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Microbial community structure in two anchialine caves on Mljet Island (Adriatic Sea)

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The microbial abundances, including bacteria, viruses, and heterotrophic nanoflagellates, were determined for two anchialine caves located on the Island of Mljet (Adriatic Sea): Bjejajka Cave and Lenga Pit. Both caves are situated approximately 100 m from the coast with extensive subterranean connections to the sea, resulting in noticeable marine and terrestrial, influences. Because of the shallow settings of the studied caves, they represent habitats with a minimal light or complete darkness where photosynthesis is minimal or not possible. Thus, during the surveys there was no evidence of cyanobacterial cells in either caves, but the presence of bacteria, viruses and heterotrophic nanoflagellates (HNF) was established. Further, bacterial abundance was higher in caves in comparison to surrounding open seawater. In the surrounding seawater the predominance of the LNA group over HNA is determined, which also indicates the differences in relation to the studied caves. In fact, the dominance of HNA group of bacteria in caves together with higher total bacterial abundance indicates that the caves are of higher trophic level than the surrounding seawater. With regard to bacterial diversity, both caves had a unique makeup of bacterial populations and low diversity, with the chemolithoautotrophic Epsilonproteobacteria representing the most abundant taxonomic group. To examine the mechanisms regulating bacterial abundance in these habitats, we observed the relationship between bacteria and heterotrophic nanoflagellates (HNF) and between bacteria and viruses. The importance of predation in controlling bacteria (top down control) and, consequently, the domination of bottom up control of HNF were observed in both caves

Key words: anchialine caves, cyanobacteria, bacteria, viruses, heterotrophic nanoflagellates

INTRODUCTION

The eastern coast of the Adriatic Sea is a karst region and over 9,000 caves are known, many of which are located on islands or along the coastline (less than 100 meters from shore), and most of these caves have a pit-like entrance. To date, only 64 anchialine caves have been partially explored and/or described (CUKROV *et al*., 2009). Particularly famous cave is Šipun cave, located in the region of Dubrovnik in Croatia, due to its long known history and research and the richness of its fauna. Šipun is the type of locality for 18 animal taxa, 14 still valid.

Recorded fauna is a mixture of troglobionts, troglophiles and trogloxenes (OZIMEC, 2012). Detailed analysis of the crustacean community in comparison to abiotic factors in the anchialine caves of the Kornati islands was conducted by GOTTSTEIN *et al.* (2007). In the last decade, 4 new species of copepods have been described from the anchialine caves of the island of Vis, the island of Hvar and the islet of Badija and Kvarner area (KRŠINIĆ, 2005a; 2005b; 2008; 2012). GOTTSTEIN *et al*. (2012) listed 34 study caves with indicated number of taxa for each anchialine cave and recorded a hot spot cave on the island of Lošinj (the cave Medvjeđa Špilja with 12 recorded taxa) rich in organic material. Ecology and thermal regime in the Živa voda anchialine cave on the island of Hvar were described by NOVOSEL *et al*. (2007). This cave contains a dense population of the deep-sea hexactinellid sponge *Oopsacas minuta* Topsent, 1927. Therefore ecology and biodiversity of anchialine systems in Croatia are the object of accelerating research efforts, but there was no information about the microbial communities in these environments.

Generally, microbial abundances in caves are poorly understood (CAMPBELL *et al*., 2011), though caves do support a complex microbial life (NORTHUP & LAVOIE, 2001). Anchialine caves represent distinctive habitats with lack of light or complete darkness, relatively constant air and water temperatures, and poor supplies of easily degradable organic matter. Consequently, most cave ecosystems depend on allochthonous organic matter for energy (POULSON & LAVOIE, 2000; SIMON *et al*., 2003). Previous investigations describing microorganisms from caves and karst settings have suggested that most cave microbes originate from surface environments (MIKELL *et al.,* 1996), but a lot of other studying microbial abundances in cave environments provide information on subterranean chemolithotrophic ecosystems (BARTON *et al.,* 2004; CHELIUS & MOORE, 2004; GROTH *et al*., 1999; LAIZ *et al*., 1999, 2003; SCHABEREITER-GURTNER *et al.,* 2002, GONZALES *et al.,* 2011). Together, these investigations suggest that without sunlight energy and through geological isolation, caves are extremely starved environments, where levels of available organic carbon, which is needed to support heterotrophic microbial growth, are often a thousand-fold lower than in starved terrestrial environments (BARTON & NORTHUP, 2007). While heterotrophy is dominant in most cave ecosystems, chemoautotrophic pathways in anchialine ecosystems have been implicated as sources of organic matter and energy that support higher trophic level fauna. However, the specific biogeochemical processes and microbes mediating these transformations are not well-understood.

This study was carried out in two anchialine caves located in the National Park Mljet on the island of Mljet on the eastern coast of the Adriatic Sea: Bjejajka Cave and Lenga Pit. Both sites are situated approximately 100 m from the seacoast and are affected by marine tides, usually with restricted exposure to open air and extensive subterranean connections to the sea, which show noticeable marine and terrestrial influences. The objectives of this study were: (1) determining the presence and abundance of different microbial groups; (2) to obtain a preliminary evaluation of the taxonomic affiliation of the prokaryotes in anchialine caves; (3) studying the relationship between bacteria and their top down controllers (HNF predators and viruses).

MATERIALS AND METHODS

Study sites

Bjejajka Cave and Lenga Pit are anchialine caves located in the Mljet National Park on the Island of Mljet (Fig. 1). Both sites are situated approximately 100 m from the seacoast.

Bjejajka Cave is 22 m high and 40 m long with a water depth of around 12 m and a water volume of roughly 300 m^3 (Fig. 2). The surface of the pool is separated into two parts by a rock ridge. Water nearest the entrance is typically less saline than the main pool, which sits above a siphon that extends further back into the karstic rock. Preliminary measurements suggest that the water within the siphon is stratified, similar to the main pool. A colony of bats seasonally inhabits Bjejajka Cave, and their droppings, which are rich in organic carbon and nitrogen, are evident in and around the shallow bottom fringing the main pool.

