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ORIGINAL ARTICLE

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CYANOBACTERIA AND ALGAE FROM BIOFILM AT THE ENTRANCE ZONE OF PETNICA CAVE

ABSTRACT: The importance of biofilms in caves, the diversity of microorganisms in them, their mutual relationship and relationship with the substratum are among the advancing research topics in microbial biospeleology. This research is making contribution to the knowledge about biofilms at cave entrances and phototrophic communities in them. In that manner, biofilms from the entrance zone of the Petnica Cave were examined. Light microscopy showed that cyanobacteria were exclusively dominant phototrophs (34 taxa out of 39 total taxa recorded) with coccoid forms prevailing (28 taxa); simple trichal forms were present to a lesser extent, while heterocytous ones were completely absent. Genera *Gloeocapsa, Chroococcus*,*Gloeothece* and *Leptolyngbya* were the most diverse. Four green algal genera characteristic for aerophytic habitats (*Apatococcus*, *Desmococcus*, *Haematococcus* and *Trentepohlia*) were also recorded, while Bacillariophyta were observed sporadically. Three groups of sampling sites were distinguished based on recorded taxa, their richness and similarity, using non-metric multidimensional scaling (NMDS). Quantitative biofilm characteristics were also assessed – the content of chlorophyll *a* (Chl *a*) was determined, as well as the contents of water, organic and inorganic matter. Chl *a* had a significant positive correlation with the content of organic matter (r=0.904, P=0.013).

KEYWORDS: aerophytic phototrophs, algae, biofilm, cyanobacteria, Petnica Cave

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INTRODUCTION

Numerous different cavities are present widely underneath the Earth's surface, with many of them being solutional – karst caves which usually occur in limestone. Those are so far the most frequently studied types. Even though many caves are explored worldwide so far, it still is a small part of the present underground passages (Klimchouk, 2004). Cave entrance habitats are a part of caves characterized by the presence of daylight and may be considered as ecotones between the surface and dark cave habitats. The depth to which light penetrates, considering cave morphology, depends on the shape, position or width of the cave opening (Sket, 2004). For caves with large entrances, the light intensity reduces 1% or less per meter toward the interior, while in caves characterized with extremely small entrances (<1 m), almost complete darkness can ensue only after 1 m from the entrance (Pentecost, 2004). Here, at cave entrances, due to the presence of light, phototrophs are present (Sket, 2004). Different photosynthetic species may differently tolerate lack of light, while cyanobacteria and algae may have some adaptations to low light intensities and some can grow on rock walls, even if the only source of water is condensation (Pentecost, 2004).

Cyanobacteria and algae in caves are often found forming biofilms with other microorganisms/organisms. The variety of biofilms in caves can be quite high, and it seems that they are "unusually diverse compared to those found on the surface" (Boston et al., 2001 cited in Boston, 2004). Since they help in the survival and growth of microorganisms and their protection, it is even suggested that the primary evolutionary units are not the organisms incorporated in biofilms, but biofilms themselves (Boston, 2004).

Cyanobacteria are quantitatively among the most important organisms on Earth: they had a key role in the oxygenation of the atmosphere in the past and today. Their ability to detect and respond to variations in the environment is of key importance for their success and their presence in a diverse range of habitats (Whitton and Potts, 2012) and among them, in caves (Albertano, 2012). They are more adaptable compared to eukaryotic algae and some of the adaptations to subaerophytic life are a better tolerance of desiccation and water stress (Whitton and Potts, 2012). The cyanobacterial community inside caves, unlike those inhabiting outside habitat, is influenced by the characteristics of this somehow confined environment and it is mostly influenced by quality and quantity of light and air humidity (Albertano, 2012). They are resistant to different conditions in caves, including darkness (Czerwik-Marcinkowska and Massalski, 2018).

The aim of this research was to explore the diversity of phototrophic microorganisms from the entrance zone of Petnica Cave.

MATERIALS AND METHODS

Sampling location

Petnica Cave is situated southeast of the town of Valjevo, near Petnica village. The entrances to the cave lie on the northern slope of the Osoj hill in the village of Petnica, at the junction of the hill area and Valjevo and the Kolubara valley. River Banja springs from the Cave and flows into the river Kolubara (Figure 1).

