



ORIGINAL ARTICLE

Open Access



# Diversity of protist genera in periphyton of tufa-depositing karstic river

Antonija Kulaš<sup>1\*</sup>, Petar Žutinić<sup>1</sup>, Vesna Gulin Beljak<sup>1</sup>, Renata Matoničkin Kepčija<sup>1</sup>, Mirela Sertić Perić<sup>1</sup>, Sandi Orlić<sup>2,3</sup>, Ines Sviličić Petrić<sup>2</sup>, Tamara Marković<sup>4</sup> and Marija Gligora Udovič<sup>1</sup>

## Abstract

**Purpose** In aquatic ecosystems, protists play a crucial role and cover numerous ecological functions. The karstic Krka River (Croatia) is a unique hotspot for high diversity of aquatic organisms, especially protists. The main objective of the present study was to obtain a detailed overview of the protist community structure in the periphyton of the Krka River and to determine the differences in protist diversity along the river.

**Methods** Protist diversity was detected by amplicon sequencing of the hypervariable region V9 of the 18S rRNA gene, using the universal eukaryotic primer pair.

**Results** The three main groups of protists were as follows: Ciliophora, Cercozoa, and Bacillariophyta. In terms of abundance of protist OTUs, the shade plot revealed an evident difference from the upstream to downstream river section, which increased between locations from Krka spring to Skradinski buk. Diversity was explored using measures of alpha and beta diversity. Alpha diversity showed an increasing trend in the downstream direction of the river. The location effect, or clustering/grouping of samples by location, was confirmed by the PERMANOVA permutation test of beta diversity.

**Conclusion** The combination of alpha and beta diversity can help provide deeper insight into the study of diversity patterns, but also point out to decline in species diversity and allow for effective ways to protect aquatic karst habitats in future management.

**Keywords** Diatoms, Protozoa, Periphyton, Karstic river, Molecular approach

## Introduction

Biodiversity is a key indicator of ecosystem health and thus the central goal of most conservation efforts (Niesenbaum 2019; Watermeyer et al. 2021). As it is important to understand biodiversity and how to

preserve it in the face of environmental change, there is one significantly overlooked category of organisms, protists (Gran-Stadniczenko et al. 2019; Metz et al. 2022). Protists serve numerous functions in aquatic ecosystems, yet they receive less attention than other aquatic organisms (e.g., macroinvertebrates) and their biodiversity is still poorly investigated (Gran-Stadniczenko et al. 2019). They play crucial ecological roles as primary producers, predators, decomposers, and parasites, which has led to great efforts in quantifying specific species and inferring their ecological functions (Massana et al. 2015). Protists can be phototrophic, heterotrophic, mixotrophic, or osmotrophic where they are referred to as microalgae and “protozoans” (Selosse et al. 2017). Microalgae contribute substantially to carbon flux through the microbial

\*Correspondence:

Antonija Kulaš  
antonija.kulas@biol.pmf.hr

<sup>1</sup> Faculty of Science, Department of Biology, University of Zagreb, Horvatovac 102a, HR-10000 Zagreb, Croatia

<sup>2</sup> Ruđer Bošković Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia

<sup>3</sup> Center of Excellence for Science and Technology Integrating Mediterranean Region (STIM), HR-10000 Zagreb, Croatia

<sup>4</sup> Department of Hydrogeology and Engineering Geology, Croatian Geological Survey, Sachsova 2, HR-10000 Zagreb, Croatia



© The Author(s) 2023. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

loop (Metz et al. 2022) and are the main supply of photosynthetic products on which the higher trophic levels of the food web depend upon. On the other hand, being the major grazers of bacteria, protozoans increase mineralization and availability of nutrients to primary producers (Koller et al. 2013). In general, protists are morphologically and genetically diverse and are common in the periphyton, where their microbial interactions are of great importance for the primary production, nutrient cycling, and food web structure (Metz et al. 2022). The increasing application of molecular methods in aquatic environments and their steady advances provide new perspectives on the protist community and allow for better understanding of the specific role of freshwater periphyton (West et al. 2018; Burki et al. 2021).

One of the main constructs in freshwater ecology is water flow—the River Continuum Concept, to understand changes in river ecology along the longitudinal gradient and show how different aspects change community composition from upstream to downstream parts of the river (Porter and Patton 2016; Bock et al. 2020; Englmaier et al. 2020). Studies on longitudinal gradient (Chen et al. 2018; Bock et al. 2020; Englmaier et al. 2020) have improved the understanding of lotic ecosystems, including numerous aspects such as energy flow, distribution, abundance, and diversity of stream and river organisms. The seasonal variations in water level also directly affect the community composition structure along the longitudinal gradient due to changes in water features, habitat structure and availability, and food resources (Porter and Patton 2016). Nevertheless, differentiation in community composition of many biota, as well as their habitat preferences, functional traits, and distribution patterns are often still poorly understood (Englmaier et al. 2020). Most conceptual studies on river zonation have addressed fish communities where fish community structure has changed along the longitudinal profile (Song et al. 2019; Sutela et al. 2020; Englmaier et al. 2020), while studies on the longitudinal dynamics of periphyton have revealed changes in their taxonomic structure and community composition (Rusanov and Khromov 2016). Typically, periphyton communities in river ecosystems show transition between habitats along the longitudinal gradient from upstream to downstream (Jäger and Borchardt 2018), where longitudinal variation can be described using integral features of community composition, such as species richness and diversity (Rusanov and Khromov 2016). However, studies on rivers are usually focused on small scales, such as specific sections or locations (Jäger and Borchardt 2018).

Karst rivers in the Mediterranean region represent unique diversity hotspots of various aquatic organisms, especially protists (Tierno de Figueroa et al. 2013; Lai

et al. 2019; Gligora Udovič et al. 2022). The pronounced process of karstification has led to distinctive climatic and environmental conditions that have resulted in habitat heterogeneity in these areas (Vilenica et al. 2018). Their geographic and hydrological uniqueness, habitat heterogeneity, high biodiversity, and conservation requirements should be a priority for the sustainable management of this sensitive region (Darwall et al. 2014). In this study, attention is focused on the Krka River, located in the Dinaric karst ecoregion in Croatia. This river has an extremely complex hydrological network (Bonacci et al. 2006, 2013) and is famous for its tufa barriers with a high diversity of freshwater taxa, such as algal species (Gligora Udovič et al. 2022, 2023), insects (Ivković and Pont 2015, 2016), or protozoa (Primc-Habdija and Matoničkin 2005). The main objective of the present study was to obtain a detailed overview of the protist diversity in periphyton along the Krka River and to determine the potential differences between upstream and downstream sections of the river by using amplicon sequencing of hypervariable region V9 of the 18S rRNA gene.

