Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET

BIOLOŠKI ODSJEK

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BIOINDIKATORSKI POTENCIJAL PERIFITONA U PRAĆENJU REVITALIZACIJE SEDRENIH BARIJERA

DOKTORSKI RAD

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FACULTY OF SCIENCE

DEPARTMENT OF BIOLOGY

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BIOINDICATOR POTENTIAL OF PERIPHYTON IN MONITORING REVITALIZATION OF TUFA BARRIERS

DOCTORAL THESIS

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Ovaj doktorski rad izrađen je u Laboratoriju za beskralješnjake Zoologijskog zavoda Biološkog odsjeka Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu, pod vodstvom prof. dr. sc. Renate Matoničkin Kepčija, u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu.

Informacije o mentorici

MATONIČKIN KEPČIJA, RENATA (Bjelovar, 6. studenog 1972.), redovita profesorica, 231956

Osnovnu školu završila je u Sedlarici i Pitomači, a srednju školu matematičko-informatičkog usmjerenja u Virovitici. Diplomirala je 1997. godine na Biološkom odsjeku Prirodoslovno-matematičkog fakulteta, Sveučilište u Zagrebu. Na istoj instituciji je obranila magistarski rad 2000. godine, a doktorirala je 2006. godine. Od 1997. do 2007. zaposlena je kao znanstveni novak na Zoologijskom zavodu pri Biološkom odsjeku PMF-a Sveučilišta u Zagrebu. Kao znanstveni novak sudjelovala je u nastavi iz kolegija Avertebrata, Biologija beskralježnjaka, Protista, Osnove biologije, Limnologija, Ekologija protista i Terenska nastava. U znanstveno-nastavno zvanje docent izabrana je 2007. godine, a od veljače 2022. godine je redovita profesorica. Na preddiplomskim i diplomskim te integriranom studijskom programu predaje kolegije Beskralješnjaci, Protista, Ekologija protista, Terenska nastava iz biološke raznolikosti protista i invertebrata, Raznolikost faune Hrvatske i Zaštićene vrste i područja RH te kolegije Biološka klasifikacija slatkih voda i funkcionalna organizacija zajednica i Stupanj trofije i protok energije u ekosustavu na doktorskom studiju Biologije. Pod njenim mentorstvom izrađeno je 16 diplomskih radova, 13 završnih radova, tri rada nagrađenih Rektorovom nagradom, jedan magistarski rad te tri doktorske disertacije. Njena znanstvena djelatnost je u području ekologije slatkih voda, primarno ekologije fagotrofnih protista u perifitonu tekućica, poplavnih područja i cretova. Pratila je učinak raznih čimbenika na strukturiranje perifitona, učinak revitalizacijskih mjera u zaštićenim područjima, organizme kao biomedijatore u procesu osedravanja u krškim tekućicama, učinke eutrofikacije na tekućice te primjenu praživotinja kao bioindikatora uzimajući u obzir biocenološke pokazatelje i njihovu funkcionalnu ekologiju. Dio njenih istraživanja ulazi u područje primijenjene ekologije te se može primijeniti u upravljanju zaštićenim područjima i u monitoringu tekućica. Objavila je više od 50 znanstvenih radova, a na znanstvenim skupovima je sudjelovala sa 60 priopćenja. Koautorica je dva sveučilišna udžbenika. Autorica je i više stručnih studija vezanih uz praćenje revitalizacije sedrenih barijera te razvoja klasifikacijskih sustava ocjene ekološkog stanja tekućica. Sudjelovala je na nizu znanstvenih projekata, vodila nekoliko stručnih projekata te sudjelovala u pripremi i provođenju jednog strateškog projekta Biološkog odsjeka PMF-a. Voditeljica je projekta cjelovite obnove jedne zgrade PMF-a na Marulićevom trgu 20. Redoviti je recenzent u desetak znanstvenih časopisa iz područja limnologije. Bila je član organizacijskih i znanstvenih odbora nekoliko stručnih i znanstvenih skupova (11th Symposium for European Freshwater Sciences, Zagreb, 2019.; 1., 2. i 3. Simpozij o biologiji slatkih voda, Zagreb, 2015., 2017., 2019.), a urednica je dvije knjige sažetaka znanstvenog skupa. Aktivno sudjeluje u međunarodnom GLOBE programu koji je pod pokroviteljstvom Agencije za odgoj i obrazovanje. Više od dva desetljeća je članica, a bila je i predsjednica Državnog povjerenstva za Smotru i natjecanje hrvatskih GLOBE škola te je sudjelovala u organizacijskom odboru GLOBE Learning Expedition (Šibenik, 2003.). Na Biološkom odsjeku PMF-a obavljala je dužnost pomoćnice pročelnice Biološkog odsjeka za znanost i zamjenice pročelnice te je bila studentski voditelj integriranog preddiplomskog i diplomskog studija biologije i kemije. Nekoliko godina je sudjelovala u radu povjerenstva za diplomski ispit te povjerenstava za upis u preddiplomske i diplomske studijske programe Biološkog odsjeka. Bila je predsjednica Hrvatskog udruženja slatkovodnih ekologa, a član je nekoliko strukovnih društava.

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Alone, a journey.

Together, an adventure.

Sveučilište u Zagrebu
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Biološki odsjek

Doktorski rad

BIOINDIKATORSKI POTENCIJAL PERIFITONA U PRAĆENJU REVITALIZACIJE SEDRENIH BARIJERA

Vesna Gulin Beljak
Prirodoslovno-matematički fakultet, Biološki odsjek

Restauracijski napori u lotičkim ekosustavima svakodnevno se povećavaju diljem svijeta. U sklopu ovog doktorskog rada prvi put je istražen bioindikatorski potencijal perifitona tj. njegove heterotrofne komponente (ameboidni protisti i trepetljikaši) u detekciji i praćenju revitalizacije vodenih tokova sedrene barijere Skradinski buk nakon provedenih mjera restauracije u vidu uklanjanja jedinki invazivne biljne vrste pajasena. Sveobuhvatnom analizom taksonomskih i funkcionalnih metrika fagotrofnih protista te pripadajućih okolišnih čimbenika revitaliziranih tokova potvrđen je odgovor perifitona na revitalizaciju. Promjenjiva hidrologija revitaliziranih tokova (bujični tokovi/presušivanje) utjecala je na većinu okolišnih parametara (fizikalno-kemijske parametre vode, podrijetlo i količinu organske tvari, količinu sedre) te posljedično taksonomski i funkcionalni sastav perifitona. U oblikovanju funkcionalne raznolikosti fagotrofnih protista osim okolišnih parametara ključnom se pokazala i interspecijska kompeticija odnosno kompetitivno isključivanje, a važnost iste potvrđena je i istraživanjem funkcionalne i filogenetske raznolikosti trepetljikaša duž rijeke Krke.

(193 stranice, 2 slike, 212 literaturnih navoda, jezik izvornika hrvatski)

Ključne riječi: restauracija, ameboidni protisti, trepetljikaši, sedra, funkcionalna raznolikost, organska tvar

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Doctoral thesis

BIOINDICATOR POTENTIAL OF PERIPHYTON IN MONITORING REVITALIZATION OF TUFA BARRIERS

Vesna Gulin Beljak

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Efforts to restore lotic ecosystems are increasing daily worldwide. In this doctoral thesis, the bioindicator potential of periphyton, i.e., its heterotrophic components (amoeboid protists and ciliates) in detection and monitoring stream revitalization at Skradinski buk tufa barrier was investigated for the first time. A comprehensive analysis of taxonomic and functional metrics of phagotrophic protists and associated environmental parameters of revitalized streams confirmed the response of periphyton to revitalization. The changing hydrology of revitalized streams (flooding/drying events) affected most environmental parameters (physicochemical parameters of water, origin and amount of organic matter, amount of tufa) and consequently the taxonomic and functional composition of periphyton. Besides environmental parameters, interspecies competition was crucial in shaping the functional diversity of phagotrophic protists, and its importance was also confirmed by a study of ciliates' functional and phylogenetic diversity along the Krka River.

(193 pages, 2 figures, 212 references, original in Croatian)

Keywords: restoration, amoeboid protists, ciliates, tufa, functional diversity, organic matter

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Sadržaj

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PROŠIRENI SAŽETAK

Lotički ekosustavi pod značajnim su antropogenim pritiskom uslijed degradacije staništa, zagađenja, prekomjernog iskorištavanja i unosa invazivnih vrsta koji nerijetko rezultiraju gubitkom bioraznolikosti (MEA, 2005). Stoga restauracijski napori u lotičkim ekosustavima svakodnevno rastu, a Europska unija (EU) ima za cilj restaurirati najmanje 20% kopnenih i vodenih površina u EU do 2030. godine u okviru globalnog pokreta restauracije degradiranih ekosustava (engl. *United Nations (UN) Decade on Ecosystem Restoration*). U svjetlu spomenutog, za očekivati je da će s restauracijskim naporima rasti i potreba za praćenjem odgovora ekosustava na restauraciju te istraživanjima procjene uspješnosti provedenih mjera pri čemu je od iznimne važnosti definirati specifične i mjerljive ciljeve restauracije te utvrditi optimalan način/metodu procjene uspješnosti samih mjera (Woolsey i sur., 2007). U razumijevanju potencijala restauracije lotičkih ekosustava i procjeni uspješnosti mjera iste, tradicionalno se najveći naglasak stavlja na ulogu strukturne složenosti, odnosno fizičke heterogenosti staništa/mikrostaništa pri čemu je biološka komponenta restauracije nerijetko zapostavljena. Ipak, ove dvije komponente blisko su povezane jer strukturna složenost pogoduje većoj brojnosti i raznolikosti vrsta pružajući veći raspon niša te osim toga podržava više izvora hrane uslijed zadržavanja čestica organske tvari (Taniguchi i sur., 2003; Frainer i sur., 2017). Povezanost složenosti mikrostaništa i retencije organske tvari posebno je važna za zajednice vezane uz podlogu poput perifitona čiji matriks zadržava i akumulira organsku tvar te tako utječe na prijenos iste između nižih i viših trofičkih razina lotičkih ekosustava (Risse-Buhl i sur., 2015; Geisen i sur., 2017), a i sam usložnjava stanište. Upravo zbog toga i niza dodatnih razloga, perifiton se često primjenjuje kao bioindikator u istraživanjima lotičkih ekosustava (Wu, 2017), a njegova heterotrofna komponenta (fagotrofni protisti) ima i dugu tradiciju istraživanja unutar sedrotvornih ekosustava Republike Hrvatske poput Nacionalnog parka Krka (NP Krka) koja osim sastava perifitona izučavaju i međuodnos perifitona, dinamike organske tvari i procesa osedravanja (Matoničkin i Pavletić, 1962; Primc-Habdija i sur., 1997, 2001; Matoničkin Kepčija i sur., 2017). U svjetlu novih izazova s kojima se susreće NP Krka, poput zarastanja sedrene barijere Skradinski buk invazivnom biljnom vrstom pajasen (*Ailanthus altissima* (Mill.) Swinge) te posljedično presušivanja dijelova barijere, bioindikatorski potencijal perifitona mogao bi se proširiti i na detekciju i praćenje uspjeha restauracije za što je potrebno istražiti i definirati metrike od interesa koje bi imale implikaciju za funkcioniranje ekosustava.

Cilj ovog doktorskog rada bio je istražiti bioindikatorski potencijal perifitona u detekciji i praćenju revitalizacije vodenih tokova sedrene barijere Skradinski buk nakon njihove aktivacije po uklanjanju pajasena, kroz četiri znanstvene publikacije (publikacije **I - IV**) koje su pokušale odgovoriti na znanstvena pitanja relevantna za tematiku istraživanja. U ovom doktorskom radu prvi put je istražen bioindikatorski potencijal perifitona u restauraciji sedrenih barijera unutar NP Krka, te u Republici Hrvatskoj. Rezultati publikacija **I** i **II** ukazuju da perifiton uspješno reflektira proces revitalizacije i uspostavu novih vodenih tokova na mikroskali, pri čemu pružaju sveobuhvatnu i komplementarnu analizu taksonomskih i funkcionalnih metrika fagotrofnih protista, uspoređujući potencijal ameboidnih protista i trepetljikaša u detekciji i praćenju revitalizacije. Dodatno, primjenom različitih metoda uzorkovanja, ove dvije publikacije omogućavaju usporedbu rezultata i kritički osvrt na prednosti i potencijalne nedostatke istih unapređujući metodologiju uzorkovanja perifitona u sedrotvornim ekosustavima. Iz rezultata publikacija **II** i **III** razvidno je da su, u odnosu na ameboidne protiste, trepetljikaši mnogo osjetljiviji na okolišne promjene, potvrđujući tako njihov bioindikatorski potencijal i u praćenju revitalizacije vodenih tokova sedrenih barijera.

Znanstveni doprinos ovog doktorskog rada je i opsežan uvid u sastav funkcionalnih značajki perifitona revitaliziranih tokova (publikacije **II** i **III**) koji je ukazao na važnost revitaliziranih tokova kao poveznice kopnenih i lotičkih ekosustava, što je dodatno potvrđeno analizom okolišnih čimbenika u preostalim publikacijama. Tako je na temelju rezultata publikacija **I** i **II** razvidno da revitalizirane tokove karakterizira promjenjiva hidrologija, koja ispiranjem tla uvjetuje unos nutrijenata te povećava strukturnu složenost/heterogenost mikrostaništa uslijed donosa dodatne alohtone organske tvari. Važnost alohtone organske tvari za perifiton sezonski se mijenja, pri čemu je od najveće važnosti u jesenskim i zimskim mjesecima uslijed pojave bujičnih tokova i veće brzine strujanja, ali ima i potencijalno veliku važnost u fazama presušivanja korita kada je onemogućena primarna produkcija. Među fagotrofnim protistima, u perifitonu revitaliziranih tokova prevladavaju bolji kompetitori, tj. omnivorni i bakteriovorni, slobodnoplivajući trepetljikaši koji koriste veći raspon različitih niša nastalih uslijed veće složenosti mikrostaništa ispiranjem okolnog tla, taloženjem kristalića kalcita tj. sedre i zadržavanja čestica dodatne alohtone organske tvari. Rezultati publikacija **I** i **II** potvrđuju i da je u revitaliziranim tokovima prisutno osedravanje koje je temeljni fenomen NP Krka, pri čemu osim kvantificiranja procesa osedravanja u perifitonu revitaliziranih tokova, pridonose i razumijevanju međuodnosa perifitona, heterogenosti mikrostaništa i procesa osedravanja. Povezanost heterogenosti staništa, funkcionalnih značajki fagotrofnih protista te podrijetla i dinamike organske tvari ukazuje na to da revitalizacija vodenih tokova utječe na funkcioniranje ekosustava kroz utjecaj na njegove funkcionalne sastavnice. Time ovaj doktorski rad doprinosi boljem razumijevanju ne samo revitaliziranih tokova Skradinskog buka, već i drugih restauriranih tekućica mediteranske regije ističući potrebu da ih se sagledava kroz prizmu lotičkih, ali i kopnenih ekosustava.

