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Microbial mats as shelter microhabitat for amphipods in an intermittent karstic spring

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Abstract – Microbial mats represent complex communities where cyanobacteria and diatoms as key organisms provide shelter for diverse assemblages of aquatic invertebrates, like the small stygophilous amphipod *Synurella ambulans*. Studies addressing such communities in the karst springs have rarely examined springheads, and have ignored intermittent springs. During high flow conditions the stygophilic crustaceans are flushed to the surface of a temporary stream Krčić where microbial mats prevent their drift and enables their successful retreat into underground in the periods of drought. The objective of this study was to characterize the microbial mat community of the Krčić Spring as a shelter for *S. ambulans* during strong current and high water level. Representative samples for diatom and cyanobacterial species identification and composition, as well as the fresh mat material for potential animal activity and cyanobacterial phylogenetic analysis were collected. The most dominant diatom was *Achnanthidium minutissimum*, whilst *Fragilaria capucina*, *Meridion circulare*, *Navicula cryptocephala* and *Nitzschia palea* had abundance greater than 0.5%. Morphological observations of cyanobacteria revealed that *Phormidium favosum* was the most dominant, with *Hydrocoleum muscicola* as a subdominant. Cyanobacterial phylogenetic relationship revealed two distinct clusters: (i) "*Phormidium* cluster", confirming morphological observations in both winter and spring samples, and (ii) "*Wilmottia* cluster", a first report for Croatia and found exclusively in the winter sample. Laboratory observations revealed a small stygophilic amphipod *S. ambulans*, hiding and feeding inside the pockets of fresh microbial mat. The intermittent Krčić Spring as a predator-free and competitor-free ecosystem provides a spatiotemporal conformity between microbial mat and stygophilous amphipod.

Keywords: Microbial mat / cyanobacteria / diatoms / amphipods / karstic spring

Résumé – Le tapis microbien comme microhabitat refuge pour les amphipodes dans une source karstique intermittente. Le tapis microbien représente des communautés complexes où les cyanobactéries et les diatomées, en tant qu'organismes clés, abritent divers assemblages d'invertébrés aquatiques, comme les petits amphipodes stygophiles *Synurella ambulans*. Les études portant sur ces communautés dans les sources karstiques ont rarement examiné ces têtes de ruisseaux et ont ignoré les sources intermittentes. Dans les conditions d'écoulement fort, les crustacés stygophiles sont chassés à la surface d'un ruisseau temporaire Krčić où les tapis microbiens empêchent leur dérive et permettent leur retrait dans le sous-sol en période de sécheresse. L'objectif de cette étude était de caractériser la communauté du tapis microbien de la source Krčić comme abri pour *S. ambulans* lors de forts courants et de niveaux d'eau élevés. La diatomée la plus dominante était *Achnanthidium minutissimum*, tandis que *Fragilaria capucina*, *Meridion circulare*, *Navicula cryptocephala* et *Nitzschia palea* avaient une abondance supérieure à 0.5%. Des échantillons représentatifs ont été prélevés pour l'identification et la composition des espèces de diatomées et de cyanobactéries, ainsi que de la matière organique du tapis servant à l'activité animale potentielle et pour l'analyse phylogénétique des cyanobactéries. Les observations morphologiques des cyanobactéries ont

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révélé que *Phormidium favosum* était le plus dominant, avec *Hydrocoleum muscicola* comme sous-dominant. L'étude phylogénétique cyanobactérienne a révélé deux groupes distincts: (i)"groupe de *Phormidium*", confirmant les observations morphologiques dans des échantillons hivernaux et printaniers, et (ii)"groupe de *Wilmottia*", une première observation pour la Croatie trouvé exclusivement dans l'échantillon hivernal. Les observations en laboratoire ont révélé qu'un petit amphipode stygophile *S. ambulans* se cachait et se nourrissait dans les poches du tapis microbien. La source Krčić intermittente en tant qu'écosystème sans prédateurs et sans concurrents assure une conformité spatio-temporelle entre le tapis microbien et l'amphipode stygophilique.

Mots-Clés : Tapis microbien / cyanobactérie / diatomée / amphipode / source karstique

1 Introduction

Microbial mats are structurally complex communities composed of physiologically and ecologically diverse micro-organisms adapted to gradients of light and nutrient accessibility. They are present in very diverse, often extreme environments with limited or even minimal top-down controls, like ephemeral and intermittent streams and springs (Stal and Caumette, 1994; Stanish *et al.,* 2013). Microbial mats can form dense micrometer-scale communities in which the full plethora of microbial metabolism can be present, usually consisting of filamentous, entangled organisms that produce a macroscopic 'mat-like' structure. These structures exhibit great variety in morphology and composition and may include diverse biofilms of immobilized microorganisms (Bauld, 1984). Cyanobacteria and diatoms are emphasized as the key organisms comprising microbial mats in spring habitats (Esposito *et al.,* 2006; Stal, 2012; Cantonati *et al.,* 2012, 2015, 2016), where these prokaryotic-eukaryotic interactions play an important role in microbial mat development by enabling significant photosynthetic activity (Elster and Komárek, 2003) and nutrient cycling and acquisition (Gooseff *et al.,* 2004; McKnight *et al.,* 2004; Mueller and Vincent, 2006). Their remarkable adaptability, through which they can overcome the extremity of environmental conditions prevailing in many carbonate-rock springs, makes them one of the key components in terms of ecology, management and conservation of spring ecosystems (Cantonati *et al.,* 2015). Taxa populating these habitats must be able to cope with rapid irradiance alterations, high water-level fluctuations and monthslong drought periods. Moreover, a diverse assemblages of aquatic invertebrates, particularly amphipods like the small stygophilous spring dwelling *Synurella ambulans* (F. Müller, 1846), live on and within the microbial mats (Camacho and Thacker, 2006, 2013; Korpinen *et al.,* 2006; Lévesque *et al.,* 2015).

A complex geological history of Croatian Dinaric karst manifests through formation of specific features, like well-developed underground drainage system, strong interactions between circulation of surface and groundwater, presence of unpredictable conduits, fissures and cavities beneath the ground, unexpected connections of water, changes of underground flow path over time, all of which shape a pronouncedly heterogeneous hydrological, hydrogeological, morphological, physicochemical and biological conditions in karst springs (Bonacci, 1993, 2015). The majority of water input into karst spring systems is caused by rainfall, which reaches groundwater *via* infiltration through karst aquifers.

