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Cultivable microbiota of *Proteus anguinus* from underground habitats and animals accidently washed to the surface in Croatia

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ABSTRACT

Proteus anguinus (olm) is an amphibian endemic to underground cave systems of the Dinaric karst of Central Europe, adapted to a life in complete darkness. Recent studies have demonstrated the global decline of amphibians due to poorly understood microbiological diseases, emphasizing just how little is known about the natural microflora of amphibians. Not much is known about the olm's microbiota in their cave habitats, and nothing is known about the microbiology of animals washed to the surface. This observational study describes the microbiological analysis of the skin, cloaca and oral cavity of six olms collected from their cave habitats, and 16 collected after being washed out by flows from two springs, to learn more about the olm's normal microbiota and possible changes after contact with the different environmental conditions on the surface. Standard microbiological procedures, MALDI-TOF and Real-Time PCR were used for microbiological species identification. All animals tested negative for *Ranavirus*, *Batrachochytrium dendrobatidis*, *Batrachochytrium salamandrivorans* and *Chlamydia* spp. The most abundant fungi isolated were *Penicillium* spp. and *Cladosporium* spp. The washed out animals showed a higher diversity of bacterial flora than those from cave habitats, with *Acinetobacter johnsonii, Aeromonas hydrophila, Bacillus* spp. and *Janthinobacterium lividum* as the most frequently identified isolates.

Key words: *Proteidae*; stygobiont; endemic; endangered; protected; *Janthinobacterium lividum*

Introduction

Proteus anguinus (olm) is a neotenic cave salamander inhabiting the deep underground cave systems of the Dinaric karst of northern Italy, southern Slovenia and south-western Croatia and Bosnia and Herzegovina (SKET, 1997). Along with the European cave fish (*Barbatula* sp.) (BEHRMANN-GODEL et al., 2017), the *olm* is the only fully adapted cave-dwelling (stygobiont)

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chordate in Europe. This species is dependent on clean water, and is therefore very susceptible to human influences on underground habitats. The olm is widely protected, and is listed as 19th on the EDGE list of the top 100 evolutionary distinct and globally threatened amphibians (SAFI et al., 2013).

The normal temperature of the olm's habitat in the underground karst is around 8-12°C (LUNGHI et al., 2017). During periods of excessive rain or snow melt, animals may be washed out of their underground habitats. On the surface, these olms are exposed to the unfavourable conditions of temperatures much higher than in their cave habitats, light, and various vectors such as other amphibians and migratory birds which could be potential sources of infectious diseases, such as chytrid fungi, *Ranavirus* and others (CAMPBELL et al., 2018; ALLAIN and DUFFUS, 2019; FISHER and GARNER, 2020). Therefore, it is important to determine how their defence mechanisms and skin microbiota composition are able to deal with opportunistic microorganisms/real pathogens under unfavourable conditions.

The general knowledge of the olm's ecology and biology is limited due to their elusive way of life. Therefore, the Croatian Herpetological Society, in association with its partners, initiated a project of field research and conservation, and *ex situ* research, where animals were kept in a specially designed "cold room" to ensure their complete requirements regarding air and water temperature, water quality, food, aeration and shelter (LUKAČ et al., 2013). The majority of the animals kept in the "cold room" were those washed out of their cave habitats at certain localities in Croatia due to heavy rains or snow melt. Additionally, some olms were taken from their cave habitats in order to compare the behaviour and microbiological findings of these animals with washed out animals. The aim of this observational study was to gain more insight into the olm's normal aerobic microbial flora in the oral cavity, cloaca and skin, and to investigate the type(s) of opportunistic or pathogenic cultivable microorganisms that may affect animals being washed from their cave habitats and exposed to surface microorganisms under unfavourable environmental conditions. For that purpose, swabs from the skin, oral cavity and cloaca were taken from 16 washed out animals and six animals in their cave habitats at five localities in Croatia. The water from those localities was also tested, to distinguish between the aquatic microorganisms and microorganisms from the animals. Standard microbiological procedures and the MALDI-TOF method for bacteria, and Real-Time PCR for *Chlamydia* sp., *Batrachochytrium dendrobatidis*, *B. salamandrivorans* and Ranavirus identification were used.