U sklopu ovog doktorskog rada (publikacija **IV**) prvi puta je istražena i funkcionalna te filogenetska raznolikost perifitonskih trepetljikaša duž rijeke Krke, od izvora prema ušću primjenom suvremenih metoda metabarkodiranja DNA pri čemu se pokazalo da, kao i u revitaliziranim tokovima, funkcionalnu raznolikost trepetljikaša značajno uvjetuje interspecijska kompeticija. Pokazavši da se filogenetska i funkcionalna raznolikost trepetljikaša mijenja duž toka rijeke Krke, odnosno da izvorišno područje Krke ima nižu funkcionalnu i filogenetsku raznolikost od nizvodne barijere Skradinski buk (što potencijalno sugerira veću podložnost okolišnim promjenama/poremećajima), ovaj doktorski rad poboljšava razumijevanje osjetljivosti jedinstvenog sedrotvornog ekosustava rijeke Krke u svjetlu sve intenzivnijih klimatskih promjena te pruža temelj za buduće konzervatorske i restauracijske napore. Komplementarnim korištenjem tradicionalne mikroskopske identifikacije i modernih metoda metabarkodiranja DNA nadilaze se potencijalna ograničenja tradicionalne mikroskopske identifikacije čime je omogućen bolji uvid u funkcionalnu raznolikost trepetljikaša perifitona sedrenih barijera.

THESIS SUMMARY

Lotic ecosystems are under significant anthropogenic pressure due to habitat degradation, pollution, overexploitation, and the introduction of invasive species that often lead to biodiversity loss (MEA, 2005). Therefore, restoration efforts in lotic ecosystems are growing every day, and the European Union (EU) has set a goal of restoring at least 20% of the EU's land and water areas by 2030, as part of the global movement to restore degraded ecosystems (United Nations (UN) Decade on Ecosystem Restoration). With this in mind, it is expected that restoration efforts will increase the need to monitor ecosystem response to restoration and to explore the success of implemented actions, with the utmost importance of defining specific and measurable restoration objectives and determining the optimal way/method to evaluate the success of the actions themselves (Woolsey et al., 2007). In understanding the restoration potential of lotic ecosystems and evaluating the success of restoration efforts, the role of structural complexity, i.e., physical heterogeneity of habitats/microhabitats, has traditionally been given the greatest importance, while the biological component of restoration is often neglected. However, these two components are closely linked, as structural complexity supports greater species abundance and diversity by providing a greater range of niches and also supports more food sources due to the retention of organic matter particles (Taniguchi et al., 2003; Frainer et al., 2017). The link between microhabitat complexity and organic matter retention is particularly important for communities that are substrate-bound, such as periphyton, whose matrix retains and accumulates organic matter, influencing its transfer between lower and higher trophic levels of lotic ecosystems (Risse-Buhl et al., 2015; Geisen et al., 2017) and increases its structural complexity. Precisely because of this and a number of other reasons, periphyton is often used as a bioindicator in the study of lotic ecosystems (Wu, 2017), and its heterotrophic component (phagotrophic protists) has a long research tradition in the tufa-depositing ecosystems of the Republic of Croatia, such as the Krka National Park (NP Krka), investigating not only the composition of periphyton but also the relationships between periphyton, organic matter dynamics, and the process of tufa deposition (Matoničkin and Pavletić, 1962; Primc-Habdija et al., 1997, 2001; Matoničkin Kepčija et al., 2017). In light of the new challenges faced by NP Krka, such as the overgrowth of the Skradinski buk tufa barrier with the invasive plant species tree of heaven (*Ailanthus altissima* (Mill.) Swinge) and the resulting desiccation of several barrier parts, the bioindicator potential of periphyton could be

extended to the detection and monitoring of restoration success, for which it is necessary to explore and define metrics from interests that would have an impact on ecosystem functioning.

The aim of this doctoral thesis was to investigate the bioindicator potential of periphyton in detecting and monitoring the revitalization of streams of the Skradinski buk tufa barrier after their activation as a result of the removal of the tree of heaven. This was done on the basis of four scientific publications (publications **I - IV**), which attempted to answer the scientific questions relevant to the research topic. This doctoral thesis represents the first attempt to use the bioindicator potential of periphyton in detecting and monitoring the restoration of tufa barriers in Krka National Park, but also in the Republic of Croatia in general. The results of publications **I** and **II** demonstrate that periphyton successfully reflects the process of revitalization and establishment of new streams at the microscale, while providing a comprehensive and complementary analysis of taxonomic and functional metrics of phagotrophic protists, comparing the potential of amoeboid protists and ciliates in detecting and monitoring revitalization. In addition, by using different sampling methods, these two publications allow for a comparison of results and a critical review of their advantages and potential disadvantages, improving periphyton sampling methodology in tufa-depositing ecosystems. From the results of the **II** and **III** publications, it appears that ciliates are much more sensitive to environmental changes compared to amoeboid protists, confirming their potential as bioindicators for monitoring tufa barrier revitalization.

The scientific contribution of this doctoral thesis is a comprehensive insight into the composition of the functional traits of the periphyton of revitalized streams (publications **II** and **III**), showing the importance of revitalized streams as a link between terrestrial and lotic ecosystems, which was additionally confirmed by the analysis of environmental factors in the other publications. From the results of publications **I** and **II**, revitalized streams are characterized by changing hydrology, which conditions nutrient uptake through soil leaching and increases the structural complexity/heterogeneity of the microhabitat due to the introduction of additional allochthonous organic matter. The importance of allochthonous organic matter to the periphyton changes seasonally, being most important during the autumn and winter months due to occasional runoff and higher flow velocities, but can also be important during periods of streambed drying when primary production is impaired. Among phagotrophic protists in revitalized streams, better competitors predominate, i.e., omnivorous and bacterivorous free-swimming ciliates that utilize a wider range of different niches created by the greater complexity of the microhabitat due to leaching of the surrounding soil, precipitation of calcite crystals, i.e. tufa, and retention of allochthonous organic matter particles. The results of publications **I** and **II** confirm that tufa deposition occurs in revitalized streams, which is a key phenomenon in Krka National Park, and in addition to quantifying the deposition process in the periphyton of revitalized streams, contribute to the understanding of the relationships between periphyton, microhabitat heterogeneity, and tufa deposition processes. The relationship between habitat heterogeneity, functional traits of phagotrophic protists, and the origin and dynamics of organic matter suggests that stream revitalization affects ecosystem functioning through its influence on its functional components. This doctoral thesis contributes to a better understanding not only of the revitalized streams of Skradinski buk, but also of other restored streams in the Mediterranean region, by emphasizing the need to investigate them through the prism of both lotic and terrestrial ecosystems.

In this doctoral thesis (publication **IV**), the functional and phylogenetic diversity of periphytic ciliates along the Krka River was studied for the first time using modern DNA metabarcoding methods. The results of this publication show that the functional diversity of ciliates is determined to a considerable extent by interspecies competition not only in revitalized streams but also along the course of the Krka River (from spring to mouth). By demonstrating that the phylogenetic and functional diversity of ciliates changes along the course of the Krka River, i.e., that the Krka River spring has a lower functional and phylogenetic diversity than the downstream Skradinski buk tufa barrier (possibly indicating greater susceptibility to environmental change/disturbance), this doctoral thesis improves understanding of the sensitivity of a unique tufa-depositing ecosystem of the Krka River in light of increasingly intense climate change and provides a basis for future conservation and restoration efforts. The complementary application of traditional microscopic identification and modern DNA metabarcoding methods overcomes the potential limitations of traditional microscopic identification, providing greater insight into the functional diversity of periphytic ciliates in tufa barriers.

UVOD

RESTAURACIJA LOTIČKIH EKOSUSTAVA

Ideja restauracije ekosustava, odnosno vraćanja istog u prirodno stanje, potječe još iz 19. stoljeća, ali kao takva nije zaživjela do 80-ih godina 20. stoljeća kada ulazi među glavne smjerove ekologije (Palmer i sur., 2014). Restauracijska ekologija je razmjerno mlada grana ekologije koja eksponencijalno i ubrzano raste. Iako je po shvaćanju primijenjena znanost, njena teorijska osnova čvrsto je ukorijenjena u klasičnoj ekologiji, a projekti restauracije pružaju jedinstvenu priliku za testiranje znanstvenih hipoteza (Palmer i sur., 2007, 2010, 2014). Pitanje je li sustav uopće moguće vratiti u prirodno stanje je u svojoj srži gotovo filozofsko pitanje, stoga procjena uspješnosti mjera restauracije predstavlja veliki izazov. U razumijevanju potencijala restauracije ekosustava i procjeni uspješnosti mjera iste, tradicionalno se najveći naglasak stavlja na ulogu fizičke heterogenosti staništa/mikrostaništa te razumijevanje čimbenika koji potiču obnavljanje bioraznolikosti. Ipak, restauracijski ekolozi svoj fokus polagano skreću i prema pitanju što restaurirani ekosustavi mogu pružiti u kontekstu podržavanja procesa ekosustava (engl. *ecosystem processes*) ili što isti mogu pružiti ljudima (usluge ekosustava, engl. *ecosystem services*) (Montoya i sur., 2012). Najveći pomak u ovom smjeru evidentan je za lotičke ekosustave poput rijeka i potoka koji su tradicionalno bili središtem ljudske aktivnosti u smislu prijevoza, izvora hrane i opskrbe pitkom vodom te su posljedično tomu pod velikim antropogenim pritiskom. Prema MEA (engl. *The Millennium Ecosystem Assessment*, 2005) izvješću, lotički ekosustavi pod značajnom su ugrozom uslijed degradacije staništa, zagađenja, prekomjernog iskorištavanja i unosa invazivnih vrsta koji nerijetko rezultiraju gubitkom bioraznolikosti.

Restauracijski napori u lotičkim ekosustavima svakodnevno se povećavaju diljem svijeta (Palmer i sur., 2014), a 2021. godina predstavlja početak Desetljeća Ujedinjenih Naroda posvećenog restauraciji ekosustava (engl. *United Nations (UN) Decade on Ecosystem Restoration*), globalnog pokreta restauracije degradiranih ekosustava. U okvirima ovog pokreta, Europska unija (EU) ima za cilj obnoviti najmanje 20% kopnenih i vodenih površina u EU do 2030. godine, a do 2050. obuhvatiti sve ekosustave kojima je potreban neki oblik restauracije. Prema Strategiji EU-a za bioraznolikost do 2030. objavljenoj 2020. godine EU već ima pravne okvire, strategije i akcijske planove za restauraciju staništa i vrsta, no postoje brojni nedostaci u provedbi i regulativi istih koji otežavaju napredak. Tako nisu definirani ostvarivi ciljevi, a nema ni obveze praćenja (monitoringa) i ocjenjivanja stanja ekosustava. Kako bi se postigli ciljevi iz Okvirne direktive o vodama (ODV) (Europska komisija, 2000) potrebno je učiniti puno više za restauraciju lotičkih i lentičkih ekosustava, stoga je u planu do 2030. godine restaurirati najmanje 25 000 km rijeka, odnosno ponovno ih pretvoriti u rijeke slobodnog toka. U svjetlu spomenutog, za očekivati je da će s restauracijskim naporima rasti i potreba za praćenjem odgovora ekosustava na restauraciju te istraživanjima procjene uspješnosti provedenih mjera. U takvim istraživanjima od iznimne je važnosti definirati specifične i mjerljive ciljeve restauracije te utvrditi optimalan način/metodu procjene uspješnosti samih mjera (Woolsey i sur., 2007).

Mjere restauracije lotičkih ekosustava većinom su koncentrirane na restauriranje prirodnog režima narušenog vodenog toka bilo rekonfiguriranjem kanala ili uklanjanjem brana, odnosno na fizičku strukturu te stabilnost toka i sedimenta pri čemu još uvijek nedovoljno uključuju i biološku komponentu. Očekivanje da složenost staništa/mikrostaništa pogoduje većoj brojnosti i raznolikosti vrsta proizlazi iz osnovne ekološke teorije da strukturno složenija staništa/mikrostaništa pružaju veći raspon niša i smanjuju vjerojatnost kompeticije, olakšavajući koegzistenciju vrsta (Snyder i Chesson, 2004; Palmer i sur., 2007). Tako su Lentendu i Dunthorn (2021) pokazali da lokalna heterogenost može utjecati na kompetitivno isključivanje, te da ono ima veći učinak u manje heterogenim staništima. Osim toga, strukturno složena staništa/mikrostaništa mogu podržavati više izvora hrane uslijed zadržavanja čestica organske tvari (Taniguchi i sur., 2003; Frainer i sur., 2017). Povezanost složenosti mikrostaništa i retencije organske tvari posebno je važna za zajednice vezane uz podlogu poput perifitona čiji matriks zadržava i akumulira organsku tvar, ali služi i kao izvor za ponovno naseljavanje nakon nepovoljnih uvjeta (Dzubakova i sur., 2018). Također, zadržavanjem organske tvari mijenja se i sastav zajednice uslijed povećane mikrobne aktivnosti (Jochum i sur., 2017). Hidro(geo)morfološka obilježja vodenih tijela prepoznata su i kao nezaobilazan element u procjeni ekološkog stanja tekućica usvajanjem Okvirne direktive o vodama (Europska komisija, 2000) prema kojoj se hidromorfološka ocjena tekućica temelji na praćenju hidrološkog režima (količine i dinamike vodenog toka te veze s podzemnim vodama), kontinuiteta toka te morfoloških uvjeta (varijacije širine i dubine korita; strukture i sedimenta na dnu korita; strukture obalnog pojasa) (Vučković i sur., 2021). U istraživanjima koja implementiraju mjere restauracije, hidrogeomorfološka složenost staništa najčešće se kvantificira kroz četiri dimenzije: raspodjelu veličine zrna sedimenta, uzdužni profil, poprečni presjek i prisutnost komada grana/drva u koritu/vodenom toku (Palmer i sur., 2014). Iako su mnoga istraživanja pokazala da je povećanje heterogenosti staništa/mikrostaništa unutar lotičkih ekosustava ključan element restauracije, fokus isključivo na jedan element neće dovesti do sveobuhvatne restauracije koja uključuje i biološku komponentu (Jähnig i sur., 2010; Palmer i sur., 2010).

