UNRAVELLING BIOINDICATORS IN FRESHWATERS THROUGH THE INTEGRATION OF eDNA METABARCODING AND MORPHOLOGICAL METHODS

Kulaš, Antonija

University of Zagreb

Faculty of Science

Department of Biology

Antonija Kulaš

UNRAVELLING BIOINDICATORS IN FRESHWATERS TROUGH THE INTEGRATION OF eDNA METABARCODING AND MORPHOLOGICAL METHODS

DOCTORAL DISSERTATION

Zagreb, 2022

Sveučilište u Zagrebu

Prirodoslovno matematički fakultet

Biološki odsjek

Antonija Kulaš

ISTRAŽIVANJE BIOINDIKATORSKIH ORGANIZAMA SLATKIH VODA INTEGRACIJOM eDNA METABARKODIRANJA I MORFOLOŠKIH METODA

DOKTORSKI RAD

Zagreb, 2022

This doctoral dissertation was carried out within the Postgraduate doctoral programme in Biology at the University of Zagreb, Faculty of Science, Department of Biology (Division of Botany), under the supervision of Assoc. prof. dr. sc. Marija Gligora Udovič. The research was performed in the frame of the „Origin, fate and TRAnsport modelling of NItrate in the Varaždin ALluvial aquifer - (TRANITAL)" project, supported by the Croatian Science Foundation (project number HRZZ-IP-2016-06-5356; project leader dr.sc. Tamara Marković) and „Assessment of the ecological status of the Krka River using DNA metabarcoding, NP Krka" project (project leader Assoc. prof. dr. sc. Marija Gligora Udovič) and from the grant agreements FEMS Research and Training Grant FEMS-GO-2018-127 and COST DNAqua-Net grant CA15219. The experimental parts of the research was carried out in part at Ruđer Bošković Institute, Zagreb, Croatia, while part of the bioinformatics analyses were carried out at University of Kaiserslautern (Germany), University of Ankara (Turkey) and University of Zagreb (Croatia).

MENTOR BIOGRAPHY

Marija Gligora Udovič (born 1th March 1977), PhD, Associate professor

She is a phycologist with a PhD in phytoplankton ecology and currently works as an associate professor at the University of Zagreb, Faculty of Science. Assoc. Prof. Gligora Udovič obtained her degree in Biology in 2001 at the Faculty of Science of the University of Zagreb. She joined the Department of Biology at the Faculty of Science since 2001, initially as a junior researcher and teaching assistant. In 2007, she received her PhD and subsequently worked as a postdoctoral researcher at the same institution. In 2009, she became an Assistant Professor and in 2018, an Associate Professor. From 2001 to 2009, she was a teaching assistant in five courses and continues to teach undergraduate and graduate courses of Botany, Protists, Ecology of Protists, Freshwater plankton, Algae and student projects, Microbiology of the ecosystem, Field Course in freshwater protist ecology, Algae in biological valorisation of freshwater ecosystems, Microbial diversity of natural and anthropogenic ecosystems, as a professor contributing to courses not only at the Faculty of Since but also at the Faculty of Agriculture and the Faculty of Forestry and Wood Technology of the University of Zagreb. She supervised 21 students in their master and bachelor theses and four PhD students.

She has received several scientific training grants in Europe and the United States and has been involved in many research projects in aquatic ecology and microorganisms during her career to date, focusing on the use of algae in standards and thresholds for impact assessment. This led to a career as an applied freshwater ecologist focusing on aquatic bioassessment issues and developing methods for classification of ecological status of surface waters in Croatia. She contributed to the development of national methods for ecosystem status assessment in relation to the objectives of the Water Framework Directive, in particular the use of phytoplankton and diatoms in ecological assessment. Currently, she has authored or co-authored 45 scientific articles, 89 conference abstracts, 5 conference papers, and four popular science publications. She was a member of the organizing committee of the 7th European Phycological Congress (7th EPC), the 15th Symposium on Aquatic Microbial Ecology (SAME15), the 12th Croatian Biological Congress with international participation, the 8th Central European Diatom Meeting (8th CEDM) and has contributed to many events and workshops on popularization of science. She is a member of COST Action Ocean4Biotech platform, and a collaborator in two research projects of HRZZ (Croatian Science Foundation) CELLSTRESS and TRANITAL. In 2018, she registered a unique diatom collection as Croatian National Diatom Collection.

ACKNOWLEDGEMENTS

I would like to thank to Igor Stanković, who was my first mentor into freshwater algae, and recommended me for this job.

My heartfelt thanks to my supervisor Marija Gligora Udovič, who patiently supported and guided me in every way, gave me space and time, and lead me in the right direction to become independent. She was also relentless in securing collaborations that were crucial for all the scientific work I needed for my PhD. Thank you, Maja.

Thanks in abundance to prof. Zlatko Levkov, who introduced me into "the world of diatoms" and provided me with his knowledge in observing diatoms; to my collaborators, Dr. Sandi Orlić, prof. Thorsten Stoeck, and Assoc. prof. Emre Keskin, who welcomed me into their laboratories and allowed me access to laboratory and research facilities, without which I would not have been able to examine my work; to Dr. Guillaume Lentendu for introducing me to bioinformatics and statistics, and for his sincere guidance and fruitful discussions; to the eGL research team for their support and help during my stay and work at Ankara University.

I owe a thank you to my lab team prof. Anđelka Plenković-Moraj, Petar and Mirela for stimulating discussions, help, support and encouragement.

There are many people who have helped me selflessly in my PhD work. Thank you, Katarina, Anamarija, Maja and Ines. I thank my colleagues: Zrinka, Sunčica, Lucija, Marta, Klara, Antonija, Vesna, Barbara and Skejo. Thank you, Martina and Sandrica for helping numerous times in lab, but most importantly, for stimulating conservations, coffee breaks and lunches. I hope I have not overlooked anyone, and of course, special thanks to the rest of my co-authors who contributed to this work, and to the thesis reviewers.

I am deeply indebted to my family and friends. Mom and dad, thank you for your encouragement, support, faith and love. Enormous thanks to my sister Ivana, who is my best friend and always understands my nonsense ideas and attitudes. And to all my friends who always supported me and believed in me, especially Dario, who encouraged me the most in my PhD process.

In the end, I would like to thank two special people in my life. The one who taught me how to believe in myself and my work; and my soulmate who is the exact opposite of me in unpredictable situations and my true support because he is simply by my side.

University of Zagreb Doctoral thesis

Faculty of Science

Department of Biology

UNRAVELLING BIOINDICATORS IN FRESHWATERS THROUGH THE INTEGRATION OF eDNA METABARCODING AND MORPHOLOGICAL METHODS

ANTONIJA KULAŠ

Faculty of Science, Department of Biology

Microbial communities are widely used in biomonitoring assessments as planktic or benthic organisms, but most attention has been given to one algal group - diatoms, while other eukaryotic and bacterial groups have been largely overlooked. The objective of this thesis was to evaluate the reliability of using measurable results of microbial diversity in ecological researches in assessing the status of aquatic ecosystems; and to propose potential bioindicators of microorganisms and water body types that are not currently included in routine monitoring. Based on the first three publications, it was shown that the comparison of the results obtained with both approaches allows a comprehensive assessment of the microbial community structure in the karstic Krka River and in small water body in the alluvial area of the Drava River. The last publication highlighted the diversity of the overall protist communities in the periphyton, showing that such information is also crucial for further effective management and protection of aquatic biodiversity.

(79 pages, 4 figures, 192 references, original in English)

Keywords: microbial community, morphological approach, eDNA, freshwater assessment.

Supervisor: Dr Marija Gligora Udovič, Associate Professor

Reviewers: Dr Petar Žutinić, Assistant Professor

Dr Sandi Orlić, Senior scientist

Dr Emre Keskin, Associate Professor

Sveučilište u Zagrebu Doktorski rad

Prirodoslovno-matematički fakultet

Biološki odsjek

ISTRAŽIVANJE BIOINDIKATORSKIH ORGANIZAMA SLATKIH VODA INTEGRACIJOM eDNA METABARKODIRANJA I MORFOLOŠKIH METODA

ANTONIJA KULAŠ

Prirodoslovno-matematički fakultet, Biološki odsjek

Mikrobne zajednice koriste se u biomonitoringu kao planktonski ili bentoski organizmi, no najviše pažnje pridaje se jednoj skupini algi - dijatomejama, dok su drugi eukariotski mikrorganizmi i bakterije uglavnom zanemarene. Cilj ove doktorske disertacije je bila usporedba mjerljivosti rezultata dobivenih molekularnim i morfološkim analizama u ekološkim istraživanjima pri procjeni stanja vodenih ekosustava; te prijedlog potencijalnih bioindikatorskih skupina mikroorganizama i tipova vodnih tijela koji trenutno nisu uključeni u rutinski monitoring. Na temelju prve tri publikacije pokazalo se da usporedba rezultata dobivenih s oba pristupa omogućuje sveobuhvatnu procjenu strukture mikrobne zajednice u krškoj rijeci Krki i u malim vodnim tijelima u aluvijalnom području rijeke Drave. Posljednja publikacija istaknula je raznolikost sveobuhvatne zajednice protista u perifitonu, pokazujući da su takve informacije također ključne za daljnje učinkovito upravljanje i zaštitu slatkovodnih sustava.

(79 stranice, 4 slike, 192 literaturna navoda, jezik izvornika: engleski)

Ključne riječi: mikrobna zajednica, morfološki pristup, okolišna DNA (eDNA), ocjena stanja vodenih sustava.

Mentor: Izv. prof. dr. sc. Marija Gligora Udovič

Ocjenjivači: Doc. dr. sc. Petar Žutinić

Dr. sc. Sandi Orlić

Izv. prof. dr. sc. Emre Keskin

TABLE OF CONTENTS

LIST OF PUBLICATIONS

I. **Kulaš, A.**, Marković, T., Žutinić, P., Kajan, K., Karlović, I., Orlić, S., Keskin, E., Filipović, V., and Gligora Udovič, M. (2020). Succession of Microbial Community in a Small Water Body within the Alluvial Aquifer of a Large River. *Water*, 13(2), 115.

II. **Kulaš, A.**, Gulin, V., Matoničkin Kepčija, R., Žutinić, P., Sertić Perić, M., Orlić, S., Kajan, K., Stoeck, T., Lentendu, G., Čanjevac, I., Martinić, I., and Gligora Udovič, M. (2021). Ciliates (Alveolata, Ciliophora) as bioindicators of environmental pressure: A karstic river case. *Ecological indicators* 124, 107430.

III. **Kulaš, A.,** Gligora Udovič, M., Tapolczai, K., Žutinić, P., Orlić, S., and Levkov, Z. (2022). Diatom eDNA metabarcoding and morphological methods for bioassessment of karstic river. *Science of the total environment* 829, 154536.

IV. **Kulaš, A.,** Žutinić, P., Gulin, V., Matoničkin Kepčija, R., Sertić Perić, M., Orlić, S., Sviličić Petrić, I., Marković, T., and Gligora Udovič, M. (2022). Diversity of protist genera in periphyton of tufa-depositing karstic river (*submitted to Annals of Microbiology*).

EXTENDED SUMMARY

Microorganisms dominate aquatic ecosystems, and their ecology is very important, encompassing studies of microbial interactions that play a critical role in regulating ecosystem productivity and stability, modulating trophic networks, and mediating global biogeochemical cycles (Liu et al., 2019; Moënne-Loccoz et al., 2015). With the exception of viruses as non-living organisms, all microbial representatives are grouped into the three major domains (Bacteria, Archaea, and Eukarya), which can also be distinguished according to their role in nutrient cycling and their lifestyle (planktic and benthic).

Microbial communities are wide use in biomonitoring assessment as planktic or benthic organisms, because of their sensitivity, ubiquitous nature, small size, high abundance, fast response to any pressures, stress or disturbance events, and detectability (Payne, 2013). Traditionally, the most attention in freshwater biomonitoring and water assessment has been given to an algal group called diatoms, whilst largely overlooking other eukaryotic and bacterial groups (Debroas et al., 2017; Simon et al., 2015). Bacterial community provides a basis for understanding the entire microbial community and carry out key processes of the nutrient cycles in aquatic environments (Findlay, 2010; Jetten et al., 2003). Understanding the bacterial community composition and its impact on ecosystem functioning can provide new insights into species ecology and groups (Anderson et al., 2002). Eukaryotic microorganisms, represented by algae as primary producers and microbial grazers and metazoans as consumers, are involved in global functioning of ecosystems (Caron et al., 2009; Debroas et al., 2017; Worden et al., 2015). Diatoms are eukaryotic, unicellular, photosynthetic organisms, widespread and receive the most attention because their representatives are used to assess the ecological status of aquatic ecosystems (Kahlert et al., 2016). As opposed to algae, protozoans have generally received less attention in elucidating their diversity, distribution and ecology. A very large and diverse group of heterotrophic microeukaryotes that occupy an essential position in the trophic web of freshwater ecosystems are ciliates. They are excellent bioindicators, but they are almost completely excluded or rarely integrated into water quality assessment.

Choosing an appropriate method is important to properly explore the still mysterious compartment of microorganisms in freshwater environments with efficient methodological tools (Joux et al., 2015). Combining new technologies such as molecular approach with traditional approaches can provide integrative taxonomical information, but also genetic and

ecological data that can stimulate interdisciplinary research in the ecology of aquatic environments (Dayrat, 2005; Warren et al., 2017).

The main aim of this thesis was to evaluate the reliability of using measurable results of microbial diversity in ecological researches in assessing the status of aquatic ecosystems; and to propose potential bioindicators of microorganisms and water body types that are not currently included in routine monitoring. The thesis addressed the following hypotheses: I) molecular methods are valuable for assessing diversity of microorganisms in the plankton and benthos of freshwater ecosystems; II) diatoms are well studied group of microorganisms in benthos and periphyton, but not the only one with good indicator potential; III) interactions between groups of organisms in the plankton and benthos in freshwater systems, controlled by anthropogenic pressure into the system, provide new insights into the indicator properties of species and communities; IV) small water bodies are important nutrient recyclers in the systems of large rivers; V) with a larger number of sampling of different microhabitats, we can get a better insight into the state of the ecosystem than with a one representative monitoring sampling point; VI) due to limitations of currently used biomonitoring methodologies, that rely on traditional taxonomic identification, methods based on eDNA allow integration of a much wider range of taxa and indicator groups into freshwater ecological assessments and VII) due to limitations of currently used biomonitoring data, including time-consumption, space and researcher availability, methods based on eDNA allow the possibility of increasing the number and types of biotopes into freshwater ecological assessments.

In this doctoral thesis, the results are presented in the form of four publications (Publication **I, II, III, IV**) together with a detailed discussion. Publications **I, II** and **III** provided answers to the main aim of using measurable results of microbial diversity in environmental assessment analysed by morphological and molecular methods. This study evaluates the reliability of the application of the eDNA metabarcoding tool in the ecological assessment of biomonitoring for the microbial community in the plankton or benthos of the karstic Krka River and in small water body in the alluvial area of the Drava River. The diversity of the microbial community was characterized using traditional morphological and molecular methods. The results of both approaches were compared depending on the studied organisms within the microbial community to determine if eDNA metabarcoding can be used as a replacement for traditional methods. Finally, Publications **I, II**, and **III** demonstrated and confirmed that the results of both methods are comparable, measurable, and have the potential to be used in biomonitoring assessments. The publication **IV** provided a deeper insight into the

protists diversity and composition in the karstic Krka River obtained by a molecular approach (using specific primers of the V9 region for 18S of rRNA). Furthermore, this publication demonstrated how molecular approach can provide valid biological data on protists diversity that can be used for conservation of karstic environments.

The scientific contribution of this thesis is that it demonstrates the applicability of molecular methods in Croatian freshwaters and provides new insights into the diversity and interactions between different groups of microbial organisms in the plankton and benthos of freshwater ecosystems. The thesis also provides new insights into the importance of microorganisms in recycling and utilization of nutrients in small water body in large river systems. At the same time, the molecular methods contribute to the expansion of monitoring through a greater number of analyses using standardized procedures, allowing the implementation and clarification of the ecological value of the microbial community in expanded indicator groups and demonstrating the possibility of increasing the number and type of water bodies in routine monitoring. Moreover, this is the first attempt of such a research example based on the expansion of indicator groups in Croatia to include organisms other than phytoplankton and phytobenthos as bioindicators and to determine the interactions between microbial communities to better understand their indicator potential. Anthropogenic impacts are causing unprecedented changes in freshwater ecosystems, characterized by biodiversity loss. Accordingly, there is a need for rapid, sensitive, cost-effective, and non-invasive monitoring such as that provided by molecular methods. This work will ultimately contribute to better freshwater management and protection of Croatian freshwaters.

PROŠIRENI SAŽETAK

Mikroorganizmi dominiraju u vodenim ekosustavima, a njihova ekologija je vrlo važna jer uključuje mikrobne interakcije, koje imaju ključnu ulogu u regulaciji produktivnosti i stabilnosti sustava, modulaciji trofičkih mreža i posredovanju globalnih biogeokemijskih ciklusa (Liu i sur., 2019; Moënne-Loccoz i sur., 2014). Svi predstavnici unutar mikrobne zajednice, isključujući viruse podijeljeni su u tri glavne domene (bakterije, arheje i eukarioti), a mogu se podijeliti i prema njihovoj ulozi u kruženju hranjivih tvari i prema životnim strategijama (planktonski i bentoski organizmi).

Mikrobne zajednice imaju široku primjenu u biomonitoringu kao planktonski ili bentoski organizmi, zbog svoje osjetljivosti, široke rasprostarnjenosti, male veličine, velike zastupljenosti, brzog odgovora na okolišne pritiske i mogućnosti njihove detekcije (Payne 2013). Tradicionalno, najviše pozornosti u biomonitoringu slatkih voda pridaje se skupini algi pod nazivom dijatomeje, dok se uglavnom zanemaruju druge eukariotske i bakterijske skupine (Debroas i sur., 2017; Simon i sur., 2015). Bakterijska zajednica pruža osnovu za razumijevanje cjelokupne mikrobne zajednice i provođenje ključnih procesa ciklusa hranjivih tvari u vodenom okolišu (Findlay i sur., 2010; Jetten i sur., 2003). Razumijevanje sastava bakterijske zajednice i razjašnjavanje odnosa između bakterijske bioraznolikosti i njezina utjecaja na funkcioniranje ekosustava može pružiti nove uvide u ekologiju vrsta i skupine (Anderson i sur., 2002). Eukariotski mikroorganizmi, alge kao primarni proizvođači i metazoa kao potrošači, uključeni su u globalno funkcioniranje ekosustava (Caron i sur., 2009; Debroas i sur., 2017; Worden i sur., 2015). Dijatomeje su eukariotski, jednostanični, fotosintetski organizmi, koji dobivaju najveću pozornost jer se pomoću njih procjenjuje ekološko stanje vodenih ekosustava (Kahlert i sur., 2016.). Za razliku od algi, protozoama se općenito pridavalo manje pozornosti u istraživanju raznolikosti, rasprostranjenosti i ekologije vrsta. Tako su cilijati vrlo velika i raznolika skupina heterotrofnih mikroeukariota koji zauzimaju bitno mjesto u trofičkoj mreži slatkovodnih ekosustava. Izvrsni su bioindikatori, ali su gotovo potpuno isključeni ili rijetko uključeni u ocjenu kakvoće vode.

Odabir odgovarajuće metode važan je za pravilno istraživanje još uvijek neotkrivenog svijeta mikroorganizama u slatkovodnim ekosustavima (Joux i sur., 2015). Kombinacija novih tehnologija, poput molekularnih, s tradicionalnim pristupima može pružiti integrativne taksonomske informacije, ali i genetičke i ekološke podatke koji mogu potaknuti

interdisciplinarna istraživanja u ekologiji vodenih ekosustava (Dayrat, 2005; Warren i sur., 2017).

Glavni cilj ove doktorske disertacije je bila usporedba mjerljivosti rezultata dobivenih molekularnim i morfološkim analizama u ekološkim istraživanjima pri procjeni stanja vodenih ekosustava; te prijedlog potencijalnih bioindikatorskih skupina mikroorganizama i tipova vodnih tijela koji trenutno nisu uključeni u rutinski monitoring. Cilj je također bio ostvariti mogućnost korištenja različitih taksonomskih skupina utvrđenih na temelju okolišne DNA (eDNA), kao pokazatelja promjene u okolišu, uzimajući u obzir skupine koje se koriste u tradicionalnom biomonitoringu, ali i one koje nisu obuhvaćene rutinskim praćenjem. Iz glavnih ciljeva ove doktorske disertacije proizašle su i navedene hipoteze: I) veća raznolikost planktonskih i bentoskih (perifitskih) svojti slatkovodnih ekosustava utvrdit će se molekularnim pristupom; II) uz dijatomeje, cilijati pokazuju velik indikatorski potencijal u slatkovodnim ekosustavima; III) u usporedbi s jednim reprezentativnim monitoring mjestom uzorkovanja, veći broj mjesta uzorkovanja na različitim mikrostaništima mogao bi pružiti bolji uvid u ekološko stanje slatkovodnih ekosustava; IV) mala vodna tijela važna su u recikliranju hranjivih tvari u sustavima velikih rijeka; V) interakcije između skupina organizama u planktonu i bentosu malih slatkovodnih sustava pod antropogenim utjecajem pružaju novi uvid u indikatorska svojstva vrsta i zajednica; VI) zbog ograničenja u postojećim metodologijama biomonitoringa, koja se oslanjaju na tradicionalnu taksonomsku identifikaciju, metode temeljene na okolišnoj DNA (eDNA) omogućuju integraciju mnogo šireg spektra svojti i indikatorskih skupina za ocjenu stanja slatkovodnih ekosustava; VII) zbog ograničenja u postojećem biomonitoringu koje uključuju vrijeme, prostor i dostupnost istraživača, metode temeljene na okolišnoj DNA (eDNA) omogućuju uključivanje mnogo šireg raspona biotopa za ocjenu stanja slatkovodnih ekosustava.

U ovoj doktorskoj disertaciji rezultati su prikazani u obliku četiri publikacije **(**Publikacija **I, II, III, IV)** uz iscrpnu raspravu. Publikacije **I, II** i **III** odgovorile su na pitanje mjerljivosti između molekularnih i morfoloških metoda u karakterizaciji mikrobnih zajednica u slatkim vodama. Istraživanje procjenjuje pouzdanost primjene metabarkodiranja okolišne DNA (eDNA) u ocjeni stanja okoliša za biomonitoring mikrobnih zajednica u planktonu ili bentosu krške rijeke Krke i malog vodnog tijela u aluvijalnom području rijeke Drave. Raznolikost mikrobnih zajednica karakterizirana je tradicionalniim morfološkim i molekularnim pristupima, a rezultati dobiveni pomoću oba pristupa međusobno su uspoređeni ovisno o proučavanim organizmima unutar mikrobnih zajednica kako bi se utvrdilo može li se

eDNA metabarkodiranje koristiti kao zamjena tradicionalnim metodama. Konačno, kroz Publikacije **I, II** i **III** prikazano je i potvrđeno da su rezultati obje metode usporedivi i mjerljivi, te da imaju potencijal za korištenje u ocjeni stanja vodenih ekosustava. Publikacija **IV** omogućila je dublji uvid u raznolikost i sastav protista u krškoj rijeci Krki dobivenih molekularnim pristupom (upotrebom specifičnih primera V9 regije, 18S rRNA). Predstavljeno je kako se molekularnim pristupom može omogućiti i istraživanje bioraznolikosti drugih skupina protista što može biti od velike važnosti za očuvanje krškog okoliša.

Znanstveni doprinos ove doktorske disertacije je u tome što prikazuje primjenjivost molekularnih metoda u hrvatskim slatkovodnim tijelima i otkrivanje novih spoznaja o odgovoru mikrobne raznolikosti na okolišne pritiske u različitim slatkovodnim tijelima. Doprinos je također i u pružanju novih spoznaja o važnosti mikroorganizama u recikliranju i korištenju hranjivih tvari u malim vodnim tijelima u sklopu velikih riječnih sustava. Osim toga, molekularne će metode, putem većeg broja analiza prema standardiziranim postupcima, pridonijeti proširenju monitoringa. Ovim načinom će se omogućiti lakša provedba i tumačenje ekološke vrijednosti mikrobne zajednice u vidu proširenih indikatorskih skupina, te će se istaknuti mogućnost povećanja broja i tipova vodnih tijela u rutinskom monitoringu. Štoviše, ovo istraživanje predstavlja prvi primjer istraživanja koje daje podlogu mogućem proširivanju indikatorskih skupina u Hrvatskoj, u svrhu uključivanja organizama koji nisu definirani kao biološki elementi kakvoće te utvrđivanja interakcija između mikrobnih zajednica s ciljem boljeg razumijevanja i definiranja njihovog indikatorskog potencijala. Antropogeni utjecaji uzrokuju promjene u slatkovodnim ekosustavima, koje karakterizira gubitak bioraznolikosti. Sukladno tome, postoji potreba za brzim, osjetljivim, ekonomičnim i neinvazivnim praćenjem, kao što to mogu omogućiti molekularne metode. Na ovaj način će ova disertacija u konačnici doprinijeti boljem upravljanu i zaštiti slatkovodnih ekosustava u Hrvatskoj.

INTRODUCTION

Microbial communities in freshwater ecosystems

There are about 1.386 billion cubic kilometres of water on Earth. Almost 97% of the water is distributed in the form of seas and oceans, around 2% exists in the form of ice caps and glaciers, and about 1% is distributed in the form of rivers, lakes, groundwater, and water vapour. When considering freshwater availability on Earth, nearly 66.7% is distributed in the form of ice caps and glaciers alone, about 30.1% is available as groundwater, directly available surface water shared by lakes, swamps, and flowing waters such as rivers accounts for 0.3%, with the remaining 0.9% present as water vapour and soil water (Balasubramanian, 2015). Although covering such a small proportion of total water on Earth, surface freshwater systems provide life with a wide range and diversity of ecological niches, including different trophic levels, light availability, temperature, and oxygen concentrations. These conditions include physical support, accessibility of three-dimensional space, passive movement by water currents, dispersal of motile elements in a liquid medium, minimal water loss, lower extremes of temperature and solar radiation, and availability of soluble organic and inorganic nutrients (Sigee, 2005). Due to numerous potentials and variations, freshwater systems can host drastically different communities as microbial communities (Boenigk et al., 2018; Debroas et al., 2017). The studies on freshwater systems have traditionally been focused on their physical, chemical and biological properties, the latter mostly referring to exploring community composition from microorganisms to fish. In the context of environmental management, changes in community composition have been used as an indicator of changes in water bodies resulting from various types of processes (Ptacnik et al., 2008; Reiss et al., 2009).

The term 'microbes' refers to all organisms smaller than 100 μm, visible only with an artificial magnification. The microbial ecology involves studies on microbial interactions, which play crucial roles in regulating ecosystem productivity and stability, modulating trophic networks and mediating global biogeochemical cycles (Liu et al., 2019; Moënne-Loccoz et al., 2015). Microbial communities dominate in aquatic ecosystems, and they are capable of flourishing in all water habitats. Excluding viruses as non-freeliving organisms, all microbial representatives are comprised in the three major domains (Bacteria, Archaea, and Eukarya). Within these domains, organisms can be distinguished in terms of several physiological, structural and biochemical characteristics, as well as in their roles in nutrient cycling as

photoaututrophs, heterotrophs or mixotrophs (Massana, 2011). Autotrophic group covers photosynthetic bacteria and microalgae, whereas heterotrophs include several subgroups such as saprotrophic organisms that obtain their nutrients from non-living material and associations with living organisms like parasitism or symbiosis; and on the other hand there are mixotrophs which have the capability to utilize autotophic and heterotrophic modes of nutrition (Berry et al., 2006; Crane and Grover, 2010; Stoeck et al., 2014). Generally, the activities of microbes have profound impact on global scales, being largely implicated in carbon fixation (Jardillier et al., 2010) and climate regulation (Simó, 2001). According to their living habits, microorganisms in freshwaters can be distinguished into planktic and benthic organisms. The term *plankton* is a collective term relating to all organisms that spend their lives floating in the water column, thus encompassing groups from viruses to bacteria, protists, fungi, and metazoans. Understanding the ecology of plankton is critical because these organisms form the basis of the entire aquatic food web (Falkowski et al., 2004). Depending on their role in the ecosystem, plankton are divided into photosynthetic primary producers (phytoplankton), phagotrophic consumers (zooplankton), and heterotrophic decomposers (bacterioplankton). Plankton can also be subdivided by size/cell length into pico- to macroplankton (pico-, nano-, micro-, meso- and macroplankton; Dipper, 2022). Phytoplankton are unicellular organisms that drift with the currents, carry out oxygenic photosynthesis, and live in the upper illuminated waters of all aquatic ecosystems. There are about 25 000 known species of phytoplankton, including eubacterial and eukaryotic species. This phylogenetically diverse group of organisms forms the base of the food chain in most aquatic ecosystems and has a profound impact on the biogeochemistry of Earth. Currently, phytoplankton taxa are responsible for the photosynthetic fixation of about 50×10^{15} g C per year, accounting for nearly half of the global net primary production on Earth (Fox et al., 2020). Benthos, on the other hand, is defined as flora and fauna that occur on, in, or close to the bottom substrate of water bodies (lakes, ponds, rivers, streams or sea). The benthic components can differ considerably, depending on the depth and speed of water. However, the most important selection criterion is often the nature of the substrate, because some benthic organisms can walk or glide on solid surface, while some are sessile and have obligate dependence on the presence of a rigid surface (Reynolds, 2006). Besides the nature of the substrate, the energetic constraints on the biotic processing of carbon profoundly affect the functional and structural organization of benthic communities, where benthic photoautotrophs known as a phytobenthos are restricted to the substrata in shallow water and littoral of larger lakes and sea (Reynolds, 2006). Phytobenthos includes cyanobacteria and representatives of most algal phyla, especially diatoms (Bacillariophyceae). The benthic

photoautotrophs provide surface habitats and food sources for benthic animal organisms, such as macroinvertebrates known as benthic herbivores and detritivores (Reynolds, 2006). Microbial communities play a crucial role in essential processes of biological production and biodegradation, moreover they are extremely important for ecosystem functioning in freshwater management (Barthel et al., 2008; Curtis et al., 2003; Ghazy et al., 2008).

Ecological water quality assesment required by the European Water Framework Directive (WFD) is based on predefined bioindicator species or biological quality elements (BQEs), which include fish, macrozoobenthos, phytoplankton, phytobenthos and macrophytes (Andersen et al., 2016; Hunting et al., 2017). The advantages of using planktic or benthic microbial communities in biomonitoring include their sensitivity, ubiquitous nature, small size, high abundance, fast response to any pressures, stress or disturbance events, and detectability (Payne, 2013). Traditionally, the most attention in freshwater biomonitoring and water assessment has been given to diatoms, whilst largely overlooking other eukaryotic and bacterial groups (Debroas et al., 2017; Simon et al., 2015).

Prokaryotic microorganisms are small, simple organisms characterized by the absence of a true nucleus and membrane-bound cell organelles, such as mitochondria or chloroplasts. They consist of two separate major groups, Bacteria and Archaea. Bacteria are the least complex living microorganisms, but offer the greatest metabolic flexibility and exhibit the greatest diversity. They control numerous environmental processes that are important not only to humans, but also to the environment (e.g. nitrogen fixation). About half of the bacterial phyla estimated at the molecular level have not yet been fully discovered (Liu et al., 2020). Based on their cell envelope architecture, Bacteria can be structurally separated into two major groups: Gram positive or Gram negative. This architectural difference helps dictate strategies for survival in the environment. For example, the thick cell wall of Gram-positive bacteria helps them withstand the harsh physical conditions found in soil environments. On the other hand, the more complex architecture of the cell envelope in Gram-negative bacteria seems to aid these microbes in interacting with mineral surfaces and solutes in the environment to obtain required nutrients for metabolism (Gupta et al., 2016). Generally, bacterial community provides a basis for understanding the entire microbial community and carry out key processes of the nutrient cycles in aquatic environments, and are responsible for a large part of organic matter breakdown (Findlay, 2010; Jetten et al., 2003). Aquatic bacterial communities are extremely diverse and highly dynamic in terms of taxonomic composition, offering a large degree of variation in community structure among different types of freshwater ecosystems (Crump et al., 2007; Liu

et al., 2015; O'Lear et al., 2013). Understanding the bacterial community composition and its influencing factors is helpful in evaluating water quality and interpreting nutrition cycle mechanisms (Anderson et al., 2002). The most abundant bacterial phyla in aquatic ecosystems are: Proteobacteria, Actinobacteria and Bacteroidetes. Routine monitoring programs of freshwater ecosystems often disregard bacterial community due to intrinsic complexity and small fraction of bacterial taxa that can be successfully cultivated (Huse et al., 2008, 2010). Recently, the wide use of molecular tools rapidly expanded knowledge on bacterial diversity, due to deep sequencing of bacterial communities and identification of rare populations in low abundance (Caporaso et al., 2011; Glenn, 2011). Elucidating the relationship between bacterial biodiversity and its impact on ecosystem functioning can provide new insights into species ecology and groups (Schmidt et al., 2020).

Eukaryotic microorganisms, represented by algae as primary producers and microbial grazers and metazoans as consumers, are involved in global functioning of ecosystems (Caron et al., 2009; Debroas et al., 2017; Worden et al., 2015) and perform a key link in aquatic food webs. Algae are oxygenic, photosynthetic organisms that include simple unicells, as well as complex multicellular structures. They can colonise a wide range of habitats, from aquatic environments (freshwater, marine, and brackish) to soils and rocks, but they are most often found in saturated environments, either suspended in the water column as plankton or living on the bottom as benthos (Pepper et al., 2015). The classification of algae is complex and includes numerous cellular characteristics. For example, algae can be classified into groups based on cell wall chemistry, cell morphology, chlorophyll and accessory pigments, number and position of flagella in the cell wall, reproductive structures, life cycle, and habitat preference. According to cell characteristics, algae are divided into green algae (Chlorophyta), euglenoids (Euglenozoa), dinoflagellates (Miozoa), golden brown algae (Ochrophyta - Chrysophyceae), diatoms (Bacillariophyta), brown algae (Ochrophyta - Phaeophyceae) and red algae (Rhodophyta). Diatoms are small sized, widespread in aquatic ecosystems and receive the most attention because their representatives are used to assess the ecological status of aquatic ecosystems (Kahlert et al., 2016). Diatoms are eukaryotic, unicellular, photosynthetic organisms with a silica cell wall called frustule, occurring either in plankton or benthos of waters as solitary cells, filaments, chains, or colonies (Round et al., 1990). Most often, they are abundant in benthic communities as periphytic photoautotrophic algae - phytobenthos. They play an important role in the biogeochemical cycling of nitrogen, phosphorus, silicon, and carbon and are responsible for at least 25% of global carbon dioxide fixation and 20% of global

net primary production (Burliga and Kociolek, 2016; Wilhelm et al., 2006). Interestingly, they have significantly higher maximum nutrient uptake rates than any other group of algae (Litchman et al., 2006). This, along with relatively high maximum growth rates, wide diversity, and ubiquitous distribution makes diatoms good nutrient competitors and "speed specialists" capable of effectively exploiting nutrient pulses (Litchman, 2007). These characteristics can steer each diatom species toward specific ecological preferences that allow for rapid and distinct responses to environmental changes, enabling the use of benthic diatoms as biological indicators in biomonitoring programmes required by the Water Framework Directive (WFD, Directive 2000/60/EC, 2000). However, the current methodology for biomonitoring, based on morphological taxonomic identification, is time-consuming (counting of 400 valves per sample under microscope) and requires extensive expertise due to a constantly evolving taxonomy (Kahlert et al., 2012). Moreover, there are taxonomic discrepancies between laboratories hampering the sharing of data, so applied high-throughput sequencing of diatom taxa in biofilm samples could overcome the limitations of traditional microscopic approach (Pawlowski et al., 2018).

As opposed to algae, protozoans have generally received less attention in elucidating their diversity, distribution and ecology. The reasons of such limited interest lie in their small size with very few unambiguous morphological differences, which makes the identification challenging, as well as poorly resolved or oversimplified taxonomic resolution (Debroas et al., 2017; Nolte et al., 2010; Simon et al., 2015). Numerous studies have confirmed protozoans to cover multiple ecological roles in ecosystems (Arndt et al., 2000; Zubkov and Tarran, 2008), whilst others have demonstrated that many groups are more flexible in their nutritional efficiencies than initially thought (McManus et al., 2018; Stoecker and Silver, 1987), as photosynthetic capability via endosymbiotic associations or chloroplast retention has been observed in a broad range of eukaryotic lineages, such as ciliates (Johnson, 2011; McManus et al., 2018). Ciliates represent a very large and diverse group of heterotrophic microeukaryotes that occupy an essential position in the trophic web of freshwater ecosystems. As one of the key players in the periphytic microbial food web, they feed on bacteria, algae, heterotrophic flagellates, and other protists, while themselves are being consumed by members of the meiofauna (Finlay and Esteban, 1998; Lear et al., 2009). However, it is still not well understood how ciliate diversity and community structure are affected by changing environmental conditions, or how ciliate communities affect other biota and processes in phytoplanktic and benthic communities of aquatic environments. Previous studies have found evidence of the

existence of diverse communities of abundant ciliates and shift in community structure in response to eco-physiological parameters (Kepner and Pratt, 1996; Lear 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. For example, light increases biomass production and favours autotrophs, directly affecting the community composition (Vermaat, 2005). Certain ciliate species exhibit photosensitive behaviour, or they can have positive phototaxis which can help in avoiding predators (Esteban et al., 2010; Lynn, 2010). Ciliates have been successfully applied in assessment of water quality using the saprobic sytem (Berger and Foissner, 2003). Despite being excellent bioindicators due to their ubiquity, abundance, and sensitivity to anthropogenic impacts (Hughes, 2018), they are almost completely excluded or rarely integrated into water quality assessment. Any detected change in the ciliate community composition in response to environmental shifts can be used as a robust bioassessment tool (Pawlowski et al., 2016). Though having a vast bioindicator potential, ciliates are largely overlooked mainly due to limitations of morphological identification, as many of them are fragile and fast moving which often require complex preserving and staining protocols for reliable identification (Hering et al., 2018; Lear et al., 2009).

Combining new technologies such as molecular approach with traditional approaches can provide integrative taxonomical information, but also genetic and ecological data that can stimulate interdisciplinary research in the ecology of aquatic environments (Dayrat, 2005; Warren et al., 2017).

Methodology for microbial community research in freshwaters

The different components of microbial community can be characterized from the point of view of their diversity, biomass, and their role and interactions in the environment. There are different types of methods in detecting microbial communities in freshwater ecosystems, with their advantages and disadvantages. The choosing of an appropriate method is important to properly explore the still mysterious compartment of microorganisms in freshwater environments with efficient methodological tools (Joux et al., 2015). Methodology also depends on the scope of study, type of water body, sampling and detection methods due to varieties in the monitoring practice of microbial communities. Thus, there are methodologies for shallow lakes, small water bodies, deep lakes, rivers, and other water reservoirs (Joux et al., 2015). The selection of method also depends on types of targeted microorganisms with respect

to their groups and living types. Collecting microbial samples can be considered as the process of obtaining an aliquot of the studied microorganisms in the aquatic environment, in which the sampling consists of preserving, conserving, and storing a portion of the collected water for analytical purposes (Joux et al., 2015).

The importance of bacterial diversity surveys of freshwaters is apparent in exploring bacterial ecology and evolution, supporting management policies, or obtaining risk assessment studies. Microbiological quality of water was traditionally based on culture-dependent methods (Mossel and Struijk, 2004; Vaz-Moreira et al., 2011). However, this culturing method has a severely limited biodiscovery potential on bacterial communities (Hobbie et al., 1977) and doesn't work on most aquatic microbes as they exist under extremely low concentrations of nutrients and sometimes cannot grow well on laboratory media (Mossel and Struijk, 2004). Other, applicable methods in bacterial characterization include epifluorescence microscopy, radioisotopic techniques, and methods for measuring bacterial exoenzymatic activity. The greatest progress occurred following the introduction of molecular biology techniques (Joint et al., 2010).

Studies on protist composition have been carried out for over a century with a focus on community diversity and dynamics based on morphological approach by using light-, electron, and epifluorescence microscopy and flow cytometry (Backe-Hansen and Throndsen, 2002; Dittami et al., 2014; Kuylenstierna and Karlson, 1994). Morphological approach has its own challenges and limitations, especially with its consistency in species identification. This process can be particularly time consuming, especially when used in monitoring assessments. Besides the aforementioned difficulties in identifying ciliates, which require complex preserving and staining protocols (Dopheide et al., 2009), the morphological determination of diatoms (algae) is based on the characterization of frustule, a siliceous skeleton that protects the cellular content of each individual cell, whose structures are hard to distinguish using light microscopy and thus require the use of scanning electron microscopy (Vasselon et al., 2017). All in all, morphological identification requires labour-intensive species identification, as well as taxonomic knowledge and expertise, which may limit taxonomic resolution due to misidentification (Rimet and Bouchez, 2012).

As the primary photosynthetic pigment in microalgae, chlorophyll-*a* is a measure of their biomass production and an indicator of their abundance. Therefore, chlorophyll-*a* concentration is used as an important indicator for rational assessment of eutrophication status, as well as for scientific prediction of development trends in freshwaters. There are myriad

methods for estimating chlorophyll in aquatic systems (Peng et al., 2013). Ranging from from simple photometric techniques to *in situ* prompt fluorescence measurement methods, high-performance liquid chromatography (HPLC; Wiltshire et al., 1998), or delayed fluorescence measurements. Although spectrophotometry is the most commonly used method for determination of chlorophyll-*a*, and while the fluorescence method is fast and simple, the HPLC method has the advantage of separation and determination of a number of chlorophylls and carotenoids that serve as indicators of microscopic algal biomass and as biological markers for algal species, recycling processes, and productivity measurements (Peng et al., 2013; Wiltshire et al., 1998). In environmental monitoring, different methods should be used for different requirements due to limited experimental conditions as well as the conditions for results in terms of accuracy, precision, and detection limit. Unfortunately, some of these methods tend to be subjective due to large variations caused by the presence of different taxa or due to environmental factors, such as irradiance or nutrient limitation (Descy et al., 2009), which have large effect on chlorophyll-*a* concentration (Leboulanger et al., 2006; Simmons et al., 2016).

Molecular methods and computational power led to easier biological identification, thus reducing the taxonomic impediments and making the characteristics of microbial organisms more accessible to ecologists (Pawlowski et al., 2016; Stoeck et al., 2010; Zimmermann et al., 2015). High-throughput methods and implementation of molecular methods have provided unprecedented insights into diversity and ecology of microbial communities. Just as the utilization of a particular molecular technique depend on the scientific aims, so does the application of an appropriate molecular sequencing tool depend on single-species or eDNA functional diversity detection (Taberlet et al., 2012). There are several options available to molecularly survey the environmental diversity, including metagenomics (DNA) and metatranscriptomics (RNA) which sequence the total nucleic acids of environmental samples, or the targeted metabarcoding approach (Figure 1; Burki et al., 2021). Metagenomics approach has been widely applied to studying prokaryote diversity, whereas metabarcoding is currently more routinely applied in analysing protist diversity and distribution (Burki et al., 2021). The sequencing of eDNA allows detection and characterisation of natural communities without a priori knowledge of what members belong to these communities and permits the identification of species with seemingly non-differentiable morphology (Burki et al., 2021). Over the last decades, DNA sequencing of environmental phylogenetic markers has changed our perception of organisms, especially microbial diversity (Massana et al., 2015). The DNA sequencing, especially gene coding for the small ribosomal subunit 18S rRNA, is mostly used for protist

diversity, while 16S rRNA is applied to target bacterial diversity. Other molecular markers have been used to study more restricted taxonomic groups, such as the internal transcribed spacer (ITS) of the ribosomal operon for fungi or oomycetes, the chloroplastic ribulose-1,5 biphosphate carboxylase–oxygenase large subunit (*rbcL*) gene which targets mainly plants, but diatoms as well, and the mitochondrial cytochrome oxidase c subunit I (COI) gene applied in detecting animals and a range of microbial groups (Burki et al., 2021). The stark differences in evolutionary rates along the rDNA operon genetic structure result in conserved and more variable regions. These variable regions can be used for the study of the diversity at different hierarchical levels, by comparing conserved regions for distantly related taxa and variable regions for closely related taxa. Molecular tools have also the potential in detecting and tracking rare or invasive species in their early stage of invasion. DNA metabarconding has become a key component in the toolbox of ecologists (Taberlet et al., 2012). Environmental DNA (eDNA) is probed for studying diversity, diet and ecological interactions, as well as biomonitoring of different ecosystem types. By definition, eDNA is a complex mixture of genomic DNA from many different organisms found in an environmental sample (Taberlet et al., 2012). Environmental DNA barcoding/metabarcoding uses short, standardized gene regions from environmental samples as internal species markers to enable rapid identification (Taberlet et al., 2012). The first metabarcoding methods (Sanger sequencing) allowed the acquisition of relatively long sequences (up to 1000 bp) which permitted phylogenetical interpretation, leading to the placement in trees of groups unseen before. However, the development of second generation high-throughput sequencing technologies (HTS) took over environmental sequencing, but in doing so introduced severe limitations on the lengths of the fragments that could be targeted. These new metabarcodes lacked the earlier phylogenetic signal of environmental clone libraries, but most of today's metabarcoding datasets are made of millions of short sequences reads (most often produced by Illumina). For protists, the most often targeted hypervariable regions are the V4 or V9 of the 18S rRNA gene. Although the V4 region is largely used, the V9 region has a relatively simple one-step-PCR amplicon library preparation method (Caporaso et al., 2012; Gilbert et al., 2010; Minerovic et al., 2020), thus offering improved detection of diversity and community structure especially of photosynthetic eukaryotes (Bradley et al., 2016), good trade-off between database coverage and taxonomic resolution and low sequencing costs (Tanabe et al., 2016). These two markers (V4 and V9) have different advantages depending on the taxonomic groups under study, suggesting that V9 provides a more comprehensive overview of the community, while V4 differentiates between closely related strains within a less comprehensive group of higher level taxa (Stoeck et al., 2010). A

small 312 bp fragment of the *rbcL* encodes the Ribulose-1,5-bisphosphate carboxylase/oxygenase is mostly used for metabarcoding diatoms. This gene shows alternations of highly conserved and polymorphic regions, which are key requirements for a successful genetic identification to the species level (Kermarrec et al., 2013). Within the 16S rRNA gene used for bacterial communities, regions V1-V2 and V3-V4 are the most employed (Santos et al., 2020). Despite the positive sides of molecular methods, biases can be introduced in every step, starting from DNA isolation, choosing primers for different target regions, amplification to the availability and updates of bioinformatics analyses, biases in taxonomic assignment etc. After completion of all molecular analyses, different bioinformatics platforms and algorithms (e.g. OBiTools, QIIME2, DADA2) are employed, followed by taxonomical assignment against different reference database (e.g. SILVA; PR2, diat.barcode database).

The traditional methods, along with their advantages and disadvantages, have permitted decades-long studies of microbial communities in freshwater systems, thus giving/providing fundamental knowledge and information about microorganisms. However, the highly efficient, technically simple and readily available molecular techniques offer the possibility to develop, improve and expand the hidden knowledge of microbial communities and simplify detection methods and increase accuracy, resolution, and speed while reducing costs (Pawlowski et al., 2016; Stoeck et al., 2014; Taberlet et al., 2012).

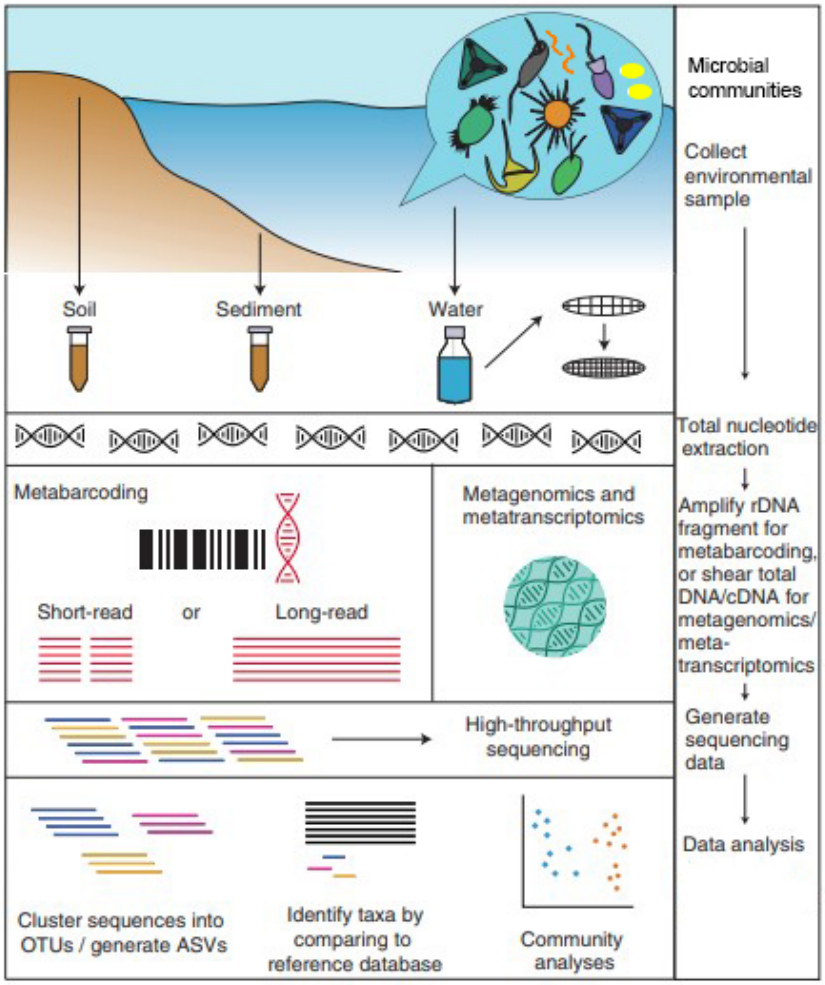


Figure 1. Environmental sequencing and metabarcoding. Overview of environmental sequeincing with emphasis on metabarcoding (box on the right side is description of the main steps). Adapted from Burki et al., 2021.

Typology of the freshwater bodies

By definition, a water body type is a group of lakes or rivers that share common natural ecological conditions in terms of geomorphological, hydrological, physico-chemical, and biological characteristics. In addition, it can be considered a homogeneous unit with limited natural environmental variability, which allows the definition of a baseline from which human-induced impacts can be identified (Lyche Solheim et al., 2019). The Water Framework Directive (WFD, Directive 2000/60/EC, 2000), the current legislation governing water management in Europe, requires EU member states to develop typologies for lakes and rivers

based on a set of environmental variables or type descriptors with either predefined or more freely defined ranges for each descriptor (European Commission, 2000). Common river and lake types have been defined within European regions to intercalibrate national classification systems for the ecological status of water bodies. However, European countries have defined > 1000 national river types and > 400 national lake types, and a small proportion of national types correspond to these common intercalibration types. This leads to uncertainty about whether ecological status classifications are consistent across countries (Lyche Solheim et al., 2019). The typology of lakes and rivers proved to be an extremely relevant concept in limnology and river ecology many decades ago (Naumann, 1932; Strahler, 1952; Thienemann, 1925). Although rivers are open and continuous systems with high temporal and spatial variability, early on river ecologists postulated the concept of isolated sections that are predictably distributed along the longitudinal dimension of a river. Reference conditions can be established exclusively for natural water bodies, while the quality of small water bodies are still not mandated and included in standard monitoring in all European countries.

Depending on the relief and hydrogeological function of the rocks, there are two major ecoregions in Croatia, the Panonian and the Dinaric, for which the uniform monitoring is required. Surface water monitoring is carried out according to the monitoring plan, which includes rivers, natural lakes, artificial and heavily modified water bodies. Pannonian and Dinaric ecoregions are also differentiated by the number of surface water types in Croatia, where the Panonian ecoregion includes ten types of watercourse habitats, while the Dinaric ecoregion is divided into three subecoregions that include nineteen watercourse habitats. The Dinaric ecoregion also comprises a Mediterranean ecoregion that includes transitional and coastal waters. Within the streams in these two ecoregions, there are also natural lakes (only in the Dinaric ecoregion) and lakes that are not of natural origin (Official gazzete, 2019).

The Pannonian part encompasses the basins of the Sava, Drava and Danube rivers, which are connected to the forming of Pannonian sea with the melting of ice in the Pleistocene, thus creating mountain torrents which brought large amounts of eroded material and formed glacial-fluvial terraces in the river valleys. A large supply of river sediments caused the gradual formation and eventual disappearance of the Pannonian Sea, after which the final shaping of the relief and hydrological system of these rivers took place. The aquifers of the Sava and Drava rivers consist of thick layers of sedimented gravel, resulting in the 1 to 20 m water depth variation along the north part of Croatia and contributing to a dense network of surface freshwaters (Šafarek and Šoltić, 2011).

With a flow of 322,8 km through Croatian territory, mainly along the Croatian-Hungarian border, Drava River represents one of the largest rivers of the Pannonian ecoregion where it forms a wide alluvial valley as part of the Black sea catchment (Figure 2). It springs in Italy (South Tirol), passes through Austria, Slovenia, Hungary, and finally Croatia, ending as the right tributary of the Danube River. The geology of Drava River basin is complex with the structure of alpine strata, tectonic processes, and varied lithology that have resulted in a diverse relief (Lóczy, 2019). The glaciated high mountains, the karst landscapes of the carbonate massifs, the low mountain topography on metamorphic rocks and exhumed intrusions, the volcanic cones, the hilly regions in the Tertiary sedimentary basins and mountain foothills, and the alluvial plains and terraces make the landscape of the Drava River Basin extremely varied and picturesque (Lóczy, 2019). Drava River lowland is characterized by intergranular porosity with many aquifers (small water bodies) such as gravel pits, which appear in the largest number in the northern Croatia around the city of Varaždin. Gravel pits represent exposed groundwater and vulnerable areas where the contamination of groundwater can occur faster from surface contaminants. In some cases, inactive (abandoned) gravel pits are used as waste (industrial or urban) disposal sites and are becoming a threat to groundwater quality (Navarro and Carbonell, 2008).

Figure 2. Drava River lowland. Photo by G. Šafarek.

As stipulated in the WFD, the assessment of ecological status of surface water bodies has to integrate the interactions between the biological quality elements (BQE) and the

supporting physical, chemical and hydro-morphological quality elements (Hanžek et al., 2021). Traditionally, the main reason for assessing the quality of the aquatic environment has been the need to verify that the observed water quality is suitable for the intended use. Monitoring helps determine trends in the water quality and how that quality is affected by pollutant releases and other anthropogenic activities. The primary media for aquatic monitoring, such as water, certain substances, and living organisms, are not the only primary methods. Thus, methods within the three major components for monitoring include proper selection of the water body for analysis because water quality is a highly variable aspect that differs more in rivers than lakes, but much less in aquifers (Chapman, 1992). Routine monitoring programs for freshwater ecosystems often overlook various types of water bodies, especially small standing water bodies such as gravel pits or ponds.

Small standing water ecosystems are characterized by a lower area ratio compared to larger lentic freshwater ecosystems, which emphasizes the contribution of ecotonal zones to their metabolism and functioning (Bolpagni et al., 2019). In general, small standing water bodies contribute significantly to global watershed functioning and maximize the importance of their role as biogeochemical reactors along hydrologic transport pathways (Søndergaard et al., 2005). They are shallow (less than 20 m deep), small, and lentic aquatic habitats with an area of less than 1 m^2, including small lakes, ponds, pools, and wetlands that can be both perennial and temporary and have an artificial or natural origin (Figure 3; Bolpagni et al., 2019). They support high metabolic rates, often coupled with naturally high nutrient levels and trophic conditions in larger freshwater systems. Their role is to modulate nutrient retention and recycling along hydrologic pathways. Although usually associated with eutrophic or hypertrophic conditions, small standing water bodies have very high overall species diversity, with the more species-rich communities often better adapted to conditions of eutrophication and a wide range of physical and chemical conditions than communities in larger waters. The distinct richness of small water bodies and their natural potential to withstand high nutrient concentrations underscore the need to include them in monitoring (Bolpagni et al., 2019; Rosset et al., 2014). High nutrient loads in small water bodies originating from anthropogenic sources may lead to different pathways in interactions among organisms in plankton or benthos and provide new insights into environmental preferences of species and traits, thus suggesting new prespectives in bioindications.

Figure 3. Small water body, gravel pit Šijanec within Drava alluavial area. Photo by A. Kulaš.

