Biopotential of microbial biofilms in the protection of subterranean habitats in Dinaric karst

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This brief overview of cave biota associated with microbial biofilms, including speleophyton, bacteria, protozoa and cave-dwelling fauna in Dinaric karst caves, is focused on taxa with potential application in biotechnology, bioremediation/ biostimulation and biomonitoring.

The review discusses the dynamics and structure of biofilms and the interspecies interactions of biofilms in a number of Dinaric karst caves, as well as a new approach to biofilm investigation.

A deep understanding of subterranean ecosystems and the unique organisms that inhabit them, connected by complex interactions, could elucidate many new aspects in the evolution of signal pathways important for producing novel biologically active molecules. Consideration of the potential applications will improve the preparation of cave management plans, especially of touristic open caves, but also help consultants to propose the most appropriate (bio)monitoring with a strong focus on habitat protection and biodiversity conservation.

Keywords: microbial biofilm, subterranean habitats, biotechnology, bioremediation, Dinaric karst

INTRODUCTION

A subterranean (hypogean) environment, including both terrestrial and aquatic habitats, *is trophically dependent* on surface ecosystems. The high constancy of abiotic factors makes hypogean habitats and their associated inhabitants among the most vulnerable on Earth (Gibert and Deharveng, 2002). Biodiversity in the hypogean realm is generally low compared to surface habitats of the same region, presumably due to the very specific and often harsh environmental conditions such as the lack of light and low nutrient availability (Juberthie, 2000). The lack of sunlight and consequent absence of photosynthetic activity, stable (low to moderate) temperature, high relative humidity and low rates of evaporation largely determine the distribution and population density of cave fauna (Holsinger, 1988). In such nutrient-poor conditions, the biome becomes increasingly dominated by microorganisms (Barr, 1967; Culver and Sket, 2000), metabolically miscellaneous and unexpectedly diverse (Barton and Jurado, 2007).

In some rare cave ecosystems energy originates from *in situ* bacterial chemoautotrophy (e.g. Movile cave in Romania (Sarbu et al., 1996), but most caves depend on nutrient input originating from the cave exterior. Nutrient transport in the dark zone relies either on allochthonous organic material (Gibert et al., 1994) sourced from penetrating plant roots, streams and percolating groundwater that enter caves, or on the decomposition of material such as bat guano, dung of birds and rodents, carcasses and migrating animals (Gunde-Cimerman et al., 1998; Culver and Pipan, 2009). In addition, chemolithoautotrophic primary production provides for the usage of sulphur-, ammonium- or nitrite-based chemolithotrophy and autochthonous organic carbon (Chen et al., 2009; Porter et al., 2009). Under environmental conditions, most bacteria persist in biofilm mode encased in an extracellular polymeric substance (EPS) matrix, responsible for architecture, morphology, coherence and activity (Flemming et al., 2000a) but also provide a beneficial structure to biofilm forming microbes in bioremediation (Flemming and Wingender, 2001; Flemming and Wingender, 2010; More et al., 2014). In fact, the variety of microniches provided by the biofilm also promotes a great diversity of microbial life and metabolic potential.

Dinaric karst is known worldwide as the richest subterranean fauna region, a hotspot of subterranean biodiversity (Vandel, 1965; Sket et al., 2004), on account of its numerous habitat types as an adaptive zone and the abundance of adaptive types inhabiting them (Gunn, 2004; White and Culver, 2012; Sket, 2017) as well as processes involved in the adaptation, speciation and evolution of subterranean animals. Notwithstanding the well documented biodiversity of cave-dweller animals (Sket, 2003; Sket, 2012), microbial communities were often neglected in past studies despite their high ecological importance (Engel, 2012a; Engel, 2012b) resulting from their biodiversity and incredible biochemical and physiological evolution in response to environmental conditions (Engel, 2010). In the Dinarides, "unusual aquatic organic formations" were first observed in Vjetrenica cave (Bosnia and Herzegovina) by the Czech speleobiologist Karel Absolon (1912), while several years later these formations were described as bacterial colonies by Olga Smolikova (1919). Over the past few decades, several studies of the bacterial diversity of microbial mats/biofilms have been reported (Pašić et al., 2010; Pleše et al., 2011; Porca et al., 2012; Kostanjšek et al., 2013; Pleše et al., 2016) revealing the diversity and uniqueness of the microbial world of caves.

Microbes, including bacteria, archaea, phytoplankton, protozoans and fungi, still catalyse the major transformations of elements, break down organic matter, and produce and consume oxygen and carbon dioxide (Smil, 2003). Thus bacteria and microbial eukaryotes, especially fungi and protists, are used for pollution removal (Bouwer and Zehnder, 1993; Cerniglia, 1997; Mitra and Mukhopadhyay, 2016) bioremediation (Balaji et al., 2014) biomonitoring (Pronk et al., 2009) and different branches of biotechonology (medical, agricultural, industrial biotechnology etc) (De Lurdes and Dapkevicius, 2013). The combination of *omics*-driven technologies and improved cultivation techniques is extending the knowledge of their biological diversity, paving the way for new biotechnological exploitations (Handelsman, 2004; Shestakov, 2012; Luna, 2015). In consequence, it has been emphasised that microbial ecologists can indeed contribute to socio-ecology and efforts toward achieving global sustainability by understanding the characteristics and processes of microbial communities that enable microbial services (Ducklow, 2008).

Our review, which focused on recent publications of the composition of microbial communities in Dinaric karst caves, suggests implementing the obtained results *in situ*, with a view to the protection of particular, critically endangered, subterranean habitats and the cave-dwelling fauna. Consideration of potential applications will improve the preparation of cave management plans, especially of touristic open caves, but also help consultants to propose the most appropriate (bio)monitoring with a strong focus on habitat conservation.

BIOLOGICAL PROPERTIES OF MICROBIAL BIOFILMS

The enormous global biodiversity of bacteria (Woese, 1987; Pace, 1997; Olsen et al., 1986; Dykhuizen et al., 1998; Schloter et al., 2000; Curtis et al., 2002; Torsvik et al., 2002; Rappé

and Giovannoni, 2003) and diverse biochemical and physiological evolution in response to environmental conditions, gave rise to an incredible diversity of adaptive solutions. The dynamics of biofilm formation are influenced by biotic and abiotic factors (Lawrence et al., 1991; Costerton et al., 1994; Wolfaardt et al, 1994; Costerton et al., 1995) whereas the nature of the carbon source has been shown to be essential in the control of the biofilm development pathways, architectural heterogeneity (Tolker-Nielsen and Molin, 2000) and species diversity (Caldwell et al., 1992; James et al., 1995a; Moller et al., 1997).

Although each biofilm is unique (Tolker-Nielsen and Molin, 2000), they share some common features and structural attributes which can generally be considered as universal, pertaining to the relatively high diversity, Proteobacteria domination and phylogenetically novel sequence types. Biofilms are very heterogeneous in both space and time, containing microcolonies of bacterial cells encased in an EPS matrix and separated from other microcolonies by interstitial voids (Lewandowski, 2000). The matrix architecture and biofilm mechanical stability are determined by a number of intrinsic factors, such as cell motility (Tolker-Nielsen et al., 2000), as well as extrinsic factors, combining to produce a dynamic, heterogeneous microenvironment for the attached and enveloped cells (Allison, 2003). Microorganisms use this polymer matrix for the concentration of nutrients used for their survival and growth while other cave-dwellers utilize these microbial formations as a food source. The three-dimensional structure of the biofilm also provides protection against predation, toxic substances and physical perturbation.

Most types of EPS are both hydrophilic and hydrophobic (Sutherland, 2001) and also highly hydrated, because EPS can incorporate large amounts of water into its structure by hydrogen bonding. EPS, primarily composed of polysaccharides, may account for 50% to 90% of the total organic carbon of biofilms (Flemming et al., 2000b). The chemical nature of the substratum may also affect the success of cell attachment and the morphology of attached cells, as well as subsequent biofilm structure (Dalton et al., 1994). The mechanisms that contribute to genetic and physiological heterogeneity (distinct metabolic pathways, stress responses, etc.) include microscale chemical gradients, adaptation to local environmental conditions and the genotypic variation that occurs through mutation and selection (Stewart and Franklin, 2008).