Većina dosadašnjih projekata restauracije lotičkih ekosustava bila je usredotočena na praćenje uskog raspona taksonomskih metrika poput bogatstva vrsta i abundancije izračunatih za makrobeskralježnjake ili ribe (Lepori i sur., 2005; Muhar i sur., 2016; Frainer i sur., 2017). Budući da se različite skupine organizama razlikuju u svojim zahtjevima za niše, stupnju ekološke specijalizacije i prostornoj skali na kojoj djeluju, malo je vjerojatno da će jedna mjera restauracije biti jednako učinkovita za sve skupine. Tako restauracija koja rezultira heterogenošću staništa postignutom dodavanjem komada drva/grana u korito rijeke može biti korisna pružajući utočište za makrobeskralježnjake ili prostor za mrijest ribama (Muhar i sur., 2016; Pilotto i sur., 2018), dok neće nužno imati pozitivan utjecaj na mikrobnu zajednicu npr. zajednicu dijatomeja (Jones i sur., 2017).

Uz procjenu sastava i bogatstva vrsta, osnovne elemente uspješne restauracije, prema organizaciji *The Society for Ecological Restoration*, čine i funkcija i stabilnost ekosustava te elementi krajolika (Palmer i sur., 2014). S obzirom na to, sveobuhvatna procjena uspjeha restauracije trebala bi povezati promjene u strukturi staništa ne samo s promjenama u taksonomskoj raznolikosti već i onoj funkcionalnoj, s posljedicama za procese ekosustava kao npr. razgradnju organske tvari, kruženje ugljika itd. Frainer i McKie (2015) pokazali su da čak i u izostanku učinka restauracije na taksonomske metrike, promjene u strukturi staništa lotičkih ekosustava mogu utjecati na sastav funkcionalnih značajki vrsta što ima implikacije i za funkcioniranje ekosustava. Funkcionalne značajke (engl. *functional traits*) su karakteristike fenotipa organizma koje određuju njegov fitnes i performanse te njegov odgovor na okolišne uvjete (Legendre i sur., 1997; Violle i sur., 2007). Ipak, njihova primjena slabo je zastupljena u procjeni uspješnosti projekata restauracije. Od 243 projekta vezana uz restauraciju raznih ekosustava diljem kontinenata, objavljena u bazi podataka organizacije *The Society for Ecological Restoration* (https://www.ser-rrc.org/project-database/) između 1992. i 2020., samo osam sadrži ključni pojam „*functional trait*", dok od 24 projekta vezana uz lotičke ekosustave diljem kontinenata, niti jedan projekt ne sadrži isti ključni pojam. Ovi rezultati ukazuju na činjenicu da se primjena funkcionalnih značajki zanemaruje u restauracijskoj praksi što potvrđuju i mnoga recentna istraživanja. Prema Merchant i sur. (2023) neki od osnovnih razloga zašto planovi restauracije ne uključuju funkcionalne značajke su nedostatna količina informacija o funkcionalnim značajkama odabranih indikatorskih skupina; činjenica da primjena ove metodologije iziskuje stručnjake koji posjeduju znanje i iskustvo iz područja funkcionalne ekologije, ali ponajviše sklonost tradicionalnoj metodologiji temeljenoj na taksonomskim metrikama. Unatoč tome, važno je naglasiti da primjenu funkcionalnih značajki treba nadopunjavati, a ne zamijeniti ostale metodološke pristupe, te u kombinaciji s njima može olakšati postizanje ciljeva u projektima restauracije pruživši sveobuhvatniju procjenu uspješnosti mjera.

RIJEKA KRKA

PRIRODNO-GEOGRAFSKA OBILJEŽJA RIJEKE KRKE

Rijeka Krka smještena je između Ravnih kotara, zaravni rijeke Čikole i Dalmatinske zagore te se u cijelosti prostire na području Šibensko-kninske županije (Marguš, 2017). Izvire tri i pol kilometra sjeveroistočno od Knina, u podnožju planine Dinare, ulijevajući se u Jadransko more u blizini Skradina (Buljan i Pavičić, 2010). Izvorište rijeke Krke čine tri stalna izvora: Glavni, Mali i Treći izvor, od kojih je najizdašniji Glavni koji čini 80-95% ukupne vode rijeke (Bonacci, 1985). Glavni izvor rijeke Krke smješten je ispod 22 m visokog slapa Topoljski buk, a vidljiv je isključivo ljeti kada slap presuši. Radi se o tipičnom reokrenom izvoru kod kojeg voda odmah nakon izlaska iz podzemlja na površinu formira tok. U porječju Krke izdvajaju se četiri reljefne cjeline: planinsko područje na sjeveroistoku kojeg tvore Dinara i jugoistočni ogranci Velebita; dolinska proširenja (Plavno, Kninsko, Kosovo i Petrovo polje); vapnenačke zaravni (sjevernodalmatinska, kistanjska i zaravan oko Krke i Čikole) te kanjoni s tokovima Krčića i Čikole (Friganović, 1990). Sa svojim pritocima (Krčić, Kosovčica, Orašnica, Butižnica, Čikola i Guduča) rijeka Krka čini sustav kompozitnih dolina u kršu, a recentna morfologija doline i okolnih područja rezultat je geološke građe i klimatskih promjena tijekom gornjeg pleistocena i holocena (Cukrov i Lojen, 2010). Nakon posljednjeg glacijalnog maksimuma na prijelazu iz pleistocena u holocen dolazi do topljenja leda te izdizanja morske razine na prostoru Jadranskog mora uslijed čega dolazi do prodiranja mora u današnji estuarij rijeke Krke (Renić, 1989; Cukrov i Lojen, 2010). Među holocenskim (kvartarnim) naslagama posebno se ističe sedra - slatkovodni karbonatni sediment zastupljen u dva varijeteta: čvrsta, kamena sedra šupljikave strukture taložena na barijerama te prašinasto-pelagička sedra taložena u ujezerenjima nizvodno od barijere, tj. na dnu jezera (Renić, 1989). Nastanak sedre uvjetuje čitav niz geoloških, hidro(geo)loških, fizikalno-kemijskih i bioloških čimbenika. Osnovni uvjet svakako je prezasićenost vode kalcijevim karbonatom do koje dolazi uklanjanjem ugljikovog dioksida iz otopine uslijed zagrijavanja, smanjenja parcijalnog tlaka pri većoj turbulenciji vode te kroz biološke fotosintetske procese (Pentecost, 2005). Najintenzivnije osedravanje bilježi se na dijelovima karakteriziranim turbulentnim tokom i većom brzinom strujanja poput slapova i barijera koji olakšavaju prozračivanje i izlazak ugljikovog dioksida iz vode (isplinjavanje) (Matoničkin i Pavletić, 1962; Zhang i sur., 2001; Pentecost i Zhaohui, 2002). Uz brzinu strujanja, među ključnim čimbenicima koji uvjetuju osedravanje su i pH te koncentracija otopljene organske tvari (Srdoč i sur., 1985). Osedravanju pogoduje i biota, odnosno brojni sedrotvorni organizmi kroz svoje fizikalno (hvatanje i vezivanje kristalića kalcita, nukleacija, inkrustacija) i kemijsko djelovanje (fotosintetska aktivnost kojom se iz vode uklanja ugljikov dioksid i izaziva prezasićenost) (Stilinović, 1994; Primc-Habdija i sur., 1997; Arp i sur., 2001). Među najvažnijim sedrotvorcima treba izdvojiti mahovine, ali i heterotrofnu komponentu perifitona (fagotrofni protisti) čiji se međuodnos s procesom osedravanja pokazao važnim elementom dinamike sedrotvornih ekosustava (Matoničkin i Pavletić, 1962; Primc-Habdija i sur., 1997, 2001; Matoničkin Kepčija, 2006; Matoničkin Kepčija i sur., 2017).

Duž toka rijeke Krke prisutni su brojni geomorfološki oblici sedre, a najčešći su pragovi i barijere koji nastaju na dnu krških brzaca te slapovi koji nastaju na mjestima na kojima se obrušava velika količina vode. U svom toku od 72,5 km, s ukupnim padom od 242 metra, rijeka Krka obuhvaća sedam sedrenih slapova te tri jezera (Visovačko jezero, Prokljansko jezero i jezero Brljan). Desetak kilometara nizvodno od Knina pojavljuje se prvi slap Bilušića buk nakon kojeg slijede Brljan, Manojlovački slap, Rošnjak te Miljacka slap. Roški slap je pretposljednja sedrena barijera, smještena 14 km nizvodno od Miljacka slapa, odnosno 35,5 km od izvora. Obilježava ga niz manjih kaskada popularno nazvanih „ogrlicama“ te manjih rukavaca i otočića. Glavni slap se nalazi na prijelazu Krke u Visovačko jezero, na samom kraju barijere te se od posljednjeg slapa Skradinskog buka razlikuje po hidrogeomorfološkim svojstvima. Voda koja se prelijeva preko Roškog slapa ima dominantno svojstva tekućice jer prije slapa protječe kanjonskim koritom rijeke Krke, dok se preko Skradinskog buka prelijeva epilimnijska voda Visovačkog jezera te ulijeva voda ujezerenog donjeg toka Čikole (Primc-Habdija i sur., 1997; Špoljar, 1998; Habdija i sur., 2002; Špoljar i sur., 2005). Skradinski buk najveća je sedrena barijera rijeke Krke, a karakterizira ga relativno visok pad (s 46 m nadmorske visine na manje od 2 metra) te manje sedrene barijere i ujezerenja (Bonacci i sur., 2017). Ispod samog Skradinskog buka počinje estuarij, odnosno potopljeno ušće rijeke Krke.

ZAŠTITA SEDROTVORNOG PROCESA U NP KRKA

Nacionalni park Krka (NP Krka) spada u najmlađe nacionalne parkove u Republici Hrvatskoj (RH) predstavljajući kategoriju zaštićenog područja usmjerenu na očuvanje i unapređenje prirodnih vrijednosti uz popratni razvoj turističke i odgojno-obrazovne djelatnosti (Jovičić i Ivanović, 2004). Proteže se pedesetak kilometara uz tok rijeke Krke, počevši dva kilometra nizvodno od Knina do Skradina te uključujući i 3,5 km donjeg toka rijeke Čikole (Marguš i sur., 2017). Površina parka mijenjala se od prvog proglašenja nacionalnim parkom 1985. godine kada je obuhvaćala 142 km^2 do danas kada obuhvaća 109 km^2 gornjeg i srednjeg toka rijeke Krke te donjeg toka rijeke Čikole od kojih 9 km^2 čine vodene površine (Bonacci i sur., 2016). NP Krka prepoznat je kao jedna od najljepših prirodnih znamenitosti RH, jedinstvenog prostorno-dinamičnog entiteta, velike biološke raznolikosti i visokog stupnja endemizma, a proces osedravanja smatra se temeljnim prirodnim fenomenom NP Krka (Bulat, 2012; Kudrnovsky i Lazowski, 2017). Drugo je najposjećenije zaštićeno područje u RH (nakon NP Plitvička jezera) s preko milijun posjetitelja godišnje (Marić, 2020) stoga usklađivanje turističke djelatnosti uz istovremeno očuvanje prirodnog bogatstva predstavlja izazov rastućih razmjera. Krški sustavi, a među njima i područja unutar NP Krka, iznimno su osjetljivi i podložni različitim vrstama vanjskih poremećaja. Porječje rijeke Krke spominje se i u EU Direktivi o staništima kao područje brojnih slatkovodnih staništa i velike bioraznolikosti dok se sedrene barijere krških Dinarida nalaze na popisu prirodnih stanišnih tipova od interesa za EU na području RH, a krški izvori kao prioritetni stanišni tip među ugroženim stanišnim tipovima u RH prema ekološkoj mreži Natura 2000 (Radović i sur., 2006).

