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Ciliates (Alveolata, Ciliophora) as bioindicators of environmental pressure: A karstic river case

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ABSTRACT

Ciliates are single celled eukaryotes recognized as key players in the microbial loop of aquatic ecosystems. The present study was carried out on the Krka River (Croatia), a karst freshwater ecosystem characterized by tufa barriers, biomineralization and highly diverse aquatic communities. The main aims of the study were to investigate ciliate community structure in the biofilm (i.e. periphyton) samples collected from light- and dark-exposed lithified tufa/stones. Furthermore, by establishing links between ciliate community patterns and environmental parameters, we aimed to assess the bioindicator potential of specific ciliate taxa for environmental monitoring of freshwater habitats. The periphyton sampling was performed at four representative sites of the river source, upstream, middle and downstream river sections. Ciliate community was investigated via traditional microscopy analyses and environmental DNA (eDNA) metabarcoding (Illumina sequencing of the hypervariable V9-region of the SSU rRNA gene). The molecular approach recorded a substantially higher number of ciliate taxa, most of which taxonomically belonging to genera typically occurring in tufa barriers. The results from microscopy analyses did not show any links between ciliate community structure and sampling location. However, eDNA approach indicated significant differences among the sampling locations regarding the ciliate community structure. Thereby, hydrological parameters and saprobiological classification of the sampling sites were the main structuring factors for ciliate community. The coupling of eDNA metabarcoding with the morphological approach provides a robust, in-depth analytical system in elucidating the bioindicator potential of ciliated protists.

1. Introduction

Global socio-economic developments have a profound effect on freshwaters, specifically on the water quality, biotic communities and ecological integrity (Vörösmarty et al., 2010). Freshwater ecosystems have high level of biodiversity, which is greatly impacted by anthropogenic activities and associated climate change (Dudgeon et al., 2006; Ormerod et al., 2010). Karst aquifers represent highly vulnerable and variable freshwater ecosystems sustaining highly diverse and threatened biota. Though being highlighted as unique biodiversity hotspots and prioritized for the protection of biodiversity on a global scale, freshwater karst habitats are still poorly inventoried and not widely acknowledged for their ecological importance (Bonacci, 2009; Barrios et al., 2014). As stipulated in the European Water Framework Directive (WFD), the ecological water quality assessments are based on predefined bioindicator taxa termed biological quality elements (BQEs), with supporting physico-chemical and hydromorphological quality elements (Andersen et al., 2016; Hunting et al., 2017). The majority ofecological assessments and biomonitoring studies on different aquatic systems explore the influence of environmental pressures (e.g. pollution and

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habitat degradation) on selected BQEs, providing a wide range of biotic metrics/indices targeted towards defining ecological status of the ecosystems (Pawlowski et al., 2018). However, such approach comes with two major drawbacks. Firstly, the use of predefined bioindicator taxa and the respective biotic indices primarily focuses on the community structure of aquatic ecosystem, whilst overlooking the ecosystem functioning, i.e. the interaction among the community components and with the wider ecosystem (Caroni & Irvine, 2010). Secondly, the use of foreordained BQEs causes loss of valuable information (e.g. interaction-based and trait-based structural information) that might constrain the conclusions on biological response of other valuable bioindicators, such as protozoan ciliates.

Protozoan ciliates represent a very large and diverse group of heterotrophic microeukaryotes that occupy an essential position in the trophic web of freshwater ecosystems (Caroni & Irvine, 2010). As one of the key players in the periphytic microbial food web they feed on bacteria, algae, heterotrophic flagellates and other protists, while themselves being consumed by members of the meiofauna (Finlay & Esteban, 1998; Hillebrand, 2002; Dopheide et al., 2009). In addition to biotic factors, their abundance and diversity also depend on several abiotic factors that affect periphyton, such as light, water flow and sedimentation. Light increases biomass production and favours autotrophs, directly affecting the community composition (Vermaat, 2005). Water flow facilitates particle movement and nutrient uptake (Saravia et al., 1998), but can also lead to siltation (Pitois et al., 2001; 2003). Certain ciliate species exhibit photosensitive behaviour, e.g. pigmented heterotrichs are often photophobic to a rapid increase in light intensity. This type of response is considered a selective advantage in avoiding predators (Lynn, 2008). Conversely, mixotrophic ciliates may exhibit positive phototaxis (Esteban et al., 2010). In this context, sampling of both light- and dark-exposed lithified tufa/stones provides greater insight into community structure by including several different factors. Ciliates have been particularly successfully applied in assessing saprobic water quality, especially in zonation of organic pollution, where four main classes can be discerned: polysaprobic (heavily polluted), alpha-mesosaprobic (highly polluted), beta-mesosaprobic (moderately polluted), oligosaprobic (clean or low polluted) (Sládeček, 1973; Kolkwitz & Marsson, 1909; Berger & Foissner, 2003). Despite being excellent bioindicators due to their ubiquity, abundance and sensitivity to anthropogenic impacts (Foissner, 2004; Hughes, 2018), they are almost completely excluded or rarely integrated into water quality assessments. Any detected change in the ciliate community composition in response to environmental shifts (e.g. climate, water quality) can be used as a powerful tool for bioassessment and biomonitoring (Pawlowski et al., 2016).

Although having a vast bioindicator potential, ciliates are largely overlooked mainly due to limitations of morphological identification, which is both time-consuming and costly (Hering et al., 2018). The main features used to identify ciliates are body shape and colour of the cytoplasm, oral and somatic ciliatures, specific movement, position and number of contractile vacuoles, as well as the position of macronucleus and shape of inclusions. Many ciliates are fragile and fast moving, and often require difficult preserving and staining protocols for reliable identification (Dopheide et al., 2009). The present-day taxonomic approach integrates different aspects of biology into one concept (Wake, 1995), which is why the emphasis is on combining new advanced technologies such as the molecular approach with traditional approaches (Dayrat, 2005; Dawson, 2005; Cedrola et al., 2015). Integrative taxonomy uses morphological and molecular methods to identify organisms (McManus and Katz, 2009), but also provides other information, such as genetic and ecological data, that can contribute to interdisciplinary research into the ecology of the aquatic environment (Warren et al., 2017). Molecular methods are less subjective as they do not depend solely on the taxonomist's expertise, as is the case for morphological determination, and can be more informative and increase the possibilities of discovering potential indicator taxa, cryptic and rare species that are unlikely to be recognised under the microscope (Amaral-Zettler et al., 2009; Nolte et al., 2010; Pawlowski et al., 2016).

In the present study we used a combination of molecular and morphological approaches to provide a more detailed overview of the structure and ecological preferences of ciliate community inhabiting different microhabitats within the karst Krka River (Croatia). The main aims were to investigate: (i) ciliate community structure in the biofilm (i.e. periphyton) samples collected from light- and dark-exposed lithified tufa/stones; (ii) ecological preferences of the present ciliate community members implementing existing saprobiological classification (Foissner et al., 1991, 1992, 1994, 1995); (iii) improving both methodologies for the analysing bioindicator potential of specific ciliate taxa in environmental monitoring of freshwater karst habitats.