As a protected mode of growth, biofilm formation is an ancient and integral component of the prokaryotic life cycle, a key factor for survival in diverse environments and crucial for the colonisation of new niches (Hall-Stoodley et al., 2004). Biofilms represent primary units of evolutionary selection (Caldwell and Costerton, 1996). They provide an ideal niche for the genetic exchange of plasmid DNA by horizontal gene transfer (HGT). It has been well documented that conjugation occurs at a greater rate between cells in biofilms than between planktonic cells (Hausner and Wuertz, 1999; Roberts et al., 1999), but also promote plasmid stability (Madsen et al., 2012). Additionally, Ghigo (2001) has suggested that medically relevant strains of bacteria that contain conjugative plasmids more readily develop biofilms, while without plasmids these same organisms produce only microcolonies devoid of any further development. Since plasmids may contain genes for resistance to multiple antimicrobial agents or heavy metals, and also many other genes crucial for survival in a particular environment, biofilm also provides a mechanism for selecting and promoting the spread of these plasmids among the members of the community.

INTRA- AND INTER-SPECIES INTERACTIONS IN MICROBIAL BIOFILM

Host-symbiont interactions are common in nature and comprise long-term associations that range from mutualism to parasitism (Brownlie and Johnson, 2009). Although competition also occurs within biofilms, which was observed on *Hyphomicrobium* sp. biofilms invaded by *Pseudomonas putida* resulting in its dominance (James et al., 2005b) and on laboratory-grown biofilms containing *Klebsiella pneumoniae and Pseudomonas aeruginosa* (Stewart et al., 2007), the generally accepted view is that subterranean, near-starvation, oligotrophy leads to replacement of selfish competition by cooperative and mutual interactions, such as observed in biofilms (Barton and Jurado, 2007).

Development of *omics* technologies have enhanced our understanding of microbial communities, and also contributed to a new holistic approach to the biofilm function. Individual microbes react individually to the host environment in the context of other microbes and their mutual interactions, producing as a result an integrated collective behaviour (Comolli, 2014). Based on the diverse metabolic processes carried out by different bacteria, the relative abundance of specific biofilm members would clearly be expected to play a role in regulating the overall metabolic potential of the biofilm system (Moller et al., 1997). Bacteria use *quorum sensing* to coordinate gene expression according to the density of their local population (Xie et al., 2000; Mangwani et al., 2016). As well as the use of chemical signals to synchronise behaviour across entire populations, the type and extent of microbe-microbe and microbe-host nutritional interactions will determine the metabolism of the entire community in a given environment (Comolli, 2014). In fact,

microbial communities are evolving entities whose driving mechanisms are the interactions among them (Kassen and Rainey, 2004). Complex intra- and inter-species interactions should be considered among the cave-dwelling animal taxa but also between them and microbial (bacterial, archaeal, fungal and protists) communities in the mats/biofilm (Pleše et al., 2016). The role of microbial biofilms in the food web of subterranean habitats was confirmed by isotopic analyses of the microbial biomass, invertebrate consumers of microbes and invertebrate predators. It was also postulated that invertebrates subsisted on microbes or other invertebrates that feed on them (Sarbu et al., 1996).

A unique example of symbiosis in the early stages of evolution is that between chemoautotrophic bacteria (belongs to *Thiothrix* phylotypes) and a freshwater cave amphipod *Niphargus ictus* G. Karaman, 1985, endemic to the sulphide-rich Frasassi Cave system in central Italy, which originated less than 1 million years ago (Dattagupta et al., 2009). An interesting feeding strategy of the cave isopod *Mesoniscus graniger* (Frivaldszky, 1865) (Crustacea: Isopoda) was observed (Šustr et al., 2005) in respect to troglobite food sources and food web in subterranean ecosystems, in addition to the utilization of guano, filter-feeding and microphagous feeding strategies. It includes organic deposits with associated microorganisms, as sources of essential polyunsaturated fatty acids (PUFA). Indeed, algae, fungi and bat guano were found to be the most important potential resources of PUFA, essential to isopod nutrition. A food web based on the rich microbial biomass of hygropetric moonmilk and troglobitic beetle *Cansiliella servadeii* Paoletti 1972 (Coleoptera: Leptodirini) (Paoletti et al., 2011) has been recorded. In spite of the limited food sources for cave-dwelling animal species, some nutritional specialisations in terms of food web and food preferences were described for selected terrestrial arthropods from Slovakian karst (Smrž et al., 2015).

Faecal deposition by bats also affects community structure in guano-based ecosystems. Bat guano forms the basis of a food web consisting of bacteria, fungi, protozoans, nematodes, and arthropods (Harris, 1970). The diversity of organisms associated with guano has been shown to vary depending on the diet of the bat producing it. Thus the structure of a guano community is more dependent on the microenvironment provided by the guano than on the cave environment as a whole (Ferreira et al., 2007). Insects on dung in caves are exposed to a high diversity and quantity of fungi, and some species are known to produce anti-microbial substances (Ribeiro et al., 2012). Moreover, it was reported that mites, as the most diverse invertebrate taxa in bat guano, especially those that eat microfungi (e.g. *Histoplasma capsulatum*), may provide natural biological control for this pathogen, as well as regulating cave-dwelling microfungi in general (Estrada-Barcenas et al., 2010). It was also reported that cave salamanders consume the guano of grey bats (*Myotis grisescens* A. H. Howell, 1909) (Fenolio et al., 2006). Though a predator, the olm *Proteus anguinus* Laurenti, 1768 has been observed to thrive on a diet of mud and associated microflora (Vandel and Bouillon, 1959; Vandel, 1964).

A comprehensive and multidisciplinary approach to the complexities and different aspects of interspecies interaction, such as host-symbiont vs. host-parasite, predator-prey interactions to some trait-mediated indirect interactions such as the ecology of fear, and the evolution of behavioural plasticity, enhance our understanding of the significant impact on species interactions, community structure, and ecosystem function (Miner et al., 2005; Agrawal, 2001).

BIOPOTENTIAL OF CAVE BIOTA IN DINARIC KARST

SPELEOPHYTON

Cave ecosystems have a simple structure and small energy flow based upon primary bioproduction of cyanobacteria and algae (Golubić, 1967; Mulec, 2008). The ecological demands of primary producers in caves, especially in the darkness zone, are extremely low. However, owing to their broad tolerance range, as a result of genome evolution and their long life history, some species of algae live at a light intensity that is even lower than their compensation point (Hill and Forti, 1997). This possibility has been observed in Dinophyta, which are able to switch to heterotrophic nutrition as an alternative mode of energy supply (Adhikari, 2002). Due to their low energy flux, cave ecosystems are extremely sensitive to changes in primary production rate, whereas in electrically lit surroundings biofilms composed of algae and cyanobacteria develop (Borderie et al., 2014). The higher input of energy driven by lamps renders allochthonous taxa more competitive in relation to native ones (Mulec and Kosi, 2009). Since lampenflora has been recognized as a main cause of rock biodeterioration (Albertano, 2012), it has to be carefully treated. The drastic example of cleaning with sodium hypochlorite in the Mammoth Cave had negative consequences for native microbes and algal diversity (Smith and Olson, 2007). A new approach using well-controlled UV-C irradiation in algal proliferation control in caves was confirmed as highly effective (Borderie et al., 2014).

DIVERSITY OF SPELEOPHYTON: AN OVERVIEW FOR SOUTH-EAST (SE) AND EAST EUROPE

Cave vegetation is divided into three zones according to light conditions, reflecting the evolutionary path of

photoautotrophs from algae in complete darkness, through mosses and ferns in the twilight area, to phanerogams which grow around the cave entrance (Latella and Stoch, 2002). The cave-dwelling flora mainly consists of cyanobacteria, green algae and diatoms (Mulec et al., 2008, Czerwik-Marcinkowska et al., 2015) while Cyanophyta were found exclusively in the darkness zone (Martínez and Asencio, 2010). In general, speleophyton diversity is low due to the factors limiting photosynthesis (Smith and Olson, 2007).