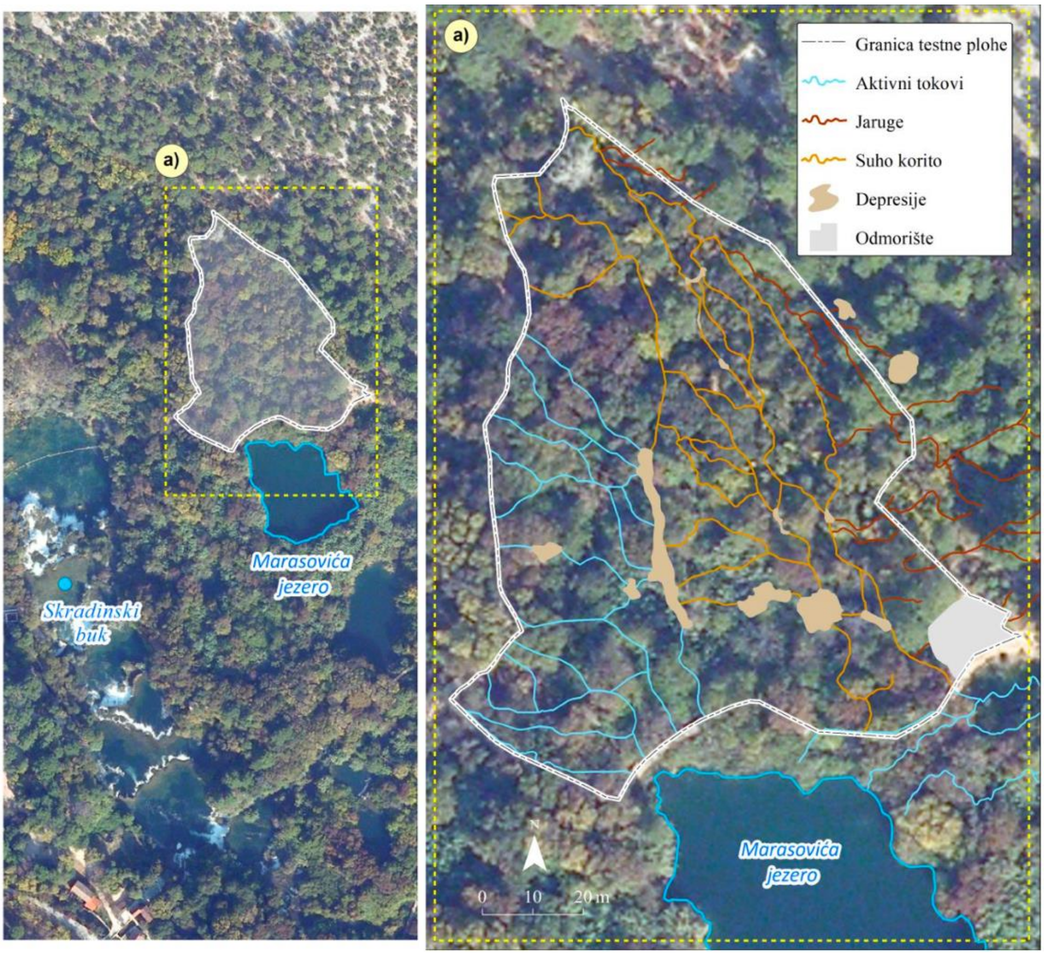
Prema Barrios i sur. (2014), antropogeni utjecaj, pojačana eutrofikacija i širenje invazivnih vrsta vegetacije među glavnim su čimbenicima koji doprinose povećanom pritisku na slatkovodne ekosustave. U području NP Krka antropogeni utjecaj bio je prisutan i mnogo prije proglašenja kategorije zaštite, manifestirajući se kroz rušenje i isušivanje sedrenih barijera u svrhu dobivanja plodnog tla, gradnju suhozida i mlinica, dok je danas vezan uz turističku aktivnost, uređivanje staza i gradnju odmorišta (Perica i sur., 2005). Najveći pritisak unutar granica NP uočen je na širem području Skradinskog buka koji uslijed svoje prirodne ljepote te brojnih pješačkih i biciklističkih staza, vidikovaca, livada s kupalištem i ugostiteljskog sadržaja predstavlja najpoznatije i najposjećenije slapište NP Krka (Klanjšček i sur., 2017). Posljednjih nekoliko godina šire područje Skradinskog buka bilježi negativne hidrološke promjene, izmjenu smjera sedrotvornih vodenih tokova te smanjen intenzitet osedravanja uslijed smanjenja srednjih i minimalnih godišnjih protoka, ali i nekontroliranog rasta i širenja invazivnih biljnih vrsta (Rubinić i sur., 2017). Pretjerani i nekontrolirani rast vegetacije, posebno viših biljaka može negativno utjecati na proces osedravanja posrednim ili neposrednim putem, ovisno o razmjerima obrastanja sedrene barijere. Za razliku od mahovina koje su među značajnim sedrotvorcima, više biljke nisu pogodne kao supstrat za nukleaciju mikrokristalića kalcita (Turner i Jones, 2005). Osim što svojim snažnim korijenjem prorastaju i mehanički uništavaju stabilnost barijere, više biljke promjenom pH-vrijednosti u blizini njihovog korijenja ili pak prekomjernim taloženjem listinca mijenjaju fizikalno-kemijske parametre vode te tako utječu i direktno na proces osedravanja inhibirajući ga (Liu i sur., 2006).

Prekomjerni razvoj makrovegetacije uslijed antropogenog djelovanja uočen je i u NP Plitvička jezera prije dvadesetak godina. Uslijed tzv. antropogene eutrofikacije, odnosno obogaćivanja vode hranjivim spojevima fosfora i dušika zbog ispuštanja gnojiva u neposrednoj blizini granica parka, domaćih i divljih životinja, kao i kupališne aktivnosti posjetitelja (Pavlus i Novosel, 2004), došlo je do nekontroliranog bujanja podvodne vegetacije, ali i prekomjernog razvoja makrovegetacije uz rub jezera i na samim sedrenim barijerama. Posebno su „štetnima“ utvrđene populacije močvarnog ljutka (*Cladium mariscus* (L.) Pohl) koje svojim habitusom ne dozvoljavaju prozračivanje čime dolazi do stvaranja anoksičnih uvjeta i retencije organske tvari, te trske (*Phragmites australis* (Cav.) Trin. Ex Steud) i vrbe (*Salix* spp.) koje formiraju guste monokulture uz/na sedrenim barijerama. Mjere restauracije provedene 2002. godine za cilj su imale uklanjanje makrovegetacije i posljedično ostvarivanje i održavanje povoljnih uvjeta za proces osedravanja. Zahvati uklanjanja makrovegetacije podrazumijevali su mehaničko odstranjivanje, a dvije godine nakon uklanjanja, uočeno je znatno povećanje protočnosti vode, bolje prozračivanje i smanjenje količine organske tvari, dok je sam proces osedravanja tekao brže na nekima od barijera (Mililiša i sur., 2006).

U granicama NP Krka do sada je utvrđeno približno 1200 biljnih svojti što je više od 20% ukupne flore Hrvatske (Milović, 2017). Među florom neofita (stranih vrsta dospjelih u Europu nakon otkrića Amerike) posebno se ističu bagrem (*Robinia pseudacacia* L.) i pajasen (*Ailanthus altissima* (Mill.) Swinge) koji su prepoznati kao invazivne biljne vrste, odnosno pokazuju invazivno ponašanje na području NP Krka (Milović, 2002, 2007). Invazivne vrste karakterizira velika genetska varijabilnost i fenotipska plastičnost te iznimna sposobnost razmnožavanja kojom postižu veliku brojnost u kratkom vremenskom periodu (Davidson i sur., 2011; Nikolić i sur., 2014; Novak i Novak, 2017). Tako pajasen uz značajnu hiperprodukciju pokazuje i maksimalnu prilagodljivost uvjetima okoliša, odnosno osim na dubokim rastresitim tlima raste i na vrlo siromašnom stjenovitom tlu. Tolerantan je na sušu i vlažnost zraka, a sposoban je rasti u širokom rasponu pH-vrijednosti tla (Nikolić i sur., 2014). Osim spomenutog, pajasen inhibira rast drugih biljaka u okolici alelopatijom, izlučujući spoj ailanton koji postiže učinak sličan herbicidu glifosatu (Heisey i Heisey, 2003; Vuković i sur., 2012). Sve navedeno učinilo je pajasen jednom od najvećih prijetnji bioraznolikosti u izoliranim sustavima kao što su otoci i zaštićena područja (Bellard i sur., 2016), pa tako i u granicama NP Krka, a posebno na širem području Skradinskog buka gdje je njegova prisutnost dovela do zarastanja sedrenih barijera (Slika 1).

Slika 1. Pajasen na Skradinskom buku (iz: Novak i Novak, 2017).

Prepoznavši važnost uočenih izazova na širem području Skradinskog buka, uprava NP Krka pokrenula je 2017. godine interdisciplinarni projekt pod nazivom *Upravljanje i održavanje makrovegetacije na Skradinskom buku – izrada višekriterijskog modela održivog upravljanja.* Na sjeveroistočnoj strani šireg područja Skradinskog buka, u neposrednoj blizini Marasovića jezera, definirana je testna ploha površine 8000 m^2 na kojoj je provedeno sustavno mapiranje vegetacije i pripremljen selektivni plan uklanjanja pajasena. S obzirom na to da NP Krka spada pod državnu razinu upravljanja, pristup uklanjanju pajasena sa Skradinskog buka bio je ograničen na mehaničko uklanjanje s kojim se započelo u srpnju i nastavilo u kolovozu i rujnu 2017. godine nakon ishođenja dozvole Hrvatske agencije za okoliš i prirodu. Uklanjanje jedinki pajasena promijenilo je hidrologiju testne plohe na Skradinskom buku i aktiviralo/revitaliziralo stare vodene tokove na barijeri (Slika 2.) koji otječu okolnim šumskim tlom.



Slika 2. Testna ploha na širem području Skradinskog buka (iz: Marić, 2020).

U sklopu krovnog interdisciplinarnog projekta restauracije/revitalizacije vodenih tokova na testnoj plohi Skradinskog buka pokrenuto je nekoliko manjih projekata, a među njima i oni posvećeni istraživanju biodinamičkih procesa osedravanja i bioindikatorskom potencijalu perifitona u praćenju revitalizacije vodenih tokova sedrenih barijera: „705509 Utjecaj uklanjanja pajasena na osedravanje prirodnih podloga i dinamiku perifitona i makrozoobentosa“ (voditeljica Renata Matoničkin Kepčija) te „7056135 Praćenje oporavka perifitona i makrozoobentosa na sedrenim barijerama u Nacionalnom parku Krka nakon uklanjanja pajasena“ (voditeljica Renata Matoničkin Kepčija), u sklopu kojih je izrađen ovaj doktorski rad. Dodatno, dio doktorskog rada izrađen je i u sklopu projekta „705532 Ocjena ekološkog stanja rijeke Krke primjenom DNA barcodinga“ voditeljice Marije Gligora Udovič. Kako je prije provođenja samih mjera restauracije važno poznavati i kako je sustav izgledao u prirodnom stanju, odnosno odrediti referentno stanje na temelju kojeg je moguće predvidjeti i procijeniti odgovor na promjene izazvane mjerama restauracije (Roje-Bonacci i Bonacci, 2016), za kontrolne tokove odabrani su dijelovi plohe s tipičnim mahovinskim pokrovom na kojima je voda bila prisutna i prije uklanjanja pajasena, a koji služe kao kontrola za usporedbu sastava perifitona i vrijednosti okolišnih parametara.

PERIFITON U LOTIČKIM EKOSUSTAVIMA

STRUKTURA I RAZVOJ PERIFITONA

Perifiton ili obraštaj podrazumijeva složenu zajednicu autotrofnih i heterotrofnih organizama na podlozi koja je stalno ili privremeno uronjena u vodu (Azim i sur., 2005). Najčešći pripadnici autotrofne komponente su cijanobakterije i alge dok su među heterotrofima zastupljene bakterije, gljive, fagotrofni protisti i beskralježnjaci (Wu, 2017). Terminologija vezana uz perifiton mijenjala se od početka 20. stoljeća do danas. Tako je terminu perifiton prethodila njemačka riječ „aufwuchs“ (*auf* - na, *der Wuchs* - rast, rasti na nečemu) koja je podrazumijevala sve organizme koji su pričvršćeni na podlogu ili se njome slobodno kreću bez prodiranja u istu (Azim i sur., 2005), da bi ju potom oko 2000. godine zamijenio termin „biofilm“ (Kalff, 2002). S obzirom na to da se u suvremenim istraživanjima termin „bioflm“ najčešće veže uz zajednice bakterija i/ili dijatomeja (Wu, 2017), u ovom doktorskom radu koristit će se termin perifiton u skladu s međunarodnom prihvaćenom terminologijom, ali i tradicijom dosadašnjih istraživanja istog u slatkovodnim ekosustavima RH.

Razvoj perifitona započinje već unutar nekoliko sati izloženosti podloge (supstrata) u vodi, taloženjem sloja organskih tvari koje pospješuju prihvaćanje bakterija, malih penatnih dijatomeja, a potom i zelenih i crvenih algi (Battin i sur., 2003; Azim, 2009). Slijedi kolonizacija od strane fagotrofnih protista, primarno bakteriovora i algivora, a potom i omnivornih i predatorskih protista i beskralježnjaka (Worner i sur., 2000). Ekstracelularni matriks (engl. *extracellular polymeric substances*, EPS) kojeg luče bakterije i alge, dominantno polisaharidne, proteinske i glikoproteinske prirode, perifitonu pruža trodimenzionalnost i heterogenost stvarajući niz mikropora i kanala kojima struji voda i koji predstavljaju utočište mnogim organizmima. Strujanje vode olakšava protok otopljenih hranjivih tvari, posredno regulirajući razvoj, metabolizam i produktivnost zajednice (Stewart i sur., 2013). Perifiton predstavlja vrlo dinamičan „mikroekosustav“ čija se biomasa u početnom periodu razvoja eksponencijalno povećava do maksimalne vrijednosti (faza prirasta) nakon koje slijedi faza gubitka, karakterizirana odumiranjem jedinki i odvajanjem od podloge uslijed stvaranja sve debljeg sloja perifitona koji onemogućava pristup svjetlosti i nutrijentima. Oporavak zajednice (sekundarna sukcesija) ostvariv je u relativno brzom roku, ovisno o biomasi preostalog perifitona te kolonizaciji od strane organizama iz okolne vode ili drugih izvora (npr. pritokom vode iz uzvodnih dionica). Lake i sur. (2007) i Sundermann i sur. (2011) pronalaze da vrste koje mogu kolonizirati novo područje proizlaze iz regionalnog fonda.