Variations in precipitation induce rapid and pronounced oscillations of groundwater, strongly affect spring flow and cause fluctuations in discharge volume, thus making catchment boundaries of springs extremely time-variant (Hao *et al.,* 2012; Bonacci, 2015). Karst spring discharges reflect periods of poor or abundant precipitations due to specific recharge conditions and the locally prevalent climate regime (Bonacci, 2015; Fiorillo *et al.,* 2015). In such conditions streamflow regime is recognized as the principal variable affecting the success and distribution of aquatic biota (Meyer and Meyer, 2000; Meyer *et al.,* 2003; Konrad *et al.,* 2008). Limited knowledge of the effects of temporary seasonal drying of karst springs on stream biota introduces numerous questions when comparing biological integrity of these specific habitats to perennially flowing streams (Reiss and Chifflard, 2015).

There is a paucity of information on the adaptations of various organisms to temporary streamflows and their use of microbial mats during these stressful conditions (McDonough *et al.,* 2011; Robson *et al.,* 2011). Most studies addressing cyanobacterial and diatom communities in karst springs have examined mostly perennial streams, rarely springheads, and have ignored intermittent springs (Cantonati *et al.,* 2012, 2016). Their ecological role as the food source and shelter for freshwater invertebrates is still poorly understood (Camacho and Thacker, 2013; Lévesque *et al.,* 2015). During high flow conditions specimens of the stygophilic amphipod *S. ambulans* are flushed to the surface of a temporary stream Krčić where they can be retained by the microbial mat and moss, thus preventing their drift and facilitating their successful retreat into underground in the periods of drought. The objective of this study was to characterize the microbial mat community in the karstic stream Krčić and its role as a feasible alternative shelter for the subterranean stygophilic crustacean *S. ambulans* during two events: (i) high water level with fast streamflow, and (ii) water level decrease preceding drought.

2 Materials and methods

2.1 Study area

Krčić is a small intermittent stream situated in the middle of Dinaric karst of the southern Croatia, confined mostly to the External Dinarides, which consists predominantly of Triassic, Jurassic and Cretaceous limestones and dolomites (Bonacci *et al.,* 2006; Hajna *et al.,* 2010). The study area falls within a continental climate influenced by the mid-Mediterranean climate with the average annual temperature of 13 °C. The
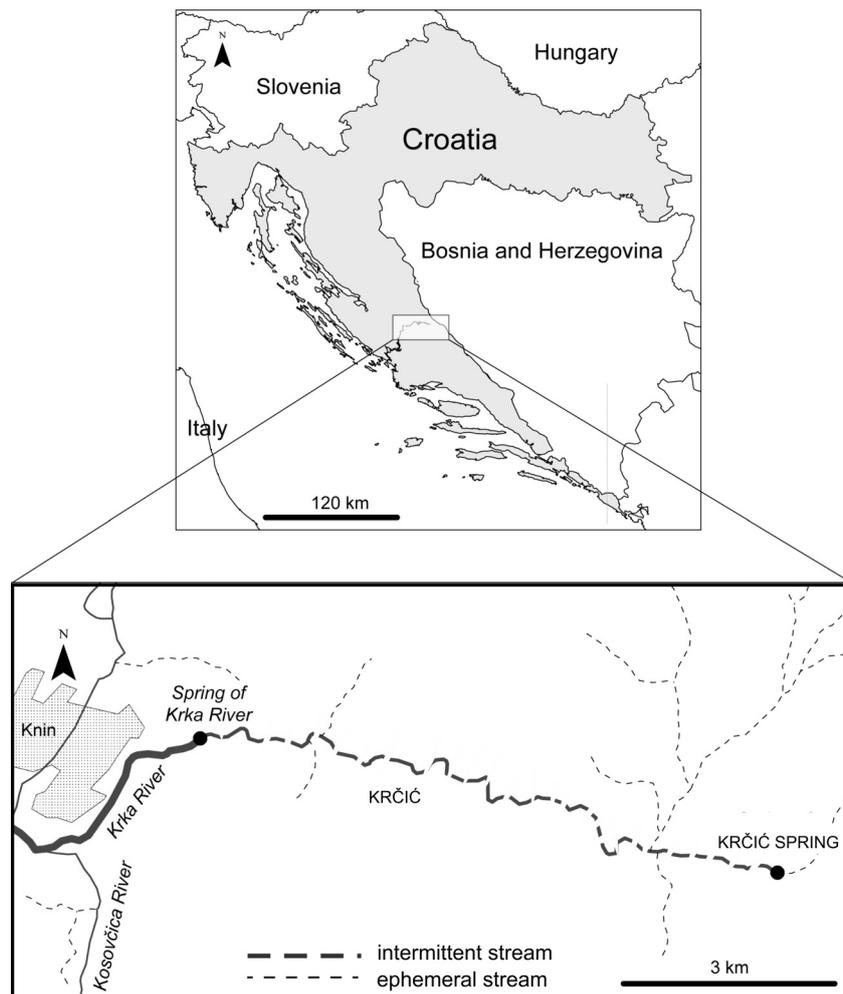
Fig. 1. Map of the investigated area.

summers are hot, with maximum temperature above 30 °C, whilst winters are cold with temperatures as low as −10 °C. The catchment area of Krčić covers 157 km² with the surface flow of 10.5 km from the springhead to the waterfall in the Krka River (Bonacci *et al.,* 2006). The spring and the mouth of the river lie between latitudes 44°1′32″N and 44°2′31″N and longitudes 16°19′53″E and 16°14′7″E, respectively (Fig. 1). Strong river bed karstification causes significant water losses along the stream causing alternating surface and subsurface flow (Bonacci, 1987, 1993; Bonacci *et al.,* 2006). Krčić functions as a descending karstic spring (Jukić and Denić-Jukić, 2006) and dries up regularly every year from June to September, usually when springwater discharge of Krka River (below the mouth of Krčić) drops below 4.20 m³ s⁻¹ (Bonacci *et al.,* 2006). However, in 2014 it retained water for the entire year because of exceptionally rainy summer (Fig. 2).

The diverse rheocrene Krčić Spring has multiple spheres of discharge rate over time due to groundwater emerges on the bottom, and along the edges of the springhead as a fast-flowing spring runs. The spring is not shaded by trees and the habitats are well preserved, although arteficial barriers of the 18th century mill building are situated 100 m downstream of the springhead.