Among Cyanophyta, the order *Chroococcales* seems to be predominant, followed by *Oscillatoriales* and *Nostocales* (Martínez and Asencio, 2010). Algae in caves occur in two living forms, aerophytic and as freshwater taxa (Kuehn et al., 1992; Sanchez et al., 2002).

Since algal flora in Dinaric caves has been neglected, records were relatively scarce. Findings from Slovenia (Mulec, 2005; Mulec et al., 2007; 2008) and recently, from Serbia (Popović et al., 2017), provide recent data on algal composition in cave biofilms, as do sources from Bulgaria (Blagoy et al., 2007), Poland (Czerwik-Marcinkowska and Mrozinska, 2009; 2011; Czerwik-Marcinkowska et al., 2015), Greece (Lamprinou et al., 2014) and Hungary (Komáromy, 1977).

In Slovenia, no fewer than 42 algal taxa were detected in Račiške ponikve (Mulec and Kosi, 2008) as well 35 from Škocjanske jame, with the most abundant being Cyanophyta (71%), Chlorophyta (19%) and Chrysophyta (10%) (Mulec et al., 2007). A previous study of Slovenian caves showed that *Aphanocapsa muscicola, Chlorella* sp., *Lyngbya* sp., *Synechocystis* sp. and *Trentepohlia aurea* are common taxa in subterranean habitats. The same study also reported the discovery of typical cave species on poorly illuminated cave walls, *Geitleria calcarea* (Mulec, 2005).

The algal flora analysis from three caves in Serbia reveals the prevalence of cyanobacteria, mostly chrococcalean taxa, with *Gloeocapsa* as the most diverse genus. In addition to cyanobacteria, Chlorophyta and Bacillariophyta were found in the sampling sites with representatives of aerophytic and freshwater forms (Popović et al., 2017).

The aerophytic speleophyton of Bulgaria displays a prevalence of cyanobacteria (59 taxa) of the total of 64 identified species (Blagoy et al., 2007). In Greece the distribution pattern of Cyanophyta has recently been analysed in two caves of the "Diros" complex in the Peloponnese (Lamprinou et al., 2014). Of the 43 detected taxa of Cyanophyta, no fewer than 23 belong to to the order *Chroococcales*, 17 to *Oscillatoriales* and three to the order *Nostocales*. The most common species were *Scytonema julianum, Iphinoe spelaeobios* and *Leptolyngbya gracillima*. In Poland, Chlorophyta, Chrysophyta, Bacillariophyta, Xanthophyta and Cyanophyta were detected as well as representatives of Dinophyta (*Gloeodinium cracoviense*, *Phytodinium aureum)*, mentioned previously regarding their ability to alter their energy supply mode (Czerwik-Marcinkowska and Mrozinska, 2009; 2011). From Hungary, five taxa of Chlorophyta, three of Xanthophyta, nine of Bacillariophyceae and four taxa of Cyanophyta were reported, while *Chlorella minutissima, Ch. zofingensis, Hantzschia amphyoxis* and *Navicula contenta* should be considered as typical representatives of algal cave flora (Komáromy, 1977).

POTENTIAL USE OF SPELEOPHYTON IN BIOENGINEERING

The bioengineering of individual microbial organisms or microbial communities has great potential in agriculture, bioremediation and industry. Algae have long been recognised as a food source in the human diet and the nutrology of domestic animals (Newton, 1951; Tseng, 1981; Aaronson, 1986; Turner, 2003; Gantar and Svircev, 2008; Dillehay et al., 2008; Craigie, 2010), which is extremely important in a global food crisis (Switzer, 1980; Jassby, 1988; Gantar and Svircev, 2008). Algae can be used in biomonitoring as *a* valuable *indicator* of even a small alteration in aquatic ecosystems (Pantle and Buck, 1955; Sladaček, 1973; Fournier et al., 2005), *and* as a source of novel bioactive compounds as well in biofuel production (Xiong et al., 2008; Griffiths and Harrison, 2009; Rodolfi et al., 2009; Rodolfi et al., 2016; Kumar et al., 2017; Arbenz et al., 2018). The biopotential of speleophyton is shown in Table 1.

Cyanobacteria have significant bioengineering potential, while the potential of other groups of cave-dwelling flora such as Chlorophyta, Xanthophyta, Chrysophyta, Bacillariophyta and Dinophyta is considered to be negligible. The mucilaginous sheath secreted by cyanobacteria provides a limited diffusion site whereas pH increases due to carbon uptake (Riding, 2011). The sheath provides support, stability and protection against physical damage, dehydration and grazers (Dudman, 1977). In the Dinaric region, cyanobacteria *Scytonema julianum* seems to be an interesting candidate for bioengineering on account of its ability to promote the formation of biofilms in a karst environment (Lamprinou et al., 2014). Further, the genus *Leptolyngbya*, already known as a producer of sheath-forming extracellular polymeric substances, has an important role in accelerating microbial biofilm formation (Albertano, 2012). In addition, *Gloeocapsa sp*. as a common species in the karst subterranean habitats may be an appropriate candidate for bioremediation/ biostimulation processes, by enriching microhabitats with autochthonous species, but without affecting a sensitive food web (Czerwik-Marcinkowska et al., 2015). It has been demonstrated that *Gloeothece sp.* may remove heavy metals (Cu2+, Pb2+) from aqueous solutions by sequestration in the extracellular matrix (Pereira et al., 2011).

Table 1. The potential usage of speleophyton in bioengineering.

Group of algae / Taxon	Potential usage in bioengineering	Reference
Cyanobacteria and algae	The important role in biofilm formation; Pioneer colonizers of bare rocks in general	Falasco et al., 2014
Scytonema julianum	Formation of biofilms on karst	Lamprinou et al., 2014
Iphinoe spelaeobios	Ability to mobilize calcium ions from calcareous substrate	Albertano, 2012 Smith and Olson, 2007
Leptolyngbya sp.	Ability to secrete sheath-forming EPS rich in polysaccharides which allows adhesion of biofilm	Albertano, 2012
Cyanobacteria	Biofilms can induce changes of chemical parameters in microhabitat	Albertano, 2012
Coccoid cyanobacteria	Promote attaching and colonization other members of biofilm	Popović et al., 2017
Cyanobacteria	Chelating agents	Falasco et al., 2014 Whitton, 2012
Aerophytic algae	The 3D biofilm structure acts as a *trap* for planktonic and phytobenthic algal species; Increase the primary bioproduction	Mulec and Kosi, 2008
Gloeocapsa sp.	Formation of biofilms on cave walls	Czerwik-Marcinkowska et al., 2015
Gloeothece sp.	Removal of heavy metals (Cu^{2}+, Pb^{2}+) from aqueous solutions by sequestration in the extracellular matrix;	Pereira et al., 2011
	The important role in biofilm formation; Pioneer colonizers of bare rocks in general	

BACTERIA

Along with previous studies of the diversity and composition of microbial biofilms from all over the world (Chelius and Moore, 2004; Gonzalezet al., 2006; Macalady et al., 2007; Shabarova and Pernthaler, 2010; Borsodi et al., 2012) and those from the Dinaric karst (Pašić et al., 2010; Porca et al., 2012; Kostanjšek et al., 2013; Pleše et al., 2016), a few studies, mostly based on microscopy and DNA metabarcoding, have analysed the taxonomical and functional characteristics of the microbiome from the surface of speleothems (Spear et al., 2007; Yücel and Yamaç, 2010; Ortiz et al., 2013; Sallstedt et al., 2014).

Comparative analysis of the results obtained from two studies of the composition of microbial communities (Kostanjšek et al., 2013; Pleše et al., 2016) from Vjetrenica cave in Bosnia and Herzegovina (from two different sampling sites) has yielded different results (Pleše et al., 2016). Kostanjšek et al. (2013) demonstrated that bacterial aggregates named "Candidatus *Troglogloea absoloni*" from Vjetrenica cave (Bosnia and Herzegovina) (sampling site: Donji Absolonov kanal) primarily consist of filamentous Betaproteobacteria, whereas other microbial populations present in the crust include members of the Bacteriodetes, Gammaproteobacteria, Actinombacteria, Alphaproteobacteria, and Planctomycetes. However, in Vjetrenica clone libraries obtained by Pleše et al. (2016) (sampling site: Donja Vjetrenica) *Nitrospirae* prevailed with 60% while neither *Alpha-* nor *Gamma-* proteobacterial sequences were retrieved, which is explained by different conductivity and consequent variations in the amount of dissolved solids, mostly nitrates (Pleše et al., 2016). Indeed, small alterations in the environment may affect the microbial biofilm composition and, consequently, influence particular interspecies interactions.