U lotičkim ekosustavima, perifiton je reguliran nizom čimbenika biotičke (npr. kompeticija, ispaša (engl. *grazing*) i predatorski pritisak) i abiotičke prirode (npr. temperatura, insolacija tj. količina i kvaliteta svjetlosti, pH-vrijednost, količina otopljenog kisika i hranjivih tvari, brzina strujanja vode te sastav i hrapavost podloge) (Wimpenny i sur., 2000). Količina svjetlosti neposredno uvjetuje strukturu zajednice rezultirajući dominacijom algi pri uvjetima dovoljne osvijetljenosti, odnosno većim udjelom heterotrofa i/ili anaerobnih bakterija pri ograničenoj svjetlosti/potpunoj tami (Vermaat, 2005), a posljedično tome mijenjaju se i uvjeti za kolonizaciju drugih organizama. Količina otopljenih hranjivih tvari (nutrijenata) poput dušikovih spojeva i ortofosfata značajno utječe na biomasu i abundanciju autotrofne i heterotrofne komponente perifitona (Gong i sur., 2005), baš kao i brzina strujanja (Saravia i sur., 2001). Ona uvjetuje mehaničke procese otplavljivanja, pričvršćivanja i prijenosa hranjivih čestica. Veća brzina strujanja poboljšava transport i difuziju hranjivih tvari, no njezin pozitivan utjecaj prestaje pri graničnim vrijednostima od 0,5 m/s (Horner i Welch, 1981) tj. 0,6 m/s (Horner i sur., 1990). Istraživanja djelovanja brzine strujanja na razvoj autotrofne komponente perifitona (algalne biomase) nerijetko su proturječna. Tako Biggs (1996), Habdija i sur. (2000) te Pérez-Calpe i sur. (2021) opažaju da se biomasa autotrofa smanjuje s većom brzinom strujanja dok Pitois i sur. (2001), Matoničkin Kepčija (2006) te Matoničkin Kepčija i sur. (2017) bilježe porast pri većim brzinama. Rezultati istraživanja utjecaja brzine strujanja na heterotrofnu komponentu perifitona (trepetljikaše) također pokazuju oprečne rezultate. Primc-Habdija i sur. (2000) bilježe manju brojnost trepetljikaša u uvjetima brže struje vode u NP Plitvička jezera dok u istraživanju u NP Krka bilježe raznolikiju zajednicu u uvjetima brže struje vode (Primc-Habdija i sur., 2001). Matoničkin Kepčija (2006) bilježi negativan utjecaj veće brzine strujanja na brojnost, biomasu i bogatstvo trepetljikaša, odnosno bilježi da su najoptimalniji uvjeti za razvoj perifitona na sedrenim barijerama NP Plitvička jezera na brzinama strujanja ispod 0,2 m/s, dok Matoničkin Kepčija i sur. (2011) bilježe da je utjecaj brzine strujanja na trepetljikaše vrsno specifičan.

BIOINDIKATORSKI POTENCIJAL FAGOTROFNIH PROTISTA PERIFITONA U LOTIČKIM EKOSUSTAVIMA

Unutar heterotrofne komponente perifitona, najveću raznolikost postižu fagotrofni protisti, odnosno jednostanični eukariotski organizmi koji hranu priskrbljuju procesom fagocitoze. S obzirom na to da su najvažniji predatori u mikrobnim zajednicama kopnenih i vodenih ekosustava, fagotrofni protisti su neizostavan i važan dio prijenosa ugljika i energije između nižih i viših trofičkih razina (Risse-Buhl i sur., 2015; Geisen i sur., 2017). Ameboidni protisti, odnosno organizmi koje karakterizira prisustvo pseudopodija ili tzv. „lažnih nožica“ česta su i brojna heterotrofna komponenta perifitona lotičkih ekosustava (Singer i sur., 2021). Pseudopodiji su najčešće prstasti ili lobozni (lobopodiji), nitasti ili filozni (filopodiji), ukrućeni mikrotubulima (aksopodiji), a rjeđe mrežastih i drugih oblika (lamelipodiji, retikulopodiji) (Habdija i sur., 2011).

Taksonomska klasifikacija ameboidnih protista značajno se mijenjala kroz prošlost. Prvi pokušaj klasifikacije proveo je Dujardin (1841) koji uvodi nazive Rhizopoda i Sarcodina čija priroda riječi na grčkom jeziku aludira na organizme nalik korijenju ili sluzavoj želatinoznoj masi. Nakon toga Schulze (1854) dijeli ameboidne protiste na „gole“ i one s kućicom uvodeći naziv „Testacea“ koji će se dugo zadržati u literaturi. Haeckel (1866) uvodi skupinu Gymnamoebae koja podrazumijeva gole amebe bez ljušturice, a potom Page (1987) predlaže i prvi sustav determinacije vrsta golih ameba baziran isključivo na morfološkim karakteristikama. Ipak, najveće promjene u taksonomskoj klasifikaciji ameboidnih protista nastupile su u moderno doba uvođenjem molekularne filogenetike kada je postalo jasno da „umjetna priroda“ skupina Rhizopoda i Sarcodina nema znanstveno uporište, tj. da su ameboidni i flagelatni protisti filogenetski isprepleteni (Cavalier-Smith, 2003). Prema najnovijoj podjeli Adl i sur. (2019), ameboidni protisti zastupljeni su u nekoliko kladova unutar Amoebozoa: Tubulinea - koji obuhvaća organizme s lobopodijima (npr. okućene amebe reda Arcellinida, gole amebe redova Euamoebida i Leptomyxida); Evosea - koji obuhvaća organizme kompleksnih životnih stadija koji često uključuju i stadije s trepetljikama ili pak potpunim izostankom ameboidnog oblika (npr. gole amebe roda *Filamoeba*) i Discosea - koji obuhvaća pretežno spljoštene gole amebe koje nikada ne stvaraju tubularne pseudopodije (npr. rodovi *Thecamoeba*, *Mayorella*, *Dermamoeba*, *Korotnevella*, *Vannella*), dok se ameboidni protisti s filopodijima mogu pronaći unutar Cercozoa (npr. okućene amebe rodova *Euglypha*, *Trinema*); Opisthokonta (npr. gole amebe roda *Nuclearia*) i zasebnog klada CRuMSs (Collodictyonid + Rigifilida + Mantamonas).

Identifikacija i klasifikacija golih ameba uvijek je predstavljala težak zadatak zbog njihove male veličine tijela te polimorfne prirode (Smirnov i sur., 2005) stoga je i njihova bioindikatorska primjena u istraživanjima skromna. Tradicionalno su gole amebe, posebice pripadnici Amoebozoa bolje proučavani u kopnenim nego vodenim ekosustavima gdje prema recentnim saznanjima čine jednu od najbrojnijih skupina fagotrofnih protista (Fiore-Donno i sur., 2016). S druge strane, okućene amebe dobro su istražene zahvaljujući specifičnoj morfologiji zaštitne kućice koja omogućuje lako određivanje svojti, kao i konzerviranje. U većine rodova kućica ima jedan otvor (pseudostom) iz kojeg izlaze lobopodiji ili filopodiji (Todorov i Bankov, 2019). Okućene amebe odlični su bioindikatori okolišnih promjena jer njihovo bogatstvo vrsta, raznolikost, abundancija i biomasa brzo odgovaraju na iste (Koenig i sur., 2018). Osim toga, ovi organizmi postižu veliku brojnost; imaju kratko generacijsko vrijeme; među glavnim su konzumentima bakterija u mikrobnim hranidbenim mrežama te igraju važnu ulogu u kruženju dušika i ugljika u kopnenim i slatkovodnim ekosustavima (Jassey i sur., 2015). Iako je tradicionalno najveći broj istraživanja okućenih ameba taksonomske prirode, recentna istraživanja okućenih ameba obuhvaćaju i njihove funkcionalne značajke, odnosno proučavaju promjene funkcionalne raznolikosti okućenih ameba u odnosu na okolišne promjene (van Bellen i sur., 2017; Koenig i sur., 2018; Marcisz i sur., 2020). Primjena funkcionalnih značajki prednjači pred tradicionalnim taksonomskim istraživanjima jer predstavlja mehanističku poveznicu sa uslugama ekosustava. Osim toga, istraživanja temeljena na funkcionalnim značajkama okućenih ameba potencijalno su i robusnija i manje podložna greškama od taksonomskih istraživanja koja se oslanjaju na morfologiju s obzirom na to da su mnoge vrste okućenih ameba kriptične, odnosno ne mogu se identificirati isključivo oslanjajući se na morfologiju (npr. rodovi *Nebela* i *Hyalosphenia* (Oliverio i sur., 2014)). Među najčešće korištenim funkcionalnim značajkama su: sastav kućice (koja može biti organska/proteinska, vapnenačka, silikatna ili aglutinirana) (Todorov i Bankov, 2019); veličina kućice i morfologija; oblik i veličina otvora (van Bellen i sur., 2017; Koenig i sur., 2018). Većina ovih funkcionalnih značajki odražavaju uvjete vlažnosti i predstavljaju prilagodbe na vlažne ili suhe uvjete (Fournier i sur., 2015; van Bellen i sur., 2017), ali mogu ukazivati na pH-vrijednost ili omjer C/N, tj. upućivati na količinu organske tvari (Mitchell i sur., 2008; Fiore-Donno i sur., 2019). Globalno se pokazalo da okućene amebe iz skupine Cercozoa preferiraju neutralni ili bazični okoliš tla (Fiore-Donno i sur., 2019; Marcisz i sur., 2020).

Trepetljikaši (Ciliophora) među najbolje su proučenim skupinama fagotrofnih protista. Od ostalih skupina razlikuju se po specifičnoj građi korteksa, jezgrenom dimorfizmu (posjedovanju mikro- i makronukleusa) te jedinstvenom spolnom procesu razmnožavanja - konjugaciji (Lynn, 2008). Upravo strukturalna obilježja korteksa, naročito somatskog (tjelesnog) i oralnog (usnog) područja, vrsno su specifična i važna za taksonomsko određivanje pojedinih vrsta trepetljikaša (Lynn, 2008). Taksonomska istraživanja trepetljikaša temeljena na morfološkim obilježjima svojevrstan su izazov koji iziskuje mnogo iskustva i vremena, no unatoč tome dostupna literatura i ključevi za određivanje vrsta detaljni su i dobro zastupljeni, stoga su rezultirali velikim brojem opisanih vrsta (Foissner i sur., 1991, 1992, 1994; Foissner i Berger, 1996). Važnost morfoloških karakteristika trepetljikaša svoju primjenu ima i u istraživanjima njihovih filogenetskih odnosa. Protistolog Denis Lynn je primjenivši tzv. hipotezu o strukturalnom konzervatizmu, prema kojoj su elementi oralne trepetljikavosti skloni većoj stopi evolucijskih promjena od konzervativnih elemenata somatske trepetljikavosti zato jer direktno utječu na fitnes pojedine vrste, revolucionirao istraživanja filogenetske raznolikosti trepetljikaša (Agatha i sur., 2020). Na temelju njegovih istraživanja utemeljena je podjela trepetljikaša na dva potkoljena: Postciliodesmatophora i Intramacronucleata te 11 razreda: Armophorea, Colpodea, Heterotrichea, Karyorelictea, Litostomatea, Nassophorea, Oligohymenophorea, Phyllopharyngea, Plagiopylea, Prostomatea i Spirotrichea (Adl i sur., 2019), koja je trenutno u primjeni.

S obzirom na to da postižu veliku brojnost i raznolikost te da imaju kratko generacijsko vrijeme, trepetljikaši imaju važnu ulogu bioindikatora u lotičkim ekosustavima (Madoni i Zangrossi, 2005; Segovia i sur., 2016). Neke od ostalih prednosti koje čine trepetljikaše pouzdanim bioindikatorom su kozmopolitska rasprostranjenost, osjetljivost na promjene stanja okoliša te raznolika i lako primjenjiva metodologija uzorkovanja. S druge strane, potencijalni izazovi primjene trepetljikaša kao bioindikatora leže u morfološkoj identifikaciji koja iziskuje iskustvo te razne metode bojenja, ali i gubitku velike količine materijala uslijed konzerviranja (Foissner i Berger, 1996). U istraživanjima perifitonskih trepetljikaša u upotrebi su različite podloge za kolonizaciju, prirodne ili umjetne. Primc (1979) te Mieczan (2007) pokazali su da nema veće razlike između zajednice trepetljikaša na umjetnoj i prirodnoj podlozi te da su sukcesije na različitim podlogama usporedive. Ipak, prirodne podloge otežavaju kvantitativne analize zajednice uslijed velike varijabilnosti u sastavu i veličini podloge. S druge strane, umjetne podloge su uniformne i ekonomične stoga omogućuju lakše praćenje dinamike naseljavanja i sukcesije organizama (Mieczan, 2007). Među najčešće korištenim umjetnim podlogama su poliuretanska pjena (Cairns i sur., 1979) i predmetna stakalca (Primc, 1979; Primc-Habdija i sur., 2001; Mieczan, 2007) umetnuta u različite nosače poput kamenja i cigli koji onemogućavaju otplavljivanje istih. Predmetna stakalca standardnih dimenzija (2,6 x 7,6 cm) možda imaju najveću prednost među umjetnim podlogama jer njihova prozirnost omogućava promatranje perifitona direktno pod mikroskopom, ostavivši uzorak neoštećenim za razliku od metoda četkanja i struganja koje zahtijevaju drugi tipovi umjetnih podloga.