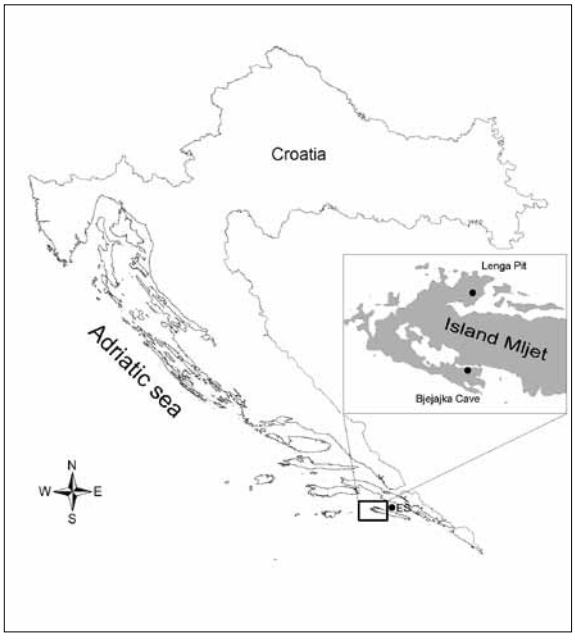

Fig.1. Location of anchialine caves (Lenga Pit and Bjejajka Cave) and seawater station (ES)

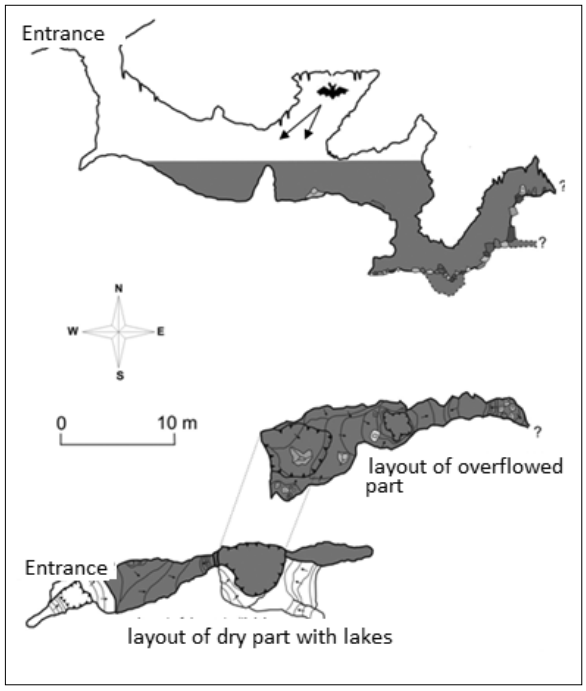

Fig. 2. Bjejajka Cave vertical transects. The arrows in Bjejajka Cave show where bat droppings occur (Topographic survey: B, Jalžić & V. Jalžić; Measured: P. Kovač-Konrad & V. Jalžić)

Lenga Pit is a 22 m deep vertical pit, of which one half is dry and is directly open to the atmosphere through a relatively narrow opening (Fig. 3). The volume of water in the pit is estimated to be ~90 m^3 with a water depth of 10.5 m. Lenga Pit is surrounded by small-scale agricultural area; therefore, this cave has some extent of antrophogenic influence. The sub-surface pit bottom is covered with leaves and twigs of decayed terrestrial plants.

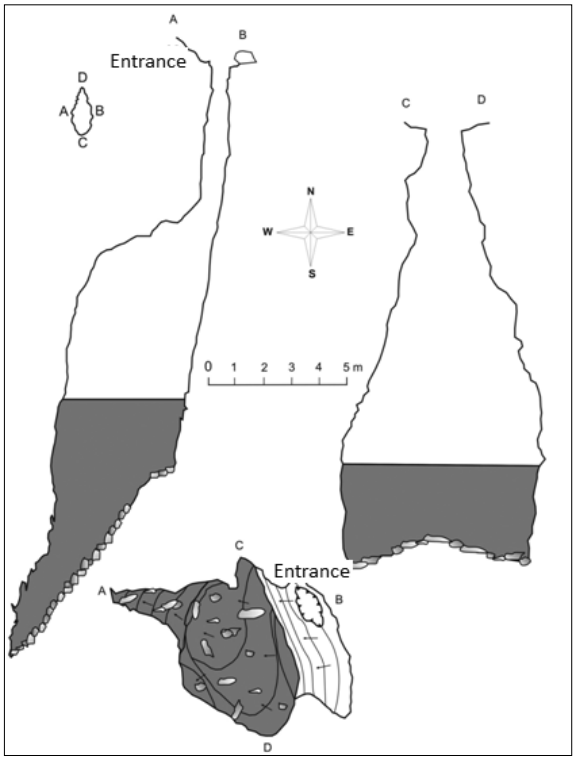

Fig. 3. Lenga Pit vertical transects (Topographic survey: B, Jalžić & B. Marković; Measured: V. Vračar)

Environmental samples from nearby sea were collected at marine station located in Mljet Channel (ES, coordinates: 42^0 47.5' N; 17^0 33.71' E).

Sampling and analysis

Sampling was conducted in April, September and November 2006 and in January 2008 during sampling expeditions of the members of Croatian Biospeleological Society. In Bjejajka Cave, the sampling depths were 0, 2.8, 4 and 10 m; in Lenga Pit the sampling depths were 0,3 and 8.5 m. Samples for measuring prokaryotes, viruses and HNF were collected in sterile glass bottles by a scuba diver. Environmental samples from nearby coastal sea were collected at 0,30 and 80 m.