2.2 Specimen collection and identification

Representative samples of the microbial mat for diatom and cyanobacterial species identification and composition were collected during two field trips in winter (February) and spring (May) of 2014 from the springhead of the Krčić stream. Months were selected based on their hydrologic conditions. February is characterized by high water level accumulating from precipitation and snowmelt. May is distinguished by a strong decrease in streamflow usually followed by a months-long drought period (Fig. 2). Algae were brushed and scraped from the stone surface (3 × 3 cm²) and rinsed into a sample jar. Samples were preserved with a buffered 4% formaldehyde solution. Diatom samples were cleaned following Hendey (1964). Acids were neutralised with distilled water until pH reached 7. Cleaned valves were mounted in Naphrax diatom mountant. Light microscope observations were conducted using an Olympus BX51 Microscope (Olympus, Japan). Diatoms were counted under oil immersion at 1000× magnification until a minimum of 400 valves have been counted. Identification of diatoms was performed using Krammer and Lange-Bertalot (1986, 1991b), Lange-Bertalot (2001), and Hofmann *et al.* (2013). For scanning electron microscopy (SEM), part of the clean diatom frustule suspension was filtered
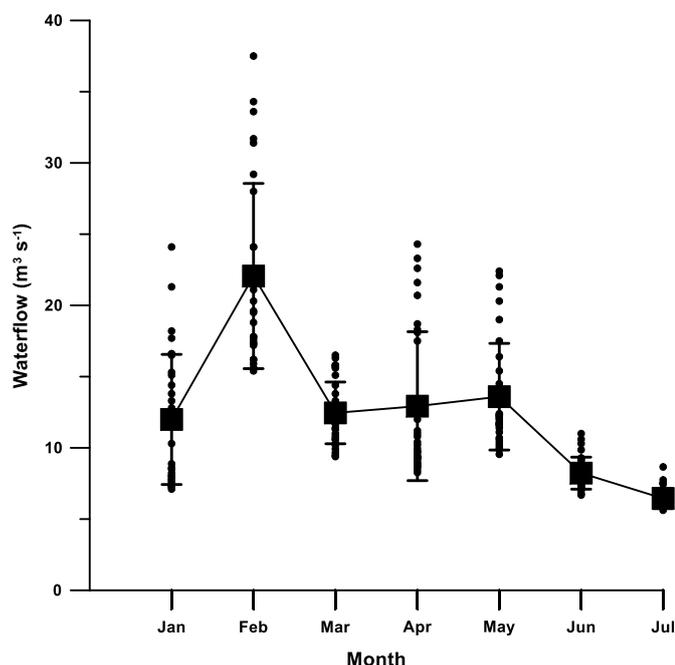
Fig. 2. Waterflow of the lower part of Krčić in the period between January and July 2014 (Meteorological and Hydrological Service, Croatia).

Table 1. Primer sequences used for amplification of cyanobacterial 16S rRNA.

Primer	Sequence (5′–3′)
CYA106F	CGC ACG GGT GAG TAA CGC GTG A
CYA359F	GGG GAA TYT TCC GCA ATG GG
CYA781R(a)[*]	GAC TAC TGG GGT ATC TAA TCC CAT T
CYA781R(b)[*]	GAC TAC AGG GGT ATC TAA TCC CAT T

[*] Reverse primer was an equimolar mixture of CYA781R(a) and CYA781R(b).

NaCl) with the addition of Sarcosyl (1%). After 1 h incubation at the room temperature, pellets were washed 4 times with TEN buffer solution. Each dry pellet, in which 2–5 small beads were added, was mechanically homogenized to fine powder using FastPrep FP120 Cell Disrupter at $4.5 \, \text{m s}^{-1}$ for 45 s (Thermo Savant Bio101, Qbiogene, France). The obtained homogenate was then resuspended in 100 μl of TEN buffer solution and incubated at 37 °C (15 min) with lysozyme (final concentration $0.5 \, \text{mg ml}^{-1}$). According to the NucleoSpin Plant protocol, Lysis Buffer PL1 and RNase were added and the sample was incubated at 65 °C (30 min). Additional step included the addition of Proteinase K enzyme to the sample and incubation at 55 °C (30 min), followed by a short incubation of the sample at 90 °C (5 min) for Proteinase K inactivation. Samples were then centrifuged (11 000 g, 5 min), after which the extraction continued on the NucleoSpin Filter according to manufacturer's instructions (Macherey-Nagel, Germany). The integrity of the DNA obtained at the end of the protocol was checked by electrophoresis on 1% agarose gel. DNA was quantified at 260 nm using a BioPhotometer (Eppendorf, Germany).