Materials and methods

Animals and localities. The animals used in this study were collected as part of a large scale conservation project entitled "The Olm (*Proteus anguinus*) in Croatia – Conservation Research Project" implemented at five Croatian localities, under the permits issued by the Ministry of Environment and Nature Protection (UO/I-612-07/11-33/0075, 532-08-01-01/1-11-02; UP/I-612-07/15-48/119, 517-07-1-1-1-15-4). All applicable institutional and/or national guidelines for the care and use of animals were followed. In total, 22 animals were analyzed, divided into the test group (washed out [WO] animals) and the control group (animals taken from their cave habitats, CH). The WO group consisted of 16 individuals washed out from cave habitats due to heavy rains/melting snow, and captured from two springs formed by occasional flows: 14 from a spring known as Vedrine (Dalmatia) and two from a spring known as Istria Fontana (Istria) (Figure 1). The olms were collected manually by local people involved in the project, trained to avoid contamination, and placed in fish transport bags (JBL, Germany) filled with water and transferred immediately to Zagreb Zoo at 4-8°C. The CH group consisted of six animals (two from each locality) collected from their cave habitats in the Pincinova Jama (Istria), Rupecica (Gorski Kotar) and Markarova (Lika) caves (Figure 1). The animals were collected by professional cave divers and placed individually into sterile plastic bags. All the animals in the study were collected at five localities from all three distinct geographical regions (Istria, Gorski Kotar/Lika, and Dalmatia) where olms are found in Croatia (Fig 1). A specially

designed cold room was prepared at Zagreb Zoo for keeping the rescued and collected olms, as described elsewhere (LUKAČ et al., 2013). Each animal was housed individually in a simple 60 liter aquarium. All aquariums and equipment were cleaned with disinfectant (F10 SC, South Africa) 24 hours prior to the animals' arrival, and control swabs were taken to check for the presence of microorganisms, using standard microbiological procedures (BROWN, 2005).

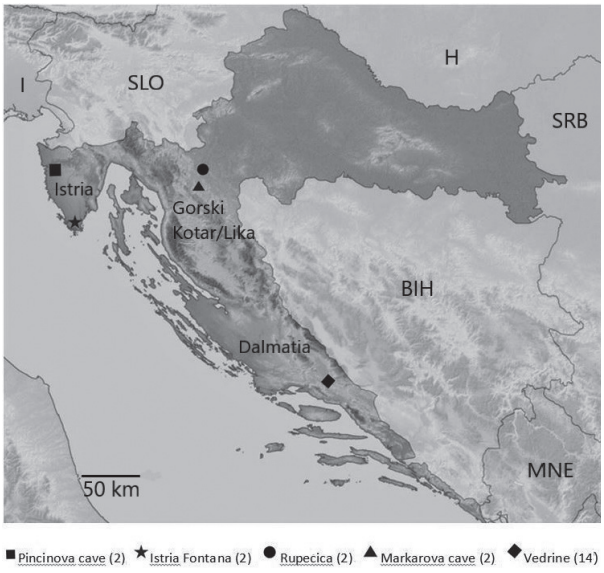

Fig. 1. Topographic map of five sampling localities in three distinct geographical regions (Istria, Gorski Kotar/Lika, Dalmatia) where olms are found in Croatia and number of animals ( ) captured at each locality: Pincinova, Rupecica and Makarova caves, and Istria Fontana and Vedrine springs.