Microbial abundance

Abundances of Sybr Green I-stained bacteria were determined using flow cytometry (MARIE *et al.* 1997). Samples of 1 mL were analysed on a Beckman Coulter EPICS XL-MCL with a high flow rate from 1 to 1.2 µL sec^{-1}, and the analysed volume was calculated by acquisition time. To standardise the fluorescence intensity of the cells, 1.0-µm yellow-green beads were added (Level-III Epics Division of Coulter Corporation Hialeah, Florida). Two subpopulations of bacteria were distinguished according to their relative green fluorescence as a proxy for the nucleic acid content (JOCHEM, 2001), referred to as high nucleic acid (HNA) and low nucleic acid bacteria (LNA) and light scattering. Autotrophic cells were separated into two groups of cyanobacteria (*Synechococcus* and *Prochlorococcus*) and were distinguished according to light scattering, red emission of cellular chlorophyll content and orange emission of phycoerythrin-rich cells.

Viral counts were carried out according to NOBLE & FUHRMAN's (1998) protocol. Preserved samples in formaldehyde (2% final concentration) were filtered onto 0.02-µm pore-size filters (Anodisc; diameter: 25 mm; Al_2O_3, Whatman) and immediately stained with SYBR Green I (stock solution diluted 1:300). Filters were incubated in the dark for 15 min and mounted on glass slides with a drop of 50% phosphate buffer (6.7 mM, pH 7.8) and 50% glycerol, containing 0.5% ascorbic acid. Slides were stored at -20°C until analysis. Viral counts were obtained by epifluorescence microscopy (Olympus BX 51, 1250 × magnification, equipped with blue excitation filter).

Heterotrophic nanoflagellates (HNF) were enumerated using standard DAPI staining technique (PORTER & FEIG, 1980). Preserved samples (10 mL) were stained with DAPI for 10 minutes and filtered through black polycarbonate filters with 0.8-µm pore size (Milipore, Ireland). Slides were examined with an Olympus BX51 microscope at 1000 × magnification.

To examine the regulation of bacteria by substrate availability (bottom up control, BU) and by predation (top down control, TD), data were analysed according to GASOL (1994). The simultaneous observations of bacterial and HNF abundance were plotted. This graph includes empirically determined maximum attainable abundance (MAA) and mean realised abundance (MRA) of HNF. The points close to the MAA line indicate strong coupling between bacteria and HNF, which, according to GASOL (1994), could be interpreted as strong TD control on bacteria and BU control on HNF. The MRA line balances the effects of both types of control. Thus, points that lie well below the MRA line indicate conditions when bacterial abundance was not controlled by HNF grazing (weak coupling between bacteria and HNF), which suggest domination of BU control on bacteria. At the same time, points below the MRA line suggest TD control of HNF.

DNA extraction and PCR amplification of 16S rRNA gene sequences

At each sample site seawater were stored until the extraction at -20°C. For phylogenetic analysis DNA was extracted from the filters using QIAamp DNA Stool kit (Qiagen, that could be used for environmental samples according the manufacturer recommendations). DNA concentration and purity for each extraction were determined using a biophotometer (Eppendorf). 16S rRNA gene sequences were PCR amplified using the primer pair 27f (f 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (r5'-GGTTACCTTGTTACGACTT-3'), which were phosphorylated at their 5'-end. The amplification profile consisted of initial denaturation for 4 min at 95°C, 35 cycles of denaturation for 1 min each at 95°C, annealing at 55°C for 1 min, elongation for 1 min at 72°C with final extension of 10 min at 72°C. Products were loaded onto a 1% agarose gel and visualised by adding SYBR Safe (1%) directly into the gel.

Amplified PCR products were sent for purification and sequencing in both directions to Macrogene Europe (Netherland). Nucleotide sequences were aligned using Clustal W within programme MEGA version 5 (TAMURA *et al.,* 2011).

Taxonomic affiliation and phylogenetic analyses

16SrRNA sequence was used for a similarity search using the SEQUENC_MATCH utility through Ribosomal Data Base Project II (www.cme.msu.edu/RDU) to determine their taxonomic identity for each sequence. Sequences that were ≥ 97% similar were grouped as a phylotype, and based on the obtained data, we performed phylogenetic analysis.

Phylogenetic analyses were performed using *MEGA* version 5 (TAMURA *et al.,* 2011). The same tool was used to construct Maximum Likelihood analyses. Reliabilities of phylogenetic relationships were evaluated using nonparametric bootstrap analysis (FELSENSTEIN, 1985) with 2,000 replicates. Bootstrap values exceeding 70 were considered to be well-supported (HILLS & BULL, 1993).

RESULTS

Hydrographical conditions, oxygen and pH regime

Hydrographical conditions, oxygen and pH regime measured during the sampling expeditions by members of the Croatian Biospeleological Society in Bjejajka Cave and Lenga Pit and were reported by ŽIC *et al.* (2008). The water in Bjejajka Cave and Lenga Pit is heavily stratified due to a strong salinity gradient. Salinity varied from approximately 5‰ at the surface in all seasons to approximately 38‰ at a depth of approximately 12 m. Observations from Bjejajka Cave suggest the existence of a halocline with a mixed layer of up to 3 m on top of a pycnocline, where salinity, and hence density, increase with depth and converge to full strength seawater at approximately 12 m. In contrast, temperature varies little, either seasonally or by depth. In Bjejajka Cave, surface water in April, September, November and January was 13.0, 14.3, 13.4 and 11.7°C, respectively. The surface water in Lenga Pit in September, November and January was 15.3, 15.5 and 14.8°C, respectively. Vertical changes in temperature were also small: in Bjejajka Cave the change from top to bottom was +1.5, +0.8 and +1.8°C in April, September and January, respectively, and in Lenga Pit this change was +0.5°C in September and +0.9°C in January.