The Dinaric ecoregion is known as a karst region, determined by geomorphological and hydrogeological features due to the solubility of the rock. The karst region occupies almost half of the total area of Croatia (about 46%) and is mainly located in the Dinaric ecoregion. The main relief features of the karst region are the result of intense tectonic movements that led to the formation of the Dinaric Mountains, and soluble carbonate rocks of different ages are responsible for the development of karst in Croatia (Šafarek and Šoltić, 2011). The rocks most commonly responsible for karstification of surface and subsurface karst structures are limestone and dolomite. These geological characteristics include both primary and secondary porosity of carbonate rocks, as well as their mineralogical composition, grain size, texture, layer thickness, and degree of tectonic deformation. Water penetrates carbonate rocks through open spaces such as layer boundaries, fractures, and faults, and at the same time enlarges them by corrosion, and dolomite dissolves more slowly than limestone and is more mechanically decomposed. Because of the fracturing of karst rock, rainwater quickly percolates through the barren karst surface or low ground cover and infiltrates into the subsurface, where it joins surface water from non-karst areas (e.g. flysch areas) that sinks into the subsurface upon contact with the karst to feed a karst aquifer. Karst aquifers are thus characterised by great diversity in terms of flow and storage of water. The permeability is extremely high, the flow rate is high, and the direction of underground water flow is usually unknown. In many regions, not only in Croatia, karst aquifers often provide the only usable water reserves, and are therefore invaluable sources for human health, food security, and the economic sector, and about a quarter of the world's population is

partially or completely dependent on drinking water supply/sources from karst aquifers (Bonacci et al., 2006; Kresic and Stevanovic, 2010; Ravbar and Kovačič, 2015). Also, due to specific geomorphological and hydrological characteristics karst areas provide the physical habitat for particular communities that are characterized by high biodiversity. A great variety of species are present both on the surface and in the underground. Unusual fauna that develop in the light-deficient subsurface environment range from microbial organisms to fish and small mammals (Culver et al., 2021). Microbial organisms are important in biological and geological processes in karst environment because they may accelerate dissolution, contribute to deposition of flowstone or may be indicators of contamination sources (Mulec, 2014). Karst systems are generally stable environments that have evolved over thousands of years. Because of their structural and hydrological characteristics, karst landscapes and their associated habitats are among the most vulnerable areas. Therefore, any inappropriate land use practices can lead to serious and irreparable changes in natural processes and pose environmental problems. Human impacts and interventions can lead to various types of pollution, natural hazards, ecosystem degradation, and biodiversity loss. Once damaged, surface and subsurface karst areas often take a long time to recover (Ford and Williams, 2007). In recent decades, pressure on karst landscapes has increased, for example, due to intensive and unsustainable expansion of settlements, infrastructure, and industry, tourism development, and intensive agricultural land use. Widespread conversion and degradation of the landscape has greatly increased, mainly due to technological development and mechanisation. Changes in natural conditions can increase natural vulnerability. Karst rivers are included in routine monitoring programmes in European countries, including Croatia, and are highlighted as unique freshwater habitats (Hartmann et al., 2014). Even so, they have not been fully explored (Lionello, 2012). Many rivers in karst areas run partly underground, flow through impressive canyons or complex systems of reservoirs, and participate in the formation of the karst relief. One of the most spectacular depositional relief forms in karst areas and landscapes are tufa barriers and cascades formed in river and lake systems (Frisia and Borsato, 2010).

One of the notable rivers in the Croatian karst ecoregion is the Krka River, famous for its tufa barriers and waterfalls (Figure 4). The Krka River is a 73 km long karst river whose 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 et al., 2006). After the spring zone, Krka flows through the Knin karst

polje, a series of valleys and canyon formations until reaching the Adriatic Sea near the city Šibenik (Perica et al., 2017). Along its course there are 7 larger tufa barriers (Bilušića buk, Brljan, Manojlovića buk, Rošnjak, Miljacka, Roški slap and Skradinski buk) forming waterfalls in the downstream direction with alternating lotic and lentic microhabitats. Some parts of the Krka River have been protected since 1948 for their special geomorphological, hydrological and landscape values. In 1985, the Krka River and its catchment area were granted status of the National Park (Official gazette, 1985).

All of the specific characteristics, as well as growing anthropogenic pressure give the Dinaric karst area its uniqueness and underline the importance of improving monitoring and assessment programmes for a viable ecological status and the conservation of natural habitats and biodiversity.

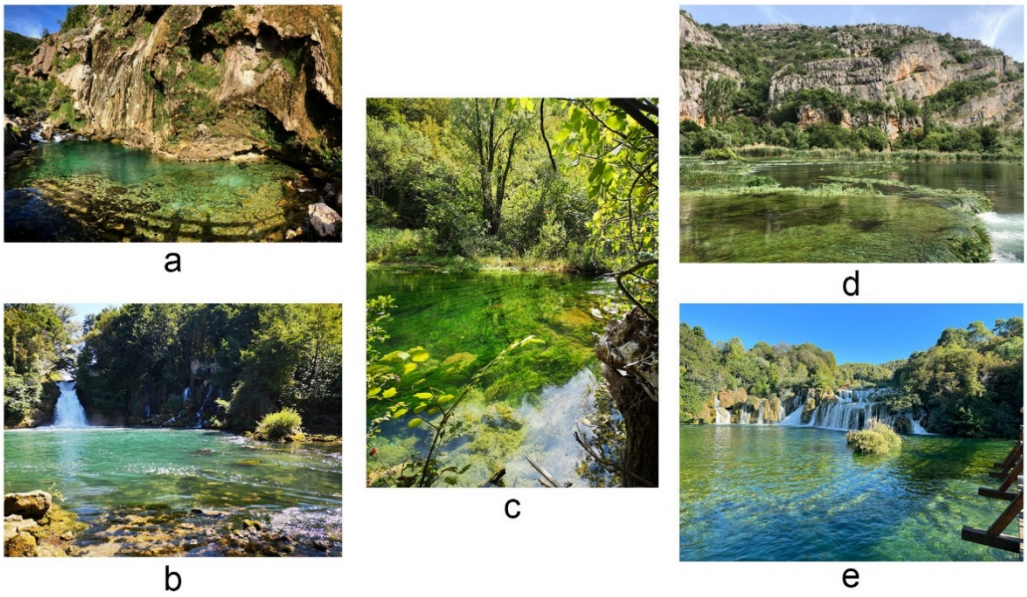


Figure 4. Krka Spring (a), Bilušića buk (b), Miljacka (c), Roški slap (d) and Skradinski buk (e). Photo by M. Gligora Udovič (a, d and e) and A. Kulaš (b and c).

Previous investigations of microorganisms in research areas

Although microbial communities are recognized as important players in freshwater ecosystems, previous research in Croatia was based on the traditional morphological approach and species cultivation as part of exploring planktic or benthic communities. Microorganisms are important for understanding the functioning of aquatic ecosystems, but our knowledge of

their biodiversity is still limited given the discrepancy between the number of species present and their estimated number (Nistal-García et al., 2021). Traditional biomonitoring of phytoplankton and phytobenthos is conducted in the rivers of Pannonian and Dinaric ecoregions in Croatia as part of the biological quality elements of the WFD (WFD, Directive 2000/60/EC, 2000). Other microbial communities, such as bacteria and protozoa, are still not recorded, even though they show good potential as bioindicators. In addition, monitoring assessment covers only large rivers and lakes, so data on small standing waters are still quite sparse.

Drava River is one of the most important source of water in the Pannonian ecoregion in Croatia. The river is a vital resource for water supply, irrigation, fishing, navigation, and recreation, but it is also a natural wastewater collector for the cities and settlements in its catchment area. Mijusković-Svetinović and Maričić (2008) stated that the Drava River is extremely important for biodiversity conservation, both at the Croatian and European levels. This can be explained by the fact that its watercourse and surrounding wetlands are among the best preserved in Central Europe (Gvozdić et al., 2012). In general, standard monitoring of physical, chemical and biological elements is carried out on the Drava River, as required by the WFD. Previous studies in this area mainly addressed water quality based on physico-chemical parameters and microbiological (bacterial) status (Dolgosné Kovács et al., 2019; Gvozdić et al., 2012) to assess the impact of anthropogenic activities on the water quality of the river and discuss its suitability for human consumption. Previous studies have indicated that the Drava River and its alluvial aquifer were an artificial reservoir for hydropower and had a full potential of surface water for drinking water consumption, but were abandoned due to anthropogenic impacts (Marković et al., 2020). Subsequently, most studies were based on the geomorphological features due to intergranular porosity of the Drava lowland (Gvozdić et al., 2012; Karlović et al., 2021a, b, c; Marković et al., 2020), and studies of anthropogenic influence, but did not consider the importance of the entire Drava aquifer, especially the importance of small water bodies within the entire alluvial system. Small water bodies in the form of gravel pits are widespread in the aquifers of rivers near the city of Varaždin, as they represent exposed groundwater and vulnerable areas where contamination of groundwater by surface contaminants can occur more quickly. Small water bodies play a role in regulating nutrient storage and recycling along hydrological pathways. They are typically associated with eutrophic conditions and host a very diverse range of species overall, often with more species-rich communities that are better adapted to eutrophic conditions and a wide range of physical and chemical conditions than communities in larger water bodies. These characteristics shed

light on the ecology and importance of small alluvial water bodies, but knowledge about them is still quite sparse, as these systems are also not included in national water resource protection strategies (Rosset et al., 2014). Most studies on microbial communities in the Drava River basin were focused on morphological characterization of phytoplankton (Plenković-Moraj et al., 2007; Stanković et al., 2012) and bacterial communities targeted for drinking water (Gvozdić et al., 2012). Plenković-Moraj et al. (2007) described the phytoplankton community in three main assemblages on the Drava River. Group Bacillariphyceae was the most abundant during the study period, dominated by *Asterionella Formosa* Hassall, *Fragillaria crotonensis* Kitton and *Melosira varians* C.Agardh as the typical species of the phytoplankton community of large rivers. Stanković et al. (2012) described the influence of hydrological characteristics and nutrient concentrations of phytoplankton at four sites on the Drava River included in the national standard monitoring. Diatom species were recorded among the dominant species. They also reported how lotic systems have extremely dynamic hydrological regimes and gained better understanding of the factors affecting phytoplankton in rivers.

The karstic tufa barriers that form along the course of the Krka River represent one of the most unique and recognizable natural features. Tufa provides a favourable substrate for colonisation and the change in composition of the periphyton is very important for the depositional processes in the tufa. In addition, trophic interactions within the periphyton communities are also very important and can influence the transport of biochemically important solutes into and within the biofilm layers (Primc-Habdija et al., 2005). The Krka River with its tufa barriers is a unique hotspot with a high biodiversity of different types of aquatic organisms, especially microbial communities, which have not yet been fully studied. Previous studies on the Krka River have been conducted on planktic or benthic communities of protists, including photosynthetic organisms and protozoa. Studies on bacterial communities were mostly conducted at the estuary, or near the spring, but not along the river course (Kolda et al., 2019; Korlević et al., 2016; Kveštak and Ahel, 1995). In general, a rich diversity of aquatic flora and fauna have been described, such as algal species (Caput Mihalić et al., 2019; Gligora Udovič et al., 2022; Žuljević et al., 2016), but also insects (Andersen et al., 2016; Ivković et al., 2012; Kvifte et al., 2013; Kvifte and Ivković, 2018; Pont and Ivković, 2013; Previšić et al., 2014) and fishes (Marčić et al., 2011; Mustafić et al., 2008) by using traditional morphological approaches, but also molecular methods such as mitochondrial and nuclear gene sequencing data. Investigations on periphytic protozoa based on morphological approach were conducted at Lake Visovac and Skradinski buk (Primc-Habdija et al., 2005; Primc-Habdija and

Matoničkin, 2005). At Lake Visovac the authors tracked seasonal changes in ciliate biomass and community, but also trophic composition associated with changes in thermal stratification and vertical oxygen gradients as important abiotic determinants of periphyton biomass as a food source, as well as tufa deposition determining substrate characteristics (Primc-Habdija et al., 2005). At Skradinski buk a new freshwater species *Lagotia dinaridica* n. sp. was detected and described within the genus *Lagotia* (Folliculinidae, Ciliophora), which was previously known only from marine habitats (Primc-Habdija and Matoničkin, 2005). In a recent study, Gulin et al. (2021) showed how tufa barriers respond to environmental changes and how microhabitat complexity directly or indirectly affects the physico and chemical conditions of calcite precipitation in karst systems. Invasive species *Ailanthus altissima* (Mill.), found on the tufa barrier, was causing the drying up of streams at the barrier. With the physical removal of invasive species, the ecosystem responded in hydromorphological changes, which can be successfully detected and monitored at the microscale level (protozoa in the periphyton). Apart from biologists, the Krka River also attracts the attention of geologists and hydromorphologists. For example, Šiljeg et al. (2020) revealed that the degradation of tufa landscape, reflected in negative hydrological changes and the intensity of tufa formation process is decreasing. All in all, the application of interdisciplinary approaches with the usage of molecular methods in the studies, especially at the microscale (microbial communities), could lead to reactivation of tufa forming watercourses and sustainable conditions for the tufa forming process.

However, the study of microbial communities is important, mainly because of their different biochemical processes and trophic conditions in aquatic ecosystems, but also because they can be very useful bioindicators for water quality assessment. New methods such as the molecular approach will help to unravel microbial communities not only in aquatic but also in other ecosystems.

THESIS OUTLINE

This doctoral thesis includes four scientific publications (**I-IV**), which are adequately addressing aims and hypothesis of the thesis.

Aims of thesis are:

1. Describe the diversity of periphytic microorganisms in river ecosystems using a molecular and morphological approach.
2. Define groups of periphytic microorganisms as bioindicators using molecular methods.
3. Define the importance of integrating large numbers of samples and microhabitats in monitoring large river ecosystems.
4. Using molecular and morphological approach, describe the biodiversity and organisms' interactions in the plankton community of the small water body system under anthropogenic influence of excessive nutrient loading.
5. Define the importance of small water body in recycling of nutrients under anthropogenic pressure and the importance of their integration in biomonitoring.
6. Validate the measurability of molecular *vs*. traditional morphological methods in the characterization of microorganisms used in freshwater biomonitoring.

Hypothesis of the thesis are:

1. Molecular methods are valuable for assessing diversify of microorganisms in the plankton and benthos of freshwater ecosystems.
2. Diatoms are well studied group of microorganisms in benthos and periphyton, but not the only one with good indicator potential.
3. Interactions between groups of organisms in the plankton and benthos in freshwater systems, controlled by anthropogenic pressure into the system, provide new insights into the indicator properties of species and communities.
4. Small water bodies are important nutrient recyclers in the systems of large rivers.
5. With a larger number of sampling of different microhabitats, we can get a better insight into the state of the ecosystem than with a one representative monitoring sampling point.
6. Due to limitations of currently used biomonitoring methodologies, that rely on traditional taxonomic identification, methods based on eDNA allow integration of a much wider range of taxa and indicator groups into freshwater ecological assessments.
7. Due to limitations of currently used biomonitoring data, including time-consumption, space and researcher availability, methods based on eDNA allow the inclusion of a much wider range of biotopes into freshwater ecological assessments.

All four publications (**I, II, III** and **IV**) answered satisfactory to the first, third, sixth and seventh hypothesis; Publication **III** pertained to second hypothesis, while Publication **II** and **III** answered to fifth hypothesis, and Publication **I** explored possibilities of fourth hypothesis. Aims of the thesis are reached: Publications **II**, **III** and **IV** described the diversity of periphytic microorganisms in river ecosystems; Publication **II** and **III** defined the importance of integrating large numbers of samples and microhabitats in monitoring large river ecosystems; Publication **I** described the biodiversity of organisms in the plankton community of the small water body and emphasised the importance of small water body in recycling of nutrients under anthropogenic pressure and the importance of their integration in biomonitoring; first three publications (**I, II** and **III**) confirmed the measurability of molecular *vs*. traditional morphological methods in characterization of microbial communities in freshwaters.

PUBLICATION I

water

MDPI

Article

Succession of Microbial Community in a Small Water Body within the Alluvial Aquifer of a Large River

Antonija Kulaš [1], Tamara Marković [2], Petar Žutinić [1,*], Katarina Kajan [3,4], Igor Karlović [2], Sandi Orlić [3,4], Emre Keskin [5], Vilim Filipović [6] and Marija Gligora Udovič [1]

[1] Department of Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, HR-10000 Zagreb, Croatia; antonija.kulas@biol.pmf.hr (A.K.); marija.gligora.udovic@biol.pmf.hr (M.G.U.)
[2] Department of Hydrogeology and Engineering Geology, Croatian Geological Survey, Sachsova 2, HR-10000 Zagreb, Croatia; tmarkovic@hgi-cgs.hr (T.M.); ikarlovic@hgi-cgs.hr (I.K.)
[3] Ruđer Bošković Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia; katarina.kajan@irb.hr (K.K.); sandi.orlic@irb.hr (S.O.)
[4] Center of Excellence for Science and Technology-Integrating Mediterranean Region (STIM), HR-21000 Split, Croatia
[5] Evolutionary Genetics Laboratory (eGL), Agricultural Faculty, Ankara University, Ankara TR-06135, Turkey; keskin@ankara.edu.tr
[6] Department of Soil Amelioration, Faculty of Agriculture, University of Zagreb, HR-10000 Zagreb, Croatia; vfilipovic@agr.hr
* Correspondence: petar.zutinic@biol.pmf.hr; Tel.: +385-(0)1-6189-706

Abstract: Nitrogen is one of the essential elements limiting growth in aquatic environments. Being primarily of anthropogenic origin, it exerts negative impacts on freshwater ecosystems. The present study was carried out at the nitrate-vulnerable zone within the alluvial aquifer of the large lowland Drava River. The main aim was to investigate the ecosystem's functionality by characterizing the bacterial and phytoplankton diversity of a small inactive gravel pit by using interdisciplinary approaches. The phytoplankton community was investigated via traditional microscopy analyses and environmental DNA (eDNA) metabarcoding, while the bacterial community was investigated by a molecular approach (eDNA). Variations in the algal and bacterial community structure indicated a strong correlation with nitrogen compounds. Summer samples were characterized by a high abundance of bloom-forming Cyanobacteria. Following the cyanobacterial breakdown in the colder winter period, Bacillariophyceae and Actinobacteriota became dominant groups. Changes in microbial composition indicated a strong correlation between N forms and algal and bacterial communities. According to the nitrogen dynamics in the alluvial aquifer, we emphasize the importance of small water bodies as potential buffer zones to anthropogenic nitrogen pressures and sentinels of the disturbances displayed as algal blooms within larger freshwater systems.

Keywords: nitrogen; alluvial aquifer; large river; small water body; phytoplankton; bacterial community

Citation: Kulaš, A.; Marković, T.; Žutinić, P.; Kajan, K.; Karlović, I.; Orlić, S.; Keskin, E.; Filipović, V.; Gligora Udovič, M. Succession of Microbial Community in a Small Water Body within the Alluvial Aquifer of a Large River. *Water* **2021**, *13*, 115. https://doi.org/10.3390/w13020115

Received: 25 November 2020
Accepted: 30 December 2020
Published: 6 January 2021

1. Introduction

Various aspects of nutrient dynamics in freshwater ecosystems are of paramount importance for understanding how the productivity of surface waters is controlled and provide the opportunity to analyse the current and future impacts of anthropogenic activities on freshwater ecosystems. In such environments, a large part of the primary production may depend on the recycling of nutrients such as nitrogen compounds [1]. Nitrogen is an essential element that often limits growth in aquatic ecosystems, and a key compound in many biochemical processes that are important for life, but can be harmful in high concentrations [2–4]. Nowadays, anthropogenic activities such as fertilizer synthesis and its widespread application on arable areas, as well as the burning of fossil fuels, significantly increase the N fluxes across different environmental compartments [3–5]. Its environmental

effects on aquatic ecosystems include acidification, anthropogenic eutrophication, degradation of water quality, biodiversity loss, and increased greenhouse gas emission [6–8]. Nitrate (NO_3^-) pollution is causing negative impacts on groundwater and surface water resources with its primary anthropogenic origin [9,10]. An elevated concentration of nitrates is associated with diffuse and point sources such as domestic or industrial wastewaters, atmospheric deposition, and animal farming waste. However, most environmental problems related to nitrate are linked to intensive agriculture production [11], as the nitrogen is used to promote crop growth [12,13]. Alluvial groundwater is particularly vulnerable to nitrate leaching from agricultural soils, since agricultural land is characterized by the presence of shallow groundwater and fertile soil suitable for farming [14,15].

The composition of the microbial community depends on environmental conditions that may affect the ecosystem's function [16–19], as they drive the various processes of recycling, dynamics, and assimilation of nitrogen compounds in freshwater habitats [18–20]. The availability of certain N forms in freshwater habitats influences the composition of the phytoplankton community, increases its productivity, and causes harmful algal blooms [6,21,22]. Bloom-forming species encompass a variety of eukaryotic algae but also Cyanobacteria, a prokaryotic algal group closely related to problematic freshwater nuisances. Cyanobacteria are extremely adaptive and competitive organisms with a long evolutionary history, which endowed them with an array of physiological, morphological, and ecological adaptations to survive in a wide variety of environmental conditions [6,23]. Many species of Cyanobacteria are capable of surviving and even thriving in extremely inhabitable conditions, tolerating desiccation, high temperatures, extreme pH, high salinity, and pesticides, thus illustrating their capacity to acclimate in different kinds of habitats [24]. They are the only planktonic group capable of utilizing atmospheric nitrogen via biological N_2 fixation, and, as such, can circumvent N-limited conditions [25,26]. Cyanobacterial genera capable of diazotrophy retain a competitive advantage over other phytoplankton groups. The ability of some Cyanobacteria to form potentially toxic surface blooms has drawn much attention from the general public [21,27,28]. Worldwide, fewer than 30 species that cause a real nuisance. It is still difficult to generalize their ecological requirements, as they can be ubiquistic, specifically preferring eutrophic conditions [29]. Anthropogenic eutrophication is recognized as a global environmental problem in terms of both freshwater biodiversity loss and harmful algal blooms due to the presence of toxins [30,31]. However, the impact of eutrophication may differ in large rivers and lakes, when compared to smaller water bodies, such as streams, ponds, bogs, and small lakes, as well as groundwater [32]. Generally, the small lowland water bodies support naturally high concentrations of nutrients and range from eutrophic to hypertrophic [33,34]. Despite high nutrient conditions, small lowland water bodies collectively support a very diverse and ofttimes unique biodiversity, often richer than the one found in big rivers or lakes [35]. The consequences of eutrophication on the biodiversity of small water bodies are poorly understood and have yet to be fully explored.

One of the systems characterized by high nitrogen inputs in Croatia is the alluvial aquifer of Drava, the second longest river in Croatia. The aquifer has dozens of small lotic and lentic ecosystems, which play a potentially important role as biogeochemical reactors in nitrogen buffering and recycling. The regime and quality of these small water bodies are under heavy anthropogenic pressure, mainly due to agriculture [36]. To investigate the role of these small water bodies the nitrogen recycling in the Drava River alluvial area, we have selected a small inactive gravel pit. By employing interdisciplinary approaches, we aim to characterize the ecosystem's functionality, emphasizing bacterial and phytoplankton diversity and its effects on nitrogen recycling along the hydrological transport pathways.

2. Materials and Methods

2.1. Study Area

The study area, situated in the Drava River valley, upstream of the town of Varaždin (NW Croatia), belongs to the Black sea catchment and covers an area of approximately

200 km^2 (Figure 1). On the NW side, the alluvial aquifer is adjoined by the Varaždin Lake, an artificial reservoir of the hydroelectric power plant Varaždin, of which the Drava River watercourse constitutes a natural border in the NE direction. The Plitvica Stream flows at the S-SE edge of the study area, while in the middle there are several active and inactive gravel pits. All gravel pits represent exposed groundwater and vulnerable areas where the contamination of groundwater can occur faster from surface contaminants. In some cases, inactive (abandoned) gravel pits are used as waste (industrial or urban) disposal sites and are becoming a threat to groundwater quality [37–39].

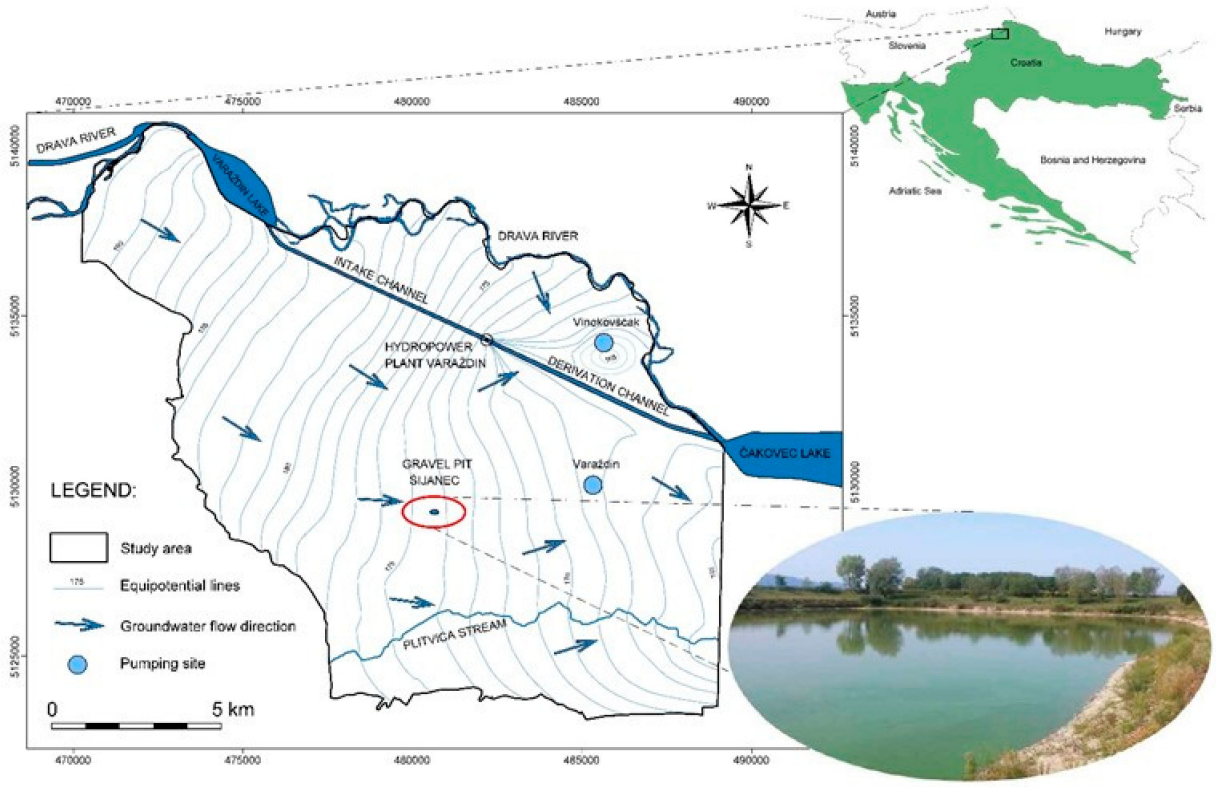


Figure 1. The geographical position of the study area with the location of the sampling area—Šijanec gravel pit—and indication of the groundwater flow direction.

The study area is densely populated, with industrial and intensive agricultural production. The most common type of crops grown are corn, cabbage, potatoes, and vegetables. Extensive poultry farming is present, especially the fattening of chickens, quails, and pheasants, and the breeding of hens [40]. The Varaždin pumping site, one of two in the area, was shut down due to high nitrogen concentrations in groundwater caused by significant anthropogenic activities [41]. Nevertheless, demands for drinking and industrial water rise because of the growing production in the area. The gravel pit in the village of Šijanec was chosen because of its inactivity and accessibility. It is a small pit covering an area of approximately 12,000 m^2 (Figure 1).

2.2. Sampling and Laboratory Analysis

Phytoplankton and bacterial samples were taken monthly from June 2017 until March 2018 on the deepest point of the gravel pit using the vertical Hydro-Bios water sampler (Hydro-Bios Apparatebau GmbH, Altenholz, Germany). Samples for chemical analysis were taken simultaneously with biological samples and transported in a portable freezer at 4 °C to the laboratory for further analysis. Before sampling, physico-chemical parameters of water, including electrical conductivity (EC), pH, temperature (T), and dissolved oxygen concentration (DO), were measured with a portable WTW Multi 3630 multimeter (Xylem

Analytics, Weilheim, Germany). Water transparency (Z_{SD}) was estimated using a Secchi Disc. Total alkalinity was measured by titration with 1.6 N H_2SO_4 using phenolphthalein and bromocresol green-methyl red as indicators. Dissolved cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) and anions ($SO_4{}^{2-}$, $NO_3{}^-$, Cl^-) were analyzed by ion chromatography using a Dionex ICS-6000 (Thermo Fisher Scientific, Waltham, MA, USA), while $NH_4{}^+$, $NO_2{}^-$, and $PO_4{}^{3-}$-P were measured spectrophotometrically. Dissolved inorganic and organic carbon (DIC, DOC) and total inorganic and organic carbon (TIC, TOC) were analysed using a HACH QbD1200 TOC analyser (Hach Company, Frederick, MD, USA). The analytical precision of the measurements of cations and anions, indicated by the ionic balance error (IBE), was computed on the basis of ions expressed in $meqL^{-1}$. The IBE value was observed to be within a limit of $<\pm 5\%$ [42,43]. The PHREEQC geochemical code [44] was used to calculate the charge balance and pCO_2 pressure and to study the saturation state of the mineral phases. The samples for Chlorophyll *a* were filtered on Whatman GF/F glass filters (Whatman International Ltd, Kent, UK), extracted in 96% ethanol, and measured for Chl *a* using a UV–VIS spectrophotometer according to compliance monitoring standards [45].

2.3. Microbial Community Analysis

Phytoplankton samples were collected for both morphological and molecular analyses. Samples for morphological analysis were fixed with acid Lugol solution and stored in 250 mL volume glass bottles in the dark at 4 °C. The morphological analysis included a qualitative and quantitative community characterization according to the Utermöhl method [46], using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Oberkochen, Germany).

Samples for molecular analysis were collected in sterile plastic bottles and preserved on ice during the transport to the laboratory. In the laboratory, they were immediately filtered on Nucleopore Track-Etch membrane filters (25 mm diameter, 0.4 µm pore size; Whatman International Ltd, Kent, UK) and stored at −20 °C until further processing. DNA was extracted from the frozen filters using DNeasy PowerWaterKit (Qiagen, Hilden, Germany). The manufacturer's instructions were followed with a slight modification in the final step, where 60 µL of sterile DNA-free PCR Grade water was added instead of Qiagen's C6 Solution. The quality of the extracted DNA was assessed with the Shimadzu BioSpec-nano spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

2.4. PCR of the Phytoplankton (Eukaryotic) Community

The hypervariable V9-region of the SSU rRNA gene (ca. 150 base pairs) was amplified from environmental DNA using the universal eukaryotic primer pair [47,48]. The forward and reverse primers were 1391F (5′-GTAC ACACCGCCCGTC-3′) and EukB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′), designed by Amaral-Zettler and colleagues [49]. Polymerase chain reactions (PCR) contained 1 U of Hot Start Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA), and for V9 amplification an initial activation step at 95 °C was employed 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, followed by a final 2 min extension at 72 °C. 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 (New England Biolabs, Ipswich, MA, USA). Libraries were sequenced on an Illumina MiSeq platform, generating 250-bp paired-end reads (SeqIT GmbH & Co. KG, Kaiserslautern, Germany).

2.5. PCR for the Bacterial Community

The V3-4 region of bacterial rRNA genes was amplified using the forward primer 341 F 5′-CCTACGGGNGGCWGCAG-3′ and reverse primer 805 R 5′-GACTACHVGGGTATCTAA TCC-3′ [50]. PCR and sequencing were performed in the LGC Genomics GmbH laboratory (Berlin, Germany). The libraries were sequenced on an Illumina MiSeq platform, generating 300-bp paired-end reads.

2.6. Bioinformatic Analysis of the Phytoplankton (Eukaryotic) Community

Sequence reads were analysed using the programs implemented in the OBITools package, as described in De Barba et al. [51]. The quality of the reads was assessed using FastQC. Paired-end reads were aligned using Illumina paired-end, and alignments with quality scores >32 were kept. The aligned data set was demultiplexed using the ngsfilter command, which identified primers and tags and assigned the sequences to each sample. For dereplication, we used the obiuniq command for clustering together strictly identical sequences and keeping the information about their distribution among samples. Sequences shorter than 10 bp, or containing ambiguous nucleotides, or with occurrence lower or equal to 10 were excluded using the obigrep command. The obiclean command was then run to assign the status of "head", "internal", or "singleton" to each sequence within a PCR product. All sequences labeled "internal", corresponding to PCR errors, were discarded. Finally, the taxonomic assignment of OTUs was performed with the ecotag command, combining two reference databases, filtered according to target taxa from NCBI taxonomy and the EMBL database, after running the ecoPCR program [52,53]. Only sequences with a 98% match to the reference sequence were kept. Single-read OTUs were removed from the samples to avoid potential false positives. The final filtered file with taxonomically assigned OTUs of eukaryotic algae groups was used as a basis for all downstream analyses. The DNA sequencing reaction on two samples (September and October 2017) did not yield valid results. A list of commands with related parameters are presented in the Supplementary Materials (S1). Raw demultiplexed reads were deposited at the ENA's Sequence Read Archive and are publicly available under project number PRJEB40961.

2.7. Bioinformatic Analysis of the Bacterial Community

The quality of the reads was assessed using FastQC. Paired-end reads were quality-trimmed using the bbduk function and merged using the bbmerge function of the BBMap package 34.48 (Lawrence Berkeley National Lab., Berkeley, CA, USA) [54]. Merged reads were quality-filtered using QIIME v1.8.0 [55]. Reads with exact barcodes and primers, unambiguous nucleotides, and a minimum length of 250 base pairs were retained. A Chimera check was done using UCHIME [56]. Non-chimeric reads were clustered with SWARM v3.0.0 [57] with default settings into Operational Taxonomic Units (OTUs). The bacterial reads were blasted against the SILVA database release 138 (Max Planck Institute for Marine Microbiology and Jacobs University, Bremen, Germany) using blastn (BLAST v2.9.0) [58]. Nontarget OTUs (chloroplasts, mitochondria), as well as singletons and doubletons, were excluded. The resulting OTUs were filtered by the quality of the blast results ($\geq$98% identity). The DNA sequencing reaction on two samples (February and March 2018) did not yield valid results. Raw demultiplexed reads were deposited at the ENA's Sequence Read Archive and are publicly available under project number PRJEB40962.

2.8. Statistical Analysis

Statistical analyses were conducted in R v. 4.0.2 [59] using the program packages "vegan", "fossil", "factoextra", "devtools", and "ggbiplot", as well as "ggplot2" for all graphical representations. To access the comparability of morphological and molecular methodologies in the phytoplankton community, the taxa lists derived from both approaches were compared with regard to the presence or absence of taxa and community composition. The bacterial community composition was analysed by using the molecular approach. The results for downstream analysis were combined into a single data set for each approach and for each community. The molecular results were transformed into relative abundances to normalize the OTU database [60]. Biomass data obtained by microscopy for the phytoplankton community were transformed following the logarithmic scale [61].

The Shannon, Simpson, and richness indices were calculated for both approaches and both communities as measures of alpha diversity using program packages "Vegan v. 2.5.6" [62].

To test the statistical significance of the environmental parameters and which parameter was singled out depending on the month studied, a principal component analysis (PCA) was performed using the R package "vegan" [62].

Canonical correspondence analyses (CCA) were performed on phytoplankton morphological data and bacterial molecular data to estimate variance in environmental variables for both communities. ANOVA test was applied to test the statistical significance of all axes, and forward selections were used to evaluate the importance of each variable. The logarithm function was used to transform environmental parameters and both community datasets for statistical analysis.

3. Results

3.1. Analysis of Environmental Parameters

The environmental variables of the investigated gravel pit are indicated in Table 1. The highest value of nitrates (NO_3^-) concentration was measured in March (38.4 mg L^{-1}) and the lowest in June (0.62 mg L^{-1}), whereas the maximum concentrations of ammonium (NH_4^+) and nitrites (NO_2^-) were recorded in July (2.75 mg L^{-1} and 0.17 mg L^{-1}, respectively). The highest value of pH was detected in August (9.48), indicating that the water was alkaline. The highest temperature (T) value was recorded in June (24.2 °C) and the lowest in February (1.2 °C), respectively. The maximum value of dissolved oxygen was measured in August (17.1 mg L^{-1}), and the minimum value was in July (7.1 mg L^{-1}). For electrical conductivity (EC) and bicarbonates (HCO_3^-), the maximum values were recorded in March (497 µS cm^{-1} and 249 mg L^{-1}, respectively) and the minimum ones in October (252 µS cm^{-1} and 107 mg L^{-1}, respectively). Silicon dioxide (SiO_2) concentrations were high during the warmer period, and the maximum was in September (26.8 mg L^{-1}). During the colder period the concentrations were much lower, and the minimum was in December (7.2 mg L^{-1}). In addition, a change was also observed in the concentrations of calcium, bicarbonates, and logpCO_2 pressure (Table 1), with the lowest concentrations detected in the summer period and the highest concentrations during winter.

Table 1. Ranges of environmental variables in the Šijanec gravel pit during the investigated period.

Variable	Min	Max	Mean	Med	SD
T (°C)	1.2	24.2	13.0	11.5	9.1
EC (µS cm^{-1})	252	497	347	316	99
pH	7.83	9.48	8.49	8.23	0.60
DO (mg L^{-1})	7.1	17.1	12.5	12.7	2.9
logpCO_2	−4.69	−2.54	−3.44	−3.15	0.74
HCO_3^- (mg L^{-1})	107	249	159	138	54
PO_4^{3-}-P (mg L^{-1})	0.01	0.32	0.10	0.05	0.11
TN (mg L^{-1})	0.28	10.15	4.57	4.45	3.54
NH_4^+ (mg L^{-1})	0.01	2.75	0.42	0.09	0.84
NO_2^- (mg L^{-1})	0.05	0.17	0.08	0.07	0.04
NO_3^- (mg L^{-1})	0.6	38.4	15.3	11.1	14.0
TIC (mg L^{-1})	18.44	29.36	23.76	23.06	4.62
DIC (mg L^{-1})	14.50	27.68	21.61	20.19	4.33
TOC (mg L^{-1})	7.20	24.66	16.61	16.25	6.68
DOC (mg L^{-1})	6.13	20.22	14.08	13.82	5.57
Ca^{2+} (mg L^{-1})	20.0	66.1	38.2	31.7	18.3
Mg^{2+} (mg L^{-1})	15.2	20.0	17.4	16.7	1.7
Na^+ (mg L^{-1})	6.9	17.4	12.2	13.3	3.7
K^+ (mg L^{-1})	0.9	1.6	1.1	1.1	0.2
Cl^- (mg L^{-1})	11.8	24.7	17.5	17.3	4.2
SO_4^{2-} (mg L^{-1})	16.0	33.1	24.3	24.8	5.6
SiO_2 (mg L^{-1})	7.2	26.8	15.6	13.0	6.2
Z_{SD} (m)	0.125	0.5	0.28	0.25	0.111
$SI_{Calcite}$	−0.2	1.1	0.5	0.5	0.4

PCA Analysis

The principal component analysis (PCA) performed for the 24 environmental variables explained 71.1% of the total variance on the first two PC axes. The overall strength of correlations between the samples and environmental parameters are summarized in Table 2. The most important variables for the PC1 axis were TIC, Ca^{2+}, and EC (intra-set correlations: 0.271, 0.269, and 0.268, respectively). Regarding the PC2 axis, NH_4^+ and DO (intra-set correlations: −0.355 and 0.407, respectively) were the variables that weighted most for the ordination. PCA arranged the samples into three groups (Figure 2): the first group consisted of samples from a warmer period of investigation (June, August, September, and October), the second group included a sample from July, while the third one comprised all samples from the colder period of investigation (November, December, January, February, and March).

Table 2. Summary statistics for the first two axes of PCA performed on the environmental variables during the investigated period.

PCA Axis	**PC1**	**PC2**
Standard deviation	3.531	2.145
Proportion of variance (%)	51.9	19.2
Cumulative proportion (%)	51.9	71.1
Eigenvalues		
T	−0.246	−0.094
EC	0.268	−0.012
pH	−0.254	0.173
DO	0.030	0.407
logpCO_2	0.256	−0.184
HCO_3^-	0.237	−0.045
PO_4^{3-}-P	−0.008	−0.126
TN	0.265	−0.021
NH_4^+	−0.089	−0.355
NO_2^-	−0.154	−0.294
NO_3^-	0.266	0.059
TIC	0.271	−0.067
DIC	0.193	−0.054
TOC	−0.260	−0.028
DOC	−0.249	−0.106
Ca^{2+}	0.269	−0.0003
Mg^{2+}	−0.205	−0.103
Na^+	−0.006	0.377
K^+	−0.182	−0.263
Cl^-	0.041	0.368
SO_4^{2-}	−0.156	0.285
SiO_2	−0.208	0.200
Z_{SD} (Secchi)	−0.079	−0.103
$SI_{Calcite}$	−0.205	0.127

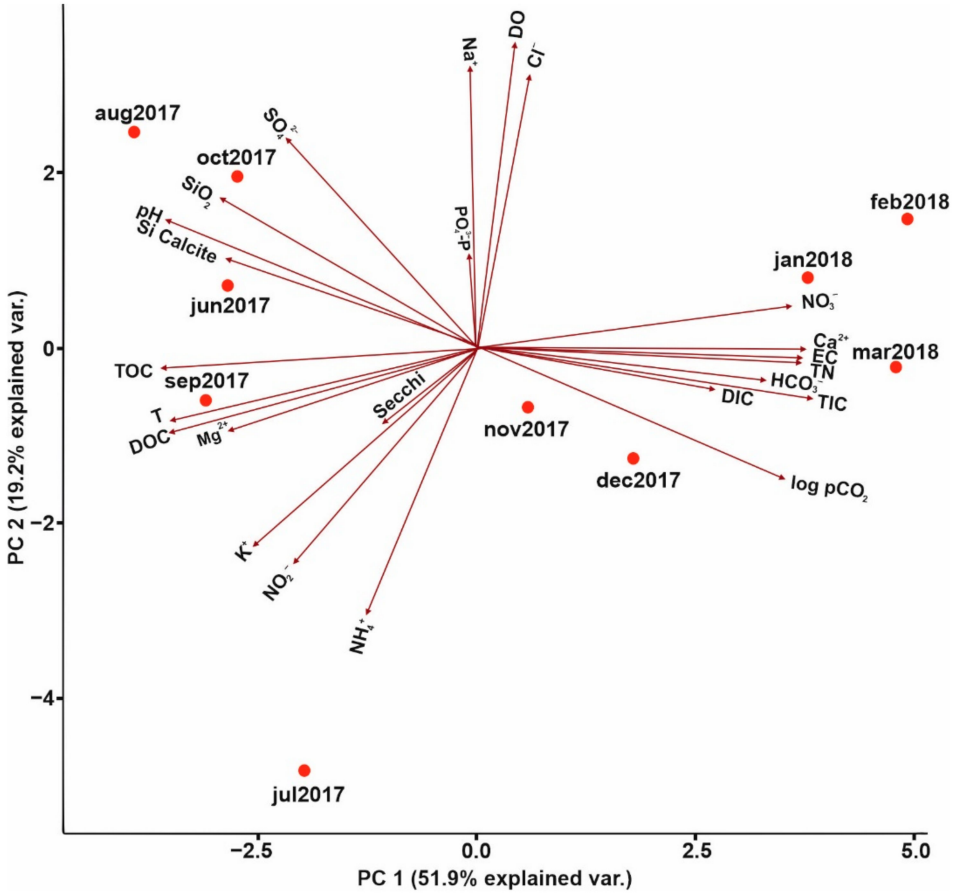


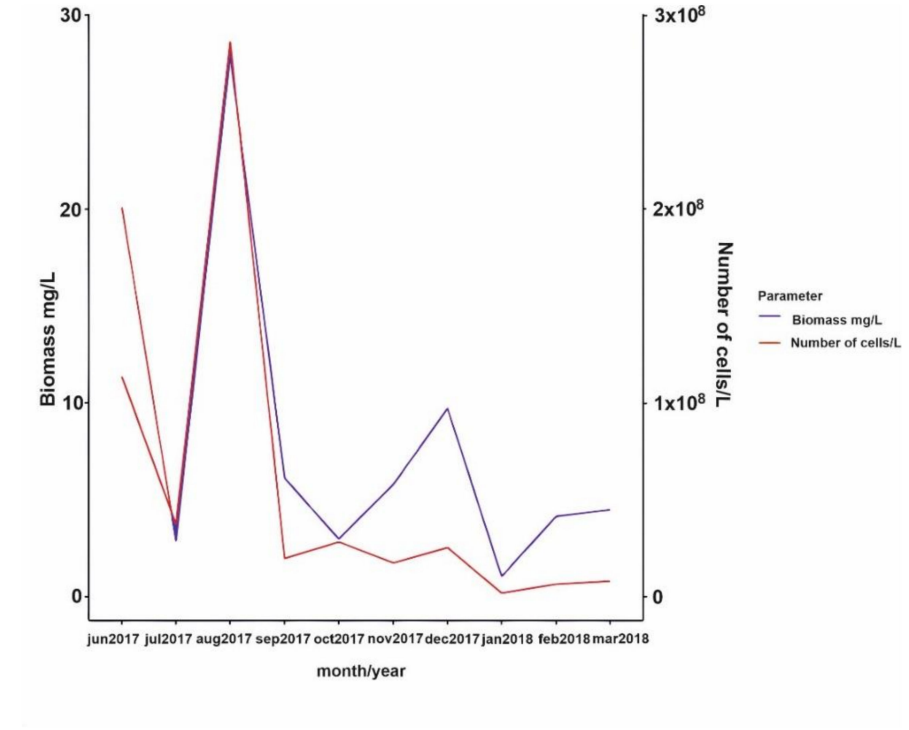
Figure 2. Principal Component Analysis (PCA) ordination of the environmental variables during the investigated period.

3.2. Phytoplankton Succession

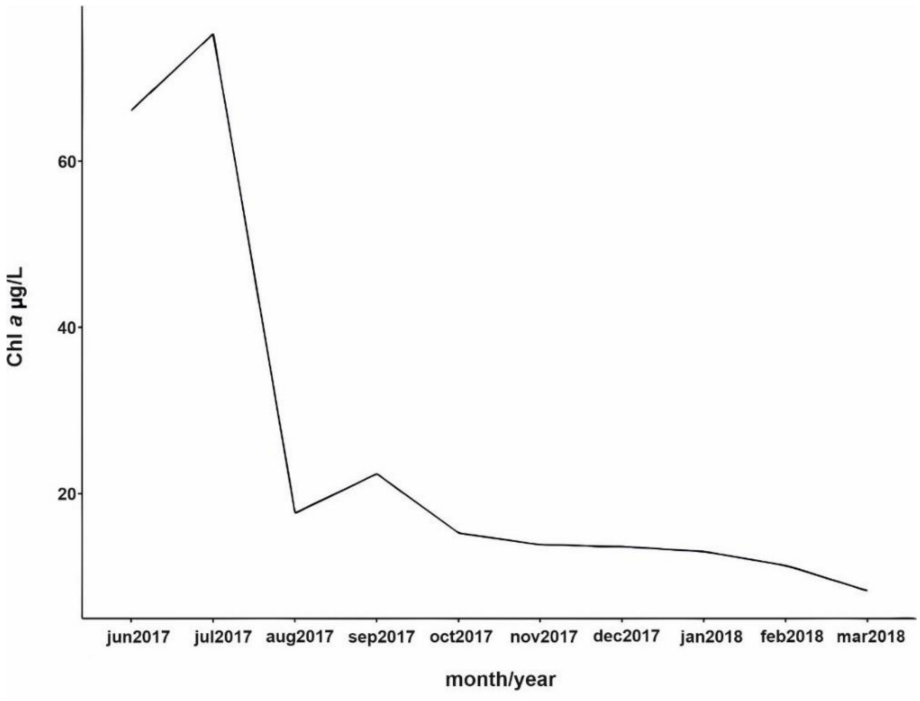
A total of 38 phytoplankton species were recorded by a morphological approach within the 10 samples collected during the investigated period. A total of 47,130 reads clustered into 88 OTUs were detected in the remaining eight samples. OTUs were taxonomically assigned into 30 eukaryotic algal taxa.

Phytoplankton abundance ranged between 1.9×10^6 cells L^{-1} in January 2018 to 2.8×10^8 cells L^{-1} in August 2017. Phytoplankton biomass ranged from 1.05 mg L^{-1} in January 2018 to 27.85 mg L^{-1} in August 2017, related to the cyanobacterial bloom of *Microcystis* spp. (Figure 3a). The chlorophyll *a* value fluctuated from 8.37 µg L^{-1} (March 2018) to 75.3 µg L^{-1} (July 2017). Concurrently, the highest peak of chlorophyll *a* concentration was not recorded during the cyanobacterial bloom in August 2017, but instead in July 2017, during the proliferation of green algae, predominantly *Scenedesmus quadricauda* (Turpin) Brébisson (Figure 3b).

As inferred from the morphological approach, the alpha diversity in the richness, Shannon, and Simpson indices of phytoplankton varied considerably during the investigated period. The maximum value of species richness was recorded in June 2017, while the minimum was noted in February 2018. The Shannon index values ranged from the maximum in October 2017 to the minimum in March 2018. The maximum value of the Simpson index was also noted in October 2017, but the minimum was recorded in June 2017 (Figure 4a). The richness, Shannon, and Simpson indices inferred from the molecular approach did not show the same pattern. The highest value of richness index was recorded in January 2018, while the lowest richness was noted in August 2017. The minimum and maximum values of the Shannon and Simpson indices were reported in July 2017 and June 2017, respectively (Figure 4b).

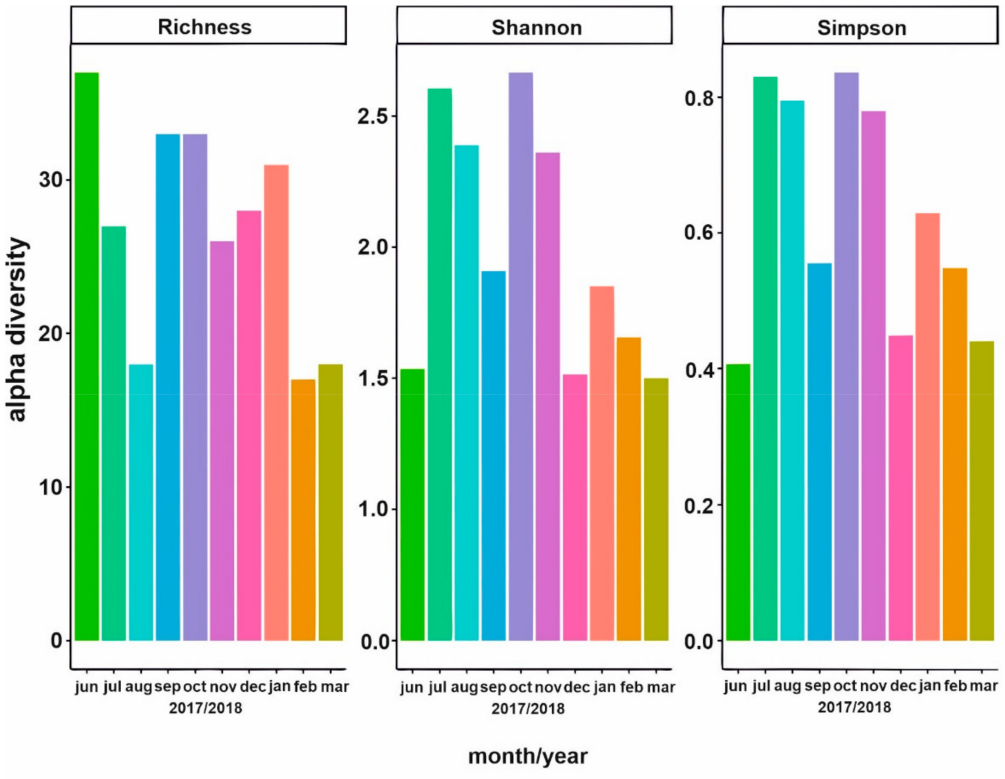


(**a**)

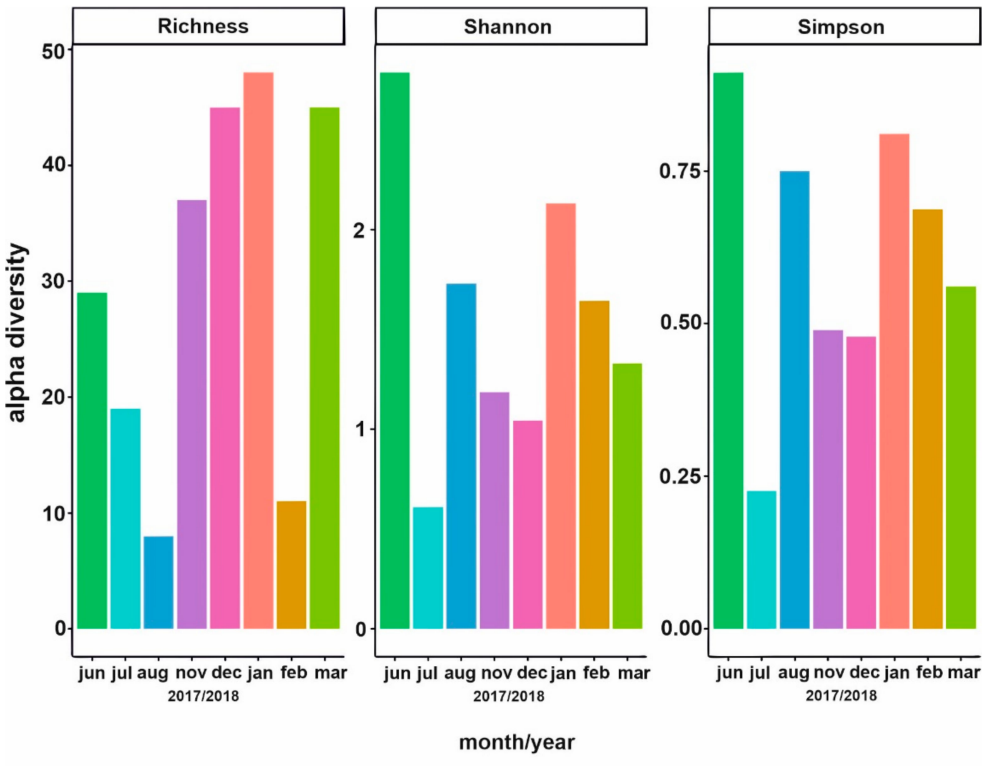


(**b**)

Figure 3. Phytoplankton: (**a**) total biomass and abundance; (**b**) chlorophyll *a* concentration during the investigated period.



(**a**)



(**b**)

Figure 4. Alpha diversity of the richness, Shannon, and Simpson indices of the phytoplankton community inferred from the morphological and molecular approach during the investigated period ((**a**) = morphological approach, (**b**) = molecular approach).

According to the frequency and biomass, the most dominant species were: filamentous cyanobacterium *Limnothrix redekei* (Goor) Meffert, colonial clathrate cyanobacteria *Microcystis aeruginosa* (Kützing) Kützing and *M. wesenbergii* (Komárek) Komárek ex Komárek, centric diatom *Aulacoseira muzzanensis* (F.Meister) Krammer, pennate diatoms *Ulnaria ulna* (Nitzsch) Compère and *Ulnaria* sp. (Kützing) Compère, cryptophyte *Cryptomonas* sp. Ehrenberg, dinoflagellate *Peridinium* sp. Ehrenberg, and colonial chlorophyte *Scenedesmus quadricauda* (Turpin) Brébisson.

A CCA was performed for the phytoplankton samples, and nine constrained environmental variables (Figure 5) indicated eigenvalues for the first two axes of 0.4328 and 0.3210, respectively, explaining 38.5% of the total variance on the first two axes. A Pearson environment-species permutation for the two significant axes indicated a significant correlation between abiotic constrained values and phytoplankton functional variables. According to the ANOVA permutation test, the ordination of both axes for environmental variables was statistically significant ($p < 0.05$). Canonical coefficients and intra-set correlations on the phytoplankton samples showed that NO_3^- and EC were the most important variables for the ordination axis 1 (intra-set correlation coefficients: 0.6332 and 0.5467, respectively). Regarding axis 2, NO_2^- and NH_4^+ (intra-set correlations −0.8155 and −0.6837, respectively) were the variables that weighted most for the ordination. At the positive end of both axes, phytoplankton samples were associated with EC, DO, HCO_3^-, and NO_3^-. At the negative end of both axes, phytoplankton samples were associated with pH, T, SiO_2, NH_4^+, and NO_2^-. Considering the environmental pressure to phytoplankton, the CCA analysis showed the separation of samples into three groups (Figure 5). The first group, comprised of summer and autumn samples (July to October 2017), correlated with high concentrations of NH_4^+ (2.75 mg L^{-1}), NO_2^- (0.173 mg L^{-1}), and SiO_2 (26.8 mg L^{-1}) and high values of pH (9.48) and T (24 °C). The most common species of the group were cyanobacteria *M. aeruginosa* and *M. wesenbergii*, green alga *Scenedesmus quadricauda* and cryptophyte *Cryptomonas* sp. According to the morphological approach, the samples collected in July were characterized by the highest Chl *a* concentration, the dominance of *S. quadricauda*, and the highest concentration of NH_4^+ (2.75 mg L^{-1}). A pronounced increase in the total phytoplankton biomass was recorded in August as a result of *Microcystis* spp. bloom. *M. aeruginosa* and *Cryptomonas* sp. were the descriptive species of the phytoplankton community in September and October, with the continuing decrease of the phytoplankton biomass. The molecular approach did not confirm the same composition, but rather detected the cyanobacterial predominance and a higher contribution of OTUs taxonomically assigned to the *Cryptomonas* genera. According to the morphological approach, the second group, consisting of an outlying sample from June 2017, was characterized by the predominance of filamentous cyanobacterium *Limnotrix redekei* (Goor) Meffert and centric diatom *Aulacoseira muzzanensis* (Meister) Krammer. The molecular approach confirmed the dominance of OTUs taxonomically assigned to the *Aulacoseira* genera as well, but also detected a high number of reads of OTUs taxonomically assigned to genus *Parvodinium*. The third group, composed of winter samples (November 2017 to March 2018), correlated with low T and higher concentrations of NO_3^- (38.4 mg L^{-1}) and HCO_3^- (249 mg L^{-1}) and with mostly constant values of DO (11.9 to 14.7 mg L^{-1}) and EC (497 µS cm^{-1}). As confirmed by both morphological and molecular approaches, the most dominant species/taxonomically assigned OTUs during the colder period were pennate diatoms (Fragilariaceae) of the genus *Ulnaria*. Dinoflagellate *Peridinium* sp. was recorded only by the morphological approach as the subdominant species in November and January. The higher contribution of the genus *Dinobryon* was confirmed by both analyses during February and March.