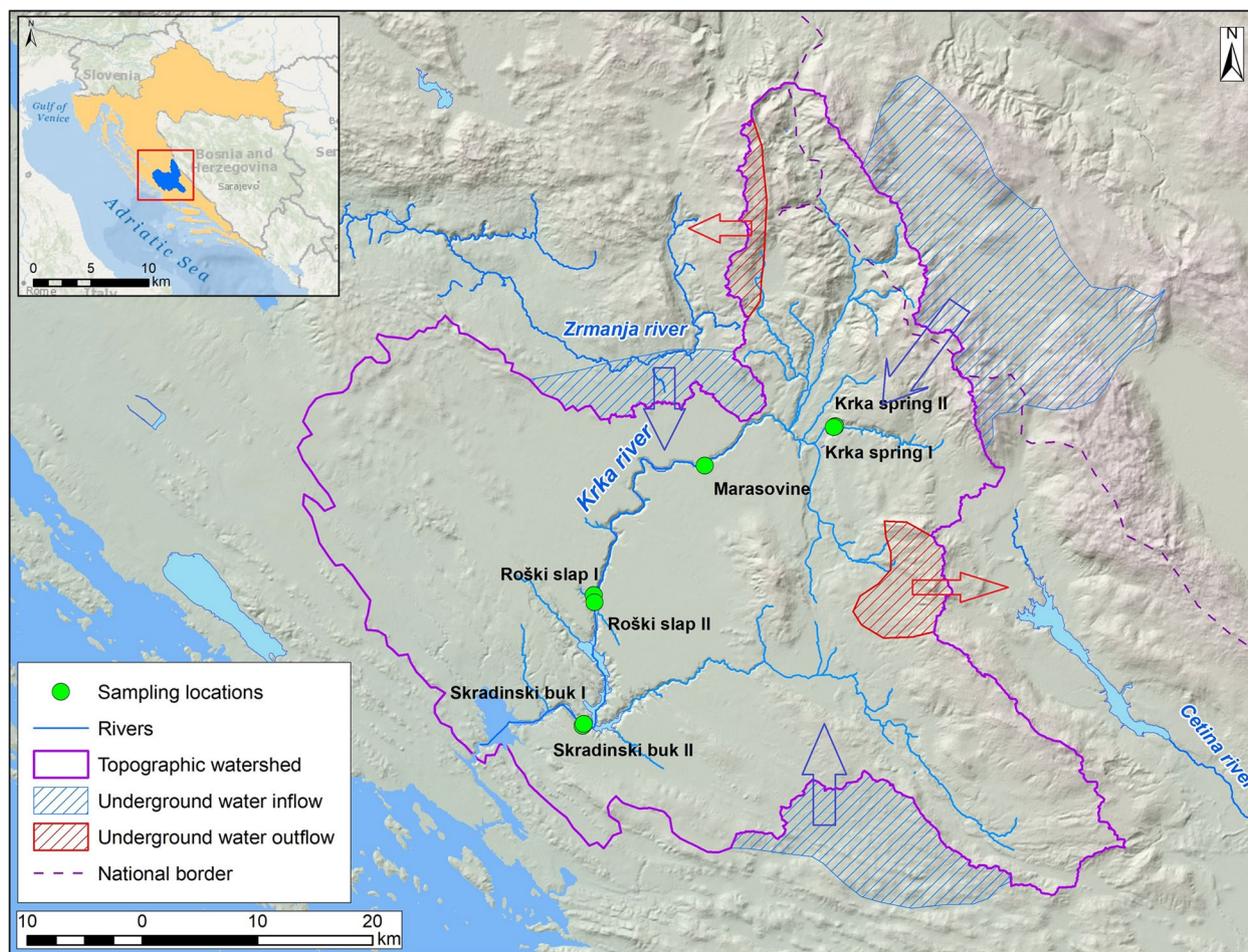
## Materials and methods

### Study area

The Krka River is a 73 km long river situated in the Dinaric region of Dalmatia, Croatia (Cukrov et al. 2008). Along its watercourse, the Krka River is characterized by tufa barriers, a unique form of deposited tufa resulting from the physical and chemical properties of water and biota (Primc-Habdija and Matoničkin 2005; Gulin et al. 2021, 2022). The Krka River springs in the vicinity of Dinara Mountain and flows through the Knin karst polje, creating a series of valleys and canyon formations until reaching the Adriatic Sea near the city of Šibenik (Perica et al. 2017). Along the Krka River, there are 7 larger tufa barriers with alternating lotic and lentic microhabitats with very high and diverse biota. Some parts of the Krka River have been placed under protection due to their special geomorphological, hydrological, and landscape values. In 1985, the Krka River and its catchment area were granted the status of a National Park (Official gazette 1985, 2019). The four sampling locations were chosen, as described in detail in Kulaš et al. (2021). Due to their heterogeneity, the locations Krka spring, Roški slap, and Skradinski buk were sampled at two representative microhabitats (Fig. 1).

### Sampling procedure

Sampling was performed between 21 and 23 September 2017 and included taking three individual samples 10 m apart at each sampling location and selecting each successive habitat upstream of the previously sampled location. At sites where longitudinal sampling was not



**Fig. 1** Map of sampling locations situated at the Krka River, Croatia (author: Ivan Martinić)

possible due to waterfalls, transverse sampling was conducted. A sample was represented by randomly collecting 5 stones or tufa (composite sample) and scraping off the substrate (periphyton) from both light- and dark-exposed sides of tufa/stones at each sampling location. In total, 42 samples for DNA extraction were stored in Falcon tubes (50 mL), placed on ice during transport to the laboratory, and stored at  $-20\text{ }^{\circ}\text{C}$  until further processing.

#### Molecular analysis and bioinformatic processing

DNA extraction, PCR reaction, and bioinformatic processing were performed as previously described in Kulaš et al. (2021). Before the first step of DNA extraction, the samples were centrifuged ( $4000 \times G$  for 1 min) to remove excess water. After the first step, DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The samples were prepared by adding C1 solution in the PowerBead tubes. After preparation of samples, the next step was cell lysis by adding C2 solution and incubating the

samples at  $2\text{--}8\text{ }^{\circ}\text{C}$ . The next step was removal of inhibitors with the C3 solution and again incubation at  $2\text{--}8\text{ }^{\circ}\text{C}$ . Then, DNA was bound with the C4 solution through the MB spin columns. The last two steps were washing the DNA with C5 solution and finally eluting the DNA with  $60\text{ }\mu\text{L}$  of sterile DNA-Free PCR Grade Water instead of the C6 solution. The quality of the extracted DNA was measured using a spectrophotometer (BioSpec Nano, Shimadzu, Kyoto, Japan). From the eDNA, the hypervariable V9 region of the SSU rRNA gene (ca. 130 bp) was amplified using the universal eukaryotic primer pair 1391F ( $5'\text{-GTACACACCGCCCGTC-3}'$ ) and EukB ( $5'\text{-TGATCCTTCTGCAGGTTACCTAC-3}'$ ; Amaral-Zettler et al. 2009), according to the protocol of Stoeck et al. (Stock et al. 2009; Stoeck et al. 2010). The usage of V9 region offers a simple one-step-PCR amplicon library preparation method (Thompson et al. 2017; Minerovic et al. 2020), the ability to capture assemblages especially of photosynthetic organisms (Bradley et al. 2016), a good trade-off between database coverage and taxonomic

resolution, and low sequencing costs (Tanabe et al. 2016). After the PCR reaction sequencing, libraries were prepared using the NEB Next<sup>®</sup> Ultra<sup>™</sup> DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Libraries were sequenced on an Illumina NextSeq platform, generating 150-bp paired-end reads (SeqIT GmbH & Co. KG, Kaiserslautern, Germany).

For demultiplexing (removing barcodes) in the 5' to 3' combination, Cutadapt v1.18 (Martin 2011) was used for raw Illumina reads. After the first step, demultiplexed reads were processed using the DeltaMP pipeline v0.3 (<https://github.com/lentendu/DeltaMP>). In the final steps, sequences were grouped into Operational Taxonomic Units (OTUs) using SWARM v2 (Mahé et al. 2015), and the global pairwise alignments of VSEARCH's were used for taxonomic assignment with the Protist Ribosomal Reference (PR2) database v.4.12.0 and a threshold value of 80% identity (Guillou et al. 2013). A consensus taxonomy with a 60% threshold was created for OTUs with multiple best matches to different taxonomy in the database. OTUs assigned to the Streptophyta, Metazoa, Fungi, unclassified Archaeplastida, unclassified Eukaryota, and unclassified Opisthokonta were removed. Protist OTUs were used for all downstream analysis. Raw demultiplexed reads were deposited at the ENA's Sequence Read Archive and are publicly available under the project number PRJEB39359.

### Statistical analysis

All community analyses were conducted using the Primer v7 software package (Clarke and Gorley 2015). The numbers of reads were transformed using the center-log ratio (clr) transformation (Gloor et al. 2017). A number of recorded taxonomically assigned OTUs ( $S$ ), Margalef ( $d$ ), Shannon-Wiener ( $H'$ ), and Simpson ( $1 - \text{Lambda}$ ) indices were calculated as measures of alpha diversity (Thukral 2017; Magurran 2021). A resemblance matrix based on Bray-Curtis similarities was constructed from the transformed (clr) protist data for the four locations. CLUSTER analysis was used to group the locations according to protist groups adding CLUSTER on shade plot. Shade plot was used to show relationships among clusters of samples and protist groups showing only the major groups which contributed for at least 10% of protist OTUs abundances as calculated by Primer7. The Bray-Curtis (BC) dissimilarity matrices were calculated on the transformed data (clr) and used to measure beta diversity as the distance from individual samples between locations. PERMANOVA permutation test (beta diversity) was assigned to test the significance of individual and combined effects of location on changes in community composition analysed using non-metric multidimensional scaling (NMDS). The ranking of the most common genera for