2.4 PCR amplification and phylogenetic analysis of cyanobacterial 16S rRNA

16S rRNA gene fragments were amplified from the DNA by using two primer sets specifically designed to target cyanobacterial 16S rRNA (Nübel *et al.*, 1997). A product of approximately 700 base pairs (bp) was targeted in the 1st PCR and of approx. 450 bp in the 2nd PCR by using primer set 1 (CYA106F/CYA781R) and primer set 2 (CYA359/CYA781R), respectively. Sequences of the primers used are given in the Table 1. PCR was performed with 100 ng of DNA used as a template under conditions identical to those reported in Srivastava *et al.* (2007). Cycling conditions were as follows: 94 °C for 3 min; followed by 35 cycles each consisting of 1 min denaturation at 94 °C; 1.5 min annealing at 59 °C; 2 min elongation at 72 °C; and a final 5 min elongation at 72 °C. Obtained PCR products were analyzed on agarose gels (2%) before being purified from the reaction using gel extraction kit (GenElute Gel extraction kit, Sigma, USA). Subsequently, purified PCR products were cloned into the plasmid vector pGEM-T Easy according to the manufacturer's instructions (Promega, France). Two libraries were established from winter sample (one from 1st PCR and one from 2nd PCR) and two from those samples collected at the spring time (one from 1st PCR and one from 2nd PCR). Clones were picked from different libraries (approx. 20 from each library) and checked for the correct insert size by vector targeted PCR. Diversity
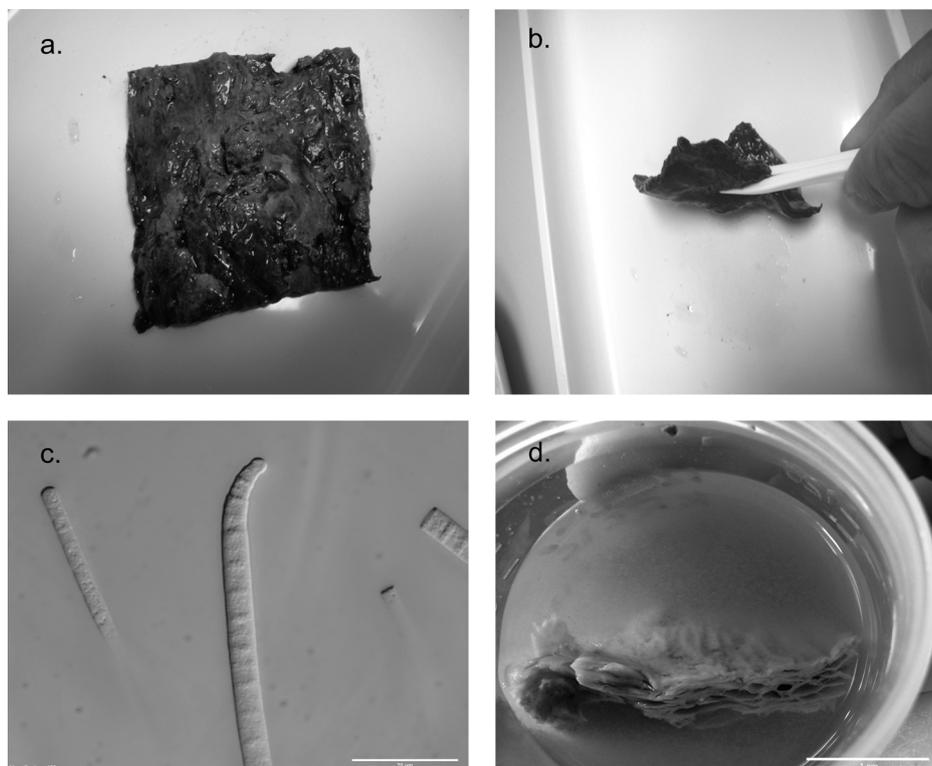
onto Whatman Nucleopore membrane filters (Sigma-Aldrich, USA) with a maximum pore diameter of 0.3 μm, pieces of which were attached to aluminum stubs after air-drying. The stubs were sputter-coated with 30 nm of gold and studied using MIRA3 FEG-SEM microscope (Tescan Orsay Holding, Czech Republic) in the Forensic Science Centre Zagreb.

For the morphological analysis of cyanobacterial samples, length and width of at least 30 cells on each trichome were measured under the 1000× magnification using the BA310 Motic optical microscope (Speed Fair Co. Ltd., China). Identification of cyanobacteria was performed following the most recent literature (Komárek and Anagnostidis, 2005; Palińska *et al.*, 2011; Hašler *et al.*, 2012; Strunecký *et al.*, 2013). Microphotographs were taken with the Optika Pro 3LT camera and processed with Optika Vision Pro Software (Optika Microscopes, Italy).

The fresh mat material, sampled directly at the springhead, was transported to the laboratory for the subtle observations of potential animal activity inside the jelly pockets. Part of fresh mat material was placed into diatom medium (Andersen, 2005) for a few days and sent for DNA extraction.

2.3 DNA extraction from cyanobacterial mats

Approximately 100 mg of the fresh mat material preserved in culture media was used as a starting material for nucleic acid extraction. DNA was extracted from winter and spring samples using NucleoSpin Plant II Kit (Macherey-Nagel, Germany) according to the manufacturer instructions with a few modifications. In order to remove the culture media, mat material was first centrifuged (600 g, 15–30 min), followed by resuspension of the obtained cell in TEN buffer (final concentration 50 mM TrisHCl pH 8; 5 mM EDTA; 50 mM

Fig. 3. Components of microbial mats: macroscopic image of microbial mat (a, b); *Phormidium favosum* (c); visible pockets in microbial mat of Krčić Spring (d).

within 16S rRNA sequences was checked by restriction fragment length polymorphism (RFLP) analysis. Amplified 16S rRNA PCR products were digested with restriction endonucleases AluI (Fermentas, Thermo Fisher Scientific, USA) following manufacturer's instructions. Digested fragments were visualized by running electrophoresis on 3% agarose gel. Clones showing different RFLP pattern were selected and further subjected to 16S rRNA gene sequencing (Macrogen, Amsterdam) using universal SP6 primer. Forward and complementary sequences were aligned using the Clustal X2 Multiple Sequence Alignment Program (Larkin *et al.,* 2007). Ambiguities and PCR errors were checked manually and the chromatograms were used to make corrections where it was appropriate. The corrected sequences were aligned with sequences from GenBank. 16S rRNA sequences were compared with known nucleotide sequences using the Nucleotide Basic Local Alignment Search Tool (Nucleotide BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed from multiple alignments drawn by using NJplot software (Perrière and Gouy, 1996) using the neighbour-joining method that included references from GenBank showing highest similarities to our sequences. Nucleotide sequences have been deposited in the Genbank database under the accession numbers KY820663-KY820690.

3 Results

Microbial mat was densely developed in the Krčić Spring during high streamflow in both winter and spring samples (Fig. 3). Total biomass of microbial mat was estimated as

chlorophyll *a* concentration. The values of chlorophyll *a* in winter sample were $6.83\,\mu g\,cm^{-2}$, while spring Chl *a* was $68.31\,\mu g\,cm^{-2}$.

3.1 Diatom assemblage

A total of 25 diatom species was recorded from samples at the Krčić Spring. A number of species was rather low and relatively constant (12–15). In every sample one or two species dominated, while other diatoms were poorly represented with only a few frustules. All samples were dominated by *Achnanthidium minutissimum* (Kützing) Czarnecki, with a very high abundance in winter (92%), as well as in spring (35%). During winter only *Fragilaria capucina* Desmazières, *Meridion circulare* (Greville) C.Agardh, *Navicula cryptocephala* Kützing and *Nitzschia palea* (Kützing) W.Smith had abundance greater than 0.5%. The same species were also more abundant in the spring samples, with subdominant *N. palea* (27%) and *M. circulare* (22%).