As confirmed by both the morphological and molecular approaches, the most dominant species during the colder period were pennate diatoms (Fragilariaceae) of the genus *Ulnaria* with dinoflagellate *Peridinium* sp. as the subdominant species.

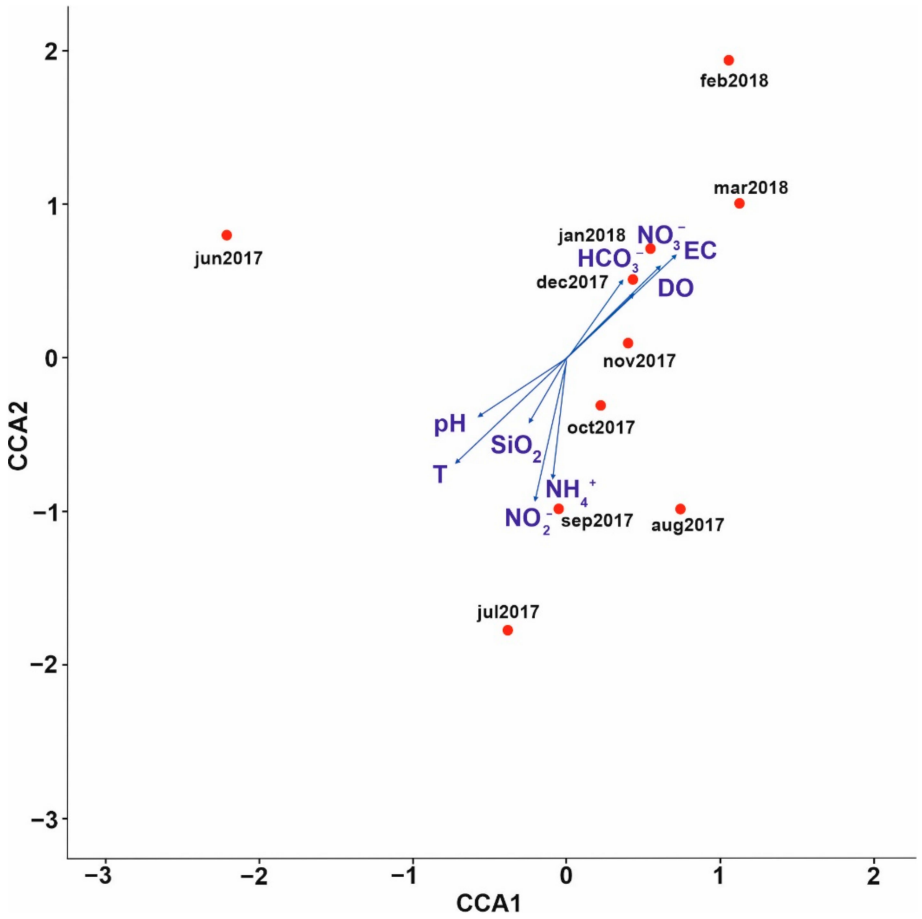


Figure 5. Ordination diagram of the canonical correspondence analysis (CCA) of the phytoplankton community inferred from the morphological approach in correlation to environmental variables during the investigated period.

3.3. Bacterial Community Composition

For the eight samples, a total of 582,900 reads clustered into 1743 OTUs were recorded. Sequence reads were taxonomically assigned into 52 bacterial phyla. The bacterial composition showed a succession of differences between months. The variations of alpha diversity in the rarefied richness, Shannon, and Simpson indices are shown in Figure 6. The highest bacterial richness was recorded in July 2017, and the lowest in October 2017. The Shannon and Simpson indices showed similar results, with the highest values in January 2018 and the lowest in August 2017. Comparing the composition of bacteria and eukaryotic algae inferred from the molecular approach, low values in richness and both indices during the summer period were noted.

According to the percentage of classified OTUs, dominant bacterial phyla were Planctomycetota (22%), Cyanobacteria (64%), Bacteroidota (11%), Actinobacteriota (45%), and Proteobacteria (56%).

The CCA analysis performed on the bacterial community and seven constrained environmental variables (Figure 7) indicated eigenvalues for the first two axes of 0.7131 and 0.6563, respectively, explaining 47.2% of the total variance on the first two axes. A Pearson environment-bacterial community permutation for the two significant axes indicated a significant correlation between abiotic constrained values and bacterial functional variables. According to the ANOVA permutation test, the ordination of both axes for environmental variables was statistically significant ($p < 0.05$). Canonical coefficients and intra-set correlations carried out on the bacterial community samples showed that pH and DO were the most important variables for the ordination axis 1 (intra-set correlation coefficients:

0.6140 and 0.3770, respectively). Regarding axis 2, NO_2^- and NH_4^+ (intra-set correlations: 0.9229 and 0.8706, respectively) were the variables that weighted most for ordination. At the positive end of both axes, the bacterial community's samples were associated with T, pH, and NO_2^-. At the negative end of both axes, the bacterial community's samples were associated with EC and HCO_3^-. Based on the position of the samples related to the CCA1 axis, the bacterial community separated into two groups (Figure 7). The first group, comprised of summer samples (June to September 2017), correlated with a high concentration of NH_4^+ and NO_2^-, and high values of pH and T. According to the percentage of classified OTUs, the most common phyla in the first group were Cyanobacteria, with the most dominant families being Pseudanabaenaceae (June 2017), Microcystaceae (July 2017), and Synechococcaceae and Microcystaceae (August 2017). This was also confirmed by a morphological approach, except for the picocyanobacterium of the genus *Cyanobium*, which is hard to detect under light microscopy. In June, Planctomycetota were codominant with Cyanobacteria. The sample from July was singled out as a result of the codominance of Cyanobacteria and Bacteroidota, which correlated with the concentration of NH_4^+. The second group, comprising samples from the colder months (October 2017 to January 2018), correlated with low T and higher concentrations of HCO_3^-, DO, and EC. Actinobacteriota was the dominant phylum in October 2017 and January 2018, whilst samples from November and December were dominated by Proteobacteria.

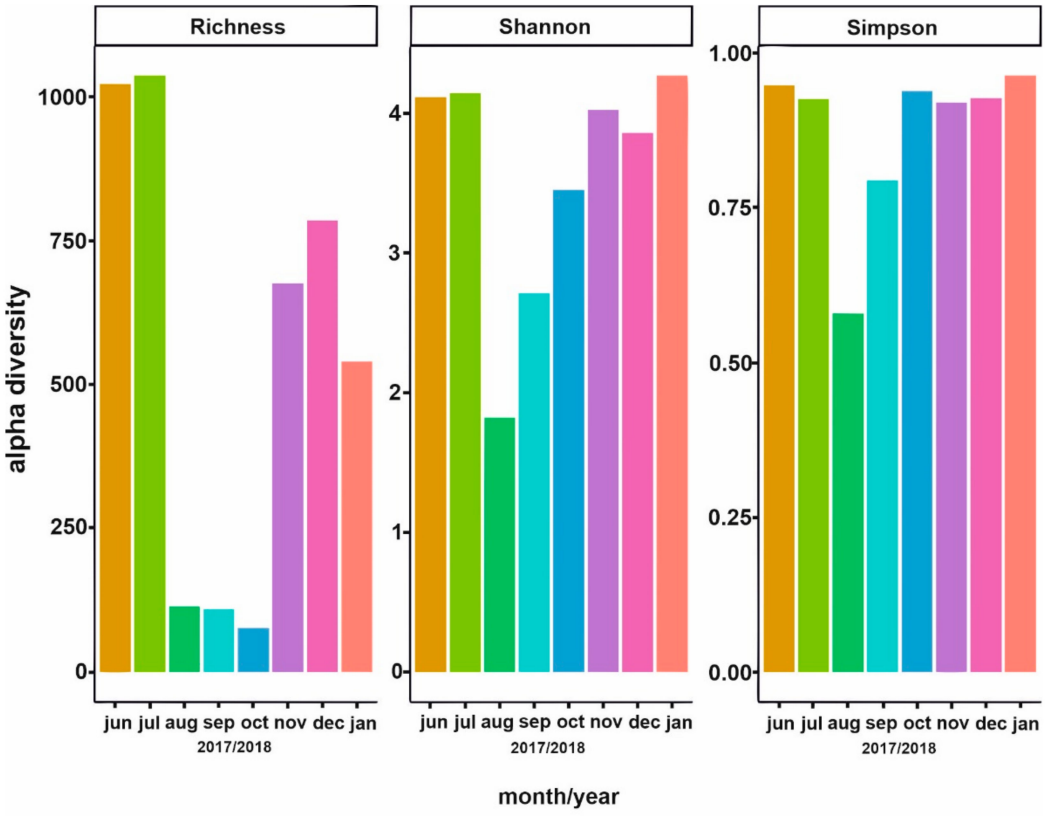


Figure 6. Alpha diversity of the richness, Shannon, and Simpson indices of the bacterial community inferred from the molecular approach during the investigated period.

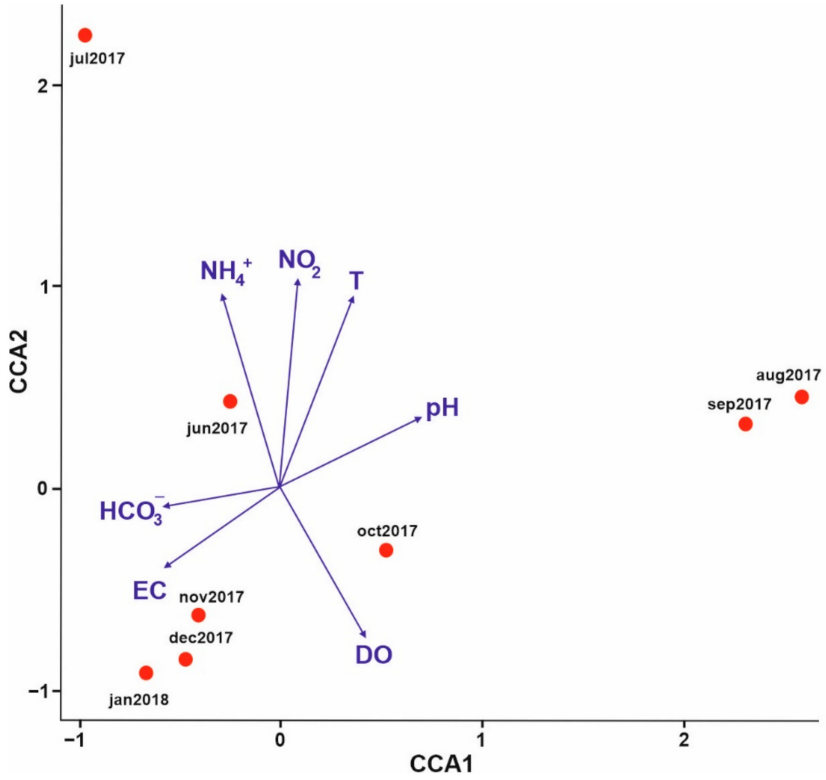


Figure 7. Ordination diagram of the canonical correspondence analysis (CCA) of the bacterial community inferred from the molecular approach in correlation with environmental variables during the investigated period.

4. Discussion

Data on ecology and the importance of small water bodies in alluvial lowlands are still quite scarce, as those systems are not included in the national strategies for the protection of water resources. Within larger freshwater systems, small water bodies act as biochemical reactors because of their potential for supporting high metabolic rates that are often paired with naturally high concentrations of nutrients and trophic conditions [33,34,63]. Their role is modulating nutrient retention and recycling along the hydrological pathways [63]. Even though usually related to eutrophic or hypertrophic conditions, small standing water bodies collectively support a very diverse biodiversity, often with species-richer communities more adapted to eutrophication conditions and to a broad range of physical and chemical conditions than the communities in larger water bodies [38,64]. Eutrophication has been described as a major stressor for the freshwater biodiversity of both large and small water bodies [65,66]. Rosset et al. [32] suggested that the eutrophication management of lowland small water bodies should be regulated differently than for larger freshwater systems, with the conservation efforts focused on the protection of small water bodies representing a mosaic of trophic conditions (and acting as hosts of regional biodiversity).

Seasonal changes with complex dynamic phases govern the high rates of biodiversity in small lowland water bodies altered by high anthropogenic pressure and climate-related impacts, such as the Šijanec gravel pit [67,68]. Previous studies on the Drava River lowland did not consider the importance of small water bodies within the whole alluvial system [69,70]. Due to hydromorphological characteristics of the catchment, Šijanec receives a high nitrogen input from the Drava River aquifer via groundwater recharging [41]. The high concentration of nitrates in the Drava River groundwater system, with an average of 60.9 mg L^{-1}, was observed by Marković et al. [41]. Nitrogen compounds can easily percolate through the soil into the groundwater either from direct terrestrial runoff or with rainfall or irrigation water [71]. A higher nitrogen concentration was noted during the colder seasons as a consequence of the recorded decrease in the phytoplankton biomass

and abundance, as well as the rise of the groundwater level due to an increase in precipitation [72,73], whereas nitrogen concentrations in the groundwater dropped during the warmer periods following the decrease of precipitation, as confirmed by the PCA analysis. Since nitrogen fertilizers are widely used in agriculture to increase crop production, the cropping practices and soil texture have been found to influence the extent of nitrate leaching [74].

The nutrient-based indication of eutrophic conditions in the gravel pit was further supported by high values of phytoplankton biomass and bacterial density throughout the investigated period, especially during the summer months, when the lowest NO_3^- concentration (June) and the highest concentrations of NH_4^+ and NO_2^- (July) were detected. This occurrence of elevated nitrogen is presumably occurring as an effect of the cropping season, e.g., fertilizer application through intense irrigation [75]. This is particularly true for nitrates, which are normally assumed not to be absorbed by soil particles and are therefore easily leached, in which case the nitrate distribution should follow the wetting front [76,77]. As found by Paredes et al. [78], high intra- and interannual hydrological fluctuations influenced nitrate occurrence in freshwater streams and ponds, with the main source of nitrate linked to agricultural practices and the use of both organic and synthetic fertilizers. Since it can be rapidly oxygenized, the concentration of NO_2^- is usually deficient [79], as was exhibited in this study. Most of the nitrogen uptake in shallow eutrophic systems is the form of nitrate, which has a positive effect on the growth of phytoplankton biomass [80,81]. A sample from June had the lowest concentrations of NO_3^-, TN, TIC, and logpCO_2, but the highest temperature and Si values. These conditions were characterized by the dominance of cyanobacteria *Limnotrix redekei* and centric diatom *Aulacoseira muzzanensis*. *Limnotrix redekei*, a species characteristic of eutrophic shallow water bodies used for recreation and fishing, often shows domination in spring and summer periods with co-occurrence of centric diatoms [82]. This species is known for its ability to adapt to low-light, cold conditions and capability to overwinter in considerable densities under the long-term ice- and snow-cover [83–86]. *Aulacoseira muzzanensis*, a species adapted to live in turbid and nutrient-rich waters [87], was described in a hyper-eutrophic lake (Lago di Muzzano) located in the Tessin region of Switzerland [88], which suffers from periodic *Microcystis* algal blooms [89–92]. During lower light conditions, both taxa can occur concomitantly with the *Microcystis* species in quantities capable of eliminating other phytoplankton taxa [93]. A sample from July in the grouped composed of summer and autumn samples was characterized by maximum concentrations of nitrites and ammonium. This suggested enhanced growth conditions for specific algal groups under a higher NH_4^+ supply, which was also evident by a recorded high concentration of the chlorophyll *a*. Certain phytoplankton taxa, such as cyanobacteria and chlorophytes, prefer a high supply of energetically favorable NH_4^+ [94], as was also noted in our investigation. These conditions can also inhibit NO_3^- uptake for other taxa, such as large diatoms [94–96]. The most abundant chlorophyte was *Scenedesmus quadricauda*, a small coenobium-forming and ammonium-tolerant species [97–99] with higher uptake abilities for ammonium under nitrogen limitation than species of the genus *Microcystis* and with competitive superiority in the large-pulse, low-frequency nutrient recharging [100], as was present in our study area. With regard to size, small algae can be more competitive than larger species, because they have high surface area to volume ratios, resulting in greater specific uptake and growth under low nutrient concentrations, when even slight NH_4^+ additions can be enough to promote the growth of small algae [101]. Secondly, the most dominant species during the summer period was *Microcystis* spp., with the culmination of dominance (surface bloom) in August, which was connected with the maximum of phytoplankton biomass and number of cells per liter. *Microcystis* is a non-N_2-fixing cyanobacteria, which dominates in highly eutrophicated, stratified ponds, rivers, or lakes receiving elevated N loadings, since its growth is dependent on nitrogen supplies [6]. A strong bloom of *Microcystis* spp. was associated with the warmer period, low nitrogen concentrations, and the subsequent water level drawdown [102]. Cyanobacteria have the ability to adapt to different environments

by adjusting their light harvesting and carbon fixation mechanisms. Adversely, a high rate of photosynthesis induced by the *Microcystis* bloom can considerably reduce dissolved CO_2 concentrations and drive up the pH value [103]. Furthermore, *Microcystis* favors more alkaline conditions as a competitive advantage over other phytoplankton groups [104,105]. Along with the *Microcystis* bloom, the molecular approach detected the cyanobacterium of the genus *Cyanobium*, which was dominant in August. *Cyanobium* is a picocyanobacterial genus with a presumably significant role in the functioning of aquatic ecosystems, but rather hard to detect microscopically due to its size and taxonomical obscurity. Both *Microcystis* and *Cyanobium* genera are less demanding on nutrients and generally demonstrate summer peaks when the concentrations of nitrogen compounds are usually lower [106]. As detected by the molecular approach, the phylum Planctomycetota was subdominant in the bacterial community in June. Members of the phylum Planctomycetota have been found in a variety of environments, including freshwater [107], and are known for their role in the anaerobic oxidation of ammonium (anammox), as part of the biogeochemical nitrogen cycle [108–112]. In oxygen-limited systems, such as biofilm aggregates, the planctomycete anammox bacteria live closely associated with aerobic ammonium oxidizers. The aerobic ammonium-oxidizing bacteria consume oxygen at the outside of the biofilm, thus keeping the inside anoxic for the anammox bacteria. Together, they create conditions in which they can convert ammonium directly into dinitrogen gas. Anammox bacteria can contribute significantly to the loss of fixed nitrogen in both natural and anthropogenic-influenced ecosystems [110,112,113]. The members of the phylum Bacteroidota recorded in July are typical for freshwater environments [114]. Their dominance correlated with OTUs taxonomically assigned in eukaryotic biflagellate cryptophytes from *Cryptomonas* genera, and with chlorophyte *Scenedesmus quadricauda*, as recorded by the morphological approach. The increase in their abundance correlated with higher algal concentrations, presumably due to their ability to establish a mutualistic relationship on the algal cell surface [115]. High densities of the *Cryptomonas* genus can occur following the period of nutrient enrichment [6]. During the colder period, the composition of the phytoplankton switched from cyanobacteria to a diatom-dominated community characterized by the genus *Ulnaria*, whose winter blooms require both biogenic silica for the formation of their outer cell wall structures (frustules) and lower basic pH conditions, as not to corrode them [116] and decrease the growth rate [117]. This resulted in a threefold drop in the SiO_2 concentration which was presumably consumed in the building of their frustules [118,119]. Diatoms are characterized as effective nitrate utilizers with high preferences to NO_3^- uptake [120]. Also subdominant in the colder period, together with diatoms, were dinoflagellate species, which were reported to have significant NO_3^- uptake rates [121]. A dense bloom of colonial chrysophyte *Dinobryon* spp. characterized the phytoplankton community in February and March. As adaptations to the lower temperature, the winter blooms of *Dinobryon* could indicate enhanced nutrient loading [6], but also the ability to obtain nutrients from bacteria by mixotrophy [122,123]. Members of Proteobacteria, as the most dominant bacterial phylum in the samples from November and December, are ubiquitous in freshwater environments, specifically in eutrophic conditions with high phytoplankton numbers [114,124]. Actinobacteriota were present throughout the investigated period, with increased abundance in October and January during the low abundance of cyanobacteria. This is plausibly correlated with the sensitivity of Actinobacteriota to conditions prevailing during the cyanobacterial blooms, such as high organic matter, inorganic nutrient availability, and high temperatures, under which the highly streamlined actinobacterial cells cannot compete [125]. Actinobacteriota can thrive under oligotrophic conditions due to their small size, high surface area-to-volume ratio, and enhanced capacity for efficient nutrient acquisition through high-affinity, broad-specific uptake systems [126]. Another important role of Actinobacteriota in freshwater habitats is connected to the proton-pumping rhodopsins (actinorhodopsins), thus revealing a photoheterotrophic lifestyle [127]. All these traits suggest that Actinobacteriota might serve as sentinels of impending ecological damage and have the potential to become standards of ecological freshwater quality [125].

Some of the OTUs were taxonomically assigned to species usually hard to detect under the microscope. These species included colonial chrysophyte *Uroglenopsis americana* (G.N.Calkins) Lemmermann, thecate dinoflagellate *Asulcocephalium miricentonis* Kazuya Takahashi, Moestrup & M. Iwataki and small green algae *Actinochloris sphaerica* Korschikov, Meyerella planktonica Fawley & K. P. Fawley, *Wislouchiella planctonica* Skvortzov and *Chloromonas subdivisa* (Pascher & Jahoda) Gerloff & Ettl. They were all detected in the winter samples, except for *Asulcocephalium miricentonis* and *Wislouchiella planctonica*, which were detected in June. Besides type locality, no further ecological data were available on *Asulcocephalium miricentonis*, a species described in a temperate freshwater artificial pond in northeastern Japan [128]. Therefore, this record presents a contribution to the ecological conditions in which the species likely occurs. *Wislouchiella planctonica* is associated with man-made reservoirs and lentic freshwater habitats with eutrophic conditions [129,130], which is in line with our findings. *Uroglenopsis americana* was detected in February together with *Dinobryon* spp., due to its ability to compete for nutrients during the colder period, unlike algal species in eutrophic conditions [131]. The picoplanktonic species *Meyerella planktonica* is a major component of aquatic systems and a significant primary producer regularly occurring during winter [132,133]. Some of the species from the *Chloromonas* genera were found in snow samples [134], as was the case with the species *Chloromonas subdivisa* detected during snowy winter conditions in Šijanec. *Actinochloris sphaerica* is a cosmopolitan species mostly recorded in soil cultures and puddles [135]. Since very scarce information is available on all these species, the presented results also contribute to elucidating their ecological preferences.

Both approaches showed variations in diversity richness. Based on the morphological approach, the maximum value of alpha diversity was recorded in June, presumably due to the favorable conditions for algal growth. The minimum value of alpha diversity was detected in February, due to the lower number of algal species, whereas by using the molecular approach, higher values of alpha diversity were detected in the colder period, which can be associated with two possible causes: (1) when cell abundances of specific taxa in the water sample drop below a specific threshold, they can still be detectable with the molecular approach, but may not be found by microcopy; and (2) the resting stages of some algal species cannot be identified and assigned correctly by microscopy, but might be more easily recorded by the molecular approach [136]. Moreover, the lowest value of alpha diversity detected in August with the molecular approach could be correlated with the cyanobacterial bloom. Some cyanobacteria are known to produce toxins and cyclic peptides which can inhibit regulatory enzymes in eukaryotic cells, thus causing PCR inhibition [137,138]. Similar variations were detected in bacterial alpha diversity, with a maximum in July and low values throughout August, September, and October. This finding also corresponds to cyanobacterial bloom, which can inhibit the stabilization of microbial diversity [139]. Surprisingly, the morphological approach, as a basic descriptive method, succeeded in recognizing a higher microalgal diversity in Šijanec than the molecular approach, which is commonly considered a more powerful detector tool [140]. Events such as cyanobacterial blooms or discrepancies in the various DNA extraction methods can also have a discernible impact on the certainty of the community analysis via the molecular approach. Eukaryotic algae have a large range of cell wall structures, thus imposing challenges to the unbiased, uniform, and universal extraction of nucleic acids from such communities [138]. In spite of this, the molecular approach proved far more effective in discerning small-sized eukaryotic algae and cyanobacterial taxa, which are generally hard to detect with microscopy due to the scarcity of taxonomic knowledge and limitations of resolving power. Molecular methods showed that they can be successfully used to complement the morphological approach for assessing the microbial community's structure [138,141], especially in these kinds of eutrophic environments.

5. Conclusions

Despite the size, small water bodies like the Šijanec gravel pit play a key role as buffer zones within alluvial areas of large rivers. Due to the hydromorphological characteristics of the catchment, Šijanec receives a high nitrate input directly from the groundwater recharging. Nitrogen compounds likely control the phytoplankton biomass, thereby influencing the complete microbial community's structure. The integration of morphological and molecular approaches facilitates the comprehensive assessment of the microbial community's structure. Interdisciplinary approaches can be successfully used to elucidate the ecological preferences of microbial species and the prediction and prevention of algal blooms. The study emphasizes the importance of small water bodies in maintaining the state of water ecosystems and stresses the need for their enlistment in biomonitoring actions.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4441/13/2/115/s1. Document S1: includes the list of commands with related parameters used in the OBITools package.

Author Contributions: Formal analysis, investigation, data curation, writing—Original draft preparation, visualization, A.K.; chemical analysis, investigation, writing—Review and editing, T.M.; investigation, validation, writing—Review and editing, P.Ž.; data curation, writing—Review and editing, K.K.; description of the study area, investigation, writing—Review and editing, I.K.; methodology, investigation, writing—Review and editing, S.O.; software, writing—Review and editing, E.K.; validation, writing—Review and editing, V.F.; conceptualization, investigation, supervision, validation, writing—Review and editing M.G.U. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Croatian Scientific Foundation (HRZZ), grant number HRZZ-IP-2016-06-5356, and by the COST DNAqua-Net grant (CA15219–"eDNA metabarcoding in providing assessment of microbial eukaryotic biodiversity in small standing-water ecosystem"). SO and KK were partially supported by the project STIM – REI, Contract Number: KK.01.1.1.01.0003., funded by the European Union through the European Regional Development Fund—the Operational Programme Competitiveness and Cohesion 2014–2020 (KK.01.1.1.01).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in: Kulaš, A.; Marković, T.; Žutinić, P.; Kajan, K.; Karlović, I.; Orlić, S.; Keskin, E.; Filipović, V.; Udovič, M.G. Succession of Microbial Community in a Small Water Body within the Alluvial Aquifer of a Large River Water *Water* **2021**, *13*, 115. https://doi.org/10.3390/w13020115.

Acknowledgments: We thank the evolutionary Genetics Laboratory (eGL) research team for their support and help in bioinformatics analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Thingstad, T.; Sakshaug, E. Control of phytoplankton growth in nutrient recycling ecosystems. Theory and terminology. *Mar. Ecol. Prog. Ser.* **1990**, *63*, 261–272. [CrossRef]
2. Chan, J.L.; Mantzoros, C.S. Role of leptin in energy-deprivation states: Normal human physiology and clinical implications for hypothalamic amenorrhoea and anorexia nervosa. *Lancet* **2005**, *366*, 74–85. [CrossRef]
3. Green, P.; Vörösmarty, C.J.; Meybeck, M.; Galloway, J.N.; Peterson, B.J.; Boyer, E.W. Pre-industrial and contemporary fluxes of nitrogen through rivers: A global assessment based on typology. *Biogeochemistry* **2004**, *68*, 71–105. [CrossRef]
4. Li, Z.G.; Lin, B.-L.; Sagisaka, M.; Yang, P.; Wu, W. Global-scale modelling of potential changes in terrestrial nitrogen cycle from a growing nitrogen deposition. *Proc. Environ. Sci.* **2012**, *13*, 1057–1068. [CrossRef]
5. Galloway, J.N.; Cowling, E.B.; Seitzinger, S.; Socolow, R.H. Reactive nitrogen: Too much of a good thing? *Ambio* **2002**, *31*, 60–63. [CrossRef]
6. Paerl, H.W.; Fulton, R.S.; Moisander, P.H.; Dyble, J. Harmful freshwater algal blooms, with an emphasis on Cyanobacteria. *Sci. World J.* **2001**, *1*, 76–113. [CrossRef]
7. Erisman, J.W.; Galloway, J.; Seitzinger, S.; Bleeker, A.; Butterbach-Bahl, K. Reactive nitrogen in the environment and its effect on climate change. *Curr. Opin. Environ. Sustain.* **2011**, *3*, 281–290. [CrossRef]

8. Dupas, R.; Delmas, M.; Dorioz, J.-M.; Garnier, J.; Moatar, F.; Gascuel-Odoux, C. Assessing the impact of agricultural pressures on N and P loads and eutrophication risk. *Ecol. Indic.* **2015**, *48*, 396–407. [CrossRef]
9. Erisman, J.W.; Galloway, J.N.; Seitzinger, S.; Bleeker, A.; Dise, N.B.; Petrescu, A.M.R.; Leach, A.M.; de Vries, W. Consequences of human modification of the global nitrogen cycle. *Philos. Trans. R. Soc. B Biol. Sci.* **2013**, *368*, 20130116. [CrossRef]
10. Mekonnen, M.M.; Hoekstra, A.Y. Global gray water footprint and water pollution levels related to anthropogenic nitrogen loads to fresh water. *Environ. Sci. Technol.* **2015**, *49*, 12860–12868. [CrossRef]
11. le Doux, E.; Gomez, E.; Monget, J.; Viavattene, C.; Viennot, P.; Ducharne, A.; Benoit, M.; Mignolet, C.; Schott, C.; Mary, B. Agriculture and groundwater nitrate contamination in the Seine basin. The STICS–MODCOU modelling chain. *Sci. Total Environ.* **2007**, *375*, 33–47. [CrossRef] [PubMed]
12. Mengel, K.; Hütsch, B.; Kane, Y. Nitrogen fertilizer application rates on cereal crops according to available mineral and organic soil nitrogen. *Eur. J. Agron.* **2006**, *24*, 343–348. [CrossRef]
13. Sete, P.B.; Comin, J.J.; Ciotta, M.N.; Salume, J.A.; Thewes, F.; Brackmann, A.; Toselli, M.; Nava, G.; Rozane, D.-E.; Loss, A.; et al. Nitrogen fertilization affects yield and fruit quality in pear. *Sci. Hortic.* **2019**, *258*, 108782. [CrossRef]
14. Jégo, G.; Sánchez-Pérez, J.M.; Justes, E. Predicting soil water and mineral nitrogen contents with the STICS model for estimating nitrate leaching under agricultural fields. *Agric. Water Manag.* **2012**, *107*, 54–65. [CrossRef]
15. Bernard-Jannin, L.; Sun, X.; Teissier, S.; Sauvage, S.; Sánchez-Pérez, J.M. Spatio-temporal analysis of factors controlling nitrate dynamics and potential denitrification hot spots and hot moments in groundwater of an alluvial floodplain. *Ecol. Eng.* **2017**, *103*, 372–384. [CrossRef]
16. Singer, G.; Besemer, K.; Schmitt-Kopplin, P.; Hödl, I.; Battin, T.J. Physical heterogeneity increases biofilm resource use and its molecular diversity in stream mesocosms. *PLoS ONE* **2010**, *5*, e9988. [CrossRef]
17. Cardinale, B.J.; Duffy, J.E.; Gonzalez, A.; Hooper, D.U.; Perrings, C.; Venail, P.; Narwani, A.; Mace, G.M.; Tilman, D.; Wardle, D.A.; et al. Biodiversity loss and its impact on humanity. *Nat. Cell Biol.* **2012**, *486*, 59–67. [CrossRef]
18. Besemer, K.; Singer, G.; Quince, C.; Bertuzzo, E.; Sloan, W.; Battin, T.J. Headwaters are critical reservoirs of microbial diversity for fluvial networks. *Proc. R. Soc. B* **2013**, *280*, 20131760. [CrossRef]
19. Widder, S.; Besemer, K.; Singer, G.A.; Ceola, S.; Bertuzzo, E.; Quince, C.; Sloan, W.T.; Rinaldo, A.; Battin, T.J. Fluvial network organization imprints on microbial co-occurrence networks. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 12799–12804. [CrossRef]
20. Peterson, B.J.; Wollheim, W.M.; Mulholland, P.J.; Webster, J.R.; Meyer, J.L.; Tank, J.L.; Martí, E.; Bowden, W.B.; Valett, H.M.; Hershey, A.E.; et al. Control of nitrogen export from watersheds by headwater streams. *Science* **2001**, *292*, 86–90. [CrossRef]
21. Paerl, H.W. Mitigating harmful cyanobacterial blooms in a human- and climatically impacted world. *Life* **2014**, *4*, 988–1012. [CrossRef]
22. Pal, R.; Choudhury, A.K. *An Introduction to Phytoplanktons: Diversity and Ecology*; Springer Science and Business Media LLC: Berlin/Heidelberg, Germany, 2014; pp. 25–52.
23. Herrero, A.; Muro-Pastor, A.M.; Flores, E. Nitrogen control in Cyanobacteria. *J. Bacteriol.* **2001**, *183*, 411–425. [CrossRef] [PubMed]
24. Stal, L. Cyanobacteria: Diversity and versatility. In *Algae and Cyanobacteria in Extreme Environments: Cellular Origin, Life in Extreme Habitats and Astrobiology*; Sedbach, J., Ed.; Springer: Dordrecht, The Netherlands, 2007; Volume 11, pp. 659–680.
25. Sellner, K.G. Physiology, ecology, and toxic properties of marine cyanobacteria blooms. *Limnol. Oceanogr.* **1997**, *42*, 1089–1104. [CrossRef]
26. Hemida, M.; Ohyam, T. Nitrogen fixing Cyanobacteria: Future prospect. *Nitrogen Fixat.* **2014**, 23–48. [CrossRef]
27. Bakker, E.S.; Hilt, S. Impact of water-level fluctuations on cyanobacterial blooms: Options for management. *Aquat. Ecol.* **2016**, *50*, 485–498. [CrossRef]
28. Hamilton, L.C. Public awareness of the scientific consensus on climate. *SAGE Open* **2016**, *6*. [CrossRef]
29. van Gremberghe, I.; Leliaert, F.; Mergeay, J.; Vanormelingen, P.; van der Gucht, K.; Debeer, A.-E.; Lacerot, G.; de Meester, L.; Vyverman, W. Lack of phylogeographic structure in the freshwater Cyanobacterium *Microcystis aeruginosa* suggests global dispersal. *PLoS ONE* **2011**, *6*, e19561. [CrossRef]
30. Gijzen, H.J.; Mulder, A. The nitrogen cycle out of balance. *Water* **2001**, *21*, 38–40.
31. Moss, B.; Barker, T.; Stephen, D.; Williams, A.E.; Balayla, D.J.; Beklioglu, M.; de Carvalho, L.P.S. Consequences of reduced nutrient loading on a lake system in a lowland catchment: Deviations from the norm? *Freshw. Biol.* **2005**, *50*, 1687–1705. [CrossRef]
32. Rosset, V.; Angélibert, S.; Arthaud, F.; Bornette, G.; Robin, J.; Wezel, A.; Vallod, D.; Oertli, B. Is eutrophication really a major impairment for small waterbody biodiversity? *J. Appl. Ecol.* **2014**, *51*, 415–425. [CrossRef]
33. Menetrey, N.; Sager, L.; Oertli, B.; Lachavanne, J.-B. Looking for metrics to assess the trophic state of ponds. Macroinvertebrates and amphibians. *Aquat. Conserv. Mar. Freshw. Ecosyst.* **2005**, *15*, 653–664. [CrossRef]
34. Søndergaard, M.; Jeppesen, E.; Jensen, J.P.; Amsinck, S.L. Water Framework Directive: Ecological classification of Danish lakes. *J. Appl. Ecol.* **2005**, *42*, 616–629. [CrossRef]
35. Williams, P.; Whitfield, M.; Biggs, J.; Bray, S.; Fox, G.; Nicolet, P.; Sear, D. Comparative biodiversity of rivers, streams, ditches and ponds in an agricultural landscape in Southern England. *Biol. Conserv.* **2004**, *115*, 329–341. [CrossRef]
36. Gvozdić, V.; Brana, J.; Puntarić, D.; Vidosavljević, D.; Roland, D. Changes in the Lower Drava River water quality parameters over 24 years. *Arch. Ind. Hyg. Toxicol.* **2011**, *62*, 325–333. [CrossRef]
37. Villanueva, J.; Rosell, A.; Grimalt, J.O.; Navarro, A.; Rosell-Melé, A. Chemical characterization of polycyclic aromatic hydrocarbon mixtures in uncontrolled hazardous waste dumps. *Chemosphere* **1991**, *22*, 317–326. [CrossRef]

38. Navarro, A.; Carbonell, M. Assessment of groundwater contamination caused by uncontrolled dumping in old gravel quarries in the Besòs aquifers (Barcelona, Spain). *Environ. Geochem. Health* **2007**, *30*, 273–289. [CrossRef]
39. Preziosi, E.; Frollini, E.; Zoppini, A.; Ghergo, S.; Melita, M.; Parrone, D.; Rossi, D.; Amalfitano, S. Disentangling natural and anthropogenic impacts on groundwater by hydrogeochemical, isotopic and microbiological data: Hints from a municipal solid waste landfill. *Waste Manag.* **2019**, *84*, 245–255. [CrossRef]
40. Ondrasek, G.; Begić, H.B.; Zovko, M.; Filipović, L.; Meriño-Gergichevich, C.; Savić, R.; Rengel, Z. Biogeochemistry of soil organic matter in agroecosystems & environmental implications. *Sci. Total Environ.* **2019**, *658*, 1559–1573. [CrossRef]
41. Marković, T.; Karlović, I.; Tadić, M.P.; Larva, O. Application of stable water isotopes to improve conceptual model of alluvial aquifer in the Varaždin area. *Water* **2020**, *12*, 379. [CrossRef]
42. Mandel, S.; Shiftan, Z.L. *Groundwater Resources: Investigation and Development*, 1st ed.; Academic Press: New York, NY, USA, 1980; pp. 180–200.
43. Domenico, P.A.; Schwartz, F.W. *Physical and Chemical Hydrogeology*, 2nd ed.; John Willey and Sons: New York, NY, USA, 1990.
44. Parkhurst, D.L.; Appelo, C. User's guide to PHREEQC (Version 2): A computer program for speciation, batch-reaction, one-dimensional transport, and inverse geochemical calculations. *Water Res. Investig. Rep.* **1999**, *99*, 4259.
45. ISO/TC 147 Water Quality. Available online: https://www.iso.org/committee/52834/x/catalogue/ (accessed on 16 April 2018).
46. Utermöhl, H. Zur vervollkomnung der quantitativen phytoplankton-methodik. Mitteilungen Internationale Vereiningung für Theoretische und Angewandte. *Limnologie* **1958**, *9*, 1–38.
47. Stock, A.; Jürgens, K.; Bunge, J.; Stoeck, T. Protistan diversity in suboxic and anoxic waters of the Gotland Deep (Baltic Sea) as revealed by 18S rRNA clone libraries. *Aquat. Microb. Ecol.* **2009**, *55*, 267–284. [CrossRef]
48. Stoeck, T.; Bass, D.; Nebel, M.; Christen, R.; Jones, M.D.M.; Breiner, H.-W.; Richards, T.A. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. *Mol. Ecol.* **2010**, *19*, 21–31. [CrossRef] [PubMed]
49. Amaral-Zettler, L.; McCliment, E.A.; Ducklow, H.W.; Huse, S.M. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. *PLoS ONE* **2009**, *4*, e6372. [CrossRef]
50. Herlemann, D.P.R.; Labrenz, M.; Jürgens, K.; Bertilsson, S.; Waniek, J.J.; Andersson, A.F. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* **2011**, *5*, 1571–1579. [CrossRef]
51. de Barba, M.; Miquel, C.; Boyer, F.; Mercier, C.; Rioux, D.; Coissac, E.; Taberlet, P. DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: Application to omnivorous diet. *Mol. Ecol. Resour.* **2013**, *14*, 306–323. [CrossRef] [PubMed]
52. Bellemain, E.; Carlsen, T.; Brochmann, C.; Coissac, E.; Taberlet, P.; Kauserud, H. ITS as an environmental DNA barcode for fungi: An in silico approach reveals potential PCR biases. *BMC Microbiol.* **2010**, *10*, 189. [CrossRef]
53. Ficetola, G.F.; Coissac, E.; Zundel, S.; Riaz, T.; Shehzad, W.; Bessière, J.; Taberlet, P.; Pompanon, F. An *in-silico* approach for the evaluation of DNA barcodes. *BMC Genom.* **2010**, *11*, 434. [CrossRef]
54. BBMap. Available online: https://sourceforge.net/projects/bbmap/ (accessed on 19 February 2019).
55. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [CrossRef]
56. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27*, 2194–2200. [CrossRef]
57. Mahé, F.; Rognes, T.; Quince, C.; de Vargas, C.; Dunthorn, M. Swarm v2: Highly scalable and high-resolution amplicon clustering. *PeerJ* **2015**, *3*, e1420. [CrossRef] [PubMed]
58. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
59. R Core Team. *R: A Language and Environment for Statistical Computing*; R Core Team: Vienna, Austria, 2020.
60. Tapolczai, K.; Vasselon, V.; Lefrançois, E.; Stenger-Kovács, C.; Padisák, J.; Rimet, F. The impact of OTU sequence similarity threshold on diatom-based bioassessment: A case study of the rivers of Mayotte (France, Indian Ocean). *Ecol. Evol.* **2019**, *9*, 166–179. [CrossRef] [PubMed]
61. Anderson, M.J. Distance-based tests for homogeneity of multivariate dispersions. *Biometrics* **2005**, *62*, 245–253. [CrossRef] [PubMed]
62. Oksanen, J.; Blanchet, F.G.; Friendly, M.; Kindt, R.; Legendre, P.; McGlinn, D.; Minchin, P.R.; O'Hara, R.B.; Simpson, G.L.; Solymos, P.; et al. Vegan: Community Ecology Package. *R Packag. Vers. 22-1.* **2015**, *2*, 1–2.
63. Bolpagni, R.; Poikane, S.; Laini, A.; Bagella, S.; Bartoli, M.; Cantonati, M. Ecological and conservation value of small standing-water ecosystems: A systematic review of current knowledge and future challenges. *Water* **2019**, *11*, 402. [CrossRef]
64. Céréghino, R.; Boix, D.; Cauchie, H.-M.; Martens, K.; Oertli, B. The ecological role of ponds in a changing world. *Hydrobiologia* **2014**, *723*, 1–6. [CrossRef]
65. Brönmark, C.; Hansson, L.-A. Environmental issues in lakes and ponds: Current state and perspectives. *Environ. Conserv.* **2002**, *29*, 290–307. [CrossRef]
66. Hering, D.; Haidekker, A.; Schmidt-Kloiber, A.; Barker, T.; Buisson, L.; Graf, W.; Grenouillet, G.; Lorenz, A.; Sandin, L.; Stendera, S. Monitoring the responses of freshwater ecosystems to climate change. *Clim. Chang. Impacts Freshw. Ecosyst.* **2010**, 84–118. [CrossRef]

67. Boix, D.; Biggs, J.; Céréghino, R.; Hull, A.P.; Kalettka, T.; Oertli, B. Pond research and management in Europe: "Small is Beautiful". *Hydrobiologia* **2012**, *689*, 1–9. [CrossRef]
68. Bagella, S.; Gascón, S.; Filigheddu, R.; Cogoni, A.; Boix, D. Mediterranean temporary ponds: New challenges from a neglected habitat. *Hydrobiologia* **2016**, *782*, 1–10. [CrossRef]
69. Stanković, I.; Vlahović, T.; Udovič, M.G.; Várbíró, G.; Borics, G. Phytoplankton functional and morpho-functional approach in large floodplain rivers. *Hydrobiologia* **2012**, *698*, 217–231. [CrossRef]
70. Kovács, A.D.; Tóth, G.; Lóczy, D. *Water Quality of the Lower Drava River*; Springer Science and Business Media LLC: Berlin/Heidelberg, Germany, 2018; pp. 231–245.
71. Gao, K.; Helbling, E.; Häder, D.; Hutchins, D. Responses of marine primary producers to interactions between ocean acidification, solar radiation, and warming. *Mar. Ecol. Prog. Ser.* **2012**, *470*, 167–189. [CrossRef]
72. van Verseveld, W.J.; McDonnell, J.J.; Lajtha, K. The role of hillslope hydrology in controlling nutrient loss. *J. Hydrol.* **2009**, *367*, 177–187. [CrossRef]
73. Jiang, R.; Woli, K.P.; Kuramochi, K.; Hayakawa, A.; Shimizu, M.; Hatano, R. Hydrological process controls on nitrogen export during storm events in an agricultural watershed. *Soil Sci. Plant Nutr.* **2010**, *56*, 72–85. [CrossRef]
74. Serio, F.; Miglietta, P.P.; Lamastra, L.; Ficocelli, S.; Intini, F.; de Leo, F.; de Donno, A. Groundwater nitrate contamination and agricultural land use: A grey water footprint perspective in Southern Apulia Region (Italy). *Sci. Total Environ.* **2018**, *645*, 1425–1431. [CrossRef] [PubMed]
75. Filipovic, V.; Kodešová, R.; Petosic, D. Experimental and mathematical modeling of water regime and nitrate dynamics on zero tension plate lysimeters in soil influenced by high groundwater table. *Nutr. Cycl. Agroecosyst.* **2012**, *95*, 23–42. [CrossRef]
76. Filipović, V.; Romić, D.; Romić, M.; Borošić, J.; Filipović, L.; Mallmann, F.J.K.; Robinson, D.A. Plastic mulch and nitrogen fertigation in growing vegetables modify soil temperature, water and nitrate dynamics: Experimental results and a modeling study. *Agric. Water Manag.* **2016**, *176*, 100–110. [CrossRef]
77. Hardie, M.; Ridges, J.; Swarts, N.; Close, D.C. Drip irrigation wetting patterns and nitrate distribution: Comparison between electrical resistivity (ERI), dye tracer, and 2D soil–water modelling approaches. *Irrig. Sci.* **2018**, *36*, 97–110. [CrossRef]
78. Paredes, I.; Otero, N.; Soler, A.; Green, A.J.; Soto, D.X. Agricultural and urban delivered nitrate pollution input to Mediterranean temporary freshwaters. *Agric. Ecosyst. Environ.* **2020**, *294*, 106859. [CrossRef]
79. Wetzel, R.G. *Limnology: Lake and River Ecosystems*, 3rd ed.; Academic Press: San Diego, CA, USA, 2001; pp. 239–288.
80. Kanda, F.; Yagi, E.; Fukuda, M.; Nakajima, K.; Ohta, T.; Nakata, O. Elucidation of chemical compounds responsible for foot malodour. *Br. J. Dermatol.* **1990**, *122*, 771–776. [CrossRef]
81. Strom, S. Novel interactions between phytoplankton and microzooplankton: Their influence on the coupling between growth and grazing rates in the sea. *Hydrobiologia* **2002**, *480*, 41–54. [CrossRef]
82. Budzyńska, A.; Gołdyn, R.; Zagajewski, P.; Dondajewska, R.; Kowalczewska-Madura, K. The dynamics of a *Planktothrix agardhii* population in a shallow dimictic lake. *Oceanol. Hydrobiol. Stud.* **2009**, *38*, 1–6.
83. Meffert, M.F. Planktic unsheathed filaments (Cyanophyceae) with polar and central gas-vacuoles. II. Biology population dynamics and biotopes of *Limnothrix redekei* (Van Goor) Meffert. *Arch. Hydrobiol.* **1989**, *116*, 257–282.
84. Trifonova, I.S. *Ecology and Succession of Lake Phytoplankton*; Nauka Press: Leningrad, Russia, 1990.
85. Wiedner, C.; Nixdorf, B. Success of chrysophytes, cryptophytes and dinoflagellates over blue green (cyanobacteria) during an extreme winter (1995/96) in eutrophic shallow lakes. *Hydrobiologia* **1998**, *369*, 229–235. [CrossRef]
86. Romanov, R.E. *Limnothrix redeckei* (Van Goor) Meffert (Cyanoprocaryota) in the potamoplankton. *Int. J. Algae* **2007**, *9*, 105–116. [CrossRef]
87. Licursi, M.; Sierra, M.V.; Gómez, N. Diatom assemblages from a turbid coastal plain estuary: Río de la Plata (South America). *J. Mar. Syst.* **2006**, *62*, 35–45. [CrossRef]
88. Meister, F. Die Kieselalgen der Schweiz. In *Beiträge zur Kryptogamenflora der Schweiz*; Wyss, K.J., Ed.; K.J. Wyss: Bern, Switzerland, 1912; Volume 4, p. 254.
89. Bottinelli, M. Fioriture di Cianobatteri della specie *Microcystis wesenbergii* nel Lago di Muzzano. Ph.D. Thesis, Tesi Sperimentale di Laurea in Scienze Naturali, Universita degli Studi di Pavia, Pavia, Italy, 1999; p. 172.
90. Bottinelli, M.; Tonolla, M.; Forlani, G.; Crivelli, C.; Sanangelantoni, A.M.; e Peduzzi, R. Fioriture di Cianobatteri della specie *Microcystis wesenbergii* nel Lago di Muzzano (Svizzera). *Boll. Della Soc. Tic. Di Sci. Nat.* **2000**, *88*, 53–61.
91. Isenburg, C.; Loizeau, J.L.; Tonolla, M.; Peduzzi, R. Aspetti limnologici e microbiologici del Laghetto di Muzzano (TI). *Boll. Soc. Tic. Sci. Nat.* **2000**, *88*, 41–51.
92. Pedrotta, T. Analisi qualitativa degli immissari e dell'emissario del Laghetto di Muzzano. Tesi di Laurea in AGRN. *Front. Ecol. Evolut.* **2009**, *3*, 70.
93. Reynolds, C. *Ecology of Phytoplankton*; Cambridge University Press: Cambridge, UK, 2006.
94. Glibert, P.M.; Wilkerson, F.P.; Dugdale, R.C.; Raven, J.A.; Dupont, C.L.; Leavitt, P.R.; Parker, A.E.; Burkholder, J.M.; Kana, T.M. Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. *Limnol. Oceanogr.* **2016**, *61*, 165–197. [CrossRef]
95. Eppley, R.W.; Coatsworth, J.L.; Solórzano, L. Studies of nitrate reductase in marine phytoplankton. *Limnol. Oceanogr.* **1969**, *14*, 194–205. [CrossRef]

96. Sommer, U. Disturbance-diversity relationships in two lakes of similar nutrient chemistry but contrasting disturbance regimes. *Hydrobiologia* **1993**, *249*, 59–65. [CrossRef]
97. Miyazaki, T.; Honjo, Y.; Ichimura, S. Applicability of the stable isotope method using 13C and 15N simultaneously to the estimation of carbon and nitrogen assimilation in a eutrophic, freshwater lake, Lake Nakanuma, Japan. *Arch. Hydrobiol.* **1985**, *102*, 355–365.
98. Karya, N.; van der Steen, N.; Lens, P. Photo-oxygenation to support nitrification in an algal–bacterial consortium treating artificial wastewater. *Bioresour. Technol.* **2013**, *134*, 244–250. [CrossRef]
99. Hou, X.L.; Ma, D.L.; Yang, K.F.; Wang, R.; Rong, D.L.; Yin, X.T. The physiological responses of one algae species *Scenedesmus quadricauda* to ammonium and alanine. *IOP Conf. Ser. Earth Environ. Sci.* **2019**, *344*, 012167. [CrossRef]
100. Watanabe, T.; Miyazaki, T. Maximum ammonium uptake rates of *Scenedesmus quadricauda* (Chlorophyta) and *Microcystis novacekii* (Cyanobacteria) grown under nitrogen limitation and implications for competition. *J. Phycol.* **1996**, *32*, 243–249. [CrossRef]
101. Trommer, G.; Poxleitner, M.; Stibor, H. Responses of lake phytoplankton communities to changing inorganic nitrogen supply forms. *Aquat. Sci.* **2020**, *82*, 22. [CrossRef]
102. Barone, R.; Naselli-Flores, L. Distribution and seasonal dynamics of cryptomonads in Sicilian water bodies. *Hydrobiologia* **2003**, *502*, 325–329. [CrossRef]
103. Paerl, H.W. Mitigating toxic planktonic cyanobacterial blooms in aquatic ecosystems facing increasing anthropogenic and climatic pressures. *Toxins* **2018**, *10*, 76. [CrossRef]
104. Hutchinson, G.E. The paradox of the plankton. *Am. Nat.* **2002**, *95*, 137–145. [CrossRef]
105. Wilhelm, S.W.; Bullerjahn, G.S.; McKay, R.M.L. The complicated and confusing ecology of *Microcystis* blooms. *mBio* **2020**, *11*, 00529-20. [CrossRef] [PubMed]
106. Belykh, O.I.; Dmitrieva, O.A.; Gladkikh, A.S.; Sorokovikova, E.G. Identification of toxigenic Cyanobacteria of the genus *Microcystis* in the Curonian Lagoon (Baltic Sea). *Oceanology* **2013**, *53*, 71–79. [CrossRef]
107. Fuerst, J.A. Planctomycetes—new models for microbial cells and activities. *Microb. Res.* **2017**, 1–27. [CrossRef]
108. Strous, M.; Kuenen, J.G.; Jetten, M.S.M. Key physiology of anaerobic ammonium oxidation. *Appl. Environ. Microbiol.* **1999**, *65*, 3248–3250. [CrossRef] [PubMed]
109. Jetten, M.S.; Wagner, M.; Fuerst, J.; van Loosdrecht, M.; Kuenen, G.; Strous, M. Microbiology and application of the anaerobic ammonium oxidation ('anammox') process. *Curr. Opin. Biotechnol.* **2001**, *12*, 283–288. [CrossRef]
110. Jetten, M.S.; Sliekers, O.; Kuypers, M.M.M.; Dalsgaard, T.K.; van Niftrik, L.; Cirpus, I.; van de Pas-Schoonen, K.; Lavik, G.; Thamdrup, B.; le Paslier, D.; et al. Anaerobic ammonium oxidation by marine and freshwater planctomycete-like bacteria. *Appl. Microbiol. Biotechnol.* **2003**, *63*, 107–114. [CrossRef]
111. Schmid, M.; Schmitz-Esser, S.; Jetten, M.; Wagner, M. 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: Implications for phylogeny and *in situ* detection . *Environ. Microbiol.* **2001**, *3*, 450–459. [CrossRef]
112. Kuypers, M.M.M.; Sliekers, A.O.; Lavik, G.; Schmid, M.; Jørgensen, B.B.; Kuenen, J.G.; Damsté, J.S.S.; Strous, M.; Jetten, M.S.M. Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nat. Cell Biol.* **2003**, *422*, 608–611. [CrossRef]
113. van de Graaf, A.A.; de Bruijn, P.; Robertson, L.A.; Jetten, M.S.M.; Kuenen, J.G. Metabolic pathway of anaerobic ammonium oxidation on the basis of 15N studies in a fluidized bed reactor. *Microbiology* **1997**, *143*, 2415–2421. [CrossRef]
114. Newton, P.N.; Amin, A.A.; E Bird, C.; Passmore, P.; Dukes, G.; Tomson, G.; Simons, B.; Bate, R.; Guerin, P.J.; White, N.J. The primacy of public health considerations in defining poor quality medicines. *PLoS Med.* **2011**, *8*, e1001139. [CrossRef] [PubMed]
115. Russo, D.A.; Couto, N.; Beckerman, A.P.; Pandhal, J. A metaproteomic analysis of the response of a freshwater microbial community under nutrient enrichment. *Front. Microbiol.* **2016**, *7*, 1172. [CrossRef] [PubMed]
116. Martin-Jezequel, V.; Hildebrand, M.; Brzezinski, M.A. Silicon metabolism in diatoms: Implications for growth. *J. Phycol.* **2000**, *36*, 821–840. [CrossRef]
117. Lundholm, N.; Moestrup, Ø.; Kotaki, Y.; Hoef-Emden, K.; Scholin, C.; Miller, P. Inter- and intraspecific variation of the *Pseudo-nitzschia delicatissima* complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and phylogenetic analyses. *J. Phycol.* **2006**, *42*, 464–481. [CrossRef]
118. Nelson, D.M.; Dortch, Q. Silicic acid depletion in the plume of the Mississippi River and limitation of Si availability to diatoms in the northern Gulf of Mexico: Evidence from kinetic studies in spring and summer. *Mar. Ecol. Prog. Ser.* **1996**, *136*, 163–178. [CrossRef]
119. Gibson, C.E.; Wang, G.; Foy, R.H. Silica and diatom growth in Lough Neagh: The importance of internal recycling. *Freshw. Biol.* **2000**, *45*, 285–293. [CrossRef]
120. Glibert, P.M.; Wilkerson, F.P.; Dugdale, R.C.; Parker, A.E.; Alexander, J.A.; Blaser, S.; Murasko, S. Phytoplankton communities from San Francisco Bay Delta respond differently to oxidized and reduced nitrogen substrates-even under conditions that would otherwise suggest nitrogen sufficiency. *Front. Mar. Sci.* **2014**, *1*, 17. [CrossRef]
121. Dortch, Q. The interaction between ammonium and nitrate uptake in phytoplankton. *Mar. Ecol. Prog. Ser.* **1990**, *61*, 183–201. [CrossRef]
122. Kamjunke, N.; Henrichs, T.; Gaedke, U. Phosphorus gain by bacterivory promotes the mixotrophic flagellate *Dinobryon* spp. during re-oligotrophication. *J. Plankton Res.* **2006**, *29*, 39–46. [CrossRef]
123. Žutinić, P.; Gligora Udovič, M.; Kralj Borojević, K.; Plenković-Moraj, A.; Padisák, J. Morpho-functional classifications of phytoplankton assemblages of two deep karstic lakes. *Hydrobiologia* **2014**, *740*, 147–166. [CrossRef]