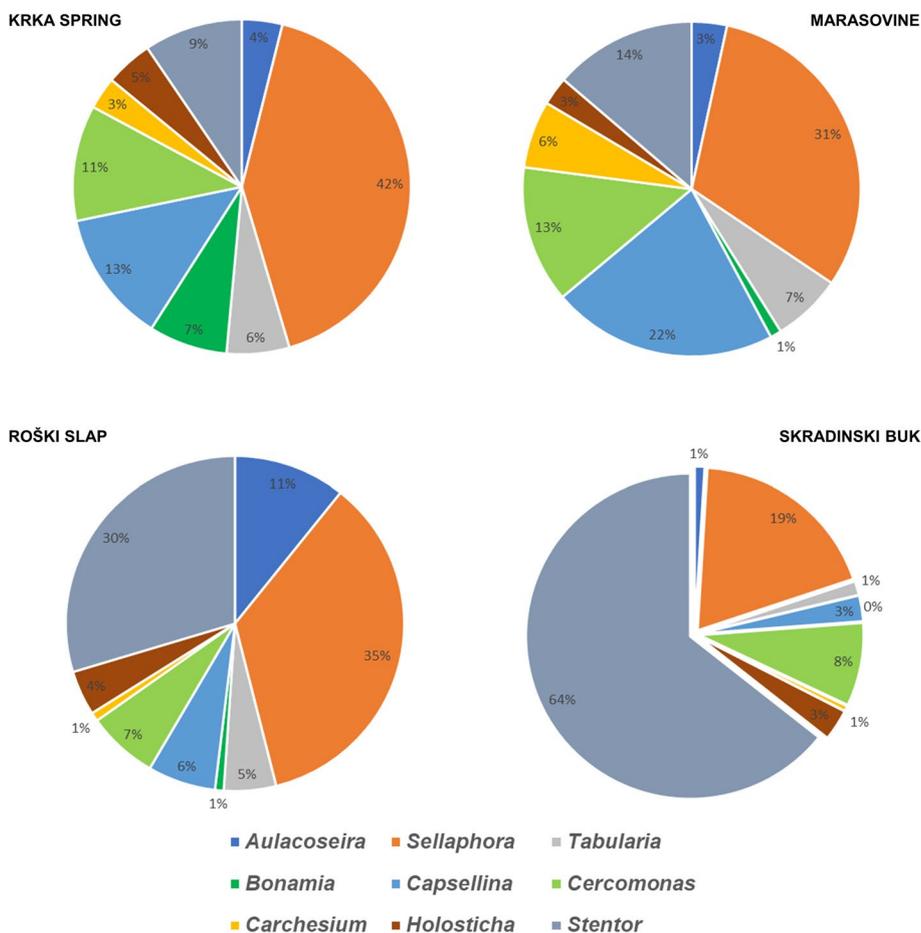
each location within a major protist group was presented in pie charts using Microsoft Office Excel 365 (Microsoft Corporation, USA). Average taxonomic distinctness ( $\Delta+$ ) was determined for each location. The branch lengths between taxonomic rank ( $\omega$ ) were weighted using the taxa richness information gained from the full taxa inventory. Higher branch lengths were assigned to successive taxonomic ranks according to differences in taxa richness, with branch lengths of zero assigned to taxonomic groups with the same taxa richness. Each location's taxa list was compared to the full taxa inventory for the study, and the resulting  $\Delta+$  values were plotted using a funnel plot under the null hypothesis that communities are a random selection from the regional taxa pool, but with probabilities adjusted to account for commonness/rarity (Jones et al. 2011).

## Results

### Diversity of taxonomically assigned protist groups

A total of 42 samples were sequenced, but in three samples, the DNA sequencing reaction failed due to poor quality of the extracted DNA. In the remaining 39 samples, approximately 5,413,607 reads were obtained within 11,295 OTUs for protists (Table S1; Kulaš et al. 2021). The three main groups of protists were taxonomically assigned as follows: Ciliophora clustered into 3724 OTUs, Cercozoa clustered into 1806 OTUs, and Bacillariophyta clustered into 1225 OTUs. Other groups within protists were as follows: Discoba (846 OTUs), Lobosa (579 OTUs), Dinoflagellata (468 OTUs), Pseudofungi (337 OTUs), Chlorophyta (254 OTUs), Mesomycetozoa (223 OTUs), Apicomplexa (216 OTUs), other Ochrophyta (191 OTUs), and other protists clustered into 1402 OTUs (Kulaš et al. 2021). The most abundant OTUs within Ciliophora corresponded to genera *Carchesium*, *Holosticha* and *Stentor*, while within Cercozoa were *Bonamia*, *Capsellina*, and *Cercomonas*. Within Stramenopiles, the most abundant group was Bacillariophyta and the most abundant OTUs corresponded to centric diatom *Aulacoseira*, araphid pennate *Tabularia*, and raphid pennate *Sellaphora* (Fig. 2). Within other recorded groups, the most abundant OTUs corresponded to the following genera: *Neobodo* (Discoba), *Vannella* and *Ptolemeba* (Lobosa), *Peridinium* (Dinoflagellata), *Pythium*, *Phytophthora* and *Saproglenia* (Pseudofungi), *Spermatozopsis* and *Chloroidium* (Chlorophyta), *Amphibocystidium*, *Anurofeca* and *Nuclearia* (Mesomycetozoa), and *Monocystis* (Apicomplexa).

The shade plot included only the major protist groups, which contributed for at least 10% of the protist OTUs abundances (Fig. 3). In terms of coverage, there was an evident difference from the upstream to downstream river section, which increased between locations from



**Fig. 2** The most abundant genera within three major groups at all sampled locations (the most abundant genera were calculated from the abundance of taxonomically assigned OTUs per location)

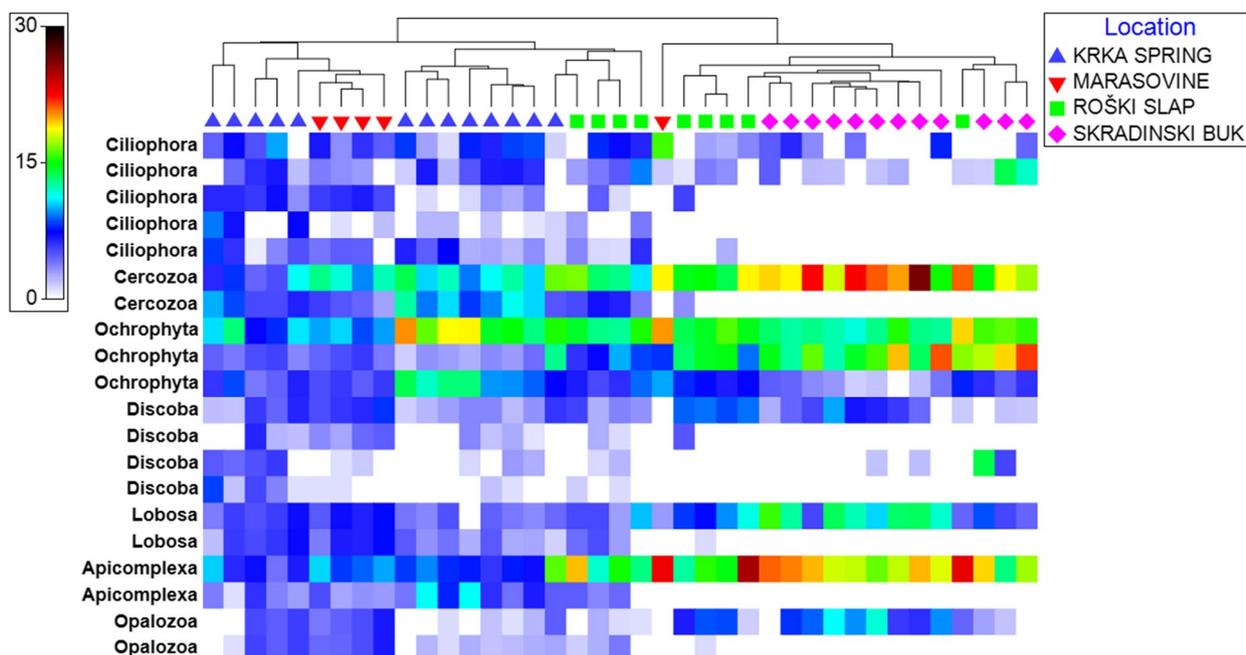
Krka spring to Skradinski buk. At Skradinski buk the most abundant taxa were the representatives of the groups Cercozoa, Ciliophora, Ochrophyta, and Apicomplexa. Taxa from the groups Cercozoa and Ochrophyta were abundant at all locations but demonstrated a downstream increase from Krka spring to Skradinski buk. However, a clear separation between the samples from the upstream to downstream river section was also evident in the remaining cluster, except for the two samples from locations Marasovine and Roški slap.

**Alpha and beta diversity of protist communities along the Krka River**

For each sampling location, the alpha diversity was expressed by calculating the number of recorded taxonomically assigned OTUs (*S*), and Margalef (*d*), Shannon-Wiener (*H'*), and Simpson ( $1 - \lambda$ ) indices (Table S2). All calculated indices showed an increasing trend in alpha diversity from upstream to downstream river section (Fig. 4). Maximum mean values of all indices

were recorded at Skradinski buk, while the minimum values were present at Krka spring. The number of recorded taxonomically assigned OTUs and the Margalef index had a very similar increasing trend, while the Shannon-Wiener index demonstrated a linear increase from Krka spring to Skradinski buk. In general, the Simpson index was the lowest at Krka spring and the highest at Skradinski buk. The differences between the alpha diversity indices were higher in the upstream locations (Krka spring and Marasovine) than in the downstream (Roški slap and Skradinski buk).

Non-metric multidimensional scaling analysis (NMDS) based on Bray-Curtis dissimilarity showed a clear separation of sampling locations for all protist groups included, which was also confirmed by the PERMANOVA test for location effect ( $p = 0.001$ ). A clear clustering on the ordination plot was observed, with the aggregation of samples from the upstream river section and grouping of samples from the downstream section closer together. Samples collected at the upper side of the Roški slap barrier were



**Fig. 3** Shade plot showing relationships among clusters of samples and major protist groups which contributed for at least 10% of the protist OTUs abundances

grouped with samples from the upstream part of the river, while samples collected at the lower side of barrier were grouped with samples from the downstream part of the river. Categorical factors in the NMDS ordination plot were also applied as the drawn trajectories, specifying all groups in the same order by the selected factors. The first factor specifies the order of sampling locations divided by river section parts and the second factor was the location name, allowing the river section progression to be tracked more clearly on the ordination (Fig. 5).