Sampling methods. Upon arrival at Zagreb Zoo, all animals were examined for any clinical signs such as wounds, loss of extremities, and the overall appearance of the skin and gills, and were manually restrained for sampling. For standard microbiology tests, sterile swabs, with transport media (Amies transport swabs, Aptaca, Italy), were taken from the oral cavity, cloaca and skin. Then samples for detection of *Batrachochytrium dendrobatidis*, *Batrachochytrium salamandrivorans* and *Ranavirus* were taken by wiping the skin of the entire body surface with sterile swabs without transport media (Copan plain swab, Italy). Oral cavity, cloacal and skin swabs for detection of *Chlamydia* spp. were taken thereafter with sterile swabs without transport media (Aptaca dry swab). All samplings were carried out before the first feeding, to avoid potential contamination by live animal food. The water from all localities was sampled the same time as the animals were captured, for standard microbiological tests using sterile test tubes (Aptaca, 50 ml conical test tubes), and kept at 4-8°C until processing.

Detection of aerobic bacteria and fungi. Standard microbiological methods as described by BROWN (2005) were used. The oral cavity, cloacal and skin swabs and water from the localities were plated on the Nutrient agar (Difco Nutrient Agar, Becton, Dickinson and Company, USA), Brilliant green agar (BGA - modified, Oxoid, England) and Sabouraud dextrose agar (Oxoid, England). Plates were incubated under aerobic conditions at two temperatures: 8–10°C and 22–24°C. All plates were checked on a daily basis for up to five days to record changes in microbial growth. Isolated bacteria were further identified microscopically to determine their morphology, by catalase and oxidase reactions described by BROWN (2005) and by Gram staining. Further bacterial identification was done by the Analytical profile index (API) (API20 E, API 20 NE, bioMérieux S.A., France). Four bacterial colonies not determined to the genus level (two from cloacal samples of one CH and one WO animal, and two from spring water samples) by the above methods were identified using the Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Those samples were analysed at the Rugjer Bošković Institute, Zagreb, Croatia, using a Microflex LT instrument (Bruker Daltonic GmbH, Germany) as previously described (ALISPAHIĆ et al., 2010). Bacterial isolates were identified based on the reference database with the log(score) values between 2.1 and 2.3 (labelled in respective tables in the Results section). Fungal identification was based on morphological appearance and microscopic characteristics using lactophenol staining (Lactophenol blue solution, Sigma-Aldrich, France).

Real-time PCR for detection of Batrachochytrium dendrobatidis (Bd), Batrachochytrium salamandrivorans

(Bsal), Chlamydia spp. and Ranavirus. DNA was extracted from the skin swabs of each animal for Bd and Bsal detection, and from the oral cavity, cloacal and skin swabs of each animal for *Chlamydia* spp. and *Ranavirus* detection using a QIAmp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions.

For Bd detection the protocol of BOYLE et al. 2004 was used. PCR reactions were run in a 25 µl volume containing 2.5 µl of each *B. dendrobatidis* specific primer, 12.5 µl Premix Ex Taq PCR Mix (Takara, Japan), 2.5 µl specific *B. dendrobatidis* probe, and 5 µl template. All samples were run in duplicate with the appropriate negative (ultrapure water) and positive controls (JEL423). The cycling profile consisted of an initial denaturation step at 95°C for 10 min, followed by melting (43 cycles, 95°C, 15 s) and annealing/extension (60°C, 60 s), when fluorescence was recorded.

The protocol of BLOOI et al. 2013 was followed for Bsal detection. PCR reactions were run in a 25 µl volume containing 0.25 µl of each *B. salamandrivorans* specific primer, 12.5 µl Premix Ex Taq PCR Mix (Takara, Japan), 6.75 µl ultrapure water with BSA, 0.25 µl specific *B. salamandrivorans* probe, and 5 µl template. All samples were run in duplicate with the appropriate negative (ultrapure water) and positive controls (AMFP13/01). The cycling profile consisted of an initial denaturation step at 95°C for 10 min, followed by melting (43 cycles, 95°C, 15 s) and annealing/extension (60°C, 60 s), when fluorescence was recorded.