BIOPOTENTIAL OF BACTERIAL BIOFILMS: EXAMPLES FROM SOME DINARIC KARST CAVES

With the focus on the possible bioremediation and biotechnological potential of microbial biofilms sampled in three Dinaric caves (Veternica, Izvor Bistrac and Vjetrenica), the list of candidate taxa were shown in Table 2A (phylotypes obtained using bacterial 16S) and Table 2B (phylotypes obtained using five protein coding genes: leucyl-tRNA synthetase (*leuS*), elongation factor G (*fusA*), CTP synthetase (*pyrG*), 50S ribosomal protein L2 (*rplB*) and ATP-dependent DNA helicase (*recG*)). In Veternica and Vjetrenica clone libraries *Nitrospirae* prevailed with 36% and 60% respectively, while in Izvor Bistrac the most abundant were *Alphaproteobacteria* (41%) followed by *Firmicutes* (32%) (Pleše et al., 2013). The *Nitrospirae* phylotypes obtained from all sampling sites showed similarity with Candidatus *Nitrospira defluvii*, which differs greatly from other known nitrite oxidizers in the key enzyme nitrite oxidoreductase (NXR) as a physiological adaptation to a substrate-limited conditions, that allow for a mixotrophic lifestyle (Lücker et al., 2010).

Table 2A. Phylogenetic affinities of phylotypes[a16S] identified from the selected caves in Dinaric karst.

Clone[a]/ Sample site[b]	NCBI Acc. No.	16S ID[c]	Closest identified sequence with ACCN	Origin of the closest identified sequence	Reference
BIOREMEDIATION					
VET_03	JQ886446	99%	*Thiobacillus thioparus* (Hm173634)	Thiocyante assimilation	Boden et al., 2012
VET_04 VJET_03	JQ886447 JQ886429	92%	Planctomycete A-2 (AM056027)	The novel anammox bacteria; Wastewater treatment plant	Jia and Xu, 2005
VET_11	JQ886454	100%	*Novosphingobium* sp. K16 (Aj000920)	Chlorophenol-degrading bacteria /polluted water	Mannisto et al., 1999
IZB_02	JQ886434	100%	*Methylobacterium radiotolerans* strain P3 (Hm192796)	Phenol degradation; Methanol-utilizing bacterium	Tuan et al., 2011
IZB_03	JQ886435	99%	*Bradyrhizobium* sp. PC34 (Jf317681)	Nitrogen-fixing bacteria	Nievas et al., 2011
IZB_08 IZB_09	JQ886439 JQ886440	100%	*Bacillus* sp. PKSWIII (Jf768714)	Sub-surface soil of uranium-rich deposits	Kumar et al., 2013
IZB_10 IZB_12	JQ886441 JQ886443	100%	*Sphingomonas* sp. SeL7 (Hm352418)	Degaradation of polycyclic hydrocarbons	Yang et al., 2010
PATHOGENS					
IZB_06	JQ886437	100%	*Anaerococcus* sp. 8405254 (HM587319)	Anaerobe human pathogen	La Scola et al., 2011
VET_01 IZB_01	JQ886444 JQ886433	100%	*Shigella boydii* (Jq315923)	Human pathogen (dysentery)	Prasanna Kumar, 2011
IZB_11	JQ886442	99%	*Acinetobacter* sp. K-1 (Hq634943)	Nosocomial pathogen	Zhang and Feng, 2010
IZB_05	JQ886436	100%	*Microbacterium* sp. PrLB9 (Hm352399)	Nosocomial pathogen	Yang et al., 2010

[a]16S ribosomal RNA gene sequences (Pleše et al., 2016)
[b]Sample sites/ habitate type: (VET) Veternica (hygropetric); (IZB) Izvor Bistrac (Endogean underground streams); (VJET) Vjetrenica (Endogean underground river; Underground lakes)
[c]According to Fig. 6 (Pleše et al., 2016)

Table 2B. Phylogenetic affinities of phylotypes[ap] identified from the selected caves in Dinaric karst.

Clonea/ Sample siteb	Acc. No	prot. IDc	Closest identified sequence with ACCNd	Origin of the closest identified sequence	Reference
VJET_fusA VET_fusA IZB_fusA	JQ973807 JQ973808 JQ973809	98-99%	*Candidatus Nitrospira defluvii* YP_003796984	Nitrite oxidizer; *N. defluvii* differs dramatically from other known nitrite oxidizers in the key enzyme nitrite oxidoreductase (NXR)	Lücker et al., 2010
VJET_pyrG IZB_pyrG_01	JQ973794	91% 88%	*Methylibium petroleiphilum* strain PM1 YP_001023228	Degradation of the fuel additive MTBE (methyl tertiary butyl ether); Aerobic degradation of aromatic hydrocarbons including benzene, toluene, xylenes and phenol.	Kane et al., 2007 Burns et al., 2001 Deeb et al., 2001
VJET_pyrG	JQ973792	86%	*Leptothrix_cholodnii*_SP-6 YP_001793015	Metal-rich aquatic environments; Oxidizes both iron and manganese; Deposits Mn- and Fe-oxides on its sheath; Sequestration of heavy metals	Takeda et al., 2005 Copeland et al., 2008
IZB_pyrg_03	JQ973796	96%	*Magnetospirillum_gryphiswaldense*_ MSR-1_CAM75505	Phosphate accumulating; Abilities for phosphate recycling from wastewater	Richter et al., 2007 Zhou et al., 2017
IZB_pyrG_04	JQ973788	94%	*Novosphingobium aromaticivorans* DSM 12444 (YP_497290)	Degradation of a wide variety of aromatic hydrocarbons and polycyclic aromatic compounds	Copeland et al., 2014
IZB_recG VET_recG VJET_recG	JQ973799 JQ973798 JQ973797	89%	*Geobacter uraniireducens* YP_001231561	Use uranium as an electron acceptor for their respiration and reduce it to insoluble form; *Geobacter* sp. can oxidize organic pollutants in the subsurface using Fe(III) as an electron acceptor	Lovely et al., 1989 Lovley, 1991 Zhang et al., 2010 Kumar et al., 2013
VET_pyrG	JQ973793	91% 86%	*Acidovorax ebreus* TPSY YP_002551754 *Variovorax paradoxus* EPS YP_004154487	Anaerobic nitrate-dependent Fe(II) oxidizer; Anaerobically oxidize iron and uranium; Degradation of a wide array of recalcitrant organic pollutants including 2,4-dinitrotoluene, aliphatic polycarbonates and polychlorinated biphenyls	Byrne-Bailey et al., 2010 Lucas et al., 2008 Satola et al., 2012

[a] Protein coding genes leucyl-tRNA synthetase (*leuS*), elongation factor G (*fusA*), CTP synthetase (*pyrG*), 50S ribosomal protein L2 (*rplB*) and ATP-dependent DNA helicase (*recG*) (Pleše et al., 2016)
[b] Sample sites/ habitate type: (VET) Veternica (hygropetric); (IZB) Izvor Bistrac (Endogean underground streams); (VJET) Vjetrenica (Endogean underground river; Underground lakes)
[c] According to Fig. 7 (Pleše et al., 2016)

Besides a few pathogenic or nosocomial bacteria important for public health and indicators of faecal contamination, such as *Shigella boydii* (Gammaproteobacteria, Enterobacteriales) (shigellosis; dysentery), one of the most important phylotypes shows marked similarity with *Methylibium petroleiphilum* strain PM1 (Betaproteobacteria; Burkholderiales) (Vjetrenica and Izvor Bistrac), which can grow on and completely degrade the fuel additive MTBE (methyl tertiary butyl ether) (Bruns et al., 2001). Furthermore, PM1 strain has a broad range of metabolic capabilities such as the ability to aerobically degrade aromatic hydrocarbons including benzene, toluene, xylenes and phenol (Deeb et al., 2001).