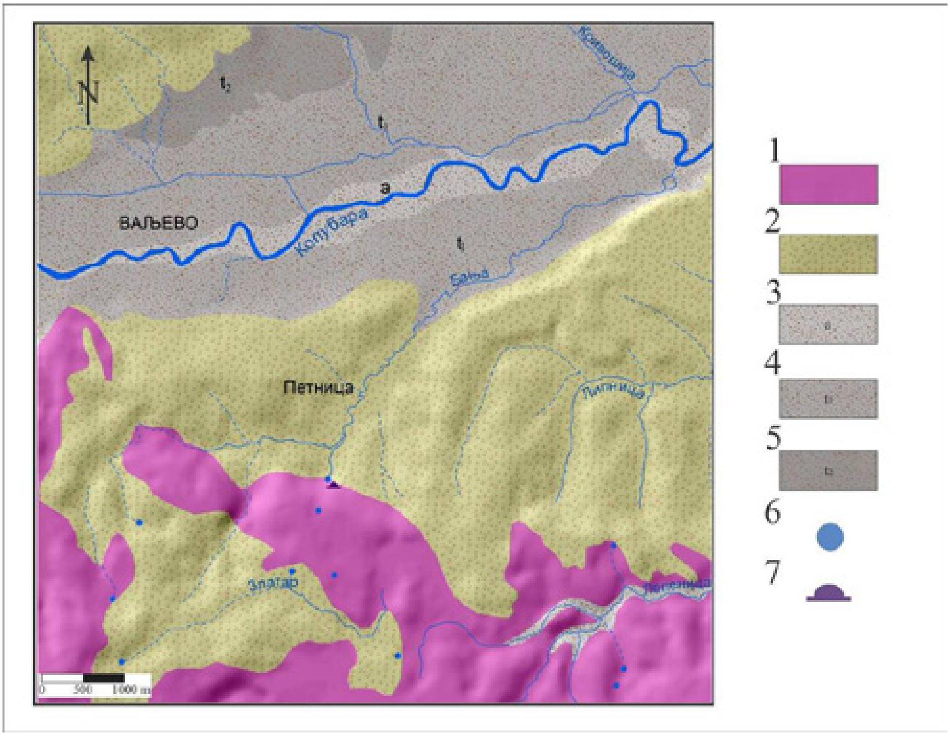

Figure 1. Relief, hydrology and geology of the wider surroundings of Petnica Cave according to geomorphological information system – ArcMap. 1 – carbonate rocks; 2 – Miocene marly-clay sediments; 3 – alluvium; 4,5 – river terraces; 6 – springs; 7 – cave.

Petnica Cave consists of two main parts: Donja pećina (lower cave), which is hydrologically active and Gornja pećina (upper cave), a dry part of the cave characterized with the occasionally present seeping water. They are connected and the total length of canals is 580 m. The entrance of the lower cave has a triangle shape, 14 m wide at the basis and 9 m high, while the entrance of the upper cave is considerably smaller. Due to large openings, external temperature and air humidity influence cave conditions at the entrance and near the entrance (Đurović, 1998; Lazarević, 1988). The sampling was performed at the entrance of the lower cave.

Petnica Cave is the oldest researched and the most famous cave in the Valjevo karst. It was first protected as an archeological site, and then it was declared a natural monument, as a speleological object and geoheritage site and is among the ten speleological monuments of nature in Western Serbia. A protection zone of 2.8 ha was established around the cave in 1969, while the latest protection study (2012) proposes to protect an area of 8.10 ha (Group of authors, 2012). Also, spring Banja ("Spring Petnica") is an extremely rare hydrological phenomenon, which, according to Simić (2008) belongs to the first groups of hydrological heritage objects of Serbia. The cave itself has so far been infrastructurally arranged and equipped for the needs of the tourist cave three times, but the number of visitors has never been enough for this function to be established. For long it has been the subject of different scientific research and educational activities (Group of authors, 2012).