124. Eiler, A.; Bertilsson, S. Flavobacteria blooms in four eutrophic lakes: Linking population dynamics of freshwater bacterioplankton to resource availability. *Appl. Environ. Microbiol.* **2007**, *73*, 3511–3518. [CrossRef] [PubMed]
125. Ghai, R.; Mizuno, C.M.; Picazo, A.; Camacho, A.; Rodriguez-Valera, F. Key roles for freshwater Actinobacteria revealed by deep metagenomic sequencing. *Mol. Ecol.* **2014**, *23*, 6073–6090. [CrossRef] [PubMed]
126. Lauro, F.M.; McDougald, D.; Thomas, T.; Williams, T.J.; Egan, S.; Rice, S.; DeMaere, M.Z.; Ting, L.; Ertan, H.; Johnson, J.; et al. The genomic basis of trophic strategy in marine bacteria. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 15527–15533. [CrossRef] [PubMed]
127. Sharma, A.K.; Sommerfeld, K.; Bullerjahn, G.S.; Matteson, A.R.; Wilhelm, S.W.; Jezbera, J.; Brandt, U.; Doolittle, W.F.; Hahn, M.W. Actinorhodopsin genes discovered in diverse freshwater habitats and among cultivated freshwater Actinobacteria. *ISME J.* **2009**, *3*, 726–737. [CrossRef] [PubMed]
128. Takahashi, K.; Moestrup, Ø.; Jordan, R.W.; Iwataki, M. Two new freshwater Woloszynskioids *Asulcocephalium miricentonis* gen. et sp. nov. and *Leiocephalium pseudosanguineum* gen. et sp. nov. (Suessiaceae, Dinophyceae) lacking an apical furrow apparatus. *Protist* **2015**, *166*, 638–658. [CrossRef] [PubMed]
129. Reveal, J.L. *Wislouchiella planctonica* Skvortz. (Chlorophyta, Volvocales), a new algal record for Nevada. *Great Basin Nat.* **1969**, *29*, 3–4.
130. Broady, P.A.; Flint, E.A.; Nelson, W.A.; Cassie Cooper, V.; de Winton, M.D.; Novis, P.M. Phylum Chlorophyta and Charophyta: Green algae. In *New Zealand Inventory of Biodiversity, Kingdoms Bacteria, Protozoa, Chromista, Plantae, Fungi*; Gordon, D.P., Ed.; Canterbury University Press: Christchurch, New Zealand, 2012; Volume 3, pp. 347–381.
131. Felip, M.; Sattler, B.; Psenner, R.; Catalan, J. Highly active microbial communities in the ice and snow cover of high mountain lakes. *Appl. Environ. Microbiol.* **1995**, *61*, 2394. [CrossRef]
132. Callieri, C.; Stockner, J.G. Freshwater autotrophic picoplankton: A review. *J. Limnol.* **2002**, *61*, 1–14. [CrossRef]
133. Fawley, M.W.; Fawley, K.P.; Buchheim, M.A. Molecular diversity among communities of freshwater microchlorophytes. *Microb. Ecol.* **2004**, *48*, 489–499. [CrossRef]
134. Muramoto, K.; Nakada, T.; Shitara, T.; Hara, Y.; Nozaki, H. Re-examination of the snow algal species *Chloromonas miwae* (Fukushima) Muramoto et al., comb. nov. (Volvocales, Chlorophyceae) from Japan, based on molecular phylogeny and cultured material. *Eur. J. Phycol.* **2010**, *45*, 27–37. [CrossRef]
135. Mikhailyuk, T.; Glaser, K.; Tsarenko, P.; Demchenko, E.; Karsten, U. Composition of biological soil crusts from sand dunes of the Baltic Sea coast in the context of an integrative approach to the taxonomy of microalgae and cyanobacteria. *Eur. J. Phycol.* **2019**, *54*, 263–290. [CrossRef]
136. Medinger, R.; Nolte, V.; Pandey, R.V.; Jost, S.; Ottenwälder, B.; Schlötterer, C.; Boenigk, J. Diversity in a hidden world: Potential and limitation of next-generation sequencing for surveys of molecular diversity of eukaryotic microorganisms. *Mol. Ecol.* **2010**, *19*, 32–40. [CrossRef]
137. Mackintosh, C.; Beattie, K.A.; Klumpp, S.; Cohen, P.; Codd, G.A. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* **1990**, *264*, 187–192. [CrossRef]
138. Eland, L.E.; Davenport, R.J.; Mota, C. Evaluation of DNA extraction methods for freshwater eukaryotic microalgae. *Water Res.* **2012**, *46*, 5355–5364. [CrossRef] [PubMed]
139. Rashidan, K.K.; Bird, D.F. Role of predatory bacteria in the termination of a cyanobacterial bloom. *Microb. Ecol.* **2001**, *41*, 97–105. [CrossRef] [PubMed]
140. Ghosh, S.; Love, N.G. Application of rbcL based molecular diversity analysis to algae in wastewater treatment plants. *Bioresour. Technol.* **2011**, *102*, 3619–3622. [CrossRef]
141. van Elsas, J.D.; Boersma, F.G.H. A review of molecular methods to study the microbiota of soil and the mycosphere. *Eur. J. Soil Biol.* **2011**, *47*, 77–87. [CrossRef]

PUBLICATION II

Ecological Indicators 124 (2021) 107430

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

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

Antonija Kulaš [a,*], Vesna Gulin [a], Renata Matoničkin Kepčija [a], Petar Žutinić [a], Mirela Sertić Perić [a], Sandi Orlić [c,d], Katarina Kajan [c,d], Thorsten Stoeck [e], Guillaume Lentendu [e,f], Ivan Čanjevac [b], Ivan Martinić [b], Marija Gligora Udovič [a]

[a] University of Zagreb, Faculty of Science, Department of Biology, Rooseveltov trg 6, HR-10000 Zagreb, Croatia
[b] University of Zagreb, Faculty of Science, Department of Geography, Marulićev trg 19/II, HR-10000 Zagreb, Croatia
[c] Ruđer Bošković Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia
[d] Center of Excellence for Science and Technology-Integrating Mediterranean Region (STIM), HR-10000 Zagreb, Croatia
[e] University of Kaiserslautern, Ecology Group, Erwin-Schrödinger-Straße, D-67663 Kaiserslautern, Germany
[f] University of Neuchâtel, Institute of Biology, rue Emile Argand 11, CH-2000 Neuchâtel, Switzerland

ARTICLE INFO

Keywords:
Ciliates
Morphology
eDNA metabarcoding
Bioindicators
Molecular OTUs
Biofilms

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

* Corresponding author.
E-mail address: antonija.kulas@biol.pmf.hr (A. Kulaš).

https://doi.org/10.1016/j.ecolind.2021.107430
Received 9 October 2020; Received in revised form 31 December 2020; Accepted 16 January 2021

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

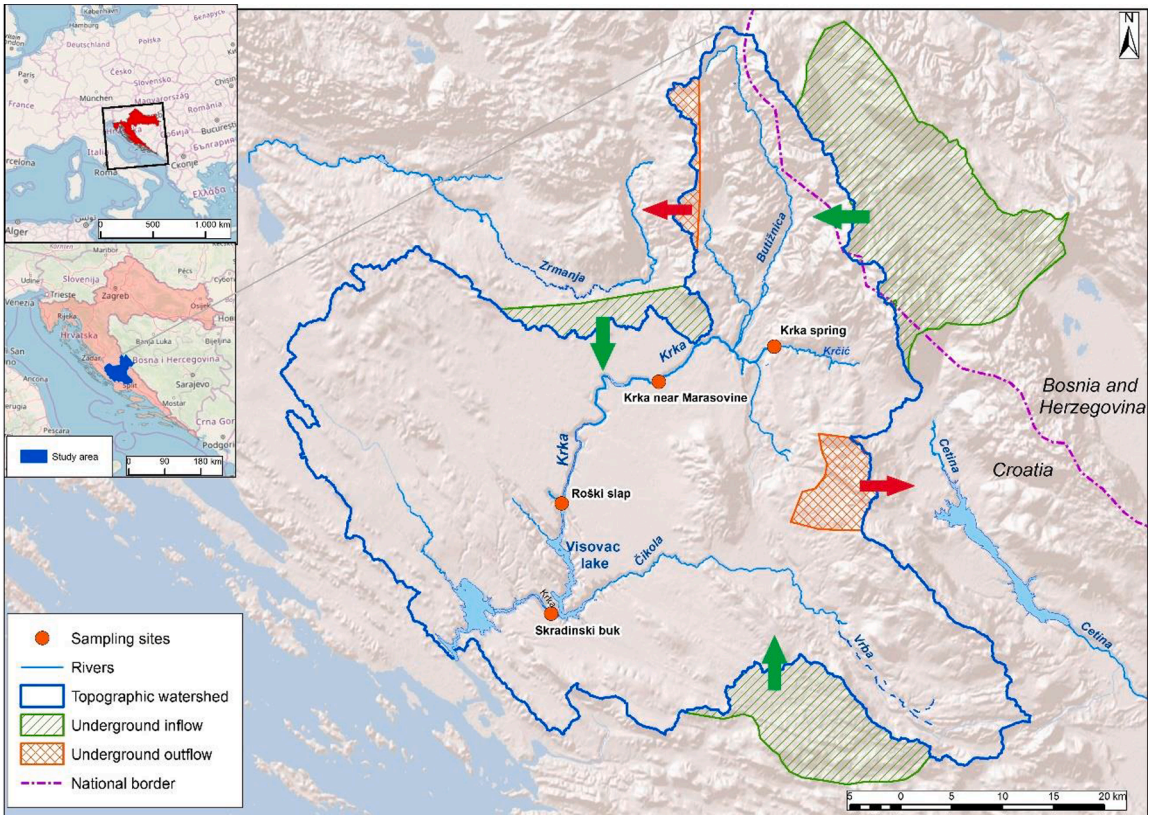


Fig. 1. Map of sampling sites situated at the Krka River, Croatia. (Underground connections according to Bonacci & Ljubenkov, 2005).

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

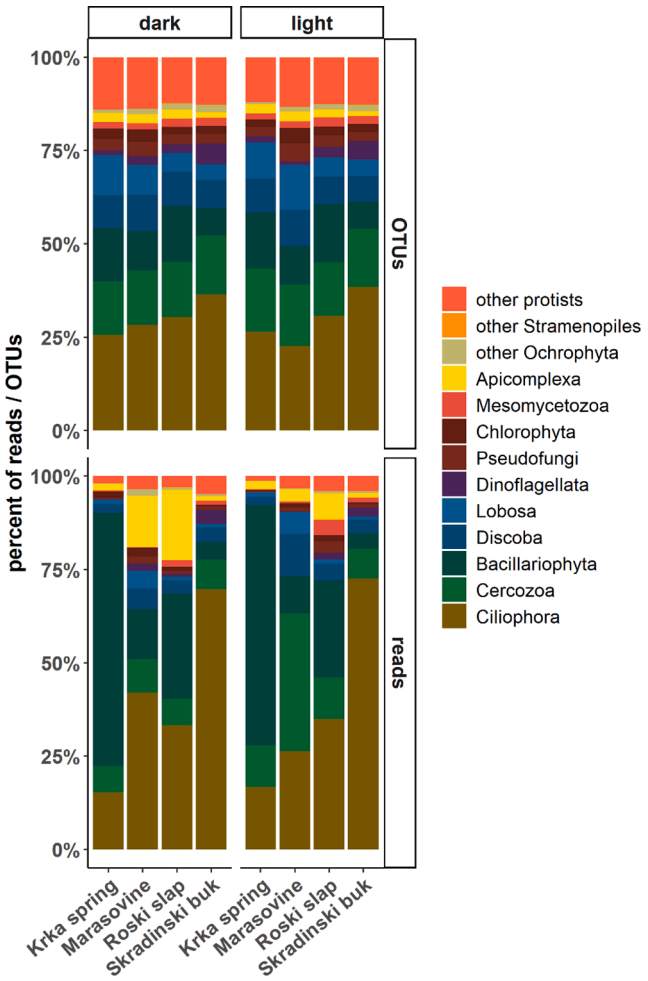


Fig. 2. Taxonomic assignment and relative abundance of protist reads and OTUs according to investigated sampling locations of light- and dark-exposed biofilms covering lithified tufa/stones.

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,

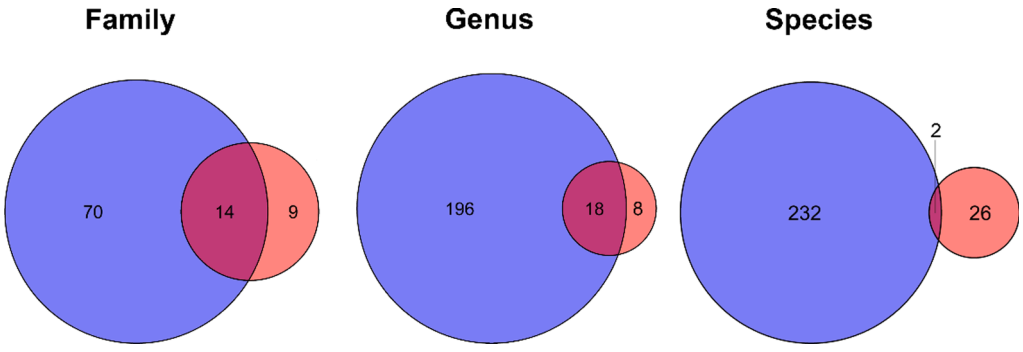


Fig. 3. Venn diagrams comparing the ciliates assigned at family, genus and species rank either by the molecular (blue circles) or by the morphological (red circles) approach. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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

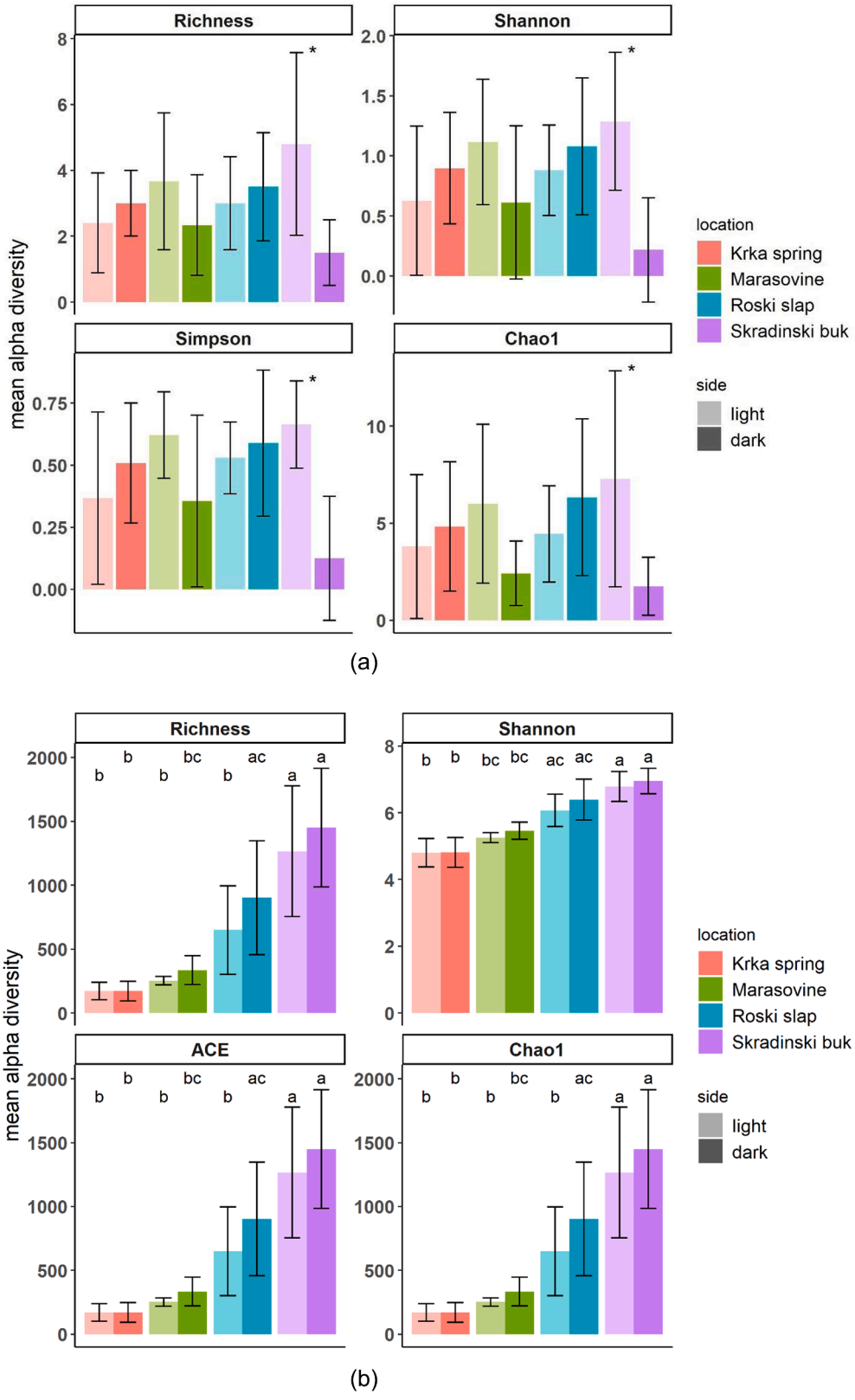


Fig. 4. Variations in alpha diversity for richness, Shannon, ACE, Chao 1 and Simpson index: a) morphological approach (asterisk next to whiskers of light-exposed side of Skradinski buk indicates significant effects exposed sides based on Mann-Whitney test); b) molecular approach (different letters above 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

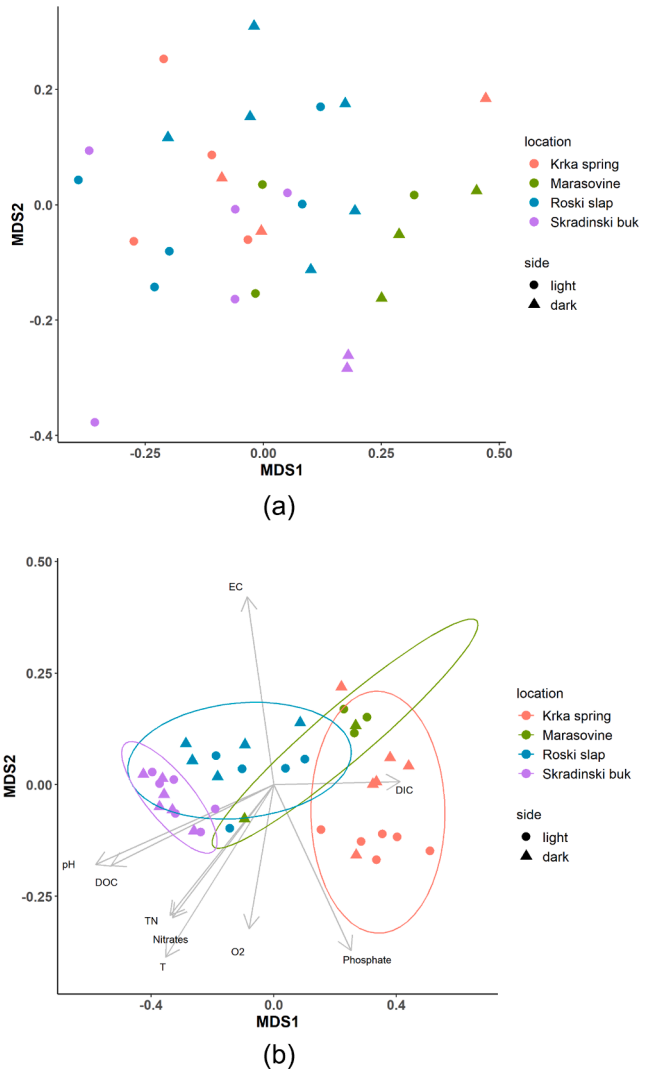


Fig. 5. Position of sampling sites in the multidimensional scaling analysis based on Bray-Curtis similarity index for ciliates: a) for morphological approach; b) for molecular approach with significant environmental parameters (EC = conductivity, pH, T = temperature, TN = total nitrogen, O_2 = oxygen 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).

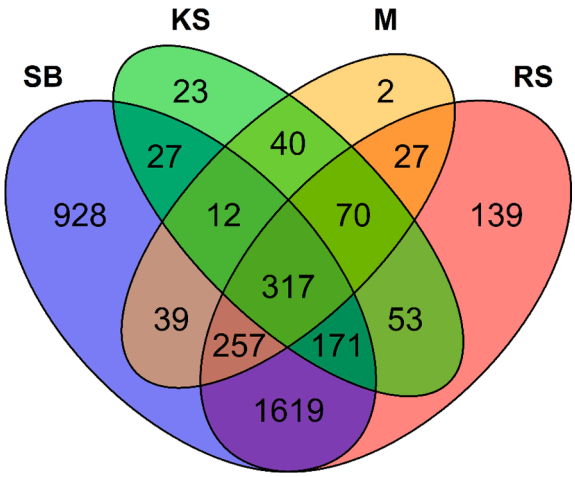


Fig. 6. Venn diagram preforming sharing and unique OTUs per locations (SB = Skradinski buk, KS = Krka spring, M = Marasovine, RS = Roški slap).

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.

Acknowledgements

Mr. sc. Gordana Goreta is thanked for her support with the survey of project at Krka River. Dr. Dominik Forster is thanked for his kind help for advices in statistical analysis.

Funding

This study was financial supported by 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.

Appendix A. Supplementary data

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

References

Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W., Huse, S.M., 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. PLoS One 4 (7), e6372.

Andersen, J.H., Aroviita, J., Carstensen, J., Friberg, N., Johnson, R.K., Kauppila, P., Lindegarth, M., Murray, C., Norling, K., 2016. Approaches for integrated assessment of ecological and eutrophication status of surface waters in Nordic Countries. Ambio 45, 681–691.

Apothéloz-Perret-Gentil, L., Cordonier, A., Straub, F., Iseli, J., Esling, P., Pawlowski, J., 2017. Taxonomy-free molecular diatom index for high-throughput eDNA biomonitoring. Mol. Ecol. Resour. 17, 1231–1242.

Barrios, V., S. Carrizo, W. R. T. Darwall, J. Freyhof, C. Numa, & K. G. Smith, 2014. Freshwater key biodiversity areas in the Mediterranean basin hotspot. https://portals.iucn.org/library/node/44936.

Berger, H., Foissner, W., 2003. Illustrated guide and ecological notes to ciliate indicator species (Protozoa, Ciliophora) in running waters, lakes, and sewage plants Handbuch Angewandte Limnologie: Grundlagen - Gewässerbelastung - Restaurierung - Aquatische Ökotoxikologie - Bewertung - Gewässerschutz. Ecomed Verlag 160 https://onlinelibrary.wiley.com/doi/abs/10.1002/9783527678488.hbal2003005.

Blagojević, S., 1974. The Development Of Periphyton In Open Devices Waterworks On Karstic Springs. PhD Thesis. Faculty of Science, University of Zagreb.

Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D. A., Caporaso, J.G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Meth. Nat. Publ. Group 10, 57–59.

Bonacci, O., 1985. Hydrological investigations of Dinaric karst at the Krčić catchment and the river Krka springs (Yugoslavia). J. Hydrol. 82, 317–326.

Bonacci, O., 2009. Karst Landscape Ecohydrology. Proceedings of Eleventh International Symposium on Water Management and hydraulic Engineering; WMHE2009 781–790.

Bonacci, O., & I. Ljubenkov, 2005. Nove spoznaje o hidrologiji rijeke Krke. Hrvatske vode: časopis za vodno gospodarstvo 13: 265–281.

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.

Bonacci, O., Ljubenkov, I., Roje-Bonacci, T., 2006. Karst flash floods: an example from the Dinaric karst (Croatia). Nat. Hazards Earth Syst. Sci. 6, 195–203.

Bradley, I.M., Pinto, A.J., Guest, J.S., 2016. Design and evaluation of Illumina MiSeqcompatible, 18S rRNA gene-specific primers for improved characterization of mixed phototrophic communities. Appl. Environ. Microbiol. 82, 5878–5891.

Čanjevac, I., Orešić, D., 2020. Surface water resources and their management in Croatia. In: Negm, A.M., Romanescu, G., Zelenakova, M. (Eds.), Water Resources Management in Balkan Countries. Springer International Publishing, Cham, pp. 159–174.

Cantonati, M., Bertuzzi, E., Spitale, D. (Eds.), 2008. The Spring Habitat: Biota and Sampling Methods. Museo Tridentino di Scienze Naturali, Trento, Italy.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. Nat. Publ. Group 6, 1621–1624.

Caroni, R., Irvine, K., 2010. The potential of zooplankton communities for ecological assessment of lakes: Redundant concept or political oversight? Biol. Environ. 110B, 35–53.

Cedrola, F., Rossi, M., Dias, R., Martinele, I., ĎAgosto, M., 2015. Methods for taxonomic studies of rumen ciliates (Alveolata, Ciliophora): A brief review. Zoolog. Sci. 32.

Chasapis, C.T., 2019. Preliminary results from structural systems biology approach in *Tetrahymena thermophila* reveal novel perspectives for this toxicological model. Arch. Microbiol. 201, 51–59.

CMHS – Croatian Meteorological and Hydrological Service, hidro.dhz.hr (February 22nd 2019).

Cukrov, N., D. Barišić, & M. Juračić, 2007. Calculated sedimentation rate in the Krka River estuary using vertical distribution of 137Cs. Rapports et procès-verbaux des réunions - Commission internationale pour l’exploration scientifique de la mer Méditerranée. Commission internationale pour l’exploration scientifique de la mer Méditerranée: 81–81.

Dawson, M.N., 2005. Renaissance taxonomy: integrative evolutionary analyses in the classification of Scyphozoa. J. Marine Biol. Assoc. UK 85, 733–739.

Dayrat, B., 2005. Towards integrative taxonomy. Biol. J. Linn. Soc. 85, 407–415.

Deiner, K., Altermatt, F., 2014. Transport distance of invertebrate environmental DNA in a natural river. PLoS ONEPubl. Lib. Sci. 9, e88786.

Dopheide, A., Lear, G., Stott, R., Lewis, G., 2009. Relative diversity and community structure of ciliates in stream biofilms according to molecular and microscopy methods. Appl. Environ. Microbiol. Am. Soc. Microbiol. 75, 5261–5272.

Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z.-I., Knowler, D.J., Lévêque, C., Naiman, R.J., Prieur-Richard, A.-H., Soto, D., Stiassny, M.L.J., Sullivan, C.A., 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biol. Rev. 81, 163–182.

Dufrene, M., Legendre, P., 1997. Species assemblages and indicator species: the need for a flexible asymmetrical approach. Ecol. Monogr. Ecol. Soc. Am. 67, 345–366.

Dunthorn, M., Klier, J., Bunge, J., Stoeck, T., 2012. Comparing the hyper-variable V4 and V9 regions of the small subunit rDNA for assessment of ciliate environmental diversity. J. Eukary. Microbiol. 59, 185–187.

Esteban, G.F., Fenchel, T., Finlay, B.J., 2010. Mixotrophy in ciliates. Protist 161 (5), 621–641.

Fenchel, T.M., 1987. Ecology of Protozoa: The Biology Of Free-Living Phagotrophic Protists. Springer-Verlag, Berlin Heidelberg https://www.springer.com/gp/book/9783662068199.
Finlay, B.J., Esteban, G.F., 1998. Freshwater protozoa: biodiversity and ecological function. Biodivers. Conserv. 7, 1163–1186.
Foissner, W., 2004. Protozoa as bioindicators in running waters. Regierung von Schwaben & Deutsche Gesellschaft für Limnologie e.V. (Ed.): Biologische Gewässeruntersuchung und Bewertung; Taxonomie und Qualitätssicherung. Symposium zur Feier des 70. Geburtstages von Dr. Erik Mauch am 6. Oktober 2005 in Augsburg: 6–10. 10.
Foissner, W., H. Berger, H. Blatterer, & F. Kohmann, 1995. Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems. Band IV: Gymnostomatea, Loxodes, Suctoria. Bayerisches Landesamt für Wasserwirtschaft, https://www.protozoology.com/ciliateatlas-IV/index.html.
Foissner, W., H. Berger, & F. Kohmann, 1992. Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems. Band II: Peritrichia, Heterotrichida, Odontostomatida by Foissner, Berger and Kohmann -. Bayerisches Landesamt für Wasserwirtschaft, Deggendorf, Germany, https://www.protozoology.com/ciliateatlas-II/index.html.
Foissner, W., H. Berger, & F. Kohmann, 1994. Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems. Band III: Hymenostomata, Prostomatida, Nassulida. Bayerisches Landesamt für Wasserwirtschaft, Deggendorf, Germany, https://www.protozoology.com/ciliateatlas-III/index.html.
Foissner, W., Berger, H., Kohmann, F., 1996. A user-friendly guide to the ciliates (Protozoa, Ciliophora) commonly used by hydrobiologist as bioindicators in rivers, lakes, and waste waters, with notes on their ecology. Freshw. Biol. 35, 375–482.
Foissner, W., Berger, H., Schaumburg, J., 1999. Identification and Ecology Of Limnetic Plankton Ciliates. Bayerisches Landesamt für Wasserwirtschaft, Munich, Germany https://www.researchgate.net/publication/277007192_Identification_and_Ecology_of_Limnetic_Plankton_Ciliates.
Foissner, W., H. Blatterer, H. Berger, & F. Kohmann, 1991. Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems. Band I: Cyrtophorida, Oligotrichida, Hypotrichia, Colpodea. Bayerisches Landesamt für Wasserwirtschaft, Deggendorf, Germany, https://www.protozoology.com/ciliateatlas-I/index.html.
Foissner, W., Müller, H., Agatha, S., 2007. A comparative fine structural and phylogenetic analysis of resting cysts in oligotrich and hypotrich Spirotrichea (Ciliophora). Eur. J Protistol. 43, 295–314.
Ford, T.D., Pedley, H.M., 1996. A review of tufa and travertine deposits of the world. Earth Sci. Rev. 41, 117–175.
Forster, D., Filker, S., Kochems, R., Breiner, H.-W., Cordier, T., Pawlowski, J., Stoeck, T., 2019. A comparison of different ciliate metabarcode genes as bioindicators for environmental impact assessments of salmon aquaculture. J. Eukaryot. Microbiol. 66, 294–308.
Forster, D., Dunthorn, M., Stoeck, T., Mahé, F., 2016. Comparison of three clustering approaches for detecting novel environmental microbial diversity. PeerJ 4, e1692.
Friganović, M., 1990. Geografske značajke i vrednote Krke Ekološke monografije, knjiga 2. Hrvatsko ekološko društvo 1–14.
Gilbert, J.A., Meyer, F., Antonopoulos, D., Balaji, P., Brown, C.T., Brown, C.T., Desai, N., Eisen, J.A., Evers, D., Field, D., Feng, W., Huson, D., Jansson, J., Knight, R., Knight, J., Kolker, E., Konstantindis, K., Kostka, J., Kyrpides, N., Mackelprang, R., McHardy, A., Quince, C., Raes, J., Sczyrba, A., Shade, A., Stevens, R., 2010. Meeting report: The terabase metagenomics workshop and the vision of an earth microbiome project. Stand. Genom. Sci. Michigan State Univ. 3, 243.
Gloor, G.B., Macklaim, J.M., Pawlowsky-Glahn, V., Egozcue, J.J., 2017. Microbiome datasets are compositional: and this is not optional. Front. Mycrobiol. 8, 2224.
Gómez-Gutiérrez, J., Kawaguchi, S., Morales-Ávila, J.R., 2017. Global Diversity And Ecological Function Of Parasites of Euphausiids. Springer International Publishing.
Gong, J., Dong, J., Liu, X., Massana, R., 2013. Extremely high copy numbers and polymorphisms of the rDNA operon estimated from single cell analysis of oligotrich and peritrich ciliates. Protist 164, 369–379.
Grattepanche, J.D., Juarez, D.L., Wood, C.C., McManus, G.B., Katz, L.A., 2019. Incubation and grazing effects on spirotrich ciliate diversity inferred from molecular analyses of microcosm experiments. PLoS One 14 (5), e0215872.
Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, C., Bittner, L., Boutte, C., Burgaud, G., de Vargas, C., Decelle, J., del Campo, J., Dolan, J.R., Dunthorn, M., Edvardsen, B., Holzmann, M., Kooistra, W.H.C.F., Lara, E., Le Bescot, N., Logares, R., Mahé, F., Massana, R., Montresor, M., Morard, R., Not, F., Pawlowski, J., Probert, I., Sauvadet, A.-L., Siano, R., Stoeck, T., Vaulot, D., Zimmermann, P., Christen, R., 2013. The protist ribosomal reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. Nucl. Acids Res. Oxford Acad. 41, D597–D604.
Gulin, V., Matoničkin Kepčija, R., 2012. Periphyton in Different Microhabitats On Tufa barrier in National Park Plitvice Lakes (Croatia). Abstract Book, 4th Congress of the Ecologists of Macedonia with International Participation. Macedonian Ecological Society, Skopje, Macedonia.
Hadziavdic, K., Lekang, K., Lanzen, A., Jonassen, I., Thompson, E.M., Troedsson, C., 2014. Characterization of the 18S rRNA gene for designing universal eukaryote specific primers. Plos One Pub. Lib. Sci. 9, e87624.
Hering, D., Borja, A., Jones, J.I., Pont, D., Boets, P., Bouchez, A., Bruce, K., Drakare, S., Hänfling, B., Kahlert, M., Leese, F., Meissner, K., Mergen, P., Reyjol, Y., Segurado, P., Vogler, A., Kelly, M., 2018. Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. Water Res. 138, 192–205.
Hillebrand, H., 2002. Top-down versus bottom-up control of autotrophic biomass—a meta-analysis on experiments with periphyton. J. North Am. Benthol. Soc. 21 (3), 349–369.
Hughes, J., 2018. Freshwater Ecology And Conservation: Approaches And Techniques. Freshwater Ecology and Conservation. Oxford University Press https://www.oxfordscholarship.com/view/10.1093/oso/9780198766384.001.0001/oso-9780198766384.
Hunting, E.R., de Jong, S., Vijver, M.G., 2017. Assessment of monitoring tools and strategies safeguarding aquatic ecosystems within the European water framework directive. Inst. Environ. Sci. (CML).
Huse, S.M., Welch, D.M., Morrison, H.G., Sogin, M.L., 2010. Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environ. Microbiol. 12 (7), 1889–1898.
Jane, S.F., Wilcox, T.M., McKelvey, K.S., Young, M.K., Schwartz, M.K., Lowe, W.H., Letcher, B.H., Whiteley, A.R., 2015. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Mol. Ecol. Resour. 15, 216–227.
Jones, H., 1997. A classification of mixotrophic protists based on their behaviour. Freshw. Biol. 37, 35–43.
Kahl, A. 1930−1935. Urtiere oder Protozoa I: Wimpertiere oder Ciliata (Infusoria). In: Dahl, F. (ed) Die Tierwelt Deutschlands. G. Fisher, J. 1−886.
Kock, C., Meyer, A., Spänhoff, B., Meyer, E., 2006. Tufa deposition in karst streams can enhance the food supply of the grazing caddisfly *Melampophylax mucoreus* (Limnephilidae). Int. Rev. Hydrobiol. 91, 242–249.
Kolkwitz, R., Marsson, M., 1909. Ökologie der tierischen Saprobien. Internationale. Revue der Hydrobiologie 2, 126–519.
Lynn, D., 2008. The Ciliated Protozoa: Characterization, Classification And Guide To The Literature. Springer, Netherlands https://www.springer.com/gp/book/9781402082382.
Mahé, F., Rognes, T., Quince, C., de Vargas, C., Dunthorn, M., 2015. Swarm v2: highly-scalable and high-resolution amplicon clustering. PeerJ PeerJ Inc. 3, e1420.
Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 17, 10–12.
Masella, A.P., Bartram, A.K., Truszkowski, J.M., Brown, D.G., Neufeld, J.D., 2012. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinf. 13, 31.
Matoničkin Kepčija, R., Miliša, M., Sertić Perić, M., Matijić Cvjetović, M., Primc-Habdija, B., 2011. Response of periphyton to nutrient addition in tufa-depositing environment. Aquat. Microb. Ecol. 65, 183–195.
Maurya, R., Pandey, A.K., 2020. Importance of protozoa tetrahymena in toxicological studies: A review. Sci. Total Environ. 741, 140058.
McManus, G.B., Katz, L.A., 2009. Molecular and morphological methods for identifying plankton: what makes a successful marriage? J. Plankton Res. 31, 1119–1129.
Medinger, R., Nolte, V., Pandey, R.V., 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. Mol. Ecol. 19, 32–40.
Minerovic, A.D., Potapova, M.G., Sales, C.M., Price, J.R., Enache, M.D., 2020. 18S–V9 DNA metabarcoding detects the effect of water-quality impairment on stream biofilm eukaryotic assemblages. Ecol. Ind. 113, 106225.
Nelson, M.C., Morrison, H.G., Benjamino, J., Grim, S.L., Graf, J., 2014. Analysis, optimization and verification of Illumina-generated 16S rRNA gene amplicon surveys. Plos One Publ. Lib. Sci. 9, e94249.
Nolte, V., Vinay Pandey, R.A.M., Jost, S., Medinger, R., Ottenwälder, B., Boenigk, J., Schlötterer, C., 2010. Contrasting seasonal niche separation between rare and abundant taxa conceals the extent of protist diversity. Mol. Ecol. 19 (14), 2908–2915.
Oksanen, J., F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, E. Szoecs, & H. Wagner, 2019. vegan: Community Ecology Package, https://CRAN.R-project.org/package=vegan.
Ormerod, S.J., Dobson, M., Hildrew, A.G., Townsend, C.R., 2010. Multiple stressors in freshwater ecosystems. Freshw. Biol. 55, 1–4.
Panov, A.G., 2019. Species Composition And Dynamics Of Ciliophora In Small Water Bodies In Downstream Basin of Taranai river (South of Sakhalin Island, Russian Far East). The Bulletin of Irkutsk State University Series Biology. Ecology, pp. 24–39.
Pawlowski, J., Kelly-Quinn, M., Altermatt, F., Apothéloz-Perret-Gentil, L., Beja, P., Boggero, A., Borja, A., Bouchez, A., Cordier, T., Domaizon, I., Feio, M.J., Filipe, A.F., Fornaroli, R., Graf, W., Herder, J., van der Hoorn, B., Iwan Jones, J., Sagova-Mareckova, M., Moritz, C., Barquín, J., Piggott, J.J., Pinna, M., Rimet, F., Rinkevich, B., Sousa-Santos, C., Specchia, V., Trobajo, R., Vasselon, V., Vitecek, S., Zimmerman, J., Weigand, A., Leese, F., Kahlert, M., 2018. The future of biotic indices in the ecogenomic era: Integrating (e)DNA metabarcoding in biological assessment of aquatic ecosystems. Sci. Total Environ. 637–638, 1295–1310.
Pawlowski, J., Lejzerowicz, F., Apotheloz-Perret-Gentil, L., Visco, J., Esling, P., 2016. Protist metabarcoding and environmental biomonitoring: Time for change. Eur. J. Protistol. 55, 12–25.
Pedroso Dias, R.J., Mendes de Souza, P., Fonseca Rossi, M., Hannemann Wieloch, A., Domingos da Silva-Neto, I., D'Agosto, M., 2020. Ciliates as bioindicators of water quality: A case study in the neotropical region and evidence of phylogenetic signals (18S-RDNA). Environ. Pollut. 115760.
Perica, D., Orešić, D., Trajbar, S., 2005. Geomorfološka obilježja doline i poriječja rijeke Krke s osvrtom na dio od Knina do Bilušića buka. Geoadria 10, 131–156.
Pitois, F., Jigorel, A., Bertru, G., 2003. Development of cyanobacterial build-up and evolution of river bed morphology in the chalk stream eaulne (Upper-Normandy, France). Biodivers. Conserv. 12 (4), 621–636.
Pitois, F., Jigorel, A., Bertru, G., 2001. Colonization dynamics of an encrusting cyanobacterial mat in a hardwater river (Eaulne, France). Geomicrobiol J. 18, 139–155.
Pitsch, G., Bruni, E.P., Forster, D., Qu, Z., Sonntag, B., Stoeck, T., Posch, T., 2019. Seasonality of planktonic freshwater ciliates: are analyses based on V9 regions of the

18S rRNA gene correlated with morphospecies counts? Front. Microbiol. Front. 10 https://www.frontiersin.org/articles/10.3389/fmicb.2019.00248/full.
Primc-Habdija, B., Habdija, I., Matoničkin, R., Špoljar, M., 2005. Development of ciliate community on artificial substrates associated with vertical gradients of environmental conditions in a karstic lake. Archiv für Hydrobiologie 164, 513–527.
Primc-Habdija, B., Matoničkin, R., 2005. A new freshwater folliculinid (Ciliophora) from the karstic region of Croatia. Eur. J. Protistol. 41, 37–43.
R Core Team, 2020. R: A Language And Environment For Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Reiss, J., Schmid-Araya, J.M., 2008. Existing in plenty: abundance, biomass and diversity of ciliates and meiofauna in small streams. Freshw. Biol. 53, 652–668.
Risse-Buhl, U., Küsel, K., 2009. Colonization dynamics of biofilm-associated ciliate morphotypes at different flow velocities. Eur. J. Protistol. 45, 64–76.
Roberts, D. W., 2019. labdsv: ordination and multivariate analysis for ecology, https://CRAN.R-project.org/package=labdsv.
Rubinić, J., Goreta, G., Giljušić, M., Marguš, D., Bošnjak, T., 2013. Characteristics of the Krka River based on assessment of data obtained from regular water monitoring. In: 3rd International Conference; Waters in Sensitive & Protected Areas, pp. 183–187.
Saravia, L.A., Momo, F., Boffi Lissin, L.D., 1998. Modelling periphyton dynamics in running water. Ecol. Model. 114, 35–47.
Sato, Y., T. Muto, Y. Endo, T. Kobayashi, N. Nakano, H. Sato, G. Nishitani, & W. Sato-Okoshi, 2015. Morphological, developmental, and ecological characteristics of the suctorian ciliate Ephelota gigantea (Ciliophora, Phyllopharyngea, Ephelotidae) found on cultured wakame seaweed in northeastern Japan. Acta Protozoologica Portal Czasopism Naukowych Ejournals.eu 2015: 295–303.
Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541.
Sládeček, V., 1973. System of water quality from the biological point of view. Archiv für Hydrobiologie 7, 1–218.
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.
Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M.D.M., Breiner, H.-W., Richards, T. A., 2010. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Mol. Ecol. 19, 21–31.
Stoeck, T., Breiner, H.-W., Filker, S., Ostermaier, V., Kammerlander, B., Sonntag, B., 2014. A morphogenetic survey on ciliate plankton from a mountain lake pinpoints the necessity of lineage-specific barcode markers in microbial ecology. Environ. Microbiol. 16, 430–444.
Stoeck, T., Kochems, R., Forster, D., Lejzerowicz, F., Pawlowski, J., 2018. Metabarcoding of benthic ciliate communities shows high potential for environmental monitoring in salmon aquaculture. Ecol. Ind. 85, 153–164.
Strmečki, S., Ciglenečki, I., Gligora Udovič, M., Marguš, M., Bura-Nakić, E., Dautović, J., Plavšić, M., 2018. Voltammetric study of organic matter components in the upper reach of the Krka River, Croatia. Croatica Chemica Acta Hrvatsko kemijsko društvo 91, 447–454.
Šemnički, P., Previšić, A., Ivković, M., Čmrlec, K., Mihaljević, Z., 2012. Tufa barriers from a caddisfly's point of view: Streams or lake outlets? Int. Rev. Hydrobiol. 97, 465–484.
Špoljar, M., Primc-Habdija, B., Habdija, $ I., 2007. Transport of seston in the karstic hydrosystem of the Plitvice Lakes (Croatia). Hydrobiologia 579, 199–209.
Tanabe, A.S., 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.
Thompson, L.R., Sanders, J.G., McDonald, D., Amir, A., Ladau, J., Locey, K.J., Prill, R.J., Tripathi, A., Gibbons, S.M., Ackermann, G., Navas-Molina, J.A., Janssen, S., Kopylova, E., Vázquez-Baeza, Y., González, A., Morton, J.T., Mirarab, S., Zech Xu, Z., Jiang, L., Haroon, M.F., Kanbar, J., Zhu, Q., Jin Song, S., Kosciolek, T., Bokulich, N. A., Lefler, J., Brislawn, C.J., Humphrey, G., Owens, S.M., Hampton-Marcell, J., Berg-Lyons, D., McKenzie, V., Fierer, N., Fuhrman, J.A., Clauset, A., Stevens, R.L., Shade, A., Pollard, K.S., Goodwin, K.D., Jansson, J.K., Gilbert, J.A., Knight, R., 2017. A communal catalogue reveals Earth's multiscale microbial diversity. Nat. Nat. Publ. Group 551, 457–463.
Thorp, J.H., Covich, A.P., 2010. Ecology and Classification of North American Freshwater Invertebrates. Ecology and Classification of North American Freshwater Invertebrates. Academic Press (Elsevier), Burlington, MA.
Vermaat, J.E. 2005. Periphyton Dynamics and Influencing factor. Periphyon: Ecology, Exploitation and Management (eds M.E. Azim et al) 35-45.
Vörösmarty, C.J., McIntyre, P.B., Gessner, M.O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S.E., Sullivan, C.A., Liermann, C.R., Davies, P.M., 2010. Global threats to human water security and river biodiversity. Nature 467, 555–561.
Wake, M.H., 1995. An integrative approach to the biology of biodiversity. Integr. Comp. Biol. 31, 1–6.
Wang, Y., Jiang, Y., Liu, Y., Li, Y., Katz, L.A., Gao, F., Yan, Y., 2020. Comparative studies on the polymorphism and copy number variation of mtSSU rDNA in ciliates (Protista, Ciliophora): implications for phylogenetic, environmental, and ecological research. Microorganisms 8, 316.
Warren, A., Patterson, D.J., Dunthorn, M., Clamp, J.C., Achilles-Day, U.E.M., Aescht, E., Al-Farraj, S.A., Al-Quraishy, S., Al-Rasheid, K., Carr, M., Day, J.G., Dellinger, M., El-Serehy, H.A., Fan, Y., Gao, F., Gao, S., Gong, J., Gupta, R., Hu, X., Kamra, K., Langlois, G., Lin, X., Lipscomb, D., Lobban, C.S., Luporini, P., Lynn, D.H., Ma, H., Macek, M., Mackenzie-Dodds, J., Makhija, S., Mansergh, R.I., Martín-Cereceda, M., McMiller, N., Montagnes, D.J.S., Nikolaeva, S., Ong'ondo, G.O., Pérez-Uz, B., Purushothaman, J., Quintela-Alonso, P., Rotterová, J., Santoferrara, L., Shao, C., Shen, Z., Shi, X., Song, W., Stoeck, T., La Terza, A., Vallesi, A., Wang, M., Weisse, T., Wiackowski, K., Wu, L., Xu, K., Yi, Z., Zufall, R., Agatha, $.S., 2017. Beyond the 'Code': A guide to the description and documentation of biodiversity in ciliated protists (Alveolata, Ciliophora). J. Eukaryot. Microbiol. 64 (4), 539–554.
Weber, A., Pawlowski, J., 2013. Can abundance of protists be inferred from sequence data: A case study of foraminifera. PLoS One 8, e56739.
Wei, M., Yuhe, Y., Yunfen, S., Xiyuan, Z., 2004. Intraspecific phylogeography of *Carchesium polypinum* (Peritrichia, Ciliophora) from China, inferred from 18S-ITS1-5.8S ribosomal DNA. Sci. China Series C, Life Sci. 47, 11–17.
Ye, Q., Feng, Y., Wang, Z., Jiang, W., Qu, Y., Zhang, C., Zhou, A., Xie, S., Zou, J., 2018. Effects of gelsemine on oxidative stress and DNA damage responses of tetrahymena thermophila. PeerJ 12, 1–17.
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 Journal of. Ecol. Environ. 22 (1), 29–44.

PUBLICATION III

Science of the Total Environment 829 (2022) 154536

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Diatom eDNA metabarcoding and morphological methods for bioassessment of karstic river

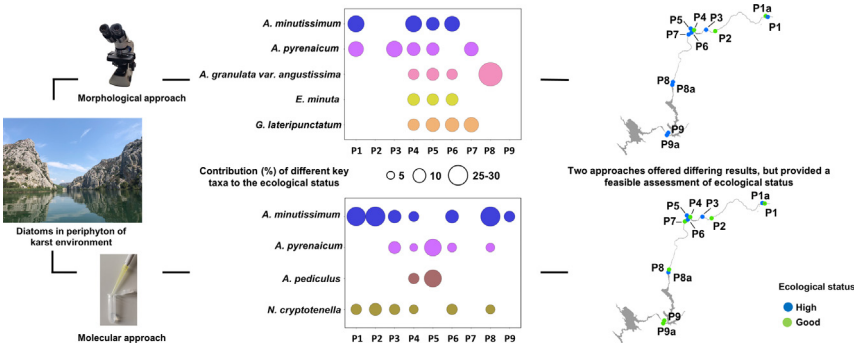
Antonija Kulaš [a,*], Marija Gligora Udovič [a], Kálmán Tapolczai [b,c,d], Petar Žutinić [a], Sandi Orlić [e,f], Zlatko Levkov [g]

[a] University of Zagreb, Faculty of Science, Department of Biology, Rooseveltov trg 6, HR-10000 Zagreb, Croatia
[b] Premium Postdoctoral Research Program, Hungarian Academy of Sciences, Budapest, Hungary
[c] Research Group of Limnology, Center for Natural Science, University of Pannonia, Egyetem u. 10, H-8200 Veszprém, Hungary
[d] Balaton Limnological Research Institute, Eötvös Loránd Research Network (ELKH), Klebelsberg Kuno u. 3, Tihany, Hungary
[e] Ruđer Bošković Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia
[f] Center of Excellence for Science and Technology Integrating Mediterranean Region (STIM), HR-10000 Zagreb, Croatia
[g] Institute of Biology, Faculty of Natural Science, Ss Cyril and Methodius University, Skopje, Macedonia

HIGHLIGHTS

- Karst ecosystems play a unique role as exceptional natural habitats.
- Periphytic diatom diversity was described by microscopy and eDNA metabarcoding.
- Considerable differences in results were presented by both approaches.
- Both approaches provided a feasible assessment of ecological status.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 December 2021
Received in revised form 20 February 2022
Accepted 8 March 2022
Available online 15 March 2022

Editor: Wei Ouyang

Keywords:
Diatom community
Periphyton
Karst river
Morphology
eDNA metabarcoding
Ecological status

ABSTRACT

Karst ecosystems play a unique role as exceptional natural habitats in sustaining biodiversity. This study focuses on diatoms, a diverse group of microeukaryotes in the periphytic community of a karstic river. In a multi-microhabitat study along the Krka River (Croatia), our goal was to obtain a detailed overview of diatom diversity and community structure using morphological and molecular approaches, and to assess the applicability of eDNA metabarcoding as a reliable tool for biomonitoring assessment. The results revealed a relatively low agreement in the diatom community composition between the two approaches, but also provided complementary information, with no differences in beta diversity detected between microhabitats. The SIMPER analysis underlined the importance of the molecular approach in identifying diatom community composition, due to errors in distinguishing between deposited diatom cells that occurred in the morphological analysis. In contrast, the morphological approach indicated a clear diatom community separation along the river with a strong location effect. Despite certain differences, both approaches provided a feasible assessment of the ecological status according to the relationship to environmental pressures, classifying the Krka River as High (morphological approach) or Good (molecular approach) throughout the most of its course. Moreover, diatom diversity based on both approaches provides a reliable dataset applicable in routine monitoring assessment and offers a deeper understanding of the presented ecological status. The incompleteness of a reference database presents one major drawback of the molecular approach, which needs further updating in order to improve routine diatom metabarcoding.

* Corresponding author.
E-mail address: antonija.kulas@biol.pmf.hr (A. Kulaš).

http://dx.doi.org/10.1016/j.scitotenv.2022.154536

1. Introduction

Karst areas account for 7–12% of the Earth's continental area, and about a quarter of the world's population relies either partly or entirely on drinking water supplies from karst aquifers (Hartmann et al., 2014). Karst takes up around 46% of the Croatian territory, mainly southern Croatia as part of the Dinaric karst belt (Matočec et al., 2002). Rivers are particularly vulnerable and fragile systems in Croatian karst (Šiljeg et al., 2020), generally heavily affected by anthropogenic activities and associated climate change (Lionello, 2012). The karst area has also been highlighted by having unique biodiversity hotspots (Smith et al., 2014). The geographic and hydrologic heterogeneity of these habitats has resulted in an ecosystem that hosts a very diverse biota, including a wide variety of protists, some of which contribute to the very important calcite precipitates in karst rivers (Ford and Pedley, 1996; Primc-Habdija and Matoničkin, 2005).

In these systems and processes, the most distinctive organisms are associated with benthic/periphytic communities (Poulíčková et al., 2008). Among the most abundant periphytic photoautotrophic algae are diatoms, whose representatives are used worldwide to assess the ecological status of rivers (Kahlert et al., 2016). The rapid and specific response of diatoms to environmental changes, their wide diversity and ubiquitous distribution, and known ecological preferences of many taxa have enabled the use of benthic diatoms as biological indicators in biomonitoring programmes required by Water Framework Directive (WFD; Directive 2000/60/EC, 2000). The use of diatoms as a biological water quality element requires highly specialised and expert morphological identification to species level, well researched areas and known operational taxa lists (Mann et al., 2016). This brings scientific research to a level where a new method and a new perspective can be applied not only to elucidate diatom diversity, but also to its more effective use in biomonitoring.

The recent development of novel molecular methods in ecological studies, such as the environmental DNA (eDNA) metabarcoding, provides an efficient way to study complex communities based on the genetic material extracted directly from environmental samples (Vasselon et al., 2019). Even though eDNA metabarcoding still requires standardization, specifically in bioinformatic pipelines (Bailet et al., 2020), this method has emerged as alternative to classical taxonomy and monitoring because it is fast, inexpensive, and requires less human effort (Kermarrec et al., 2013; Zimmermann et al., 2015). An increasing number of studies have collected taxonomic and genetic information, from which diatom quality indices are calculated to assess the ecological status of rivers in national biomonitoring networks (Bailet et al., 2019; Kelly et al., 2008; Mortágua et al., 2019; Pérez-Burillo et al., 2020; Pissaridou et al., 2021; Rivera et al., 2020; Vasselon et al., 2017a). The eDNA metabarcoding offers key advantages for large-scale surveys, such as an easier control and comparability of results, which emanate from comparable sequencing data and parallel processing of high numbers of samples (Pawlowski et al., 2018). However, implementing this method as a standardized biomonitoring tool has its own challenges, as protocols and methods still vary between laboratories and require standardization to define good practices and methodological uniformity (Leese et al., 2016; Rivera et al., 2020). This challenge includes statements indicating that both species composition and relative abundance data obtained by eDNA metabarcoding can be limited by the incompleteness of the reference library (Bailet et al., 2019; Rivera et al., 2018; Vasselon et al., 2017a), the DNA extraction method (Pawlowski et al., 2018; Vasselon et al., 2017b), the DNA barcode used (Elbrecht and Leese, 2015; Keck et al., 2018; Kermarrec et al., 2013), the bioinformatics treatment (Bailet et al., 2019; Rivera et al., 2020; Tapolczai et al., 2019, 2021) or the gene copy number per cell (Pérez-Burillo et al., 2020; Rimet et al., 2018; Vasselon et al., 2018).

To improve and test the potential of diatom eDNA metabarcoding for assessing ecological status, the aforementioned biases need to be addressed, particularly their impact on the final index scores prior to reliable utilization of molecular methods in routine biomonitoring.

Research on heterogeneous habitats with high diversity and specificity may provide new insights that will complement the process of standard setting and good comparable practice (Xie et al., 2021). Therefore, the first objective of the present study on the karst Krka River (Croatia) was to attain a detailed overview of the structure and diversity of the diatom community inhabiting different microhabitats, as well to elucidate the differences along the river course through a combination of molecular and morphological approaches. The second objective was to analyse the applicability of eDNA metabarcoding as a reliable tool for biomonitoring of the karst river by comparing index values obtained from morphological and molecular approaches.

2. Materials and methods

2.1. Study area

The Krka River is a 73 km long karst river situated in the Dinaric region of Dalmatia, Croatia. It is characterized by tufa barriers, where "tufa" designates porous $CaCO_3$ deposits forming under specific physical and chemical conditions which host very diverse biota (Primc-Habdija and Matoničkin, 2005). Krka River is part of the Dinaric Western Balkan ecoregion (ER5; sensu Illies, 1978), pertaining to national type HR-R_12: medium and large upland rivers (Official gazette, 2019). According to the intercalibration river typology (Schöll et al., 2012) it belongs into the IC type R-M2: Mediterranean rivers with catchment between 100 and 1000 km^2, mixed geology (except non-siliceous) and high seasonality. 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). After the spring zone, Krka flows through the Knin karst polje, a series of valleys and canyon formations until reaching the Adriatic Sea near the city of Šibenik (Perica et al., 2005). Along the course of the Krka River there are 7 larger tufa barriers shaping waterfalls in the downstream direction. Some of them form lacustrine sections in the river and all of them influence dynamics of the river by creating parts with alternating lotic and lentic microhabitats. Some parts of the Krka River have been protected since 1948 for their special geomorphological, hydrological and landscape values. In 1985, the Krka River and its catchment area were granted status of the National Park (Official gazette, 1985).

The nine sampling locations (Krka spring, Krka near Marasovine, Bilušića buk, Brljan, Manojlovića buk, Rošnjak, Miljacka, Roški slap and Skradinski buk) were chosen to represent sections of the river in the downstream direction (Fig. 1). The main idea of chosen representative locations was to cover all locations with tufa barriers from spring until the last section of the river before estuary. Due to heterogeneity the locations 1, 8 and 9 were sampled on two representative habitats (1, 1a, 8, 8a, 9, 9a). The list of sampling locations with microhabitats was adapted from Žutinić et al. (2020a).