2. Materials and methods

2.1. Study area

The Krka River is situated in the Dinaric region of Dalmatia, Croatia. It is a specific karst river with high interconnection of surface and groundwater depending on lithological formations, tectonics, level of karstification, groundwater connections and hydrological conditions which are still not fully elucidated. Along its watercourse, Krka is characterized by tufa barriers, where "tufa" designates porous $CaCO_3$ deposits forming under specific physical and chemical conditions, and hosting very diverse biota, including high diversity of protists, partly contributing in calcite precipitation (Ford & Pedley, 1996; Primc-Habdija et al., 2005). The freshwater length from the Krka River source to the last tufa barrier Skradinski buk is 49 km, after which the river forms around 25 km long brackish estuary into the Adriatic Sea. The topographic catchment between the Krka River spring to the Skradinski buk covers 2450 km^2 (Perica et al., 2005), whilst its hydrological catchment includes parts of the Zrmanja River (the Miljacka spring zone in the middle section of the Krka River valley) and extends into the Bosnia and Herzegovina covering up to 2788 km^2 (Bonacci et al., 2006). The Krka River spring zone lies in the vicinity of Dinara Mountain and consists of several more or less independent springs: Main spring (80–90% of the total spring zone discharge) located in the cave beneath the Krčić stream waterfall at 225 m a.s.l., Little spring (5–15% contribution) and the Third spring (Bonacci, 1985; Bonacci et al., 2006). The spring zone also includes Krčić stream, a 10 km long intermittent tributary hydrologically connected with the Krka River, which is most likely a morphogenic spring of the Krka River (Friganović, 1990). After the spring zone, Krka flows through the Knin karst polje receiving several surface tributaries (Kosovčica, Orašnica, Butižnica) and further on across the North Dalmatian karst plateau. This zone is a deep composite valley consisting of longer, narrow canyon parts and smaller and larger, wider, less steep valley parts formed by the river flow. The composite character is a result of interaction of lithology and tectonics (Perica et al., 2005). Along the composite valley of the Krka River there are 7 larger tufa barriers forming waterfalls in the downstream direction as follows: Bilušića buk, Brljan, Manojlovića buk, Rošnjak, Miljacka, Roški slap and Skradinski buk. Some of them form lacustrine sections in the river and all of them influence dynamic of the river by creating parts with alternating (faster and slower) currents. The Visovac Lake, a 3.6 km long lentic dilatation of the Krka River situated between the last two barriers, receives additional water from its longest tributary, the Čikola River. The mean discharge at the spring zone (Topolje hydrological station) is around 12 $m^3 s^{-1}$, and at the Skradinski buk around 51 $m^3 s^{-1}$ (Bonacci & Ljubenkov, 2005; Rubinić et al., 2013). Due to the total gradient of about 200 m, the Krka River has been used for hydroelectric power generation since 1895, when the HPP Jaruga on the Skradinski buk was built as the first hydroelectric power plant in Europe and the second in the world (Čanjevac & Orešić, 2020). Since then, two more plants have been built on the river (HPP Miljacka and HPP Roški slap) and two more in the topographic catchment (HPP Krčić and HPP

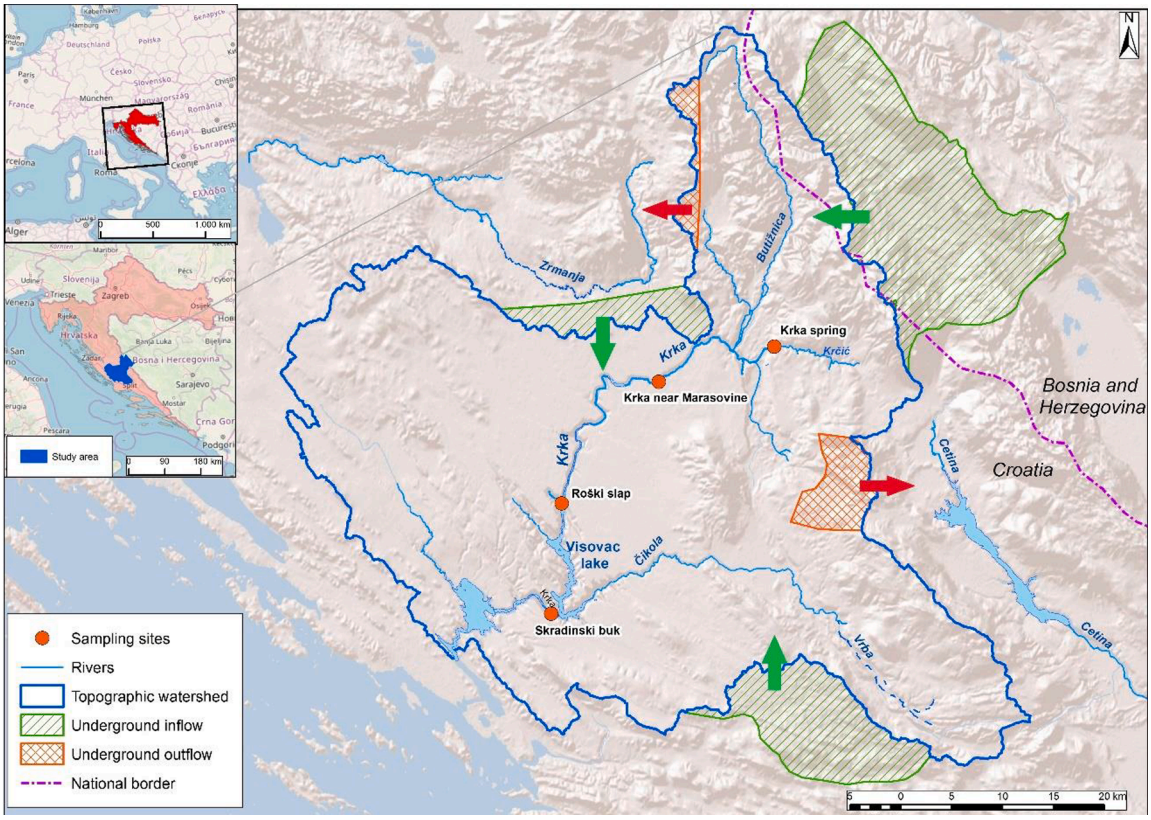

Fig. 1. Map of sampling sites situated at the Krka River, Croatia. (Underground connections according to Bonacci & Ljubenkov, 2005).

Golubić).

The four sampling sites (Krka spring, Krka near Marasovine, Roški slap, Skradinski buk) were chosen to represent the upstream, middle stream and downstream sections of the river (Fig. 1). Sampling was conducted from September 21 to 23 2017. The first sampling site, Krka spring, consisted of 2 subsampling sites and 6 microhabitats (P13-P18). The discharge measured at the HPP Krčić (upstream of the Main spring) on 22 September 2017 was 4.86 $m^3\ s^{-1}$ (CMHS, 2019). The second sampling site, represented by 3 microhabitats (P19-P21) near the settlement of Marasovine about 35 km upstream of the Skradinski buk, is located in a small valley characterized by slower water flow and small agricultural areas on the left bank of the river. The third sampling site consisted of 2 subsampling sites and 6 microhabitats (P7-P12), downstream of the tufa barrier Roški slap , which thus represents the transition from the middle to the downstream part of the river. The water discharge measured at the HPP Roški slap (upstream of the Roški slap barrier) on 22 September 2017 was 15.05 $m^3\ s^{-1}$ (CMHS, 2019). The fourth sampling site, represented by 2 subsampling sites and 6 microhabitats (P1-P6) , was located at the Skradinski buk tufa barrier complex. Upstream, at HS Skradinski buk gornji, measured discharge on 21 September 2017 was 37.11 $m^3\ s^{-1}$ (CMHS, 2019).