3.2 Morphological characterization of cyanobacterial taxa

According to microscopic and morphological observations, dominant species in the microbial mat of the Krčić Spring in both winter and spring samples was *Phormidium favosum* Gomont (Fig. 3). The second most dominant species in the community was *Hydrocoleum muscicola* Hansgirg ex Forti.
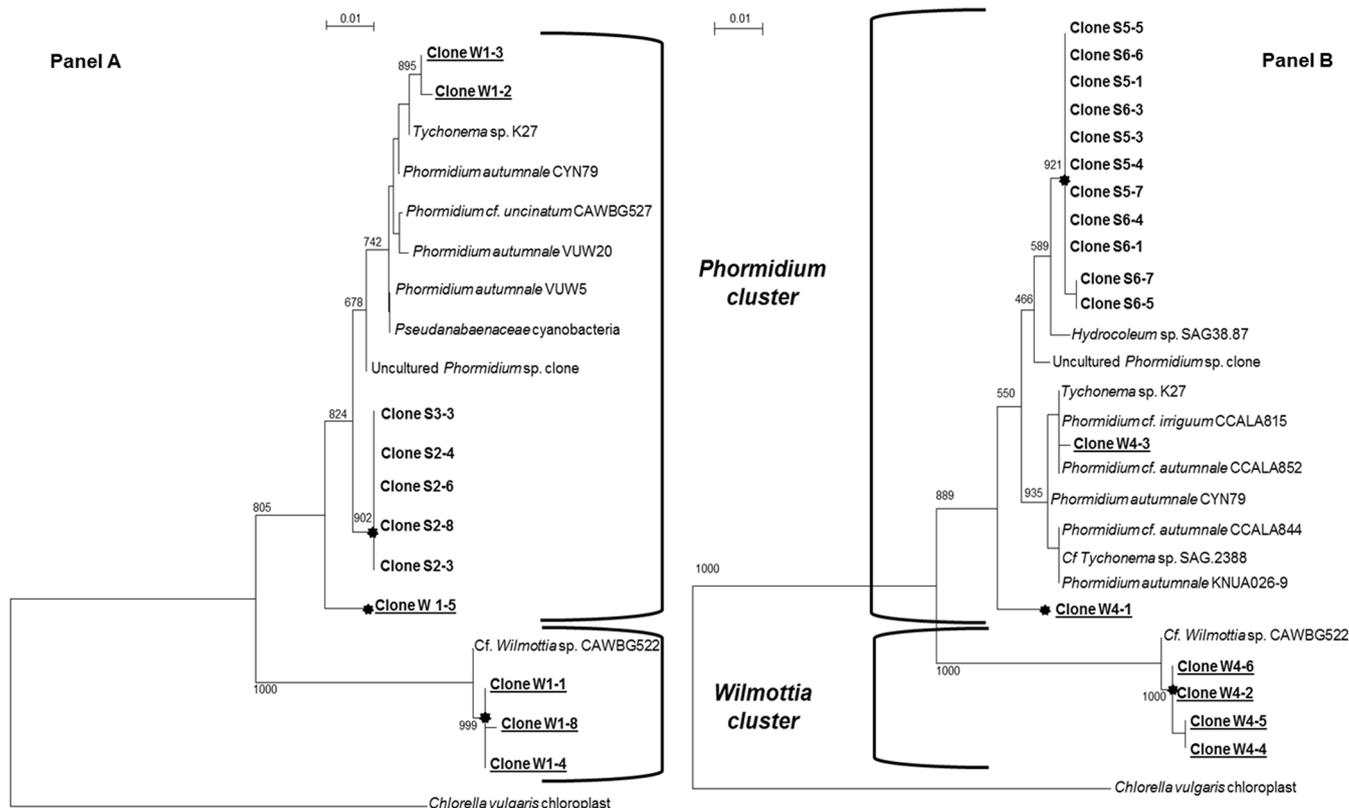
Fig. 4. Phylogenetic relationship of 16S rRNA larger fragment (A) and smaller fragment amplicon (B) amplified in clones originating from the Krčić Spring mat to the most related strains selected from GenBank database. Sequences obtained from the winter sample clone libraries are underlined and marked with "Clone W", while those from spring clone libraries with "Clone S". Sub-clusters are marked with asterisk. Molecular phylogenetic analyses were conducted in Clustal X2. The bootstrap consensus tree was inferred from 1000 replicates.

3.3 *Phormidium favosum* Gomont

Thallus blue-green, layered and soft. Trichomes blue-green, narrow, curved at both ends, unconstricted at the cross walls. Apical cells well developed, capitate, with well-formed wall thickenings (calyptrae). Cells with distinct granules agglomerated at the cross walls. Cell width from 3.1 to 5.6 µm (avg. 4.7 µm), length between 2.9 and 4.5 µm (avg. 3.7 µm); cells only slightly wider than long (length/width ratio 1.3). Atypical morphotypes also present, with capitate and widely rounded apical cells.

3.4 *Hydrocoleum muscicola* Hansgirg ex Forti

Thallus light brown, soft, placed underneath blue-green layers of *Phormidium*. Sheaths thin, colourless, usually enclosing one or up to several trichomes. Trichomes pale green, 2.9–4.5 µm (avg. 3.4 µm) wide. Cells 2.2–4.1 µm (avg. 3.2 µm) long, on average almost isodiametric (length/width ratio 1.1). Apical cells widely rounded, without calyptrae.

3.5 Molecular phylogeny of cyanobacterial mats

By using modified extraction protocol 24–113 ng µl^{-1} of high-quality DNA was successfully obtained from cyanobacterial mats collected in the Krčić Spring at both winter and spring sampling times. PCR amplification of the cyanobacterial 16S rRNA gene gave amplicons of the expected sizes: (i) in 1st PCR larger fragment of 650 bp, and (ii) in 2nd PCR smaller fragment of 450 bp. After cloning into appropriate vector four separate clone libraries were established: (i) winter sample/larger fragment, (ii) winter sample/smaller fragment, (iii) spring sample/larger fragment, and (iv) spring sample/smaller fragment. Clones were further subjected to RFLP analysis in order to check the diversity of cyanobacterial communities. Clones showing different restriction patterns were assembled into different RFLP families (results not presented). For further sequencing, at least one representative member of each of the RFLP family (altogether 28 clones) was chosen. Based on the sequences pairwise alignment phylogenetic tree was built (Fig. 4). Majority of sequences shared high homology (98–99%) with the 16S rRNA gene of the Oscillatoriales genera *Tychonema K. Anagnostidis* and *J. Komárek*, *Pseudanabaena Lauterborn* and *Hydrocoleum Kützing* ex Gomont but mainly matched to *Phormidium autumnale* Gomont. Similarities between 16S rRNA sequences of these genera did not allow more clear identification of the species. A smaller number of sequences were identified as *Wilmottia* O. Strunecký, J. Elster and J. Komárek.