Sampling sites and sampling procedure

Signs of the presence of phototrophic microorganisms were visible on the walls of the entire entrance zone of the lower level of Petnica Cave. Six sam-

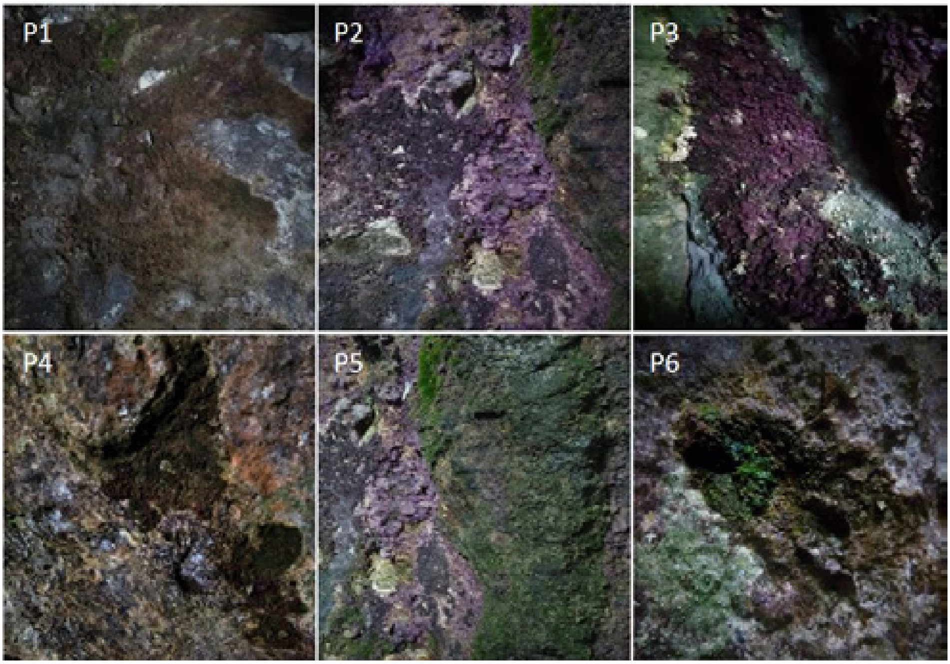

Figure 2. Sampling sites in Petnica Cave: P1) brownish coloured biofilm; P2) light purple gelatinous biofilm; P3) dark purple gelatinous biofilm, P4) brown coloured biofilm; P5) light green coloured biofilm; P6) dark green coloured biofilm.

pling sites (P1–P6) showing variously coloured biofilms, which photographs are shown in Figure 2, are chosen for biofilm sampling. All sampling sites were located, at the distance of approximately 20 m from the entrance of the cave (on the cave wall opposite to the cave entrance), but at different heights from the ground: P1 – 1.4 m, P2 – 1.4 m, P3 – 1.4 m, P4 – 1.65 m, P5 – 1.65 m and P6 – 1.7 m.

Biofilm samples for algological analyses were sampled as described in Popović et al. (2017). A flame sterilized scalpel was used to collect biofilm directly from the rock substratum, by gently scraping the material from the stone surface. The samples of biofilm were stored in labeled sterile polyethylene bags and as such transported to the laboratory for further processing – making of slides for microscopical analysis and identification of cyanobacteria and algae.

Algological analyses

For microscopical analyses of phototrophic microorganisms, two kinds of slides were made: temporarily wet mount slide (a piece of biofilm mixed with water) and semi permanent slide (a piece of biofilm mixed with glycerine). The slides were observed using the light microscope Zeiss Axio-Imager M.1 with software AxioVision 4.8. The identification of cyanobacteria and algae was performed by using the standard literature: John et al. (2003), Komárek and Anagnostidis (1998; 2005) and Starmach (1972).

Determination of chlorophyll *a*, water content, organic and inorganic matter in biofilms

Biofilm was sampled from a certain surface of 3.8 cm^2 using the round matrix as described in Popović et al. (2017), both for the determination of chlorophyll *a* (Chl *a*) content, as well as for biofilm parameters – the content of water, organic and inorganic matter. Biofilm was kept in sterile polyethylene bags until arrival in the laboratory. Chl *a* extraction and calculation were done as described in Popović et al. (2015), after which this parameter was expressed per surface area, as μg Chl *a* cm^{-2}. The content of water, organic and inorganic matter were determined based on the difference between fresh sample weight and weights after drying at 105 °C and ashing at 550 °C (Popović et al., 2017) and were expressed in percentages.