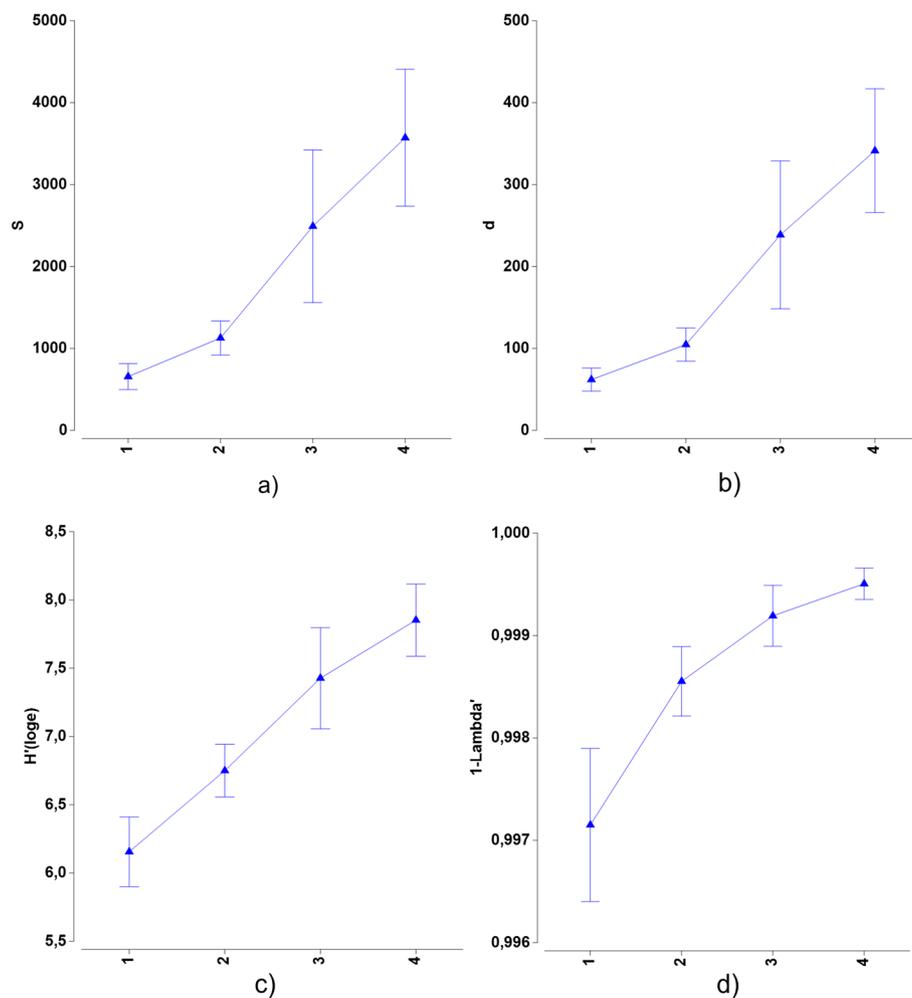
**Average taxonomic distinctness AvTD ( $\Delta+$ )**

According to the number of taxonomically assigned OTUs and taxonomic distinctness, there was a gradient of recorded OTUs and taxonomic distinctness across all four locations (Fig. 6). Krka spring had a total of 2658 recorded OTUs, Marasovine had a total of 2897 OTUs, Roški slap contained 8276 OTUs, and Skradinski buk 9511 OTUs. It was clearly shown that the number of total recorded OTUs increased from the upstream to the downstream river section. In contrast to the increase in the number of recorded OTUs, the taxonomic distinctness decreased from the upstream to the downstream river section. The first two locations of the upstream section (Krka spring and Marasovine) had frequency-based values of  $\Delta+$  above the mean (around 90%) including samples from the upper part of Roški slap. The values of the downstream samples of Roški slap were equal or

above the mean value of  $\Delta+$ , while Skradinski buk had the lowest taxonomic distinctness (below 90%).

**Discussion**

The Krka River is a hotspot for a wide variety of aquatic organisms, especially protists. Tufa barriers provide a favorable substrate for colonization and growth of periphyton, which is an important biomediator in the tufa deposition process (Risse-Buhl and Küssel 2009; Matoničkin Kepčija et al. 2011; Gulin et al. 2021, 2022). Previous studies in the Krka River were based solely on morphological identification of particular protists using the light microscope, such as diatoms and ciliates (Primc-Habdija and Matoničkin 2005; Primc-Habdija et al. 2005; Kralj et al. 2006; Žutinić et al. 2020). Other protist groups are even less studied, especially some groups of algae in the Krka River. Molecular methods provide a powerful tool to facilitate the process and uncover the hidden diversity and ecology of protists (Burki et al. 2021). Nevertheless, the accuracy of taxonomic assignments from short amplicon reads to the species level is still problematic because too many species are missing from the reference database and the target sequences are too small to allow consistent and correct species assignments (Amaral-Zettler et al. 2009; Stoeck et al. 2010). However, it is recognized that metabarcoding on V9 region of the SSU rRNA genes only allows correct identification down

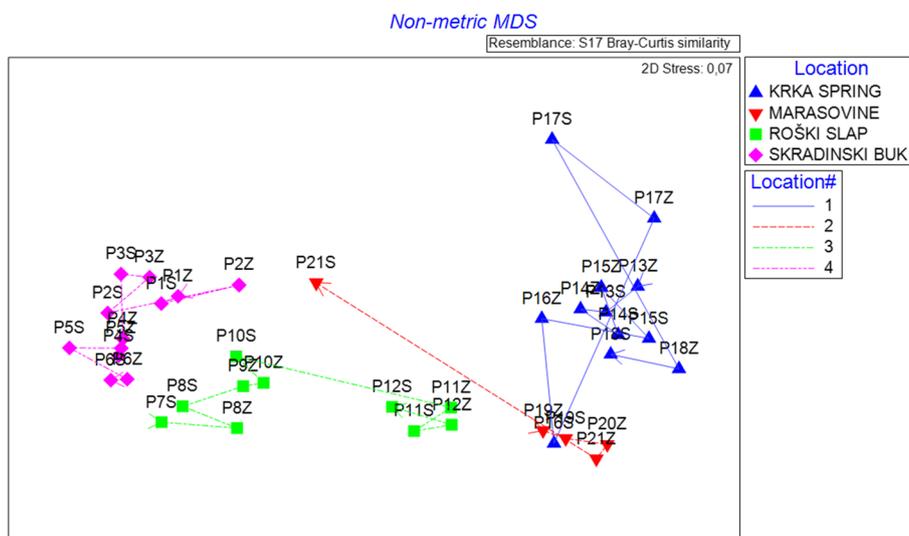


**Fig. 4** The average alpha diversity on each sampling location was expressed by the number of taxonomically assigned OTUs ( $S$ ), Margalef ( $d$ ), Shannon-Wiener ( $H'$ ), and Simpson ( $1-\text{Lambda}'$ ) indices. Error bars denote mean SD, numbers 1 to 4 on x axis denote different sampling locations: 1 = Krka spring, 2 = Marasovine, 3 = Roški slap, and 4 = Skradinski buk

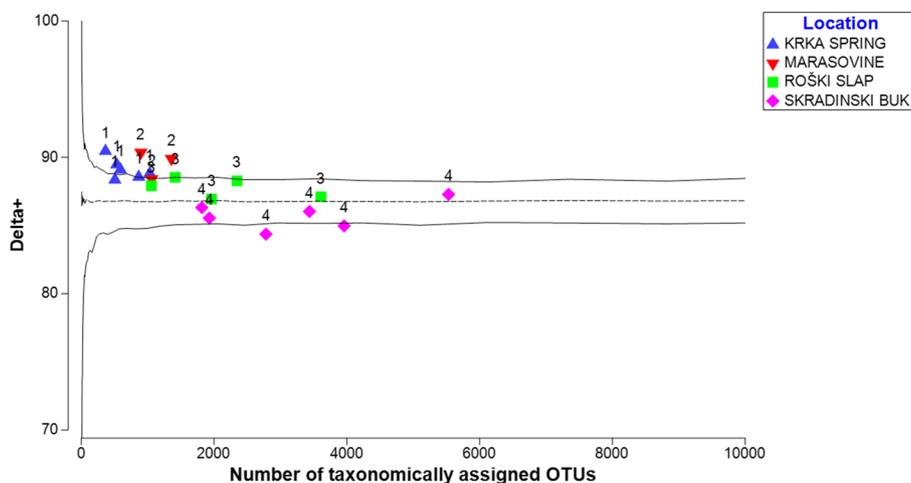
to the genus level, so our analyses of protist diversity were based on genera.