Analogous phylogenies were obtained by analysing both smaller and larger 16S rRNA gene fragments sequences (Fig. 4). Sequences clearly grouped into 2 separate clusters: (i) "Phormidium cluster", and (ii) "Wilmottia cluster", with the
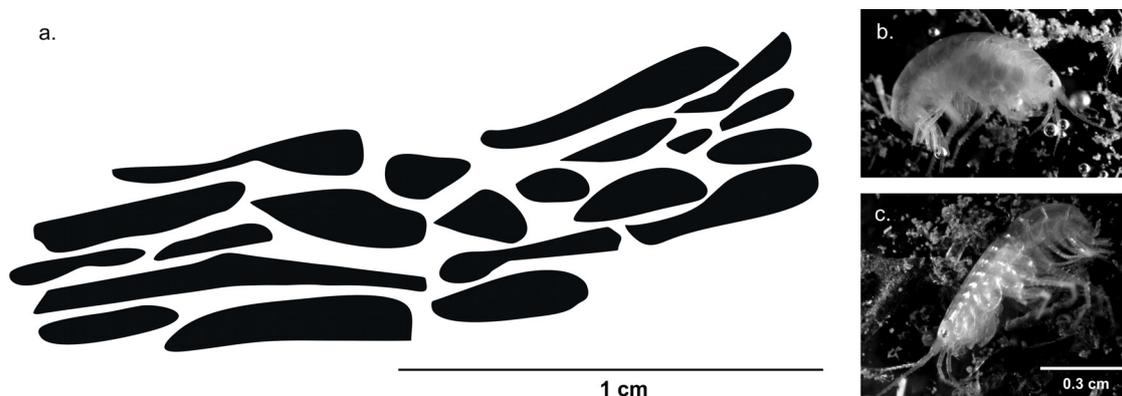
Fig. 5. Pockets of microbial mat, detailed scheme (a); *Synurella ambulans* (b) female specimen; (c) male specimen.

majority of sequences falling into "Phormidium cluster". Sequence comparison showed 7–10% difference in 16S rRNA among the clusters, thus indicating a distinct phylogenetic separation. Cyanobacteria selected from the GenBank were shown to originate from geographically distinct regions, such as German, Spanish and Swedish streams, but also streams from New Zealand and Polar Regions. Phylogenetic tree revealed that the representatives of the "Phormidium cluster" could be found in the Krčić Spring in both spring (Clones S3-3, S2-4, S2-6, S2-8, S2-3, S5-5, S6-6, S5-1, S6-3, S5-3, S5-4, S5-7, S6-4, S6-1, S 6-7, S6-5) and winter (Clones W1-5, W1-3, W1-2, W4-1, W4-3) seasons. However, clones forming "Wilmottia cluster" originated exclusively from the sample collected at the winter time. These winter clones (clones W1-1, W1-8, W1-4, W4-6, W4-5 and W4-4) showed 99% homology to *Wilmottia* sp. CAWBG522 isolated from benthic freshwater habitats in New Zealand. In addition to main clusters two sequences W1-5 (larger 16S rRNA fragment) and W4-1 (smaller 16S rRNA fragment, based on the 97% similarity cut-off value, formed distinct operational taxonomic units (OTUs) inside "*Phormidium* cluster".

3.6 Microbial mat as a shelter for crustaceans

Laboratory observations on fresh microbial mat revealed a small stygophilic amphipod species *S. ambulans*. The adult specimens were less than 7 mm in size, hiding and feeding on fine particulate organic matter (FPOM) inside the pockets of thin cyanobacteria-diatom jelly coating in size of cca 1–8 mm (Fig. 5). The average densities of crustaceans inside the pockets were presented in Table 2.

4 Discussion

Recent studies on cyanobacterial representatives of lotic aquatic systems emphasize the problems of distinguishing cyanobacterial species based on morphological features and ecological preferences (Komárek, 2016), specifically referring to the *Phormidium sensu lato* group (Palińska and Marquardt, 2007; Strunecký *et al.*, 2013). The classification of *P. sensu lato* by Komárek and Anagnostidis (2005) has recently been supplemented by several new genera based on the combination of both morphological and genetic features, namely the 16S

Table 2. Number of specimens of *Synurella ambulans* per square 10×10 cm in mucilage microbial mat at the Krčić Spring.

Month	Avg ± SD	Min	Max
Winter (February 2014)	6.75 ± 4.50	3	12
Spring (May 2014)	4.50 ± 2.38	2	7

rRNA gene sequencing (Komárek, 2010; Strunecký *et al.,* 2010; Chatchawan *et al.,* 2012). *P. autumnale* Gomont, a key species within the complex, encompasses a cluster denominated as a group VII (Komárek and Anagnostidis, 2005), comprising species characterized by very similar morphological traits and widely diverse, ubiquitous morphotypes adapted to various biotopes (Strunecký *et al.,* 2013). Subtle differences in colony formation, trichome organization and exploitation of distinct habitats can be noted amid the cluster.

Microbial mats predominately composed of *P. autumnale* complex prevail and dominate in fast flowing streams with rapid water current and stony bottom (Komárek *et al.,* 2012), as was the case in the Krčić Spring. *P. favosum*, which is included into group VII, is attributed with isodiametric cells, trichomes shortly attenuated towards the trichome ends, and the presence of well-developed calyptrate apical cells. The ecotype-based differences between *P. favosum* and *P. autumnale*, where the former is a stenothermal species inhabiting cold oligotrophic streams and rivers (Strunecký *et al.,* 2012), while the other is typical for meso- to eutrophic waters (Izaguirre *et al.,* 2001; Palińska and Marquardt 2007; Strunecký *et al.,* 2010; Hašler *et al.,* 2012; Du, 2013; Loza *et al.,* 2013) corresponded to morphological observations of species forming mats, as well as habitat characteristics of the Krčić Spring.