Chlamydia sp. was detected by the protocol of EHRICHT et al. 2006. PCR reactions were run in a 20 µl volume containing 0.5 µl of each *Chlamydia* sp. specific primer, 10 µl Premix Ex Taq PCR Mix (Takara, Japan), 1.8 µl ultrapure water, 2 µl specific *Chlamydia* sp. probe, and 5 µl template. Each sample was run in duplicate, with positive (*C. psittaci* strain) and negative (ultrapure water) controls, also run in duplicate. The cycling profile consisted of an initial denaturation step at 95°C for 10 min, followed by melting (50 cycles, 95°C, 10 min) and annealing/extension (60°C, 60 s), when fluorescence was recorded.

For *Ranavirus* detection, the protocol of PICCO et al. 2007 was followed. PCR reactions were run in a 20 µl volume containing 0.5 µl of each *Ranavirus* specific primer, 10 µl Premix Ex Taq (Probe qPCR) (Takara, Japan), 1.8 µl ultrapure water, and 2 µl specific *Ranavirus* probe per sample. All samples were run in duplicate with the appropriate negative (ultrapure water) and positive controls (FV3 strain). The cycling profile consisted of the following conditions: 95°C 10 min, 50 replication cycles, denaturation for 15 s at 95ºC, and primer annealing at 60ºC for 60 s, when fluorescence was recorded. All samples were analyzed with an Mx3005P (Stratagene, USA) instrument with the TaqMan system for replicated segment identification.

Statistics. As the data did not show normal distribution, the number of isolates per sample and per animal was analysed with the Mann-Whitney U test using the MedCalc for Windows package, Statistics for Biomedical Research, Version 9 (MedCalc Software Ltd, Belgium). P values <0.05 were considered statistically significant.

Results

This study presents the results of viral and aerobic bacterial, and fungal flora analysis performed by standard microbiology and/or Real-Time PCR, of the oral cavity, skin and cloaca of 22 olms (*Proteus anguinus*). Sixteen animals, collected at two localities, had been washed out (WO group), while six more were captured from three cave habitats as a control (CH) group (Figure 1).

At 22–24°C incubation temperature (IT), bacteria were isolated from 100.0% WO and 66.7% CH animals (Table 2). Significantly more isolates were recovered per sample and per animal in the WO than the CH group (P=0.010 and P=0.047, respectively; Mann-Whitney U test). In total, 14 and seven bacterial species were identified in the WO and CH groups, respectively (Table 2). Three of the seven species detected in the CH group (*Aeromonas bestiarum, Micrococcus luteus, Pseudomonas gessardii*) were not present in the WO group (Table 2). The most common species, isolated from ≥ 50% animals were: *Acinetobacter johnsonii, Aeromonas*

hydrophila, Bacilllus spp., *Buttiauxella agrestis* and *Janthinobacterium lividum* in the WO group, and *Bacillus* spp. and *Pseudomonas fluorescens* in the CH group (Table 2).

At 8–10°C IT, bacteria were isolated from 2 of 16 (12.5%) WO animals but from none of the CH animals (Table 1). Three bacterial species were detected in the WO group: *Bacillus* spp. and *Escherichia coli* with two isolates each, and *Pseudomonas fluorescens* with one isolate (Table 2).

Table 1. Number of olms with positive bacterial and fungal finding(s) after incubation at two temperatures

Animals	Oral cavity	Cloaca	Skin	Total
Bacteria, 22–24°C				
All animals	14/22[a]	19/22	10/22	20/22
Cave habitat	3/6	4/6	2/6	4/6
Washed out	11/16	15/16	11/16	16/16
Bacteria, 8–10°C				
All animals	2/22	1/22	0/22	2/22
Cave habitat	0/6	0/6	0/6	0/6
Washed out	2/15	1/15	0/15	2/15
Fungi, 22–24°C				
All animals	3/22	3/22	2/22	3/22
Cave habitat	0/6	0/6	0/6	0/6
Washed out	3/16	3/16	2/16	3/16
Fungi, 8–10°C				
All animals	0/22	0/22	0/22	0/22