Data analysis

Correlations (Pearson coefficient) between Chl *a* and biofilm parameters were demonstrated using the statistical package XLSTAT (Addinsoft, 2020). Considering sampling sites, non-metric multidimensional scaling (NMDS) based on Bray-Curtis distance was done to see potential similarity/dissimilarity between them, based on documented cyanobacterial and algal taxa. For this purpose, the Canoco 5 Software Package (Ter Braak and Šmilauer, 2012) was used.

RESULTS AND DISCUSSION

In the aerophytic phototrophic community from the entrance zone of Petnica Cave, a total of 39 cyanobacterial and algal taxa was documented (Table 1). Cyanobacteria prevailed with 34 taxa, but algae belonging to Chlorophyta were also found, though in a lesser extent. Bacillariophyta were spotted too. The dominant Cyanobacteria were coccoid forms with 28 identified representatives (genus or species level) from 12 genera. However, simple trichal forms of Cyanobacteria were recorded too, but heterocytous ones were completely absent at these sampling sites. The most diverse cyanobacterial genera were *Gloeocapsa, Chroococcus* and *Gloeothece* considering coccoid forms and *Leptolyngbya* considering simple trichal forms. *Gloeobacter violasceus* was documented on all sampling sites, while *Chroococcus ercegovicii, Gloeocapsa biformis* and *G.nigrescens* were found on five. The only green algal representatives that were recorded in biofilm samples were members of *Apatococcus*, *Desmococcus*, *Haematococcus* and *Trentepohlia* genera. Few Bacillariophyta were spotted sporadically in biofilm. Some cyanobacterial representatives are shown in Figure 3.

Table 1. List of cyanobacterial and algal taxa from biofilm samples from the entrance of Petnica Cave.

	P1	P2	P3	P4	P5	P6
Cyanobacteria						
Coccoid						
Aphanocapsa fusco-lutea Hansgirg					+	+
Aphanothece saxicola Nägeli				+		
Aphanothece sp. Nägeli					+	
Asterocapsa sp. H.-J.Chu				+	+	+
Chondrocystis dermochroa (Nägeli ex Kützing) Komárek & Anagnostidis			+			
Chroococcidiopsis sp. Geitler						+
Chroococcus cohaerens (Brébisson) Nägeli	+					+
Chroococcus ercegovicii Komárek & Anagnostidis	+	+	+	+	+	
Chroococcus sp. (Kützing) Nägeli	+					
Chroococcus tenax (Kirchner) Hieronymus					+	+
Chroococcus turgidus (Kützing) Nägeli				+	+	+
Gloeobacter violasceus Rippka, J. B. Waterbury & Cohen-Bazire	+	+	+	+	+	+
Gloeocapsa aeruginosa Kützing			+			+
Gloeocapsa atrata Kützing	+			+	+	+

Gloeocapsa biformis Ercegovic	+		+	+	+	+
Gloeocapsa cf. *alpina* Nägeli				+		
Gloeocapsa nigrescens Nägeli	+	+		+	+	+
Gloeocapsa novacekii Komárek & Anagnostidis				+		
Gloeocapsa punctata Nägeli			+			+
Gloeocapsa reicheltii P.G.Richter				+		
Gloeocapsa sp. Kützing		+				
Gloeocapsopsis sp. Geitler ex Komárek					+	+
Gloeothece cf. *palea* (Kützing) Nägeli				+	+	+
Gloeothece confluens Nägeli				+		
Gloeothece rupestris (Lyngbye) Bornet				+	+	+
Gloeothece sp. Nägeli				+		
Pseudocapsa dubia Ercegovic	+				+	
Synechococcus cf. *elongatus* (Nägeli) Nägeli			+			
Unidentified coccoid Cyanobacteria			+	+	+	
Simple trichal						
Leptolyngbya foveolarum (Gomont) Anagnostidis & Komárek						+
Leptolyngbya perforans (Geitler) Anagnostidis & Komárek			+	+	+	
Leptolyngbya spp. Anagnostidis & Komárek	+		+	+	+	
Tapinothrix bornetii Sauvageau						+
Wolskyella cf. *floridana* Maresš & Kaštovský			+			+
Chlorophyta						
Apatococcus lobatus (Chodat) J.B.Petersen					+	
Desmococcus olivaceus (Persoon ex Acharius) J. R. Laundon					+	+
Haematococcus pluvialis Flotow					+	+
Trentepohlia aurea (Linnaeus) C.Martius				+		
Bacillariophyta						
Unidentified Bacillariophyta					+	+