P. autumnale and other species within the group VII share similar features with the *Microcoleus sensu stricto* complex, *i. e.* homogeneous cell division and thylakoid structure, as noted by Boyer *et al.* (2002) and Komárek and Anagnostidis (2005). Consequent research on *Microcoleus vaginatus* and *P. autumnale* samples from different aquatic systems yielded a taxonomic revision of *P. autumnale* into *Microcoleus autumnalis* (Trevisan former Gomont) Strunecký, Komárek and Johansen comb. nov., and *P. favosum* into *Microcoleus favosus* (Gomont) Strunecký, Komárek and Johansen comb.

nov. (Strunecký *et al.,* 2013). Still, the taxonomic uncertainty of identifying morphospecies could produce spurious phylogenetic inferences (Bagley *et al.,* 2015), specially when RNA sequencing of samples classified as *P. autumnale* indicates the presence of several different genotypes (Strunecký *et al.,* 2013). Furthermore, when compared with the results of a partial 16S rRNA phylogeny, ecotype preferences used to separate *M. vaginatus* from *P. autumnale* proved fairly inaccurate. In addition, all subsequent taxonomic revisions of *Microcoleus* complex should include more genetic markers due to high similarity of the 16S rRNA genes. Due to aforementioned reasons the authors have decided to use the nomenclature following the sequence names in the NCBI database.

As in the previous case, genera *Microcoleus* and *Hydrocoleum* are also morphologically poorly delineated. Although Komárek and Anagnostidis (2005) made distinctions based on the sheath structure, as being hyaline in *Microcoleus* opposed to lengthwise striated in *Hydrocoleum*, they stated that a phenotype with both features may appear, thus recommending a detailed molecular review in both genera.

H. muscicola is a freshwater species characterized by olive green to reddish, often warty thallus with trichomes 3–4 µm wide. According to Komárek and Anagnostidis (2005), it was reported from the hilly and mountainous streams in Austria (Carinthia, Tyrol) and Croatia (Dalmatia), and from tufa barrier waterfalls in Greece (Edessa, Macedonia). From the metaphyton samples collected in Nahuel Huapi National Park (Patagonia, Argentina) Wenzel and Díaz (2008) described following morphological characteristics of *H. muscicola*: trichomes unconstricted, roughly parallel, 3–4 µm in width, gradually attenuated at the ends; sheath hyaline, unbranched, narrow; cells 1.5–3 µm in length, with granulations near the transverse walls; apical cells conical or rounded, sometimes capitated. This was in accordance with morphological observations on *H. muscicola* described herein, specifically the width of thalli, the size and structure of individual cells, and the shape of apical cells.

Results of the phylogenetic analysis confirmed presence of the Oscillatoriales cyanobacteria in the microbial mats of the Krčić Spring. Even though two different primers were used in order to elucidate cyanobacteria to the species level, cyanobacterial genera *Phormidium, Hydrocoleum* and *Tychonema* were not readily distinguished by their 16S rRNA gene sequences. These species showed high sequence similarity form a single clade with 80–90% bootstrap support, as was previously shown by Harland *et al.* (2014). Nevertheless, clear separation between two phylogenetically distinct clusters implied that two cyanobacterial populations dominate microbial mats in the Krčić Spring with clear seasonal differentiation. The *Phormidium* population, found in both winter and spring samples, supports characterization of dominant species appertaining to P. *autumnale* complex. Other population comprised cyanobacteria identified as *Wilmottia* species. This finding was surprising since the cyanobacterial ribotypes corresponding to *Wilmottia* sp., to our knowledge, have not yet been reported in Croatia. Interestingly, this population was found exclusively in the winter sample. Ecology of *Wilmottia* species is considered to be very distinct, with low temperatures and long frozen periods. However, cyanobacteria isolated from cold environments have temperature optima growth rates in the

range 15–20 °C, suggesting that they likely had their evolutionary origins within temperate latitudes (Jungblut *et al.,* 2009), and only subsequently colonized perennial cold habitats. Considering this, *Wilmottia* in the Krčić Spring could be endemic for this area, although a broader investigation on the topic is needed for further conclusions. In addition, sub-clustering inside *Phormidium* population (clones W4-1, W1-5) indicated a possibile existence of cyanobacteria genotypes specific for Croatian region. Moreover, those strains were found to be exclusively related to winter season period.

M. circulare is a pennate araphid diatom with thickened silica ribs (costae) oriented transversely across the valve (Kociolek *et al.,* 2011). It is a widely distributed member of benthic and periphytic (epilithic and epiphytic) communities of various freshwater ecosystems including brooks (Krejci and Lowe, 1987), streams (B-Béres *et al.,* 2016), karstic springs and spring-fed streams (Cantonati and Ortler, 1998; Delgado *et al.,* 2013) and alpine springs (Cantonati and Lange-Bertalot, 2010; Mogna *et al.,* 2015). It forms fan-shaped colonies with cells enclosed within a mucilaginous polysaccharide matrix (Lock *et al.,* 1984). Ecologically, *M. circulare* is classified as a crenophilic (Krammer and Lange-Bertalot, 1991a), alkaliphilous and oligo-eutraphentic species (Van Dam *et al.,* 1994; Cantonati and Ortler, 1998), commonly present in calcareous medium-to-high-altitude (500–1500 m a.s.1.) springs and streams (Margalef, 1949; Symoens, 1957). It is a microthermal species growing abundantly in the cold water within a temperature range of 7–15 °C (Whitford, 1960; Krejci and Lowe, 1987), with phosphorus-poor, high-light conditions (Cox, 1993; Hill *et al.,* 2011), all of which conformed to the Krčić Spring.

As one of the most widely recognized freshwater diatoms, *A. minutissimum*, a species corresponding to a still largely unresolved species complex, is frequently dominating benthic algal communities in the upper courses of streams and rivers (Virtanen *et al.,* 2011; Noga, 2012; Novais *et al.,* 2015; Cyr, 2016). Despite its omnipresence and importance in water quality assessments, *A. minutissimum* is attributed to a rather difficult identification due to small cell size, inter- and intra-specific varieties and insufficiently studied ecology (Potapova and Ponader, 2004; Ponader and Potapova, 2007; Novais *et al.,* 2015). It is an early colonizing species (Falasco *et al.,* 2012) reported to successfully recolonize and develop dense populations in the fast water substrata after repeated annual dry phases (Della Bella *et al.*, 2017). The properties of Krčić Spring corresponded to water associated with high abundance and frequency of *A. minutissimum*, like streamflow variability and slighty alkaline conditions (Noga *et al.,* 2014; Della Bella *et al.*, 2017; Jakovljević *et al.,* 2016). *A. minutissimum* is reported as a pioneering species during the initial processes of biofilm formation (Sekar *et al.,* 2004; Leinweber and Kroth, 2015). It is often the most numerous diatom occuring in *P. autumnale* and *P. favosum*-dominated microbial mats (Kelly, 2012; Brasell *et al.,* 2015), as was the case in Krčić Spring, where it could efficiently utilise potency by forming pads and stalks, thus additionally anchoring the biofilm.