2.2. Field sampling

The sampling was performed in triplicates. Individual subsamples at each sampling site were 10 m apart. During sampling, each successive habitat upstream of the previously sampled site was selected. The exception (transverse sampling) was made at those sites where longitudinal sampling was not possible due to waterfalls. In each habitat, 5 stones were randomly collected at the sampling site on each sampling date. Samples were collected by brushing and/or scraping the substrate (biofilm) from the light- and dark-exposed sides of the lithified tufa/stones and rinsing with water. Live samples of ciliates were stored in 100 mL plastic containers filled with a small amount of ambient water without fixative (sample to water ratio ca. 1:4) and transported to laboratory using a portable freezer (stored on ca. 4 °C). Subsamples of biofilm were stored for eDNA metabarcoding in Falcon tubes (50 mL), kept on ice during transportation to the laboratory and stored at −20 °C until further processing. The following spot measurements of physical and chemical variables were taken using a portable multimeter (Hach HQ40d, Germany): temperature (T), pH, electrical conductivity (EC), dissolved oxygen concentration (DO) and oxygen saturation. For water chemistry, the following parameters were quantified in the samples according to compliance monitoring standards (https://www.iso.org/committee/52834/x/catalogue): nitrites ($N\text{-}NO_2^-$), nitrates ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$), phosphates ($P\text{-}PO_4^{3-}$), total nitrogen (TN), silicon dioxide (SiO_2), total inorganic carbon (TIC), dissolved inorganic carbon (DIC), total organic carbon (TOC) and dissolved organic carbon (DOC).

2.3. Laboratory analyses

2.3.1. Sample processing and molecular analysis

Live samples of ciliates were stored at 4 °C and morphologically identified within 4 to 10 h from sampling. The samples were gently shaken, followed by subsampling. Three subsamples (0.4 mL each) were analysed, and the abundance was expressed in ind./cm^2 using known sample volume and area sampled. Identification was conducted to the lowest possible taxonomic level using Jenaval binocular microscope (Carl Zeiss AG, Germany) with 125×, 250× and 400× magnification and relevant literature (Kahl, 1930-1935; Foissner et al., 1991, 1992, 1994, 1995).

Since DNA was extracted from frozen epilithic biofilms, the sample material was first centrifuged (4000×*g* for 1 min) to remove excess water. Total DNA was isolated using DNeasy PowerSoil Kit (Qiagen, Germany) following the manufacturer's instructions with slight modification in the final step, where 60 µl of sterile DNA-Free PCR Grade

Water was added instead of Qiagen's C6 Solution. Quality of the extracted DNA was assessed with NanoDrop spectrophotometer (Bio-Spec – nano, Schimadzu, Kyoto, Japan).

The hypervariable V9-region of the SSU rRNA gene (ca. 130 base pairs) was amplified from environmental DNA using the universal eukaryotic primer pair according to the protocol of Stoeck et al. (Stock et al., 2009; Stoeck et al., 2010). Primers were 1391F (5′-GTACACACCGCCCGTC-3′) and EukB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′), designed by Amaral-Zettler et al. (2009). Polymerase chain reactions (PCR) contained 1 U of Hot Start Taq DNA Polymerase (New England Biolabs, USA) and for V9 amplification employed an initial activation step at 95 °C for 5 min, followed by 30 three-step cycles consisting of 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 1 min; then a final 2 min extension at 72 °C (Stoeck et al., 2018). PCR products were assessed by visualizing on a 1% agarose gel. Sequencing libraries were constructed using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA). Libraries were sequenced on an Illumina NextSeq platform, generating 150-bp paired-end reads (SeqIT GmbH & Co. KG, Kaiserslautern, Germany).

2.3.2. *Sequence data processing*

Raw Illumina reads were demultiplexed with Cutadapt v1.18 (Martin, 2011), removing barcodes in combination 5′ to 3′ and then processed using the DeltaMP pipeline v0.3 (https://github.com/lentendu/DeltaMP). Reads were trimmed and retained if they contained both primers (minimum overlaps set to 2/3 the primer length, linked adapter strategy), had a minimum length of 70 nucleotides and had no ambiguous positions using Cutadapt. Reads were pair-end assembled using the "simple Bayesian" algorithm in PandaSeq v2.10 with a minimum overlap of 50 nucleotides and a default minimum similarity of 0.6 (Masella et al., 2012). Reads were dereplicated with VSEARCH and clustered using Swarm v2.1.5 (Mahé et al., 2015), with the d = 1 and the fastidious options on. The most abundant amplicon in each Operational Taxonomic Unit (OTU) was searched for chimeric sequences using UCHIME as implemented in Mothur v1.40.5 (Schloss et al., 2009); chimeric sequences and their OTUs were subsequently removed. Taxonomic assignment used VSEARCH's global pairwise alignments with the Protist Ribosomal Reference (PR2) database v.4.12.0 and threshold value of 80% identity (Guillou et al., 2013). A consensus taxonomy with a 60% threshold was created for OTUs with multiple best match with different taxonomy in the database. To retain only protist OTUs, OTUs assigned to the following taxa were removed: Streptophyta, Metazoa, Fungi, unclassified Archaeplastida, unclassified Eukaryota, and unclassified Opisthokonta. Low abundance OTUs consisting of only one, two or three amplicons and occurring exclusively in one sample were also removed, as they were most likely erroneous sequencing products (Bokulich et al., 2013; Nelson et al., 2014). Ciliophora, Cercozoa and Bacillariophyta comprised the majority of the protists reads and OTUs in this data set (Fig. 2). Further statistical analyses were only conducted on OTUs taxonomically assigned to Ciliophora. Raw demultiplexed reads were deposited at the ENA's Sequence Read Archive and are publicly available under project number PRJEB39359.

2.4. *Community statistical analyses*

All statistical analyses were conducted in R v. 4.0.2 (R Core Team, 2020) using the packages "vegan", "fossil", "labdsv", as well as "ggplot2" and "VennDiagram" for graphical representation. To allow comparability between the two methods, taxa lists derived from the molecular and morphological approaches were compared in terms of the presence or absence of taxa and the composition of the ciliate community. Results for downstream analysis were combined into a single dataset for each approach, with molecular results transformed using the center-log ratio transformation (Gloor et al., 2017). Relative abundance data obtained by microscopywere not transformed. Correlation of sequences versus cell counts was tested using a Mantel test with 10 000

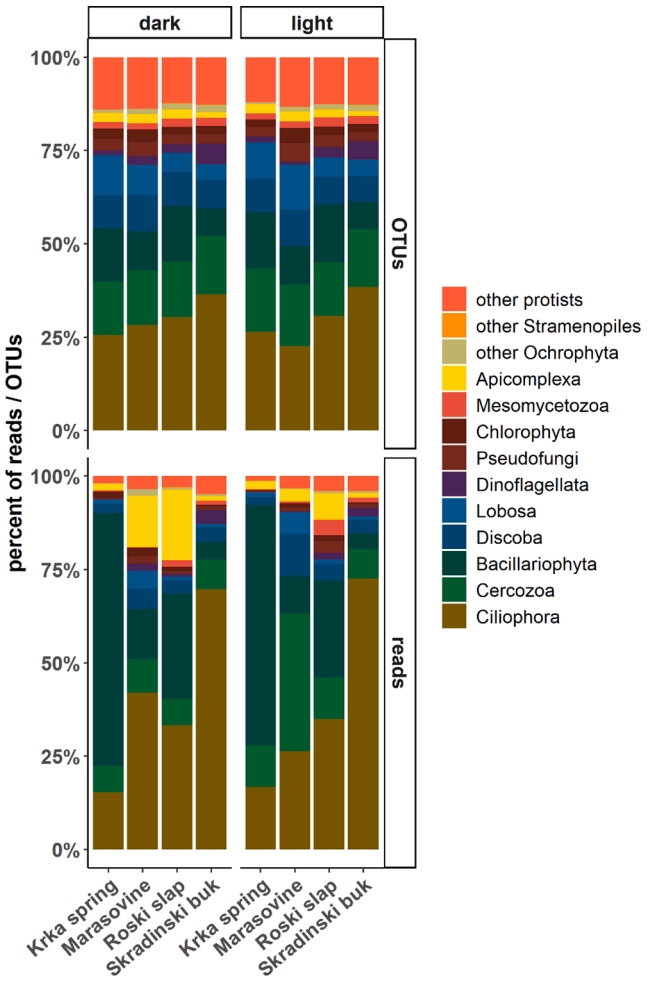

Fig. 2. Taxonomic assignment and relative abundance of protist reads and OTUs according to investigated sampling locations of light- and dark-exposed biofilms covering lithified tufa/stones.

permutations. Shannon, Simpson and Jaccard indices, ICE (incidence-based coverage estimator), Chao1 (estimator based on abundance) and richness were calculated for both data as measures of alpha diversity using vegan v.2.5.6 (Oksanen et al., 2019). The effect of exposed sides in alpha diversity were tested separately for both data using the non-parametric Mann-Whitney test. Location effect in alpha diversity was tested using Tukey's HDS parametric test for the molecular approach.