[a]Positive/total number of animals

Table 2. Bacterial species isolated and the number of isolates identified in oral cavity, cloacal and skin samples of cave habitat and washed out animals incubated at two temperatures

All sites		Oral cavity		Cloaca		Skin	
Cave habitat, 22–24°C							
Aeromonas bestiarum[b]	1[a]	*Bacillus* spp.	2	*Aeromonas bestiarum*	1	*Bacillus cereus*	2
Bacillus cereus	2	*Pseudomonas fluorescens*	1	*Bacillus* spp.	2	*Bacillus* spp.	2
Bacillus spp.	6			*Enterobacter* spp.	2		
Enterobacter spp.	2			*Micrococcus luteus*	2		
Micrococcus luteus	2			*Pseudomonas fluorescens*	2		
Pseudomonas fluorescens	3			*Pseudomonas gessardii*	1		
Pseudomonas gessardii	1						
Total	17		3		10		4
Washed out, 22–24°C							

Table 2. Bacterial species isolated and the number of isolates identified in oral cavity, cloacal and skin samples of cave habitat and washed out animals incubated at two temperatures (continued)

All sites		Oral cavity		Cloaca		Skin	
Acinetobacter johnsonii	13	*Acinetobacter johnsonii*	3	*Acinetobacter johnsonii*	6	*Acinetobacter*	4
Aeromonas hydrophila	14	*Aeromonas hydrophila*	3	*Aeromonas hydrophila*	5	*johnsonii*	6
Bacillus cereus	5	*Bacillus cereus*	2	*Bacillus cereus*	2	*Aeromonas hydrophila*	1
Bacillus spp.	13	*Bacillus* spp.	3	*Bacillus* spp.	6	*Bacillus cereus*	4
Buttiauxella agrestis	12	*Buttiauxella agrestis*	3	*Buttiauxella agrestis*	5	*Bacillus* spp.	4
Chryseobacterium indologenes	6	*C. indologenes*	2	*C. indologenes*	2	*Buttiauxella agrestis*	2
						C. indologenes	
	1	*Enterobacter cloacae*	1	*Enterobacter* spp.	*2*		*2*
Enterobacter cloacae	3	*Enterobacter* spp.	1	*Escherichia coli*	4	*Escherichia coli*	4
Enterobacter spp.	7	*Escherichia coli*	1	*Janthinobacterium*	5	*Janthinobacterium*	1
Escherichia coli	13	*Janthinobacterium*	4	*lividum*	3	*lividum*	2
Janthinobacterium	1	*lividum*	4	*Pseudomonas fluorescens*	4	*Micrococcus roseus*	
lividum	9	*Pseudomonas fluorescens*	1	*P. frederiksbergensi*[b]		*Pseudomonas*	
Micrococcus roseus	4	*Staphylococcus* spp.				*fluorescens*	
Pseudomonas fluorescens	1						
Pseudomonas frederiksbergensi							
Staphylococcus spp.							
Total	102		28		44		30
Cave habitat, 8–10°C							
NBI		NBI		NBI		NBI	
Washed out, 8–10°C							
Bacillus spp.	2	*Bacillus* spp.	1	*Bacillus* spp.	1		0
Escherichia coli	2	*Escherichia coli*	1	*Escherichia* coli	1		
Pseudomonas fluorescens	1	*Pseudomonas fluorescens*	1				
Total	5		3		2		0

[a]Number of isolates; [b]One isolate identified by MALDI-TOF; NBI = None of bacteria were isolated.

At 22–24°C IT, nine bacterial species were identified in the water from the five localities. The highest number of species was found at the Vedrine locality (Table 3). All bacteria identified in the water, with the exception of *Microbacterium maritypicum*, were also present in the animals included in the study. There were no positive findings at 8–10°C IT at any locality.