Geobacter uranireducens Rf4 (Deltaproteobacteria; Desulfomonadales) in the subsurface environment is the primary bacterium able to use uranium as an electron acceptor for respiration and to reduce it to insoluble form (Lovley, 1991; Palmisano and Hazen, 2003). Additionally, species of the genus *Geobacter* can oxidize organic pollutants in the subsurface using Fe(III) as an electron acceptor (Lovely et al., 1989; Zhang et al., 2010). It is important to note that Izvor Bistrac is contaminated by metal waste as well as explosive remnants of war (ERW).

Acidovorax ebreus (strain TPSY) (Betaproteobacteria; Burkholderiales) is able to anaerobically oxidise iron and uranium when coupled with nitrate reduction. These bacteria may be important for the remediation of uranium in contaminated environments.

Variovorax paradoxus (Betaproteobacteria; Burkholderiales) is abundant in numerous environments that are contaminated with either recalcitrant organic compounds or heavy metals. The diverse metabolic capabilities of *V. paradoxus* enable it to degrade a wide array of recalcitrant organic pollutants including 2,4-dinitrotoluene, aliphatic polycarbonates and polychlorinated biphenyls. Both its catabolic and anabolic capabilities have been suggested for biotechnological use, such as to neutralise or degrade pollutants at contaminated sites (Satola et al., 2012).

Pseudomonas aeruginosa (Gammaproteobacteria; Pseudomonadales) is a ubiquitous environmental bacterium as well as an important opportunistic human pathogen. Certain isolates infect a broad range of host organisms, from plants to humans (He et al., 2004). As an important soil bacterium, it possesses a complex metabolism capable of degrading polycyclic aromatic hydrocarbons and producing biologically active secondary metabolites including quinolones, rhamnolipids, lectins, hydrogen cyanide, and phenazines. Its metabolism complexity is reflected in a relatively large genome size of about 6.5 Mb. PA14 is also an excellent model for the study of pathogenesis and biofilm formation, adaptable for a free-living and pathogenic life style. Comparative genomic analysis of the virulent strain PA14 and the less pathogenic strain PAO1 reveals that the virulence of *P. aeruginosa* is the result of a pool of pathogenicity-related genes that interact in various combinations in different genetic backgrounds (Lee et al., 2006). Significant similarity (app. 90%) of phylotypes isolated from subterranean habitats in Croatia (Veternica and Izvor Bistrac) was shown with both strains of *P. aeruginosa*, UCBPP-PA14 and PAO1 (Pleše et al., 2016).

An interesting finding was the similarity (app. 90%) with two species of the genus *Desulfovibrio*: *D. vulgaris* (YP_967305) and *D. magneticus* (ZP_11310108). Rajeev et al. (2014) reported on two diguanylate cyclases (DGCs) in the bacterium *Desulfovibrio vulgaris* Hildenborough that function as part of a two-component signalling pathway, each one specific for one choice of growth fate. Once the fate has been committed, the type of interactions, topological relationships and constraints, and the physical means to establish them determine metabolic strategies.

Bacteria are also a significant source of biotechnologically important substances. A recent study from 19 karstic caves in Turkey yielded 180 isolates (62%; out of 290) which exhibited antimicrobial activity against bacteria and fungi, even against multiresistant strains of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterobacter faecium* (VRE), and *Acinetobacter baumanii* (Ortiz et al., 2013). Novel phylotypes isolated from Izvor Bistrac (Pleše et al., 2016), showing similarity to *Stigmatella aurantiaca* and *Myxococcus xantus* (Deltaproteobacteria; Myxococcales), known for producing antimicrobial compounds, may play a role in maintaining the balance of the microbial population in its habitat (Dworkin, 2007).

Sulphur cave bacteria are of particular interest. These were observed for the first time from sulphurous karstic cave springs in Split, where several new bacterial species have been described: *Thiothrix longiarticulata*, *T. voukii, Beggiatoa gigantea* and *Thiosiphon adriaticum* (Gammaproteobacteria, Thiotrichales) (Klas, 1936a; 1936b; 1937; 1938). *Thiophysa gigantea* was found in the Sumporače sulphur caves in Mokošica near Dubrovnik in 1948 and described by biologist Zora Klas (Klas 1949; 1950; 1951). Later, *Thiogloea*, a new genus from the same cave, with two species, *T. ruttneri* and *T. ragusina*, was described by academician Zvonimir Dévidé (Dévidé, 1952). The list of recorded bacterial taxa in Sumporača mala and Sumporača velika caves, with the capacity to oxidise sulphur compounds, comprises at least 14 species of the genera *Beggiatoa, Thiothrix, Thiophysa, Thiovulum, Thiospira* and *Thiogloea* (Cukrov et al., 2012; Cukrov and Ozimec, 2012).

PROTOZOA

Protozoans, as an important indicator of biotic and abiotic changes in the environment, are an integral part of all ecosystems, as regards their dynamics and community structure. Although cave-dwelling protozoa have been investigated since 19th century, data still remain scarce (Mulec, 2008). In caves, protozoa are usually isolated from the water column, guano or sediments. Another important but barely studied group consists of protozoan epibionts and parasites on cave animals (Golemansky and Bonnet, 1994; McAllister and Bursey, 2004). A comprehensive study on protozoa composition from four caves including nine different habitats in Slovenian karst first reported on the diversity of small free-living amoebae (FLA) in carbonate precipitating habitats in karst caves. The potentially pathogenic *Acanthamoeba castellanii* genotype T4 and *Hartmannella vermiformis* were identified from a cave pool with calcite rafts in Pečina v Borštu cave. Both amoebae can serve as vectors for intracellular pathogenic bacteria. A new vahlkampfiid amoeba, *Allovahlkampfia spelaea* gen. nov., sp. nov. was also described from weathered limestone in the same cave (Mulec and Walochnik, 2007; Walochnik and Mulec, 2009).

Murga et al. (2001) demonstrated the colonisation and subsequent predation of heterotrophic biofilms by *Hartmannella vermiformis*, a free-living protozoon. Predation has also been demonstrated with *Acanthamoeba* spp. in contact lens storage case biofilms (McLaughlin-Borlace et al., 1998).

The temporal and spatial distribution and biodiversity analysis of protozoa in Veternica Cave (Croatia) yielded 47 protozoan taxa on three types of microhabitats (microcaverns, mud puddles and hygropetric), while the lowest biodiversity was recorded on hygropetric habitats (Kajtezović, 2012).

POTENTIAL USE OF PROTOZOA IN BIOTECHNOLOGY AND BIOMONITORING

Small free-living amoebae (FLA) are ubiquitous protozoans that feed on bacteria, algae, fungi and detritus (Laybourn-Parry, 1992; Foissner and Berger, 1996). They are widely distributed in terrestrial and aquatic habitats and may play a significant role at the base of food webs, but also help to maintain soil fertility by making nutrients available. Moreover, protozoan excretory products include dissolved phosphorus and nitrogen compounds and other organic molecules (Laybourn-Parry, 1992).

Protozoa, especially ciliates, are recognised as excellent bioindicators for water quality in different habitats, and their characteristics give them a distinct advantage over higher organisms (Laybourn-Parry, 1992; Foissner and Berger, 1996). Similarly, cave protozoa can be used as indicator organisms for monitoring water quality, due to their relative ease of culturing, their short life cycle, their cosmopolitan distribution, and their sensitivity and quick response to environmental changes (Guidolin and Coppellotti Krupa, 1999). Moreover, symbiotic associations of protozoa and endosymbiotic methanogens have been reported in groundwater communities whereas, under certain conditions, the protozoa hosting endosymbionts become important members of the microbial community. As they feed on moribund biomass and produce methane, their system-wide conclusions are relevant for engineered bioremediation approaches in general (Holmes et al., 2014).