Taksonomska istraživanja perifitonskih trepetljikaša svoju su primjenu pronašla u bioindikaciji kvalitete vode u raznim lotičkim ekosustavima (Madoni i Zangrossi, 2005; Debastiani i sur., 2016). Tako Sladeček (1973) sastavlja popis vrsta trepetljikaša kao bioindikatora kvalitete vode nakon čega su uslijedile brojne revizije i dopune, a među njima i ona Foissnerova (1988, 2016) sa sistematičnim pregledom primjene trepetljikaša kao bioindikatora u aktivnom mulju. Unatoč već dugoj tradiciji primjene kao bioindikatora kvalitete vode te uvrštavanju u Hrvatski indikatorski sustav (HRIS) koji je obuhvatio sve vodene zajednice, trepetljikaši nisu pronašli svoje mjesto u Okvirnoj direktivi o vodama (Europska komisija, 2000). Ipak, njihova primjena u praćenju međuodnosa zajednice perifitona i procesa osedravanja u sedrotvornim ekosustavima RH seže daleko unatrag. Prvo opsežnije istraživanje perifitona rijeke Krke provedeno je krajem prošlog stoljeća kada Primc-Habdija i sur. (1997) postavljaju temelje razumijevanja međuodnosa perifitona i osedravanja te dinamike razvoja istog na postajama Visovačkog jezera. Uočena je pozitivna korelacija između rasta perifitona i stope osedravanja te su postavljene prve teze o važnoj ulozi biote u procesu osedravanja unutar NP Krka. Rezultati istraživanja Primc-Habdija i sur. (2001), koje je obuhvaćalo perifitonske trepetljikaše na plohama Skradinskog buka, potvrdilo je da i unutar sedrotvornih staništa NP Krka morfologija podloge značajno utječe na kolonizaciju, što se već pokazalo relevantnim u mnogim istraživanjima bakterijske komponente perifitona (Baker, 1984) te peritrihnih trepetljikaša u drugim lotičkim ekosustavima (Harmsworth i Sleigh, 1993). Uz lotičke, mnoga istraživanja obuhvatila su i lentičke dijelove rijeke Krke (Primc-Habdija i sur., 1997; Primc-Habdija i sur., 2005). Matoničkin Kepčija i sur. (2017) nastavljaju tradiciju istraživanja perifitona unutar granica NP Krka, fokusirajući se na sezonsku dinamiku razvoja perifitonskih trepetljikaša i procesa osedravanja na sedrenim barijerama Roškog slapa i Skradinskog buka.

Za razliku od taksonomskih istraživanja perifitonskih trepetljikaša, ona temeljena na njihovoj funkcionalnoj raznolikosti, odnosno sastavu njihovih funkcionalnih značajki, još uvijek su u počecima, barem u lotičkim ekosustavima. Dosadašnja istraživanja većinom provedena u obalnim morima ukazala su na to da funkcionalne značajke trepetljikaša temeljene na izvoru hrane i načinu hranjenja, veličini i obliku tijela, mobilnosti i načinu kretanja imaju potencijalni utjecaj na funkcioniranje ekosustava (Xu i sur., 2017; Zhong i sur., 2019). Iako i razlike u veličini tijela mogu odražavati varijacije u funkcionalnoj strukturi i raznolikosti zajednice, budući da su kategorije veličine tijela u određenoj mjeri povezane s trofičkim razinama (npr. manji trepetljikaši obično se hrane bakterijama dok su oni veći algivori ili predatori nad drugim fagotrofnim protistima i mikro metazoa (Norf i sur., 2009; Zhao i sur., 2016)), stavljanjem veće važnosti na izvor hrane kao funkcionalnu značajku može se dobiti uvid u povezanost izvora hrane s mjerama heterogenosti mikrostaništa s obzirom na to da su se bakteriovorni trepetljikaši pokazali osjetljivima na sastav čestica tla, odnosno prostor između pora tla, a slobodno plivajući trepetljikaši pokazuju sklonost većim porama unutar supstrata (Fiore-Donno i sur., 2019). U opsežnom pregledu literature s osvrtom na funkcionalne značajke trepetljikaša vodenih ekosustava, Weisse (2017) ističe kako pri svrstavanju trepetljikaša u funkcionalne kategorije treba biti oprezan jer se korištenjem „preširokih“ kategorija, odnosno kategorija „širokog konteksta“ može previdjeti odgovor na razini ekosustava. Takav primjer je istraživanje van Wichelen i sur. (2013) u kojem među 66 plitkih jezera zapadne Europe nije detektirana promjena funkcionalne raznolikosti perifitonskih trepetljikaša vjerojatno zbog korištenja kategorija „preširokog konteksta“. S obzirom na to da je prisustvo neke vrste u zajednici povezano s funkcionalnim značajkama vrste stečenim tijekom evolucije filogenetskih linija, izazov suvremenih grana ekologije je utvrditi koji su čimbenici odgovorni za evoluciju raznolikosti značajki i obrnuto, kako evolucija značajki utječe na zajedničko pojavljivanje (koincidenciju) vrsta u postojećim lokalnim zajednicama.

Ameboidni protisti i trepetljikaši svoju primjenu pronalaze i u istraživanjima utjecaja mjera restauracije na biotu i procjeni uspješnosti iste. Ipak, većina takvih istraživanja usredotočena je na restauraciju cretova i tresetišta (Ternjej i sur., 2015; Jassey i sur., 2016; Mieczan i sur., 2018, 2019), dok su istraživanja utjecaja mjera restauracije na perifiton lotičkih ekosustava većinom još uvijek temeljena na bakterijskoj komponenti mikrobne zajednice (Lin i sur., 2020). Funkcionalni odgovor perifitona pokazao se obećavajućim u procjeni oporavka lotičkih ekosustava nakon određenog poremećaja kao što je isušivanje, poplava ili organsko obogaćivanje hranjivim tvarima (Timoner i sur., 2012, 2014), a novije istraživanje Atristain i sur. (2023) pokazalo je da funkcionalni parametri perifitona potoka brzo odgovaraju na mjere restauracije koje uključuju uklanjanje brana, otvarajući tako prostor novim istraživanjima ove tematike.

U istraživanjima fagotrofnih protista postoji veliki potencijal za primjenu suvremenih metoda istraživanja. Ipak, primjena metabarkodiranja u istraživanjima okolišne DNA protista još uvijek zaostaje za istraživanjima prokariota i gljiva (Geisen i sur., 2019), iako se globalna raznolikost protista procjenjuje u milijunima, a njihova biomasa i dvostruko većom od svih životinja (Burki i sur., 2021). Ovaj nedostatak posljedica je metodoloških ograničenja jer u molekularnim istraživanjima protista još uvijek ne postoji konsenzus o usporedivom pristupu, odnosno ne postoji standardizirani set početnica (engl. *primer*) za razliku od dobro poznatih početnica koje se koriste u istraživanjima bakterija i gljiva (Geisen i sur., 2019). U istraživanjima protista za sada najveću primjenu ima metabarkodiranje V4 i V9 regije 18S rRNA gena čija prednost leži u dužini amplikona kraćoj od 200 bp koji omogućava učinkovito sekvenciranje uz optimalan trošak (Amaral-Zettler i sur., 2009). Istovremeno, korištenje kratkog amplikona predstavlja i novi izazov zbog povezivanja s taksonomskom informacijom jer referentne taksonomske baze nisu kompletne stoga mogu voditi pogrešnoj identifikaciji (Burki i sur., 2021). Ipak, posljednjih 15 godina metabarkodiranja omogućilo je uvid u ogromnu raznolikost protista, pogotovo onih čija je identifikacija zahtjevna, a kultivacija otežana (npr. golih ameba). S obzirom na to da je duga povijest tradicionalne mikroskopske identifikacije rezultirala opsežnom literaturom, uključujući identifikacijske ključeve (Foissner i sur., 1991, 1992, 1994, 1995; Foissner i Berger, 1996), suvremene metode metabarkodiranja predstavljaju veliki potencijal za komplementarno korištenje metoda koje će omogućiti sveobuhvatno i preciznije predviđanje odgovora zajednica i čitavih ekosustava na izazove i poremećaje, pogotovo u kontekstu sve izraženijih klimatskih promjena (Cavicchioli i sur., 2019).

PREGLED DOKTORSKOG RADA

Ovaj doktorski rad obuhvaća četiri izvorne znanstvene publikacije (**I – IV**) u kojima su navedeni i obrađeni ciljevi i znanstvena pitanja.

Ciljevi ovog doktorskog rada su:

1. istražiti odgovor perifitona na revitalizaciju vodenih tokova sedrene barijere Skradinski buk kroz sveobuhvatnu analizu zajednice i okolišnih parametara i usporedbu s postojećim podacima
2. istražiti bioindikatorski potencijal taksonomskih i funkcionalnih metrika fagotrofnih protista (ameboidnih protista i trepetljikaša) u praćenju revitalizacije vodenih tokova Skradinskog buka
3. utvrditi mijenjaju li se taksonomske i funkcionalne metrike fagotrofnih protista u perifitonu s promjenom duljine ekspozicije podloga
4. istražiti i identificirati potencijalno indikatorske vrste trepetljikaša revitaliziranih tokova sedrenih barijera
5. usporediti različite metode uzorkovanja perifitona na sedrenim barijerama i optimizirati korištenje umjetnih podloga u svrhu primjene u restauracijskoj praksi
6. istražiti količinu i dinamiku sedre i organske tvari u revitaliziranim tokovima
7. istražiti funkcionalnu i filogenetsku raznolikost trepetljikaša duž rijeke Krke (od izvora do Skradinskog buka)

Znanstvene hipoteze ovog doktorskog rada su:

1. revitalizacija vodenih tokova će se odraziti u sastavu i strukturi perifitona, a s vremenom će razlika između postojećih i revitaliziranih tokova postajati sve manje izražena
2. funkcionalna raznolikost fagotrofnih protista pokazat će jasniji odgovor na promjene nastale uslijed revitalizacije u odnosu na taksonomsku raznolikost
3. metodu uzorkovanja perifitona treba prilagoditi specifičnim ciljevima istraživanja pri čemu uzorkovanje prirodne podloge daje bolji uvid u dinamiku supstrata revitaliziranih tokova dok primjena umjetnih podloga omogućava kvantifikaciju istaložene sedre te precizniji uvid u sastav i strukturu perifitona uslijed manje invazivnosti metode

4. taloženje sedre i organske tvari te struktura perifitona na barijerama određeni su specifičnim okolišnim čimbenicima koji su promjenjivi u prostornom i vremenskom kontekstu

Publikacije **I**, **II** i **III** direktno su vezane uz revitalizirane tokove sedrene barijere Skradinski buk. Publikacija **I** predstavlja prvu fazu istraživanja u kojoj je perifiton uzorkovan s prirodne podloge unutar prve godine aktivacije vodenih tokova, po uklanjanju jedinki invazivne biljne vrste pajasen. Provedeno istraživanje je interdisciplinarne prirode koja je podrazumijevala mjerenje niza okolišnih parametara (fizikalno-kemijski parametri vode, količina organske/anorganske tvari, granulometrijski i mineraloški sastav) uz određivanje sastava i brojnosti svojti perifitona. Rezultati ovog istraživanja pokazali su da perifiton uspješno reflektira proces revitalizacije i uspostavu novih vodenih tokova na mikroskali. U njemu prevladavaju fagotrofni protisti poput okućenih ameba i trepetljikaša čiji je broj svojti gotovo dvostruko veći u revitaliziranim tokovima u odnosu na kontrolne. Promjenjiva hidrologija revitaliziranih tokova u vidu povremenih bujičnih tokova i veće brzine strujanja pospješuje ispiranje okolnog šumskog tla te tako uvjetuje unos nutrijenata i povećava strukturnu složenost/heterogenost mikrostaništa uslijed donosa dodatne alohtone organske tvari, čineći istu važnim dodatnim izvorom organske tvari za zajednicu. Također, utjecaj promjenjive hidrologije i ispiranja vidljiv je i u granulometrijskom i mineraloškom sastavu podloge koju čini dominantno šljunkovita komponenta sastavljena od većih komada sedre za vrijeme veće brzine strujanja tijekom zimskih mjeseci, odnosno sitnozrnata komponenta za vrijeme ljetnih mjeseci. Uz kalcit kao dominantnu mineralnu fazu u svim skupnim uzorcima, prisutnost kvarca s tinjcima u revitaliziranim tokovima ukazuje na utjecaj ispiranja okolnog tla.

Publikacija **II** predstavlja nastavak istraživanja prezentiranog u publikaciji **I**, ali za razliku od prijašnjeg temelji se na praćenju kolonizacije perifitona odnosno njegove heterotrofne komponente (fagotrofni protisti) na umjetnim podlogama postavljenim unutar revitaliziranih tokova. Rezultati publikacije **II** pokazuju da se zajednice ameboidnih protista i trepetljikaša revitaliziranih tokova značajno razlikuju od onih kontrolnih tokova u taksonomskim i funkcionalnim metrikama. Promjenjiva hidrologija revitaliziranih tokova obilježava i ovu fazu istraživanja pri čemu strukturna složenost uslijed taloženja alohtone i autohtone organske tvari te kristalića kalcita uvjetuje taksonomske i funkcionalne metrike perifitonskih ameboidnih protista i trepetljikaša u revitaliziranim vodenim tokovima. Prisutnost algalnog pokrova povećava taksonomsku i funkcionalnu raznolikost trepetljikaša zbog povećanja heterogenosti mikrostaništa, smanjujući abundanciju ameboidnih protista uslijed umanjene sposobnosti hranjenja bakterijama. Osedravanje pridonosi trodimenzionalnoj strukturi i heterogenosti mikrostaništa revitaliziranih tokova, no taloženje sedre iznad granične vrijednosti uzrokuje zatrpavanje trepetljikaša koji su mnogo osjetljiviji na osedravanje u odnosu na ameboidne protiste. Taksonomske i funkcionalne metrike trepetljikaša ovisne su o dužini trajanja ekspozicije umjetnih podloga odnosno vremenu dostupnom za kolonizaciju istih dok se metrike ameboidnih protista ne mijenjaju značajnije s promjenom duljine trajanja ekspozicije. Ova publikacija pruža i važna metodološka saznanja i ističe važnost primjereno odabrane metodologije u istraživanjima perifitona postavljajući temelje za buduća istraživanja tog tipa u sedrotvornim sustavima NP Krka i mediteranske regije.