2.2. Sampling procedure

Sampling was performed in triplicates, during the low water period for two consecutive years: from 21st to 23rd September 2017 and from 10th to 11th September 2018 along the Krka River course. Individual subsamples at each sampling location were 10 m apart, where each successive habitat was selected in the upstream direction of the previously sampled location. The exception (transverse sampling) was made at those locations where longitudinal sampling was not possible due to waterfalls. Periphytic diatom samples were scrubbed with new toothbrushes from at least five randomly collected tufa or stone substrates on each microhabitat and rinsed with water from the river. Each initial sample was divided into two aliquots (subsamples), where one was stored to be used in the morphological approach, while the other aliquot was stored for the molecular approach. Samples for the morphological approach were placed into 50 mL plastic vials and preserved in a 4% final concentration formaldehyde solution (samples from 2017) or in a 70% final concentration ethanol (samples from 2018). Samples for the molecular approach were placed into Falcon tubes (50 mL) without addition of preservatives, kept on ice during

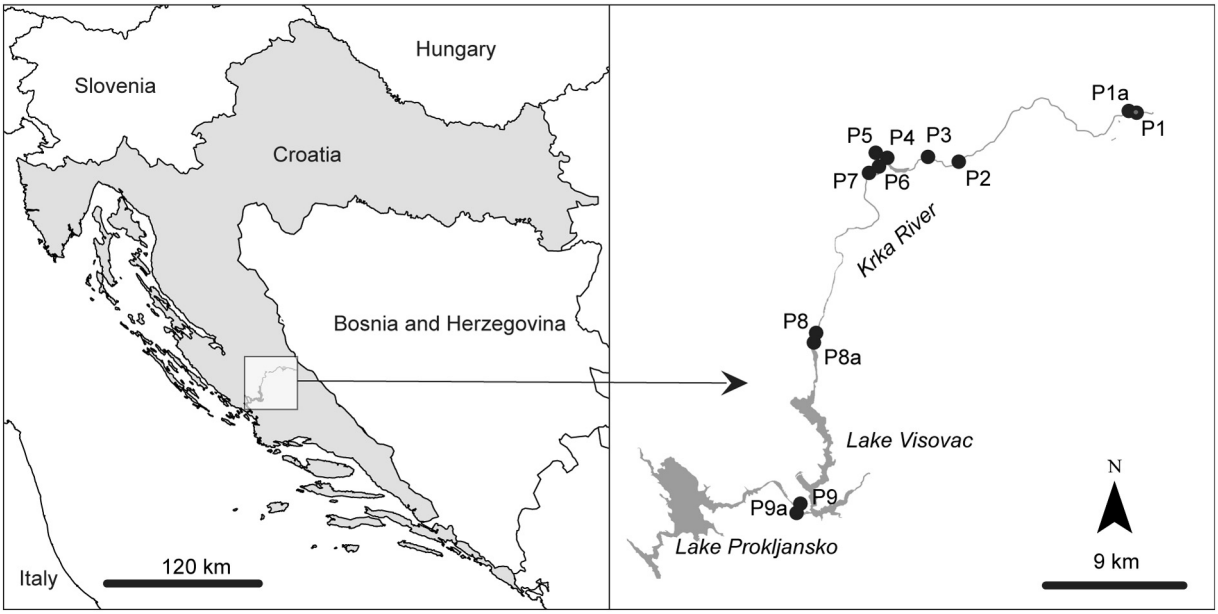


Fig. 1. Map of sampling locations situated at the Krka River, Croatia (P1, P1a = Krka spring, P2 = Marasovine, P3 = Bilušića buk, P4 = Brljan, P5 = Manojlovića buk, P6 = Rošnjak, P7 = Miljacka, P8, P8a = Roški slap, P9, P9a = Skradinski buk).

transportation to the laboratory and stored at −20 °C until further processing. For the physical and chemical analysis of water, in situ measurements of water temperature, pH, conductivity, oxygen concentration and saturation were done with a portable multimeter (Hach HQ40d, Germany). Samples for water chemistry analysis were collected, kept on ice and transported simultaneously with biological samples and stored at −20 °C until laboratory processing. The following parameters were quantified according to compliance monitoring standards (CEN – EN 15708, 2009): nitrites (NO_2^--N), nitrates (NO_3^--N), ammonium (NH_4^+-N), phosphates (PO_4^{3-}-P), 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. Microscopical analysis

The morphological analysis was performed following the protocol described in Žutinić et al. (2020a). Diatom samples were cleaned by removing all organic material. Afterwards, cleaned diatom material was mounted in Naphrax (Brunel Microscopes, UK) where at least 400 valves were counted on each slide.

2.4. Molecular analysis

DNA extraction was performed from the biofilm pellet obtained after centrifugation to remove excess water (4000 ×*g* for 1 min) using DNeasy PowerSoil Kit (Qiagen, Germany). During the isolation the manufacturer's instructions were followed 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 (BioSpec – nano, Schimadzu, Japan).

A 312 bp part of the Ribulose Bisphosphate Carboxylase Large subunit (*rbc*L) chloroplastic gene was used as the marker gene for PCR amplification. Primers used for amplification were the equimolar mix of three forward primers (Diat_rbcL_708F_1, Diat_rbcL_708F_2, Diat_rbcL_708F_3) and two reverse primers (R3_1, R3_2) according to Vasselon et al. (2017a). Amplification was conducted in a two-step process, where DNA samples were amplified (PCR1) in triplicates in a final volume of 25 μL. The replicates were pooled together and a second PCR (PCR2) was conducted with the purified amplicons of PCR1. In the end, the libraries preparation and final pool for the sequencing was prepared using Illumina MiSeq platform, generating 2 × 250-bp paired-end reads. All of these steps were performed in The Bordeaux Transcriptome Genome Platform (PGTB, Bordeaux, France).

2.5. Bioinformatics processing

Demultiplexed MiSeq reads were processed using the DADA2 pipeline (Callahan et al., 2016). A pipeline adapted to diatom metabarcoding sequence data was applied with the following steps available at https://github.com/fkeck/DADA2_diatoms_pipeline. Primer sequences from R1 (forward) and R2 (reverse) reads were removed using cutadapt v3.0 (Martin, 2011). The quality profile of the reads was checked and R1 and R2 reads were truncated to 170 and 150 nucleotides in order to remove the last, poor quality nucleotides (Fig. A.1a and b). Truncated sequences were filtered using the standard criteria of 0 ambiguities ("N") and a maximum of expected errors (maxEE) of 2. An error model, which was executed to show that estimated error rates fit well to the observed rates, is presented in the supplementary figure (Fig. A.2a and b). R1 and R2 reads were dereplicated into individual sequence units (ISUs). Exact sequence variants (ESVs) were selected based on the error rate models determined by the DADA2 denoising algorithm and paired reads were merged into one sequence. Chimeras and singletons were then removed from the dataset and read numbers in each sample were tracked after each step of the bioinformatic pipeline, as summarized in Table B.1. Taxonomic assignment of ESVs was performed using an adapted version (version 7) of the diat.barcode reference database according to the European standards for reference barcoding library management (CEN, 2018; Keck et al., 2019; Rimet et al., 2019), with the R package "diat.barcode" (Keck, 2020), using a minimum bootstrap confidence of 75 for the assignment of a taxonomic level. Raw demultiplexed reads were deposited at the ENA's Sequence Read Archive and are publicly available under project number PRJEB48565.

2.6. Statistical analysis

All statistical analyses were conducted in R v.4.1.0 (R Core Team, 2021) with the packages ("fossil", "dplyr", and "tidyverse" for basic data handling; "vegan", "stats" and "pls" for statistical analyses; "ggplot2" and "VennDiagram" for graphical representations). The SIMPER analysis was performed with the computer program PRIMER v7 for Windows (Primer-E Ltd., UK; Clarke and Gorley, 2015). All the pertaining maps were made in Arc GIS program (version 10; ESRI, 2011).

To allow comparability between the two approaches, results from both methods presented as taxa lists were compared in terms of a) presence or absence of taxa, and b) composition of diatom community. Results of the downstream analysis were combined into a single dataset for each approach, with molecular results normalized using the center-log ratio transformation (Gloor et al., 2017). Morphological data (valve counts) were not transformed. Correlation of sequences versus valve counts was tested using a Mantel test with 10,000 permutations using the R package "vegan" (Oksanen et al., 2019).

Valve counts (morphological data) and center-log ratio transformed molecular data were used to compute measures of beta diversity. Beta diversity was constrained by Permanova permutation test for both datasets separately to test the significance and to detect individual and combined effects of locations and microhabitats. The Bray-Curtis (BC) index was used as a measure of dissimilarity in community composition between the locations and microhabitats (stone or tufa). Non-metric multidimensional scaling (NMDS) was used to investigate the change in community composition linked to locations and microhabitats in the downstream flow direction. Environmental vectors were fitted to the ordination for both datasets separately 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.

The *rbc*L gene copy number per cell varies among diatom species according to their biovolume. In order to handle quantification bias and to make the molecular dataset comparable to the morphological dataset, a correction factor (CF; Vasselon et al., 2018) based on species' biovolume was applied on the abundance data (relative read number) of the molecular dataset (Rivera et al., 2020; Vasselon et al., 2018). The CFs were extracted from Diat.barcode (Rimet et al., 2019). Venn diagrams were used to visualize comparison overlapping in genus and species ranks recorded by both approaches.

Ecological status (EQR) for molecular and morphological data was assessed separately by calculating the Croatian Trophic Diatom Index (TDI_{HR}). TDI_{HR} values for the morphological data were already shown in Žutinić et al. (2020a). The difference between EQR classes of the two identification methods was tested using a pair-wise Wilcoxon test (Bauer, 1972) following the hypothesis that samples from the same population do not differ significantly. The EQR scores generated by morphological and molecular approaches were compared using Student's paired *t*-test (STUDENT, 1908; Zabell, 2008). Relevant ecological status per location was considered as the average according to microhabitat at each location and correlated with environmental parameters. Partial least squares regression (PLS regression; Chambers and Pope, 1992) was used to observe which environmental parameter correlated significantly with the observed EQR average obtained by the morphological and molecular approaches separately and for the differences (delta) in EQR values between the approaches. Then, a SIMPER analysis was carried out on species relative abundance data (Clarke, 1993) to address which of the taxa were the most abundant and responsible for the most relevant ecological status per location. This was tested with a comparison between the most abundant taxa according to the SIMPER analyses and the relevant EQR score per location. In the end, four cluster groups were used to define the deviation from the expected value of the morphological intercalibrated method: 'positive deviation', 'negative deviation', 'no deviation' (Bailet et al., 2019) or 'not available' (NA) in the scores or the absence of data in the molecular approach.

3. Results

3.1. Morphological analysis

A total of 62 genera and 239 species were identified using the morphological approach in 36 samples (Table B.2). The most abundant species at Krka spring were *Cocconeis placentula* var. *euglypta* (Ehrenberg) Grunow, *Odontidium mesodon* (Kützing) Kützing, *Meridion circulare* (Greville) C. Agardh, *Staurosirella pinnata* (Ehrenberg) D.M.Williams & Round and *Achnanthidium minutissimum* (Kützing) Czarnecki. *Rhoicosphenia abbreviata* (C.Agardh) Lange-Bertalot was the most abundant species at Marasovine. Bilušića buk was characterized by species *Achnanthidium pyrenaicum* (Hustedt) Kobayasi, *Cocconeis placentula var. euglypta* (Ehrenberg) Grunow and *Staurosirella pinnata* (Ehrenberg) D.M.Williams & Round. The most abundant species at Brljan were *Achnanthidium minutissimum* and *A. neomicrocephalum* Lange-Bertalot & F. Staab, while *Navicula* sp. and *Gomphonema lateripunctatum* E.Reichardt & Lange-Bertalot were the most abundant at Manojlovića buk. *Gomphonema lateripunctatum* and *Achnanthidium minutissimum* were recorded as the most abundant at Rošnjak, and *Amphora indistincta* Levkov at the sampling location Miljacka. For Roški slap, the most abundant species were *Aulacoseira granulata* var. *angustissima* (O.Müller) Simonsen and *Diatoma ehrenbergii* Kützing, while Skradinski buk was characterized by *Pantocsekiella ocellata* (Pantocsek) K.T.Kiss & Ács and *Navicula cryptotenella* Lange-Bertalot (Fig. 2).

3.2. Molecular analysis

Of the 36 samples collected, the DNA sequencing reaction failed for samples P3-3, P4-2, P7-1, P7-2 and P8-1 due to the poor quality of the extracted DNA. A total of 1,082,487 reads were obtained within 1642 ESVs, and after normalization 867,355 reads were taxonomically assigned to 1007 ESVs for diatoms. Within the 31 samples, a total of 62 genera and 169 species were taxonomically assigned to diatom ESVs (Table B.3). From a total of 1007 diatom ESVs, 347 ESVs (34.45%) could not be assigned from a diat.barcode v7 reference database into the species rank, 102 ESVs could not be assigned into the genus rank and 39 ESVs could not be assigned into the family rank and remained unclassified. According to the relative abundance of ESVs present after applying CFs, *Achnanthidium minutissimum* was present at all sampling locations and was also the most abundant species in the vast majority of microhabitats (Fig. 3). The most abundant ESVs at Krka spring corresponded to *Discostella woltereckii* (Hustedt) Houk & Klee, *Amphora pediculus* (Kützing) Grunow and *A. minutissimum*. For Marasovine, along with *A. minutissimum* the most abundant ESVs also corresponded to *Discostella woltereckii*. ESVs taxonomically assigned to *Amphora pediculus* and *A. minutissimum* were the most abundant at locations Bilušića buk and Brljan. The most abundant ESVs taxonomically assigned to *A. minutissimum*, *Amphora pediculus*, *Achnanthidium pyrenaicum*, *Discostella woltereckii* and *Amphora indistincta* occurred at Manojlovića buk. Rošnjak and Miljacka were characterized by ESVs corresponding solely to *A. minutissimum*. Roški slap was characterized with ESVs corresponding to *A. minutissimum* and *A. eutrophilum* (Lange-Bertalot), while species *A. minutissimum*, *Reimeria sinuata* (W.Gregory) Kociolek & Stoermer, *Planothidium victorii* P.M.Novis, J.Braidwood & C.Kilroy, *Sellaphora nigri* (De Notaris) Wetzel & Ector and *Sellaphora* sp. Mereschowsky were characterizing the location Skradinski buk (Fig. 3).

3.3. Comparison of the two methods

Comparison of results from both approaches presented a total of 46 overlaps at genus rank between the two methods (total of 64 based on morphological vs. 62 based on molecular approach), and 64 at species rank (total of 237 based on morphological vs. 169 based on molecular approach). Mantel test indicated a significant relationship for 64 species matches between Bray-Curtis distance based on the relative abundance of valve counts and relative abundance of reads ($r = 0.1529, p = 0.0032$).

The overlap between both methods is shown by Venn diagram (Fig. 4a and b) and list of overlaps at genus and species ranks are listed in Table B.4.

Comparison of the results generated by both methods showed that genus and species ranks were recognized more by using the morphological approach. Molecular results recognized many more ESVs at each sampling location, nevertheless, from the total recorded number up to 61% of ESVs could not be taxonomically assigned, even to the family or genus rank. Although a large percentage of taxonomically unassigned ESVs was present, they did not reflect the same percentage or number of unidentified species.

Non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis similarity showed that the resolution power of diatom community

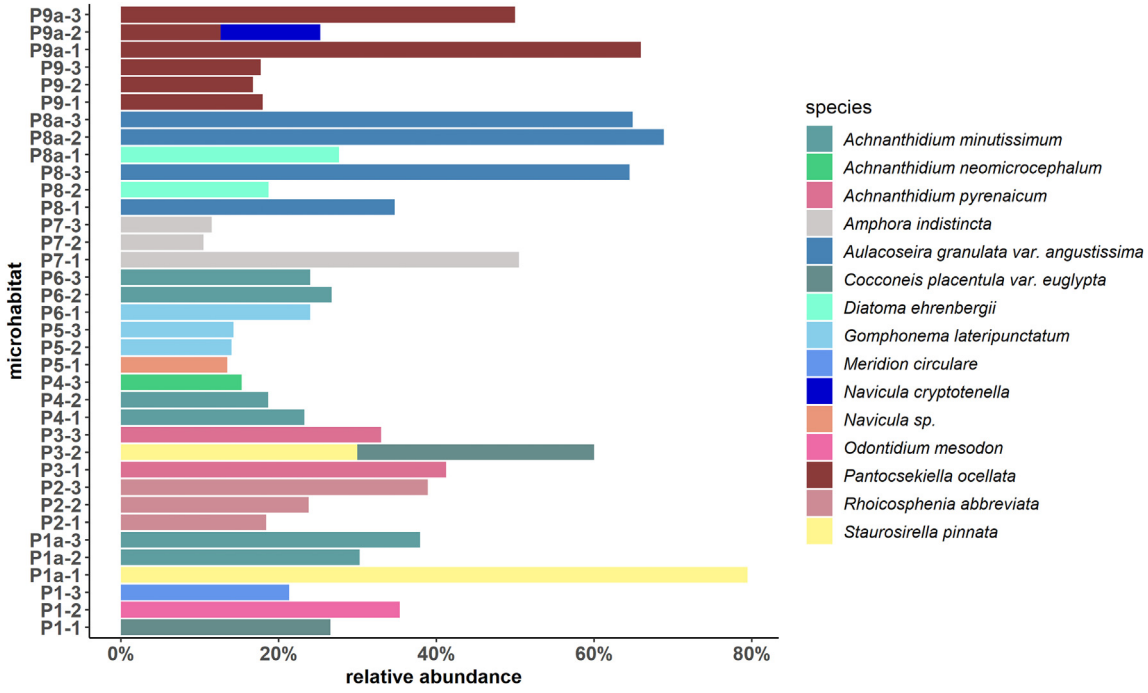


Fig. 2. The most abundant species per microhabitat identified by morphological approach (P1* and P1a* - Krka spring; P2* - Marasovine; P3* - Bilušića buk; P4* - Brljan; P5*- Manojlovića buk; P6* - Rošnjak; P7* - Miljacka; P8* and P8a* - Roški slap; P9* and P9a* - Skradinski buk).

at the sampling locations was higher when applying the morphological approach as opposed to the molecular approach. For the morphological approach, the Permanova test confirmed the significance for location ($p = 0.001$) and no significance for the microhabitats ($p = 0.067$). The low resolution power of diatom ESVs presented by the molecular approach was further confirmed by Permanova test, where no significances for both location ($p = 0.747$) and microhabitats ($p = 0.518$) were found. The overall strength of correlations between the morphologically detected diatoms community and its significant physical and chemical parameters (Table B.5; Žutinić et al., 2020a) is summarized in Table B.6. The main parameters that showed significant ($p = 0.001$) negative correlation with both axes MDS1 and MDS2 were pH, N-NO_3^-, TN, TOC and DOC. Oxygen saturation, DO and DIC showed significant positive correlation with both axes ($p = 0.001$), EC, T, SiO_2 and TIC showed significant negative correlations with MDS1 axis ($p = 0.001$), whilst P-PO_4^{3-} showed significant positive correlation ($p = 0.001$) with MDS1 axis (Fig. 5a). As for the molecular

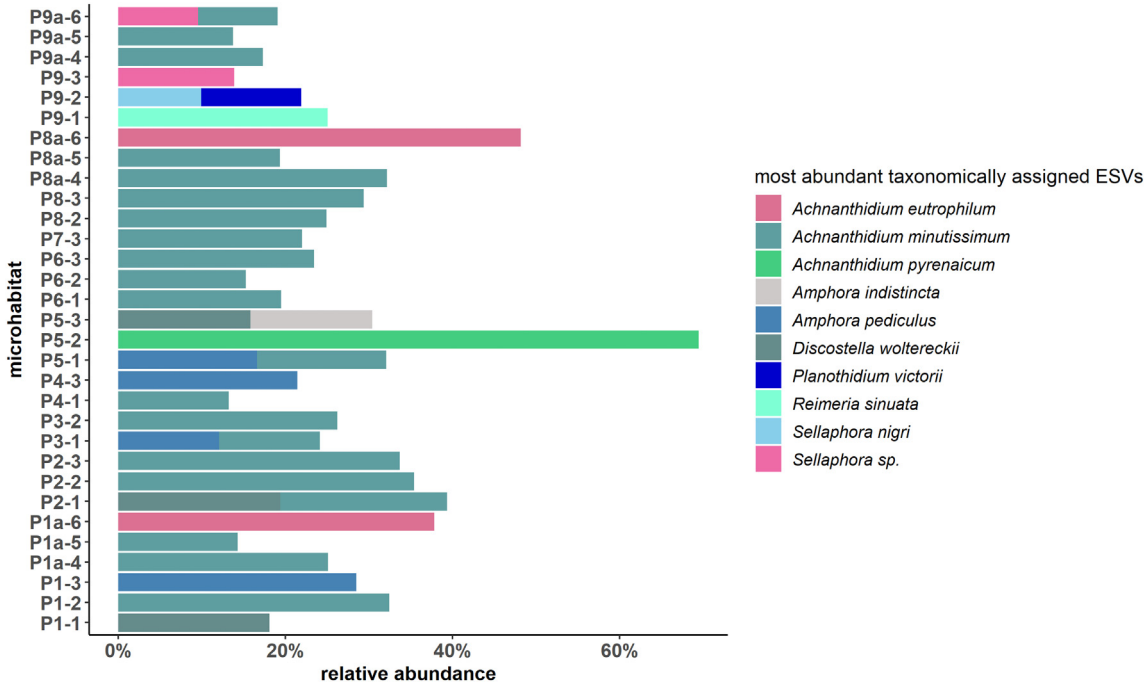


Fig. 3. The most abundant ESVs taxonomically assigned to species, per microhabitat identified by molecular approach (P1* and P1a* - Krka spring; P2* - Marasovine; P3* - Bilušića buk; P4* - Brljan; P5*- Manojlovića buk; P6* - Rošnjak; P7* - Miljacka; P8* and P8a* - Roški slap; P9* and P9a*- Skradinski buk).

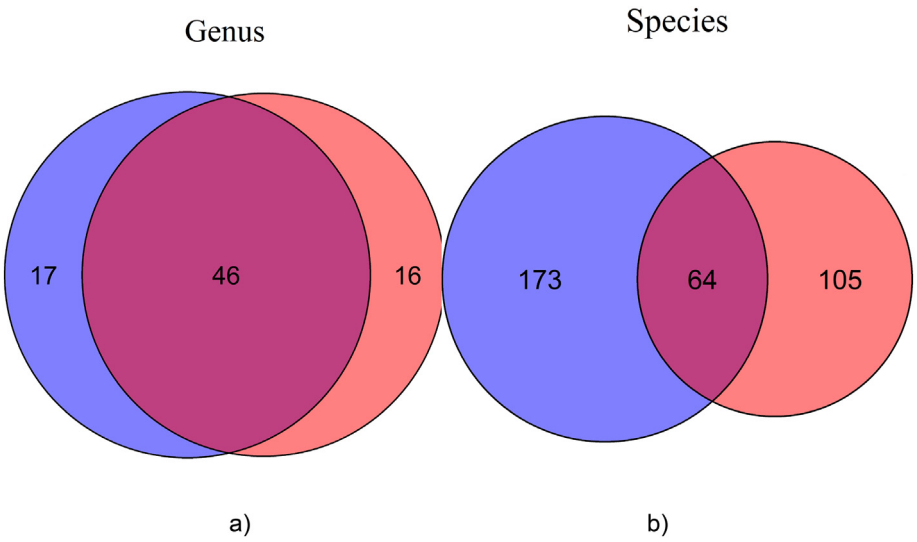


Fig. 4. Venn diagram comparing the diatoms assigned at genus (a) and species (b) rank either by the morphological (blue circles) or by the molecular (red circles) approach.

approach, NMDS analysis indicated that no parameter has demonstrated a significant correlation with MDS1 and MDS2 axes (Fig. 5b). The overall strength of correlations between the molecularly detected diatom ESVs and significant physical and chemical parameters in NMDS is summarized in Table B.7.

3.4. Ecological status

The ecological status (EQR) of Krka River based on the taxa list generated by morphological and molecular approach was assessed by separate calculation of the Croatian Trophic Diatom Index (TDI_{HR}). Results of all sampled locations with their associated microhabitats are presented in Tables B.8 and B.9, respectively. The EQR derived from the morphological approach ranged from 0.45 to 1.1, classifying the Krka River as High in most of its course. As for the molecular approach, the EQR ranged from 0.64 to 1.32, thus classifying Krka River as Good in the most of its course (Fig. 6).

According to the defined deviation, 38.9% of the samples contributed to the same ecological status in both approaches ('positive deviation'). Furthermore, lower ecological status was imparted by 33.3% of the samples attributed by the molecular approach, while higher ecological status was given by 13.9% of the samples from the molecular analyses as compared to the morphological approach ('negative deviation'). No comparison was shown in 13.9% of samples from the molecular approach due to missing results ('no deviation' or NA). A pair-wise Wilcoxon test indicated that the two approaches generated significantly different ecological status classes ($p < 0.001$).

No correlation (Pearson correlation, $R = -0.14, p > 0.05$) and a significant difference (Student's paired *t*-test, $p < 0.01$) was detected between the EQR scores calculated from taxa list generated by morphological and molecular approaches.

Partial least square regression (PLS) indicated four environmental parameters which correlated significantly with the observed EQR average. Parameters NO_2^--N, NO_3^--N and NH_4^+-N were excluded from the PLS regression because the standard deviation in each case was scaled near to zero. The PLS regression marked pH (26.46%), EC (53.46%), DO (65.88%) and T (80.24%) as significant predictors for the delta EQR status classes obtained by both approaches. For EQR averages based on the morphological approach and environmental variables, PLS regression indicated PO_4^{3-}-P ($p < 0.01, p = 0.0316$) and TN ($p < 0.01, p = 0.0855$) as significant parameters, but for EQR averages based on the molecular approach no significant parameters emerged. When considering the morphological species community, the similarity between microhabitats per locations attained in the SIMPER analysis ranged from 30.15% to 65.85%, indicating higher similarity than molecular taxa community, which ranged from 10.26% to 69.92%. The Simper analysis has identified 24 species using the morphological approach and 19 taxa using the *rbcL* marker as descriptive species/taxa which contributed to more than 50% per location (Fig. 7a and b). Additionally, the SIMPER analysis highlighted 6 species for morphological approach which were not present in the diat.barcode reference database (v7): *Aulacoseira granulata* var. *angustissima* (O.Müller) Simonsen, *Cocconeis lineata* Ehrenberg, *Cocconeis pseudolineata* (Geitler) Lange-Bertalot, *Encyonopsis krammeri* E.Reichardt, *Gomphonema lateripunctatum* E.Reichardt & Lange-Bertalot and *Planothidium hauckianum* (Grunow) Bukhtiyarova. One of the examples of major gaps in the EQR values was also noted at Skradinski buk, where the planktic species *P. ocellata* contributed most to EQR values in the morphological approach, but which did not appear as the most contributing taxon in the molecular analysis.

4. Discussion

The karst tufa barriers arising along the course of Krka River represent one of the most unique and recognizable natural attributes, where tufa provides a favourable substrate for the colonization of many protists like diatoms (Kulaš et al., 2021; Žutinić et al., 2020a). Although diatoms are important organisms for understanding the functioning of aquatic ecosystems, our knowledge of diatom biodiversity is still limited given the discrepancy between extant and estimated number of species (Nistal-García et al., 2021). Previous studies in the Krka River were based solely on morphological identification of diatom species using light microscope (Kralj et al., 2006; Žutinić et al., 2020a). However, eDNA metabarcoding provides a powerful tool to examine unknown diatom diversity and explain our knowledge about their distribution patterns (Nistal-García et al., 2021; Vasselon et al., 2019).

Comparison between morphological and molecular approaches of diatoms in the Krka River revealed 58% agreement on the genus rank and a relatively low agreement of about 20% on the species rank, but both methods provided an in-depth insight into the community complexity. The genera *Achnanthidium* and *Amphora* had the highest number of matches, which was not surprising since taxa at these ranks were the most abundant in both approaches and were recognized as ubiquitous. The most common diatom species recognized by both approaches was *A. minutissimum*, often described as tolerant of "chemical insults", but also considered an indicator of nutrient-poor waters or generally good water quality (Potapova and Hamilton, 2007). Second in the species rank was *A. pyrenaicum*, a diatom that prefers streams and springs with faster current velocities and limestone habitats (Cantonati and Spitale, 2009), as this species was most abundant at waterfalls and tufa barriers at Bilušića buk, Manojlovića buk and Roški slap.

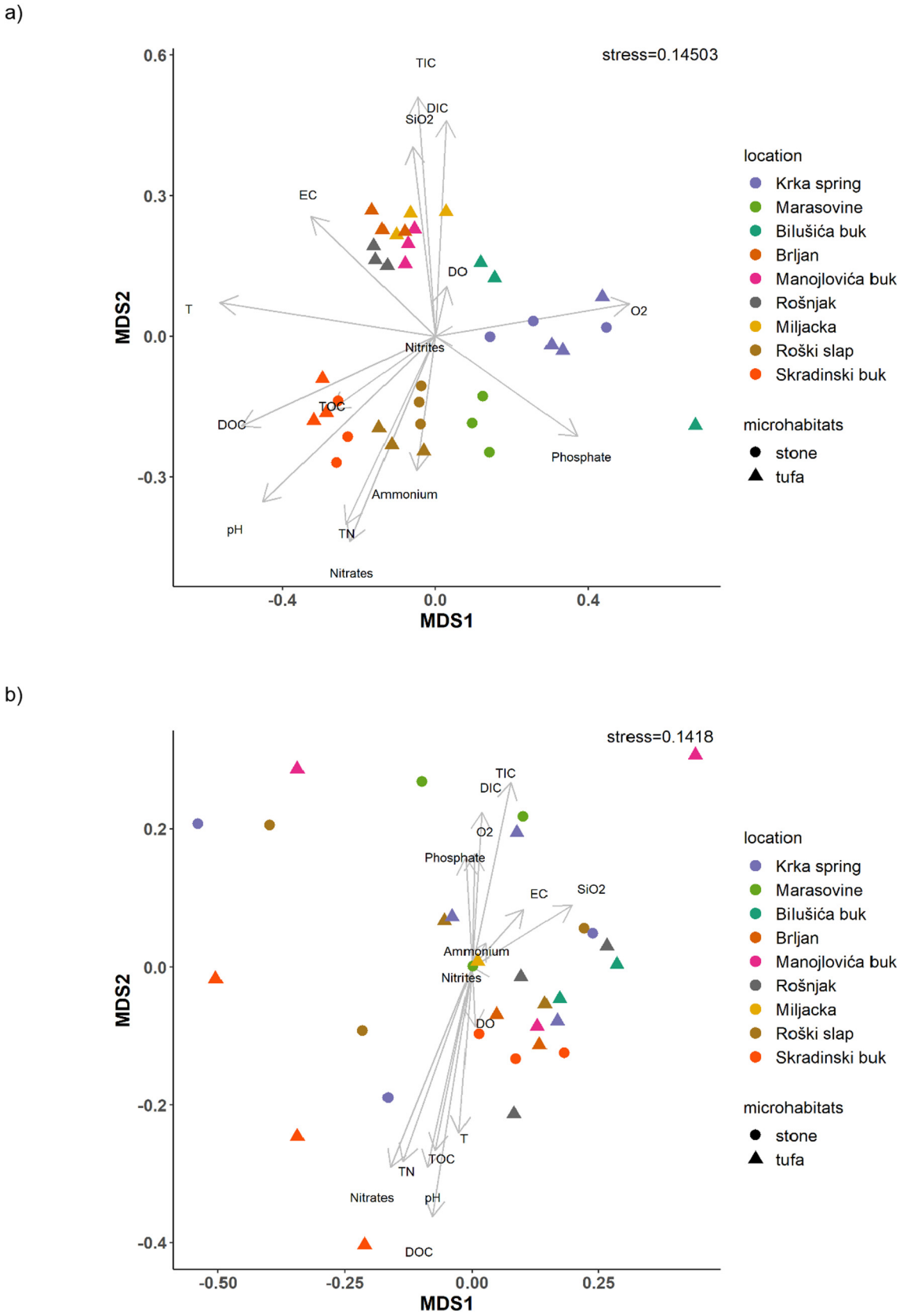


Fig. 5. Position of sampling locations in the multidimensional scaling analysis based on Bray-Curtis similarity index for diatoms a) for morphological approach; b) for molecular approach with environmental parameters (T = temperature, EC = conductivity, pH, DO = dissolved oxygen, O_2 = oxygen saturation, SiO_2 = silicon dioxide, TIC = total inorganic carbon, DIC = dissolved inorganic carbon, TN = total nitrogen, TOC = total organic carbon, DOC = dissolved organic carbon).

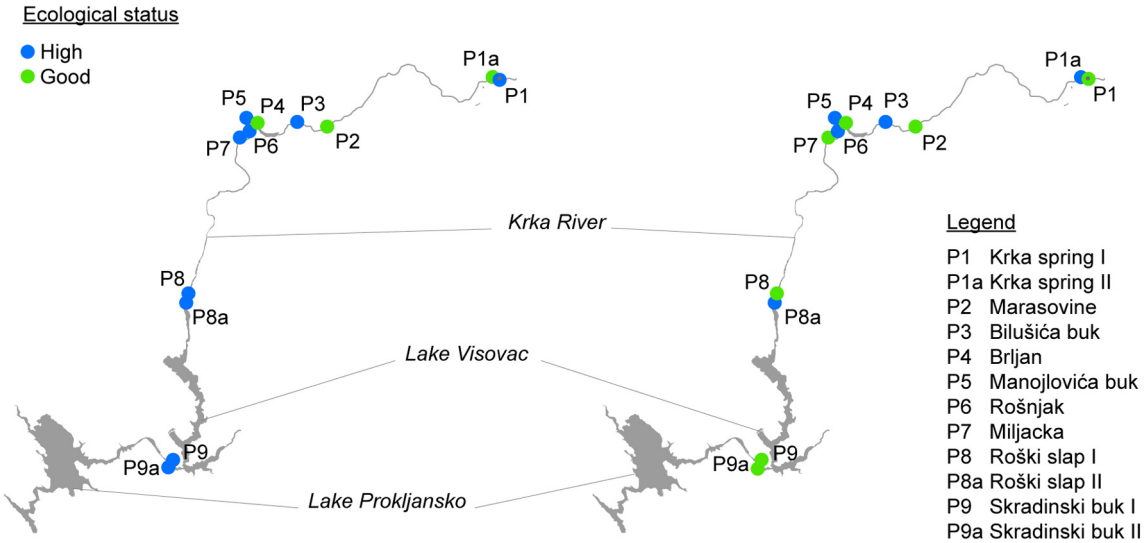


Fig. 6. Map of sampling locations with their ecological status based on morphological (left side) and molecular (right side) approaches.

Species from the genus *Amphora*, such as *A. indistincta* and *A. pediculus*, were respectively present as they prefer nutrient-poor waters (Hofmann et al., 2013).

Even though the molecular approach can give an idea of the overall richness within the sample, the comparison of inventories obtained by both methods revealed some discrepancies in the relative abundance, beta diversity and ecological status scores. Similar results, with an even larger gap from the molecular analysis, were also given by Nistal-García et al. (2021). Our comparison of both approaches showed that the molecular approach detected a much higher number of ESVs per location, with the exception of Miljacka and Manojlovića buk. Nonetheless, most of these ESVs were not assigned either to species or genus rank, and it was unclear how many species they could correspond to. The incompleteness of the reference database is a key factor which severely limits the taxonomic assignment of ESVs. Specifically, 73 species (17 genera) in this study were identified only by morphological approach and could not be molecularly detected due to the lack of reference sequences in the diat.barcode reference database (v7), as many species are still lacking barcode information (Nistal-García et al., 2021; Visco et al., 2015).

Accordingly, several species detected only by light microscopy differed from those detected by the molecular approach. Previous studies (Nistal-García et al., 2021; Jahn et al., 2007) hypothesized that taxa whose sequences are missing from the reference database could be compensated by taxa of the same genus whose sequences are available in the reference database or by a taxon not expected in the habitat studied, which may explain the relatively small discrepancies between both approaches at the genus level. As an example, the species *Cyclotella plitvicensis* Hustedt was only identified microscopically and was morphologically clearly distinguished from *C. distiguenda* Hustedt, which was detected only by molecular approach. *Cyclotella plitvicensis* was first described by Hustedt as an endemic planktic species from the Plitvice Lakes in Croatia (Hustedt, 1945), and was subsequently identified in other deep karst Croatian lakes (Gligora Udovič et al., 2017). This discrepancy can be explained by close morphological similarity between the two species and potentially close genetical relation (Hustedt, 1945), with a need to address this molecular gap in the diatom database. Many other species from the genus *Achnanthidium* have been identified only by morphological approach (*Achnanthidium catenatum* (Bily & Marvan) Lange-Bertalot, *Achnanhtidium exile* (Kützing) Round & Bukhtiyarova, *Achnanthidium affine* (Grunow) Czarnecki, *Achnanthidium gracillimum* (F.Meister) Lange-Bertalot, *Achnanthidium neomicrocephalum* Lange-Bertalot & F. Staab, *Achnanthidium rosenstockii* (Lange-Bertalot) Lange-Bertalot). A corresponding case lies within the genus *Amphora*, as several species are also not available in diatom database (e.g. *Amphora neglectiformis* Levkov & Edlund, *A. alpestris* Levkov, *A. inariensis* Krammer and *A. lange-bertalotii* Levkov & Metzeltin). Many species from the genera *Cymbella* and *Gomphonema* were identified only morphologically. Likewise, the species *Gomphosphenia plenkoviciae* Gligora Udovič & Žutinić, a recently described pennate diatom from Crveno jezero in Croatia by using light microscopy, was detected in the Krka River (Gligora Udovič et al., 2018).

In contrast to morphological identification, a certain number of species was detected exclusively by molecular approach. One such species was *Achnanthidium straubianum* (Lange-Bertalot) Lange-Bertalot, which usually co-occurs with *A. minutissimum* and prefers calcium-bicarbonate-rich, meso- to eutrophic lakes and is rarely found in oligotrophic conditions (Lange-Bertalot et al., 2017). It is a rather small diatom (maximum length up to 10 μm) belonging to the *A. minutissimum* group, which implies a high probability of mutual morphological similarities. According to Pinseel et al. (2017), several *Achnanthidium* sequences are available on GenBank (mainly of the *A. minutissium* complex) and species boundaries of the genus *Achnanthidium* are missing entirely. Also, various morphodemes of *A. minutissimum* complex have been noted to show distinct ecological preferences. The implementation of molecular data will be essential in solving the taxonomic problems associated with this group, eventually resulting in a better understanding of the biogeography and niche differentiation of different species within *A. minutissimum* complex. *Amphora atomoides* Levkov was recognized only by the molecular approach, as its small size usually conditions its limitation in morphological identification. Another small species noted as a peculiar detection by molecular approach was *Planothidium victorii* P.M.Novis, J.Braidwood & C.Kilroy. Besides falling within the size range of *P. frequentissimum*–complex, this species appears to be particularly sensitive to contamination and could be regarded as a potentially useful indicator (Novis et al., 2012). The species differs slightly from *P. frequentissimum* sensu stricto, so it can be easily replaced in morphological approach. Here molecular approach shows an advantage in detecting species that are morphologically very similar (Wetzel et al., 2019). *Dorofeyukea indokotschyi* Kulikovskiy, Maltsev, Andreeva & Kociolek, a recently described species from a tropical, shallow Lake Sentani in Indonesia (Kulikovskiy et al., 2019) also represents a potential mismatch or an uncertain interpretation by molecular detection. Another such example is *Fragilaria heatherae* Kahlert & M.G.Kelly, a species described from the lotic environments in the UK and Italy, characterized by preference towards relatively soft water and low nutrient concentrations (Kahlert et al., 2019). The species *Lemnicola hungarica* (Grunow) Round & Basson and *Luticola goeppertiana* (Bleisch) D.G.Mann ex J.Rarick, S.Wu, S.S.Lee & Edlund were also detected only by using the molecular approach. Both species are characterized by their relocation from other genera, the former being named as *Achnanthes hungarica* (Round and Basson, 1997) and the latter having an older scientific name as *Stauroneis goeppertiana* Bleisch (Rarick et al.,

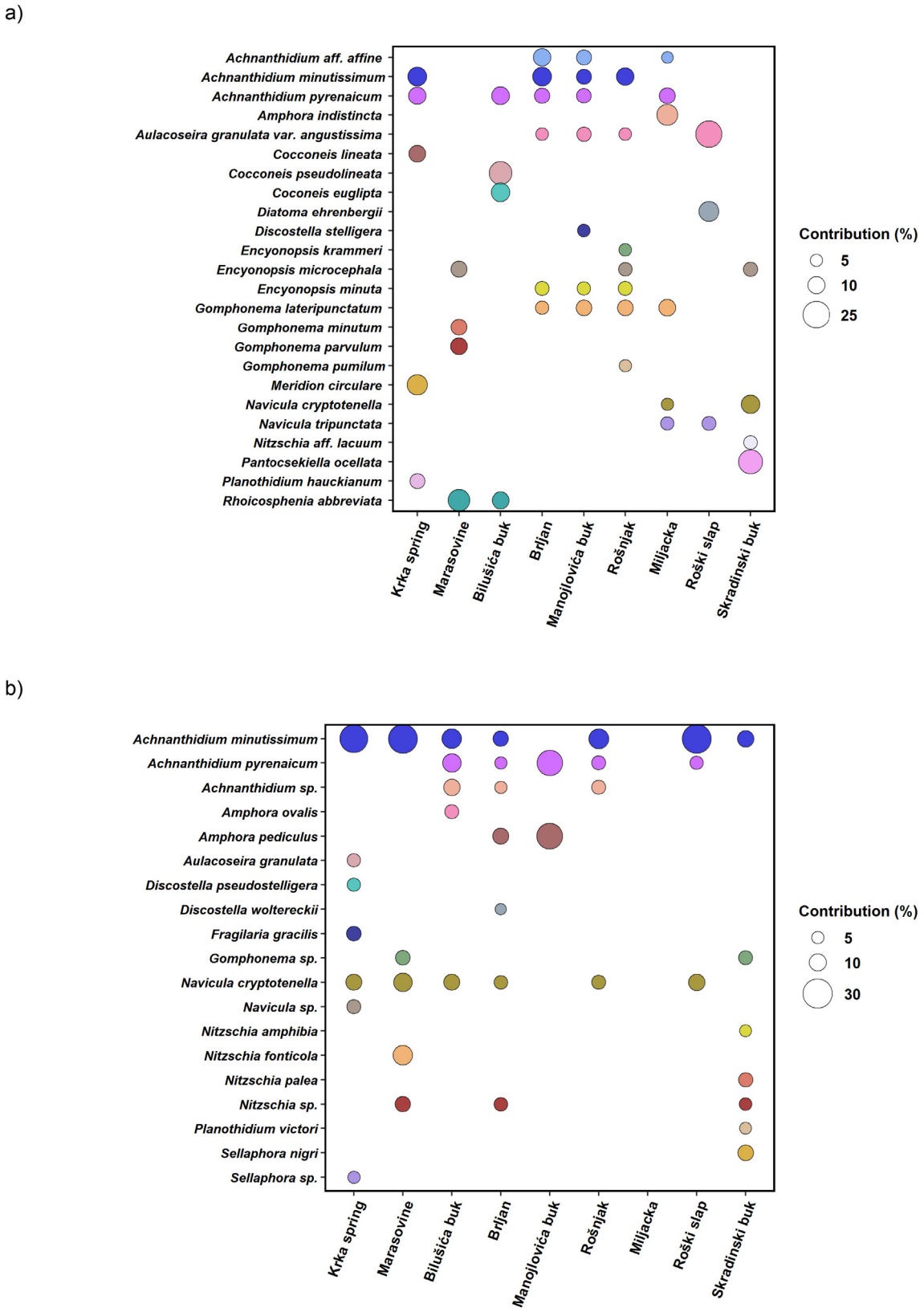


Fig. 7. Contribution of different taxa obtained by SIMPER analysis per sampling location for: a) morphological, and b) molecular approaches.

2020). The molecular detection of *Nitzschia acidoclinata* Lange-Bertalot, a diatom preferring circumneutral to slightly acidic waters, did not conform in particular to the ecological characteristics of the study area (Krammer and Lange-Bertalot, 1986). Analogously to the aforementioned *Amphora atomoides*, many species from the genus *Pinnularia* (*P. acuminata* W.Smith, *P. brebissonii* (Kützing) Rabenhorst, *P. divergens* W. Smith, *P. grunowii* Krammer, *P. neomajor* Krammer, *P. parvulissima* Krammer, *P. peracuminata* Krammer, *P. subgibba* Krammer, *P. substreptoraphe* Krammer, *P. viridiformis*

Krammer) have been detected by the molecular approach, most likely due to their large size. Interestingly, the molecular approach revealed a presence of a marine genus *Chaetoceros*. This may reflect either inaccurate taxonomic assignment or, possible "marine hits". The latter possibility results from a deep seawater intrusion into the Krka River estuary extendings up to the base of Skradinski buk, so that eDNA may be transported upstream either passively as extracellular DNA or by active or passive dispersal of organisms (Deiner and Altermatt, 2014).

There are several reasons that could explain the discrepancy in diatom taxa listed by both approaches: i) the isolation of DNA can be a challenging task, as the silicate cell wall presents an obstacle to the direct application of molecular techniques (Annunziata et al., 2021); ii) the PCR reaction used to amplify the barcode region can be inhibited by contaminants and produce chimeric DNA molecules (Hugerth and Andersson, 2017) iii) calcium, a known inhibitor of Taq polymerase that binds competitively to the polymerase during PCR rather than magnesium, thus reducing the efficiency of amplification, especially owing to the fact that the karstic river is rich in calcium carbonate (Kuffel et al., 2021; Opel et al., 2010); iv) the majority of species identified only by the morphological approach are still not present in the diatom database, which severely limits the taxonomic assignment of ESVs and could be avoided by using a taxonomy-free approach proposed by Tapolczai et al. (2021); v) the morphological approach has a lower ability to detect rare, smaller or morphologically very similar species than the molecular approach, whereas the molecular method allows detection of a wider range of species, thus providing a more distinct species richness and avoiding the underrepresentation of species in samples (Rimet et al., 2018); vi) the species with lightly silicified frustules, such as *Urosolenia eriensis* (H.L.Smith) Round & R.M.Crawford, are usually overlooked under the microscope as they are easily destroyed during the preparation of permanent slides, and are therefore more readily detected by the molecular approach (Pérez-Burillo et al., 2020; Zgrundo et al., 2013), as confirmed in this study; vii) the resting stages and spores are also readily detectable by molecular method, as they may be mistaken for different species by the morphological method unless the spores are not found attached to vegetative parents (McQuoid and Hobson, 1996); viii) other factors, such as the presence of extracellular DNA, may also influence the molecular method, whereas the extracellular DNA from diatom species may be detected in a sample even if their cells are not physically present, adding additional taxa to the molecular inventory (Rimet et al., 2018).

Non-metric multidimensional scaling (NMDS) analysis of morphologically identified data showed a clear separation of diatom community according to locations and significant correlations with various environmental parameters. The grouping by locations can be interpreted as a strong location effect on the community composition along the Krka River flow (Kulaš et al., 2021; Žutinić et al., 2020a), which was confirmed by Permanova tests. The Krka spring correlated strongly with DO, a particularly important parameter for the assessment of water quality (American Public Health Association et al., 2017; Žutinić et al., 2020a). This location is characterized by the higher values of the oxygen content due to higher solubility of calcium carbonate in the lower water temperatures, narrow streambed and fast waterflow, and is characterized by species typical for spring areas such as *Meridion circulare* (Greville) C.Agardh, *Odontidium mesodon* (Kützing) Kützing and *Planothidium hauckianum* (Grunow) Bukhtiyarova (Kulaš et al., 2020; Žutinić et al., 2020a). Even though exhibiting low concentrations of phosphorus, the strong correlation of location Marasovine with phosphorus can be construed by the geographic situation. This upper section of the river is located downstream from the town of Knin, indicating anthropogenic pressure (Žutinić et al., 2020a), which was corroborated with the presence of *Rhoicosphenia abbreviata* (C.Agardh) Lange-Bertalot, a species preferring waters of high trophic states, (Lange-Bertalot et al., 2017). Locations Brljan, Manojlovića buk, Rošnjak and Miljacka correlated with TIC, DIC and SiO_2. DIC is an indicator of primary productivity, specifically bioavailable carbon source for photosynthesis (Jarvie et al., 2017). Diatoms are a crucial biological component involved in the process of tufa formation by excreting mucus for plastering calcite microcrystals and embedding themselves into barriers, a fact demonstrated by the highest concentration of SiO_2 and the highest number of recorded diatoms (Chafetz et al., 1994; Žutinić et al., 2020a). Roški slap was correlated with NO_3^- and TN, nitrogen compounds which showed a clear trend of increasing concentrations in the downstream direction suggesting enhanced bacterial denitrification processes (Scholten and Stams, 1995; Žutinić et al., 2020a). Besides, the concentrations of nitrogen in rivers usually originate from direct terrestrial runoff compared to the atmospheric sources. As it can be rapidly oxygenized (Li et al., 2021), low nitrogen concentrations detected in this study were already confirmed by other studies (Sertić Perić et al., 2018). Dissolved organic carbon (DOC), defined mainly as input from allochthonous material, and total organic carbon (TOC) as a measure of total organic matter were very low and did not differ significantly along the sampling locations, indicating a very low presence of organic substance in the sediments (Srdoč et al., 1985). However, Skradinski buk correlated with higher values of DOC, likely due to its geographic setting downstream of Lake Visovac. Since lakes tend to be more productive systems, the influence of Lake Visovac is evident in the higher amount of dissolved organic matter and accordingly higher abundance of protists, not just algae (Kulaš et al., 2021; Špoljar et al., 2005). As opposed to morphological approach, the beta diversity calculated from the molecular approach did not clearly separate species according to locations or microhabitats. Nonetheless, from a metacommunity perspective the concept of beta diversity is essential in understanding ecosystem functioning, as it provides important information about patterns of diversity and the processes that modify ecosystems. Still, the spatial structure may account for a significant portion of community variance, suggesting that diatoms do not have a strict ubiquitous distribution and therefore exhibit biogeographic patterns (Florencio et al., 2014; Leboucher et al., 2019; Soininen, 2007). Although the molecular approach did not show clear results in beta diversity, it did capture many more taxa that could be taxonomically assigned than the species obtained by the morphological approach. This was particularly confirmed by the NMDS analysis at Skradinski buk, where the molecular approach showed three outliers corresponding to differences in the most abundant species recorded in contrast to the morphological results. This result is consistent with the previous studies that have shown that the molecular approach can definitely identify the underestimated hidden diversity of diatoms (Rivera et al., 2018; Trobajo et al., 2010).

When comparing the ecological index values from the molecular and morphological approaches, a number of studies confirmed a positive correlation between the results of both methods as also their complementarity (Duleba et al., 2021; Pérez-Burillo et al., 2020; Tapolczai et al., 2019; Vasselon et al., 2017a). However, several studies highlighted the discrepancies between the results corresponding to our results (Bailet et al., 2019; Nistal-García et al., 2021; Rivera et al., 2018). The Pearson correlation of the ecological assessment results of both methods confirmed no correlation between the methods and the Wilcoxon test showed a significant difference between the EQR scores for the Krka River. Status differences can be explained by the uniformity of the selected stone microhabitat and the stochasticity of the selection process without prior selection, which has already been discussed in Žutinić et al. (2020a). Also, diatom species diversity is generally different at locations of Poor and Good ecological status. Locations with Poor ecological status are generally represented by a few very abundant opportunistic diatom species (Stevenson et al., 2010), whereas locations with Very good status tend to be represented by communities with moderate to high diversity (Whitton and Kelly, 1995). For instance, by using the morphological approach we detected *Staurosirella pinnata* (Ehrenberg) D.M.Williams & Round, a species with high trophic diatom index, as the most abundant species in the Krka spring microhabitat with 80% presence of the total diatom abundance, whilst the molecular approach underlined *A. minutissimum*, a species with much lower trophic index, as the most abundant. In addition, 6 taxa recorded as indicator species by the morphological approach were highlighted by the SIMPER analysis as not present in the diat.barcode reference database (v7), confirming that species abundance can have a strong impact the index calculation (Bailet et al., 2019). One such species missing from the database is *Planothidium hauckianum*, with an occurrence contribution of 7.71% to

the EQR values at the Krka spring. This may also lead to the discrepancy in results, as this species has been described as having very specific ecological preferences in karstic environments and a very localised geographical distribution restricted to the Balkan region (Kulaš et al., 2020). For the morphological approach, the SIMPER analysis indicated centric diatom species *Aulacoseira granulata* var. *angustissima* and *P. ocellata* as indicator species. *A. granulata* var. *angustissima* was detected at the tufa barriers (Brljan, Manojlovića buk and Rošnjak) of the upstream course, most probably as a result of the downstream transport of the species at Roški slap. *P. ocellata* was detected at Skradinski buk, and its presence can be interpreted by the influence of Lake Visovac, where this species is the most abundant (Hanžek et al., 2021). Highlighting centric diatoms in the morphological approach by the SIMPER analysis can lead to misleading or incorrect interpretations of EQR scores, as these species are planktic and may have deposited cells that are difficult to distinguish under the light microscope due to downstream transport or they can already be bound in sediment particles (Gons, 1991). In such cases, the molecular approach has advantages in interpreting EQR scores more correctly. In the downstream section the river shows signs of significant self-purification through several small lakes formed by tufa barriers (Cukrov et al., 2008), especially waterfalls Bilušića buk, Brljan and Manojlovića buk (Žutinić et al., 2020a). The PLS regression showed significant predictors for the EQR status classes identified by both approaches that played a role in describing the community, which was also confirmed by significant parameters in the NMDS analysis obtained by the morphological approach. Linear correlations in PLS regression between EQR scores based on the morphological approach confirmed nutrients as significant variables, especially TN (nitrogen compounds). This is consistent with the settings of the Croatian Trophic Diatom Index (TDI_{HR}), a modification of the Trophic Diatom Index (Rott et al., 1999) which indicates the nutrient load of the water body (Žutinić et al., 2020a, 2020b).

In conclusion, our results showed a relatively low agreement between the morphological and molecular approach in the variation of diatom community composition. According to the beta diversity, the morphological approach indicated a clear separation of the diatom community along the river, with a strong location effect caused by the various environmental parameters. However, both methods gave complementary information, within beta diversity showing no differences in diatom composition detected between microhabitats, as they belong to the same calcium carbonate substrate/stone or deposit (tufa). In addition, morphological and molecular results provided a feasible but statistically different assessment of the ecological status, thus fitting response to the environmental pressures. Ultimately, diatom diversity based on both approaches allowed a reliable dataset that can be used in routine monitoring assessment which provides a deeper understanding of ecological status. To further strengthen the correlation between the two presented approaches, a comprehensive reference database needs to be established, which is crucial for improving metabarcoding in routine monitoring.

CRediT authorship contribution statement

Antonija Kulaš: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Marija Gligora Udovič:** Conceptualization, Funding acquisition, Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing. **Kálmán Tapolczai:** Formal analysis, Visualization, Writing – review & editing. **Petar Žutinić:** Investigation, Conceptualization, Writing – review & editing. **Sandi Orlić:** Investigation, Methodology, Writing – review & editing. **Zlatko Levkov:** Investigation, Methodology, Supervision, 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.

Acknowledgements

Mr. sc. Gordana Goreta is thanked for her support and assistance with the survey of project at the Krka River. Dr. Igor Stanković is thanked for his kind help in preparing the maps of the study area.

Funding

This study was financial supported by project “Assessment of the ecological status of the Krka River using DNA metabarcoding”, financed by the NP Krka. SO was partially supported by 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) and the DNKVODA project, Contract Number: KK.01.2.1.02.0335.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154536.

References

American Public Health Association, American Water Works Association, Water Environment Federation, 2017. Standard Methods for the Examination of Water and Wastewater. 23rd edition. American Public Health Association, American Water Works Association, Water Environment Federation.

Annunziata, R., Balestra, C., Marotta, P., Ruggiero, A., Manfellotto, F., Benvenuto, G., Biffali, E., Ferrante, M.I., 2021. An optimised method for intact nuclei isolation from diatoms. Sci. Rep. 11, 27–29. https://doi.org/10.1038/s41598-021-81238-z.

Bailet, B., Bouchez, A., Franc, A., Frigerio, J.M., Keck, F., Karjalainen, S.M., Rimet, F., Schneider, S., Kahlert, M., 2019. Molecular versus morphological data for benthic diatoms biomonitoring in northern Europe freshwater and consequences for ecological status. Metabarcoding Metagenomics 3, 21–35. https://doi.org/10.3897/mbmg.3.34002.

Bailet, B., Apothéloz-Perret-Gentil, L., Baričević, A., Chonova, T., Franc, A., Frigerio, J.-M., Kelly, M., Mora, D., Pfannkuchen, M., Proft, S., Ramon, M., Vasselon, V., Zimmermann, J., Kahlert, M., 2020. Diatom DNA metabarcoding for ecological assessment: comparison among bioinformatics pipelines used in six european countries reveals the need for standardization. Sci. Total Environ. 745, 140948. https://doi.org/10.1016/j.scitotenv.2020.140948.

Bauer, D.F., 1972. Constructing confidence sets using rank statistics. J. Am. Stat. Assoc. 67, 687–690. https://doi.org/10.1080/01621459.1972.10481279.

Bonacci, O., 1985. Hydrological investigations of dinaric karst at the krcic catchment and river Krka Springs (Yugoslavia). J. Hydrol. 82, 317–326. https://doi.org/10.1016/0022-1694(85)90024-1.