All the bacterial species identified in both CH animals and the underground water (Pincinova, Makarova and Rupecica caves) belonged to three phyla, most of them to Proteobacteria, followed by Firmicutes and Actinobacteria (Table 4).

At 22–24°C IT, fungal species were isolated from the WO group from the Vedrine locality. Seven species were identified, *Penicillium* spp. being the most common isolate (4/11; 36.4% isolates) present in 4 of 16 (25%) animals (Table 5). At 8–10°C IT, no fungal isolates were identified from either group (Table 5).

Two fungal species were identified in the water from all five localities at 22–24°C IT: one isolate of *Penicillium* spp. and one isolate of *Aspergillus* spp. No fungal species were isolated at 8–10°C IT (Table 5).

All sampled animals were negative for *Chlamydia* spp., *B. dendrobatidis, B. salamandrivorans* and *Ranavirus*. The tested samples did not show any ct values, and did not generate any peak in the melting curves.

Control swabs of aquariums and equipment taken after disinfection revealed negative findings of bacteria and fungi.

Table 3. Bacterial species isolated and the number of isolates identified in the water from five localities incubated at two temperatures

Locality	Bacterial species	Number of isolates
22–24°C		
All localities	*Acinetobacter johnsonii*	1
	Aeromonas hydrophila	2
	Bacillus cereus	2
	Bacillus spp.	3
	Buttiauxella agrestis	1
	Janthinobacterium lividum	3
	Microbacterium maritypicum	1
	Micrococcus luteus	1
	Staphylococcus spp.	1
Istria Fontana spring	*Janthinobacterium lividum* *Bacillus* spp. *Staphylococcus* spp.	
Makarova cave	*Janthinobacterium lividum* *Bacillus cereus* *Micrococcus luteus*	
Pincinova cave	*Aeromonas hydrophila* *Bacillus* spp.	
Rupecica cave	*Bacillus cereus* *Bacillus* spp.	
Vedrine spring	*Janthinobacterium lividum*[a] *Aeromonas hydrophila* *Acinetobacter johnsonii* *Buttiauxella agrestis* *Microbacterium maritypicum*[a]	
8–10°C		
All localities: none of bacteria were isolated		

[a]Identified by MALDI-TOF

Table 4. Bacterial phyla identified in cave habitat olms and in cave and spring water

Species	Number of species	Phylum
Cave habitat olms		
Aeromonas bestiarum *Enterobacter* spp. *Pseudomonas fluorescens* *Pseudomonas gessardii*	4	Proteobacteria
Bacillus cereus *Bacillus* spp.	2	Firmicutes
Micrococcus luteus	1	Actinobacteria
Cave water		
Aeromonas hydrophila *Janthinobacterium lividum*[a]	2	Proteobacteria
Bacillus cereus *Bacillus spp.*	2	Firmicutes
Micrococcus luteus[a]	1	Actinobacteria

Table 4. Bacterial phyla identified in cave habitat olms and in cave and spring water (continued)

Species	Number of species	Phylum
Spring water		
Acinetobacter johnsonii Aeromonas hydrophila Buttiauxella agrestis Janthinobacterium lividum	4	Proteobacteria
Bacillus spp. *Staphylococcus* spp.	2	Firmicutes
Microbacterium maritypicum	1	Actinobacteria

[a]Identified by MALDI-TOF

Discussion

To learn more about the microbiology of olms, 22 animals from five different Croatian localities were included in this study. Skin, cloacal and oral cavity swabs of 16 washed out (WO) animals and six animals from three different cave habitats (CH) were examined. A higher number of washed animals was examined because these animals could not be returned to their underground habitats. All the CH animals were captured at the localities with the highest population abundance, respecting the fact that olms are rare and endangered animals. Using methods for detection of cultivable bacteria, the aim of the study was to gather information potentially useful primarily to conservators and others included in investigations and protection of olms, who work in the field and need rapid information on the microorganisms that are causing infection or disease in a given situation and environment.