With respect to oxygen the waters in both caves were strongly under-saturated, with concentrations routinely falling between ~ 0.6–4.0 mg L^{-1} compared to strongly saturated oxygen concentrations of 8.2–8.5 mg L^{-1} existing contemporaneously immediately outside the caves in the surface seawater (ŽIC *et al*., 2008). The surface samples from Bjejajka Cave in November and January were more highly oxygenated (6.7 and 7.7 mg L^{-1}). The results show an overall tendency for an oxygen minimum to be established at 3–5 m depth. Meanwhile, pH ranged from 6.76–7.73 with some tendency for a pH minimum at a depth of approximately 5 m. These measurements contrast with consistently higher values of 8.15–8.20 that were encountered routinely at that time in South Adriatic coastal seawater around the Island of Mljet (ŽIC *et al*., 2008). In both caves, pH was systematically lower in September than in November. The lowest values of both pH and oxygen were present at the halocline.

Abundance of prokaryotes

The abundances of cyanobacteria (*Synechococcus* and *Prochlorococcus)* have not been determined in both caves. Average monthly bacterial abundance in Bjejajka Cave ranged from 0.03×10^6 mL^{-1} to 0.53×10^6 mL^{-1} (Fig. 4). Bacteria were more abundant in Lenga Pit, with average monthly values of 0.61×10^6 mL^{-1} to 0.72×10^6 mL^{-1} (Fig. 4). Vertical gradients of bacterial abundance were pronounced in both caves with maximum values in the surface layers and minimum values at the bottom. The decrease of bacterial abundance from the surface to the bottom constituted an order of magnitude (Fig. 4). An exception to this pattern was recorded in January in Bjejajka Cave, in which the highest abundance of bacteria was recorded at a depth of 4 m (halocline) (Fig. 4).

Monthly fluctuations in the abundance of total bacteria during the investigated period were more pronounced in Bjejajka Cave com-

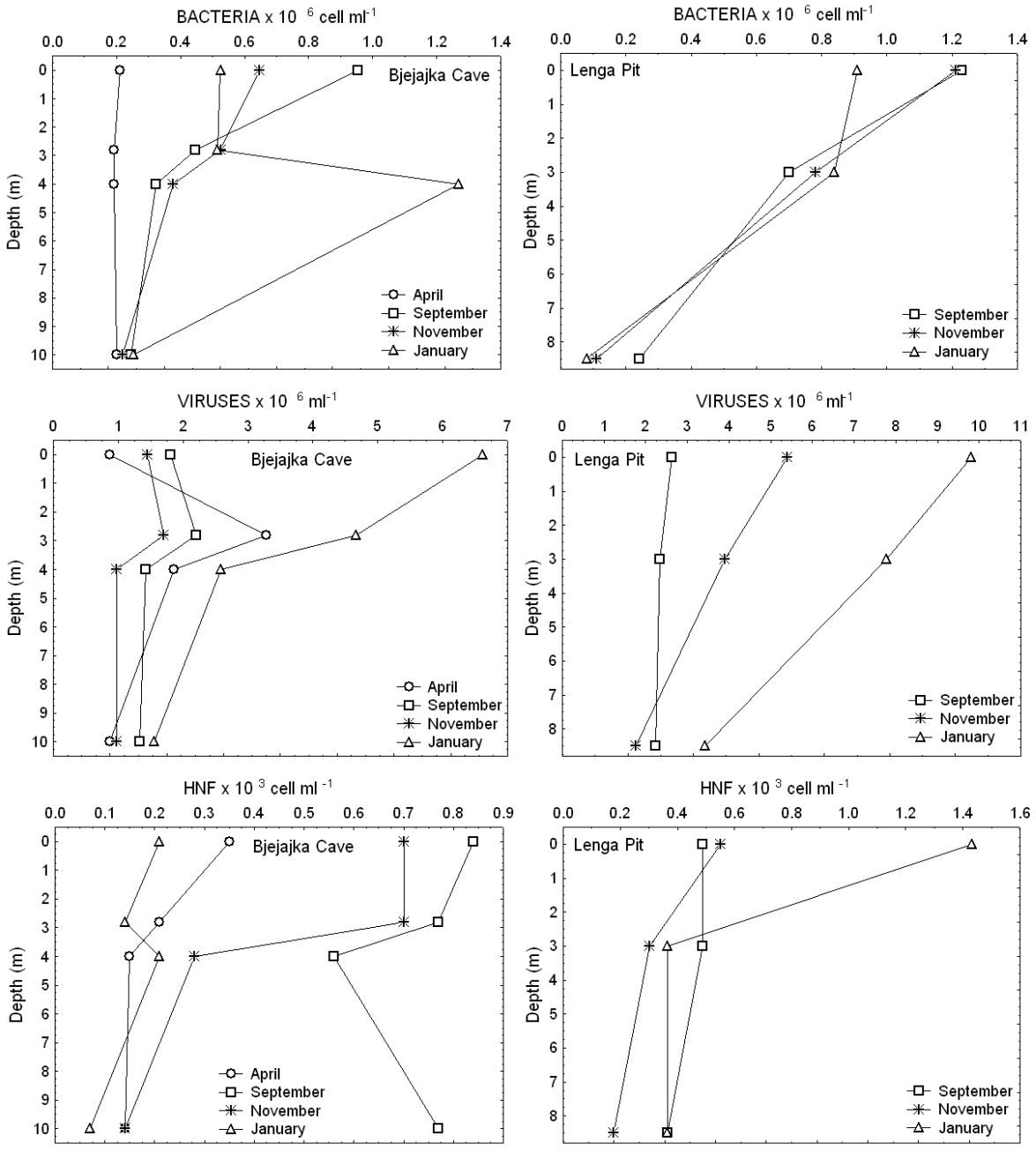

Fig. 4. Abundance of bacteria, viruses and HNF at depth profiles in Bjejajka Cave and Lenga Pit

pared to Lenga Pit (Fig. 4). Bacterial abundance in Bjejajka Cave in April was lower by an order of magnitude than in other months. Unfortunately, sampling in Lenga Pit in April was not performed, due to technical diving problems.