Biodiversity and environmental properties are the fundamental for ecosystem describing (Protasov et al. 2019), and they are highly relevant for environmental protection. The present study provides a deeper insight into the complexity of protists within the periphyton of a karstic river. The Margalef index and the number of recorded taxonomically assigned OTUs showed a very similar trend, as the Margalef index comprises species richness as a measure of biodiversity (Gamito 2010). Species richness can often reflect an independent component of species diversity and variation in abundance of different species/taxa (Hillebrand et al. 2008). The Simpson index and Shannon-Wiener index also displayed a positive linear trend increasing from Krka spring to Skradinski buk, thus indicating that alpha diversity indices provide vital

information in defining species/taxa richness (Jianshuang et al. 2012). The Simpson and Shannon-Wiener indices did not differ between the samples of Roški slap and Marasovine (upstream section). Generally, alpha diversity for all calculated indices increased in the downstream river direction, which may be attributed to increasing habitat diversity downstream. In addition, certain protist groups could be associated with specific areas/microhabitats where there is significant exchange of species from the upstream to the downstream section of the river (Porter and Patton 2016). The main reason of samples at Roški slap barrier being separated can be explained by the structure of the waterfall itself, which is 22.5 m high and extends over a length of 650 m and a width of 400 m, with a special formation called “the cascade” on the upper side and a new additional spring water inflow (Štambuk-Giljanović 2006). Therefore, the first part of the sampling



**Fig. 5** Position of sampling locations in the non-metric multidimensional scaling analysis (NMDS) based on Bray-Curtis similarity index for all protist groups with categorical factor applied as the drawn trajectories, specifying all groups in the same order by the selected factors: order of sampling locations—river section parts (1–4) and location names (line type and color represent different locations). Letters in sample name denote “Z” as light- and “S” as dark-exposed side from sampled tufa/stone



**Fig. 6** Funnel plots for average taxonomic distinctness ( $\Delta+$ ) versus number of taxonomically assigned OTUs. The dashed line indicates the mean  $\Delta+$  for the taxonomically assigned OTUs and the full lines represent 100% probability limits. Color symbols and numbers represent different locations of the river section (1 = Krka spring, 2 = Marasovine, 3 = Roški slap, 4 = Skradinski buk)

was done in the upper part of the Roški slap, which is more connected to the upstream section of the river. The second group of samples was taken in the downstream part of Roški slap, which is more connected with the downstream section of the river up to the last tufa barrier. The first part of Roški slap is geographically closer to the upstream part of the river so the community structure is more similar to location Marasovine, while the second part of Roški slap flows into Lake Visovac, which has the greatest influence on Skradinski buk.

According to the most abundant genera, Roški slap showed similarity with Marasovine in terms of diatom and ciliate dominance. The observed results for the different diversity indices indicated a strong competition between taxa distributed within the two protist groups on these two locations (Estrada-Villegas et al. 2012). In contrast, diatoms demonstrated a clear dominance at the Krka spring, whilst ciliates dominated at Skradinski buk. The dominance of ciliates in the downstream sections of the river may be related to local microhabitat complexity,

as the abundance of various tufa-depositing forms is much higher at the downstream locations, especially Skradinski buk (Bonacci et al. 2017). Although this study did not focus on community structure in terms of defining each species' occurrence and abundance, it could still be observed that over 60% of relative abundance of the genus *Stentor* was detected in Skradinski buk. This location is situated downstream of Lake Visovac and represents a unique lake outlet characterized by higher temperature and pH values and high DOC (dissolved organic carbon) values (Kulaš et al. 2021). As lakes are generally more productive systems (Špoljar et al. 2005), the influence of Lake Visovac is reflected in a higher amount of dissolved organic matter and a correspondingly higher abundance of ciliate OTUs corresponding to filter feeders (Kulaš et al. 2021). These conditions may reflect biotic interactions that depend on DOC and the availability of bacteria as a food source for ciliates (Hauptmann et al. 2016). Caution should always be exercised in interpreting the most common taxa recorded by amplicon sequences. There are still problems in translating abundance from sequence data to biological abundance, as variation in rDNA copy number among taxa may be one of the main reasons for incongruent results for Alveolata sequences (ciliates and dinoflagellates), as they make up the largest proportion of sequence data (Medinger et al. 2010). Thus, the highest abundance of ciliates in a data set does not necessarily mean that ciliates are so abundant here. However, our results were confirmed and compared with the microscopic analysis in the earlier study by Kulaš et al. (2021), where ciliates dominated at Skradinski buk. The dominance of one protist group at Skradinski buk may also represent a forewarning to future studies on the decrease of species diversity, which has become a global problem in river ecosystems (Ge et al. 2022).

In addition to ciliates, Skradinski buk was also dominated by Cercozoa, which was in agreement with the study of Gulin et al. (2021), observed by morphological approach. Cercozoa are one of the most abundant protists in aquatic and soil ecosystems (Fiore-Donno et al. 2020). Being predominantly bacterivorous, it is not uncommon for them to co-occur with bacterivorous ciliates as they share the same food source (Fiore-Donno et al. 2019), especially since Skradinski buk has the highest diversity of microhabitats among the four sampling locations (Bonacci et al. 2017) and parts with newly revitalized streams with intensive soil drainage (Gulin et al. 2021). The dominance of these protist groups at Skradinski buk can also be linked to environmental change or regional species pools (Sundermann et al. 2011), which could alter environmental conditions to make them unsuitable to other groups (Bini et al. 2014; Graco-Roza et al.

2020), limit resource availability (Silva et al. 2018), or facilitate the spread of invasive species that may increase competitive exclusion (Albano et al. 2018). For example, the expansion of invasive plant species *Ailanthus altissima* (Mill.), at Skradinski buk resulted in changes in hydromorphology and a decrease in the abundance of the protozoan community in the periphyton (Gulin et al. 2021). Consequently, the anthropogenic interventions due to increasing influence of tourism in this part of the Krka River (Bonacci et al. 2017) may filter out most of the functional traits and sensitive species resulting in biodiversity loss (Silva et al. 2017). Additionally, there is anthropogenic impact from technological and municipal wastewater, located 2 km upstream from the border of the Krka National Park near the city of Knin. Previous studies (Cukrov et al. 2008; Filipović Marijić et al. 2018) showed that physico-chemical and microbial water parameters indicated that technological and municipal wastewater was a continuous source of nutrients and bacteria, which also posed a risk to the National Park. The main reason for the risk lies in the special characteristics of karstic areas (geomorphology, hydrology), which can contribute to the fact that the sources of pollution can act many kilometers away through a well-developed network of underground watercourses.

The number of taxonomically assigned OTUs and taxonomic distinctness showed an opposing gradient across all sampled locations. These contrasting results may be related to the taxonomic assignment of OTUs, because the number of recorded OTUs does not reflect the same number of different recorded genus or species ranks. Moreover, similarly contrasting results observed by Jones et al. (2011) were interpreted as a result of anthropogenic disturbance through displacement with stress-tolerant species or with competitive interactions among species. Since Skradinski buk is one of the most touristically attractive parts of the Krka National Park, it could be assumed that anthropogenic pressure also affects the protist community.

Generally, beta diversity indicates the level of variation in composition (Koleff et al. 2003). One of its driving factors is habitat heterogeneity, which can create niches favoring certain species over others, as some of the samples were collected on stones and others on tufa (Astorga et al. 2014). It can help clarify processes associated with community composition, which typically break down into taxa exchange and richness differences in gain or loss (Ge et al. 2022). Interestingly, the location effect was also confirmed by NMDS analysis using the PERMANOVA test, which grouped/clustered samples according to locations. The location effect can be explained by the physical structure of the habitat. Tufa barriers are a product of calcium carbonate deposition where physical and chemical properties of water, geologic substrate, and biota play

an intertwining role. All of these can influence the biotic community through hydrogeological processes that include both subsurface and surface water flow, often with high flow velocities and discharge (Tamburini and Menichetti 2020).

The next main driver of diversity composition is productivity, where more productive areas support higher regional diversity. Productivity is linked to beta diversity and can result in high regional diversity and increased niche specialization, especially for particularly rare species (Currie et al. 2004). In the current case, this driver is connected with the Krka spring zone area. Previous studies have confirmed the spring zones of karstic rivers being inhabited by various organisms (Mogna et al. 2015; Lai et al. 2020), particularly diatoms as one of the most diverse groups (Cantonati et al. 2012). The group Ochrophyta were recorded as the most abundant primary producers at the Krka spring. About 50% in relative abundance of the most abundant protist genera in Ochrophyta belonged to diatoms, with the genus *Sellaphora* accounting for the largest proportion, as was also confirmed with DNA sequencing using the *rbcl* gene marker (Kulaš et al. 2022). Genus *Sellaphora* belongs to small-growing diatoms, and small-sized species usually occur in conditions under lower nutrient conditions which is the case with spring areas (Cantonati et al. 2012; Kulaš et al. 2020).

## Conclusion

This study provided a deeper insight into protist diversity based on genus rank from the upstream to downstream parts of the karstic Krka River observed using a molecular approach. Previous studies have already shown that analyses based on genera mirror those based on species and may be sufficient for studying community structure (Bevilacqua et al. 2012; Smith et al. 2014). In addition, genera have inherently larger ranges than species, so regional/location similarities are necessarily greater at the genus level than at the species level (Bevilacqua et al. 2012; Smith et al. 2014). In this study, however, analyses were not based on longitudinal gradient, but protist community composition showed differences along the river between upstream and downstream through an increasing trend in alpha diversity indices and grouping by location in beta diversity. Combining alpha and beta diversity can provide better insight into protist community structure. This kind of valid biological data is of great importance for the conservation of karstic environments but can also indicate declines in biodiversity and allow for effective protection of aquatic karst habitats in future management.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13213-023-01712-z>.

**Additional file 1: Table S1.** Operational Taxonomic Units (“OTUs”) of 18S-V9 rRNA from 39 samples. **Table S2.** Results of alpha diversity indices per samples and sampling location.