Bacteria were recovered from 100.0% of WO and 66.7% of CH animals, and twice as many bacterial species were identified in WO as in CH animals (14 vs. 7 species, respectively), although these differences did not reach statistical significance. However, the total number of isolates per sample and per animal was significantly higher in the former group at higher IT. This was likely due to the exposure of WO animals to bacteria from the field, since no isolates were identified in CH animals at lower IT, closer to the temperature in their underground habitat. Still, a few isolates were found at the lower IT in the WO group, which may reflect the pollution of underground water with bacteria from landfills.

Table 5. Fungal species isolated and the number of isolates identified in the oral cavity, cloacal and skin samples of cave habitat and washed out animals, as well as in water samples from five localities incubated at two temperatures

Animals, 22–24°C							
Cave habitat							
None of bacteria were isolated							
Washed out							
All sites		Oral cavity		Cloaca		Skin	
Aspergillus flavus *Aspergillus fumigatus* *Aspergillus* spp. *Cladosporium* spp. *Fusarium roseum* *Penicillium* spp. *Rhodotorula rubra*	1[a] 1 1 2 1 4 1	*Aspergillus* spp. *Cladosporium* spp. *Penicillium* spp.	1 1 2	*Aspergillus flavus* *Penicillium* spp. *Rhodotorula rubra*	1 1 1	*Aspergillus fumigatus* *Cladosporium* spp. *Fusarium roseum* *Penicillium* spp.	1 1 1 1
Total	11		4		3		4
Animals, cave habitat & washed out, 8–10°C							
None of bacteria were isolated							
Water from all localities, 22–24°C							
Penicillium sp., 1 isolate *Aspergillus* sp., 1 isolate							
Water from all localities, 8–10 °C							
None of bacteria were isolated							

[a]Number of isolates

All bacterial species identified in the CH animals have already been detected in amphibians (HUBBARD, 1981; HIRD et al., 1983; LOUDON et al., 2014; CHAI et al., 2018; MULETZ-WOLZ et al., 2018), most of them (4/7, 57%) belonging to Proteobacteria. This is in line with the recently published results of KOSTANJŠEK et al. (2019), who reported that the three most abundant bacterial families identified in seven olms from all five distinct genetic lineages from Slovenia also belonged to the same phylum. Using the 16S rRNA metagenomics approach, those authors identified more bacterial species in skin samples from their animals than we did by standard microbiological methods. The composition of microbiota might also be influenced by different genetic lineages of olms (VOROS et al., 2019) and this should probably be considered in future microbiological studies.

In general, all the bacteria and fungi isolated in the whole study group were soil microorganisms, bioindicators and opportunistic microbes (TALWAR and CHATLI, 2018). *Bacillus* spp., the most abundant isolate in the CH, and one of the five most represented isolates in the WO group, is ubiquitous in nature. Some species, particularly *B. cereus*, are occasional pathogens of humans and livestock (TURNBULL, 1996), but there is no information on their potential pathogenicity in amphibians. The remaining four species most represented, *Aeromonas hydrophila, Acinetobacter johnsonii, Buttiauxella agrestis* and *Janthinobacterium lividum*, were only identified in the WO group. *Aeromonas hydrophila* can be a causative agent of bacterial dermatosepticemia in frogs (DENSMORE and GREEN, 2007; MAUEL et al., 2002), while *A. johnsonii* and *B. agrestis* have not been described as pathogens in amphibians. Some bacterial symbionts on the skin of amphibians were demonstrated to have protective properties by acting against certain microbes including chytrid fungi (WALKE et al., 2015; MULETZ-WOLZ et al., 2017; ZHIMIN et al., 2020). *Janthinobacterium lividum*, one of the four species most represented in the WO animals was demonstrated to produce antifungal metabolites, such as violacein, at concentrations lethal to *B. dendrobatidis* (BRUCKER et al., 2008). All the tested animals were negative for the presence of *B. dendrobatidis* and *B. salamandrivorans*. This finding is consistent with the results of Slovenian researchers who also did not detect *B. dendrobatidis* in any of the animals tested (KOSTANJŠEK et al., 2019). One possible explanation could be the composition of the skin bacterial community that includes bacteria already described to have antifungal properties, such as *J. lividum* and *Pseudomonas* spp. (BECKER et al., 2009; HARRIS et al., 2009; LOUDON et al., 2014). Still, the low prevalence of chytrid infection, if present, could be missed due to the limited number of animals included in our study.