The percentage of HNA and LNA bacteria in the community of total bacteria highlighted differences between the investigated caves. The average monthly percentage of HNA bacteria from the Bjejajka Cave ranged from 13.97–78.87%, while LNA bacteria ranged from 21.13–86.03% (Fig.5). The highest proportion of LNA bacteria to total bacteria was detected in September, and the highest proportion of HNA bacteria to total bacteria was in April. There were no statistically significantly differences in abundance of LNA and HNA bacteria related to depth (t-test, P>0, 05). In Lenga Pit, a slight

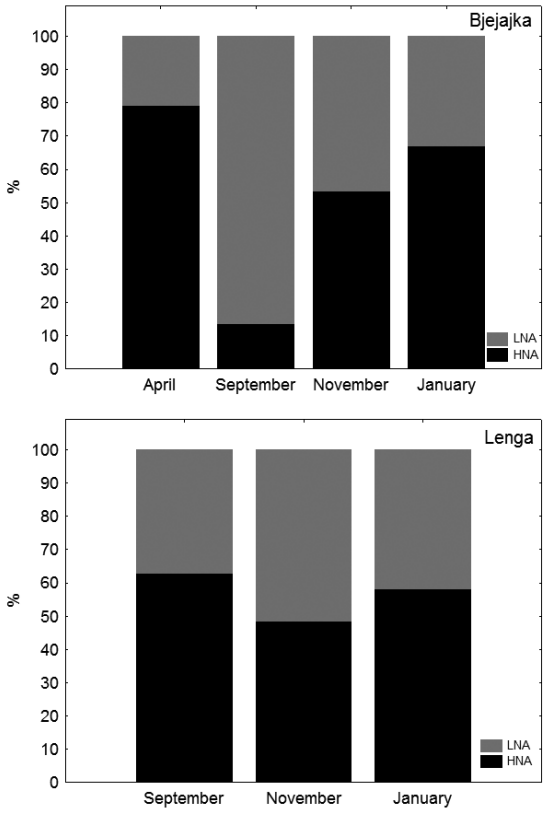

Fig. 5. Proportion of High Nucleic Acid (HNA) and Low Nucleic Acid bacteria (LNA) (average values) in Bjejajka Cave and Lenga Pit

prevalence of HNA bacteria was recorded relative to all investigated periods, ranging in average from 50.4–62.6% (Fig. 5). In contrast with Bjejajka Cave, the difference in abundance of HNA bacteria related to depth was statistically significant (t-test, $P<0.05$) in Lenga Pit. In the surface layer, the proportion of HNA bacteria to total bacteria ranged from 61.31–81.68%, whereas in the layer at 8.5 m depth it ranged from 49.2–59.81%.

Bacterial diversity

A maximum likelihood tree for both caves is reported in Figure 6. The highest number of matches (n=26) in Bjejajka Cave was assigned to the phylum *Proteobacteria,* class *Epsilonproteobacteria*. In this cave, we also determined domain Archeae (n=2), belonging to the phylum *Crenarcheota,* (Fig. 6). Identified bacteria were clustered in three groups (Fig. 6) showing bacterial genetic differentiation between cave and marine environment populations. In Lenga Pit, bacteria belonging to the phylum *Proteobacteria,* class *Epsilonproteobacteria*, were determined. The sequences were clustered in two groups and suggested bacterial genetic differences between the cave and marine environment populations (Fig. 6). In both caves, there was no difference in bacterial species related to depth.

In marine environmental samples from the surface (0 m), class *Gammaproteobacteria* was determined, and as depth increased below 30 m, *Deltaproteobacteria*, was also found.

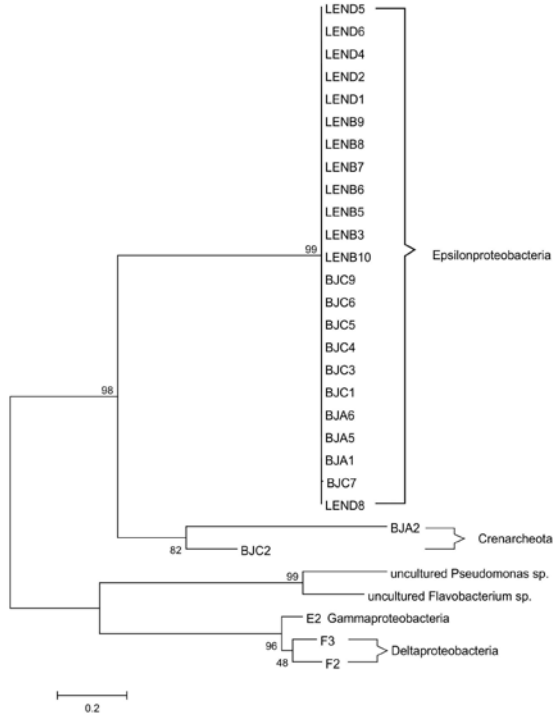

Fig. 6. 16S rRNA gene based phylogenetic tree of the Lenga Pit (LEN) and Bjejaka Cave (BJ) prokaryotic community. Tree were rooted with marine environmental uncultured bacteria Pseudomonas sp. *(GenBank: JF 968476.1) and* Flavobacterium sp. *(GenBank: JF 96848.1). (Letters and numbers by the LEN and BJ represent samples from studied caves; E,F – marine environmental samples)*

Viral abundance

Viral abundance in Bjejajka Pit ranged from 0.45×10^6 to 3.41×10^6 mL $^{-1}$. These values were about half of those recorded in Lenga Pit during the same period of investigation. In Lenga Pit,

values of viral abundance ranged from 1.06 × 10^6 to 6.91 × 10^6 mL^{-1} with pronounced vertical gradients in the abundance of viruses, decreasing from the surface to the bottom by an order of magnitude (Fig. 4). Fluctuations in viral abundance during the study period were much more pronounced in both caves than was observed for bacteria. In Bjejajka Cave, the number of viral particles oscillated in ratio of 1:7.5, and for bacteria 1:1.7; whereas in Lenga Pit the ratio for viruses was 1:6.5, and for bacteria 1:1.18. The Virus Bacteria Ratio (VBR) in Bjejajka Cave ranged from 1.3 to 32, while in Lenga Pit this ratio was much lower and ranged from 1.47 to 11.34 (Fig.7).