Cell counts (morphological data) and center-log ratio transformed molecular data were used to compute measures of beta diversity. To test significance and to detect individual and combined effects of locations and exposed sides, beta diversity was constrained by Permanova permutation test for both data separately. The Bray-Curtis (BC) index was used as a measure of dissimilarity in community composition between the locations and light- and dark-exposed biofilms covering lithified tufa/stones. Non-metric multidimensional scaling (NMDS) was used to investigate the change in community composition linked to location and exposure. Environmental vectors were fitted only for molecular approach, to the ordination using the *envfit* function. The fit (R^2) of each variable to the ordination was assessed with a Monte Carlo analysis of 10 000 permutations.

Venn diagrams were used to graphically visualize the proportions of shared and unique OTUs between the four different sampling locations. Finally, identified OTUs were associated with indicator values for each location using an Indicator Species analysis (Dufrene & Legendre, 1997) as implemented in the package "labdsv" (Roberts, 2019). The indicator value was calculated for an "i" OTU in relation to a "j" type of location:

Table 1
Physical and chemical variables at the investigated sampling sites.

	Krka spring I	Krka spring II	Marasovine	Roški slap I	Roški slap II	Skradinski buk I	Skradinski buk II
T (°C)	10.3	10.4	–	15.4	15.4	20.6	20.2
DO (mg L^{-1})	10.26	10.4	–	9.75	9.5	9.16	8.19
O_2 (%)	94.5	95.4	–	97.2	95.2	101.5	98.1
pH	7.75	7.76	7.88	8.35	7.96	8.58	8.53
EC (μS cm^{-1})	391	405	690	648	653	505	523
N-NO_3^- (mg L^{-1})	<0.1	<0.1	<0.1	6.6	<0.1	6.2	1.8
N-NO_2^- (mg L^{-1})	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
N-NH_4^+ (mg L^{-1})	<0.01	<0.01	<0.01	0.02	<0.01	<0.01	<0.01
P-PO_4^{3-} (mg L^{-1})	0.31	0.31	<0.01	0.27	<0.01	<0.01	<0.01
SiO_2 (mg L^{-1})	0.9	0.8	1.7	2	2.4	0.8	1.2
TN (mg L^{-1})	<0.1	<0.1	<0.1	7.1	<0.1	6.4	2
TIC (mg L^{-1})	10.77	10.78	10.46	10.79	11.06	10.55	9.78
DIC (mg L^{-1})	10.53	10.64	10.2	10.45	10.73	10.15	8.88
TOC (mg L^{-1})	0.61	1.44	0.96	0.61	0.72	1.37	2.17
DOC (mg L^{-1})	0.26	0.23	0.46	0.45	0.44	1.09	1.1

$$IndVal_{ij} = Specificity_{ij} * Fidelity_{ij} * 100$$

where $IndVal_{ij}$ is the indicator value of an "i" OTU (species) in relation to a "j" type of location, $Specificity_{ij}$ is the proportion of sites of type "j" with OTU (species) "I", and $Fidelity_{ij}$ is the proportion of the number of individuals (in this case the number of transformed reads) OTUs "i" that are in a "j" type of location. Used ranges of indicator values were compared with the results of IndVal analysis in Minerovic et al. (2020).

3. Results

3.1. Analyses of environmental parameters

The environmental variables of Krka River are listed in Table 1. The values of DO decreased from the spring zone in the downstream direction. Conversely, water temperature and pH showed an increase in the downstream direction. The highest concentration of phosphates was observed at the Krka spring. Concentrations of nitrogen compounds were very low at all sampling sites, except for higher TN and nitrates measured at Roški slap and Skradinski buk. Skradinski buk was characterized with slightly higher concentrations of DOC and TOC, while the highest values of TIC and DIC were measured at Roški slap.

3.2. Sequencing and morphological identification of ciliates

A total of 26 genera and 28 species were identified by using morphological approach (Supplementary Material 1). For two light-exposed and five dark-exposed samples no ciliate species were recorded. Ciliate species with the highest number of occurrences were *Aspidisca lynceus* O.F. Müller, 1773, *Aspidisca cicada* O. F. Müller, 1786, *Cinetochilum margaritaceum* Ehrenberg, 1838 and *Glaucoma scintillans* Ehrenberg, 1830 recorded at Krka spring, then *Vorticella convallaria* Linnaeus, 1758 at Marasovine and *Stylonychia mytilus* (Müller, 1773) Ehrenberg, 1830 at Skradinski buk. At genera level, with the highest number of occurrences belonged to *Euplotes* at Krka spring, *Oxytricha* and *Urostyla* at Roški slap.

Of the 42 samples collected, the DNA sequencing reaction failed for samples P7Z, P9S and P20S due to the poor quality of extracted DNA. From the remaining 39 samples, around 5,413,607 reads within 11,295 OTUs for protists were obtained. Reads taxonomically assigned to Ciliophora (466,344 reads, which clustered into 3724 OTUs) were extracted and further analysed in detail (Supplementary Material 2). The most represented OTUs at all sampling sites were taxonomically assigned to the subclass Suctoria, especially at Roški slap. OTUs present at all sampling sites corresponded to genera *Stentor*, *Holosticha*, *Anteholosticha*, *Euplotes* and *Oxytricha*. The most abundant OTUs at Skradinski buk corresponded to genus *Stentor*, while genus *Limnostrombidium* was the most abundant OTUs present at Roški slap. Marasovine was characterized by OTUs corresponding to genus *Tetrahymena,* while OTUs describing Krka spring belonged to families Foettingeriidae and Chilodonellidae, specifically genera *Carchesium* and *Urocentrum*, respectively.

3.3. Abundance of taxonomically identified species vs. numbers of sequence reads

The comparison of both methodological approaches resulted in the following outcomes: i) 26 genera were identified based on microscope counts, while Ciliophora OTUs could be assigned to 214 genera; ii) after aggregation and comparison of the results a total of 14 OTUs were associated with both approaches at family rank (83% based on molecular vs. 11% based on morphological approach), 18 at genus rank (91% molecular vs. 4% morphological) and 2 at species rank (99% molecular vs. 10% morphological), with overlaps between both methods shown by Venn diagram (Fig. 3); iii) overlaps on family rank were: Stentoridae,