Publikacija **III** nastavak je publikacije **II** i pruža dublji uvid u zajednicu trepetljikaša revitaliziranih tokova analizom sastava zajednice na razini pojedinih svojti te analizom pripadajućih funkcionalnih značajki. Rezultati ovog istraživanja ukazuju na to da su revitalizirani tokovi poveznica kopnenih i lotičkih ekosustava. Perifiton revitaliziranih tokova naseljavaju vrste trepetljikaša koje osim u kopnenim vodama veliku brojnost postižu i u tlu, poput vrste *Platyophrya vorax* koja je identificirana kao potencijalno indikatorska vrsta trepetljikaša revitaliziranih tokova sedrene barijere Skradinski buk uz implikacije za važnost u kruženju ugljika i ciklusu organske tvari u revitaliziranim tokovima. U vidu funkcionalnih značajki, perifitonom revitaliziranih tokova dominiraju pokretni (slobodnoplivajući), pretežno bakteriovorni i omnivorni trepetljikaši koji koriste veći raspon različitih niša nastalih uslijed veće složenosti mikrostaništa u perifitonu revitaliziranih tokova pri čemu interspecijska kompeticija, odnosno kompetitivno isključivanje, igra važnu ulogu u strukturiranju funkcionalnog sastava zajednice.

Uloga kompeticije u strukturiranju funkcionalne raznolikosti trepetljikaša dodatno je istražena u publikaciji **IV** ovog doktorskog rada, koja premda nije direktno vezana uz revitalizirane tokove Skradinskog buka, istražuje perifitonske trepetljikaše na četiri lokacije od izvora prema ušću rijeke Krke (izvor Krke, Marasovine, Roški slap, Skradinski buk) pruživši prvi uvid u filogenetsku i funkcionalnu raznolikost trepetljikaša duž rijeke Krke. Dodatno, u istraživanje je uključena i komponenta raznolikosti mikrostaništa budući da je perifiton uzorkovan s ploha s gornje i donje strane supstrata (svjetlo/tamno izložena strana sedre). U ovom istraživanju funkcionalne značajke dodijeljene su na temelju molekularne identifikacije trepetljikaša na razini roda. Rezultati ove publikacije pokazali su da izvorišno područje rijeke Krke ima nižu filogenetsku raznolikost i funkcionalnu raznolikost od nizvodnih sedrenih barijera poput barijere Skradinski buk. Okolišni uvjeti izvorišnog područja poput niže koncentracije otopljenih hranjivih tvari i nižeg protoka favoriziraju uspješnije kompetitore poput sjedilačkih i polu-sjedilačkih kolonijalnih trepetljikaša koji su uspješni bakteriovorni filtratori rezultirajući filogenetski divergentnom zajednicom. Pokazavši da se filogenetska i funkcionalna raznolikost trepetljikaša mijenja duž toka rijeke Krke, publikacija **IV** poboljšava razumijevanje osjetljivosti jedinstvenog sedrotvornog ekosustava rijeke Krke u svjetlu sve intenzivnijih klimatskih promjena te pruža temelj za buduće konzervatorske i restauracijske napore.

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Environmental and periphyton response to stream revitalization – A pilot study from a tufa barrier

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A R T I C L E I N F O

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A B S T R A C T

The formation of tufa barriers is driven by specific physicochemical and biological factors which make them very sensitive to environmental changes. The barriers provide great microhabitat complexity and are therefore inhabited by various periphytic organisms, whose metabolic activity alters the microenvironment, directly or indirectly affecting the physicochemical conditions of calcite precipitation. After the invasive plant species *Ailanthus altissima* (Mill.) Swinge was identified as one of the main factors causing current dryness of streams at the Skradinski buk barrier (Krka National Park, Croatia), the invasive species was removed, resulting in changes in the hydromorphology of the barrier and reactivating previously dry streams. The objective of this study was to determine potential differences between control and revitalized sites (reactivated streams) with regards to periphyton and the associated environmental changes over an annual cycle. Clear patterns were observed between sites reflecting the substantial changes in the landscape following invasive species removal. Revitalized sites had lower flow velocity, conductivity, pH, alkalinity and total water hardness values while exhibiting higher values of dissolved organic matter, nitrites and orthophosphates due to the presence of soil-derived organic material and intensive soil drainage. Organic matter content was generally higher at revitalized sites but did not inhibit tufa deposition. The influence of the surrounding forest soil was reflected not only in the mineralogical analysis of tufa sediment, but also in the abundance and diversity of periphyton. Revitalized site communities exhibited higher abundance and diversity and were dominated by bacterivorous and omnivorous ciliates and cercozoans due to the abundant food resources of the soil. The results presented show that ecosystem responses to hydromorphological changes generated by invasive species removal can be efficiently detected and monitored at microscale (i.e., periphyton scale) through a comprehensive interdisciplinary approach. As the first study to examine the relationship between aquatic ecosystems and sediments in the context of tufa stream revitalization, this pilot study provides data and guidelines for future management plans applicable to various tufa depositing systems facing invasive species propagation, allowing better understanding and protection of this unique and vulnerable karstic feature.

1. Introduction

The Adriatic karst area is represented by a mosaic of different karst landforms (Woodward, 2009), mostly developed on mechanically strong, but chemically soluble rocks, predominantly limestone. Tufa deposits formed by precipitation of carbonate minerals from these oversaturated karst waters usually occur at ambient or higher temperatures (>20 °C), slightly alkaline pH and can reach impressive sizes, as in the case of tufa barriers along the Krka River in Croatia (Jennings, 1985; Ford and Pedley, 1996; Glover and Robertson, 2003). Precisely, tufa represents deposits of inorganic matter (i.e., calcite) with remnants of organisms (leaf litter, branches, fine detritus, larval cases, shells) (Pedley, 2000; Wu, 2016). Such deposits provide a high degree of microhabitat complexity and a large number of niches and refugia for the organisms (Beisel et al., 2000; Bednar et al., 2017), promoting spatial resilience (i.e., an ability to cope with disturbance on various spatial scales), particularly for microbial (e.g., periphyton) communities (Dzubakova et al., 2018).

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Periphyton represents a complex community comprising autotrophic and heterotrophic microorganisms, their extracellular products and organic detritus attached to submerged surfaces in permanent or temporal aquatic habitats (Palmer and White, 1997). Because of the dimensions and life cycles of the organisms involved, periphyton can rapidly (within 24 h to several weeks) grow or disappear in respond to environmental changes, and can thus serve as an early indicator of changing landscapes over a wide range of spatial scales (metres to tens of kilometres) (Wu, 2016). In addition to its rapid response, there are several other desirable features making periphyton a reliable ecological indicator: (1) ubiquitous distribution, 2) quantifiability at different levels of biological organization (individual, species, population and community) and (3) connectivity to levels above and below its placement in the food web (Karr, 1999). Periphyton is also useful in assessing the recovery of lotic ecosystems following a particular disturbance such as desiccation, flooding or organic nutrient enrichment (Steinman and McIntre, 1990; Timoner et al., 2012, 2014). A range of studies that have examined the effects of dry periods and flow resumption (i.e., stream intermittency) on micro-metazoan communities suggest that dry period duration is the primary driver of periphyton community structure and functional diversity (Vadher et al., 2018).

Unlike intermittent (seasonal) or ephemeral (precipitation-dependent) streams (Lake et al., 2017), some tufa-depositing streams have been dry for centuries due to various factors of landscape degradation (e.g., climate change, local anthropogenic interventions in the environment, rapid tourism expansion) (Goudie et al., 1993; Gu et al., 2013; Qiao et al., 2016; Šiljeg et al., 2020). Uncontrolled growth and spread of invasive plant species *Ailanthus altissima* (Mill.) Swinge (tree of heaven), has been identified as one of the main factors causing current dryness of streams at the Skradinski buk barrier, the final and longest tufa barrier in the Krka National Park (Šiljeg et al., 2020). Whereas bryophytes (moss) are recognized as important biomediators of the tufa deposition, higher (vascular) plants can cause sparse calcite mineralization (Turner and Jones, 2005), overgrowth of tufa barriers (due to their strong root system), water flow cessation and eventually barrier dryness within tufa-precipitating environments. The negative effects of vascular plant overgrowth are particularly pronounced in the case of invasive plants, especially in protected areas such as national parks (Šiljeg et al., 2020). Previous interventions of the vascular plant removal in the tufa depositing system of the Plitvice Lakes National Park resulted in a better aeration and reduction in the organic matter load, which consequently led to enhanced tufa deposition and higher abundance of macrozoobenthos (Miliša et al., 2006; Pavlus and Novosel, 2004).

The present pilot-study builds on the previous findings, investigating the response of periphyton to newly emerging water pathways and associated environmental changes following the removal of the invasive plant species - tree of heaven, *A. altissima* at a tufa barrier in Krka National Park, Croatia. The study is a part of an interdisciplinary project aimed towards developing a model for a sustainable and long-term management of tufa-precipitating hydrosystems in protected areas. In such areas, a balance must be found between the preservation of natural processes and necessary interventions such as the control of invasive species. Specific aims of this pilot-study were to examine the influence of the invasive plant (*A. altissima*) removal on stream revitalization and its consequences on: 1) physicochemical parameters of water, organic and inorganic (i.e., tufa) matter content in periphyton; 2) abundance and diversity of periphytic taxa; and 3) granulometric composition and mineral constituents of the stream substrate. In terms of community structure, emphasis was placed on phagotrophic protists and micrometazoans, recognized to considerably alter the surrounding microenvironment with their metabolic activity (Primc-Habdija et al., 2001; Matoničkin Kepčija et al., 2006), thus affecting the physicochemical conditions (i.e., near-surface gradients of pH and carbonate, organic matter and nutrient contents) for calcite precipitation.

We hypothesized that there would be significant differences between control sites (located within permanent streams) and revitalized sites (located within reactivated streams) with respect to flow velocity, pH, organic matter content and nutrient concentrations due to considerable landscape changes caused by the invasive plant removal. Because of the drainage of the surrounding forest soil, we presumed that water at the revitalized sites would have higher nutrient concentrations and consequently lower pH values. We also anticipated that organic matter content in periphyton would be higher within revitalized sites, but still low enough to allow for tufa deposition. We further hypothesized that periphyton colonization would occur within the first few months of invasive plant removal, although exhibiting lower taxa diversity and abundance within revitalized sites due to the strong fluctuations in environmental conditions (especially flow velocity) and lower microhabitat heterogeneity compared to control sites. To date, few studies have examined periphyton on porous tufa sediments (Wu, 2016), and to our knowledge, no study has yet examined the relationship between the in-stream environmental conditions and periphyton community development in the context of invasive plant removal followed by stream revitalisation within a tufa-precipitating environment. The results of this pilot-study will primarily provide guidelines for future management plans applicable in Krka National Park, but they could ultimately be useful in tailoring management guidelines for other protected areas and tufa depositing systems facing invasive plant propagation.

2. Methods

2.1. Study area

The Krka River is a karst river located in the central part of the northeastern Adriatic coast. It drains predominantly carbonate terrains of the Croatian Dinaric karst area with a catchment area of 2427 km^2 (Bonacci and Ljubenkov, 2005). Due to its average annual discharge of 47.4 m^3 s^{-1} (1990–2009), Krka is considered a medium-size river in Croatia (Čanjevac and Orešić, 2015). With its seven large waterfalls and a total elevation drop of 242 m, the Krka River is a natural karstic phenomenon that was proclaimed a national park in 1985. Near the town of Knin, Krka enters a canyon where its course is characterized by many lentic areas, as a consequence of tufa precipitation processes and tufa barrier formation. One of the longest tufa barriers within the Park is Skradinski Buk at the southern border of Visovac Lake (Fig. 1), a large lake-like expansion of the river course. This barrier was selected as the study area in this study.

2.2. Sampling sites

An experimental 1 ha plot was defined at the Skradinski Buk tufa barrier (Fig. 1). A comprehensive aerial survey (Phantom 4) and extensive vegetation mapping indicated that the invasive plant *A. altissima* was found to dominate the surface area. In August 2017, individuals of the invasive tree were mechanically removed in accordance with a permit from the Croatian Agency for the Environment and Nature. Within two months of the tree removal, five previously dry streams in the experimental plot were found to be reactivated. Within the experimental plot, seven sampling sites were selected: two control sites located within permanent streams, C1 (15.966381, 43.805752) – control site where water had been present before and after *A. altissima* removal and displaying well-developed moss cover; C2 (15.965213, 43.806690) – control site characterized by occasional drying-out-phases before and after *A. altissima* removal with patch-like moss cover; and five revitalized sites: N1 (15.965449, 43.806438), N2 (15.965324, 43.806624), N3 (15.965246, 43.806541), N4 (15.965186, 43.806489), N5 (15.965538, 43.806225), representing newly reactivated streams (Fig. 1).

2.3. Sampling design and measurements of environmental parameters

Field sampling was conducted over a one-year period from October

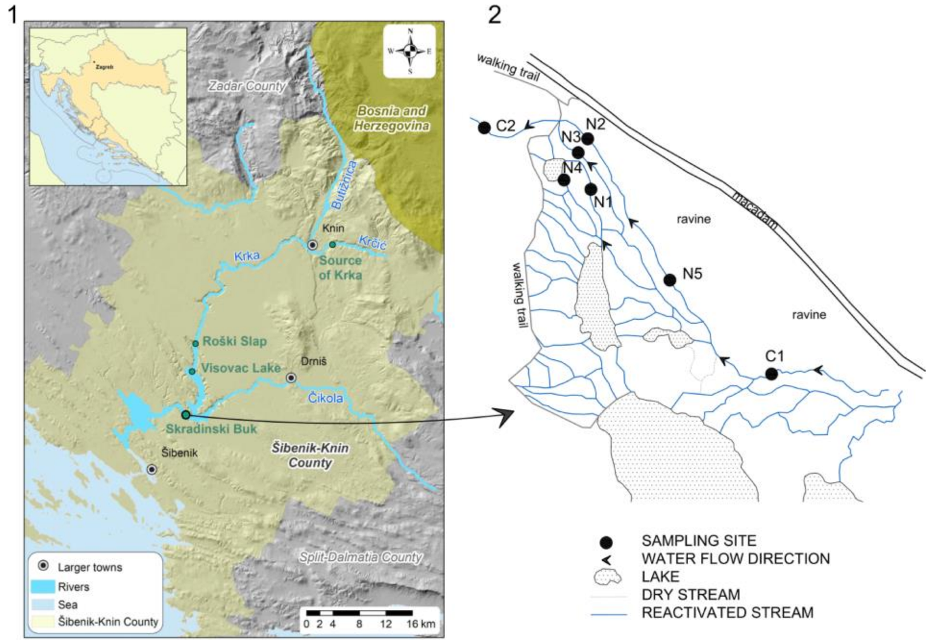

Fig. 1. Map of Croatia and the Krka River catchment (1) with marked sampling sites at the Skradinski Buk barrier (2). Description of the sampling site codes is given in the Methods.