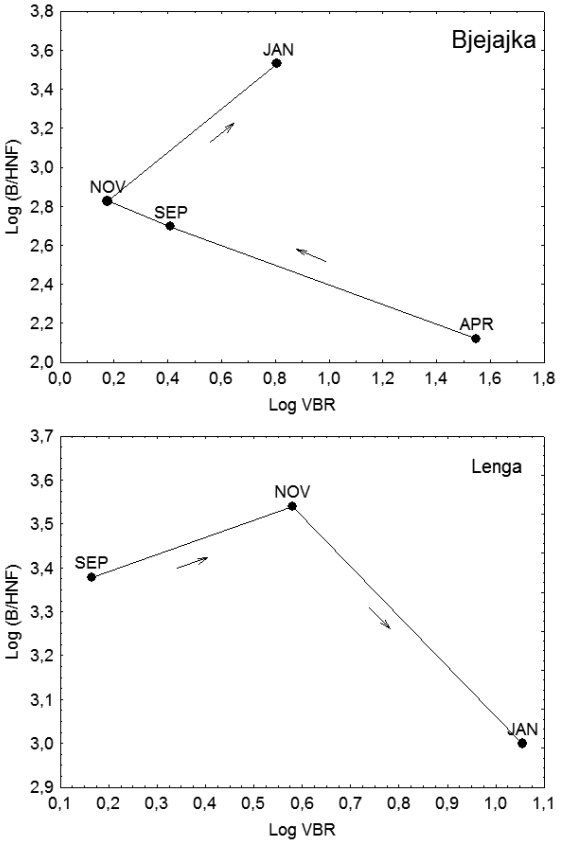

Fig. 7. Trajectory plot of temporal changes in virus to bacteria ratio (VBR) vs. bacteria to HNF ratio (B: HNF) in Bjejajka Cave and Lenga Pit

Heterotrophic nanofllagelate abundance

The abundance of HNF in Bjejajka Cave ranged, on average, from 0.16 × 10^3 to 0.73 × 10^3 mL^{-1}, and in Lenga Pit, the abundance ranged from 0.18 × 10^3 to 0.61 × 10^3 mL^{-1} (Fig. 4). Therefore, the difference in the abundance of HNF between the two investigated caves was less pronounced than the difference observed for bacteria and viruses. However, the variations in abundance of HNF throughout the investigated period were more pronounced than the variations observed for bacteria, and less pronounced than those observed for viruses (oscillated in ratio 1:4.6 in Bjejajka Cave and 1:3.4 in Lenga Pit, respectively).

DISCUSSION

Bjejajka Cave and Lenga Pit are typical anchialine environments as defined by STOCK *et al.* (1986) and ILIFFE (2000). Thus, these caves are under-saturated with respect to oxygen and are stratified due to a density gradient between the upper lens of fresh or brackish water and the underlying seawater, resulting in a marked halocline (ŽIC *et al*., 2008). The caves represent habitats with minimal light or complete darkness and during these surveys there was no evidence of cyanobacterial groups, even they only require a low intensity of light for photosynthesis (PARTENSKY *et al.,* 1999). However, microbial community point to the considerable abundance of bacteria, viruses and HNF as the main predators of bacteria. Average monthly abundance of bacteria obtained as the average value from the surface to the bottom layers in Bjejajka Cave ranged from 0.03 × 10^6 $mL^{-1}$to 0.53 × 10^6 mL^{-1}. In Lenga Pit, bacteria were more abundant with average monthly values of 0.61 × 10^6 $mL^{-1}$to 0.72 × 10^6 mL^{-1} (Fig. 4). Vertical gradients of bacterial abundance were pronounced in both caves with maximum values in the surface layers and minimum values at the bottom. An exception to this pattern was recorded in Bjejajka Cave in January, in which the highest abundance of bacteria was recorded at a depth of 4 m (halocline). Significant decrease of bacterial abundance from the surface to the bottom is probably due to the hydrographical features which drastically changed from the surface to the bottom, and possibly more important, to the vertical concentrations of DOC that generally

decreased linearly with depth and with increasing salinity in all investigated seasons in both caves (CUCULIĆ *et al*., 2011). Similar was recorded by ŽIC *et al.* (2011) studying the nitrogen and phosphorus nutrient systems within the caves. The high nitrate and phosphate concentrations in the surface waters and the peaks in ammonium and nitrate at 4 m at the halocline are the most notable features of the nutrient distributions in studied caves.

Significant differences have been identified by comparing the abundance of bacteria in the caves and open sea (Fig. 8). The surrounding sea water, as well as the entire area of the south Adriatic, has been characterized as oligotrophic area in which the abundance of bacteria does not exceed the values of 10^5 cells mL^{-1} (ŠANTIĆ *et al.,* 2013), while in the caves abundance of bacteria occasionally exceeds the values of 10^6 cells mL^{-1}, especially in the surface layers. Based on these values, according to COTNER & BIDDANDA (2002) the explored caves can be classified into systems of higher trophic level in relation to the surrounding sea. It is possible that these differences arise from the presence of different groups of bacteria in the caves and in the surrounding sea water, but also from the nutrient availability. CUCULIĆ *et al.* (2011) found DOC amounts up to 5-fold higher in both investigated anchialine caves compared to their concentrations in the open sea water. Similar was found by ŽIC *et al.* (2011) for nutrient concentrations, which were also higher in the caves than in the surrounding sea water. These differences point to the possibly stronger terrestrial than marine influence on supplying of caves by nutrients. The contribution to this hypothesis are also the data on the presence of terrestrial organic material (leaves and twigs) accumulated on the bottom of Lenga Pit, and some bat guano entered the water of Bjejajka Cave.