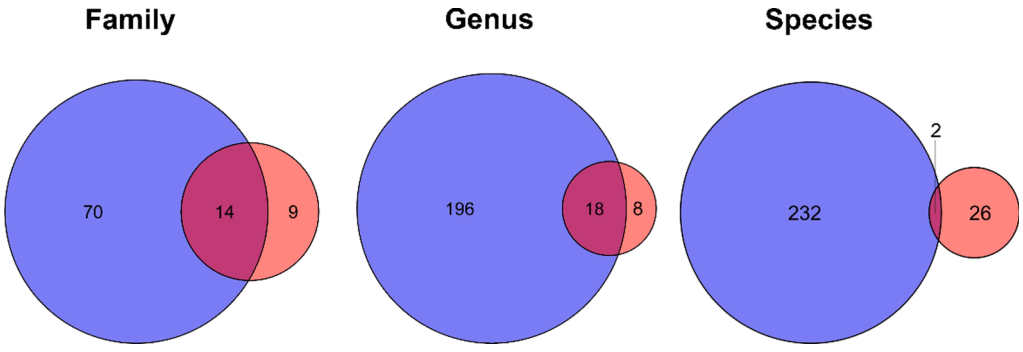

Fig. 3. Venn diagrams comparing the ciliates assigned at family, genus and species rank either by the molecular (blue circles) or by the morphological (red circles) approach. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Euplotidae, Oxytrichidae, Holostichidae, Chilodonellidae, Tetrahymenidae, Tracheliidae, Spirostomidae, Aspidiscidae, Dysteriidae, Lembadionidae, Pseudomicrothoracidae, Glaucomidae and Lynchellidae; on genus rank were: *Chilodonella*, *Stentor*, *Trithigmostoma*, *Vorticella*, *Oxytricha*, *Euplotes*, *Coleps*, *Lembadion*, *Tetrahymena*, *Spirostomum*, *Loxophyllum, Holosticha, Dileptus, Aspidisca, Litonotus, Pseudomicrothorax, Trochilia* and *Chlamydonellopsis*; for species rank only two matches were detected: *Trithigmostoma cucullulus* (Müller, 1786) Jankowski, 1967 (100% similarity) and *Vorticella campanula* Ehrenberg, 1831 (80% similarity); iv) Mantel test indicated no correlation (no statistical significance) for any of the 18 genera matches between Bray-Curtis distances based on the number of reads and cell counts ($r = 0.047$, $p = 0.258$); v) in percentage proportion of ciliates, molecular results showed much higher number of represented ciliate OTUs on sampling sites.

To analyse the effects of exposed sides of biofilms covering lithified tufa/stones on ciliate abundance, a non-parametric Mann-Whitney test was performed on the mean values of alpha diversity for both approaches. ACE index for morphological approach and Simpson index for molecular approach were excluded from the graphical representation, as they were not representative for the analyses. For the morphological approach (Fig. 4a), significant differences were shown in light-exposed samples only for Skradinski buk for richness ($p = 0.03$) and all indices tested; Shannon, Chao1 ($p = 0.03$) and Simpson ($p = 0.02$). The results of effects for other locations were not significant ($p > 0.05$). In contrast, for the molecular approach for the exposed sides (light/dark), there were no significant effects for richness and all tested indices (Mann-Whitney test, $p > 0.05$), but they revealed a significant increase in OTU richness from Krka spring downstream to Skradinski buk (Tukey's HSD test, $p < 0.05$, Fig. 4b).

NMDS analysis (stress 0.1027) based on Bray-Curtis similarity obtained for the morphological approach showed that the resolution power of ciliate community at sampling sites was lower than for the molecular approach. This was further corroborated by Permanova test, which did not show significance for location ($p = 0.093$), exposed sides ($p = 0.133$) or combined ($p = 0.633$) effects (Fig. 5a). NMDS analysis (stress 0.0864) for the molecular approach showed a clear separation of sampling sites, which was also confirmed by Permanova test for location effect ($p = 0.001$), while side ($p = 0.822$) and combined effects ($p = 0.669$) were not significant (Fig. 5b). Because the morphological approach showed low abundance and resolution power of ciliates in NMDS analysis for sampling sites, correlations with significant environmental parameters were performed only for molecular approach. By fitting the environmental variables into the NMDS analysis based on Bray-Curtis similarity, samples were separated into three distinct groups. The overall strength of correlations between the molecular characterization of the ciliate community and its significant physico-chemical parameters was summarized in Table 2. The most important parameters showing a significant ($p = 0.001$) negative correlation with both axes MDS1 and MDS2 were pH, T, N-NO_3^-, TN, DOC and oxygen saturation ($p = 0.004$). DIC showed a significant ($p = 0.001$) positive correlation with both axes, EC showed a significant ($p = 0.001$) negative correlation with MDS1 axis, whilst P-PO_4^- showed a significant ($p = 0.001$) positive correlation with MDS1 axis. NMDS ordination revealed that the environmental conditions consistently affected the ciliate community composition at all sites, resulting in a clear separation of biofilm samples along the NMDS1 axis (Fig. 5b).

From a total of 3724 OTUs assigned to ciliate species, 317 (8%) OTUs were present in all four sampling sites. Skradinski buk and Roški slap shared 1619 (43%) identical OTUs. A total of 928 (25%) unique OTUs were recorded at Skradinski buk, Roški slap had 139 (3.7%), at the Krka spring there were 23 (0.6%), whilst only 2 (0.05%) unique OTUs were recorded for Marasovine (Fig. 6).

OTUs with significant indicator value at Krka spring (IV ≥ 0.7, $p = 0.001$) corresponded to families Dysteriidae (OTU_004842) and Loxodidae (OTU_004936). OTU with a very low indicator value, but significant (IV = 0.5, $p = 0.004$) corresponded to genus *Tokophrya* (OTU_037562) showed overlapping with Venn analysis for Krka spring. At Marasovine, the OTUs with significant indicator values (IV ≥ 0.7, $p = 0.001$) corresponded to genera *Carchesium* (OTU_016887, OTU_009806, OTU_018926), *Tetrahymena* (OTU_017391, OTU_024708) and to the orders Sessilida (OTU_005871, OTU_013800, OTU_050015, OTU_020151) and Pleurostomatida (OTU_038817). Significant OTUs singled out by IndVal analysis did not show overlapping with two OTUs singled out by Venn analysis. Indicator values of OTUs detected at Roški slap were similar to the ones present at Krka spring and Marasovine (IV ≥ 0.7, $p = 0.001$) and corresponded to genera *Acineta* (OTU_002992, OTU_008552, OTU_010097), *Stentor* (OTU_025821), *Loxophyllum* (OTU_007711), *Cyclotrichium* (OTU_002713) and to the order Philasterida (OTU_004600). None of these OTUs showed overlapping in the Venn analysis. OTUs with higher indicator values (IV = 0.9, $p = 0.001$) reported at Skradinski buk were assigned to genera *Stentor* (OTU_009596, OTU_009865, OTU_019902, OTU_12099), *Enchelys* (OTU_0106489), *Prorodon* (OUT_011975), *Epalxella* (OTU_017568), *Vorticella* (OTU_019870) and to the order Sessilida (OTU_010408). Several of these OTUs (OTU_009865, OTU_019902, OTU_0106489 and OTU_019870) were also singled out by the Venn analysis as unique to this location.

4. Discussion

4.1. Comparison of morphological and molecular results

Although freshwater ciliates have been recognized as important biomediators in tufa depositing process, data on their biodiversity and ecology are still quite scarce (Kock et al., 2006; Reiss & Schmid-Araya, 2008). Biofilm-inhabiting ciliates prosper from tufa deposition, since sites of active deposition tend to have rough surface suitable for biofilm colonization and growth (Risse-Buhl & Küsel, 2009). Hence, tufa acts not only as a favourable substrate for colonization, but also becomes embedded in the matrix (Matoničkin Kepčija et al., 2011). Previous studies in the Krka River estuary (Primc-Habdija et al., 2005; Primc-Habdija & Matoničkin, 2005) were based solely on morphological identification of species using light microscopy. Impediments in the morphological approach can be surpassed with molecular approach, thus allowing the successful implementation of ciliates as freshwater bioindicators.

Comparison of results attained by both approaches at distinct taxonomic ranks, as evidenced by the present study, enables a more detailed insight into the community complexity. Unsurprisingly, most of the matches at family rank were spirotrichs. These ciliates are quite abundant in diverse freshwater habitats, especially in plankton (as they can consume up to 100% of the standing stock of nanoplankton every day) (Thorp and Covich, 2010; Grattepanche et al., 2019). Also, they can be easily morphologically identified due to their prominent adoral zone of membranelles.