All the species identified in cave and spring water, with the exception of *Microbacterium maritypicum*, were detected in olms as well. Most of them belonged to Proteobacteria, followed by Firmicutes and Actinobacteria, similar to the same level of abundance as in olms. KOSTANJŠEK et al. (2019) also reported two Proteobacteria families as the most abundant in cave water from five Slovenian localities.

Fungi were not isolated from CH animals, likely owing to the animal's protective mechanisms discussed above. In addition, the relatively constant low temperature of animals and their habitat make olms unpleasant hosts for fungi. Fungal species were isolated from WO animals, but only at the higher IT. This suggests the colonization of animals by external microbes after being washed out. Since fungi were recovered from 19% of the WO animals, and not from all of them, the extent of fungal colonization probably depends on the length of time the animals spent out in the field, and on the presence/activity of protective bacteria discussed above.

All the animals tested negative for *Ranavirus*. The results of testing probably depended on the time the WO animals had spent in the field. The relatively low number of study animals could also be of relevance.

In conclusion, this study demonstrated a very low number of bacterial and no fungal organisms present on the olms and in the water taken from five localities, cultivable at an incubation temperature close to that in caves of the Croatian karst. However, a large number of bacterial and a small

number of fungal isolates were identified in washed out animals and from the water from five localities, at IT approaching the outside environmental temperature, indicating the colonization of those animals and the pollution of underground water with external microbes. To our knowledge, this is the first report on the cultivable microbial community present on olms washed to the surface. The results will hopefully support further olm conservation attempts, particularly by people working in the field.

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SAŽETAK

Proteus anguinus (čovječja ribica) endemski je vodozemac podzemnih voda Dinarskog krša središnje Europe, prilagođen životu u potpunoj tami. Novija su istraživanja pokazala globalno smanjenje populacije vodozemaca, prvenstveno zbog nedovoljno istraženih bolesti, ističući manjkavo poznavanje mikroflore vodozemaca. Ne zna se mnogo ni o mikroflori čovječjih ribica s njihovih prirodnih staniša, a podataka o mikrobiologiji jedinki poplavama izbačenih na površinu uopće nema. Ovim opservacijskim istraživanjem dobiveni su rezultati mikrobiološke analize kože, kloake i usne šupljine šest čovječjih ribica s njihovih prirodnih staništa, te 16 jedinki prikupljenih nakon što su poplavama izbačene na površinu iz dvaju izvora. Željelo se saznati više o normalnoj mikroflori čovječjih ribica i o mogućim promjenama mikroflore nakon dodira životinja s različitim vanjskim utjecajima na površini. Identifikacija mikroba obavljena je standardnim mikrobiološkim metodama, te tehnikama MALDI-TOF i Real-Time PCR. Ni u jedne životinje nisu izolirani *Ranavirus, Batrachochytrium dendrobatidis, Batrachochytrium salamandrivorans* ni *Chlamydia* spp. Među gljivicama, najzastupljenije su bile *Penicillium* spp. i *Cladosporium* spp. U životinja izbačenih na površinu bakterijska je flora bila znatno raznolikija negoli u onih s prirodnih staništa, pri čemu su najzastupljeniji izolati bili *Acinetobacter johnsonii, Aeromonas hydrophila, Bacillus* spp. i *Janthinobacterium lividum*.

Ključne riječi: *Proteidae*; stiobionti; endemski; ugroženi; zaštićeni; *Janthinobacterium lividum*