Some data considering phototrophs from cave entrances in Serbia that are published so far indicate similar findings as in Petnica Cave (Popović et al., 2015; 2017; 2019; 2020). Cyanobacteria, more precisely coccoid forms were, for example, dominant in Božana Cave (Popović et al., 2015), Vernjikica and Degurić caves (Popović et al., 2019), Samar and Jezava caves (Popović et al., 2020). In majority, *Gloeocapsa* and *Chroococcus* were recognized as the most diverse genera. Genus *Gloeocapsa* was recognized as the most diverse genera when three caves in western Serbia were explored (Popović et al., 2017). In general genus *Gloeocapsa* is reported from various habitats characterized with variable ecological characteristics, which indicate its tolerance to a wide specter of environmental conditions (Cennamo et al., 2012).

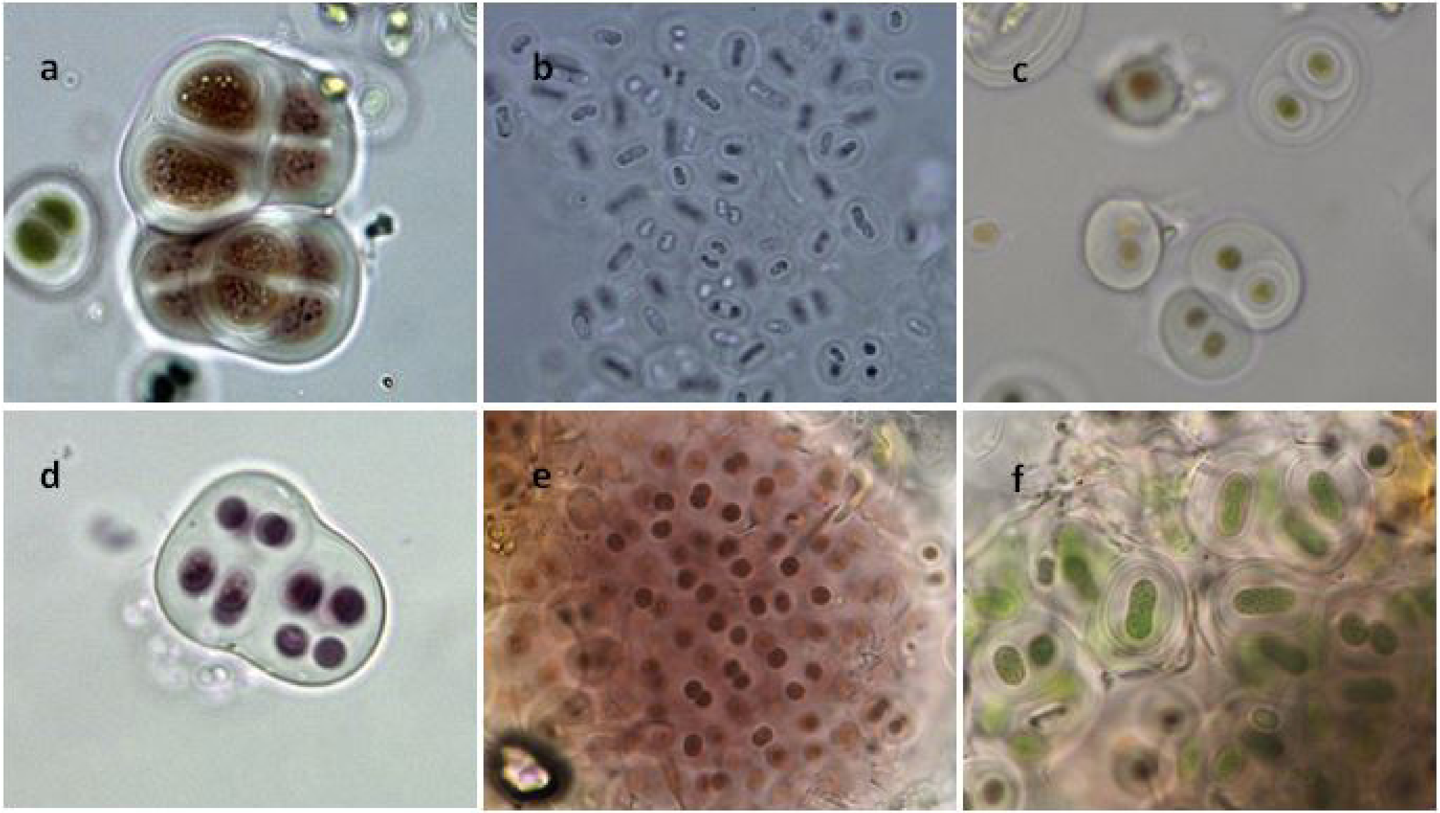

Figure 3. Example of some cyanobacterial representatives recorded in biofilm samples from Petnica Cave. a – *Chroococcus tenax*; b – *Gloeobacter violasceus*; c – *Gloeocapsa atrata*; d – *Gloeocapsa nigrescens*; e – *Gloeocapsa novacekii*; f – *Gloeothece* cf. *palea*

According to Czerwik-Marcinkowska and Massalski (2018), caves represent biodiversity centers for different types of microorganisms, especially for cyanobacteria, which can be very widespread inhabitants of rock surfaces in caves (Albertano, 2012), often making the main part of the phototrophic community in biofilms. Coccoid cyanobacterial representatives are frequent inhabitants of subaerophytic surfaces due to their simplicity and are in general better adapted to lower light conditions since they tolerate low irradiance more easily and thus often can be dominant (Mulec et al., 2008 cited in Popović et al., 2015). The presence of coccoid forms is even seen in fossils where they range from “isolated single cells, not uncommonly enveloped by multilamellar sheaths, to pairs or quartets of sheath-enveloped or sheath-lacking spheroidal cells” or “occur in large aggregates of geometrically ordered or irregularly distributed close-packed colonial cells” (Schopf, 2012) indicating that they were present many time ago and have time to adapt to various kinds of environment.