From a total of 18 matches at genus rank, most of them were filter feeders such as *Lembadion*, *Tetrahymena*, *Spirostomum, Euplotes* and *Vorticella,* who generate water currents by membranelle, relishing the minute particles of food brought by the water current (Fenchel, 1987). While *Vorticella* was mostly detected at dark-exposed biofilms, spirotrich *Euplotes* predominantly occurred at light-exposed biofilms, accompanied by *Tetrahymena* and *Spirostromum,* who occurred on both biofilm sides. This is probably a result of diverse filter feeding strategy: stalked peritrichs such as *Vorticella* who propel water perpendicular to the surface tend to attach to a solid surface when feeding in order to minimize the viscous-drag of the cilia and maximize the feeding current (Fenchel, 1987). By colonizing the dark-exposed biofilms, *Vorticella* is protected from strong water current and thus allowed to easily filter water. On the other hand, vagile filter-feeders (e.g. *Tetrahymena, Euplotes*) possess cilia to rise sufficiently above the substrate surface, enabling them to feed in a faster water current. In addition to filter feeders, histophagous and predatory genera *Lembadion*, *Coleps* and *Loxophyllum* were detected

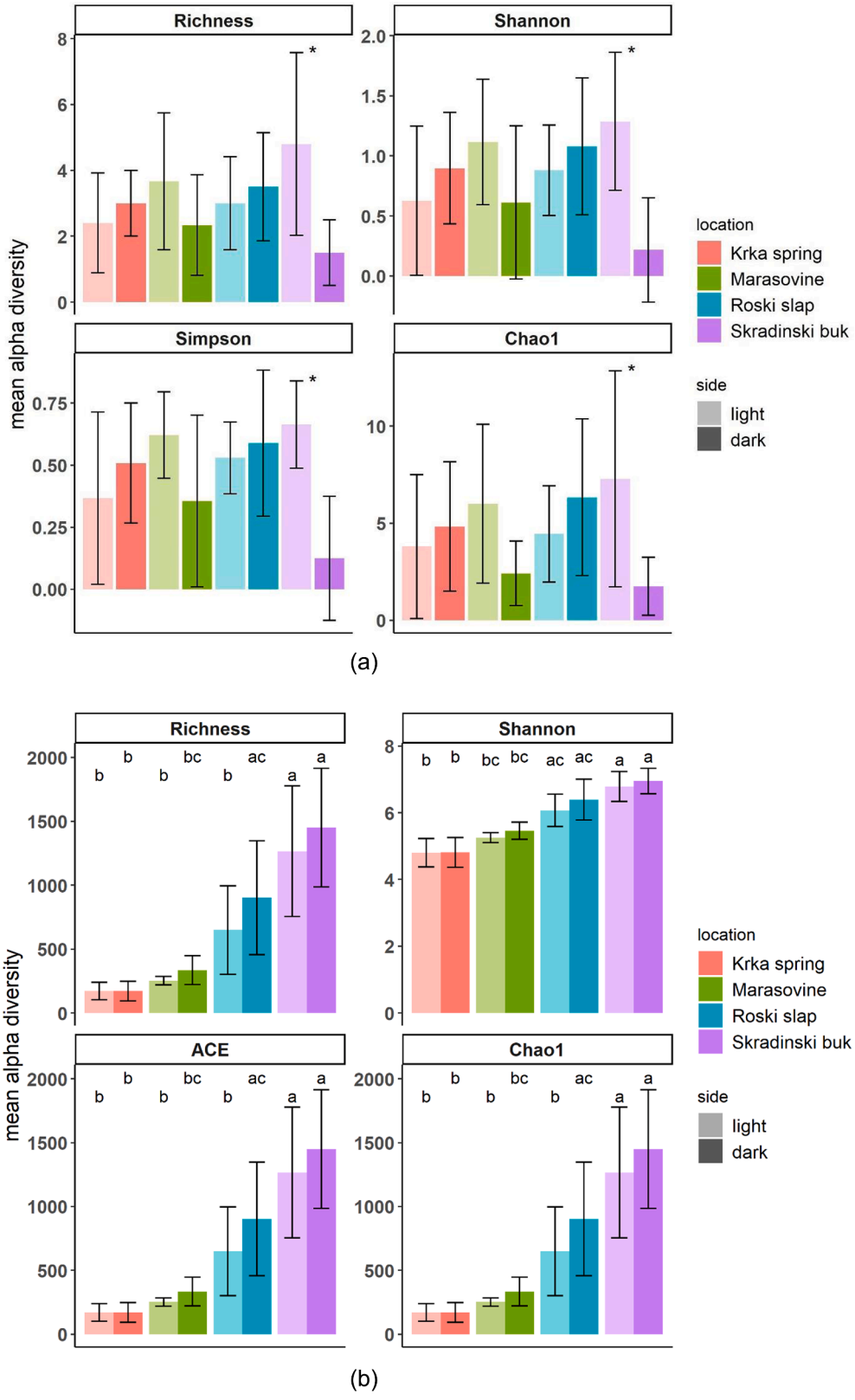

Fig. 4. Variations in alpha diversity for richness, Shannon, ACE, Chao 1 and Simpson index: a) morphological approach (asterisk next to whiskers of light-exposed side of Skradinski buk indicates significant effects exposed sides based on Mann-Whitney test); b) molecular approach (different letters above whiskers indicate significant differences among location based on Tukey's HDS test). Columns denote mean SE, and whiskers denote mean SD. The lighter colours denote light-exposed samples, whilst darker colours denote dark-exposed samples.

mostly on light-exposed biofilms, likely due to high availability of food. These results emphasize the importance of microhabitat conditions structuring the ciliate communities and reflect the same habitat preference (predominance of attached forms such as peritrichs in sheltered microhabitats) recorded by Gulin & Matoničkin Kepčija (2012). The significant effect of location and side exposition on alpha diversity, recorded only for the light-exposed biofilms at Skradinski buk using morphological approach reflects the barrier's geomorphological complexity as it comprises of numerous cascades, islands and lakes (Bonacci et al., 2017). Although molecular approach did not show significant effect on side exposition, it revealed a significant downstream increase in OTU richness, which is concordant with higher nutrient

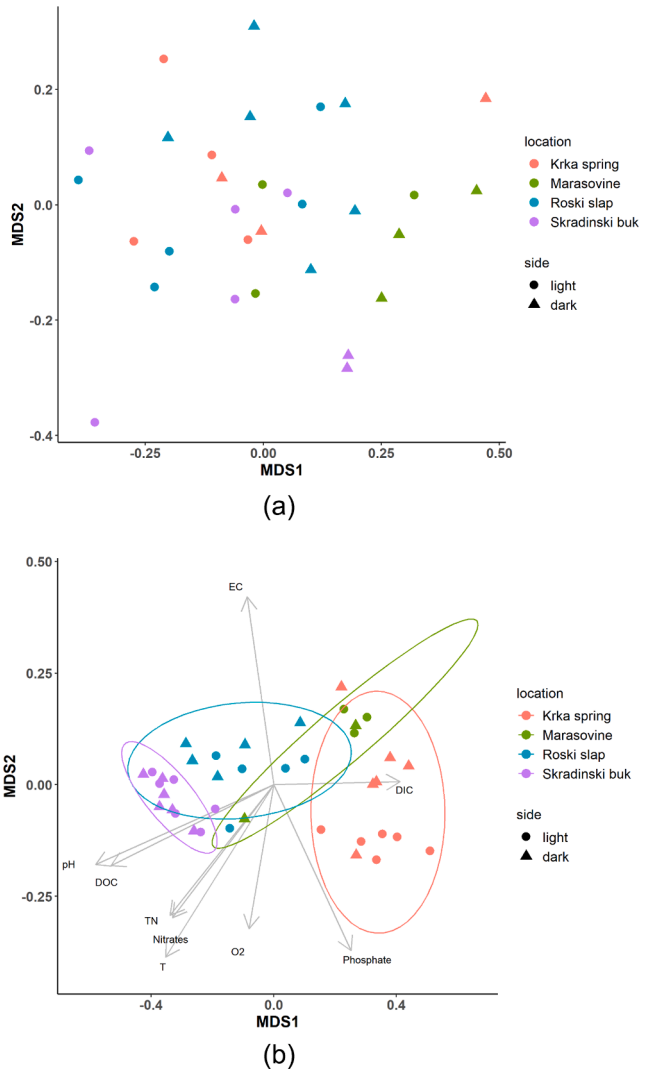

Fig. 5. Position of sampling sites in the multidimensional scaling analysis based on Bray-Curtis similarity index for ciliates: a) for morphological approach; b) for molecular approach with significant environmental parameters (EC = conductivity, pH, T = temperature, TN = total nitrogen, O_2 = oxygen saturation, DOC = dissolved organic carbon, DIC = dissolved inorganic carbon). Ellipses were drawn at a 90% confidence level.