N. palea is a pennate diatom with the nitzschioid eccentically positioned raphe within a keel supported by fibulae (Spaulding and Edlund, 2008), lanceolate valves, barely visible striae with parallel sides and poles terminating with subcapitate apices (Kociolek *et al.,* 2011). It is a benthic diatom with wide geographical distribution in freshwater lentic

and lotic habitats (Trobajo *et al.,* 2010). *N. palea* is a taxonomically problematic species encompassing a multitude of cryptic and pseudocryptic species into a *N. palea* complex (Trobajo *et al.,* 2009). It is capable of surviving extreme environmental conditions prevailing in the Krčić Spring, like high streamflow variability and annual periods of drought, as was also noted in Australian dryland river systems (McGregor *et al.,* 2006). *N. palea* was also found in tufa-forming microbial mats from the Harz Mountains karst streams in Germany (Arp *et al.,* 2010). Due to its exceptional tolerance to organic pollution and heavy metals (Palmer, 1969), biomass increase of *N. palea* is an indication of anthropogenic pollution (Tornés *et al.,* 2007; Bere *et al.,* 2014). The low abundance of *N. palea* recorded in Krčić Spring during the study period suggests minimum anthropogenic pressure on the stream. In that regard, close monitoring of *N. palea* could provide valuable information of a possible increase of human-induced impacts on the extremely vulnerable karst environments (Trobajo *et al.,* 2009; Delgado *et al.,* 2012).

N. cryptocephala is a benthic solitary pennate diatom with symmetrical biraphid morphological features (Potapova, 2011). Although common in a multitude of freshwater habitats, this polymorphic diatom is comprised of a complex of pseudo-cryptic species with presumably restricted distributions (Poulíčková *et al.,* 2010). It is a common member of metaphytic communities in well illuminated, alkaliphilous, lotic habitats with relatively low nutrient concentrations (Blinn and Bailey, 2001; Cox *et al.,* 2011).

F. capucina is an araphid pennate diatom frequently noted in benthic assemblages (Tuji and Williams, 2006; Boulêtreau *et al.,* 2010) with many recognized varieties. Although tolerant to increase in concentrations of heavy metals, it usually favours rheophilic, alkaliphilic habitats with lower water temperature, and low to moderate nutrient conditions (Kelly *et al.,* 2005). The high abundance of *F. capucina* was recorded in several Croatian karst aquatic systems, like the karst stream Jankovac (Špoljar *et al.,* 2012) and karst sub-mountain river Lika (Mejdandžić *et al.,* 2015). The most dominant epilithic taxa recorded in the Su Gologone karst spring in Italy were alkaliphilous, oligotrophic species, including *F. capucina* and *N. cryptocephala* (Lai *et al.,* 2016). Delgado *et al.* (2012) have split diatom taxa from 60 Mediterranean temporary karst streams into two groups based on their sensitivity or tolerance. *F. capucina* and *N. cryptocephala* were placed into sensitive taxa indicating reference sites, while *N. palea* was grouped into tolerant taxa, in which case the abundance of species was referring to disturbance. Moreover, a study on the 371 streams from Portugal on both siliceous and carbonate (karst) bedrock (Feio *et al.,* 2012) indicated that all three aforementioned diatoms preferred streams on non-siliceous substrate, with mean water temperature of 15 °C, increased hardness and alkalinity, and higher degree of temporality.

4.1 Interspecies cohabitation on the edge

Pockets of microbial mat in Krčić Spring serve as an important shelter for stygophilic amphipod *S. ambulans* when moss as the primary substratum is not fully developed. The cyanobacteria-diatom mat protects amphipods from drift, usually during the flow activation in autumn or from the Dinara mountain snowmelt in spring. Therefore, the amphipods are presumably well adapted to: (1) changing currents caused by seasonal drying of the spring, and (2) changing of living substrates.

The low dispersal ability of the stygophilous amphipod *S. ambulans* causes specific selection of offered substrates, such as microbial mat, within the relatively small scale of spring patches. Since the inhabitants of subterranean ecosystems might be poor competitors in more ecologically complex surface environments (Fišer *et al.,* 2014), surface-dwelling species may limit the extant of the distribution of *S. ambulans* in pockets of the microbial mat in photic habitats. Moreover, the intermittent Krčić Spring as a predator-free (fishes) and competitor-free (epigean amphipods) ecosystem provides a spatiotemporal conformity between microbial mat and stygophilous amphipod. Furthermore, microbial mat provides a large surface for small biota in a relatively stable habitat transition of aquatic-terrestrial ecotones (Cantonati *et al.,* 2016) and can serve as a capturer of organic matter between the stony substrate of the Krčić Spring. On the other hand, flow rate variability and successive community dynamic and competition between the microbial mat and moss community in intermittent spring could cause changes in the choice of neighbouring shelters by amphipods, choosing moss cushions at the top of the stony substrate as a more dominant organic substrate in the Krčić Spring.

5 Conclusion

Succession of cyanobacteria and diatoms causes shift in food web structure of intermittent springs, not only when the spring dry up or during the flow oscillation, but moreover as a consequence of evolutionary internal clock in species adapted to temporary environment. Microbial mat most likely uses chemical signals in intra- and inter- species communication about physico-chemical conditions of the environment, but the information flow could also come from animals such as crustaceans and aquatic plants, playing a significant role in the organization and function of superorganism structure such as microbial mat in intermittent springs.

A huge negative impact of global warming on temporary karstic streams may be critical for the survival of some isolated and rare species of cyanobacteria and diatoms, as well as some sophistically adapted aquatic invertebrates living on the ecotone between groundwater and surface water. Moreover, any disturbance across a wide range of spatiotemporal scale of the aquifer-spring-terrestrial system can change the community structure or even induce removal of species.

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