Differences in both abundance and fluctuations of bacteria between the caves were followed by other members of microbial community. In Lenga Pit, a prevalence of HNA bacteria was recorded during all investigated periods, especially in the surface layer, ranging from 61.31 to 81.68% (average values from 50.4 to 62.6%), while in Bjejajka Cave, LNA bacteria dominated in September, only (Fig. 5). Recent research showed that the HNA bacteria comprise an active group of bacteria in different marine ecosystems (GASOL & DEL GIORGIO, 2000), while in oligotrophic environments, LNA bacteria may represent the most active and predominating bacterioplankton community (ZUBKOV *et al.,* 2001; JOCHEM *et al.,* 2004; LONGNECKER *et al.,* 2005). In the surrounding seawater the predominance of the LNA group over HNA is determined, which also indicates the differences in relation to the studied caves. In fact, the dominance of HNA group of bacteria in caves indicates that the caves are of higher trophic level than the surrounding sea.

Regarding the bacterial diversity in both Bjejajka Cave and Lenga Pit, chemolithoautotrophic *Epsilonproteobacteria* was identified. These bacteria inhabit a wide variety of ecological niches ranging from deep-sea hydrothermal

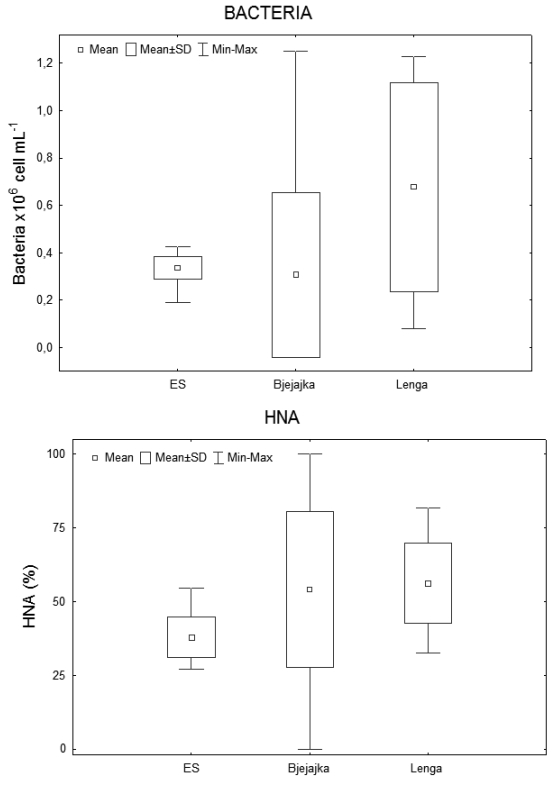

Fig. 8. Comparison of bacterial abundance and percentage contribution of High Nucleic Acid (HNA) in total bacterial counts between two studied caves and surrounding seawater (ES)

vents to hypogenic caves (ENGEL, 2010). The bacteria's ability to carry out different types of metabolism using a variety of alternative electron donors and acceptors means that they play an important role in the carbon, nitrogen and sulphur cycles within the cave. ŽIC *et al.* (2008, 2011) reported interesting iodine chemistry in these caves with iodide oxidation in bottom waters, iodate reduction and iodide re-mineralisation in mid-water, at the halocline. The oxidation of iodide to iodate in the bottom waters of these caves is possibly most easily explained as an effect of chemolithoautotrophic bacteria.

ENGEL *et al*. (2004) found this group to be the most abundant in a cave with high sulphide and low oxygen concentrations, suggesting that these organisms play a significant role in providing chemolithoautotrophic energy to the otherwise nutrient-poor cave habitat. In Bjejaka cave, in addition to *Epsilonproteobacteria*, the other taxonomic group was the Archaea. Archaeal species are often present in marine environments and surprisingly there were found in unusual and extreme niches, such as anchialine caves (McINERNEY *et al*., 2002). Recent studies have also suggested that the Archaea may play an important role in cave microbial ecosystems (CHELIUS & MOORE, 2004; GONZALEZ *et al.,* 2006; NORTHUP *et al*., 2003), although an identifiable metabolic role for these microorganisms has yet to be determined. These results indicate that anchialine caves have unique microbial makeup and low bacterial diversity (ENGEL *et al.,* 2004), which is phylogenetically differentiated from marine environmental samples. In marine samples phylogenetic differences have shown changes throughout the water-column. FUHRMAN *et al.* (1993) conclude that marine prokaryotic assemblages contain both Bacteria and Archaea. Bacteria were most common in euphotic zone and Archaea below that zone. In our marine environmental sample from the surface layers, bacterial sequence phylogenetically linked to the class *Gammaproteobacteria* was found, whereas, samples from below 30 m depth were dominated by class *Deltaproteobacteria*. (Fig. 6). It should be emphasised that this paper brings only the preliminary taxonomic affiliation of prokaryotes in caves and this aspect of research remain to be investigated in future study.

Viral abundance and dynamics followed the bacterial abundance, as bacteria were the most abundant host in the water-column of the investigated caves. The abundance of viruses in the caves was greater than that of bacteria, as observed in the marine environment (JACQUET *et al.,* 2010). The predominance of viral abundance over that of bacteria was observed using viruses to bacteria ratios (VBR). The VBR in Bjejajka Cave ranged from 1.3 to 32. In Lenga Pit this ratio was much lower, ranging from 1.47 to 11.34 (Fig.7), even though the overall abundance of bacteria in Lenga Pit was higher compared to that of Bjejajka Cave. The low VBR observed in Lenga Pit suggests that viral-mediated mortality of bacteria may be less important in Lenga Pit compared to Bjejajka Cave. Fluctuations in viral abundance during the investigated period were much more pronounced at both temporal and vertical levels in both caves than was observed for bacteria. From literature data is known that the turnover times of these communities are notably short, ranging from a few hours to a few days (FUHRMAN, 1999; WEINBAUER, 2004), and future samplings should focus on this short time-scale variability.