## Acknowledgements

Mr. sc. Gordana Goreta is thanked for her support with the survey of project at Krka River. We thank Professor Thorsten Stoeck for generously providing laboratory facilities and for sharing his invaluable knowledge and experience in the application of metabarcoding. Dr. Guillaume Lentendu is thanked for his help in bioinformatics analysis. Our colleague Ivan Martinić is thanked for his kind help in preparing the map of study area.

## Authors' contributions

A.K. performed the formal analysis, conceptualization, data curation, and visualization and was a major contributor in writing the manuscript—preparation of the original draft; P.Ž. contributed to the validation, investigation, review, and editing of the manuscript; V.G.B. contributed to the methodology preparation, review, and editing of the manuscript; R.M.K. contributed to the investigation, review, and editing of the manuscript; M.S.P. contributed to the methodology preparation, review, and editing of the manuscript; S.O. contributed to the methodology preparation, review, and editing of the manuscript; I.S.P. contributed to the validation, review, and editing of the manuscript; T.M. contributed to the review and editing of the manuscript; M.G.U., supervised and contributed to the conception of the project, validation, finding resources and acquisition, and preparation of the original draft. The authors read and approved the final manuscript.

## Funding

This study was financially supported by the project “Assessment of the ecological status of the Krka River using DNA metabarcoding,” financed by the NP Krka, SO, and KK were partially supported under the project STIM – REI, Contract Number: KK.01.1.1.01.0003, a project funded by the European Union through the European Regional Development Fund—the Operational Programme Competitiveness and Cohesion 2014–2020 (KK.01.1.1.01). Part of this study was financial supported by FEMS grant FEMS-GO-2018-127.

## Availability of data and materials

Raw demultiplexed reads were deposited at the ENA's Sequence Read Archive and are publicly available under the project number PRJEB39359.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

Received: 28 September 2022 Accepted: 23 January 2023

Published online: 18 February 2023

## References

Albano PG, Gallmetzer I, Haselmair A, Tomašových A, Stachowitsch M, Zschin M (2018) Historical ecology of a biological invasion: the interplay of eutrophication and pollution determines time lags in establishment and detection. *Biol Invasions* 20:1417–1430. <https://doi.org/10.1007/s10530-017-1634-7>

- Amaral-Zettler LA, McCliment EA, Ducklow HW, Huse SM (2009) A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. *PLoS ONE* 4:e6372. <https://doi.org/10.1371/journal.pone.0006372>
- Astorga A, Death R, Death F, Paavola R, Chakraborty M, Muotka T (2014) Habitat heterogeneity drives the geographical distribution of beta diversity: the case of New Zealand stream invertebrates. *Ecol Evol* 4:2693–2702. <https://doi.org/10.1002/ece3.1124>
- Bevilacqua S, Terlizzi A, Claudet J, Fraschetti S, Boero F (2012) Taxonomic relatedness does not matter for species surrogacy in the assessment of community responses to environmental drivers: *taxonomic relatedness and species surrogates*. *J Appl Ecol* 49:357–366. <https://doi.org/10.1111/j.1365-2664.2011.02096.x>
- Bini LM, Landeiro VL, Padiál AA, Siqueira T, Heino J (2014) Nutrient enrichment is related to two facets of beta diversity for stream invertebrates across the United States. *Ecology* 95:1569–1578. <https://doi.org/10.1890/13-0656.1>
- Bock C, Jensen M, Forster D, Marks S, Nuy J, Psenner R, Beisser D, Boenigk J (2020) Factors shaping community patterns of protists and bacteria on a European scale. *Environ Microbiol* 22:2243–2260. <https://doi.org/10.1111/1462-2920.14992>
- Bonacci O, Ljubenković I, Roje-Bonacci T (2006) Karst flash floods: an example from the Dinaric karst (Croatia). *Nat Hazards Earth Syst Sci* 6:195–203. <https://doi.org/10.5194/nhess-6-195-2006>
- Bonacci O, Željković I, Galić A (2013) Karst rivers' particularity: an example from Dinaric karst (Croatia/Bosnia and Herzegovina). *Environ Earth Sci* 70:963–974. <https://doi.org/10.1007/s12665-012-2187-9>
- Bonacci O, Andrić I, Roje-Bonacci T (2017) Hydrological analysis of Skradinski Buk tufa waterfall (Krka River, Dinaric karst, Croatia). *Environ Earth Sci* 76:669. <https://doi.org/10.1007/s12665-017-7023-9>
- Bradley IM, Pinto AJ, Guest JS (2016) Design and evaluation of illumina MiSeq-Compatible, 18S rRNA gene-specific primers for improved characterization of mixed phototrophic communities. *Appl Environ Microbiol* 82:5878–5891. <https://doi.org/10.1128/AEM.01630-16>
- Burki F, Sandin MM, Jany M (2021) Diversity and ecology of protists revealed by metabarcoding. *Curr Biol* 31:R1267–R1280. <https://doi.org/10.1016/j.cub.2021.07.066>
- Cantonati M, Füreder L, Gerecke R, Jüttner I, Cox EJ (2012) Crenic habitats, hotspots for freshwater biodiversity conservation: toward an understanding of their ecology. *Freshw Sci* 31:463–480. <https://doi.org/10.1899/11-111.1>
- Chen J, Wang P, Wang C, Wang X, Miao L, Liu S, Yuan Q (2018) Bacterial communities in riparian sediments: a large-scale longitudinal distribution pattern and response to dam construction. *Front Microbiol* 9:999. <https://doi.org/10.3389/fmicb.2018.00999>
- Clarke KR, Gorley RN (2015) PRIMER v7: user manual/tutorial. PRIMER-E Plymouth
- Cukrov N, Čmuk P, Plakar M, Omanović D (2008) Spatial distribution of trace metals in the Krka River, Croatia: an example of the self-purification. *Chemosphere* 72:1559–1566. <https://doi.org/10.1016/j.chemosphere.2008.04.038>
- Currie DJ, Mittelbach GG, Cornell HV, Field R, Guegan JF, Hawkins BA, Kaufman DM, Kerr JT, Oberdorff T, O'Brien E, Turner JRG (2004) Predictions and tests of climate-based hypotheses of broad-scale variation in taxonomic richness. *Ecol Letters* 7:1121–1134. <https://doi.org/10.1111/j.1461-0248.2004.00671.x>
- Darwall W, Carrizo S, Numa C, Barrios V, Freyhof J, Smith K (2014) Freshwater Key Biodiversity Areas in the Mediterranean Basin Hotspot: Informing species conservation and development planning in freshwater ecosystems. Cambridge, UK and Malaga, Spain: IUCN. x, pp 86. <https://doi.org/10.2305/IUCN.CH.2014.SSC-OP.52.en>
- Englmaier GK, Hayes DS, Meulenbroek P, Terefe Y, Lakew A, Tesfaye G, Waidbacher H, Malicky H, Wubie A, Leitner P, Graf W (2020) Longitudinal river zonation in the tropics: examples of fish and caddisflies from the endorheic Awash River, Ethiopia. *Hydrobiologia* 847:4063–4090. <https://doi.org/10.1007/s10750-020-04400-0>
- Estrada-Villegas S, McGill BJ, Kalko EKV (2012) Determinants of species evenness in a neotropical bat ensemble. *Oikos* 121:927–941. <https://doi.org/10.1111/j.1600-0706.2011.19837.x>
- Filipović Marijić V, Kapetanović D, Dragun Z, Valić D, Krasnići N, Redžović Z, Grgić I, Žunić J, Kružličević D, Nemeček P, Ivanković D, Vardić Smržlić I, Erk M (2018) Influence of technological and municipal wastewaters on vulnerable karst riverine system, Krka River in Croatia. *Environ Sci Pollut Res* 25:4715–4727. <https://doi.org/10.1007/s11356-017-0789-1>
- Fiore-Donno AM, Richter-Heitmann T, Degruno F, Dumack K, Regan KM, Marhan S, Boeddinghaus RS, Friedrich MW, Kandel E, Bonkowski M (2019) Functional traits and spatio-temporal structure of a major group of soil protists (Rhizaria: Cercozoa) in a temperate grassland. *Front Microbiol* 10:1332. <https://doi.org/10.3389/fmicb.2019.01332>
- Fiore-Donno AM, Richter-Heitmann T, Bonkowski M (2020) Contrasting responses of protistan plant parasites and phagotrophs to ecosystems, land management and soil properties. *Front Microbiol* 11:1823. <https://doi.org/10.3389/fmicb.2020.01823>
- Gamito S (2010) Caution is needed when applying Margalef diversity index. *Ecological Indicators* 10:550–551. <https://doi.org/10.1016/j.ecolind.2009.07.006>
- Ge F, Ma Z, Chen B, Wang Y, Lu X, An S, Zhang D, Zhang W, Yu W, Han W, Yu X, Liu Z, Kuang F (2022) Phytoplankton species diversity patterns and associated driving factors in China's Jiulong River estuary: roles that nutrients and nutrient ratios play. *Front Mar Sci* 9:829285. <https://doi.org/10.3389/fmars.2022.829285>
- Gloriga Udovič M, Kulaš A, Šušnjara M, Arapov J, Blanco S, Levkov Z (2022) *Cymbopleura amacula* stat nov. et. nom. nov. (Bacillariophyceae)—a rare diatom species from a karst river in Croatia. *Phytotaxa* 532:2. <https://doi.org/10.11646/phytotaxa.532.2>
- Gloriga Udovič M, Šušnjara M, Kulaš A, Goretta G, Arapov J, Levkov Z (2023) One new species of *Aneumastus* D.G. Mann et Stickle (Bacillariophyceae) from Krka River, Croatia. *Fottea* 23(1):1–X. <https://doi.org/10.5507/fot.2022.010>
- Gloor GB, MacKlaim JM, Pawlowsky-Glahn V, Egozcue JJ (2017) Microbiome datasets are compositional: and this is not optional. *Front Microbiol* 8:2224. <https://doi.org/10.3389/fmicb.2017.02224>
- Graco-Roza C, Santos JBO, Huszar VLM, Domingos P, Soininen J, Marinho MM (2020) Downstream transport processes modulate the effects of environmental heterogeneity on riverine phytoplankton. *Sci Total Environ* 703:135519. <https://doi.org/10.1016/j.scitotenv.2019.135519>
- Gran-Stadniczeško S, Egge E, Hostyeva V, Logares R, Eikrem W, Edvardsen B (2019) Protist diversity and seasonal dynamics in skagerrak plankton communities as revealed by metabarcoding and microscopy. *J Eukaryot Microbiol* 66:494–513. <https://doi.org/10.1111/jeu.12700>
- Guillou L, Bachar D, Audic S, Bass D, Berny C, Boute C, Burgaud G, de Vargas C, Decelle J, Del Campo J, Dolan JR, Dunthorn IM, Edvardsen B, Holzmann M, Kooistra WHCF, Lara E, Le Bescot N, Logares R, Mahé F, Montresor M, Morard R, Not F, Pawlowski J, Probert I, Sauvadet AL, Siano R, Stoeck T, Vaulot D, Zimmermann P, Christenc R (2013) The protist ribosomal reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. *Nucleic Acids Res* 41:D597–604. <https://doi.org/10.1093/nar/gks1160>
- Gulin V, Matoničkin Kepčija R, Sertić Perić M, Felja J, Fajković H, Križnjak K (2021) Environmental and periphyton response to stream revitalization – a pilot study from a tufa barrier. *Ecol Indic* 126:107629. <https://doi.org/10.1016/j.ecolind.2021.107629>
- Gulin V, Vlaičević B, Sertić Perić M, Rebrina F, Matoničkin Kepčija R (2022) Taxonomic and functional metrics of ciliates and amoeboid protists in response to stream revitalization. *Front Microbiol* 13:842395. <https://doi.org/10.3389/fmicb.2022.842395>
- Hauptmann AL, Markussen TN, Stibal M, Olsen NS, Elberling B, Bælum J, Sicheritz-Pontén T, Jacobsen CS (2016) Upstream freshwater and terrestrial sources are differentially reflected in the bacterial community structure along a small arctic river and its estuary. *Front Microbiol* 7:1474. <https://doi.org/10.3389/fmicb.2016.01474>
- Hillebrand H, Bennett DM, Cadotte MW (2008) Consequences of dominance: a review of evenness effects on local and regional ecosystem processes. *Ecology* 89:1510–1520. <https://doi.org/10.1890/07-1053.1>
- Ivković M, Pont A (2015) New records of Muscidae (Diptera) from Mediterranean countries. *ZooKeys* 496:131–144. <https://doi.org/10.3897/zookeys.496.9445>
- Ivković M, Pont AC (2016) Long-time emergence patterns of Limnophora species (Diptera, Muscidae) in specific karst habitats: tufa barriers. *Limnologica* 61:29–35. <https://doi.org/10.1016/j.limno.2016.09.003>
- Jäger CG, Borchardt D (2018) Longitudinal patterns and response lengths of algae in riverine ecosystems: a model analysis emphasising benthic-pelagic interactions. *J Theor Biol* 442:66–78. <https://doi.org/10.1016/j.jtbi.2018.01.009>