Bonacci, O., Jukić, D., Ljubenkov, I., 2006. Definition of catchment area in karst: case of the rivers Krčić and Krka, Croatia. Hydrol. Sci. J. 51, 682–699. https://doi.org/10.1623/hysj.51.4.682.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Cantonati, M., Spitale, D., 2009. The role of environmental variables in structuring epiphytic and epilithic diatom assemblages in springs and streams of the dolomiti bellunesi National Park (south-Eastern Alps). Fundam. Appl. Limnol. 174, 117–133. https://doi.org/10.1127/1863-9135/2009/0174-0117.

CEN, 2018. Water Quality - CEN/TR 17244:2018 Technical Report for the Management of Diatom Barcodes.

CEN – EN 15708, 2009. Water Quality - Guidance Standard for the Surveying, Sampling and Laboratory Analysis of Phytobenthos in Shallow Running Water (EN 15708:2009). European Committee for Standardization (CEN), Brussels, Belgium.

Chafetz, H., Srdoc, D., Horvatinčić, N., 1994. Early diagenesis of Plitvice Lakes waterfall and barrier treavertine deposits. Géog. Phys. Quatern. 48, 247. https://doi.org/10.7202/033006ar.

Chambers, R., Pope, R., 1992. Engel’s law and linear-in-moments aggregation. Am. J. Agric. Econ. 74. https://doi.org/10.2307/1242581.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18, 117–143. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x.

Clarke, K.R., Gorley, R.N., 2015. PRIMER v7: User Manual/Tutorial. PRIMER-EPlymouth.

Cukrov, N., Cmuk, P., Mlakar, 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.

Deiner, K., Altermatt, F., 2014. Transport distance of invertebrate environmental DNA in a natural river. PLoS One 9. https://doi.org/10.1371/journal.pone.0088786.

Duleba, M., Földi, A., Micsinai, A., Várbíró, G., Mohr, A., Sipos, R., Szabó, G., Buczkó, K., Trábert, Z., Kiss, K.T., Bíró, T., Vadkerti, E., Ács, É., 2021. Applicability of diatom metabarcoding in the ecological status assessment of hungarian lotic and soda pan habitats. Ecol. Indic. 130. https://doi.org/10.1016/j.ecolind.2021.108105.

Elbrecht, V., Leese, F., 2015. Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass—sequence relationships with an innovative metabarcoding protocol. PLoS One 10, e0130324.
ESRI, 2011. ArcGIS Desktop: Release 10. Environmental Systems Research Institute, Redlands, CA.
Florencio, M., Díaz-Paniagua, C., Gómez-Rodríguez, C., Serrano, L., 2014. Biodiversity patterns in a macroinvertebrate community of a temporary pond network. Insect Conserv. Divers. 7, 4–21. https://doi.org/10.1111/icad.12029.
Ford, T.D., Pedley, H.M., 1996. A review of tufa and travertine deposits of the world. Earth Sci. Rev. 41, 117–175. https://doi.org/10.1016/S0012-8252(96)00030-X.
Gligora Udovič, M., Cvetkoska, A., Žutinić, P., Bosak, S., Stanković, I., Špoljarić, I., Mršić, G., Kralj Borojević, K., Ćukurin, A., Plenković-Moraj, A., 2017. Defining centric diatoms of most relevant phytoplankton functional groups in deep karst lakes. Hydrobiologia 788, 169–191. https://doi.org/10.1007/s10750-016-2996-z.
Gligora Udovič, M., Žutinić, P., Piltaver, I.K., Kulaš, A., Ozimec, R., Tofilovska, S., 2018. *Gomphosphenia plenkoviciae* sp. nov. a new species from crveno jezero, Croatia. Phytotaxa 351. https://doi.org/10.11646/phytotaxa.351.3.4.
Gloor, G.B., Macklaim, J.M., Pawlowsky-Glahn, V., Egozcue, J.J., 2017. Microbiome datasets are compositional: and this is not optional. Front. Microbiol. 8, 1–6. https://doi.org/10.3389/fmicb.2017.02224.
Gons, H.J., 1991. Diatom sedimentation loss reduction by resuspension in shallow lakes. SIL Proc. 1922–2010 (24), 729–733. https://doi.org/10.1080/03680770.1989.11898838.
Hanžek, N., Gligora Udovič, M., Kajan, K., Borics, G., Várbíró, G., Stoeck, T., Žutinić, P., Orlić, S., Stanković, I., 2021. Assessing ecological status in karstic lakes through the integration of phytoplankton functional groups, morphological approach and environmental DNA metabarcoding. Ecol. Indic. 131, 108166. https://doi.org/10.1016/j.ecolind.2021.108166.
Hartmann, A., Goldscheider, N., Wagener, T., Lange, J., Weiler, M., 2014. Karst water resources in a changing world. Rev. Geophys. 52, 218–242. https://doi.org/10.1002/2013RG000443.
Hofmann, G., Werum, M., Lange-Bertalot, H., 2013. Diatomeen im Süßwasser - Benthos von Mitteleuropa. Bestimmungsflora Kieselalgen für die ökologische Praxis. Koeltz Scientific Books, Königstein.
Hugerth, L.W., Andersson, A.F., 2017. Analysing microbial community composition through amplicon sequencing: from sampling to hypothesis testing. Front. Microbiol. 8, 1561. https://doi.org/10.3389/fmicb.2017.01561.
Hustedt, F., 1945. Diatomeen aus Seen und Quellgebieten der Balkan-Halbinsel. Arch. Hydrobiol. 40 (4), 867–973 12 pls.
Jahn, R., Zetzsche, H., Reinhardt, R., Gemeinholzer, B., 2007. Diatoms and DNA Barcoding: A Pilot Study on an Environmental Sample. https://doi.org/10.3372/cediatom.113.
Jarvie, H.P., King, S.M., Neal, C., 2017. Inorganic carbon dominates total dissolved carbon concentrations and fluxes in british rivers: application of the THINCARB model – thermodynamic modelling of inorganic carbon in freshwaters. Sci. Total Environ. 575, 496–512. https://doi.org/10.1016/j.scitotenv.2016.08.201.
Kahlert, M., Acs, E., Almeida, S., Blanco, S., Dreßler, M., Ector, L., Karjalainen, S., Liess, A., Mertens, A., Wal, J., Vilbaste, S., 2016. Quality assurance of diatom counts in Europe: towards harmonized datasets. Hydrobiologia https://doi.org/10.1007/s10750-016-2651-8 in press.
Kahlert, M., Kelly, M.G., Mann, D.G., Rimet, F., Sato, S., Bouchez, A., Keck, F., 2019. Connecting the morphological and molecular species concepts to facilitate species identification within the genus *Fragilaria* (Bacillariophyta). J. Phycol. 55, 948–970. https://doi.org/10.1111/jpy.12886.
Keck, F., 2020. diatbarcode: Access the diat.barcode Database. R Package Version 0.0.0.9000.
Keck, F., Vasselon, V., Rimet, F., Bouchez, A., Kahlert, M., 2018. Boosting DNA metabarcoding for biomonitoring with phylogenetic estimation of operational taxonomic units' ecological profiles. Mol. Ecol. Resour. 18, 1299–1309. https://doi.org/10.1111/1755-0998.12919.
Keck, F., Rimet, F., Vasselon, V., Bouchez, A., 2019. A Ready-to-use Database for DADA2: Diat.barcode_rbcL_312bp_DADA2 Based on Diat.barcode v7. https://doi.org/10.15454/HNI1EK.
Kelly, M.G., King, L., Jones, R.I., Barker, P.A., Jamieson, B.J., 2008. Validation of diatoms as proxies for phytobenthos when assessing ecological status in lakes. Hydrobiologia 610, 125–129. https://doi.org/10.1007/s10750-008-9427-8.
Kermarrec, L., Franc, A., Rimet, F., Chaumeil, P., Humbert, J.F., Bouchez, A., 2013. Next-generation sequencing to inventory taxonomic diversity in eukaryotic communities: a test for freshwater diatoms. Mol. Ecol. Resour. 13, 607–619. https://doi.org/10.1111/1755-0998.12105.
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.
Krammer, K., Lange-Bertalot, H., 1986. Bacillariophyceae. 1. Teil: Naviculaceae. Gustav Fischer Verlag, Stuttgart, New York.
Kuffel, A., Gray, A., Daeid, N.N., 2021. Impact of metal ions on PCR inhibition and RT-PCR efficiency. Int. J. Legal Med. 135, 63–72. https://doi.org/10.1007/s00414-020-02363-4.
Kulaš, A., Gligora Udovič, M., Ector, L., Van de Vijver, B., 2020. Analysis of the type material of *Achnanthes hauckiana* Grunow (Achnanthaceae, Bacillariophyceae). Bot. Lett. 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. https://doi.org/10.1016/j.ecolind.2021.107430.
Kulikovskiy, M., Maltsev, Y., Andreeva, S., Glushchenko, A., Gusev, E., Podunay, Y., Ludwig, T.V., Tusset, E., Kociolek, J.P., 2019. Description of a new diatom genus *Dorofeyukea* gen. nov. with remarks on phylogeny of the family stauroneidaceae. J. Phycol. 55, 173–185. https://doi.org/10.1111/jpy.12810.
Lange-Bertalot, H., Werum, M., Cantonati, M., Hofmann, G., 2017. Freshwater benthic diatoms of Central Europe: over 800 common species used in ecological assessment. English Edition With Updated Taxonomy and Added Species. Koeltz Botanical Books, Schmitten Oberreifenberg.
Leboucher, T., Budnick, W.R., Passy, S.I., Boutry, S., Jamoneau, A., Soininen, J., Vyverman, W., Tison-Rosebery, J., 2019. Diatom β-diversity in streams increases with spatial scale and decreases with nutrient enrichment across regional to sub-continental scales. J. Biogeogr. 46, 734–744. https://doi.org/10.1111/jbi.13517.
Leese, F., Altermatt, F., Bouchez, A., Ekrem, T., Hering, D., Meissner, K., Mergen, P., Pawlowski, J., Piggott, J., Rimet, F., Steinke, D., Taberlet, P., Weigand, A., Abarenkov, K., Beja, P., Bervoets, L., Björnsdóttir, S., Boets, P., Boggero, A., Hellström, M., 2016. DNAqua-net: developing new genetic tools for bioassessment and monitoring of aquatic ecosystems in Europe. Res. Ideas Outcomes 2, e11321. https://doi.org/10.3897/rio.2.e11321.
Li, J.-J., Dong, F., Huang, A.-P., Lian, Q.-Y., Peng, W.-Q., 2021. The migration and transformation of nitrogen in the Danjiangkou reservoir and upper stream: a review. Water https://doi.org/10.3390/w13192749.
Lionello, P., 2012. The Climate of the Mediterranean Region: From the Past to the Future.
llies, J., 1978. Limnofauna Europaea. Eine Zusammenstellung aller die europäischen Binnengewässer bewohnenden mehrzelligen Tierarten mit Angaben über ihre Verbreitung und Ökologie. A checklist of the animals inhabiting European inland waters, with accounts of their distribution and ecology (except Protozoa). Gustav Fischer Verlag, Stuttgart, New York, Amsterdam.
Mann, D.G., Crawford, R.M., Round, F.E., 2016. Bacillariophyta. In: Archibald, J.M., Simpson, A.G.B., Slamovits, C.H., Margulis, L., Melkonian, M., Chapman, D.J., Corliss, J.O. (Eds.), Handbook of the Protists. Springer, Cham, New York, pp. 1–62 https://doi.org/10.1007/978-3-319-32669-6_29-1.
Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 17 (1). https://doi.org/10.14806/ej.17.1.200 Next Gener. Seq. Data Anal.
Matočec, S.G., Bakran-Petricioli, T., Bedek, J., Bukovec, D., Buzjak, S., Franičević, M., Jalžić, B., Kerovec, M., Kletečki, E., Kralj, J., Kružić, P., Kučinić, M., Kuhta, M., Matočec, N., Ozimec, R., Rada, T., Štamol, V., Ternjej, I., Tvrtković, N., 2002. An overview of the cave and interstitial biota of Croatia. Nat. Croat. 11, 1–102.
McQuoid, M.R., Hobson, L.A., 1996. Diatom resting stages. J. Phycol. 32, 889–902. https://doi.org/10.1111/j.0022-3646.1996.00889.x.
Mortágua, A., Vasselon, V., Oliveira, R., Elias, C., Chardon, C., Bouchez, A., Rimet, F., João Feio, M., Almeida, F.P., S, 2019. Applicability of DNA metabarcoding approach in the bioassessment of Portuguese rivers using diatoms. Ecol. Indic. 106, 105470. https://doi.org/10.1016/j.ecolind.2019.105470.
Nistal-García, A., García-García, P., García-Girón, J., Borrego-Ramos, M., Blanco, S., Bécares, E., 2021. DNA metabarcoding and morphological methods show complementary patterns in the metacommunity organization of lentic epiphytic diatoms. Sci. Total Environ. 786, 147410. https://doi.org/10.1016/j.scitotenv.2021.147410.
Novis, P.M., Braidwood, J., Kilroy, C., 2012. Small diatoms (Bacillariophyta) in cultures from the Styx River, New Zealand, including descriptions of three new species. Phytotaxa 64, 11–45. https://doi.org/10.11646/phytotaxa.64.1.3.
Official gazette, 1985. Law on the Proclamation of the National Park "Krka".
Official gazette, 2019. Regulation on water quality standard 45 pp (in Croatian). https://narodne-novine.nn.hr/clanci/ sluzbeni/2019_10_96_1879.html.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2019. vegan: community ecology package. https://CRAN.R-project.org/package=vegan.
Opel, K.L., Chung, D., McCord, B.R., 2010. A study of PCR inhibition mechanisms using real time PCR. J. Forensic Sci. 55, 25–33. https://doi.org/10.1111/j.1556-4029.2009.01245.x.
Pawlowski, J., Kelly-Quinn, M., Altermatt, F., Apothéloz-Perret-Gentil, L., Beja, P., Boggero, A., Borja, A., Bouchez, A., Cordier, T., Domaizon, I., Feio, M.J., Filipe, A.F., Fornaroli, R., Graf, W., Herder, J., van der Hoorn, B., Iwan Jones, J., Sagova-Mareckova, M., Moritz, C., Barquín, J., Piggott, J.J., Pinna, M., Rimet, F., Rinkevich, B., Sousa-Santos, C., Specchia, V., Trobajo, R., Vasselon, V., Vitecek, S., Zimmerman, J., Weigand, A., Leese, F., Kahlert, M., 2018. The future of biotic indices in the ecogenomic era: integrating (e)DNA metabarcoding in biological assessment of aquatic ecosystems. Sci. Total Environ. 637–638, 1295–1310. https://doi.org/10.1016/j.scitotenv.2018.05.002.
Pérez-Burillo, J., Trobajo, R., Vasselon, V., Rimet, F., Bouchez, A., Mann, D.G., 2020. Evaluation and sensitivity analysis of diatom DNA metabarcoding for WFD bioassessment of Mediterranean rivers. Sci. Total Environ. 727, 138445. https://doi.org/10.1016/j.scitotenv.2020.138445.
Perica, D., Orešić, D., Trajbar, S., 2005. Geomorfološka obilježja doline i poriječja rijeke krke s osvrtom na dio od knina do Bilušića Buka. Geoadria 10, 131–156.
Pinseel, E., Vanormelingen, P., Hamilton, P.B., Vyverman, W., Van de Vijver, B., Kopalova, K., 2017. Molecular and morphological characterization of the *Achnanthidium minutissimum* complex (Bacillariophyta) in petuniabukta (Spitsbergen, high Arctic) including the description of *A. digitatum* sp. nov. Eur. J. Phycol. 52, 264–280. https://doi.org/10.1080/09670262.2017.1283540.
Pissaridou, P., Vasselon, V., Christou, A., Chonova, T., Papatheodoulou, A., Drakou, K., Tziortzis, I., Dörflinger, G., Rimet, F., Bouchez, A., Vasquez, M.I., 2021. Cyprus' diatom diversity and the association of environmental and anthropogenic influences for ecological assessment of rivers using DNA metabarcoding. Chemosphere 272, 129814. https://doi.org/10.1016/j.chemosphere.2021.129814.
Potapova, M., Hamilton, P.B., 2007. Morphological and ecological variation within the *Achnanthidium minutissimum* (Bacillariophyceae) species complex. J. Phycol. 43, 561–575. https://doi.org/10.1111/j.1529-8817.2007.00332.x.
Poulíčková, A., Špačková, J., Kelly, M.G., Duchoslav, M., Mann, D.G., 2008. Ecological variation within *Sellaphora* species complexes (Bacillariophyceae): specialists or generalists? Hydrobiologia 614 (1), 373–386. https://doi.org/10.1007/s10750-008-9521-y.

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.

R Core Team, 2021. R: a language and environment for statistical computing. URLR Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rarick, J., Wu, S., Worth, F., Lee, S.S., 2020. EPA Public Access. 1, pp. 10–12.

Rimet, F., Vasselon, V., A.-Keszte, B., Bouchez, A., 2018. Do we similarly assess diversity with microscopy and high-throughput sequencing? Case of microalgae in lakes. Org. Divers. Evol. 18, 51–62. https://doi.org/10.1007/s13127-018-0359-5.

Rimet, F., Gusev, E., Kahlert, M., Kelly, M.G., Kulikovskiy, M., Maltsev, Y., Mann, D.G., Pfannkuchen, M., Trobajo, R., Vasselon, V., Zimmermann, J., Bouchez, A., 2019. Diat. Barcode, an open-access curated barcode library for diatoms. Sci. Rep. 9, 15116. https://doi.org/10.1038/s41598-019-51500-6.

Rivera, S.F., Vasselon, V., Jacquet, S., Bouchez, A., Ariztegui, D., Rimet, F., 2018. Metabarcoding of lake benthic diatoms: from structure assemblages to ecological assessment. Hydrobiologia 807, 37–51. https://doi.org/10.1007/s10750-017-3381-2.

Rivera, S., Vasselon, V., Bouchez, A., Rimet, F., 2020. Diatom metabarcoding applied to large scale monitoring networks: optimization of bioinformatics strategies using mothur software. Ecol. Indic. 109. https://doi.org/10.1016/j.ecolind.2019.105775.

Rott, E., Pfister, P., van Dam, H., Pipp, E., Pall, K., Binder, N., Ortler, K., 1999. Indikationslisten für Aufwuchsalgen. Teil 2: Trophieindikation sowie geochemische Präferenz, taxonomische und toxikologische Anmerkungen. Bundesministerium für Land- und Forstwirtschaft, Wien (248 pp).

Round, F.E., Basson, P.W., 1997. A new monoraphid diatom genus (*Pogoneis*) from Bahrain and the transfer of previously described species *A. hungarica* and *A. taeniata* to new genera. Diatom Res. 12, 71–81. https://doi.org/10.1080/0269249X.1997.9705403.

Schöll, F., Birk, S., Böhmer, J., 2012. XGIG Large River Intercalibration Exercise – WFD Intercalibration Phase 2: Milestone 6 Report – BQE: Phytobenthos. European Commission Directorate General JRC Joint Research Centre Institute of Environment and Sustainability (73 pp).

Scholten, J.C.M., Stams, A.J.M., 1995. The effect of sulfate and nitrate on methane formation in a freshwater sediment. Antonie Van Leeuwenhoek 68, 309–315. https://doi.org/10.1007/BF00874141.

Sertić Perić, M., Kepčija, R.M., Miliša, M., Gottstein, S., Lajtner, J., Dragun, Z., Marijić, V.F., Krasnići, N., Ivanković, D., Erk, M., 2018. Benthos-drift relationships as proxies for the detection of the most suitable bioindicator taxa in flowing waters – a pilot-study within a Mediterranean karst river. Ecotoxicol. Environ. Saf. 163, 125–135. https://doi.org/10.1016/j.ecoenv.2018.07.068.

Šiljeg, A., Marić, I., Cukrov, N., Domazetović, F., Roland, V., 2020. A multiscale framework for sustainable management of tufa-forming watercourses: a case study of National Park "Krka", Croatia. Water https://doi.org/10.3390/w12113096.

Smith, K., Darwall, W., Carrizo, Numa, C., Barrios, V., Freyhof, J., 2014. Freshwater Key Biodiversity Areas in the Mediterranean Basin Hotspot: Informing Species Conservation and Development Planning in Freshwater Ecosystems. https://doi.org/10.2305/IUCN.CH.2014.SSC-OP.52.en.

Soininen, J., 2007. Environmental and spatial control of freshwater diatoms - a review. Diatom Res. 22, 473–490. https://doi.org/10.1080/0269249X.2007.9705724.

Špoljar, M., Habdija, I., Primc-Habdija, B., Sipos, L., 2005. Impact of environmental variables and food availability on rotifer assemblage in the karstic barrage Lake Visovac (Krka River, Croatia). Int. Rev. Hydrobiol. 90, 555–579. https://doi.org/10.1002/iroh.20051079.

Srdoč, D., Horvatinčić, N., Obelic, B., Krajcar Bronić, I., Sliepčević, A., 1985. Calcite deposition processes in karst waters with special emphasis on the Plitvice Lakes, Yugoslavia. Carsus Iugosl. 11, 101–204.

Stevenson, R.J., Pan, Y., van Dam, H., 2010. Assessing environmental conditions in rivers and streams with diatoms. In: Stoermer, E.F., Smol, J.P. (Eds.), The Diatoms: Applications for the Environmental and Earth Sciences. Cambridge University Press, Cambridge, pp. 57–85 https://doi.org/10.1017/CBO9780511763175.005.

Student, 1908. The probable error of a mean. Biometrika 6, 1–25. https://doi.org/10.1093/biomet/6.1.1.

Tapolczai, K., Vasselon, V., Bouchez, A., Stenger-Kovács, C., Padisák, J., Rimet, F., 2019. The impact of OTU sequence similarity threshold on diatom-based bioassessment: a case study of the rivers of Mayotte (France, Indian Ocean). Ecol. Evol. 9, 166–179. https://doi.org/10.1002/ece3.4701.

Tapolczai, K., Selmeczy, G.B., Szabó, B., B-Béres, V., Keck, F., Bouchez, A., Rimet, F., Padisák, J., 2021. The potential of exact sequence variants (ESVs) to interpret and assess the impact of agricultural pressure on stream diatom assemblages revealed by DNA metabarcoding. Ecol. Indic., 122 https://doi.org/10.1016/j.ecolind.2020.107322.

Trobajo, R., Mann, D.G., Clavero, E., Evans, K.M., Vanormelingen, P., McGregor, R.C., 2010. The use of partial *cox*1, *rbc*L and LSU rDNA sequences for phylogenetics and species identification within the *Nitzschia palea* species complex (Bacillariophyceae). Eur. J. Phycol. 45, 413–425. https://doi.org/10.1080/09670262.2010.498586.

Vasselon, V., Domaizon, I., Rimet, F., Kahlert, M., Bouchez, A., 2017a. Application of high-throughput sequencing (HTS) metabarcoding to diatom biomonitoring: do DNA extraction methods matter? Freshw. Sci. 36, 162–177. https://doi.org/10.1086/690649.

Vasselon, V., Rimet, F., Tapolczai, K., Bouchez, A., 2017b. Assessing ecological status with diatoms DNA metabarcoding: scaling-up on a WFD monitoring network (Mayotte island, France). Ecol. Indic. 82, 1–12. https://doi.org/10.1016/j.ecolind.2017.06.024.

Vasselon, V., Bouchez, A., Rimet, F., Jacquet, S., Trobajo, R., Corniquel, M., Tapolczai, K., Domaizon, I., 2018. Avoiding quantification bias in metabarcoding: application of a cell biovolume correction factor in diatom molecular biomonitoring. Methods Ecol. Evol. 9, 1060–1069. https://doi.org/10.1111/2041-210X.12960.

Vasselon, V., Rimet, F., Domaizon, I., Monnier, O., Reyjol, Y., Bouchez, A., 2019. Assessing pollution of aquatic environments with diatoms' DNA metabarcoding: experience and developments from France water framework directive networks. Metabarcoding Metagenomics 3, 101–115. https://doi.org/10.3897/mbmg.3.39646.

Visco, J.A., Apothéloz-Perret-Gentil, L., Cordonier, A., Esling, P., Pillet, L., Pawlowski, J., 2015. Environmental monitoring: inferring the diatom index from next-generation sequencing data. Environ. Sci. Technol. 49, 7597–7605. https://doi.org/10.1021/es506158m.

Wetzel, C.E., Van de Vijver, B., Blanco, S., Ector, L., 2019. On some common and new cavum–bearing *Planothidium* (Bacillariophyta) species from freshwater. Fottea 19, 50–89. https://doi.org/10.5507/FOT.2018.016.

WFD, 2000. Directive 2000/60/ec of the European Parliament and of the Council 22.12.2000. Off. J. Eur. Communities L327 (1-72 pp).

Whitton, B.A., Kelly, M.G., 1995. Use of algae and other plants for monitoring rivers. Aust. J. Ecol. 20, 45–56. https://doi.org/10.1111/j.1442-9993.1995.tb00521.x.

Xie, R., Zhao, G., Yang, J., Wang, Zhihao, Xu, Y., Zhang, X., Wang, Zijian, 2021. eDNA metabarcoding revealed differential structures of aquatic communities in a dynamic freshwater ecosystem shaped by habitat heterogeneity. Environ. Res. 201, 111602. https://doi.org/10.1016/j.envres.2021.111602.

Zabell, S.L., 2008. On Student's 1908 article "The probable error of a mean". J. Am. Stat. Assoc. 103, 1–7. https://doi.org/10.1198/016214508000000030.

Zgrundo, A., Lemke, P., Pniewski, F., Cox, E., Latała, A., 2013. Morphological and molecular phylogenetic studies on *Fistulifera saprophila*. Diatom Res. 28. https://doi.org/10.1080/0269249X.2013.833136.

Zimmermann, J., Glöckner, G., Jahn, R., Enke, N., Gemeinholzer, B., 2015. Metabarcoding vs. morphological identification to assess diatom diversity in environmental studies. Mol. Ecol. Resour. 15, 526–542. https://doi.org/10.1111/1755-0998.12336.

Žutinić, P., Kulaš, A., Levkov, Z., Šušnjara, M., Orlić, S., Kukić, S., Goreta, G., Valić, D., Udovič, M.G., 2020a. Ecological status assessment using periphytic diatom communites - case study Krka River. Maced. J. Ecol. Environ. 22, 29–44.

Žutinić, P., Gligora Udovič, M., Šušnjara, M., Kulaš, A., 2020b. Report on Fitting of Phytobenthos Classification Method With the Results of the Completed Intercalibration of the EC GIG (R-E2, R-E3, R-EX5, R-EX6, R-EX7 and R-EX8). Croatian Waters, Zagreb (21 pp).

PUBLICATION IV

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

Antonija Kulaš[1*], Petar Žutinić[1], Vesna Gulin[1], Renata Matoničkin Kepčija[1], Mirela Sertić Perić[1], Sandi Orlić[2,3], Ines Sviličić Petrić[2], Tamara Marković[4], Marija Gligora Udovič[1]

[1]University of Zagreb, Faculty of Science, Department of Biology, Rooseveltov trg 6, HR-10000 Zagreb, Croatia

[2]Ruđer Bošković Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia

[3]Center of Excellence for Science and Technology Integrating Mediterranean Region (STIM), HR-10000 Zagreb, Croatia

[4]Croatian Geological Survey, Department of Hydrogeology and Engineering Geology, Sachsova 2, HR-10000 Zagreb, Croatia

*Correspondence: Antonija Kulaš: antonija.kulas@biol.pmf.hr

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. The shade plot, in terms of relative abundance of the major protist groups, showed that there was 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-Stadniczeñko 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-Stadniczeñko 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 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).

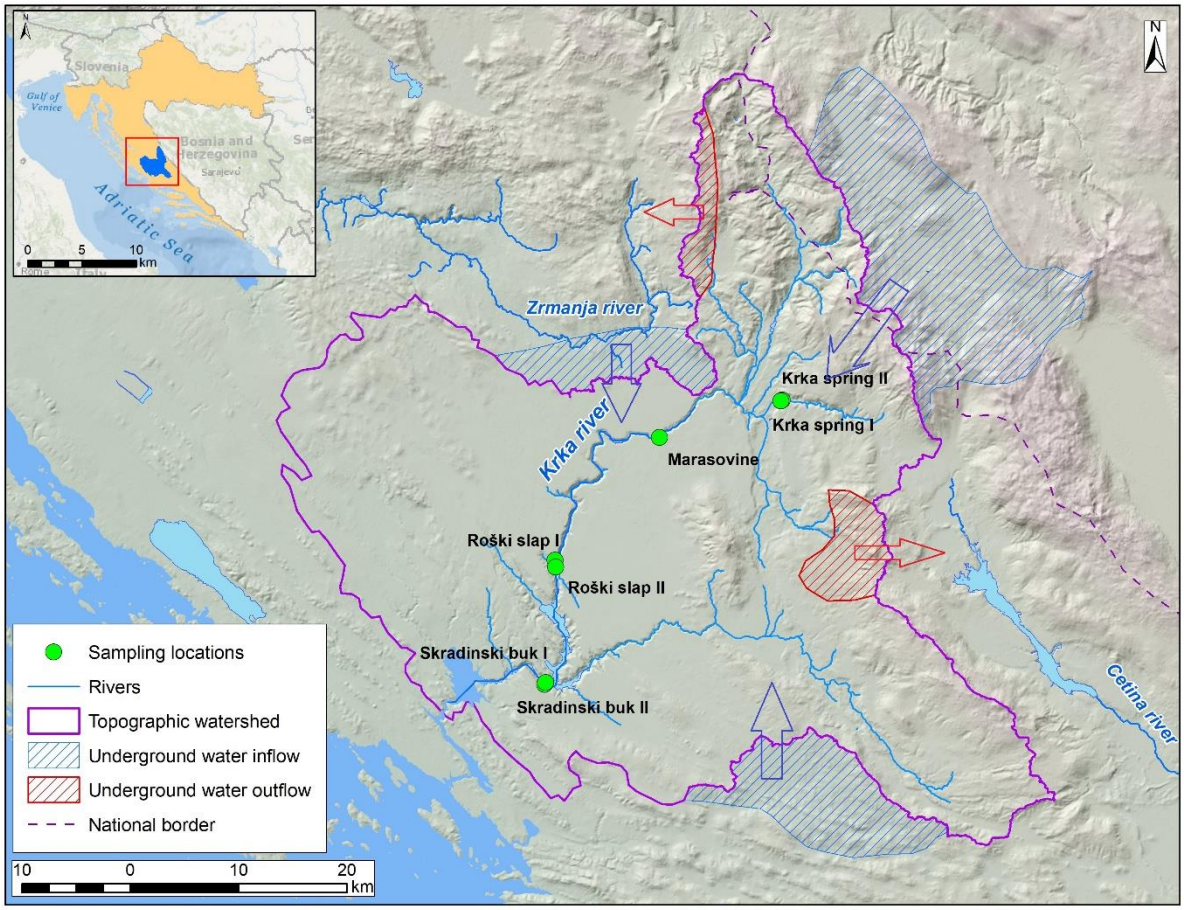
Karst rivers in the Mediterranean region represent unique diversity hotspots of various aquatic organisms, especially protists (Tierno de Figueroa et al. 2013; Lai et al. 2019; Gligora Udovič et al. 2022). The pronounced process of karstification has led to distinctive climatic and environmental conditions 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.

2015). 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; Gligora Udovič et al. in press), 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 characterised 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).



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

101

102 ***Sampling procedure***

103 Sampling was performed between 21 – 23 September 2017 and included taking three individual
104 samples 10 m apart at each sampling location and selecting each successive habitat upstream of the
105 previously sampled location. At sites where, longitudinal sampling was not possible due to waterfalls,
106 transverse sampling was conducted. A sample was represented by randomly collecting 5 stones or tufa
107 (composite sample) and scraping off the substrate (periphyton) from both light- and dark- exposed sides
108 of tufa/stones at each sampling location. In total, 42 samples for DNA extraction were stored in Falcon
109 tubes (50 mL), placed on ice during transport to the laboratory, and stored at -20 °C until further
110 processing.

111

112

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 x 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 2-8°C. The next step was removal of inhibitors with the C3 solution and again incubation at 2-8°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 µl 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′-GTACACACCGCCCGTC-3′) and EukB (5′-TGATCCTTCTGCAGGTTCACCTAC-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® Ultra™ 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). Number of recorded taxonomically assigned OTUs (*S*), Margalef (*d*), Shannon-Wiener (*H'*) and Simpson (*1- 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 (ω) 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), *Amphibiocystidium*, *Anurofeca* and *Nuclearia* (Mesomycetozoa), *Monocystis* (Apicomplexa).

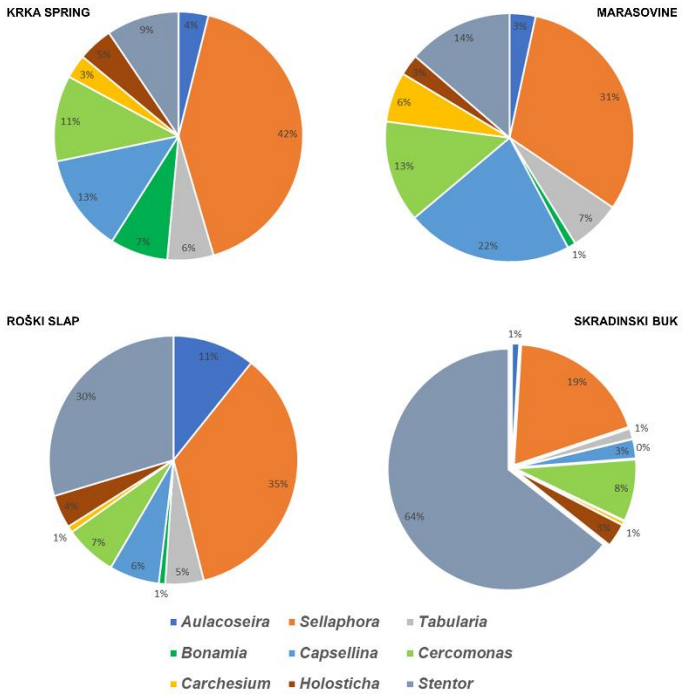


Fig. 2 The most abundant genera within three major groups at all sampled locations (the most abundant genera were calculated from the abundance of taxonomically assigned OTUs per location)

The shade plot included only the major protist groups, which contributed for at least 10% of the protists 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 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.

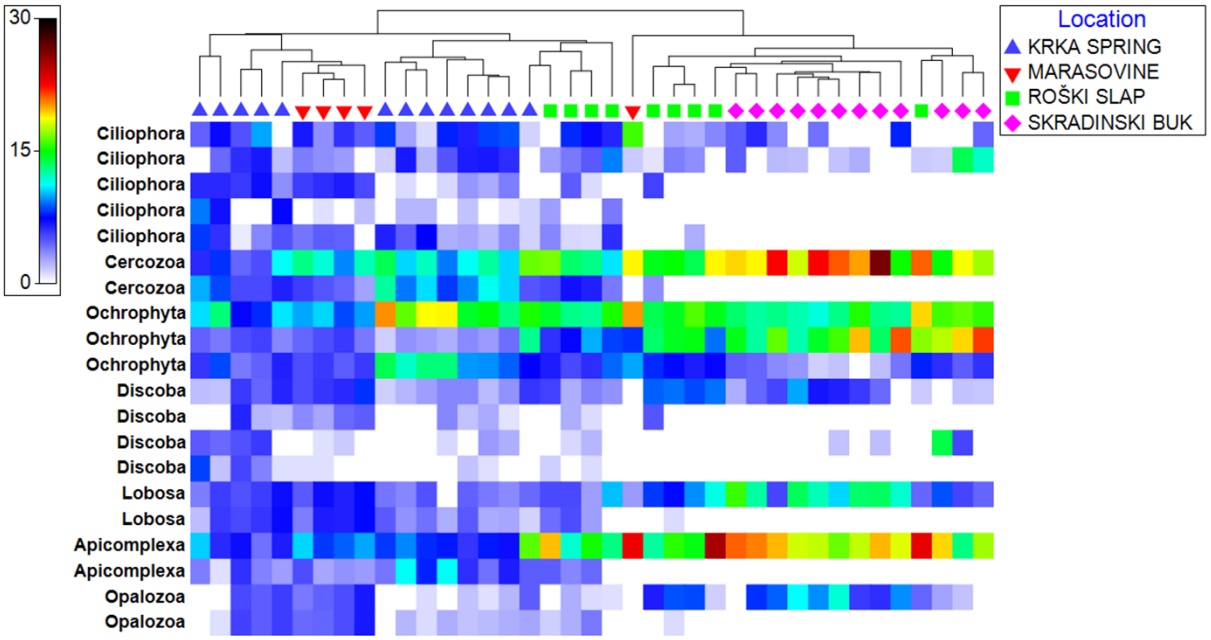


Fig. 3 Shade plot showing relationships among clusters of samples and major protist groups which contributed for at least 10% of the protists OTUs abundances

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 Maraovine) than in the downstream (Roški slap and Skradinski buk).

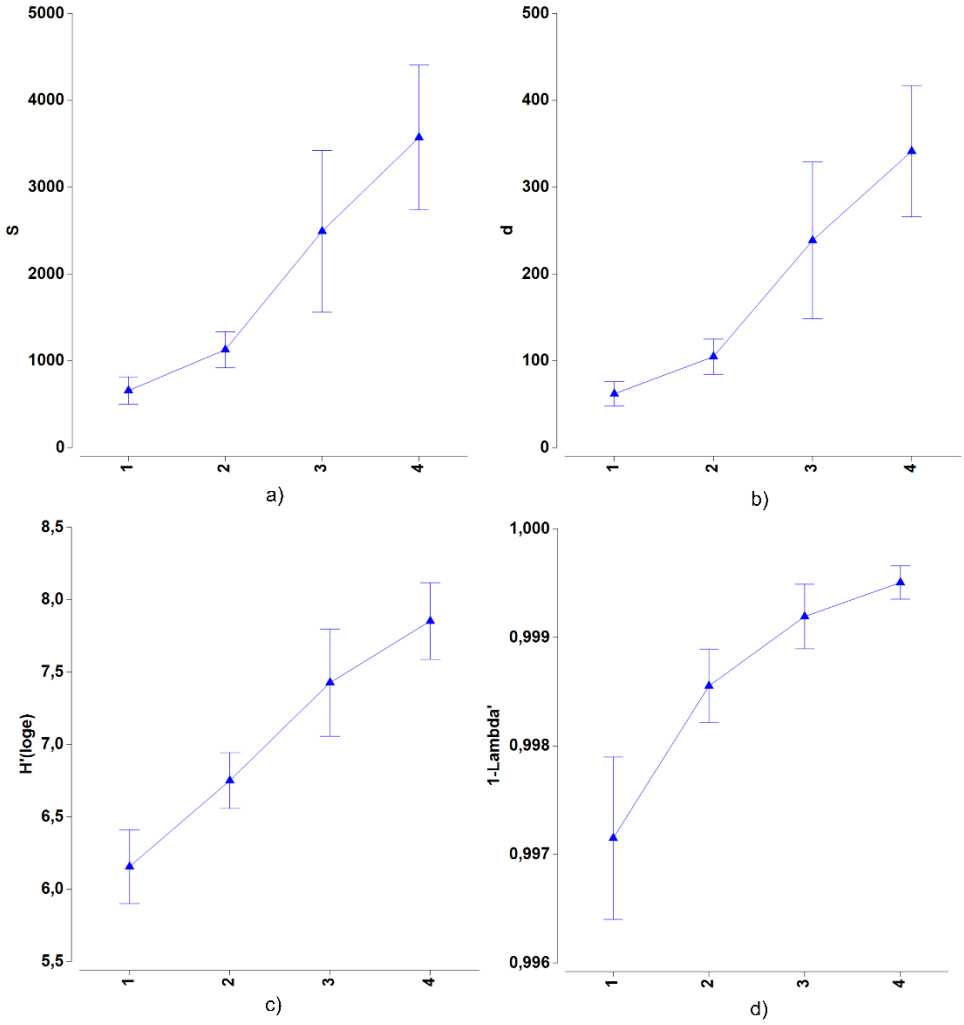


Fig. 4 The average alpha diversity on each sampling location was expressed by the number of taxonomically assigned OTUs (*S*), Margalef (*d*), Shannon-Wiener (*H'*) and Simpson (*1- ambda'*) indices. Error bars denote mean SD, numbers 1 to 4 on x axis denote different sampling locations: 1 = Krka spring;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

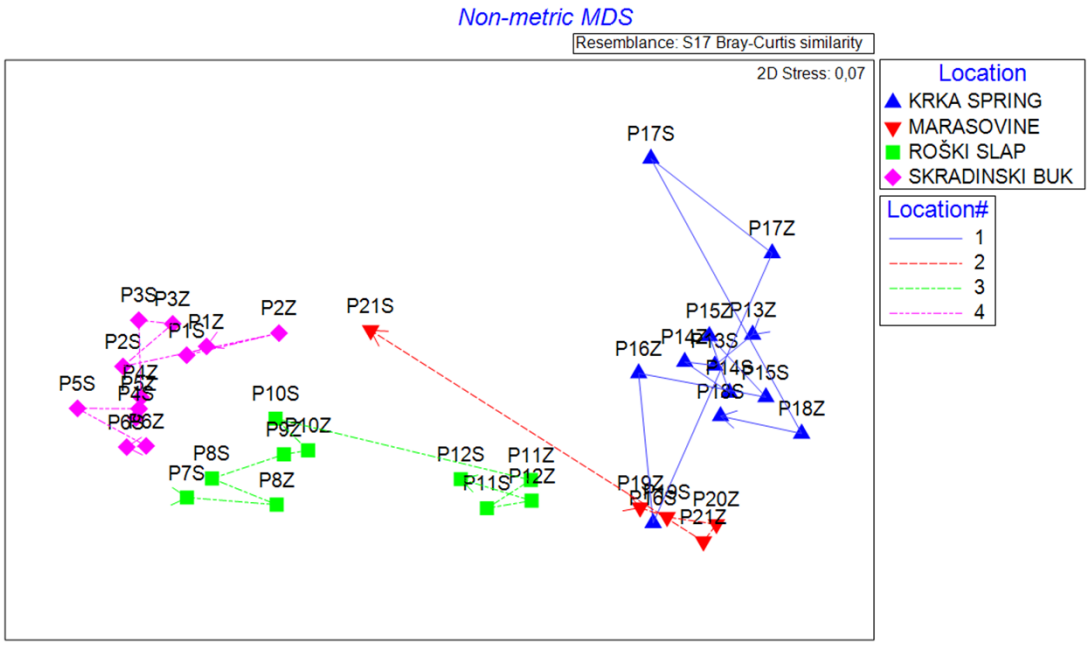


Fig. 5 Position of sampling locations in the non-metric multidimensional scaling analysis (NMDS) based on Bray-Curtis similarity index for all protist groups with categorical factor applied as the drawn trajectories, specifying all groups in the same order by the selected factors: order of sampling locations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

Average Taxonomic Distinctness AvTD (Δ+)

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 Δ+ 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 Δ+, while Skradinski buk had the lowest taxonomic distinctness (below 90%).

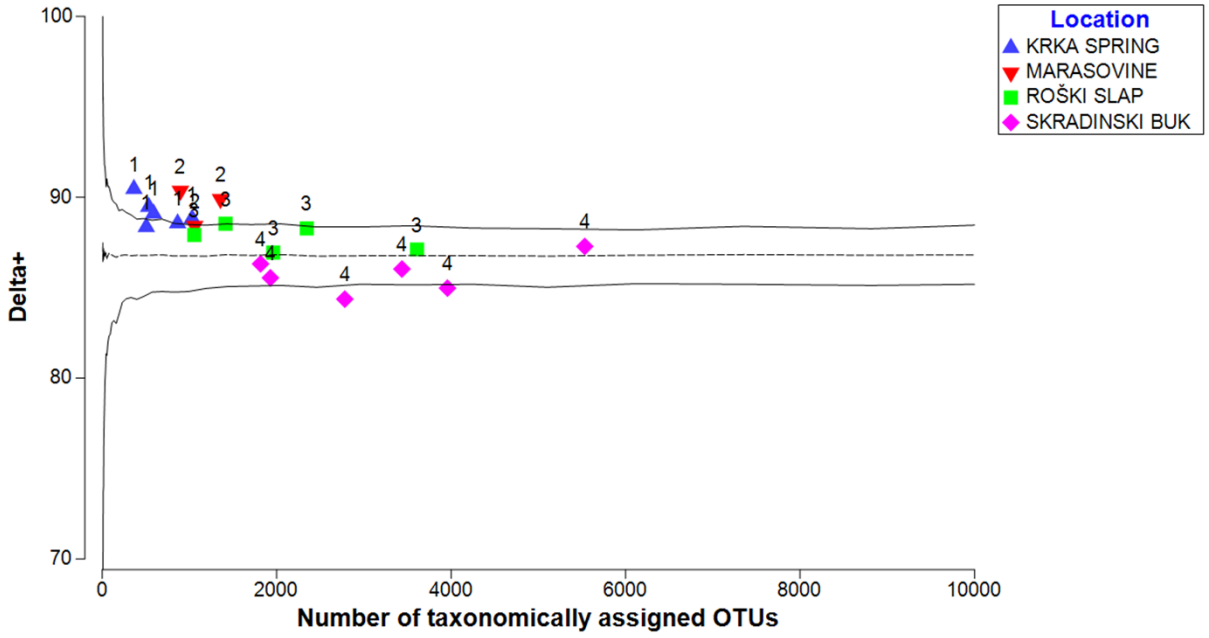


Fig. 6 Funnel plots for average taxonomic distinctness (Δ+) versus number of taxonomically assigned OTUs. The dashed line indicates the mean Δ+ for the taxonomically assigned OTUs and the full lines represent 100% probability limits. Colour symbols and numbers represent different locations of the river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

Discussion

The Krka River is a hotspot for a wide variety of aquatic organisms, especially protists. Tufa barriers provide a favourable substrate for colonisation and growth of periphyton, which is an important biomediator in the tufa deposition process (Risse-Buhl and Küsel 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 recognised that metabarcoding on V9 region of the SSU rRNA genes only allows correct identification down 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 meters high and extends over a length of 650 meters and a width of 400 meters, with a special formation called “the cascade” on the upper side and a new additional spring water inflow (Štambuk-Giljanović 2006). Therefore, first part of the sampling 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

294 geographically closer to the upstream part of the river so the community structure is more similar to
295 location Marasovine, while the second part of Roški slap flows into Lake Visovac, which has the greatest
296 influence on Skradinski buk.

297 According to the most abundant genera, Roški slap showed similarity with Marasovine in terms
298 of diatom and ciliate dominance. The observed results for the different diversity indices indicated a
299 strong competition between taxa distributed within the two protist groups on these two locations
300 (Estrada-Villegas et al. 2012). In contrast, diatoms demonstrated a clear dominance at the Krka spring,
301 whilst ciliates dominated at Skradinski buk. The dominance of ciliates in the downstream sections of
302 the river may be related to local microhabitat complexity, as the abundance of various tufa-depositing
303 forms is much higher at the downstream locations, especially Skradinski buk (Bonacci et al. 2017).
304 Although this study did not focus on community structure in terms of defining each species' occurrence
305 and abundance, it could still be observed that over 60% of relative abundance of the genus *Stentor* was
306 detected in Skradinski buk. This location is situated downstream of Lake Visovac and represents a
307 unique lake outlet characterised by higher temperature and pH values and high DOC (dissolved organic
308 carbon) values (Kulaš et al. 2021). As lakes are generally more productive systems (Špoljar et al. 2005),
309 the influence of Lake Visovac is reflected in a higher amount of dissolved organic matter and a
310 correspondingly higher abundance of ciliate OTUs corresponding to filter feeders (Kulaš et al. 2021).
311 These conditions may reflect biotic interactions that depend on DOC and the availability of bacteria as
312 a food source for ciliates (Hauptmann et al. 2016). Caution should always be exercised in interpreting
313 the most common taxa recorded by amplicon sequences. There are still problems in translating
314 abundance from sequence data to biological abundance, as variation in rDNA copy number among taxa
315 may be one of the main reasons for incongruent results for Alveolata sequences (ciliates and
316 dinoflagelates), as they make up the largest proportion of sequence data (Medinger et al. 2010). Thus,
317 the highest abundance of ciliates in a data set does not necessarily mean that ciliates are so abundant
318 here. However, our results were confirmed and compared with the microscopic analysis in the earlier
319 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 revitalised 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.), Gulin et al. (2021) at Skradinski buk resulted in changes in hydromorphology and a decrease in the abundance of the protozoan community in the periphyton. 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 town 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.

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

355 Generally, beta diversity indicates the level of variation in composition (Koleff et al. 2003). One
356 of its driving factors is habitat heterogeneity, which can create niches favouring certain species over
357 others, as some of the samples were collected on stones and others on tufa (Astorga et al. 2014). It can
358 help clarify processes associated with community composition, which typically break down into taxa
359 exchange and richness differences in gain or loss (Ge et al. 2022). Interestingly, the location effect was
360 also confirmed by NMDS analysis using the PERMANOVA test, which grouped/clustered samples
361 according to locations. The location effect can be explained by the physical structure of the habitat. Tufa
362 barriers are a product of calcium carbonate deposition where physical and chemical properties of water,
363 geologic substrate, and biota play an intertwining role. All of these can influence the biotic community
364 through hydrogeological processes that include both subsurface and surface water flow, often with high
365 flow velocities and discharge (Tamburini and Menichetti 2020).

366 The next main driver of diversity composition is productivity, where more productive areas
367 support higher regional diversity. Productivity is linked to beta diversity and can result in high regional
368 diversity and increased niche specialisation, especially for particularly rare species (Currie et al. 2004).
369 In the current case, this driver is connected with the Krka spring zone area. Previous studies have
370 confirmed the spring zones of karstic rivers being inhabited by various organisms (Mogna et al. 2015;
371 Lai et al. 2020), particularly diatoms as one of the most diverse groups (Cantonati et al. 2012). The
372 group Ochrophyta were recorded as the most abundant primary producers at the Krka spring. About
373 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 ---- **Additional file**: 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.

Acknowledgments: 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.

Author Contributions:

"A.K. - performed formal analysis, conceptualisation, data curation, visualisation, 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. – contributed with 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 with methodology preparation, review and editing of the manuscript; S.O. - contributed with methodology preparation, review and editing of the manuscript; I.S.P. - contributed with the validation, review and editing of the manuscript; T.M. - contributed with the review and editing of the manuscript; M.G.U. - as supervisor contributed with the conception of the project, validation, finding resources and acquisition, and preparation of the original draft. All authors read and approved the final manuscript."

Funding: This study was financial supported by 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.

Data Availability: 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 no competing interests.

References

Albano PG, Gallmetzer I, Haselmair A, Tomašových A, Stachowitsch M, Zuschin 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 N ew Z ealand 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*. Journal of Applied Ecology 49:357–366. https://doi.org/10.1111/j.1365-2664.2011.02096.x

Bini LM, Landeiro VL, Padial 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, 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

Bonacci O, Ljubenkov 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

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, Jamy M (2021) Diversity and ecology of protists revealed by metabarcoding. Current Biology 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. Freshwater Science 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-EPlymouth.

Cukrov N, Cmuk P, Mlakar 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 (2015) Freshwater Key Biodiversity Areas in the Mediterranean Basin Hotspot. International Union for Conservation of Nature

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, Kružlicová D, Nemeček P, Ivanković D, Vardić Smrzlić 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, 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

Fiore-Donno AM, Richter-Heitmann T, Degrune F, Dumack K, Regan KM, Marhan S, Boeddinghaus RS, Friedrich MW, Kandeler 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

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

Gligora Udovič M, Kulaš A, Šušnjara M, Arapov J, Blanco S, Levkov Z (2022) *Cymbopleura amicula* stat nov. et. nom. nov. (Bacillariophyceae)—a rare diatom species from a karst river in Croatia. Phytotaxa 532:139–151. https://doi.org/10.11646/phytotaxa.532.2.2

Gligora Udovič M, Šušnjara M, Kulaš A, Goreta G, Arapov J, Levkov Z (2023) One new species of *Aneumastus* D.G. Mann & Stickle (Bacillariophyta) from River Krka, Croatia. Fottea (accepted)

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. Science of The Total Environment 703:135519. https://doi.org/10.1016/j.scitotenv.2019.135519

Gran-Stadniczeñko 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, Berney C, Boutte C, Burgaud G, de Vargas C, Decelle J, Del Campo J, Dolan JR, Dunthorn M, 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 I, Fajković H, Križnjak K (2021) Environmental and periphyton response to stream revitalization – A pilot study from a tufa barrier. Ecological Indicators 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:. 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. Journal of Theoretical Biology 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. Journal of Resources and Ecology 3:236. https://doi.org/10.5814/j.issn.1674-764x.2012.03.006

Jones AM, Berkelmans R, Houston W (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 (Achnanthaceae, Bacillariophyceae). Botany Letters 167:439–452. https://doi.org/10.1080/23818107.2020.1808527

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. Science of The Total Environment 829:154536. https://doi.org/10.1016/j.scitotenv.2022.154536

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. Ecological Indicators 124:107430. https://doi.org/10.1016/j.ecolind.2021.107430

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 Biosystems - An International Journal Dealing with all Aspects of Plant Biology 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, Pawlowski 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

592 diversity of eukaryotic microorganisms. Molecular Ecology 19:32–40.
593 https://doi.org/10.1111/j.1365-294X.2009.04478.x

594 Metz S, Huber P, Accattatis V, Lopes dos Santos A, Bigeard E, Unrein F, Chambouvet A, Not F, Lara
595 E, Devercelli M (2022) Freshwater protists: unveiling the unexplored in a large floodplain
596 system. Environmental Microbiology 24:1731–1745. https://doi.org/10.1111/1462-2920.15838

597 Minerovic AD, Potapova MG, Sales CM, Price JR, Enache, MD (2020) 18S-V9 DNA metabarcoding
598 detects the effect of water-quality impairment on stream biofilm eukaryotic assemblages.
599 Ecological Indicators 113:106225. https://doi.org/10.1016/j.ecolind.2020.106225

600 Mogna M, Cantonati M, Andreucci F, Angeli N, Berta G, Miserere L (2015) Diatom communities and
601 vegetation of springs in the south-western Alps. Acta Botanica Croatica 74:265–285.
602 https://doi.org/10.1515/botcro-2015-0024

603 Niesenbaum RA (2019) The Integration of Conservation, Biodiversity, and Sustainability. Sustainability
604 11:4676. https://doi.org/10.3390/su11174676

605 Official gazette (1985) Law on the Proclamation of the National Park "Krka"

606 Official gazette (2019) Regulation on water quality standard (45 pp). https://narodne-
607 novine.nn.hr/clanci/ sluzbeni/2019_10_96_1879.html.