FUNGI: MYCOTECHNOLOGY

Generally, entomopathogenic fungi are a rich source of natural bioactive compounds. A comprehensive study on 47 typical entomopathogenic fungi tested for their ability to produce antibacterial compounds indicated that the majority of them possessed the ability to produce anti-*Bacillus* and anti-*Staphylococcus* compounds (81% and 64 %, respectively), but only in the presence of insect-derived materials (Lee et al., 2005). Entomopathogenic fungi have been used as a traditional Chinese herbal remedy for more than 1500 years in both China and Japan (Shah and Pell, 2003). Antitumour, antimetastatic, immunomodulatory, antioxidant, anti-inflammatory, insecticidal, antimicrobial, hypolipidaemic, hypoglycaemic, antiaging, neuroprotective, and renoprotective effects are some of the main effects shown by *Cordyceps* fungi (Ng and Wang 2005). The most common example is entomopathogen ascomycetes *Ophiocordyceps sinensis* (Berk.) G.H.Sung, J.M.Sung, Hywel-Jones & Spatafora (2007) (Sung et al., 2007), also known as Himalayan Gold (Gould, 2007). The physiologically active compounds in extracts from Tochukaso [Tochu-Kaso (*jpn.*) *summer grass winter worm* fungus; Dong Chong Xia Cao (*chi*)], product of an *Ophiocordyceps sinensis*-infected insect body from which a fruiting body emerges, promote overall vitality and human longevity by enhancing the immune system (Samuels and Paterson, 1995; Liu et al., 2002). The other potential species is *Cordyceps militaris* (L.) Fr. (1818), which has been successfully grown on artificial media for commercial purpose (Shrestha et al., 2012). Important active compounds have been isolated from *C. militaris*, such as adenosine, cordycepin, polysaccharides, mannitol, superoxide dismutase (SOD), and carotenoids (Guo et al., 2016). *C. militaris* has been recorded in 14 localities in Slovenia (Ogris, 2013) but is not traditionally used as a medicinal mushroom (Gregori, 2013).

Although cave fungi in Dinaric karst are poorly known, several important parasitic troglobionts and troglophilic species on cave coleopterans and troglophilic moths in

Croatian caves have been recorded. The Preliminary Checklist of troglobiotic fungi and fungi occurring both in and outside the cave underground in Croatia (Matočec, 2002), shows two potential genera from the family Laboulbeniaceae with a few species, as possible candidates for producing bioactive compounds: (1) genus *Laboulbenia* Mont. et C. P. Robin including tree species, ectotrophic commensals on carabids: troglophile (TP) *L. flagellata* Peyritsch commensal on TP *Laemostenus cavicola* (Schaum, 1858), troglobitic (TB) *L. shanorii* Bánhegyi commensal on TB *Neotrechus dalmatinus* (L. Miller, 1861) and *N. suturalis* (Schaufuss, 1864) and TB *L. subterranea* Thaxt. commensal on TB *Neotrechus dalmatinus* (L. Miller, 1861); (2) genus *Rhachomyces* Thaxt. with two TB species: *R. hypogaeus* (Thaxt.) Thaxt, commensal on TB *Typhlotrechus bilimekii* (Sturm, 1847) and *R. stipitatus* Thaxt. on TB *Typhlotrechus velebiticus* (Ganglbauer, 1904) and cave carabid *Duvalius reitteri* Miller, 1881. Moreover, TB *Hirsutella* sp., parasite on TB *Leptodirus hochenwartii* Schmidt, 1832 and *Parapropus sericeus* (Schmidt, 1852), was recorded (Gottstein Matočec S. et al., 2002). Impressive bioactivity by hirsutellones A and B, isolated from entomopathogenic *Hirsutella nivea* BCC 2594, was shown against *Mycobacterium tuberculosis* (Isaka et al., 2005; 2006).

Two species of the genus *Cordyceps* have been recorded in the caves of Croatia: (1) TB *Cordyceps riverae* Pacioni, found only in European cave habitats [while geometrid moths *Triphosa dubitata* (Linnaeus, 1758) and *T. sabaudiata* (Duponchel, 1831) are the only certainly known hosts]; and (2) *Cordyceps sphingum* (Tul.) Sacc., widespread in Europe and North America outside of cave habitats. Interestingly, the only record of *C. sphingum* in Croatia is one from Tučepska Vilenjača cave, Mt. Biokovo, Dalmatia (Matočec and Ozimec, 2001).

Analysis of the mycoflora of the nocturnal troglophilic cave cricket *Troglophilus neglectus* Krauss, 1879 cadavers, collected in Kozlov rog tunnel (Slovenia), revealed no fewer than 80 strains of fungi (30 species of 12 genera), belonging to entomopathogenic, opportunistic pathogenic and saprophytic fungi (Gunde-Cimerman N et al., 1998). Among opportunistic pathogenic fungi, a few species of genus *Mucor* could be considered due to its pathogenic and biotoxic activity on humans, cattle, pigs, poultry and insects such as Lepidoptera, Coleoptera and Diptera species (Prevoo et al., 1991; Reiss, 1993).

SUBTERRANEAN FAUNA

Ecological categories are defined as stygoxene and trogloxene species, which spend their complete life cycle in surface environments and are only accidentally found in subterranean habitats; stygobite and troglobite species, which spend their complete life cycle in subterranean environments; and stygophiles and troglophiles, which may have several kinds of life cycles—some populations live in surficial habitats and others in subterranean habitats, or have individual life cycles necessitating the use of both surface and subterranean environments (Gibert and Deharveng, 2002). Europe is a hotspot both of subterranean biodiversity and of research into subterranean biology, both historically and at present (Deharveng et al., 2009). The Dinaric karst in the western Balkan Peninsula is a global hotspot of subterranean biodiversity, with more than 900 aquatic and terrestrial obligate subterranean (troglobiotic) species recorded (Sket, 2005). Troglobiotic beetles are considered the most important contributors to terrestrial subterranean biodiversity in most temperate karst regions, including the Dinaric karst, where they represent about 42% of the terrestrial troglobionts (Sket et al., 2004). Subterranean biodiversity in Europe is actually higher than on other continents as indicated by (Culver and Sket, 2000) while some of the real biodiversity hotspots are western Balkans (northeast Italy, Slovenia, Croatia, Bosnia and Herzegovina, and Serbia) and the Pyrenees (France and Spain) (Sket, 1999).

Associated fauna was evaluated in five Dinaric caves where bacterial communities were analysed in a previous study (Pleše et al., 2016) in order to provide insights into ecological concepts and their connection to microbial ecosystems as a nutrition resource (Table 3).

Fauna is selected according to underground water habitats: hygropetric, endogean streams, endogean underground rivers, exogean underground rivers and underground lakes, defined according to NATURA 2000, Pal. Class. and Croatian National Habitat Classification (CNHC), as reported previously (Pleše et al., 2016). With the notable exception of Veternica, high biodiversity was observed in the Checklist of the most prominent species (taxa) inhabiting the adaptive zone of bacterial communities/biofilm, with a significant number of relict and endemic taxa. Most of the representatives associated with bacterial communities belong to aquatic and/or hygropetric fauna.

Veternica, a complex cave with several independent underground streams but scarce stygobitic fauna, is located in the southwest part of NP Mt Medvednica. With a total length of 7128 m, it is the sixth longest cave in Croatia, and an important bat habitat in NW Croatia, with 14 species of bats recorded (Žvorc et al., 2005). *Mesoniscus graniger* (Frivaldszky, 1865) was found in the hygropetric habitat of Veternica, unique to

Table 3. Checklist of fauna associated with microbial communities from selected Dinaric caves in Croatia and Bosnia and Herzegovina (or caves in the adjacent region*).