Considering Chlorophyta, *Desmococcus* was most frequently reported in aerophytic habitats in general (Lopez-Bautista et al. 2007).

Biofilm parameters (the content of water, organic and inorganic matter), together with the concentration of Chl *a*, are demonstrated in Figure 4. Considering the water content in biofilm samples, the highest was determined in the biofilm from sampling site P3. Water content was higher than 40% in all other samples too, except in biofilm from P5. On the other hand, P5 stands out as the sampling site where the highest content of organic matter was determined. The inorganic matter was highest at P1, and lowest at P6. In the time of sampling, it was noticed that biofilms at the selected sites at the cave entrance were well hydrated, which contributed to higher content in water in almost all samples. However, according to Czerwik-Marcinkowska and Massalski (2018), most European caves are moist and their walls at the entrance zone are frequently covered with cyanobacterial dominated biofilms (Pouličkova and Hašler, 2007). Cyanobacterial dominated biofilms are rich in extracellular polymeric substances (EPS) that are is secreted by the microorganisms as the glycocalyx, sheath or envelope and which one of the main role is water retaining (Albertano, 2012).

Considering the determined values of Chl *a*, the lowest was determined in biofilm taken from P1, and the highest in the biofilm from P5. In general, the highest recorded value of Chl *a* coincides with the highest determined value of organic matter. By observing correlation coefficients (Table 2), Chl *a* was significantly positively correlated with the content of organic matter (r=0.904, P=0.013) in Petnica Cave. As reported by Popović et al. (2017), the highest Chl *a* was also determined on a sampling site where the highest content of organic matter was recorded. However, it happens very often that a positive correlation between these two parameters is not observed, when organic matter in biofilms originate not exclusively from phototrophic organisms, but also from non-photosynthetic ones (bacteria, fungi, different organic particles that are deposited from air, dust, water, etc.).