608 Perica D, Orešić D, Trajbar S (2017) Geomorfološka obilježja doline i poriječja rijeke Krke s osvrtom
609 na dio od Knina do Bilušića buka. Geoadria 10:131–156. https://doi.org/10.15291/geoadria.55

610 Porter CP, Patton T (2016) Patterns of Fish Diversity and Community Structure Along the Longitudinal
611 Gradient of the Kiamichi River in Southeastern Oklahoma. Proc Okla Acad Sci 95:104–118

612 Primc-Habdija B, Habdija I, Matoničkin R, Špoljar, Maria (2005) Development of ciliate community
613 on artificial substrates associated with vertical gradients of environmental conditions in a karstic
614 lake. Arch. Hydrobiol. 164:513–527. https://doi.org/10.1127/0003-9136/2005/0164-0513

615 Primc-Habdija B, Matoničkin R (2005) A new freshwater folliculinid (Ciliophora) from the karstic
616 region of Croatia. European Journal of Protistology 41:37–43.
617 https://doi.org/10.1016/j.ejop.2004.09.004

618 Protasov A, Barinova S, Novoselova T, Sylaieva A (2019) The Aquatic Organisms Diversity,
619 Community Structure, and Environmental Conditions. Diversity 11:190.
620 https://doi.org/10.3390/d11100190

621 Risse-Buhl U, Küsel K (2009) Colonization dynamics of biofilm-associated ciliate morphotypes at
622 different flow velocities. Eur J Protistol 45:64–76. https://doi.org/10.1016/j.ejop.2008.08.001

623 Rusanov AG, Khromov VM (2016) Longitudinal distribution of periphyton algae in the Moskva river
624 under eutrophication. Water Resour 43:513–521. https://doi.org/10.1134/S0097807816030131

625 Selosse M-A, Charpin M, Not F (2017) Mixotrophy everywhere on land and in water: the *grand écart*
626 hypothesis. Ecol Lett 20:246–263. https://doi.org/10.1111/ele.12714

627 Silva DRO, Herlihy AT, Hughes RM, Macedo DR, Callisto M (2018) Assessing the extent and relative
628 risk of aquatic stressors on stream macroinvertebrate assemblages in the neotropical savanna.
629 Science of The Total Environment 633:179–188.
630 https://doi.org/10.1016/j.scitotenv.2018.03.127

631 Silva DRO, Herlihy AT, Hughes RM, Callisto M (2017) An improved macroinvertebrate multimetric
632 index for the assessment of wadeable streams in the neotropical savanna. Ecological Indicators
633 81:514–525. https://doi.org/10.1016/j.ecolind.2017.06.017

634 Smith HL, Anderson MJ, Gillanders BM, Connell SD (2014) Longitudinal variation and effects of
635 habitat on biodiversity of Australasian temperate reef fishes. J Biogeogr 41:2128–2139.
636 https://doi.org/10.1111/jbi.12359

637 Song Y, Cheng F, Ren P, Wang Z, Xie S (2019) Longitudinal recovery gradients of drifting larval fish
638 assemblages in the middle reach of the Yangtze River: impact of the Three Gorges Dam and
639 conservation implementation. Can J Fish Aquat Sci 76:2256–2267.
640 https://doi.org/10.1139/cjfas-2018-0409

641 Špoljar M, Habdija I, Primc-Habdija B, Sipos L (2005) Impact of Environmental Variables and Food
642 Availability on Rotifer Assemblage in the Karstic Barrage Lake Visovac (Krka River, Croatia).
643 Internat Rev Hydrobiol 90:555–579. https://doi.org/10.1002/iroh.200510791

644 Štambuk-Giljanović N (2006) Vode Dalmacije. Zavod za javno zdravstvo Županije splitsko-
645 dalmatinske (588 pp), Split

646 Stock A, Jürgens K, Bunge J, Stoeck T (2009) Protistan diversity in suboxic and anoxic waters of the
647 Gotland Deep (Baltic Sea) as revealed by 18S rRNA clone libraries. Aquat Microb Ecol 55:267–
648 284. https://doi.org/10.3354/ame01301

649 Stoeck T, Bass D, Nebel M, Christen R, Jones MDM, Breiner HW, Richards TA (2010) Multiple marker
650 parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in
651 marine anoxic water. Molecular Ecology 19:21–31. https://doi.org/10.1111/j.1365-
652 294X.2009.04480.x

653 Sundermann A, Stoll S, Haase P (2011) River restoration success depends on the species pool of the
654 immediate surroundings. Ecol Appl 21:1962–1971. https://doi.org/10.1890/10-0607.1

655 Sutela T, Vehanen T, Jounela P (2020) Longitudinal patterns of fish assemblages in European boreal
656 streams. Hydrobiologia 847:3277–3290. https://doi.org/10.1007/s10750-020-04330-x

657 Tamburini A, Menichetti M (2020) Groundwater Circulation in Fractured and Karstic Aquifers of the
658 Umbria-Marche Apennine. Water 12:1039. https://doi.org/10.3390/w12041039

659 Tanabe AS, Nagai S, Hida K, Yasuike M, Fujiwara A, Nakamura Y, Takano Y, Katakura S (2016)
660 Comparative study of the validity of three regions of the 18S-rRNA gene for massively parallel
661 sequencing-based monitoring of the planktonic eukaryote community. Mol Ecol Resour
662 16:402–414. https://doi.org/10.1111/1755-0998.12459

663 Thompson LR, Sanders JG, McDonald D, Amir A, Ladau J, Locey K, Prill RJ, Tripathi A, Gibbons SM,
664 Ackermann G, Navas-Molina JA, Janssen S, Kopylova E, Vázquez-Baeza Y, González A,
665 Morton JT, Mirarab S, Zech Xu Z, Jiang L, Haroon MF, Kanbar J, Zhu Q, Jin Song S,
666 Kosciolek T, Bokulich NA, Lefler J, Brislawn CJ, Humphrey G, Owens SM, Hampton-Marcell
667 J, McKenzie V, Fierer N, Fuhrman JA, Clauset A, Stevens RL, Shade A, Pollard KS,
668 Goodwin KD, Jansson JK, Knight R, Earth Microbiome Project Consortium (2017) A
669 communal catalogue reveals Earth's multiscale microbial diversity. Nature 551:457–463.
670 https://doi.org/10.1038/nature24621

671 Thukral AK (2017) A review on measurement of Alpha diversity in biology. Intern Jour Contemp
672 Microbiol 54:1. https://doi.org/10.5958/2395-146X.2017.00001.1

673 Tierno de Figueroa JM, López-Rodríguez MJ, Fenoglio S, Sánchez-Castillo P, Fochetti R (2013)
674 Freshwater biodiversity in the rivers of the Mediterranean Basin. Hydrobiologia 719:137–186.
675 https://doi.org/10.1007/s10750-012-1281-z

676 Vilenica M, Bilić M, Mičetić Stanković V, Kučinić M (2018) Mayfly ecological traits in a European
677 karst spring: species, microhabitats and life histories. Community Ecology 19:248–258.
678 https://doi.org/10.1556/168.2018.19.3.6

679 Watermeyer KE, Guillera-Arroita G, Bal P, Burgass MJ, Bland LM, Collen B, Hallam C, Kelly LT,
680 McCarthy MA, Regan TJ, Stevenson S, Wintle BA, Nicholson E (2021) Using decision science
681 to evaluate global biodiversity indices. Conservation Biology 35:492–501.
682 https://doi.org/10.1111/cobi.13574

683 West PT, Probst AJ, Grigoriev IV, Thomas BC, Banfield JF (2018) Genome-reconstruction for
684 eukaryotes from complex natural microbial communities. Genome Res 28:569–580.
685 https://doi.org/10.1101/gr.228429.117

686 Žutinić P, Kulaš A, Levkov Z, Šušnjara M, Orlić S, Kukić S, Goreta G, Valić D, Gligora Udovič M
687 (2020) Ecological status assessment using periphytic diatom communites - case study Krka
688 River. Macedonian Journal of Ecology and Environment 22:29–44.

689

Supplement information: Table S1. List of taxonomically assigned OTUs and number of reads from 39 obtained samples.

Supplement information: Table S2. Results of alpha diversity indices per samples and sampling location.

Table S2.

Sample	Location	S	d	*H'*	1- Lambda'
P13Z	Krka spring	505	47,80	5,965	0,9967
P13S	Krka spring	837	77,50	6,484	0,9981
P14Z	Krka spring	864	81,87	6,519	0,9982
P14S	Krka spring	618	58,87	6,162	0,9973
P15Z	Krka spring	589	56,69	6,131	0,9973
P15S	Krka spring	424	40,22	5,756	0,9960
P16Z	Krka spring	1030	94,63	6,680	0,9984
P16S	Krka spring	1110	103,04	6,794	0,9987
P17Z	Krka spring	363	35,95	5,540	0,9948
P17S	Krka spring	383	40,54	5,667	0,9957
P18Z	Krka spring	533	49,54	5,976	0,9967
P18S	Krka spring	621	57,97	6,186	0,9974
P19Z	Marasovine	1355	126,83	6,968	0,9988
P19S	Marasovine	1166	108,36	6,822	0,9987
P20Z	Marasovine	896	82,87	6,561	0,9983
P21Z	Marasovine	1060	96,32	6,732	0,9986
P21S	Marasovine	1161	109,13	6,664	0,9981
P7S	Roški slap	4965	476,98	8,265	0,9997
P8Z	Roški slap	1960	190,11	7,240	0,9991
P8S	Roški slap	4064	392,42	8,036	0,9996
P9Z	Roški slap	2345	190,11	7,457	0,9993
P10Z	Roški slap	3609	352,37	7,905	0,9995
P10S	Roški slap	2198	218,75	7,428	0,9992
P11Z	Roški slap	1056	98,92	6,698	0,9984
P11S	Roški slap	1200	115,10	6,828	0,9986
P12Z	Roški slap	1413	127,76	7,0001	0,9988
P12S	Roški slap	2101	191,06	7,388	0,9992
P1Z	Skradinski buk	2781	261,99	7,652	0,9994
P1S	Skradinski buk	3371	316,70	7,844	0,9995
P2Z	Skradinski buk	1815	173,13	7,158	0,9990
P2S	Skradinski buk	4068	397,21	8,032	0,9996
P3Z	Skradinski buk	1928	191,77	7,309	0,9991
P3S	Skradinski buk	1989	200,72	7,323	0,9992
P4Z	Skradinski buk	3958	378,20	8,051	0,9996
P4S	Skradinski buk	4625	442,43	8,162	0,9996
P5Z	Skradinski buk	3436	333,50	7,903	0,9995
P5S	Skradinski buk	3627	363,63	7,945	0,9996
P6Z	Skradinski buk	5532	507,77	8,393	0,9997
P6S	Skradinski buk	5729	528,84	8,443	0,9997

DISCUSSION

The measurability of molecular vs. traditional morphological methods in characterization of microbial communities in freshwaters

First three Publications (**I, II** and **III**) answered the dissertation objective on measurability between molecular and morphological methods in characterizing microbial communities in freshwaters. The investigation assesses the reliability of applying eDNA metabarcoding tool in the ecological assessment of biomonitoring for the microbial community in the plankton or benthos of the karstic Krka River and small water body in the alluvial area of the Drava River. The diversity of the microbial community was characterized using traditional morphological and molecular approaches, and the results obtained using both approaches were compared depending on the organisms studied within the microbial community to determine whether eDNA metabarcoding can be used as a replacement or complement to traditional methods. Also, it was assessed the ability of using eDNA genera groups or species as indicators by comparing environmental association between 18S or 16S nuclear small subunit of universal molecular markers and molecular marker that targets traditional biomonitoring organisms – diatoms (*rbcl*). Finally, through the first three Publications was showed and confirmed that the results from both methods are comparable and measurable, and have potential to be used in biomonitoring assessment even results were with some thresholds of divergence. This type of research is the first type of showing applicability of molecular methods in Croatian freshwaters and it demonstrated that validation and implementation of the molecular approach to monitoring assessment of freshwaters will help identify the ecological preferences of microbial species, including other organisms or water bodies, in biomonitoring.

The first hypothesis: "*Molecular methods are valuable for assessing diversify of microorganisms in the plankton and benthos of freshwater ecosystems*" was confirmed, and all four Publications addressed it in detail. From the main ways in which eDNA can be used in aquatic biomonitoring, two were validated and their applicability was demonstrated in all four Publications, namely biodiversity survey (community composition) and bioassessment (biotic indices). Publication **I** highlighted the importance of small water body as a buffer zone in alluvial area of the Drava River, and showed the measurability of the two approaches to

characterize the bacterial and phytoplankton diversity in the presence of high nitrate inputs due to anthropogenic impact. Results obtained with traditional morphological and molecular (using specific primers for V9 region 18S and V3-4 region for 16S of rRNA) approaches showed some variation in the diversity richness of the microbial plankton community. However, both approaches confirmed that nitrate inputs control phytoplankton biomass and influence the structure of the overall microbial community. Publication **II** provided more detailed overview of the structure and ecological preferences of the ciliate community inhabiting different microhabitats in the karstic Krka River using morphological and molecular (using specific primers of V9 region for 18S of rRNA) approaches. Accordingly, it was shown that ciliates, as part of the periphyton microbial community, exhibit high ecological sensitivity and should undoubtedly be considered as important organisms for monitoring tufa-forming rivers. In addition, this study demonstrated that eDNA metabarcoding and traditional approaches can be considered complementary, depending on the objectives of the study, either in listing species (including rare and/or uncommon species) or in adding other important data (developmental stages, some species characteristics). Publication **III** described detailed overview of the structure and diversity of the diatom community inhabiting different microhabitats, along the river course through a combination of morphological and molecular (*rbcL*, a chloroplast gene) approaches. Comparison of morphological and molecular approaches for diatoms in the Krka River revealed 58% agreement on the genus rank and a relatively low agreement of about 20% on the species rank, but both methods provided an in-depth insight into the community complexity. Ultimately, diatom diversity based on both approaches allowed a reliable dataset that can be used in routine monitoring assessment which provides a deeper understanding of ecological status. The last, Publication **IV** provided a deeper insight into the diversity and composition of protists in the karstic Krka River obtained by molecular approach (using specific primers of the V9 region for 18S of rRNA). Furthermore, the publication demonstrated how molecular approach can provide valid biological data on protists diversity that can be used for conservation of karstic environments.

Microbial organisms in plankton or benthos of freshwaters can provide new insights into the indicator properties of species and communities

Protist as microorganisms 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-Stadniczeñko 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, and they are mostly dominant in the periphyton of freshwaters (Massana et al., 2015). In the Publication **II**, using a molecular approach was observed that the most abundant protist groups in the periphyton were protozoan ciliates and diatoms. In general, diatoms receive the most attention as one of the biological quality elements, while ciliates are largely overlooked even though they have good bioindicator potential. The Publication **II** addressed the importance of the bioindicator potential of ciliates for environmental monitoring of karstic freshwater habitats (Kahlert et al., 2016). Krka River was selected as one of the representative karstic rivers in the Dinaric ecoregion in Croatia, with tufa barriers and waterfalls, choosing representative locations from its spring until last tufa barrier, before estuary. Most of the river area are protected and have the status of a National Park. The non-protected parts are affected by anthropogenic impacts as well as the parts of the Park that are more intensively visited by tourists during the high season. To investigate ciliate community structure in periphyton along river, sampling was conducted from lithified tufa/stones, and determined by morphological and molecular methods. Previous studies in the Krka River (Primc-Habdija et al., 2005; Primc-Habdija and Matoničkin, 2005) were based solely on morphological identification of species using light microscopy and this is the first investigation using a molecular approach in this study area. Morphological analysis revealed a total of 26 genera and 28 species where the most abundant ciliate species were: *Aspidisca lynceus* O.F. Müller, 1773, *Aspidisca cicada* O. F. Müller, 1786, *Cinetochilum margaritaceum* Ehrenberg, 1838 and *Glaucoma scintillans* Ehrenberg, 1830, *Vorticella convallaria* Linnaeus, 1758 and *Stylonychia mytilus* (Müller, 1773) Ehrenberg. Molecular analysis identified 3724 OTUs taxonomically assigned to Ciliophora, with the most representative OTUs corresponding to the genera *Holosticha, Anteholosticha, Euplotes, Oxytricha, Limnostrombidium, Tetrahymena, Carchesium and Urocentrum.* The molecular results showed a much higher number of represented ciliates OTUs and an increasing trend in OTUs richness downstream (from spring to the last tufa barrier). Comparing the results

obtained with both approaches at different taxonomic levels provides a more detailed insight into the complexity of the community. Foissner's saprobiological classification (Foissner et al., 1991, 1992, 1994, 1995) was used to assess saprobic water quality and indicator value analysis (IndVal) was used to determine the bioindicator potential of ciliates. Results indicated that ciliates are good ecological indicators of karstic environments commonly found in alpha- to beta-mesosaprobic freshwaters. Ciliates exhibit high ecological sensitivity and should undoubtedly be considered as important organisms for monitoring tufa-forming rivers. The Publication **II** clearly confirmed that metabarcoding provides an effective approach to overcoming difficulties in morphological identification of ciliates. This study showed how metabarcoding allows extends the range of bioindicators but also increase the knowledge about ciliates ecology and their sensitivity to ecological stressor.

Second hypothesis: "*Diatoms are well studied group of microorganisms in benthos and periphyton, but not the only one with good indicator potential*", was confirmed by Publication **II**, as was shown that ciliates within periphyton have also a good indicator potential, but they are still overlooked in monitoring analysis. Publication **III** and **IV** are also related with second hypothesis, where Publication **III**, presented a detailed overview of the diatom community in the periphyton obtained by morphological and molecular (chloroplast gene *rbcL*) approaches at different microhabitats of Krka River. In this Publication, the applicability of eDNA metabarcoding as a reliable tool for biomonitoring of diatoms in karstic river was analysed by comparing index values from morphological and molecular approaches. The rapid and specific response of diatoms to environmental changes, their wide diversity and ubiquitous distribution, and known ecological preferences of many taxa have enabled the use of benthic diatoms as biological indicators in biomonitoring programmes required by Water Framework Directive (WFD, Directive 2000/60/EC, 2000). The use of diatoms as a biological water quality element requires highly specialised and expert morphological identification to species level, well researched areas and known operational taxa lists (Mann et al., 2017), so new methods as molecular method, brings a new perspective to elucidate diatom diversity but also to use them more effectively in biomonitoring. Morphological approach detected most abundant genera: *Achnanthidium, Amphora, Aulacoseira, Cocconeis, Diatoma, Gomphonema, Meridion, Navicula, Odontidium, Pantocsekiella, Rhoicosphaenia and Staurosirella,* while molecular approach detected as most abundant: *Achnanthidium, Amphora, Discostella, Planothidium, Reimeria and Sellaphora.* Comparison of the results from both approaches presented a total of 46 overlaps at genus rank between the two methods and 64 at species rank. Mantel test indicated

a significant relationship for 64 species matches between Bray-Curtis distance based on the relative abundance of valve counts and relative abundance of reads. Results generated by both approaches showed that genus and species ranks were recognized more by using the morphological approach. Molecular results recognized many more ESVs at each sampling location, nevertheless, from the total recorded number up to 61% of ESVs could not be taxonomically assigned, even to the family or genus rank. Although a large percentage of taxonomically unassigned ESVs was present, they did not reflect the same percentage or number of unidentified species. The results showed a relatively low agreement between the morphological and molecular approach in the variation of diatom community composition, but both methods gave complementary information of diatom community structure of karstic Krka River. The results from the Publications **II** and **III** served as a starting point for the more detailed overview of the diversity of other protist groups in the periphyton along the Krka River given in the Publication **IV**. The differences in protist diversity between upstream and downstream sections of the river were highlighted. Protist diversity was determined by amplicon sequencing of the hypervariable region V9 of the 18S rRNA gene (molecular approach). The major protist groups, representing at least 10% of the total protist communities, were Ciliophora, Cercozoa, Ochrophyta, Apicomplexa, Discoba, Lobosa, and Opalozoa. Data on the biodiversity and ecology of protists in karstic environments are still quite sparse, and previous studies in the Krka River were based solely on morphological identification of specific protists under the light microscope, such as diatoms and ciliates (Kralj et al., 2006; Primc-Habdija et al., 2005; Žutinić et al., 2020). Other protist groups are even less studied, especially some algal groups in the Krka River. However, this study provided a deeper insight into the protist diversity of the Krka River by combining alpha and beta diversity indices using molecular approach. All three Publications contributed into that the molecular approach can be a powerful tool to facilitate the process and reveal the hidden diversity and ecology of the protists in a biodiversity survey way, opening the doors to a more holistic view of an entire ecosystem.

Ciliates and diatoms were found to be the main protist groups at all four sampling sites, with ciliates dominating at Skradinski buk, in contrast to the spring area where diatoms dominated. These results could lead to a dependence between these two groups, since algivorous ciliates can be selective predators of diatoms. In general, the periphyton represents a complex community of microorganisms attached to the substrate, and studying the entire periphytic community, not just algae, may improve our understanding of their functional role

in freshwaters (Gubelit and Grossart, 2020). This is consistent with the third hypothesis: "*Interactions between groups of organisms in the plankton and benthos in freshwater systems, controlled by anthropogenic pressure into the system, provide new insights into the indicator properties of species and communities*". Publication **I** is also consistent with the third hypothesis, which demonstrated ecosystem functionality by characterising bacterial and algal diversity in the plankton of a small inactive gravel pit under anthropogenic pressure. One of the most important components in small inactive gravel pit is the prokaryotic microbial community, which plays a crucial role in nutrient recycling. In this sense, the bacterial community provides a basis for understanding the entire microbial community (Jetten et al., 2003), and the interactions between bacterio- and phyto- plankton component in pelagic and benthic zones can provide new insights into species ecology and the functionality of organisms. Variations were confirmed for algal community using morphological and molecular method, and for bacterial community only using molecular method. Results showed a strong correlation between microbial communities and eutrophication of small water body under high pressure of nitrogen compounds. To test the comparability of the two methods in the phytoplankton community, taxa lists derived from both approaches were compared in terms of the presence or absence of taxa. Phytoplankton and bacterial community composition were analysed using alpha diversity indices. To show correlation with environmental variables, both communities were plotted using canonical correspondence analysis (CCA) ordination diagrams. The nutrient-based indication of eutrophic conditions in the gravel pit was further supported by high values of phytoplankton biomass and bacterial density. The dominant species during summer period was cyanobacteria *Microcystis* spp., with biomass pick in August (surface bloom), associated with the maximum phytoplankton total biomass. *Microcystis* bloom can significantly reduce dissolved CO_2 concentrations and drive up the pH value and favours more alkaline conditions as a competitive advantage over other plankton species (Paerl, 2018). For example, during the summer period (in June) the phylum Planctomycetota was subdominant in the bacterial community. The planctomycete anammox bacteria live in close association with aerobic ammonium oxidizers, and these bacteria consume oxygen on the outside of the biofilm, keeping the inside anoxic for the anammox bacteria. Together, they create conditions in which they can convert ammonium directly into dinitrogen gas. Anammox bacteria can contribute significantly to the loss of fixed nitrogen in both natural and anthropogenic-influenced ecosystems. In contrast to the warm period, the colder period was characterised by diatoms (genus *Ulnaria*) and dinoflagellates (genus *Peridinium*). Although, Actinobacteriota were present throughout the investigated period, with increased abundance in October and January during the low

abundance of cyanobacteria, which is likely related to the sensitivity of Actinobacteriota to conditions present during cyanobacterial bloom (high levels of organic matter, availability of inorganic nutrients, and high temperatures; Ghai et al., 2014). This may also be a good contribution to the fact that Actinobacteriota have good bioindicator potential. The molecular method reconfirmed its potential for broadening the range of bioindicators, including the prokaryotic microbiota, for biomonitoring assessment. It was, also showed how the dominance of one species can causally control the disappearance or decline of another species within microbial community and their inter dependence. The Publication **I** also demonstrated that interdisciplinary approaches can be successfully used to explore the ecological preferences of microbial species and to predict and prevent algal blooms. In addition to emphasizing changes within the planktic microbial community, the study also highlighted the importance of small water bodies, as sampling was conducted in a small water body - Šijanec gravel pit in the Drava River alluvial area under anthropogenic influence. This is consistent with the fourth hypothesis: "*Small water bodies are important nutrient recyclers in the systems of large rivers*".

Small water bodies are important nutrient recyclers in the systems of large rivers

Studies on the ecology and importance of small water bodies in alluvial lowlands are still quite rare. These systems are still not included in national water resource protection strategies, despite their small size, they account for a high proportion of the global freshwater habitat, representing up to 30% of standing freshwater by area (Harper et al., 2019). Because of their high potential for high metabolic rates as biochemical reactors within larger freshwater systems, they have ecological, aesthetic, and recreational value (Menetrey et al., 2005). They are associated with eutrophic or hypertrophic conditions as they modulate nutrient retention and recycling along hydrologic pathways, but they still harbour a very diverse biodiversity with many rare, protected, and unique species (Navarro and Carbonell, 2008). In addition, small water bodies support more species at landscape-scale than lakes and rivers. Eutrophication has been described as a major stressor for the freshwater biodiversity not just for large but also small water bodies (Hering et al., 2010). Nowadays, eutrophication management suggests that lowland small water bodies should be regulated differently than longer freshwater systems with an emphasis that they are hosts of regional biodiversity (Rosset et al., 2014). One of the systems

characterized by high nitrogen inputs is the alluvial aquifer of the Drava River in Croatia, within Pannonian ecoregion. The entire part of the aquifer is under strong anthropogenic pressure, mainly due to agriculture, where dozens of small water bodies play a potentially important role as biogeochemical reactors in nitrogen buffering and recycling (Gvozdić et al., 2011). In Publication **I**, the main objective was to characterize ecosystem functionality, focusing on the change in diversity of bacterial and phytoplankton communities under higher nitrogen concentrations as a result of anthropogenic pressure in the small gravel pit, Šijanec. Another focus of the study was to investigate the role of this small water body in nitrogen recycling in the Drava River alluvial area. Even small water bodies have enormous scientific value and abundant ecosystems, previous studies of the Drava River lowland have not considered their importance within the overall alluvial system (Dolgosné Kovács et al., 2019; Stanković et al., 2012), as was done in this study by selecting the Šijanec gravel pit. The gravel pit in the village of Šijanec was chosen due to its inactivity and accessibility. It is a small pit with an area of about 12,000 m^2. In general, nitrogen compounds in gravel pit can easily percolate through the soil to groundwater, either by direct terrestrial runoff or with precipitation or irrigation water (Gao et al., 2012). In this study, the changes in environmental variables were investigated using principal component analysis (PCA). Higher nitrogen concentrations were observed in the colder seasons due to the observed decrease in phytoplankton biomass resulting from an increase in precipitation and a rise in groundwater level. In contrast to the colder periods, the nitrogen concentration in groundwater decreased during the warmer periods due to the decrease in precipitation and increase in phytoplankton biomass (surface bloom of cyanobacteria - eutrophic conditions). The results confirm that nitrogen compounds likely control and influence microbial community structure.

Publication **I** conveyed the message that the gravel pit in the alluvial area not only serves as a buffer zone, but also maintains the condition of the aquatic ecosystem and high species diversity. This study contributed to knowledge of these important small ecosystems and confirmed that sensitive and robust method as molecular tool can revolutionize the study of small water bodies and bring them into the focus of monitoring assessment.

Differences between morphological and molecular approaches

Management of freshwater ecosystems is extremely important because of their high biological diversity as well as their sensitivity to numerous anthropogenic stressors (Dudgeon et al., 2006; Ormerod et al., 2010). The long tradition of freshwater management results from the importance of the variables studied and the methods used. Ecological Water Quality Assessment is defined as the monitoring of changes in populations or communities in the system, as required by the European Water Framework Directive (WFD). The WFD requires characterization of biological communities, along with physiochemical and hydromorphological conditions. Freshwater aquatic ecosystems can be assessed based on abiotic aspects, including water chemistry and physical structure, or on biotic aspects, including diversity and composition of different organisms (Pawlowski et al., 2020). Importantly, all of these monitoring approaches assume that the measurement of a few key variables describes the state and potential direction of change of the entire ecosystem (Pawlowski et al., 2020).

The development and application of monitoring approaches has a long history and has grown gradually in recent decades. While initially dominated by simple chemical assessments of macronutrients, they have been complemented by biological quality elements (BQEs) that characterize nutrient loading to freshwater systems (Pawlowski et al., 2020). BQEs include fish, benthic macroinvertebrates, phytoplankton, phytobenthos and macrophytes, each requiring unique sampling, analysis, and computational approaches (Simboura et al., 2005). Derived results are used to compute biotic metrics/indices to define the ecological quality status, which are defined as measures of the structure, functions or some other characteristics of biological communities that show a predictable response to anthropogenic disturbance (Bonada et al., 2006). The terms metrics or indices depend on the definition of these terms. In general, metrics/indices are classified according to their structure, from simply calculating the number of certain groups of organisms to combining several individual metrics into a multimetric index (Birk et al., 2012; Hering et al., 2006). Developed and used over 100 years, biological monitoring has notable successes in significant improvements of the detection of multiple stressors in aquatic ecosystems (Pawlowski et al., 2018).

However, traditional biological monitoring has inherent limitations and challenges, that are related to their structure, general implementation and use in the assessment system. Some of the methodologies that are currently used under the WFD assessment may be too complex or even inadequate, turning the whole process of ecological assessment into a slow and complex

procedures (Pawlowski et al., 2020). Traditional monitoring approach relies on sampling, sorting, and morphological identification of organisms, where sometimes sampling effort may not involve complicated methods. Sample processing and the analysis of the results derived from the enforcement of the metrics can be quite challenging and some of the traditional techniques have sometimes proven to be invasive on the species or ecosystem. Usually sampling can be simple (depending on the biological elements) but sorting and sample preparation are time-consuming. Traditional monitoring relies on morphological identification of species and counting of individuals under microscopic observation. Morphological identification of species can be time-consuming and requires an excellent taxonomic expertise of scientist, which is increasingly rare. Furthermore, only those species that are successfully characterized by morphological features are used as bioindicators, while those which are not successfully characterized are mostly overlooked. This limits also, the range of potential indicator taxa and large-scale assessment when greater number of samples should be applied instead of one independent sample (Pawlowski et al., 2016).

The limitations of traditional biodiversity monitoring and the novelty of the science have created demand for alternative approaches that have been introduced in the last decade, particularly a molecular approach (eDNA metabarcoding) that has the potential to overcome some of these limitations and revolutionize monitoring assessment.

The use of eDNA-based approaches has numerous advantages over traditional methods based on direct sampling of organisms and morphological identification. This method allows faster sampling of large numbers of specimens, reduction in cost per sample, detection of trace species, juveniles, and reproductive stages, identification of inconspicuous and fragmented specimens (broadening the range of indicator taxa), detection of rare, invasive, and pathogenic species, non-invasive sampling, and it could be automated (Pawlowski et al., 2020).

Considering the advantages of eDNA metabarcoding, the fifth hypothesis refers on it: "*With a larger number of sampling of different microhabitats, we can get a better insight into the state of the ecosystem than with a one representative monitoring sampling point*". Traditional monitoring requires sampling at one representative point to obtain a representative independent sample. eDNA metabarcoding allows sampling of a larger number of different microhabitats because sampling is non-invasive and faster; and results can be obtained more quickly. More data on the spatial distribution of species throughout the habitat can be obtained with a larger number of samples, which also allows, a larger number of subsamples. Biological replication is important because it is critical for robust statistical analyses that can be more

easily incorporated into the survey design with eDNA metabarcoding (Bruce et al., 2021). This hypothesis was demonstrated and discussed in the Publications **II**, **III** and **IV** obtained at karstic Krka River. In Publication **II** sampling was performed on four locations in individual triplicates. During sampling, each successive habitat upstream of the previously sampled location was selected. Five stones were randomly collected at the sampling location and samples were performed by brushing and/or scraping the substrate (biofilm) from the light- and dark-exposed sides of the lithified tufa/stone. Sampling was done on light- and dark-exposed sides to get a better insight into the ciliate community structure, since the abundance and diversity of ciliates depends on this abiotic factor (Vermaat, 2005). Ultimately, a total of 42 samples were collected, instead of four representative samples, one for each location. Ciliate diversity was represented by alpha and beta diversity indices. 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. For the morphological approach, significant differences in light-exposed samples were evident only at Skradinski buk for all alpha diversity indices presented. In contrast, the molecular approach for exposed sides (light/dark) showed that there were no significant effects for all tested indices, but they showed a significant increase in OTUs richness from Krka spring downstream to Skradinski buk, which was confirmed by Tukey's HSD test. In contrast to alpha diversity indices, beta diversity showed that the resolution power of ciliates community at sampling locations for molecular approach was greater than for the morphological approach. The Permanova test was used to test the significance of location and side effects, and only significant effect for the molecular approach was location effect. This was also evident in the NMDS ordination plot as a clear separation of sampling locations. Even there was no statistical significance between exposed sides in both diversity indices for morphological and molecular approaches, molecular approach provided better insight into the ciliate community and confirmed that there is no need for sampling on both exposed sides due to ciliates preference. Morphological approach recorded only few species in all 42 samples, even the analysis was performed within 4 to 10 h from sampling. Molecular approach captured much greater number of taxa, and taking 42 samples instead of four independent samples, showed a great coverage of ciliates community from spring to downstream part of Krka River.

In Publication **III** sampling was performed also in triplicates at nine representative locations to capture structure and diversity of the diatom community inhabiting different microhabitats. In total 36 periphytic diatom samples were scrubbed with new toothbrushes from

at least five randomly collected tufa or stone substrates on each microhabitat and rinsed with water from the river. Consistent with the beta diversity in the NMDS ordination plot, the morphological approach showed a clear separation of the diatom community along the river. Statistically, a strong location effect caused by the different environmental parameters, while the microhabitat effect showed no significant result. In contrast to morphological approach, molecular showed low resolution power of diatom community, with no statistical significance found for location and microhabitat effect. Generally, beta diversity did not show differences in diatom composition obtained by both approaches at different microhabitats, as they belong to the same calcium carbonate substrate/stone or deposit tufa. These results demonstrate that robust statistical analyses can be better confirmed by the largest possible number of samples on different microhabitats.

Publication **IV** presented a detailed overview of the protist diversity in periphyton along the Krka River from upstream to downstream river section. A sample was represented in individual triplicates on four locations, by randomly collecting 5 stones or tufa and scraping off the substrate (periphyton) from both light- and dark- exposed sides of tufa/stones at each sampling location (the same as in Publication **II**). Protist diversity was presented by alpha and beta diversity indices, where alpha diversity for all calculated indices increased in the downstream river direction. According to the most abundant genera two protist groups diatoms and ciliates showed different domination at all sampled locations. The observed results for the different diversity indices indicated a strong competition between taxa distributed within these two protist groups in the middle part of the river section. In contrast, diatoms demonstrated a clear dominance in the upstream part, whilst ciliates dominated in the downstream part of the river (Skardinski buk location). The dominance of ciliates in the downstream sections of the river could be related to local microhabitat complexity, as the abundance of various tufa-depositing forms is much higher at the downstream locations, especially at Skradinski buk (Bonacci et al., 2017). This confirmed that Skradinski buk location has the greatest diversity of microhabitats among the four sampling locations in the river section (Bonacci et al. 2017). Interestingly, beta diversity showed the location effect that was statistically confirmed and shown by NMDS ordination plot. This can be explained by habitat heterogeneity as one of the main driving factors for beta diversity (Astorga et al., 2014) which was associated with habitat physical structure in this study. Tufa barriers are a product of calcium carbonate deposition where physical and chemical properties of water, geological substrate, and biota play an

interrelated role. All of these factors can influence the biotic community through hydrogeological processes (Tamburini and Menichetti, 2020).

The research on heterogeneous microhabitats or different exposed sides may provide better insights into diversity and species distribution (Xie et al., 2021). This is not possible with standard monitoring because traditional morphological identification requires a great deal of time to identify individual organisms and a high level of taxonomic expertise, which is difficult when there are many samples instead of one independent sample per location. In this case, eDNA metabarcoding has been shown in all presented publications to be not only suitable for taxonomic identification of organisms in specific habitats, but also challenging in capturing diverse communities in complexity of habitats in large river basins (i.e. from the downstream to upstream reaches of multiple river parts or microhabitats). While independent samples for each biological element are required for traditional biomonitoring assessments, eDNA allows for comprehensive sampling at many different locations/microhabitats, resulting in one independent sample for the study of all biological elements and species distribution (Harper et al., 2020). In addition, it is very important and necessary to consider habitat variation when studying the effects of natural or anthropogenic factors on biotic communities because different microhabitats may have different sensitive response to changes in environmental conditions (Xie et al., 2021). This is very important for monitoring karstic rivers such as the Krka River, as in this case some parts of the river belong to the National Park and due to the increasing influence of tourism (anthropogenic interventions) a possible loss of biodiversity can be prevented.

eDNA metabarcoding allows integration of a much wider range of taxa and indicator groups into freshwater ecological assessments

All four Publications demonstrated how eDNA metabarcoding expands the range of bioindicators correlated with environmental factors, facilitating the identification of key environmental stressors. This is related to the sixth hypothesis: „*Due to limitations of currently used biomonitoring methodologies, that rely on traditional taxonomic identification, methods based on eDNA allow integration of a much wider range of taxa and indicator groups into freshwater ecological assessments*". Publication **I** highlighted the importance of not only phytoplankton community, but also bacterial. The bacterial species are one of the major

components of the overall microbial community (Jetten et al., 2003), but it is still overlooked and not included in standard monitoring, even it can provide new insights into species ecology and the functionality of taxonomic groups. According to the results in Publication **I**, it was demonstrated how bacterial community has importance in utilization of nitrate compounds in small water body. Bacterial communities are still not part of standard monitoring because of their limited ability to grow on laboratory media (Mossel and Struijk, 2004). Nowadays, as was showed in Publication **I**, molecular methods can provide detection and implementation bacterial community in standard monitoring as they can be used as an important tool for assessing environmental changes. In most cases, the ecological preferences of species can be related to their environmental preferences, as shown by the results using the Actinobacteriota as an example. This bacterial community was presented throughout the whole investigated period but with a low abundance during cyanobacteria surface bloom, which is connected with the sensitivity of Actinobacteriota. These preferences/traits suggested that Actinobacteriota might serve as sentinels of impending ecological damage and have the potential to become standards of ecological freshwater quality (Ghai et al., 2014). Opposite to bacterial community, much more species of phytoplankton was detected with morphological approach during the warmer period of investigation, while molecular approach detected much lower taxa in warmer period. This can be explained by cyanobacterial bloom, as some cyanobacteria are known to produce toxins that can inhibit regulatory enzymes in eukaryotic cells, causing PCR inhibition (Eland et al., 2012). The molecular approach has proven to be an effective tool for detecting small-size eukaryotic algae in the colder season. These reasons can be associated with possible causes as: when cell abundances of specific taxa in the water sample drop below a specific threshold, they can still be detectable with the molecular approach, but may not be found by microscopy; and the resting stages of some algal species cannot be identified and assigned correctly by microscopy, but might be more easily recorded by the molecular approach (Medinger et al., 2010), and small-sized algae are generally hard to detect with microscopy due to the scarcity of taxonomic knowledge and limited resolving power. Again, it was shown that the molecular approach can be successfully used not only to allow a broader range of indicator taxa, but also to better assess the microbial community structure of species that are difficult to detect with morphological methods. Usually information and ecological preferences of small-sized species within microbial communities are very scarce, so these results also contributed in elucidating their ecological preferences.

In the Publication **II** it was shown that the molecular approach, in contrast to the morphological approach, is a very powerful tool for detecting ciliates, in the periphyton. Ciliates are difficult to determine in the conventional morphological manner because they are sensitive to preservation and must be determined alive; some of them are fast-moving and small, what makes them difficult to detect under the microscope. In the Publication **II**, the morphological approach showed a much lower number of species based on single-cell determination, and the analysis showed statistically insignificant results. The molecular approach showed much better coverage of the ciliate community, as the hypervariable region V9 of the 18S rRNA gene has good potential for protist detection. Taxonomically assigned OTUs corresponded to the ciliates genera, generally occur in freshwater systems as Krka River. The results were also correlated with environmental variables, showing that ciliates can be used as good ecological indicators of karst environments. Here, the potential of the molecular approach to detect a much broader range of taxa for freshwater monitoring assessment was again confirmed.

The Publication **III** showed the applicability of the results of the detection of diatoms in the periphyton and the ecological status obtained by diatom community in the Krka River. The ecological status (EQR) of the Krka River based on the taxa list compiled using the morphological and molecular approach was assessed by a separate calculation of the Croatian Trophic Diatom Index (TDI_{HR}). In comparison, the morphological and molecular results provided a feasible, but statistically different, assessment of ecological status and thus an appropriate response to environmental stresses. Ultimately, the molecular approach again demonstrated its potential in detecting species and potential gaps between the two methods, as the most abundant species are the largest contributors to EQR values. One of the examples of major gaps in EQR values was detected in Skradinski buk, where the planktic species *Pantocsekiella ocellata* (Pantocsek) K.T.Kiss & Ács was the largest contributor to EQR values in the morphological approach, but did not appear as the most contributing taxon in the molecular analysis. This species was detected in the downstream part of the river section, and its occurrence can be interpreted by the influence of the upstream Lake Visovac, where this species is most abundant (Hanžek et al., 2021). Highlighting centric diatoms in the morphological approach may lead to misleading or incorrect interpretations of EQR values, as these species are planktic and may have deposited cells that are difficult to distinguish under the light microscope due to downstream transport, or they may already be bound in sediment particles (Gons, 1991). In such cases, the molecular approach again demonstrated the

advantages of more correct interpretation of EQR values and provided a reliable data set that can be used for routine monitoring.

The Publication **IV** gave a detailed overview of the protist diversity in the periphyton of the Krka River using a molecular approach. The use of alpha and beta diversity indices in the analysis showed that they can give a better insight into the diversity of the community. Also, the use of different genera in the molecular approach, rather than species rank, can well represent ecological functions that tend to reflect specific morphological or physiological characteristics of species. This is important to capture not just one group of organisms in a microbial community, but the entire community, allowing a better understanding of the biodiversity of aquatic ecosystems. In this case, the biological data are of great importance for karst conservation, but can also reveal declines in biodiversity and allow for effective protection of aquatic habitats in future management such as karstic area.

Publications have confirmed that eDNA metabarcoding data have the potential to cover a much broader range of taxa, which is important especially when comparing neighbouring sites or moderate changes in environmental conditions where morphological approaches may fail (Cordier et al., 2017). It is also confirmed that eDNA data encompass not only the concept of environmental filtering to individual indicator taxa, but also the interactive qualities also associated with habitat stability and ecological complexity (Cadotte and Tucker, 2017). Here, was showed how to assess interactions among taxa in high diversity areas that can be directly linked to biomonitoring (Araújo et al., 2011). In this case, the molecular approach allows us to capture the entire community structure, which can contribute to a broader approach to freshwater assessment, as it is better to see the entire biodiversity rather than focusing on a one group of organisms within the microbial community.

eDNA metabarcoding allows the inclusion of a much wider range of biotopes into freshwater ecological assessments

The seventh hypothesis: "*Due to limitations of currently used biomonitoring data, including time-consumption, space and researcher availability, methods based on eDNA allow the inclusion of a much wider range of biotopes into freshwater ecological assessments*" was verified in all four publications. All four Publications demonstrated the potential of eDNA metabarcoding to include not only a wider range of indicator groups, but also a wider range of

samples and biotopes. With eDNA metabarcoding, the limiting factors of traditional monitoring can be circumvented, allowing monitoring to be further improved in this way. Publication **I** demonstrated the applicability of the molecular method in monitoring of small water bodies. In general, small water bodies represent a high proportion of global freshwater habitat with very diverse biota (Downing et al., 2006). Despite its scientific values that small water bodies possess, there are few studies on them and they are not included in traditional monitoring standards. The importance of their environmental regulation is that they are also threatened by anthropogenic activities and environmental change, and have a greater vulnerability to environmental stressors than larger water bodies with larger alluvial ecosystems (Biggs et al., 2017). The reason that these ecosystems are poorly studied and not included in freshwater assessments may be due to a lack of appropriate monitoring tools and the fact that abundance and biodiversity assessments alone can be costly (Hill et al., 2018). In this context, molecular tools offer a solution through rapid, sensitive, cost-effective, non-invasive monitoring and promise to improve our understanding of the global biodiversity of small standing waters, which was also confirmed in Publication **I**. The small and shallow nature of small water bodies therefore exposes these systems to more extreme conditions than deeper water bodies, including greater variation in temperature range and potentially greater exposure to ultraviolet (UV) light, although turbidity is higher, which can result in a highly diverse biota from invertebrates to the microbial community (Harper et al., 2020). All of these characteristics underscore the importance of small water bodies, and eDNA metabarcoding can enable their conservation and management by providing a tool for rapid, sensitive cost-effective, non-invasive biomonitoring (Harper et al., 2020).

Another point to discuss can also be related to the seventh hypothesis by the other three Publications (**II**, **III** and **IV**) at the Krka River. Since traditional monitoring requires only one representative sample to be taken for each biological element, eDNA showed its potential to increase the number of samples, number of habitats and microhabitats or exposed sides of sampling substrate as well. In all three Publications, results are shown that highlight the importance of microhabitat or other scale variability in environmental conditions. Microhabitat preferences of freshwater microbial communities are essential for studying correlations between species and their environment, and thus for providing an adequate basis for the conservation of aquatic habitats and their biodiversity (Álvarez-Troncoso et al., 2017; Sarr et al., 2013; Vilenica et al., 2018). Microhabitat heterogeneity, along with physical and chemical properties of the water, is a key factor affecting the composition of benthic organisms

(microbial community), as individual species can often be associated with specific microhabitat types. In contrast to traditional sampling monitoring, variability at small spatial scales can rival or exceed differences observed at much larger biogeographic scales, and these differences can have significant consequences (Denny et al., 2011; Rapacciuolo et al., 2014). A good reason for examining small spatial scales in monitoring is the ability of some species to move between microhabitats, which can significantly affect species vulnerability to environmental change (Thakur et al., 2020). In all three Publications, results showed no statistically significant differences in exposed sides of stone or microhabitats, likely because they belong to the same calcium carbonate substrate/stone or deposit tufa. Overall, these results demonstrate the potential of eDNA metabarcoding in ability to sample at small spatial scales, in contrast to traditional monitoring methods and also allowed good significant statistical results according to the greater number of samples. eDNA metabarcoding could improve our understanding of freshwater networks-particularly a broader range of biotopes, to enable more effective monitoring, protection, and management of aquatic biodiversity.

CONCLUSION

The main final remarks of this thesis can be summarized in the following conclusions:

- Molecular methods are valuable for assessing diversity of microorganisms in the plankton and benthos of small water body and karstic Krka River.
- Diatoms are well studied group of microorganisms in benthos and periphyton which are used to assess the ecological status of aquatic ecosystems. Molecular approach conmfirmed that within periphyton there are other groups of organisms as ciliates with also good indicator potential due to their ubiquity, abundance and sensitivity to anthropogenic impact.
- By analysing different components of the aquatic ecosystem within the plankton and benthos, it was gained better insight into other species within microbial communities that also responded to environmental changes and anthropogenic pressure, which provided new insights into their bioindicator potential.
- Small water bodies are important nutrient recyclers in the systems of large rivers and although they are associated with eutrophic conditions, they have a very diverse biodiversity with many rare and unique species.
- Standard methods of traditional monitoring rely on a one independent sample due to limited factors such as time and scientists' expertise in taxonomic morphological identification, whereas the molecular approach allows for a greater number of samplings, yields representative results more quickly, and provides greater insight into ecosystem health.
- Standard methods of traditional monitoring provide a good insight into the ecological status of freshwaters, but using groups of organisms that have been well studied according their morphology, such as diatoms. Here, molecular methods offer the opportunity to incorporate overlooked taxa and new indicator groups into the ecological assessment of freshwaters. As eDNA metabarcoding continues to evolve, it also allows to extend traditional monitoring to a larger number of samples and a broader range of water bodies and microhabitats than the traditional monitoring.

LITERATURE

Álvarez-Troncoso, R., Benetti, C. J., Sarr, A. B., and Garrido, J. (2017). The microhabitat preferences of Trichoptera in rivers in north-western Spain. *Marine and Freshwater Research*, *68*(9), 1686. https://doi.org/10.1071/MF16246

Andersen, J. H., Aroviita, J., Carstensen, J., Friberg, N., Johnson, R. K., Kauppila, P., Lindegarth, M., Murray, C., and Norling, K. (2016). Approaches for integrated assessment of ecological and eutrophication status of surface waters in Nordic Countries. *Ambio*, *45*(6), 681–691. https://doi.org/10.1007/s13280-016-0767-8

Andersen, T., Baranov, V., Hagenlund, L. K., Ivković, M., Kvifte, G. M., and Pavlek, M. (2016). Blind Flight? A New Troglobiotic Orthoclad (Diptera, Chironomidae) from the Lukina Jama – Trojama Cave in Croatia. *PLOS ONE*, *11*(4), e0152884. https://doi.org/10.1371/journal.pone.0152884

Anderson, D. M., Glibert, P. M., and Burkholder, J. M. (2002). Harmful algal blooms and eutrophication: Nutrient sources, composition, and consequences. *Estuaries*, *25*(4), 704–726. https://doi.org/10.1007/BF02804901

Araújo, M. B., Rozenfeld, A., Rahbek, C., and Marquet, P. A. (2011). Using species co-occurrence networks to assess the impacts of climate change. *Ecography*, *34*(6), 897–908. https://doi.org/10.1111/j.1600-0587.2011.06919.x

Arndt, H., Dietrich, D., Auer, B., Cleven, E.J., Gräfenhan, T., Weitere, M., and Mylnikov, A. (2000). Functional diversity of heterotrophic flagellates in aquatic ecosystems. In: Leadbeater B. S. C., and Green, J. C. (Eds) The Flagellates. Taylor & Francis Ltd, London, 240-268

Astorga, A., Death, R., Death, F., Paavola, R., Chakraborty, M., and Muotka, T. (2014). Habitat heterogeneity drives the geographical distribution of beta diversity: The case of New Zealand stream invertebrates. *Ecology and Evolution*, *4*(13), 2693–2702. https://doi.org/10.1002/ece3.1124

Backe-Hansen, P., and Throndsen, J. (2002). Pico- and nanoplankton from the inner Oslofjord, eastern Norway, including description of two new species of Luffisphaera (incerta sedis). *Sarsia*, *87*(1), 55–64. https://doi.org/10.1080/003648202753631730

Balasubramanian, A. (2015). The World's Water. University of Mysore.

Barthel, L., de Oliveira, P. A. V. de, and da Costa, R. H. R. (2008). Plankton biomass in secondary ponds treating piggery waste. *Brazilian Archives of Biology and Technology*, *51*(6), 1287–1298. https://doi.org/10.1590/S1516-89132008000600025

Berger, H. and Foissner, W. (2003). Illustrated guide and ecological notes to ciliate indicator species (Protozoa, Ciliophora) in running waters, lakes, and sewage plants. In: Steinberg, C. (Ed.): Handbuch Angewandte Limnologie. Ecomed, 160

Berry, D., Xi, C., and Raskin, L. (2006). Microbial ecology of drinking water distribution systems. *Current Opinion in Biotechnology*, *17*(3), 297–302. https://doi.org/10.1016/j.copbio.2006.05.007

Biggs, J., von Fumetti, S., and Kelly-Quinn, M. (2017). The importance of small waterbodies for biodiversity and ecosystem services: Implications for policy makers. *Hydrobiologia*, *793*(1), 3–39. https://doi.org/10.1007/s10750-016-3007-0

Birk, S., Bonne, W., Borja, A., Brucet, S., Courrat, A., Poikane, S., Solimini, A., van de Bund, W., Zampoukas, N., and Hering, D. (2012). Three hundred ways to assess Europe's surface waters: An almost complete overview of biological methods to implement the Water Framework Directive. *Ecological Indicators*, *18*, 31–41. https://doi.org/10.1016/j.ecolind.2011.10.009

Boenigk, J., Wodniok, S., Bock, C., Beisser, D., Hempel, C., Grossmann, L., Lange, A., and Jensen, M. (2018). Geographic distance and mountain ranges structure freshwater protist communities on a European scale. *Metabarcoding and Metagenomics*, *2*, e21519. https://doi.org/10.3897/mbmg.2.21519

Bolpagni, R., Poikane, S., Laini, A., Bagella, S., Bartoli, M., and Cantonati, M. (2019). Ecological and Conservation Value of Small Standing-Water Ecosystems: A Systematic Review of Current Knowledge and Future Challenges. *Water*, *11*(3), 402. https://doi.org/10.3390/w11030402

Bonacci, O., Andrić, I., and Roje-Bonacci, T. (2017). Hydrological analysis of Skradinski Buk tufa waterfall (Krka River, Dinaric karst, Croatia). *Environmental Earth Sciences*, *76*(19), 669. https://doi.org/10.1007/s12665-017-7023-9

Bonacci, O., Ljubenkov, I., and Roje-Bonacci, T. (2006). Karst flash floods: An example from the Dinaric karst (Croatia). *Natural Hazards and Earth System Sciences*, *6*(2), 195–203. https://doi.org/10.5194/nhess-6-195-2006

Bonada, N., Prat, N., Resh, V. H., and Statzner, B. (2006). Developments in aquatic insect biomonitoring: A Comparative Analysis of Recent Approaches. *Annual Review of Entomology*, *51*(1), 495–523. https://doi.org/10.1146/annurev.ento.51.110104.151124

Bradley, I. M., Pinto, A. J., and Guest, J. S. (2016). Design and Evaluation of Illumina MiSeq-Compatible, 18S rRNA Gene-Specific Primers for Improved Characterization of Mixed Phototrophic Communities. *Applied and Environmental Microbiology*, *82*(19), 5878–5891. https://doi.org/10.1128/AEM.01630-16

Bruce, K., Blackman, R., Bourlat, S. J., Hellström, A. M., Bakker, J., Bista, I., Bohmann, K., Bouchez, A., Brys, R., Clark, K., Elbrecht, V., Fazi, S., Fonseca, V., Hänfling, B., Leese, F., Mächler, E., Mahon, A. R., Meissner, K., Panksep, K., Pawlowski, J., Schmidt Yáñez, P.L., Seymour, M., Thalinger, B., Valentini, A., Woodcock, P., Traugott, M., Vasselon, V., and Deiner, K. (2021). A practical guide to DNA-based methods for biodiversity assessment. Pensoft Publishers, 1-90. https://doi.org/10.3897/ab.e68634

Burki, F., Sandin, M. M., and Jamy, M. (2021). Diversity and ecology of protists revealed by metabarcoding. *Current Biology*, *31*(19), R1267–R1280. https://doi.org/10.1016/j.cub.2021.07.066

Burliga, A. L., and Kociolek, J. P. (2016). Diatoms (Bacillariophyta) in Rivers. In: Necchi, O. Jr. (Ed.) River Algae. Springer International Publishing, 93-128. https://doi.org/10.1007/978-3-319-31984-1_5

Cadotte, M. W., and Tucker, C. M. (2017). Should Environmental Filtering be Abandoned? *Trends in Ecology & Evolution*, *32*(6), 429–437. https://doi.org/10.1016/j.tree.2017.03.004

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G., and Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal*, *6*(8), 1621–1624. https://doi.org/10.1038/ismej.2012.8

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., and Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, *108*, 4516–4522. https://doi.org/10.1073/pnas.1000080107

Caput Mihalić, K., Gligora Udovič, M., Galović, I., Stanković, I., Šušnjara, M., Žutinić, P., Kulaš, A., Špoljarić, I., and Levkov, Z. (2019). *Tetramphora croatica* sp. Nov.—A new brackish-water species from Lake Vransko, Croatia. *Phytotaxa*, *401*(4), 276. https://doi.org/10.11646/phytotaxa.401.4.5

Caron, D. A., Worden, A. Z., Countway, P. D., Demir, E., and Heidelberg, K. B. (2009). Protists are microbes too: A perspective. *The ISME Journal*, *3*(1), 4–12. https://doi.org/10.1038/ismej.2008.101

Chapman, D. (1992). Water Quality Assessment: A Guide to the Use of Biota, Sediment and Water in Environmental Monitoring. WHO, Geneva, 585 pp.

Cordier, T., Esling, P., Lejzerowicz, F., Visco, J., Ouadahi, A., Martins, C., Cedhagen, T., and Pawlowski, J. (2017). Predicting the Ecological Quality Status of Marine Environments from eDNA Metabarcoding Data Using Supervised Machine Learning. *Environmental Science & Technology*, *51*(16), 9118–9126. https://doi.org/10.1021/acs.est.7b01518

Crane, K. W., and Grover, J. P. (2010). Coexistence of mixotrophs, autotrophs, and heterotrophs in planktonic microbial communities. *Journal of Theoretical Biology*, *262*(3), 517–527. https://doi.org/10.1016/j.jtbi.2009.10.027

Crump, B. C., Adams, H. E., Hobbie, J. E., and Kling, G. W. (2007). Biogeography of bacterioplankton in lakes and streams of an arctic tundra catchment. *Ecology*, *88*(6), 1365–1378. https://doi.org/10.1890/06-0387

Culver, D. C., Deharveng, L., Pipan, T., and Bedos, A. (2021). An Overview of Subterranean Biodiversity Hotspots. *Diversity*, *13*(10), 487. https://doi.org/10.3390/d13100487

Curtis, T. P., Head, I. M., and Graham, D. W. (2003). Peer Reviewed: Theoretical Ecology for Engineering Biology. *Environmental Science & Technology*, *37*(3), 64A-70A. https://doi.org/10.1021/es0323493

Dayrat, B. (2005). Towards integrative taxonomy: Integrative Taxonomy. *Biological Journal of the Linnean Society*, *85*(3), 407–415. https://doi.org/10.1111/j.1095-8312.2005.00503.x

Debroas, D., Mone, A., and Ter Halle, A. (2017). Plastics in the North Atlantic garbage patch: A boat-microbe for hitchhikers and plastic degraders. *Science of The Total Environment*, *599–600*, 1222–1232. https://doi.org/10.1016/j.scitotenv.2017.05.059

Denny, M. W., Dowd, W. W., Bilir, L., and Mach, K. J. (2011). Spreading the risk: Small-scale body temperature variation among intertidal organisms and its implications for species persistence. *Journal of Experimental Marine Biology and Ecology*, *400*(1–2), 175–190. https://doi.org/10.1016/j.jembe.2011.02.006

Descy, J. P., Sarmento, H., and Higgins, H. W. (2009). Variability of phytoplankton pigment ratios across aquatic environments. *European Journal of Phycology*, *44*(3), 319–330. https://doi.org/10.1080/09670260802618942

Dipper, F. (2022). Elements of Marine Ecology (fifth edition). Elsevier. https://linkinghub.elsevier.com/retrieve/pii/C20170039939

Dittami, S. M., Eveillard, D., and Tonon, T. (2014). A metabolic approach to study algal-bacterial interactions in changing environments. *Molecular Ecology*, *23*(7), 1656–1660. https://doi.org/10.1111/mec.12670

Dolgosné Kovács, A., Tóth, G., and Lóczy, D. (2019). Water Quality of the Lower Drava River. In: Lóczy, D. (Ed.) The Drava River. Springer International Publishing, 231-245. https://doi.org/10.1007/978-3-319-92816-6_15

Downing, P. E., Chan, A. W. Y., Peelen, M. V., Dodds, C. M., and Kanwisher, N. (2006). Domain Specificity in Visual Cortex. *Cerebral Cortex*, *16*(10), 1453–1461. https://doi.org/10.1093/cercor/bhj086

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z. I., Knowler, D. J., Lévêque, C., Naiman, R. J., Prieur-Richard, A. H., Soto, D., Stiassny, M. L. J., and Sullivan, C. A. (2006). Freshwater biodiversity: Importance, threats, status and conservation challenges. *Biological Reviews*, *81*(02), 163. https://doi.org/10.1017/S1464793105006950

Eland, L. E., Davenport, R., and Mota, C. R. (2012). Evaluation of DNA extraction methods for freshwater eukaryotic microalgae. *Water Research*, *46*(16), 5355–5364. https://doi.org/10.1016/j.watres.2012.07.023

Esteban, G. F., Fenchel, T., and Finlay, B. J. (2010). Mixotrophy in Ciliates. *Protist*, *161*(5), 621–641. https://doi.org/10.1016/j.protis.2010.08.002

European Commission. (2000). Directive 2000/60/EC of the European Parliament and of the council of 23rd October 2000 establishing a framework for community action in the field of water policy. Off. J. Eur. Communities, L327/1 (2000), Brussels.

Falkowski, P. G., Katz, M. E., Knoll, A. H., Quigg, A., Raven, J. A., Schofield, O., and Taylor, F. J. R. (2004). The Evolution of Modern Eukaryotic Phytoplankton. *Science*, *305*(5682), 354–360. https://doi.org/10.1126/science.1095964

Findlay, S. (2010). Stream microbial ecology. *Journal of the North American Benthological Society*, *29*(1), 170–181. https://doi.org/10.1899/09-023.1

Finlay, B. J., and Esteban, G. F. (1998). Freshwater protozoa: Biodiversity and ecological function. *Biodiversity and Conservation*, *7*(9), 1163–1186. https://doi.org/10.1023/A:1008879616066

Foissner, W., Berger, H., and Kohmann, F. (1992). Taxonomische und okologische Revision der Ciliaten des Saprobiensystems. Band II: Peritrichia, Heterotrichida, Odontostomatida.. Bayerisches Landesamt für Wasserwirtschaft.