- Jianshuang W, Xianzhou Z, Zhenxi S, Peili S, Chengqun Y, Minghua S, Xiaojia L (2012) Species richness and diversity of alpine grasslands on the northern tibetan plateau: effects of grazing exclusion and growing season precipitation. *J Resour Ecol* 3:236. <https://doi.org/10.5814/j.issn.1674-764x.2012.03.006>
- Jones AM, Berkelmans R, Houston WF (2011) Species richness and community structure on a high latitude reef: implications for conservation and management. *Diversity* 3:329–355. <https://doi.org/10.3390/d3030329>
- Koleff P, Gaston KJ, Lennon JJ (2003) Measuring beta diversity for presence-absence data. *J Anim Ecology* 72:367–382. <https://doi.org/10.1046/j.1365-2656.2003.00710.x>
- Koller R, Robin C, Bonkowski M, Ruess L, Scheu S (2013) Litter quality as driving factor for plant nutrition via grazing of protozoa on soil microorganisms. *FEMS Microbiol Ecol* 85:241–250. <https://doi.org/10.1111/1574-6941.12113>
- Kralj K, Plenković-Moraj A, Gligora M, Primc-Habdija B, Šipoš L (2006) Structure of periphytic community on artificial substrata: influence of depth, slide orientation and colonization time in karstic Lake Visovačko, Croatia. *Hydrobiologia* 560:249–258. <https://doi.org/10.1007/s10750-005-1330-y>
- Kulaš A, Gligora Udovič M, Ector L, Van de Vijver B (2020) Analysis of the type material of *Achnanthes hauckiana* Grunow (Achnanthesaceae, Bacillariophyceae). *Botany Letters* 167:439–452. <https://doi.org/10.1080/23818107.2020.1808527>
- Kulaš A, Gulin V, Matoničkin Kepčija R, Žutinić P, Sertić Perić M, Orlić S, Kajan K, Stoeck T, Lentendu G, Čanjevac I, Martinić I, Gligora Udovič M (2021) Ciliates (Alveolata, Ciliophora) as bioindicators of environmental pressure: A karstic river case. *Ecol Indic* 124:107430. <https://doi.org/10.1016/j.ecoli.2021.107430>
- Kulaš A, Gligora Udovič M, Tapolczai K, Žutinić P, Orlić S, Levkov Z (2022) Diatom eDNA metabarcoding and morphological methods for bioassessment of karstic river. *Sci Total Environ* 829:154536. <https://doi.org/10.1016/j.scitotenv.2022.154536>
- Lai GG, Burato S, Padedda BM, Zorza R, Pizzul E, Delgado C, Lugliè A, Cantonati M (2019) Diatom biodiversity in Karst Springs of Mediterranean Geographic Areas with Contrasting Characteristics: Islands vs Mainland. *Water* 11:2602. <https://doi.org/10.3390/w11122602>
- Lai GG, Padedda BM, Ector L, Wetzel CE, Lugliè A, Cantonati M (2020) Mediterranean karst springs: diatom biodiversity hotspots under the pressure of hydrological fluctuation and nutrient enrichment. *Plant Biosyst Int J Deal Aspect Plant Biol* 154:673–684. <https://doi.org/10.1080/11263504.2019.1674402>
- Magurran AE (2021) Measuring biological diversity. *Current Biology* 31:R1174–R1177. <https://doi.org/10.1016/j.cub.2021.07.049>
- Mahé F, Rognes T, Quince C, de Vargas C, Dunthorn M (2015) Swarm v2: highly-scalable and high-resolution amplicon clustering. *PeerJ* 3:e1420. <https://doi.org/10.7717/peerj.1420>
- Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J* 17:10. <https://doi.org/10.14806/ej.17.1.200>
- Massana R, Gobet A, Audic S, Bass D, Bittner L, Boutte C, Chambouvet A, Christen R, Claverie JM, Decelle J, Dolan JR, Dunthorn M, Edvardsen B, Forn I, Forster D, Guillou L, Jaillon O, Kooistra WHCF, Logares R, Not F, Ogata H, Pawłowski J, Pernice MC, Probert I, Romac S, Richards T, Santini S, Shalchian-Tabrizi K, Simon N, Stoeck T, Vaulot D, Zingone A, de Vargas C (2015) Marine protist diversity in European coastal waters and sediments as revealed by high-throughput sequencing: protist diversity in European coastal areas. *Environ Microbiol* 17:4035–4049. <https://doi.org/10.1111/1462-2920.12955>
- Matoničkin Kepčija R, Miliša M, Sertić Perić M, Matijić Cvjetović M, Primc-Habdija B (2011) Response of periphyton to nutrient addition in a tufa-depositing environment. *Aquat Microb Ecol* 65:183–195. <https://doi.org/10.3354/ame01545>
- Medinger R, Nolte V, Pandey RV, Jost S, Ottenwälder B, Schlötterer C, Boenigk J (2010) Diversity in a hidden world: potential and limitation of next-generation sequencing for surveys of molecular diversity of eukaryotic microorganisms. *Molecular Ecology* 19:32–40. <https://doi.org/10.1111/j.1365-294X.2009.04478.x>
- Metz S, Huber P, Accattatis V, Lopes dos Santos A, Bigeard E, Unrein F, Chambouvet A, Not F, Lara E, Devercelli M (2022) Freshwater protists: unveiling the unexplored in a large floodplain system. *Environ Microbiol* 24:1731–1745. <https://doi.org/10.1111/1462-2920.15838>
- Minerovic AD, Potapova MG, Sales CM, Price JR, Enache MD (2020) 18S–V9 DNA metabarcoding detects the effect of water-quality impairment on stream biofilm eukaryotic assemblages. *Ecol Indic* 113:106225. <https://doi.org/10.1016/j.ecolind.2020.106225>
- Mogna M, Cantonati M, Andreucci F, Angeli N, Berta G, Miserere L (2015) Diatom communities and vegetation of springs in the south-western Alps. *Acta Botanica Croatica* 74:265–285. <https://doi.org/10.1515/botcro-2015-0024>
- Niesenbaum RA (2019) The integration of conservation, biodiversity, and sustainability. *Sustainability* 11:4676. <https://doi.org/10.3390/su11174676>
- Official gazette, (1985) Law on the Proclamation of the National Park “Krka” Official gazette (2019) Regulation on water quality standard. pp 45 ([https://narodne-novine.nn.hr/clanci/sluzbeni/2019\\_10\\_96\\_1879.html](https://narodne-novine.nn.hr/clanci/sluzbeni/2019_10_96_1879.html))
- Perica D, Orešić D, Trajbar S (2017) Geomorfološka obilježja doline i poriječja rijeke Krke s osvrtom na dio od Knina do Bilušića buka. *Geodria* 10:131–156. <https://doi.org/10.15291/geoadria.55>
- Porter CP, Patton T (2016) Patterns of fish diversity and community structure along the longitudinal gradient of the Kiamichi River in Southeastern Oklahoma. *Proc Okla Acad Sci* 95:104–118
- Primc-Habdija B, Matoničkin R (2005) A new freshwater folliculinid (Ciliophora) from the karstic region of Croatia. *Eur J Protistol* 41:37–43. <https://doi.org/10.1016/j.ejop.2004.09.004>
- Primc-Habdija B, Habdija I, Matoničkin R, Špoljar Maria (2005) Development of ciliate community on artificial substrates associated with vertical gradients of environmental conditions in a karstic lake. *Arch. Hydrobiol.* 164:513–527. <https://doi.org/10.1127/0003-9136/2005/0164-0513>
- Protasov A, Barinova S, Novoselova T, Sylaieva A (2019) The aquatic organisms diversity, community structure, and environmental conditions. *Diversity* 11:190. <https://doi.org/10.3390/d11100190>
- Risse-Buhl U, Küsel K (2009) Colonization dynamics of biofilm-associated ciliate morphotypes at different flow velocities. *Eur J Protistol* 45:64–76. <https://doi.org/10.1016/j.ejop.2008.08.001>
- Rusanov AG, Khromov VM (2016) Longitudinal distribution of periphyton algae in the Moskva river under eutrophication. *Water Resour* 43:513–521. <https://doi.org/10.1134/S0097807816030131>
- Selosse MA, Charpin M, Not F (2017) Mixotrophy everywhere on land and in water: the *grand écart* hypothesis. *Ecol Lett* 20:246–263. <https://doi.org/10.1111/ele.12714>
- Silva DRO, Herlihy AT, Hughes RM, Callisto M (2017) An improved macroinvertebrate multimetric index for the assessment of Wadeable streams in the neotropical savanna. *Ecol Indic* 81:514–525. <https://doi.org/10.1016/j.ecolind.2017.06.017>
- Silva DRO, Herlihy AT, Hughes RM, Macedo DR, Callisto M (2018) Assessing the extent and relative risk of aquatic stressors on stream macroinvertebrate assemblages in the neotropical savanna. *Sci Total Environ* 633:179–188. <https://doi.org/10.1016/j.scitotenv.2018.03.127>
- Smith HL, Anderson MJ, Gillanders BM, Connell SD (2014) Longitudinal variation and effects of habitat on biodiversity of Australasian temperate reef fishes. *J Biogeogr* 41:2128–2139. <https://doi.org/10.1111/jbi.12359>
- Song Y, Cheng F, Ren P, Wang Z, Xie S (2019) Longitudinal recovery gradients of drifting larval fish assemblages in the middle reach of the Yangtze River: impact of the Three Gorges Dam and conservation implementation. *Can J Fish Aquat Sci* 76:2256–2267. <https://doi.org/10.1139/cjfas-2018-0409>
- Špoljar M, Habdija I, Primc-Habdija B, Šipoš L (2005) Impact of environmental variables and food availability on rotifer assemblage in the Karstic Barrage Lake Visovac (Krka River, Croatia). *Internat Rev Hydrobiol* 90:555–579. <https://doi.org/10.1002/iroh.200510791>
- Štambuk-Giljanović N (2006) *Vode Dalmacije*. Institute for Public Health of Split, 588 pp, Split, Croatia (in Croatian language)
- Stock A, Jürgens K, Bunge J, Stoeck T (2009) Protistan diversity in suboxic and anoxic waters of the Gotland Deep (Baltic Sea) as revealed by 18S rRNA clone libraries. *Aquat Microb Ecol* 55:267–284. <https://doi.org/10.3354/ame01301>
- Stoeck T, Bass D, Nebel M, Christen R, Jones MDM, Breiner HW, Richards TA (2010) Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. *Molecular Ecology* 19:21–31. <https://doi.org/10.1111/j.1365-294X.2009.04480.x>