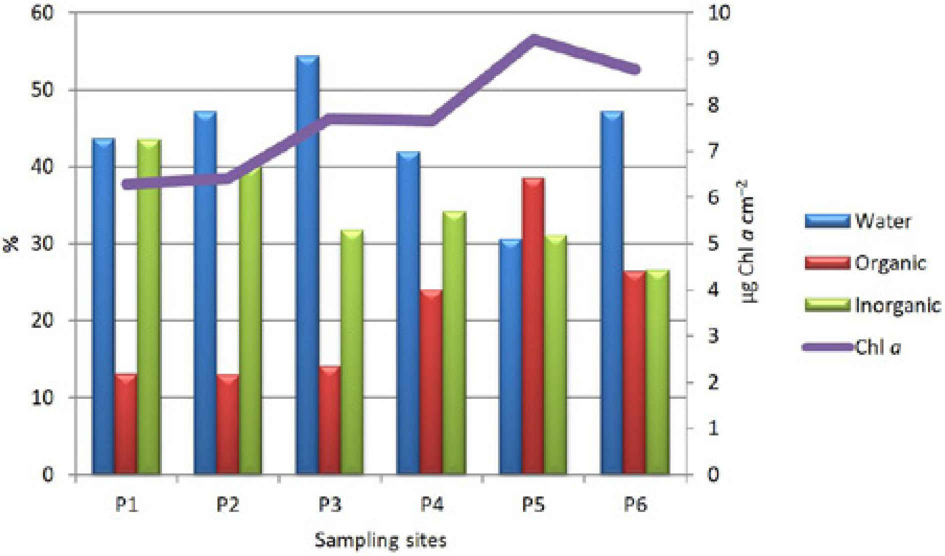

Figure 4. Chl *a* concentration (secondary axis), the content of water, organic and inorganic matter (primary axis) in biofilm samples from the entrance of Petnica Cave (P1–6, sampling sites).

Table 2. Correlation matrix between Chl *a* and biofilm parameters (the content of water, organic and inorganic matter in biofilm) showing correlation coefficients and significance

Correlation matrix (Pearson):				
Variables	Chl *a*	Water (%)	Organic (%)	Inorganic (%)
Chl *a*	1	-0.472	**0.904**	**-0.881**
Water (%)	-0.472	1	-0.791	0.027
Organic (%)	**0.904**	-0.791	1	-0.633
Inorganic (%)	**-0.881**	0.027	-0.633	1
Values in bold are different from 0 with a significance level alpha=0.05				
p-values (Pearson):				
Variables	Chl *a*	Water (%)	Organic (%)	Inorganic (%)
Chl *a*	0	0.344	0.013	0.021
Water (%)	0.344	0	0.061	0.959
Organic (%)	0.013	0.061	0	0.178
Inorganic (%)	0.021	0.959	0.178	0

In this study, we also wanted to represent which sampling sites were the most similar to each other, based on the species composition. Considering NMDS (Figure 5), three groups of sampling sites are distinguished on the ordination diagram. The first group is composed of P1 and P2 sites, and the second group of P4, P5 and P6 sites. The sites within the same group are mutually similar. However, the most distant of all, in other words, the most dissimilar of others is P3 (upper part of the ordination diagram). P1 and P2 contain a lower number of recorded taxa compared to other sites and the majority of them (almost all) belong to coccoid Cyanobacteria. On the other hand, P4, P5 and P6 are very rich in recorded taxa: at all sites, coccoid, simple trichal Cyanobacteria and Chlorophyta are recorded. P5 and P6 (very close to each other on the ordination diagram) are additionally characterized with the presence of Bacillariophyta. Sampling site P3 is the most distant from others because the number of recorded taxa is somewhere between the previously mentioned two groups and at this site only Cyanobacteria are recorded – coccoid, as well as simple trichal representatives. This sampling site was also characterized with the highest content of water in biofilm (Figure 4).