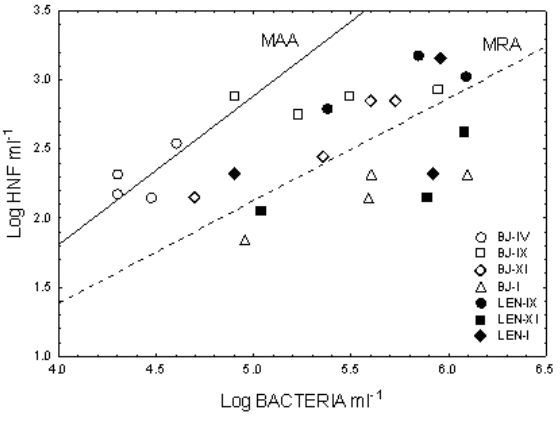

Fig. 9. Relationship between bacterial and HNF abundance at Bjejajka Cave (BJ) and Lenga Pit (LEN), plotted in a theoretical model (GASOL, 1984) (MAA = maximum attainable abundance; MRA = mean realised abundance)

To examine the regulation of bacteria and HNF by substrate availability (bottom up control, BU) and predation (top down control, TD), simultaneous observations of bacteria and HNF abundance were plotted in Fig. 9 according to the framework proposed by GASOL (1994). The samples from January at Bjejajka Cave and from November at Lenga Pit resulted in data points below the MRA line, whereas all other samples gave results above the MRA (mean realised abundance) line. This pattern suggests BU regulation of bacteria and TD control of HNF at Bjejajka Cave in January only and at Lenga Pit in November only. In all the other sampled periods, HNF predation pressure on bacteria was higher, suggesting a greater importance for TD control of bacteria and BU control of HNF, supporting the theory of ENGEL *et al.* (2004) that chemoautotrophic pathways in anchialine ecosystems are sources of organic matter and energy that support higher trophic level fauna.

Additionally, apart from the importance of HNF in the control of bacteria, the ratio of viruses to total bacterial abundance clearly points to the importance of viruses in the control of bacteria as well. In the period of the highest BU control of bacteria, the highest abundance of viruses was also observed. This could point to the fact that under these conditions, bacterial lysis is higher, leading to environmental enrichment by dissolved organic matter originating from these bacteria, and this organic matter could be a substrate for bacterial growth. The importance of this process is that it removes carbon from the classical grazing food chain and diverts it to the microbial loop. Conversely, viruses could have an important role in TD control of bacteria, which is the case in Lenga Pit in January, where a high abundance of HNF and viruses were followed by notably low numbers of bacteria (Fig. 7). During the last decades, marine viruses have been recognized to be an important component of microbial food webs. It is known that viral lysis, along with grazing by protists, can be an important source of mortality in aquatic bacterial communities (WOMMACK & COLWELL, 2000). It's important to stress that dominance of those two mortality processes varies among ecosystems. In marine habitats, bacteriophages were found to be the main factor of bacterial losses (GUIXA-BOIXEREU *et al*., 1996; WELLS & DEMING, 2006; BORAS *et al.,* 2009), to have similar significance (FUHRMAN & NOBLE, 1995; HWANG & CHO, 2002) or to be less significant than protists (GUIXA-BOIXEREU *et al*., 1996; CHOI *et al.,* 2003). Moreover, as the proportion of viral infections versus grazing can vary even within the hours (WINTER *et al.,* 2004), there is a need of simultaneously run experiments for relatively realistic prokaryotes loss processes evaluation.

CONCLUSIONS

This study presents the first investigation of the microbial abundances in the anchialine caves on the eastern coast of the Adriatic Sea. The results demonstrate that anchialine environments are inhabited by specific microbial communities. Cyanobacterial cell was not found in either caves, but the presence of prokaryotes, viruses and HNF was established. Further, bacterial abundance was higher in caves in comparison to surrounding open seawater. Prokaryotic diversity was unique, with the chemolithoautotrophic *Epsilonproteobacteria* being the most abundant taxonomic group. The high importance of predation by HNF in controlling bacteria (domination of top down control) was observed in both caves. Further, the ratio of viruses to total bacterial abundance suggests that the role of viruses in controlling bacteria could also be important.

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Struktura mikrobne zajednice u anhialinim špiljama na otoku Mljetu (Jadransko more)

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SAŽETAK

Istraživanja abundancije mikroorganizama, uključujući bakterije, viruse i heterotrofne nanoflagelate (HNF) su provedena u anhialinim špiljama Bjejajka i Lenga na otoku Mljetu. Špilje su smještene na oko 100 m udaljenosti od obalne linije s podzemnom vezom s morem te su pod znatnim utjecajem mora kao i kopnenih staništa. S obzirom na položaj istraživane špilje predstavljaju staništa s minimalnim prodorom svjetlosti ili potpunim mrakom što ukazuje na izostanak procesa fotosinteze. Tijekom istraživanja ni u jednoj špilji nije utvrđeno prisustvo cijanobakterija odgovornih za procese fotosinteze, ali je utvrđena značajna brojnost bakterija, virusa i heterotrofnih nanoflagelata. Štoviše brojnost bakterija u špiljama je bila veća od brojnosti bakterija u okolnom moru. Jednako i odnosi HNA i LNA bakterija ukazuju na razlike između špilja i okolnog mora. U okolnom moru je utvrđena dominacija LNA bakterija u odnosu na HNA dok je u špiljama utvrđeno prevladavanje HNA bakterija što zajedno s većom ukupnom brojnošću bakterija ukazuje na viši trofički stupanj u špiljama u odnosu na okolno more. Preliminarnim ispitivanjem raznolikosti bakterija u špiljama je utvrđeno najbrojnije prisustvo kemolitoautotrofnih bakterija taksonomske skupine *Epsilonproteobacteria.* U svrhu studiranja mehanizama regulacije bakterijske abundancije u istraživanim su špiljama analizirani odnosi između bakterija i heterotrofnih nanoflagelata i između bakterija i virusa. Važnost predacije u kontroli bakterija (top down kontrola) je utvđena u obje ispitivane špilje.

Ključne riječi: anhialine špilje; cijanobakterije, bakterije, virusi, heterotrofni nanoflagelati