Sites	The Fauna Check List	Ecology	Endemity	References
Veternica (Croatia)	*Niphargus stygius likanus* S. Karaman, 1952[+] *Mesoniscus graniger* (Frivaldsky,1865)[++]	SB SB	NE 0	Karaman, 1952 ([+]) Ozimec, 2003 ([+,++]) Bedek, 2007 ([+,++]) Bedek et al., 2011 ([+,++])
Izvor Bistrac (Croatia)	*Eunapius subterraneus* Sket and Velikonja, 1984 *Marifugia cavatica* Absolon and Hrabe, 1930 *Velkovrhia enigmatica* Matjašič and Sket, 1971* *Niphargus croaticus* (Jurinac, 1887) *Niphargus jalzici* G. Karaman, 1989* *Niphargus stygius* Schiødte, 1847 *Niphargus steueri subtypicus* Sket, 1960* *Typhlogammarus mrazeki* (Schäferna, 1906)* *Troglocaris intermedia Babić*, 1922 *Proteus anguinus* Laurenti, 1768*	SB SB SB SB SB SB SB SB/HY SB SB	NE e e NE NE NE NE e NE e	Kovač Konrad et al., 2014 Fišer et al., 2007
Miljacka 4 (Croatia)	*Marifugia cavatica* Absolon and Hrabe, 1930* *Hadziella sketi* Bole, 1961 *Sphaeromides virei mediodalmatina Sket*, 1964 *Monolistra (Microlistra) pretneri spinulosa Sket*, 1965 *Proasellus anophthalmus rhausinus* (Remy, 1941) *Alpioniscus balthasari* (Frankenberger, 1937) *Cyphopleon kratochvili* (Frankenberger, 1939) *Niphargus* sp. *Typhlogammarus mrazeki* (Schäferna, 1906) *Proteus anguinus* Laurenti, 1768*	SB SB SB SB SB TB TB SB SB/HY SB	e ME ME ME e ME e ? e e	Ozimec et al., 2012
Rokina Bezdana (Croatia)	*Eunapius subterraneus* Sket and Velikonja, 1984 *Typhlogammarus mrazeki* (Schäferna, 1906) *Niphargus* sp. *Titanethes dahli* Verhoeff, 1926 *Troglocaris intermedia* Babić, 1922 *Proteus anguinus* Laurenti, 1768	SB SB/HY SB SB SB SB	NE e ? NE NE e	Ozimec, 2009
Vjetrenica (Bosnia and Herzegovina)	*Marifugia cavatica* Absolon and Hrabe, 1930 *Velkovrhia enigmatica* Matjašič and Sket, 1971 *Prostoma hercegovinense* Tarman, 1961 *Lanzaia vjetrenicae* Kuščer, 1933 *Zavalia vjetrenicae* Radoman, 1973 *Typhlogammarus mrazeki* (Schäferna, 1906) *Acanthocyclops troglophilus* (Kiefer, 1932) *Diacyclops karamani* (Kiefer, 1932) *Eucyclops inarmatus* Kiefer, 1932 *Pseudocypridopsis hartmanni* Petkovski et al, 2009 *Troglocaris hercegovinensis* (Babić, 1922) *Troglomysis vjetrenicensis* Stammer, 1936 *Monolistra (Pseudomonolistra hercegoviniensis* Absolon, 1916 *Proasellus hercegovinensis* (S. Karaman , 1933) *Niphargus balcanicus* (Absolon, 1927) *Niphargus boskovici* S. Karaman, 1952 *Niphargus cvijici* S. Karaman, 1950 *Niphargus factor* G. Karaman and Sket, 1991 *Niphargus steueri kolombatovici* S. Karaman, 1950 *Niphargus trullipes* Sket, 1958 *Niphargus vjeternicensis* S. Karaman, 1932 *Niphargus zavalanus* S. Karaman, 1950 *Hadzia fragilis* S. Karaman, 1932 *Cyphonethes herzegowinensis* (Verhoeff, 1900) *Hadesia vasiceki* (J. Müller, 1911) *Nauticiella stygivaga* Moravec and Mlejnek, 2002 *Proteus anguinus* Laurenti, 1768	SB SB SB SB SB SB/HY SB SB SB SB SB SB SB SB SB SB SB SB SB SB SB SB SB SB SB SB SB SB TB SB SB	e e SE SE SE e SE SE SE SE SE SE SE SE SE SE SE SE SE SE SE SE SE SE SE SE SE SE SE SE e	Ozimec and Lučić, 2010

Legend: (TB) Troglobiont; (SB) Stygobiont; (Hy) Hygropetric (habitat type); (NE) North Dinaric endem; (ME) Midle Dinaric endem; (SE) South Dinaric endem; (0) Not Dinaric endem.

running water films or small springs over exposed bedrock. This troglophilic isopod inhabits the area from the Northern Carpathians over the Bihor and the Banat Mountains to the North Dinarids and the Julian Alps (Bedek et al., 2011).

The Izvor Bistrac is part of the Đulin ponor – Medvedica cave system in Ogulin (Croatia), characterised by its unique fauna with a number of endemic species, including the blind cave beetle *Leptodirus hochenwartii* Schmidt, 1832; the cave dwelling tube worm *Marifugia cavatica* Absolon and Hrabe, 1930, the only freshwater serpulid in the world; and the only stygobitic sponge *Eunapius subterraneus* Sket and Velikonja, 1984 as well as olm *Proteus anguinus.*

Miljacka 4 is part of the very peculiar cave system of the Miljacka river sinkhole, which conducted water from Zrmanja River to Krka River in north Dalmatia (Croatia). The cave is located on the river Krka. At least five caves, recognised as Miljacka caves 1-5, belong to the area where the Miljacka rises, with strong indications of connections between them. Fauna includes several snails, crustaceans *Sphaeromides*, *Monolistra* and *Alpioniscus Typhlogammarus mrazeki* but also *M. cavatica* and *P. anguinus* in adjacent region (Jalžić et al., 2007).

Rokina Bezdana is part of a huge underground sinkhole river in the Lika region (Croatia), of which up to 1016 m has so far been explored. This large underground cave with pronounced hydrogeological activity has been only partly investigated, but is recognised as an important cave habitat for stygobiotic taxa as: *Eunapius subterraneus, Typhlogammarus mrazeki, Niphargus* sp., *Titanethes dahli* and *P. anguinus.*

The endogean river of Rokina Bezdana and Izvor Bistrac belong to the Ogulin-Plaški plateau, recognised as refugia where many rare and relict species have survived (Ozimec et al., 2009). These caves are inhabited by sessile organisms such as the only freshwater stigobitic sponge *Eunapius subterraneus* Sket and Velikonja, 1984, a filter feeder endemic for the region, as well as *M. cavatica, Velkovrhia enigmatica,* and mobile as *T. mrazeki* and *P. anguinus* (with the exception of the sponge, all were also found in Vjetrenica).

Vjetrenica, a complex cave with a total length of 7,013.90, is located in the southern part of the Dinaric Karst, on Popovo polje (Eastern Herzegovina). Vjetrenica is one of the world's most prominent hotspots of biodiversity with cave-dwelling fauna, and is the type locality for 38 endemic taxa. The most recent list of the Vjetrenica Cave fauna, dating from December 2009, includes 219 taxa: 37 protists and 182 animals with 101 cave-dwelling taxa (49 troglobitic and 52 sygobitic) (Ozimec and Lučić, 2010). The fauna associated with the bacterial communities in Vjetrenica, characteristic of an endogean river, is composed of sessile invertebrates (*Marifugia cavatica* Absolon and Hrabe, 1930*, Velkovrhia enigmatica* Matjašič and Sket, 1971) and the motile, as a nemertean worm *Prostoma hercegovinense* Tarman, 1961, several aquatic snails, isopod crustaceans of the genera *Cyphonethes, Alpioniscus*, *Proasellus* and *Monolistra,* several species of the amphipod genus *Niphargus,* the *decapod genera Troglocaris,* and *Speleocaris*, the unique myside *Troglomysis vjetrenicensis* Stammer, 1936 as well as *Proteus anguinus*. In addition, the typical hygropetric representatives *Hadesia vasiceki* (J. Müller, 1911), *Typhlogammarus mrazeki* (Schäferna, 1906) and recently *Nauticiella stygivaga* Moravec and Mlejnek, 2002, were found. Despite their diverse phylogenetic origins, hygropetric species share a number of common features that give them a characteristic resemblance (Ribera et al., 2003) allowing them to feed on solvent particles in water film.

Dinaric karst is the habitat of one of the best-known representatives of stygofauna, the cave-dwelling amphibian, olm *Proteus anguinus* found only in the Dinaric karst region of the Balkan Peninsula (Italy, Slovenia, Croatia and Bosnia and Herzegovina), which is a globally vulnerable species (VU). *P. anguinus* was recorded in four of the five investigated caves, which dramatically increases the urgency of its habitat protection.