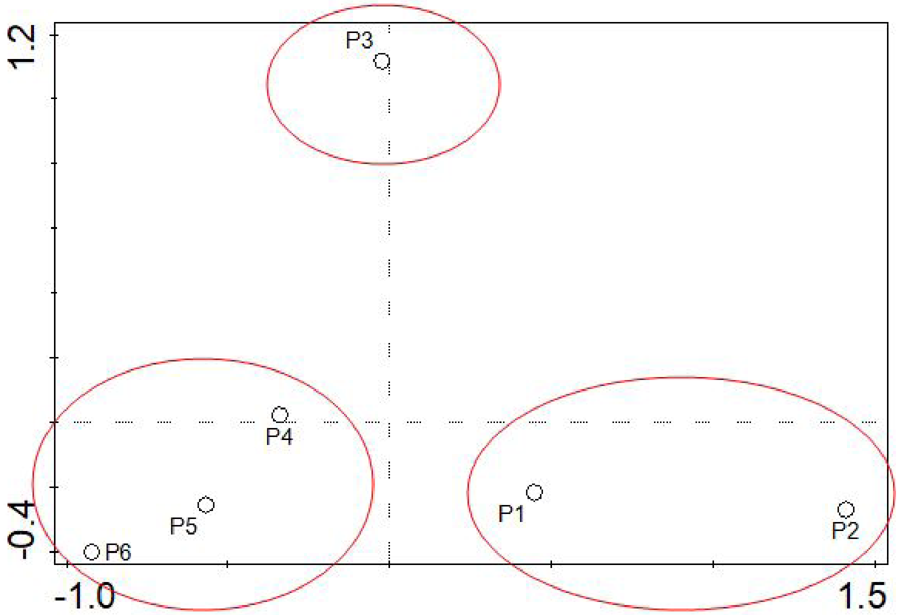

Figure 5. Non-metric multidimensional scaling (NMDS) showing similarity/dissimilarity between the sampling sites P1–6 in Petnica Cave based on recorded cyanobacterial and algal taxa.

CONCLUSIONS

The scientific value of caves due to the development of many different research fields related to them increased during recent decades and many caves are due to their unique characteristics recognized as protected sites. Research of phototrophs can be of greater importance due to their conservation. Phototrophic microorganisms, cyanobacteria and algae, were explored in biofilm samples collected at the entrance zone of Petnica Cave. Cyanobacteria were exclusively dominant, but Chlorophyta and Bacillariophyta were present too, though to a lesser extent. Coccoid Cyanobacteria prevailed where genera *Gloeocapsa, Chroococcus* and *Gloeothece* were the most diverse. Simple trichal representatives were also present, but heterocytous ones were completely absent from examined samples. By non-metric multidimensional scaling (NMDS) potential similarity/dissimilarity was observed between sampling sites based on recorded taxa. Three groups of sites were distinguished, one with the lower number of recorded taxa (consisting of almost only coccoid Cyanobacteria), second with a high number of recorded taxa (all divisions present) and third, made of only one site, was characterized with the presence of all cyanobacterial groups. Determined Chl *a* was significantly positively correlated with the content of organic matter.

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ЦИЈАНОБАКТЕРИЈЕ И АЛГЕ ИЗ БИОФИЛМА СА УЛАЗНЕ ЗОНЕ ПЕТНИЧКЕ ПЕЋИНЕ

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РЕЗИМЕ: Значај биофилмова у пећинама, разноврсност микроорганизама у њима, њихов међусобни однос, као и однос са супстратом, спадају у водеће теме истраживања у пољу микробијалне биоспелеологије. Ово истраживање доприноси општем познавању биофилмова на улазима пећина, као и познавању фототрофних заједница које се налазе у њиховом саставу. Имајући то у виду, испитивани су биофилмови са улазне зоне Петничке пећине. Светлосна микроскопија је показала да су цијанобактерије искључиво доминантни фототрофи (забележено је 34 од укупно 39 таксона), са изузетном доминацијом кокоидних форми (28 таксона); трихалне форме (хомоцитни представници) су у биофилму заступљене у мањој мери, док су хетероцитне потпуно одсутне. У родовима *Gloeocapsa*, *Chroococcus*, *Gloeothece* и *Leptolyngbya* документован је највећи број таксона. Четири рода зелених алги која су иначе карактеристична за аерофитска станишта (*Apatococcus*, *Desmococcus*, *Haematococcus* и *Trentepohlia*) су такође забележена, док су представници раздела Bacillariophyta нађени спорадично. Статистичка анализа NMDS је показала да су се према сличности, односно на основу таксона који су идентификовани, одвојиле три групе тачака узорковања. Квантитативне карактеристике биофилма су такође процењене – одређен је садржај хлорофила *a* (Chl *a*), као и садржај воде, органске и неорганске материје. Значајна позитивна корелација ($r=0.904$, $P=0.013$) уочена је између садржаја органске материје и Chl *a*.

КЉУЧНЕ РЕЧИ: аерофитски фототрофи, алге, биофилм, цијанобактерије, Петничка пећина