Foissner, W., Berger, H., and Kohmann, F. (1994). Taxonomische und okologische Revision der Ciliaten des Saprobiensystems. Band III: Hymenostomata, Prostomatida, Nassulida. Bayerisches Landesamt für Wasserwirtschaft.

Foissner, W., Berger, H., and Kohmann, F. (1995). Taxonomische und okologische Revision der Ciliaten des Saprobiensystems. Band IV: Gymnostomatea, Loxodes, Suctoria. Bayerisches Landesamt für Wasserwirtschaft.

Foissner, W., Blatterer, H., Berger, H., and Kohmann, F. (1991). Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems. Band I: Cyrtophorida, Oligotrichida, Hypotrichia, Colpodea. (Vol. 1/91). Bayerisches Landesamt für Wasserwirtschaft.

Ford, D. and Williams, P. (2007). Karst hydrogeology and geomorphology. John Wiley & Sons, LTD; West Sussex, England.

Fox, J., Behrenfeld, M. J., Haëntjens, N., Chase, A., Kramer, S. J., Boss, E., Karp-Boss, L., Fisher, N. L., Penta, W. B., Westberry, T. K., and Halsey, K. H. (2020). Phytoplankton Growth and Productivity in the Western North Atlantic: Observations of Regional Variability From the NAAMES Field Campaigns. *Frontiers in Marine Science*, *7*, 24. https://doi.org/10.3389/fmars.2020.00024

Frisia, S. and Borsato, A. (2010). Karst. In: Alonso-Zarza, A. M., Tanner, L. H. (Eds.) *Carbonates in continental settings* (Vol. 61). Elsevier.

Gao, K., Helbling, E., Häder, D., and Hutchins, D. (2012). Responses of marine primary producers to interactions between ocean acidification, solar radiation, and warming. *Marine Ecology Progress Series*, *470*, 167–189. https://doi.org/10.3354/meps10043

Ghai, R., Mizuno, C. M., Picazo, A., Camacho, A., and Rodriguez-Valera, F. (2014). Key roles for freshwater A ctinobacteria revealed by deep metagenomic sequencing. *Molecular Ecology*, *23*(24), 6073–6090. https://doi.org/10.1111/mec.12985

Ghazy, M. M. E. D., Senousy, W. M. E., Aatty, A. M. A., and Kamel, M. (2008). Performance Evaluation of a Waste Stabilization Pond in a Rural Area in Egypt. *American Journal of Environmental Sciences*, *4*(4), 316–325. https://doi.org/10.3844/ajessp.2008.316.325

Gilbert, J. A., Meyer, F., Antonopoulos, D., Balaji, P., Brown, C. T., Brown, C. T., Desai, N., Eisen, J. A., Evers, D., Field, D., Feng, W., Huson, D., Jansson, J., Knight, R., Knight, J., Kolker, E., Konstantindis, K., Kostka, J., Kyrpides, N., Mackelprang, R., McHardy, A., Quince, C., Raes J., Sczyrba, A., Shade, A., and Stevens, R. (2010). Meeting Report: The Terabase Metagenomics Workshop and the Vision of an Earth Microbiome Project. *Standards in Genomic Sciences*, *3*(3), 243–248. https://doi.org/10.4056/sigs.1433550

Glenn, T. C. (2011). Field guide to next-generation DNA sequencers. *Molecular Ecology Resources*, *11*(5), 759–769. https://doi.org/10.1111/j.1755-0998.2011.03024.x

Gligora Udovič, M., Kulaš, A., Šušnjara, M., Arapov, J., Blanco, S., and Levkov, Z. (2022). *Cymbopleura amicula* stat nov. Et. Nom. Nov. (Bacillariophyceae)—A rare diatom species

from a karst river in Croatia. *Phytotaxa*, *532*(2), 139–151. https://doi.org/10.11646/phytotaxa.532.2.2

Gons, H. J. (1991). Diatom sedimentation loss reduction by resuspension in shallow lakes. *SIL Proceedings, 1922-2010*, *24*(2), 729–733. https://doi.org/10.1080/03680770.1989.11898838

Gran-Stadniczeñko, S., Egge, E., Hostyeva, V., Logares, R., Eikrem, W., and Edvardsen, B. (2019). Protist Diversity and Seasonal Dynamics in Skagerrak Plankton Communities as Revealed by Metabarcoding and Microscopy. *Journal of Eukaryotic Microbiology*, *66*(3), 494–513. https://doi.org/10.1111/jeu.12700

Gubelit, Y. I., and Grossart, H. P. (2020). New Methods, New Concepts: What Can Be Applied to Freshwater Periphyton? *Frontiers in Microbiology*, *11*, 1275. https://doi.org/10.3389/fmicb.2020.01275

Gulin, V., Matoničkin Kepčija, R., Sertić Perić, M., Felja, I., Fajković, H., and Križnjak, K. (2021). Environmental and periphyton response to stream revitalization – A pilot study from a tufa barrier. *Ecological Indicators*, *126*, 107629. https://doi.org/10.1016/j.ecolind.2021.107629

Gupta, A., Gupta, R., and Singh, R. L. (2016). Microbes and Environment. *Principles and Applications of Environmental Biotechnology for a Sustainable Future*, *15*, 43-84. 10.1007/978-981-10-1866-4_3. PMCID: PMC7189961.

Gvozdić, V., Brana, J., Malatesti, N., and Roland, D. (2012). Principal component analysis of surface water quality data of the River Drava in eastern Croatia (24 year survey). *Journal of Hydroinformatics*, *14*(4), 1051–1060. https://doi.org/10.2166/hydro.2012.063

Gvozdić, V., Brana, J., Puntarić, D., Vidosavljević, D., and Roland, D. (2011). Changes in the Lower Drava River Water Quality Parameters Over 24 Years. *Archives of Industrial Hygiene and Toxicology*, *62*(4), 325–333. https://doi.org/10.2478/10004-1254-62-2011-2128

Hanžek, N., Gligora Udovič, M., Kajan, K., Borics, G., Várbíró, G., Stoeck, T., Žutinić, P., Orlić, S., and Stanković, I. (2021). Assessing ecological status in karstic lakes through the integration of phytoplankton functional groups, morphological approach and environmental DNA metabarcoding. *Ecological Indicators*, *131*, 108166. https://doi.org/10.1016/j.ecolind.2021.108166

Harper, L. R., Buxton, A. S., Rees, H. C., Bruce, K., Brys, R., Halfmaerten, D., Read, D. S., Watson, H. V., Sayer, C. D., Jones, E. P., Priestley, V., Mächler, E., Múrria, C., Garcés-Pastor, S., Medupin, C., Burgess, K., Benson, G., Boonham, N., Griffiths, R. A., Lawson Handley, L., and Hänfling, B. (2019). Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds. *Hydrobiologia*, *826*(1), 25–41. https://doi.org/10.1007/s10750-018-3750-5

Harper, L. R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H. C., Lewis, E., Adams, I. P., Brotherton, P., Phillips, S., and Hänfling, B. (2020). Generating and testing ecological hypotheses at the pondscape with environmental DNA metabarcoding: A case study on a threatened amphibian. *Environmental DNA*, *2*(2), 184–199. https://doi.org/10.1002/edn3.57

Hartmann, A., Goldscheider, N., Wagener, T., Lange, J., and Weiler, M. (2014). Karst water resources in a changing world: Review of hydrological modeling approaches: karst water

resources prediction. *Reviews of Geophysics*, *52*(3), 218–242. https://doi.org/10.1002/2013RG000443

Hering, D., Borja, A., Jones, J. I., Pont, D., Boets, P., Bouchez, A., Bruce, K., Drakare, S., Hänfling, B., Kahlert, M., Leese, F., Meissner, K., Mergen, P., Reyjol, Y., Segurado, P., Vogler, A., and Kelly, M. (2018). Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. *Water Research*, *138*, 192–205. https://doi.org/10.1016/j.watres.2018.03.003

Hering, D., Feld, C. K., Moog, O., and Ofenböck, T. (2006). Cook book for the development of a Multimetric Index for biological condition of aquatic ecosystems: Experiences from the European AQEM and STAR projects and related initiatives. *Hydrobiologia*, *566*(1), 311–324. https://doi.org/10.1007/s10750-006-0087-2

Hering, D., Haidekker, A., Schmidt-Kloiber, A., Barker, T., Buisson, L., Graf, W., Grenouillet, G., Lorenz, A., Sandin, L., and Stendera, S. (2010). Monitoring the Responses of Freshwater Ecosystems to Climate Change. In: Kernan, M., Battarbee, R. W., and Moss, B. (Eds.) Climate Change Impacts on Freshwater Ecosystems. Wiley-Blackwell, 84-118 https://doi.org/10.1002/9781444327397.ch5

Hill, M. J., Hassall, C., Oertli, B., Fahrig, L., Robson, B. J., Biggs, J., Samways, M. J., Usio, N., Takamura, N., Krishnaswamy, J., and Wood, P. J. (2018). New policy directions for global pond conservation. *Conservation Letters*, *11*(5), e12447. https://doi.org/10.1111/conl.12447

Hobbie, J. E., Daley, R. J., and Jasper, S. (1977). Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology*, *33*(5), 1225–1228. https://doi.org/10.1128/aem.33.5.1225-1228.1977

Hughes, J. M. R. (2018). Freshwater Ecology and Conservation: Spproaches and techniques (first edition). Oxford, Unversity Press, United Kingdom.

Hunting E.R., de Jong S., and Vijver M.G. (2017). Assessment of monitoring tools and strategies safeguarding aquatic ecosystems within the European water framework directive (No. 192). Institute of Environmental Sciences (CML), Leiden.

Huse, S. M., Dethlefsen, L., Huber, J. A., Welch, D. M., Relman, D. A., and Sogin, M. L. (2008). Exploring Microbial Diversity and Taxonomy Using SSU rRNA Hypervariable Tag Sequencing. *PLoS Genetics*, *4*(11), e1000255. https://doi.org/10.1371/journal.pgen.1000255

Huse, S. M., Welch, D. M., Morrison, H. G., and Sogin, M. L. (2010). Ironing out the wrinkles in the rare biosphere through improved OTU clustering: Ironing out the wrinkles in the rare biosphere. *Environmental Microbiology*, *12*(7), 1889–1898. https://doi.org/10.1111/j.1462-2920.2010.02193.x

Ivković, M., Plant, A., and Horvat, B. (2012). A new species of Wiedemannia (Diptera: Empididae: Clinocerinae) from Balkan Peninsula. *Zootaxa*, *3478*(1), 581–585. https://doi.org/10.11646/zootaxa.3478.1.46

Jardillier, L., Zubkov, M. V., Pearman, J., and Scanlan, D. J. (2010). Significant CO2 fixation by small prymnesiophytes in the subtropical and tropical northeast Atlantic Ocean. *The ISME Journal*, *4*(9), 1180–1192. https://doi.org/10.1038/ismej.2010.36

Jetten, M. S. M., Sliekers, O., Kuypers, M., Dalsgaard, T., van Niftrik, L., Cirpus, I., van de Pas-Schoonen, K., Lavik, G., Thamdrup, B., Le Paslier, D., Op den Camp, H. J. M., Hulth, S., Nielsen, L. P., Abma, W., Third, K., Engström, P., Kuenen, J. G., Jørgensen, B. B., Canfield, D. E., Sinninghe Damsté, J. S., Revsbech, N. P., Fuerst, J., Weissenbach, J., Wagner, M., Schmidt, I., Schmid, M., and Strous, M. (2003). Anaerobic ammonium oxidation by marine and freshwater planctomycete-like bacteria. *Applied Microbiology and Biotechnology*, *63*(2), 107–114. https://doi.org/10.1007/s00253-003-1422-4

Johnson, M. D. (2011). Acquired Phototrophy in Ciliates: A Review of Cellular Interactions and Structural Adaptations1: mixotrophic ciliates. *Journal of Eukaryotic Microbiology*, *58*(3), 185–195. https://doi.org/10.1111/j.1550-7408.2011.00545.x

Joint, I., Mühling, M., and Querellou, J. (2010). Culturing marine bacteria - an essential prerequisite for biodiscovery: Culturing marine bacteria. *Microbial Biotechnology*, *3*(5), 564–575. https://doi.org/10.1111/j.1751-7915.2010.00188.x

Joux, F., Bertrand, J.-C., De Wit, R., Grossi, V., Intertaglia, L., Lebaron, P., Michotey, V., Normand, P., Peyret, P., Raimbault, P., Tamburini, C., and Urios, L. (2015). Methods for Studying Microorganisms in the Environment. In: Bertrand, J. C., Caumette, P., Lebaron, P., Matheron, R., Normand, P., and Sime-Ngando, T. (Eds.) Environmental Microbiology: Fundamentals and Applications. Springer Netherlands, 757-829. https://doi.org/10.1007/978-94-017-9118-2_17

Kahlert, M., Ács, É., Almeida, S. F. P., Blanco, S., Dreßler, M., Ector, L., Karjalainen, S. M., Liess, A., Mertens, A., van der Wal, J., Vilbaste, S., and Werner, P. (2016). Quality assurance of diatom counts in Europe: Towards harmonized datasets. *Hydrobiologia*, *772*(1), 1–14. https://doi.org/10.1007/s10750-016-2651-8

Kahlert, M., Kelly, M., Albert, R.-L., Almeida, S. F. P., Bešta, T., Blanco, S., Coste, M., Denys, L., Ector, L., Fránková, M., Hlúbiková, D., Ivanov, P., Kennedy, B., Marvan, P., Mertens, A., Miettinen, J., Picinska-Fałtynowicz, J., Rosebery, J., Tornés, E., Vilbaste, S., and Vogel, A. (2012). Identification versus counting protocols as sources of uncertainty in diatom-based ecological status assessments. *Hydrobiologia*, *695*(1), 109–124. https://doi.org/10.1007/s10750-012-1115-z

Karlović, I., Marković, T., Šparica Miko, M., and Maldini, K. (2021a). Geochemical Characteristics of Alluvial Aquifer in the Varaždin Region. *Water*, *13*(11), 1508. https://doi.org/10.3390/w13111508

Karlović, I., Marković, T., Vujnović, T., and Larva, O. (2021b). Development of a Hydrogeological Conceptual Model of the Varaždin Alluvial Aquifer. *Hydrology*, *8*(1), 19. https://doi.org/10.3390/hydrology8010019

Karlović, I., Pavlić, K., Posavec, K., and Marković, T. (2021c). Analysis of the hydraulic connection of the Plitvica stream and the groundwater of the Varaždin alluvial aquifer. *Geofizika*, *38*(1), 15–35. https://doi.org/10.15233/gfz.2021.38.3

Kepner, R. L., and Pratt, J. R. (1996). Characterization of Surface-Associated Protozoan Communities in a Lake Erie Coastal Wetland (Old Woman Creek, Ohio). *Journal of Great Lakes Research*, *22*(1), 63–76. https://doi.org/10.1016/S0380-1330(96)70935-8

Kermarrec, L., Bouchez, A., Rimet, F., and Humbert, J.-F. (2013). First Evidence of the Existence of Semi-Cryptic Species and of a Phylogeographic Structure in the Gomphonema parvulum (Kützing) Kützing Complex (Bacillariophyta). *Protist*, *164*(5), 686–705. https://doi.org/10.1016/j.protis.2013.07.005

Kolda, A., Petrić, I., Mucko, M., Gottstein, S., Žutinić, P., Goreta, G., Ternjej, I., Rubinić, J., Radišić, M., and Udovič, M. G. (2019). How environment selects: Resilience and survival of microbial mat community within intermittent karst spring Krčić (Croatia): Microbial mat community within Krčić spring. *Ecohydrology*, *12*(2), e2063. https://doi.org/10.1002/eco.2063

Korlević, M., Šupraha, L., Ljubešić, Z., Henderiks, J., Ciglenečki, I., Dautović, J., and Orlić, S. (2016). Bacterial diversity across a highly stratified ecosystem: A salt-wedge Mediterranean estuary. *Systematic and Applied Microbiology*, *39*(6), 398–408. https://doi.org/10.1016/j.syapm.2016.06.006

Kralj, K., Plenković-Moraj, A., Gligora, M., Primc-Habdija, B., and 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*(1), 249–258. https://doi.org/10.1007/s10750-005-1330-y

Kresic, N. and Stevanovic, Z. (2010). Groundwater Hydrology of Springs. Elsevier. https://doi.org/10.1016/C2009-0-19145-6

Kuylenstierna, M., and Karlson, B. (1994). Seasonality and Composition of Pico- and Nanoplanktonic Cyanobacteria and Protists in the Skagerrak. *Botanica Marina*, *37*(1). https://doi.org/10.1515/botm.1994.37.1.17

Kveštak, R., and Ahel, M. (1995). Biotransformation of nonylphenol polyethoxylate surfactants by estuarine mixed bacterial cultures. *Archives of Environmental Contamination and Toxicology*, *29*(4), 551–556. https://doi.org/10.1007/BF00208388

Kvifte, G. M., and Ivković, M. (2018). New species and records of the Pericoma trifasciata group from Croatia (Diptera: Psychodidae). *Zootaxa*, *4486*(1), 76. https://doi.org/10.11646/zootaxa.4486.1.5

Kvifte, G. M., Ivković, M., and Klarić, A. (2013). New records of moth flies (Diptera: Psychodidae) from Croatia, with the description of Berdeniella keroveci sp.nov. *Zootaxa*, *3737*(1), 57. https://doi.org/10.11646/zootaxa.3737.1.4

Lear, G., Stott, R., and Lewis, G. (2009). Relative Diversity and Community Structure of Ciliates in Stream Biofilms According to Molecular and Microscopy Methods. *Applied and Environmental Microbiology*, *75*(16), 5261–5272. https://doi.org/10.1128/AEM.00412-09

Leboulanger, C., Quiblier, C., and Dufour, P. (2006). Rapid assessment of multiple-limiting factors of phytoplankton biomass: Bioassays, in vivo chlorophyll-a fluorescence, and factorial design. *Archiv Für Hydrobiologie*, *166*(4), 433–451. https://doi.org/10.1127/0003-9136/2006/0166-0433

Lionello, P. (2012). The Climate of the Mediterranean Region (fisrt edition). Elsevier.

Litchman, E. (2007). Resource Competition and the Ecological Success of Phytoplankton. In Falkowski, P. G., and Knoll, A. H. (Ed.) Evolution of Primary Producers in the Sea (first edition, Vol. 16). Elsevier, 351-375

Litchman, E., Klausmeier, C. A., Miller, J. R., Schofield, O. M., and Falkowski, P. G. (2006). Multi-nutrient, multi-group model of present and future oceanic phytoplankton communities. *Biogeosciences*, *3*(4), 585–606. https://doi.org/10.5194/bg-3-585-2006

Liu, J., Meng, Z., Liu, X., and Zhang, X.-H. (2019). Microbial assembly, interaction, functioning, activity and diversification: A review derived from community compositional data. *Marine Life Science & Technology*, *1*(1), 112–128. https://doi.org/10.1007/s42995-019-00004-3

Liu, L., Yang, J., Yu, Z., and Wilkinson, D. M. (2015). The biogeography of abundant and rare bacterioplankton in the lakes and reservoirs of China. *The ISME Journal*, *9*(9), 2068–2077. https://doi.org/10.1038/ismej.2015.29

Liu, Q., Zhang, Y., Wu, H., Liu, F., Peng, W., Zhang, X., Chang, F., Xie, P., Zhang, H. (2020). A Review and Perspective of eDNA Application to Eutrophication and HAB Control in Freshwater and Marine Ecosystems. *Microorganisms*, *8*(3), 417. doi: 10.3390/microorganisms8030417. PMID: 32188048; PMCID: PMC7143994.

Lóczy, D. (2019). The Drava River: Environmental Problems and Solutions. Springer International Publishing. https://doi.org/10.1007/978-3-319-92816-6

Lyche Solheim, A., Globevnik, L., Austnes, K., Kristensen, P., Moe, S. J., Persson, J., Phillips, G., Poikane, S., van de Bund, W., and Birk, S. (2019). A new broad typology for rivers and lakes in Europe: Development and application for large-scale environmental assessments. *Science of The Total Environment*, *697*, 134043. https://doi.org/10.1016/j.scitotenv.2019.134043

Lynn, D.H. (2010). The Ciliated Protozoa (third edition). Springer, Dordreht.

Mann, D. G., Crawford, R. M., and Round, F. E. (2017). Bacillariophyta. In: Archibald, J. M., Simpson, A. G. B., and Slamovits, C. H. (Eds.) Handbook of the Protists. Springer International Publishing,205-266. https://doi.org/10.1007/978-3-319-28149-0_29

Marčić, Z., Buj, I., Duplić, A., Ćaleta, M., Mustafić, P., Zanella, D., Zupančič, P., and Mrakovčić, M. (2011). A new endemic cyprinid species from the Danube drainage. *Journal of Fish Biology*, *79(2)*, 418-430. https://doi.org/10.1111/j.1095-8649.2011.03038.x

Marković, T., Karlović, I., Perčec Tadić, M., and Larva, O. (2020). Application of Stable Water Isotopes to Improve Conceptual Model of Alluvial Aquifer in the Varaždin Area. *Water*, *12*(2), 379. https://doi.org/10.3390/w12020379

Massana, R. (2011). Eukaryotic Picoplankton in Surface Oceans. *Annual Review of Microbiology*, *65*(1), 91–110. https://doi.org/10.1146/annurev-micro-090110-102903

Massana, R., Gobet, A., Audic, S., Bass, D., Bittner, L., Boutte, C., Chambouvet, A., Christen, R., Claverie, J.-M., Decelle, J., Dolan, J. R., Dunthorn, M., Edvardsen, B., Forn, I., Forster, D., Guillou, L., Jaillon, O., Kooistra, W. H. C. F., Logares, R., Mahé, F., Not, F., Ogata, H., Pawlowski, P., Pernice, M. C., Probert, I., Romac, S., Richards, T., Santini, S., Shalchian-

Tabrizi, K., Siano, R., Simon, N., Stoeck, T., Vaulot, D., Zingone, A., and 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. *Environmental Microbiology, 17*(10), 4035–4049. https://doi.org/10.1111/1462-2920.12955

McManus, G. B., Liu, W., Cole, R. A., Biemesderfer, D., and Mydosh, J. L. (2018). Strombidium rassoulzadegani: A Model Species for Chloroplast Retention in Oligotrich Ciliates. *Frontiers in Marine Science, 5*, 205. https://doi.org/10.3389/fmars.2018.00205

Medinger, R., Nolte, V., Pandey, R. V., Jost, S., Ottenwälder, B., Schlötterer, C., and 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

Menetrey, N., Sager, L., Oertli, B., and Lachavanne, J.-B. (2005). Looking for metrics to assess the trophic state of ponds. Macroinvertebrates and amphibians. *Aquatic Conservation: Marine and Freshwater Ecosystems, 15*(6), 653–664. https://doi.org/10.1002/aqc.746

Mijusković-Svetinović, T., and Maričić, S. (2008). Low flow analysis of the lower Drava River. *IOP Conference Series: Earth and Environmental Science, 4*, 012011. https://doi.org/10.1088/1755-1307/4/1/012011

Minerovic, A. D., Potapova, M. G., Sales, C. M., Price, J. R., and Enache, M. D. (2020). 18S-V9 DNA metabarcoding detects the effect of water-quality impairment on stream biofilm eukaryotic assemblages. *Ecological Indicators, 113*, 106225. https://doi.org/10.1016/j.ecolind.2020.106225

Moënne-Loccoz, Y., Mavingui, P., Combes, C., Normand, P., and Steinberg, C. (2015). Microorganisms and Biotic Interactions. In: Bertrand, J. C., Caumette, P., Lebaron, P., Matheron, R., Normand, P., and Sime-Ngando, T. (Eds.) Environmental Microbiology: Fundamentals and Applications. Springer Netherlands, 395-444. https://doi.org/10.1007/978-94-017-9118-2_11

Mossel, D. A. A., and Struijk, C. B. (2004). Assessment of the microbial integrity, sensu G.S. Wilson, of piped and bottled drinking water in the condition as ingested. *International Journal of Food Microbiology, 92*(3), 375–390. https://doi.org/10.1016/j.ijfoodmicro.2003.08.015

Mulec, J. (2014). Human impact on underground cultural and natural heritage sites, biological parameters of monitoring and remediation actions for insensitive surfaces: Case of Slovenian show caves. *Journal for Nature Conservation, 22*(2), 132–141. https://doi.org/10.1016/j.jnc.2013.10.001

Mustafić, P., Marčić, Z., Duplić, A., Mrakovčić, M., Ćaleta, M., Zanella, D., Buj, I., Podnar, M., and Dolenec, Z. (2008). A new loach species of the genus Cobitis in Croatia. *Folia Zoologica-Praha, 57*(1-2), 4–9.

Naumann, E. (1932). Grundzüge der regionalen Limnologie. Die Binnengewässer,Stuttgart.

Navarro, A., and Carbonell, M. (2008). Assessment of groundwater contamination caused by uncontrolled dumping in old gravel quarries in the Besòs aquifers (Barcelona, Spain). *Environmental Geochemistry and Health, 30*(3), 273–289. https://doi.org/10.1007/s10653-007-9123-z

Nistal-García, A., García-García, P., García-Girón, J., Borrego-Ramos, M., Blanco, S., and Bécares, E. (2021). DNA metabarcoding and morphological methods show complementary patterns in the metacommunity organization of lentic epiphytic diatoms. *Science of The Total Environment*, *786*, 147410. https://doi.org/10.1016/j.scitotenv.2021.147410

Nolte, V., Pandey, R. V., Jost, S., Medinger, R., Ottenwälder, B., Boenigk, J., and Schlötterer, C. (2010). Contrasting seasonal niche separation between rare and abundant taxa conceals the extent of protist diversity: High seasonal protist abundance turnover. *Molecular Ecology*, *19*(14), 2908–2915. https://doi.org/10.1111/j.1365-294X.2010.04669.x

Official gazette. (1985). Law on the Proclamation of the National Park "Krka."

Official gazzete. (2019). Regulation on water quality standards.

O'Lear, S., Briggs, C. M., and Denning, G. M. (2013). Environmental Security, Military Planning, and Civilian Research: The Case of Water. *Environment: Science and Policy for Sustainable Development*, *55*(5), 3–13. https://doi.org/10.1080/00139157.2013.824327

Ormerod, S. J., Dobson, M., Hildrew, A. G., and Townsend, C. R. (2010). Multiple stressors in freshwater ecosystems. *Freshwater Biology*, *55*, 1–4. https://doi.org/10.1111/j.1365-2427.2009.02395.x

Paerl, H. (2018). Mitigating Toxic Planktonic Cyanobacterial Blooms in Aquatic Ecosystems Facing Increasing Anthropogenic and Climatic Pressures. *Toxins*, *10*(2), 76. https://doi.org/10.3390/toxins10020076

Pawlowski, J., Apothéloz-Perret-Gentil, L., Mächler, E., and Altermatt, F. (2020). Environmental DNA applications for biomonitoring and bioassessment in aquatic ecosystems. Guidelines. Federal Office for the Environment, Bern. Environmental Studies. no. 2010, 71 pp. https://doi.org/10.5167/UZH-187800

Pawlowski, J., Kelly-Quinn, M., Altermatt, F., Apothéloz-Perret-Gentil, L., Beja, P., Boggero, A., Borja, A., Bouchez, A., Cordier, T., Domaizon, I., Feio, M. J., Filipe, A. F., Fornaroli, R., Graf, W., Herder, J., van der Hoorn, B., Iwan Jones, J., Sagova-Mareckova, M., Moritz, C., Barquín, J., Piggott, J. J., Pinna, M., Rimet, F., Rinkevich, B., Sousa-Santos, C., Specchia, V., Trobajo, R., Vasselon, V., Vitecek, S., Zimmerman, J., Weigand, A., Leese, F., and Kahlert, M. (2018). The future of biotic indices in the ecogenomic era: Integrating (e)DNA metabarcoding in biological assessment of aquatic ecosystems. *Science of The Total Environment*, *637–638*, 1295–1310. https://doi.org/10.1016/j.scitotenv.2018.05.002

Pawlowski, J., Lejzerowicz, F., Apotheloz-Perret-Gentil, L., Visco, J., and Esling, P. (2016). Protist metabarcoding and environmental biomonitoring: Time for change. *European Journal of Protistology*, *55*, 12–25. https://doi.org/10.1016/j.ejop.2016.02.003

Payne R.J. (2013). Seven reasons why protists make useful bioindicators. *Acta Protozoologica*, *52*(3), 105–113. https://doi.org/10.4467/16890027AP.13.0011.1108

Peng, F., Liu, S. B., Xu, H. Y., and Li, Z. Q. (2013). A Comparative Study on the Analysis Methods for Chlorophyll-*a*. *Advanced Materials Research*, *726–731*, 1411–1415. https://doi.org/10.4028/www.scientific.net/AMR.726-731.1411

Pepper I.L., Gerba C.P., and Gentry T.J. (2015). *Environmental Microbiology* (third edition). Elsevier.

Perica, D., Orešić, D., and Trajbar, S. (2017). Geomorfološka obilježja doline i poriječja rijeke Krke s osvrtom na dio od Knina do Bilušića buka. *Geoadria, 10*(2), 131–156. https://doi.org/10.15291/geoadria.55

Plenković-Moraj, A., Gligora, M., Kralj, K., and Mustafić, P. (2007). Diatoms in monitoring of Drava River, Croatia. *Arch. Hydrobiol. Suppl., 17*(3-4), 511–525.

Pont, A. C., and Ivković, M. (2013). The hunter-flies of Croatia (Diptera: Muscidae: genus *Limnophora* Robineau-Desvoidy). *Journal of Natural History, 47*(15–16), 1069–1082. https://doi.org/10.1080/00222933.2012.750775

Previšić, A., Graf, W., Vitecek, S., Kučinić, M., Bálint, M., Keresztes, L., Pauls, S. U., and Waringer, J. (2014). Cryptic diversity of caddisflies in the Balkans: The curious case of Ecclisopteryx species (Trichoptera: Limnephilidae). *Arthropod Systematics & Phylogeny*, *72*(3), 309–329.

Primc-Habdija, B., Habdija, I., Matoniĉkin, R., and Špoljar, M. (2005). Development of ciliate community on artificial substrates associated with vertical gradients of environmental conditions in a karstic lake. *Archiv Für Hydrobiologie, 164*(4), 513–527. https://doi.org/10.1127/0003-9136/2005/0164-0513

Primc-Habdija, B., and Matoničkin, R. (2005). A new freshwater folliculinid (Ciliophora) from the karstic region of Croatia. *European Journal of Protistology, 41*(1), 37–43. https://doi.org/10.1016/j.ejop.2004.09.004

Ptacnik, R., Solimini, A. G., Andersen, T., Tamminen, T., Brettum, P., Lepistö, L., Willén, E., and Rekolainen, S. (2008). Diversity predicts stability and resource use efficiency in natural phytoplankton communities. *Proceedings of the National Academy of Sciences*, *105*(13), 5134–5138. https://doi.org/10.1073/pnas.0708328105

Rapacciuolo, G., Maher, S. P., Schneider, A. C., Hammond, T. T., Jabis, M. D., Walsh, R. E., Iknayan, K. J., Walden, G. K., Oldfather, M. F., Ackerly, D. D., and Beissinger, S. R. (2014). Beyond a warming fingerprint: Individualistic biogeographic responses to heterogeneous climate change in California. *Global Change Biology*, *20*(9), 2841–2855. https://doi.org/10.1111/gcb.12638

Ravbar, N., and Kovačič, G. (2015). Vulnerability and protection aspects of some Dinaric karst aquifers: A synthesis. *Environmental Earth Sciences*, *74*(1), 129–141. https://doi.org/10.1007/s12665-014-3945-7

Reiss, J., Bridle, J. R., Montoya, J. M., and Woodward, G. (2009). Emerging horizons in biodiversity and ecosystem functioning research. *Trends in Ecology & Evolution*, *24*(9), 505–514. https://doi.org/10.1016/j.tree.2009.03.018

Reynolds, C. S. (2006). The Ecology of Phytoplankton. Cambridge University Press, Cambridge.

Rimet, F., and Bouchez, A. (2012). Life-forms, cell-sizes and ecological guilds of diatoms in European rivers. *Knowledge and Management of Aquatic Ecosystems*, *406*, 01. https://doi.org/10.1051/kmae/2012018

Rosset, V., Angélibert, S., Arthaud, F., Bornette, G., Robin, J., Wezel, A., Vallod, D., and Oertli, B. (2014). Is eutrophication really a major impairment for small waterbody biodiversity? *Journal of Applied Ecology*, *51*(2), 415–425. https://doi.org/10.1111/1365-2664.12201

Round, F.E., Crawford, R.M., and Mann, D.G. (1990). *The Diatoms. Biology and Morphology of the Genera*. Cambridge University Press, Cambridge.

Šafarek, G., and Šoltić, T. (2011). Rijeke Hrvatske. Veda d.o.o.

Santos, A., van Aerle, R., Barrientos, L., and Martinez-Urtaza, J. (2020). Computational methods for 16S metabarcoding studies using Nanopore sequencing data. *Computational and Structural Biotechnology Journal*, *18*, 296–305. https://doi.org/10.1016/j.csbj.2020.01.005

Sarr, A. B., Benetti, C. J., Fernández-Díaz, M., and Garrido, J. (2013). The microhabitat preferences of water beetles in four rivers in Ourense province, Northwest Spain. *Limnetica*, *32*, 1–10. https://doi.org/10.23818/limn.32.01

Schmidt, K., Birchill, A. J., Atkinson, A., Brewin, R. J. W., Clark, J. R., Hickman, A. E., Johns, D. G., Lohan, M. C., Milne, A., Pardo, S., Polimene, L., Smyth, T. J., Tarran, G. A., Widdicombe, C. E., Woodward, E. M. S., and Ussher, S. J. (2020). Increasing picocyanobacteria success in shelf waters contributes to long-term food web degradation. *Global Change Biology*, *26*(10), 5574–5587. https://doi.org/10.1111/gcb.15161

Sigee, D. C. (2005). Freshwater microbiology: Biodiversity and Dynamic Interactions of Microorganisms in the aquatic Environment. John Wiley & Sons, LTD; West Sussex, England.

Šiljeg, A., Marić, I., Cukrov, N., Domazetović, F., and Roland, V. (2020). A Multiscale Framework for Sustainable Management of Tufa-Forming Watercourses: A Case Study of National Park "Krka", Croatia. *Water*, *12*(11), 3096. https://doi.org/10.3390/w12113096

Simboura, N., Panayotidis, P., and Papathanassiou, E. (2005). A synthesis of the biological quality elements for the implementation of the European Water Framework Directive in the Mediterranean ecoregion: The case of Saronikos Gulf. *Ecological Indicators*, *5*(3), 253–266. https://doi.org/10.1016/j.ecolind.2005.03.006

Simmons, L. J., Sandgren, C. D., and Berges, J. A. (2016). Problems and pitfalls in using HPLC pigment analysis to distinguish Lake Michigan phytoplankton taxa. *Journal of Great Lakes Research*, *42*(2), 397–404. https://doi.org/10.1016/j.jglr.2015.12.006

Simó, R. (2001). Production of atmospheric sulfur by oceanic plankton: Biogeochemical, ecological and evolutionary links. *Trends in Ecology & Evolution*, *16*(6), 287–294. https://doi.org/10.1016/S0169-5347(01)02152-8

Simon, M., López-García, P., Deschamps, P., Moreira, D., Restoux, G., Bertolino, P., and Jardillier, L. (2015). Marked seasonality and high spatial variability of protist communities in shallow freshwater systems. *The ISME Journal*, *9*(9), 1941–1953. https://doi.org/10.1038/ismej.2015.6

Søndergaard, M., Jeppesen, E., Peder Jensen, J., and Lildal Amsinck, S. (2005). Water Framework Directive: Ecological classification of Danish lakes: Water Framework Directive and Danish lakes. *Journal of Applied Ecology*, *42*(4), 616–629. https://doi.org/10.1111/j.1365-2664.2005.01040.x

Stanković, I., Vlahović, T., Gligora Udovič, M., Várbíró, G., and Borics, G. (2012). Phytoplankton functional and morpho-functional approach in large floodplain rivers. *Hydrobiologia*, *698*(1), 217–231. https://doi.org/10.1007/s10750-012-1148-3

Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M. D. M., Breiner, H.-W., and Richards, T. A. (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

Stoeck, T., Breiner, H., Filker, S., Ostermaier, V., Kammerlander, B., and Sonntag, B. (2014). A morphogenetic survey on ciliate plankton from a mountain lake pinpoints the necessity of lineage-specific barcode markers in microbial ecology. *Environmental Microbiology*, *16*(2), 430–444. https://doi.org/10.1111/1462-2920.12194

Stoecker, D. K., and Silver, M. W. (1987). Chloroplast Retention by Marine Planktonic Ciliates. *Annals of the New York Academy of Sciences*, *503*(1), 562–565. https://doi.org/10.1111/j.1749-6632.1987.tb40646.x

Strahler, A. N. (1952). Hypsometric (Area-Altitude) Analysis Of Erosional Topography. *Geological Society of America Bulletin*, *63*(11), 1117. https://doi.org/10.1130/0016-7606(1952)63[1117:HAAOET]2.0.CO;2

Taberlet, P., Coissac, E., Hajibabaei, M., and Rieseberg, L. H. (2012). Environmental DNA. *Molecular Ecology*, *21*(8), 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Tamburini, A., and Menichetti, M. (2020). Groundwater Circulation in Fractured and Karstic Aquifers of the Umbria-Marche Apennine. *Water*, *12*(4), 1039. https://doi.org/10.3390/w12041039

Tanabe, A. S., Nagai, S., Hida, K., Yasuike, M., Fujiwara, A., Nakamura, Y., Takano, Y., and 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. *Molecular Ecology Resources*, *16*(2), 402–414. https://doi.org/10.1111/1755-0998.12459

Thakur, M. P., Bakker, E. S., Veen, G. F. (Ciska), and Harvey, J. A. (2020). Climate Extremes, Rewilding, and the Role of Microhabitats. *One Earth*, *2*(6), 506–509. https://doi.org/10.1016/j.oneear.2020.05.010

Thienemann, A. (1925). Die Binnengewässer Mitteleuropas Eine limnologische Einführung. Die Binnengewässer, Stuttgart.

Vasselon, V., Rimet, F., Tapolczai, K., and Bouchez, A. (2017). Assessing ecological status with diatoms DNA metabarcoding: Scaling-up on a WFD monitoring network (Mayotte island, France). *Ecological Indicators*, *82*, 1–12. https://doi.org/10.1016/j.ecolind.2017.06.024

Vaz-Moreira, I., Nunes, O. C., and Manaia, C. M. (2011). Diversity and Antibiotic Resistance Patterns of Sphingomonadaceae Isolates from Drinking Water. *Applied and Environmental Microbiology*, *77*(16), 5697–5706. https://doi.org/10.1128/AEM.00579-11

Vermaat, J. E. (2005). Periphyton Dynamics and Influencing factor. Periphyon. In: Azim, M. E., Verdegem, M. C. J., van Dam, A. A., Beveridge, M. C. M. (Eds) Periphyon: Ecology, Exploitation and Management. CABI Publisihing, Oxfordshire, 35–45.

Vilenica, M., Brigić, A., Sartori, M., and Mihaljević, Z. (2018). Microhabitat selection and distribution of functional feeding groups of mayfly larvae (Ephemeroptera) in lotic karst habitats. *Knowledge & Management of Aquatic Ecosystems*, *419*, 17. https://doi.org/10.1051/kmae/2018011

Warren, A., Patterson, D. J., Dunthorn, M., Clamp, J. C., Achilles-Day, U. E. M., Aescht, E., Al-Farraj, S. A., Al-Quraishy, S., Al-Rasheid, K., Carr, M., Day, J. G., Dellinger, M., El-Serehy, H. A., Fan, Y., Gao, F., Gao, S., Gong, J., Gupta, R., Hu, X., Kamra, K., Langlois, G., Lin, X., Lipscomb, D., Lobban, C., S., Luporini, P., Lynn, D. H., Ma, H., Macek, M., Mackenzie-Dodds, J., Makhija, S., Mansergh, R. I., Martín-Cereceda, M., McMiller, N., Montagnes, D. J. S., Nikolaeva, S., Ong'ondo, G. O., Pérez-Uz, B., Purushothaman, J., Quintela-Alonso, P., Rotterová, J., Santoferrara, L., Shao, C., Shen, Z., Shi, X., Song, W., Stoeck, T., La Terza, A., Vallesi, A., Wang, M., Weisse, T., Wiackowski, K., Wu, L., Xu, K., Yi, Z., Zufall, R. and Agatha, S. (2017). Beyond the "Code": A Guide to the Description and Documentation of Biodiversity in Ciliated Protists (Alveolata, Ciliophora). *Journal of Eukaryotic Microbiology*, *64*(4), 539–554. https://doi.org/10.1111/jeu.12391

WFD. (2000). Directive 2000/60/ec of the European Parliament and of the Council 22.12.2000. Off. J. Eur. Communities L327, 1-72.

Wilhelm, K., Mitchell, P. B., Niven, H., Finch, A., Wedgwood, L., Scimone, A., Blair, I. P., Parker, G., and Schofield, P. R. (2006). Life events, first depression onset and the serotonin transporter gene. *British Journal of Psychiatry*, *188*(3), 210–215. https://doi.org/10.1192/bjp.bp.105.009522

Wiltshire, K. H., Harsdorf, S., Smidt, B., Blöcker, G., Reuter, R., and Schroeder, F. (1998). The determination of algal biomass (as chlorophyll) in suspended matter from the Elbe estuary and the German Bight. *Journal of Experimental Marine Biology and Ecology*, *222*(1–2), 113–131. https://doi.org/10.1016/S0022-0981(97)00141-X

Worden, A. Z., Follows, M. J., Giovannoni, S. J., Wilken, S., Zimmerman, A. E., and Keeling, P. J. (2015). Rethinking the marine carbon cycle: Factoring in the multifarious lifestyles of microbes. *Science*, *347*(6223), 1257594. https://doi.org/10.1126/science.1257594

Xie, R., Zhao, G., Yang, J., Wang, Z., Xu, Y., Zhang, X., and Wang, Z. (2021). eDNA metabarcoding revealed differential structures of aquatic communities in a dynamic freshwater ecosystem shaped by habitat heterogeneity. *Environmental Research*, *201*, 111602. https://doi.org/10.1016/j.envres.2021.111602

Zimmermann, J., Glöckner, G., Jahn, R., Enke, N., and Gemeinholzer, B. (2015). Metabarcoding vs. Morphological identification to assess diatom diversity in environmental studies. *Molecular Ecology Resources*, *15*(3), 526–542. https://doi.org/10.1111/1755-0998.12336

Zubkov, M. V., and Tarran, G. A. (2008). High bacterivory by the smallest phytoplankton in the North Atlantic Ocean. *Nature*, *455*(7210), 224–226. https://doi.org/10.1038/nature07236

Žuljević, A., Kaleb, S., Peña, V., Despalatović, M., Cvitković, I., De Clerck, O., Le Gall, L., Falace, A., Vita, F., Braga, J. C., and Antolić, B. (2016). First freshwater coralline alga and the role of local features in a major biome transition. *Scientific Reports*, *6*(1), 19642. https://doi.org/10.1038/srep19642

Žutinić, P., Kulaš, A., Levkov, Z., Šušnjara, M., Orlić, S., Kukić, S., Goreta, G., Valić, D., and Gligora Udovič, M. (2020). Ecological status assessment using periphytic diatom communities—Case study Krka River. *Macedonian Journal of Ecology and Environment, 22*(1), 29–44.

CURRICULUM VITAE

Antonija Kulaš was born on April 5, 1989 in Slavonski Brod (Croatia). She finished elementary and high school in Slavonski Brod (Croatia) and continued her education in Zagreb (Croatia) at the University of Zagreb, Faculty of Science, Department of Biology. She completed her undergraduate studies in Environmental Sciences in 2011 with the title of Bachelor of Environmental Sciences (univ. bacc. oecol.) and also finished the graduate programme in Ecology and Nature Preservation in 2014 with the title of Master of Ecology and Nature Preservation (mag. eocol. et prot. nat.). In April 2016, she started her internship as a biological engineer at the Central Water Management Laboratory, Croatian Waters in Zagreb. During her internship she started her work in ecology and taxonomy of freshwater algae, and soon, in July 2017, she started working as a research assistant in laboratory for freshwater algae and enrolled in a PhD programme in Biology at the University of Zagreb, Faculty of Natural Sciences, Department of Biology. Her PhD work was within the Crotian Science Foundation project „Origin, fate and TRAnsport modelling of NItrate in the Varaždin ALluvial aquifer - (TRANITAL)", led by dr. sc. Tamara Marković and within „Assessment of the ecological status of the Krka River using DNA metabarcoding, NP Krka" project, led by Assoc. prof. dr. sc. Marija Gligora Udovič. She has published 11 scientific papers and participated in 15 scientific conferences, of which she actively contributed to 7. She received the Rector's Award in 2013 and two research and training grants (FEMS Research and Training Grant and COST DNA - qua-Net grant). She has also served as a teaching assistant in the undergraduate and graduate courses in Protists, Algae, Applied phycology, and Field course in invertebrate and protist biodiversity. During her doctoral studies, she has undergone several training courses abroad and in Croatia, and participated in workshops and seminars on next generation sequencing and statistical processing of data in the PRIMER v7 and R programming language. She also contributed to scientific popularization in events as Night of Biology and Festival of Science.

CROSBI PROFILE: Antonija Kulaš
(CROSBI Profile: 34311, MBZ: 363705)

Scientific Publications

Gulin Beljak, V., **Kulaš, A**., Lentendu, G., Vlaičević, B., Gligora Udovič, M., Sertić Perić, M., Rebrina, F., Žutinić, P., Orlić, S., Matoničkin Kepčija, R. (2022). Changes in Phylogenetic and Functional Diversity of Ciliates along the Course of a Mediterranean Karstic River. *Microorganisms*, 10(12), 2493. 15 doi:10.3390/microorganisms10122493

Gligora Udovič, M., Šušnjara, M., **Kulaš**, **A**., Goreta, G., Arapov, J., Levkov, Z. (2022). One new species of *Aneumastus* D.G. Mann & Stickle (Bacillariophyceae) from River Krka, Croatia. *Fottea*, 23-1.

Gligora Udovič, M., **Kulaš**, **A**., Šušnjara, M., Arapov, J., Blanco, S., Levkov Z. (2022). *Cymbopleura amicula* stat nov. et. nom. nov. (Bacillariophyceae)—a rare diatom species from a karst river in Croatia. *Phytotaxa*, 532(2), 139-151. doi:10.11646/PHYTOTAXA.532.2.2

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. *Science of the total environment*, 829, 154536. doi:10.1016/j.scitotenv.2022.154536

Šimunović, M., Kulaš, A., Žutinić, P., Goreta, G., Gligora Udovič, M. (2022). Phytoplankton metrics for trophic and ecological status assessment of a natural karstic lake. *Acta Botanica Croatica*, 81(2), 25. doi:10.37427/botcro-2022-016

Kulaš, **A**., Marković, T., Žutinić, P., Kajan, K., Karlović, I., Orlić, S., Keskin, E., Filipović, V., Gligora Udovič, M. (2021). Succession of Microbial Community in a Small Water Body within the Alluvial Aquifer of a Large River. *Water*, 13(2), 115. 23 doi:10.3390/w13020115

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. *Ecological indicators*, 124,107430. doi:10.1016/j.ecolind.2021.107430

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 communites - case study Krka River. *Macedonian Journal of Ecology and Environment*, 22(1), 29-44.

Kulaš, **A**., Gligora Udovič, M., Ector, L., Van de Vijver, B. (2020). Analysis of the type material of *Achnanthes hauckiana* Grunow (Achnanthaceae, Bacillariophyceae). *Botany Letters*, 167(4), 439-452. doi:10.1080/23818107.2020.1808527

Caput Mihalić, K., Gligora Udovič, M., Galović, I., Stanković, I., Šušnjara, M., Žutinić, P., **Kulaš**, **A**., Špoljarić, I., Levkov, Z. (2019). *Tetramphora croatica* sp. nov.- A new brackish-water species from Lake Vransko, Croatia. *Phytotaxa*, 401(4), 276-286. doi:10.11646/phytotaxa.401.4.5

Gligora Udovič, M., Žutinić, P., Kavre Piltaver, I., **Kulaš**, **A**., Ozimec, R., Tofilovska, S. (2018). *Gomphosphenia*

plenkoviciae sp. nov. A new species from Crveno jezero, Croatia. *Phytotaxa*, 351(3), 229-238. doi:10.11646/phytotaxa.351.3.4

Conference Proceedings

Kulaš, **A**., Žutinić, P., Gulin, V., Matoničkin Kepčija, R., Sertić Perić, M., Orlić, S., Sviličić Petrić, I., Marković, T., Šušnjara, M., Gligora Udovič M. (2022). Protist diversity in periphyton of tufa-depositing system. 14th Croatian Biological Congress

Šimunović, M., **Kulaš**, **A**., Žutinić, P., Goreta, G., Šušnjara, M., Gligora Udovič, M. (2022). Establishing reliable phytoplankton metrics for bioassessment of a karstic lake. 14th Croatian Biological Congress

Kamberović, J., Gligora Udovič, M., **Kulaš**, **A**., Žutinić, P., Šušnjara, M., Ahmić, A., Jusufović, A., Gajić, A., Kalamujić Stroil, B. (2022). Ecological status of the tufa-depositing Una River based on diatom eDNA metabarcoding approach. 14th Croatian Biological Congress

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 as bioindicators of environmental pressure in a karstic river. DNAQUA Conference

Kamberović, J., Gligora Udovič, M., Kahlert, M., Tapolczai, K., Lukić, Z., Ahmić, A., Dedić, A., **Kulaš**, **A**., Kajan, K., Hanjalić, J., Lasić, L., Kalamujić Stroil, B. (2021). Composition of diatom communities on travertine barriers of the Una River (Bosnia and Herzegovina) obtained by DNA metabarcoding and morphological analysis. DNAQUA Conference

Stanković, I., **Kulaš**, **A**., Šušnjara, M., Hanžek, N., Gligora Udovič, M., Levkov, Z. (2019). First occurrence of *Synedropsis roundii* Torgan, Menezes, & Melo (Bacillariophyta) in Europe with description of habitat and ecological preferences. 7th European Phycological Congress

Kulaš, **A**., Gligora Udovič, M., Žutinić, P., Kavre Piltaver, I., Ozimec, R., Tofilovska S., Šušnjara, M., Levkov, Z. (2019). A new diatom species from Lake Crveno jezero, Croatia (*Gomphosphenia plenkoviciae* sp. nov.). 3rd Symposium of Freshwater Biology - SOBS

Kulaš, **A**., Šušnjara, M., Žutinić, P., Gligora Udovič, M., Kukić, S., Plenković-Moraj, A., Goreta, G., Orlić, S., Valić, D., Levkov, Z. (2019). Diatoms of Krka River, Croatia. 12th Central European Diatom meeting, Belvaux, Luxembourg

Kulaš, **A**., Gulin, V., Matoničkin Kepčija, R., Sertić Perić, M., Žutinić, P., Šušnjara, M., Orlić, S., Kajan, K., Stoeck, T., Lentendu, G., Martinić, I., Čanjevac, I. Gligora Udovič, M. (2019). Diversity and ecological preference of ciliates assemblage in a freshwater karstic river. SEFS11 Symposium for European Freshwater Sciences.

Kulaš, **A**., Žutinić, P., Orlić, S., Karlović, I., Gligora Udovič, M., Marković, T. (2019). Nitrate concentration in alluvial aquifer of the Drava River in correlation with toxic algal bloom. 7th European Phycological Congress.

Šimunović, M., Šušnjara, M., Žutinić, P., **Kulaš**, **A**., Plenković-Moraj, A., Orlić, S., Kajan, K., Valić, D., Žunić, J., Goreta, G., Gligora Udovič, M. (2019). Application of new tools in biomonitoring of a freshwater karstic Lake Visovac (Croatia). 7th European Phycological Congress.

Kulaš, **A**., Marković, T., Žutinić, P., Gligora Udovič, M. (2018). Changes of phytoplankton community structure in correlation with different concentrations of nitrates in surface water of Varaždin alluvial aquifer system. Simpozij studenata doktorskih studija PMF-a.

Šimunović, M., **Kulaš**, **A**., Šušnjara, M., Žutinić, P., Plenković-Moraj, A., Gligora Udovič, M. (2018). HPLC analiza - brza metoda za praćenje dinamike fitoplanktona. 13. Hrvatski biološki kongres.

Marković, T., Šparica Miko, M., Đumbir, A.M., Gligora Uvodič, M., Kulaš, A., Larva, O., Brkić, Ž. (2018). Variations of nitrate concentrations in ground and surface waters of Varaždin alluvial system. 5. Slovenski geološki kongres.

Pjevac, P., Žutinić, P., Gligora Udovič, M., Stević, F., Špoljarić, D., Žuna, T., Špoljarić Maronić, D., Stanković, I., Schmidt, H., Goreta, G., **Kulaš**, **A**., Plenković-Moraj, A., Orlić S. (2018). Community composition in lakes and reservoirs along a trophic gradient. ASLO 2018

Other papers

Mustafić, P., Plenković-Moraj, A., Mihaljević, Z., Kerovec, M., Alegro, A., Marčić, Z., Zanella, D., Ćaleta, M., Buj, I., Gligora Udovič, M., Žutinić, P., **Kulaš**, **A**., Šušnjara, M., Horvatić, S. (2022). Biološka ispitivanja nadzemnih voda na HE Varaždin, HE Čakovec i HE Dubrava u 2021. godini. (elaborate).

Mustafić, P., Plenković-Moraj, A., Mihaljević, Z., Kerovec, M., Alegro, A., Marčić, Z., Zanella, D., Ćaleta, M., Buj, I., Gligora Udovič, M., Žutinić, P., **Kulaš**, **A**., Šušnjara, M., Horvatić, S. (2021). Biološka ispitivanja nadzemnih voda na HE Varaždin, HE Čakovec i HE Dubrava u 2020. godini. (elaborate).

Mustafić, P., Mrakovčić, M., Plenković-Moraj, A., Mihaljević, Z., Kerovec, M., Algero, A., Marčić, Z., Zanella, D., Ćaleta, M., Buj, I., Gligora Udovič, M., Žutinić, P., **Kulaš**, **A**., Horvatić, S., Šušnjara, M. (2019). Biološka ispitivanja voda na HE Varaždin, HE Čakovec i HE Dubrava u 2018. godini. (elaborate).

Mustafić, P., Mrakovčić, M., Plenković-Moraj, A., Mihaljević, Z., Kerovec, M., Alegro, A., Zanella, D., Marčić, Z., Ćaleta, M., Gligora Udovič, M., Žutinić, P., **Kulaš**, **A**., Horvatić, S. (2018). Biološka ispitivanja nadzemnih voda na HE Varaždin, HE Čakovec i HE Dubrava u 2017. godini. (elaborate).

Mustafić, P., Mrakovčić, M., Plenković-Moraj, A., Mihaljević, Z., Ternjej, I., Kerovec, M., Zanella, D., Marčić, Z., Ćaleta, M., Žutinić, P., Gligora Udovič, M., **Kulaš**, **A**., Horvatić, S. (2017). Istraživanja i optimizacija ihtiocenoze u svrhu smanjenja trofije akumulacije Butoniga tijekom 2017. godine. (elaborate).

Workshops & Trainings

2021 - „Workshop on Multivariate Analysis in Ecology(& Other Sciences), using PRIMER version 7", Australia, Plymouth Marine Laboratory (online course).

2021 - „Introduction to Geographic Analyses of Biodiversity", Barcelona, Spain, Transmitting Science.

February/March 2020 - COST- DNA grant for one month stay at Ankara University, Department of Fisheries and Aquaculture, Evolutionary Genetics Laboratory), host: Assoc. prof. Emre Keskin

October/November 2018 - Erasmus mobility stuff, , Technische Universität Kaiserslautern, Germany, host: prof. Thorsten Stoeck

2018 - Microbial ecology: Hands-on training in prokaryotic and eukaryotic metagenomics (ICME-9), Universite Libre de Bruxelles, Bruxelles, EMBO organization.

2018 - International Diatom Taxonomy Workshop, Botanical Garden Meise, Meise, Belgium.

2017 - Bioinformatics and statistics for Next generation sequencing. BIOCentre, Zagreb, Croatia.

2017 - Statistics and work in R programme. BIOCentre, Zagreb, Croatia.

2017 - Determination Course of Freshwater and Terrestrial Cyanobacteria, University of South Bohemia, Češke Budejovice, Czech Republic.

Activities

2019 - participation at exibition of „Festival of Science“

2019 - Technical team member at the 7th European Phycological Congress (EPC7)

2018 - member of „Urban Algae“ project, https://freshproject-urbanalgae.jimdofree.com/citizen-science/

Awards & Grants

2020 - COST DNAqua-Net grant „eDNA metabarcoding in providing assessment of microbial eukaryotic biodiversity in small standing-water ecosystem”.

2018 - FEMS, Research and Training Grant “Metabarcoding monitoring analysis of changes in the microbial community structure in correlation with different concentrations of nitrates in alluvial aquifer of Drava River“.

2013 - Rector's award for research - „Višegodišnje promjene u sastavu i strukturi hematofagnih mušica svrbljivica (Diptera, Simuliidae) na sedrenim barijerama Plitvičkih jezera".

2012 – Special Rector's award for participating in scientific popularization event „Night of Biology“.