BIOPOTENTIAL OF CAVE-DWELLING FAUNA

Cave-dwelling invertebrates also represent promising sources of bioactive compounds. The saliva of all blood-sucking arthropods contains different bioactive lipids and proteins with anti-clotting, anti-platelet, vasodilatory, anti-inflammatory and immunomodulatory features. Sialotranscriptome (transcriptome of salivary glands) of several ixodid and argasid ticks show a broad spectrum of antimicrobial proteins (Francischetti et al., 2009). Recent progress in transcriptome research has revealed that hard ticks have hundreds of different proteins expressed in their salivary glands, the majority of which have no known function, and include many novel protein families (e.g., their primary structure is unique to ticks).

Nine genera with 22 species have been recorded in the subterranean acarine fauna of Croatia. *Eschatocephalus vespertilionis* (Koch, 1844) is a parasite on bats *Rhinolophus* spp.*,* and a guanophilic species *Macrocheles penicilliger* (Berlese, 1904) was found in the Kordun area (Ozimec, 2002).

ECOSYSTEM SERVICES PROVIDED BY HYPOGEAN MICROBIAL BIOFILMS

Ecosystem services are the benefits that humans obtain from ecosystems that enhance their well-being (Kunz et al., 2011). The ecological benefits of microbial biofilms and their impact on hypogean ecosystems have been widely applied for bioremediation, biotechnology, bioconservation and

biomonitoring. The vulnerability of hypogean ecosystems is the outcome of their inhabitants' adaptation to constant conditions in the natural environment, and consequent complex interspecies interactions among the members of the three domains of life. According to the Millennium Ecosystem Assessment project (2005), biodiversity is a necessary underlying component of ecological goods and services. Biodiversity supports ecological goods and services such as biological control and genetic resources (Constanza et al., 1997). For instance, bats provide many ecosystem services; humans derive direct benefits from bats as food, guano for fertilizer, and through contributions to medicine and culture (Kunz et al., 2011).

CONSERVATION OF SUBTERRANEAN FAUNA

Subterranean ecosystems are generally oligotrophic habitats, receiving poor supplies of degradable organic matter from the surface, but anthropogenic impacts on underground ecosystems (intensive tourism and recreational caving) cause major alterations to the whole subterranean environment. Caves open to tourist traffic offer an opportunity to study the impacts of organic matter amendments in the form of human and rodent hair and dander, clothing lint, material from rodent activity (nesting materials and faeces), and algal growth in and around artificial lighting (Chelius et al., 2009). In spite of their high levels of biodiversity, caves and other subterranean habitats are still rarely considered in conservation and management plans (Medellin et al., 2017).

Water resources in the karst are extremely sensitive to all kinds of pollution, especially during dry seasons. Surface streams may often disappear completely, and underground waters may dwindle, with a consequent higher concentration of pollutants. The impact of climate change to these fragile ecosystems through the reduction of water in aquifers and lack of seasonal flooding means that cave temperature is generally strictly connected with the external climate (Baldino, 2004).

Fauna is extremely sensitive throughout, since subterranean species are ecologically specialised and are mainly endemics tending to extinction. In some areas, cave fauna may be an important indicator of the health of the habitat (Sket, 2017). Increased contamination of the environment with toxic pollutants has paved the way for efficient strategies which can be implemented for environmental restoration. Bioremediation is a growing technology with advanced potential for cleaning pollutants, while biofilm formed by various microorganisms potentially provides a suitable microenvironment for efficient bioremediation processes (Mangwani et al., 2016).

A NEW APPROACH IN MICROBIAL BIOFILM INVESTIGATION

The application of molecular techniques to the study of microbial diversity (Olsen et al., 1986; Pace et al., 1986) has revealed the existence of an incredible variety of genotypes and species in all known habitats. However, molecular ecology has moved beyond the use of a relatively small number of markers, towards a sophisticated estimation of gene expression at the whole genome level. Thus the use of microarrays and RNAseq (i.e. transcriptomics) makes it possible to capture the molecular response to environmental challenges. Transcriptome analysis helps in the elucidation of possible adaptations to ecosystems with a strong anthropogenic impact, given that variation in gene expression affected by environmental stimuli (Banskar et al., 2016) suggests that transcription may be affected by even small changes in environment (De Mandal et al., 2017).

Next generation sequencing platforms are rapidly changing the approach to characterising microbial communities from various sources and different habitats. Metagenomic studies, using these sequencing technologies, have contributed enormously to our understanding of the structure and functioning of microbial biofilms, as well as of subterranean ecosystems as a complex entity. In this regard, using Ion Torrent sequencing, it was observed that two guano samples had more than 98% sequences representing Proteobacteria (Alvarez et al., 2015), which is the most abundant phyla in the composition of microbial biofilms. Analysis of the taxonomic composition and presumed functional diversity of cave sediment metagenomes, using paired end Illumina sequencing, yielded a representation of gene sets involved in amino acid metabolism (20.9%) and carbohydrate metabolism (20.4%) in the KEGG pathways. The identification of genes responsible for carbon degradation, carbon fixation, methane metabolism, nitrification, nitrate reduction and ammonia assimilation (Richards et al., 2009) will undoubtedly enhance our understanding of the structure, regulation and evolution of subterranean ecosystem and their inhabiting biota.

Community-level physiological profiling (CLPP) using BIOLOG® EcoPlates™ has recently become a popular method for characterizing and comparing the functional diversity, functional potential, and metabolic activity of heterotrophic microbial communities. The method was described in 1991 by Garland and Mills (Button et al., 2016) and created specifically for community analysis and microbial ecological studies. Its application has expanded to many fields such as ecotoxicology, agronomy, and the monitoring and profiling of microbial communities in various wastewater treatment

systems, including constructed wetlands for water pollution control (Garland et al., 1991).

CONCLUSION

A deep understanding of vulnerable subterranean ecosystems and the unique organisms that inhabit them could shed light on many new aspects in the phylogeny and evolution of microorganisms as well as animal taxa, adjacent to their energy suppliers in microbial communities and biofilms. Bacteria present in subterranean ecosystem often survive by modifying their metabolic pathway, which doubtless affects the other cave-dwelling organisms and the entire ecosystem inside the cave. Understanding their excellent survival strategies will help to discover some new (bio)active molecules and develop an environmentally-friendly technique for the removal of toxic substances. Only an interdisciplinary approach in microbial ecology will make it possible to enhance the protection of subterranean habitats and biodiversity conservation.

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ABBREVIATIONS:

CLPP	Community-level physiological profiling
EPS	Extracellular Polymeric Substances
ERW	Explosive remnants of war
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTBE	Methyl tertiary butyl ether
PUFA	Polyunsaturated fatty acids
SOD	Superoxide dismutase
VRE	Vancomycin resistant *Enterobacter faecium*
TB	Troglobiont
SB	Stygobiont
Hy	Hygropetric (habitate type)
NE:	North Dinaric endem
ME:	Midle Dinaric endem
SE:	South Dinaric endem
E:	Dinaric endem
0:	Not Dinaric endem

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SAŽETAK

Kratki pregled živog svijeta koji naseljava podzemna staništa Dinarida, uključujući cijanobakterije i alge te bakterije, protozoa i špiljsku faunu, usmjeren je na moguću primjenu spomenutih vrsta u biotehnologiji, bioremedijaciji / biostimulaciji i biomonitoringu. U članku se razmatra dinamika i struktura biofilmova na primjeru nekoliko dinarskih špilja u kršu, inter- i intraspecijske interakcije unutar biofilmova te novi pristupi i metode u istraživanju mikrobnog biofilma.

Sustavno istraživanje podzemnih ekosustava i njihovog jedinstvenog živog svijeta, povezanog složenim interakcijama, doprinosi razumijevanju njihove evolucije, biokemije i fiziologije te otvara mnoge nove aspekte korištenja određenih taksa važnih za proizvodnju novih bioaktivnih molekula. Također, multidiscplinarnost u istraživanju s fokusom na moguće primjene mikrobnih biofilmova doprinijet će učinkovitijem provođenju planova upravljanja špiljama, osobito turističkih otvorenih špilja uz obavezni, stručno vođeni monitoring, s ciljem zaštite staništa i očuvanje biološke raznolikosti.

Ključne riječi: mikrobni biofilm, podzemni habitati, biotehnologija, bioremedijacija, Dinarski krš