- Sundermann A, Stoll S, Haase P (2011) River restoration success depends on the species pool of the immediate surroundings. *Ecol Appl* 21:1962–1971. <https://doi.org/10.1890/10-0607.1>
- Sutela T, Vehanen T, Jounela P (2020) Longitudinal patterns of fish assemblages in European boreal streams. *Hydrobiologia* 847:3277–3290. <https://doi.org/10.1007/s10750-020-04330-x>
- Tamburini A, Menichetti M (2020) Groundwater Circulation in Fractured and Karstic Aquifers of the Umbria-Marche Apennine. *Water* 12:1039. <https://doi.org/10.3390/w12041039>
- Tanabe AS, Nagai S, Hida K, Yasuike M, Fujiwara A, Nakamura Y, Takano Y, Katakura S (2016) Comparative study of the validity of three regions of the 18S-rRNA gene for massively parallel sequencing-based monitoring of the planktonic eukaryote community. *Mol Ecol Resour* 16:402–414. <https://doi.org/10.1111/1755-0998.12459>
- Thompson LR, Sanders JG, McDonald D, Amir A, Ladau J, Locey K, Prill RJ, Tripathi A, Gibbons SM, Ackermann G, Navas-Molina JA, Janssen S, Kopylova E, Vázquez-Baeza Y, González A, Morton JT, Mirarab S, Zech XuZ, Jiang L, Haroon MF, Kanbar J, Zhu Q, Jin Song S, Kosciolk T, Bokulich NA, Lefler J, Brislawn CJ, Humphrey G, Owens SM, Hampton-Marcell J, McKenzie V, Fierer N, Fuhrman JA, Clauet A, Stevens RL, Shade A, Pollard KS, Goodwin KD, Jansson JK, Knight R, Earth Microbiome Project Consortium (2017) A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* 551:457–463. <https://doi.org/10.1038/nature24621>
- Thukral AK (2017) A review on measurement of Alpha diversity in biology. *Intern Jour Contemp Microbiol* 54:1. <https://doi.org/10.5958/2395-146X.2017.00001.1>
- Tierno de Figueroa JM, López-Rodríguez MJ, Fenoglio S, Sánchez-Castillo P, Fochetti R (2013) Freshwater biodiversity in the rivers of the Mediterranean Basin. *Hydrobiologia* 719:137–186. <https://doi.org/10.1007/s10750-012-1281-z>
- Vilenica M, Bilić M, Mičetić Stanković V, Kučinić M (2018) Mayfly ecological traits in a European karst spring: species, microhabitats and life histories. *Commun Ecol* 19:248–258. <https://doi.org/10.1556/168.2018.19.3.6>
- Watermeyer KE, Guillera-Arroita G, Bal P, Burgass MJ, Bland LM, Collen B, Hallam C, Kelly LT, McCarthy MA, Regan TJ, Stevenson S, Wintle BA, Nicholson E (2021) Using decision science to evaluate global biodiversity indices. *Conserv Biol* 35:492–501. <https://doi.org/10.1111/cobi.13574>
- West PT, Probst AJ, Grigoriev IV, Thomas BC, Banfield JF (2018) Genome-reconstruction for eukaryotes from complex natural microbial communities. *Genome Res* 28:569–580. <https://doi.org/10.1101/gr.228429.117>
- Žutinić P, Kulaš A, Levkov Z, Šušnjara M, Orlić S, Kukić S, Goreta G, Valić D, Gligora Udovič M (2020) Ecological status assessment using periphytic diatom communities - case study Krka River. *Macedonian J Ecol Environ* 22:29–44

## Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more [biomedcentral.com/submissions](https://biomedcentral.com/submissions)

