survival time and spatio-temporal dynamics of their introduction to the cave environment. As some of these microorganisms could be novel heterotrophic or mixotropic species, caves could be used as polygons for research of their diversity, metabolism, and potential biotechnology application.

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