Table 2
Summary statistics of statistically significant physical and chemical variables in the Non-metric Multidimensional Scaling Analysis based on Bray-Curtis similarity ($p \leq 0.005$).

	NMDS1	NMDS2	r^2	p
T	−0.67459	−0.73820	0.61	0.001
O_2 (%)	−0.24145	−0.97041	0.24	0.004
pH	−0.95596	−0.29349	0.83	0.001
EC	−0.20178	0.97943	0.41	0.001
N-NO_3^-	−0.74330	−0.66896	0.44	0.001
P-PO_4^{3-}	0.56216	−0.82703	0.45	0.001
TN	−0.75683	−0.65361	0.45	0.001
DIC	0.99983	0.01822	0.38	0.001
DOC	−0.94648	−0.32277	0.70	0.001

levels likely due to its geographical position between the upstream Visovac Lake and the Krka River estuary, located downstream (Cukrov et al., 2007). This increase could also result from the extracellular DNA accumulation, which is passively trasported downstream (Deiner & Altermatt, 2014; Jane et al., 2015).

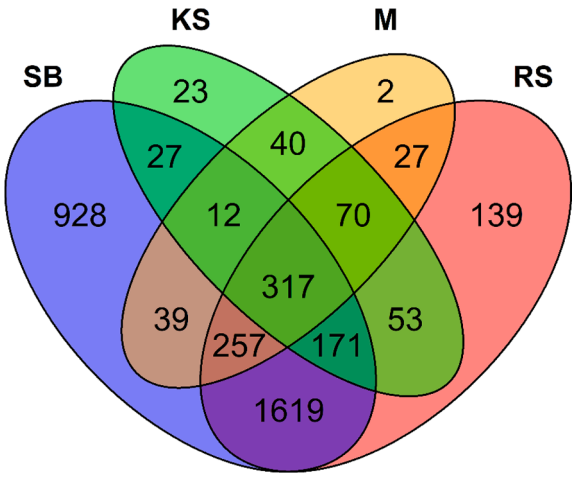

Fig. 6. Venn diagram preforming sharing and unique OTUs per locations (SB = Skradinski buk, KS = Krka spring, M = Marasovine, RS = Roški slap).

Comparison at species rank indicated 2 matches, with OTUs taxonomically assigned to *Trithigmostoma cucullulus* and *Vorticella campanula*. These species were already commonly found in diverse freshwater habitats including biofilm of tufa barriers (Matoničkin Kepčija et al., 2011; Gulin & Matoničkin Kepčija, 2012). *Trithigmostoma cucullulus* is highly characteristic for biofilm communities in alpha-mesosaprobic running waters (Foissner et al., 1999) and in our research was recorded by both approaches only at Skradinski buk and Roški slap, characterized with the highest measured nutrient levels suitable for algal and bacterial proliferation. *Vorticella campanula* is characteristic for beta-mesosaprobic to alpha-mesosaprobic (Stentoretum) communities (Foissner et al., 1996), where it occurs together with genus *Stentor,* the main indicator of this type of community. The presence of *Vorticella campanula* together with members of genus *Stentor* was recorded at Skradinski buk, which is in accordance with previously described nutrient levels.

The use of molecular approach facilitates detecting a higher number of ciliate OTUs, most of them belonging to genera quite common for tufa barriers, but often overlooked, possibly due to low abundance or difficult morphological identification. This approach revealed a significant ciliate genetic diversity in the Krka River biofilms, which was not evident upon microscopic examination. Similar results were obtained by testing both approaches considering ciliate diversity in the biofilms of streams impacted by different land use types (Dopheide et al., 2009) and in the mountain lake plankton community (Stoeck et al., 2014), where the most abundant morphotype genera were not correspondingly represented in the molecular ciliate profiles. Other sources for discrepancies in the results may also be technical artefacts related to PCR or sequencing conditions (Weber and Pawlowski, 2013), amplicon clustering (Huse et al., 2010; Forster et al., 2016), and incompleteness and errors in the reference database (Stoeck et al., 2014). Similar discrepancy between high-throughput sequencing and traditional morphological analyses in characterization of environmental eukaryotic communities was reported by Medinger et al. (2010), who concluded that rDNA copy number variation among taxa could be one of the main reasons for incongruent results of the two approaches. Moreover, Gong et al. (2013) detected a high number of rDNA copies even among closely related morphospecies accompanied with substantial sequence polymorphism, thus demonstrating the dynamic nature of ciliate genomes. Generally, ciliates have much more rDNA copies in single cells than other protists, which easily leads to overestimation of their relative abundance (Wang et al., 2020). In this study, for the genera detected simultaneously by both approaches, Mantel test did not show correlation in abundance distribution, i.e. the taxon-assigned amplicon abundances did not reflect the true taxon abundances in the considered

samples. Thus, our results have to be interpreted with caution, since highly sensitive molecular tool can detect cell abundances of a specific taxon or taxa which cannot be found by microscopy, when they persist in the sample drop below a specific threshold (Stoeck et al., 2014). Also, the resting stages of ciliates that cannot be identified and assigned correctly by microscopy, might be more easily recorded by molecular approach (Medinger et al., 2010; Stoeck et al., 2014).

4.2. Physico-chemical parameters as a reflection of the karstic environment

The measured values of most physico-chemical parameters corresponded to late summer values, which was in agreement with earlier studies on Krka River (Primc-Habdija et al., 2005; Cukrov et al., 2007; Strmečki et al., 2018; Žutinić et al., 2020).

NMDS analysis of molecular-inferred data showed a clear separation of OTUs per locations and significant correlations with several environmental parameters. Grouping of OTUs to locations can be explained by position along the Krka River flow, where Permanova testing confirmed strong significant location effect on community composition. Skradinski buk, a station characterized by significantly more site-specific genera in comparison to Roški slap and Krka spring, is located downstream of Visovac Lake and represents a unique lake outlet reach characterized by higher temperature and pH and high DOC values. Since lakes tend to be more productive systems (Špoljar et al., 2007), the influence of Visovac Lake is evident in higher amount of dissolved organic matter and accordingly higher abundance of ciliate OTUs, specifically corresponding to filter-feeders. Similar influence of tufa barrage lakes was recorded for caddisfly assemblages in Plitvice Lakes, where the filter-feeding caddisflies dominated on the most downstream tufa barriers (Šemnički et al., 2012). Consequently, a large number of reads for predatory ciliates (Litostomatea, Haptoria) were also detected at Skradinski buk. Suctorians (Phyllopharyngea) accounted for 39% of recorded OTUs at all sampling sites, most notably at Skradinski buk. They are common residents of freshwater systems and can be found in various damp/wet environments with sufficient food sources (Sato et al., 2015). Suctorians are often used as indicators of water quality - whilst being parasitic in some cases, they are mostly carnivorous (Gómez-Gutiérrez et al., 2017). Considering their carnivorous nature, their presence could be attributed to diverse community of mobile species on which they feed, in particular nassulids (Nassophorea) which were also recorded at all sampling sites, with the highest number of reads at Roški slap. OTUs belonging to suctorians were also found at Marasovine and Krka spring, along with peritrich OTUs belonging to genera *Zoothamnium*, *Vorticella*, *Pseudovorticella* and *Carchesium*. The high number of peritrichs at these sites might be explained by the availability of sheltered microhabitats and lower water currents, allowing the community to thrive (Gulin & Matoničkin Kepčija, 2012). The lentic characteristics noted at Marasovine were even more pronounced at Roški slap and Skradinski buk and reflected in the number of detected reads comprising several OTUs corresponding to euplanktonic genera (*Halteria, Rimostrombidium*, *Strobilidium*, *Tintinnidium*). Species within euplanktonic genera were considered as euplanktonic if they matched at least one of the following criteria: special morphological features (e.g. small size, lorica forming, bell-shaped); originally described from the pelagial of large water bodies; several reliable pelagic records available; the whole group lives pelagically (Foissner et al., 1999). Generally, euplanktonic ciliates live as heterotrophs, while at times a considerable part of the community can resort to mixotrophy (Jones, 1997; Foissner et al., 2007). High number of such genera at Skradinski buk is presumably influenced by the upstream Visovac Lake.

4.3. Ciliates as a bioindicators in the karstic environment

In order to broaden the use of ciliates as one of the key components in describing the karstic environment, a consistent approach should be established and further implemented. In our research, we have embraced a combination of Foissner's saprobiological classification (Foissner et al., 1991, 1992, 1994, 1995) for describing the site-unique OTUs indicated by Venn Diagram and the potential indicator OTUs acknowledged by the indicator value analysis (IndVal). For all sampling sites IndVal values were slightly lower, but significant ($IV \leq 0.9$, $p \geq 0.001$) than the values for diatom community ($IV > 0.98$, $p < 0.005$) in streams biofilms proposed by Minerovic et al. (2020). Krka spring was singled out by one OTU, which had matching by Venn and IndVal analysis with very low IndVal value ($IV = 0.5$, $p = 0.004$), corresponding to genus *Tokophrya*. Members of the genus *Tokophrya* usually occur in alpha-mesosaprobic to beta-mesosaprobic running waters with sufficient oxygen supply (Foissner et al., 1996) and Krka spring measured the highest dissolved oxygen concentration among all sampling sites. Generally, this can be explained by the characterization of karstic springs by their physico-chemical stability and tendention to have high concentrations (8–12 mg L^{-1}) of dissolved oxygen (Blagojević, 1974; Cantonati et al., 2008). For Marasovine, IndVal analysis singled out ten OTUs corresponding mostly to genera *Carchesium* and *Tetrahymena*. While members of the genus *Tetrahymena* occur in a wide range of saprobity levels, from oligosaprobic to polysaprobic (Foissner et al., 1996), members of the genus *Carchesium* are an indicator of alpha-/beta-mesosaprobic ecological conditions, with species living in freshwaters with slightly eutrophic conditions, at pH values between 6.4 and 8.7 and EC around 390–850 μS cm^{-1} (Foissner et al., 1992; Wei et al., 2004), as recorded at Marasovine. Genus *Carhesium* is generally found in different freshwater bodies under anthropogenic pressure (Panov, 2019, Pedroso Dias et al., 2020), while the genus *Tetrahymena* can provide quantitative information on water quality by changing its behaviour in the presence of various toxins (Ye et al., 2018; Chasapis, 2019; Maurya and Pandey, 2020). IndVal analysis singled out seven OTUs at Roški slap, corresponding to genera *Stentor* and *Loxophyllum*. Members of the genus *Stentor* occur in a wide range of conditions, from alpha to beta-mesosaprobic, with some species even occurring in oligosaprobic waters (such as *Stentor niger,* Foissner et al., 1996), while members of the genus *Loxophyllum* are highly characteristic for beta-mesosaprobic waters, usually occurring in low abundances (Foissner et al., 1996). These diverse saprobic conditions correlate with the highest concentrations of nitrogen compounds and higher temperatures at Roški slap, where the barrier acts as a natural funnel between riverine sections, causing the accumulation of organic matter (Strmečki et al., 2018). From 928 unique OTUs recorded by Venn analysis at Skradinski buk, IndVal analysis singled out four OTUs corresponding to genera *Stentor*, *Enchelys*, *Prorodon*, *Epalxella* and *Vorticella*. Most of these genera are often found in benthos and periphyton of stagnant and running waters, where they indicate alpha- to beta-mesosaprobic community (Foissner et al., 1999). This is in an accordance of highest values of water temperature at Skradinski buk, likely due to its geographical position as the longest and last tufa barrier, consequently resulted in a higher amount of organic matter (i.e. TOC and DOC), but also can reflect the influence of the upstream Visovac Lake.

4.4. Advantages of molecular approach and V9 region as a marker

V9 region was selected by virtue of a relatively simple one-step-PCR amplicon library preparation method (Gilbert et al., 2010; Caporaso et al., 2012; Thompson et al., 2017; Minerovic et al., 2020), as well as potential for simultaneously characterizing multiple groups of eukaryotic organisms in a cost-effective way (Hadziavdic et al., 2014). Previous studies have used different hypervariable regions for monitoring eukaryotic benthic communities, and their utility has been discussed in research (Stoeck et al., 2010; Forster et al., 2019; Pitsch et al., 2019). Some authors recommended longer V4 region as the preferred marker for detecting eukaryotic diversity (Dunthorn et al., 2012). We choose V9 region because of its ability to better capture diversity and community structure of photosynthetic eukaryotes (Bradley et al., 2016), as well as

its good trade-off between database coverage and taxonomic resolution (Tanabe et al., 2016), low sequencing costs and usage of shorter marker which is especially relevant in studies with high sample numbers or monitoring studies (Dunthorn et al., 2012; Pitsch et al., 2019).

In conclusion, the results indicated ciliates as good ecological indicators of karstic environments. These organisms are widely distributed in benthic and planktonic communities along the Krka River, and are commonly found in alpha- to beta-mesosaprobic freshwaters. Ciliates exhibit high ecological sensitivity and should undoubtedly be considered important organisms for monitoring tufa-forming rivers and streams. We have shown that eDNA metabarcoding and traditional approaches can be considered complementary, depending on the objectives of the study, whether in listing species (including rare and/or secretive species) or in adding other essential data (developmental stages, some species traits). The present study has shown that metabarcoding can be directly used for genus-level bioassessment (Apothéloz-Perret-Gentil et al., 2017; Hering et al., 2018). Further development of the molecular approach in parallel with the morphological approach on a large dataset towards assigning indicator values to genera and calculating new ciliate indices, should allow implementation in monitoring assessments. Validation of such an approach would result from a clear response of the metric to environmental pressures.

CRediT authorship contribution statement

Antonija Kulaš: Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Vesna Gulin:** Methodology, Writing - original draft. **Renata Matoničkin Kepčija:** Investigation, Writing - review & editing. **Petar Žutinić:** Investigation, Conceptualization, Writing - review & editing. **Mirela Sertić Perić:** Conceptualization, Writing - review & editing. **Sandi Orlić:** Conceptualization, Writing - review & editing. **Katarina Kajan:** Investigation. **Thorsten Stoeck:** Supervision, Data curation, Validation, Writing - review & editing. **Guillaume Lentendu:** Formal analysis, Methodology, Software, Visualization, Writing - review & editing. **Ivan Čanjevac:** Writing - original draft. **Ivan Martinić:** Writing - original draft, Visualization. **Marija Gligora Udovič:** Conceptualization, Funding acquisition, Investigation, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2021.107430.

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