2017 to November 2018 on a monthly or bimonthly basis (8 sampling sessions in total: October 30, 2017; December 4, 2017; January 29, 2018; March 27, 2018; May 14, 2018; June 26, 2018; September 5, 2018; November 5, 2018). During each field visit, the following measurements were taken using portable field meters: water temperature, dissolved oxygen and oxygen saturation (OXI 96, WTW GmbH, Weilheim, Germany), pH (330i, WTW GmbH, Weilheim, Germany), conductivity (Sension 5, Hach, Loveland, Colorado, USA) and flow velocity (flow velocity meter P600, Dostmann electronic GmbH, Wertheim-Reicholzheim, Germany). Conductivity measurements were missing for October and December 2017 sampling dates due to technical difficulties of the portable field meter. In addition to *in situ* measurements, a 1 L water sample was collected at each site for further laboratory analysis, which comprised measurements of alkalinity, total water hardness, concentrations of nitrite (N-NO_2^-), nitrate (N-NO_3^-) and orthophosphate (P-PO_4^{3-}) (according to APHA, 1985), and chemical oxygen demand (COD; according to Deutsches Institut für Normung, 1986).

2.4. Periphyton sampling and analysis

Periphyton samples were collected in triplicate at each sampling site using a metal corer with a diameter of 4 cm and an effective surface area of 12.57 cm^2. The depth of sampling was approximately 3 cm, in order to obtain representative and comparable samples. Samples were transferred to plastic containers filled with a small amount of ambient water and stored at 4 °C. The total volume of each replicate was recorded prior to homogenization by vigorous shaking, which was followed by microscopic examination of the phagotrophic protists and micrometazoans. From each replicate, three subsamples of 200 μL were examined, i.e., a total of 600 μL per replicate. Living phagotrophic protists and micrometazoans were identified to the lowest possible taxonomic level (genus in most cases except for some distinct protists and rotifers, which were identified to species level) within 48 h of sampling, using 125x, 250x and 400x magnification (Jeneval binocular microscope and Zeiss Axioimager A2 with DIC objectives and Axiocam 305 digital camera). Species were identified using Zen 2.4 Imaging software and the appropriate literature (Kahl, 1930; Koste, 1978; Foissner et al., 1991, 1992, 1994, 1995; Page, 1991; Todorov and Bankov, 2019). Per each taxa, 10 – 15 photomicrographs were taken and subjected to morphometric measurements. For ciliates, additional video clips were used to record movements and distinguishing features. A total of 1512 individual phagotrophic protists were counted, of which 315 belonged to Amebozoa, 444 to Ciliophora, 21 to Dinoflagellata, 120 to Excavata, 54 to Heliozoa and 558 to Rhizaria. Out of total 737 micro-metazoans, 4 belonged to Crustacea, 54 to Gastrotricha, 18 to Hydrachnidia, 69 to Insecta, 357 to Nematoda, 6 to Oligochaeta, 222 to Rotifera, 4 to Tardigrada and 3 to Turbellaria. Abundance of organisms was expressed as the number of individuals per cm^2 (the number of individuals in sample volume divided by the product of subsample volume and surface of the sampling corer).

2.5. Organic and inorganic matter content

Upon microscopic examination, periphyton samples were dried at 104 °C until constant weight, subsequently ashed at 400 °C for 4 h and reweighted to estimate organic matter content as ash-free dry mass (AFDM; g/g dry mass of sample, expressed as %). The remaining content was weighted, then acidified in 250 ml 5% HCl solution to dissolve $CaCO_3$ and ashed at 400 °C until constant weight, according to Primc-Habdija et al. (2001). The difference in weight before and after acidification was used to estimate the amount of inorganic matter, i.e. tufa, which was expressed as the mass of tufa per mass of dry sample, in percentages.

2.6. Grain size analysis

For grain size analysis, tufa samples were collected in December 2017 and May, June, September and November 2018. Sample collection was again done in triplicate, using a metal corer with an effective surface area of 19.63 cm^2. Subsamples were transferred to plastic containers filled with a small amount of ambient water and dried shortly upon arrival to the laboratory, at 100 °C until constant weight. Initial masses of 10, 15 or 20 g were taken for wet sieving analyses, depending on the available material. Samples were sieved through a set of 7 standard ASTM sieves (4–0.063 mm mesh) with one Φ interval. The fraction passing the 0.063 mm sieve was collected in suspension, and a subsample taken for sedigraph analysis using the standard procedure (Micrometrics, 2002). The material retained on sieves was dried and weighed, and the data obtained by both techniques were merged to obtain the overall grain size range. Sediments were classified according to Folk (1954) scheme based on the gravel-sand-mud ratio.

2.7. Mineral analysis

The qualitative mineral phase (i.e. mineral constituent) composition was determined using the X-ray diffraction technique on the Philips X'pert powder diffractometer with CuKα radiation from the tube operating at 40 kV and 45 mA. The X-ray powder diffraction (XRPD) data set was collected from 4° to 63° 2Θ. Analyses were carried out on bulk samples collected from the C1 and N1 sampling sites for May, September and December sampling dates, for a total of 12 bulk samples. Where possible (due to the amount of collected finer sediment, particles smaller than 0.063 mm), additional analyses were performed. These particles (<0.063 mm) were additionally treated with hydrochloric acid solution (1:10), drop by drop, to remove the carbonate component and collect insoluble residue for further XRPD analyses.

2.8. Data analysis

The data on environmental parameters, organic and inorganic matter content and community metrics (periphyton abundance and number of taxa) were summarized as means (average of values) and standard errors (SE) for all sampling sites and seasons and graphically presented to illustrate possible spatio-temporal trends using R v.4.0.3. (R Core Team, 2020) and the following packages: 'ggplot2' v. 3.3.3 (Wickham, 2016), 'RColorBrewer' v. 1.1.2 (Neuwirth, 2014) and 'ggpbur' v. 0.4.0 (Kassambara, 2020). All data exploration (distribution, sphericity) and statistical analyses were conducted in R using the appropriate functions and packages. Since the original data were not normally distributed (Shapiro test, $p < 0.05$) and had violated sphericity (Mauchly test, $p < 0.05$), the differences between sites and dates for all measured parameters and community data were tested by the non-parametric Friedman's test, followed by Nemenyi post-hoc test. The difference between control and revitalized sites (control sites C1 and C2 grouped together; revitalized sites N1-N5 grouped together) with regards to periphytic taxa abundance was tested using analysis of similarity (ANOSIM) and visualized using non-metric multidimensional scaling (NMDS) analysis from the 'vegan' v. 2.5.7 package (Oksanen et al., 2020). Absolute abundance data was converted to relative abundance using the *decostand* function from R 'vegan' package while the Bray-Curtis (BC) index was used as a measure of community similarity. BC similarity values were transformed to distance matrices and significant environmental vectors ($p < 0.05$) and taxa ($p < 0.01$) were fitted to the ordination using the *envfit* function of the R 'vegan' package. Correlations between measured environmental (i.e., flow velocity, pH, temperature, orthophosphates, nitrites, COD, tufa content) and community (i.e., periphyton abundance and number of taxa) variables were tested using *cor.test* function on untransformed data.

3. Results

3.1. Environmental parameters

A detailed representation of environmental data is given in Supplementary Table 1, while mean, minimum and maximum values were presented in Table 1. In general, revitalized (N-sites) displayed lower mean values of flow velocity, conductivity, total water hardness, dissolved oxygen, pH and alkalinity, but higher values of temperature, COD (used as a proxy for dissolved organic matter in water), nitrites and orthophosphates (Table 1) compared to control sites (C-sites). Significant between-site differences were observed only for total water hardness. Total water hardness was significantly higher at C2 than at N2, but significantly higher at N2 than at N1 and N5 (Friedman's test ($\chi2$ (6) = 18.701, $p < 0.05$; Nemenyi post-hoc, $p < 0.05$). Although Friedman's test also indicated significant differences between sampling sites with respect to conductivity ($\chi2$ (6) = 15.26, $p < 0.05$), Nemenyi post-hoc test did not reveal it ($p > 0.05$). Conductivity measurements for sampling dates were also found significantly different ($\chi2$ (5) = 33.48, $p < 0.001$) due to lower values observed in autumn than in spring and summer (Nemenyi post-hoc, $p < 0.05$), whereas total water hardness was significantly lower in spring than in autumn and winter ($\chi2$ (7) = 34.60, $p < 0.001$; Nemeyi post-hoc, $p < 0.05$).

Water temperature displayed seasonality with minimum values in winter and maximum values in late summer (Table 1). Friedman's and post-hoc Nemenyi tests revealed significant differences in water temperature among all dates ($\chi2$ (7) = 48.71, $p < 0.001$; Nemenyi post-hoc, $p < 0.05$). Dissolved oxygen values displayed significantly higher values in winter than in other seasons ($\chi2$ (7) = 48.38, $p < 0.001$; Nemenyi post-hoc, $p < 0.05$). Considering pH, winter dates had significantly lower pH values in comparison to summer and autumn dates ($\chi2$ (7) = 36.29, $p < 0.05$; Nemenyi post-hoc, $p < 0.05$).

Flow velocity fluctuated among sites and dates, reaching maximum values at C2 during summer (Table 1). At revitalized (N) sites, water flow was generally low, with N5-site drying out in October 2017. Significant temporal differences ($\chi2$ (7) = 24.06, $p < 0.05$) in flow velocity were observed among winter (December 2017), autumn (October 2017) and spring (March 2018) – winter recorded significantly higher flows

Table 1
Environmental parameters at control (C) and revitalized (N) sites during the study period (October 2017 - November 2018). SE - standard error, MIN - MAX - minimal to maximal values measured at sampling sites during the study period.

Physicochemical variable	C		N	
	MEAN ± SE	MIN–MAX	MEAN ± SE	MIN–MAX
Water temperature (°C)	15.77 ± 1.24	9.90–23.80	16.08 ± 0.78	9.9–24.15
O_2 (mg L^{-1})	9.87 ± 1.21	8.43–11.26	9.80 ± 0.16	8.28–11.22
Flow velocity (m s^{-1})	0.72 ± 0.12	0.07–1.66	0.60 ± 0.06	0–1.36
Conductivity (μS cm^{-1})	448.42 ± 23.65	354–554	446.20 ± 13.74	357–535
pH	8.17 ± 0.08	7.81–9.04	8.10 ± 0.04	7.35–9.02
COD (mg O_2 L^{-1})	1.35 ± 0.19	0.31–2.79	1.63 ± 0.22	0.31–8.53
Alkalinity (mg $CaCO_3$ L^{-1})	140.94 ± 8.85	90–210	138.44 ± 6.24	70–210
Total water hardness (mg $CaCO_3$ L^{-1})	201.47 ± 9.77	137.06–256.32	190.82 ± 5.67	89–242.08
N-NO_2^- (mg L^{-1})	0.03 ± 0.01	0–0.13	0.04 ± 0.01	0–0.14
N-NO_3^- (mg L^{-1})	0.67 ± 0.10	0–1.21	0.60 ± 0.06	0–1.25
P-PO_4^{3-} (mg L^{-1})	0.02 ± 0.01	0–0.10	0.02 ± 0.01	0–0.11

than autumn and spring (Nemenyi post-hoc, $p < 0.05$).

COD levels were generally low during the entire study period (Table 1) with the exception of the N1 in December 2017, where COD reached almost 9 mg O_2 L^{-1}. Significant differences among sampling dates ($\chi2$ (7) = 31.18, $p < 0.001$) referred to differences between spring and autumn dates, with spring having generally higher COD values when compared to autumn dates (Nemenyi post-hoc, $p < 0.05$). As in the case of COD, alkalinity values were also relatively lower at revitalized sites. Significant temporal differences ($\chi2$ (7) = 37.94, $p < 0.001$) in alkalinity were observed among winter (December 2017), autumn (October 2017), spring (March 2018) and summer (June 2018) – spring and summer recorded significantly higher values than autumn and winter (Nemenyi post-hoc, $p < 0.05$).

Nitrite values were rather low during the whole study period, with the exception of autumn dates which measured significantly higher values when compared to other dates ($\chi2$ (7) = 43.64, $p < 0.001$; Nemenyi post-hoc, $p < 0.05$). Orthophosphate values followed a similar trend with significantly higher values in late summer and autumn when compared to other dates ($\chi2$ (7) = 46.15, $p < 0.001$; Nemenyi post-hoc, $p < 0.05$). Nitrate values displayed unclear trends, varying widely between sampling sites and dates. Spring sampling in March 2018 had significantly lower nitrate values when compared to other dates ($\chi2$ (7) = 39.81, $p < 0.05$; Nemenyi post-hoc, $p < 0.05$).

3.2. Periphyton: Diversity and abundance

A total of 60 phagotrophic protist and 14 micro-metazoan taxa were recorded in the collected periphyton samples (Table 2). The most diverse group among phagotrophic protists were Ciliophora (35 taxa), followed by Amebozoa (13 taxa) and Rhizaria (6 taxa) (Fig. 2). No significant differences concerning the number of taxa were detected between sampling sites (Friedman's test, $p > 0.05$) nor sampling dates ($p > 0.05$). However, some spatial trends were observed. On average, revitalized sites displayed a higher number of taxa, with the maximum of 35 taxa recorded at sites N2 and N4. In comparison to control sites, which supported 57% (42) of all recorded taxa, revitalized sites (when grouped together) supported 86% (64) of all recorded taxa.

The abundance of periphytic organisms did not differ significantly between sampling sites ($p > 0.05$), but it was evident that revitalized sites, especially N1, had higher abundance compared to control sites with average values almost three times higher. Concerning the sampling dates, a significantly higher abundance was observed in October 2017 than in January 2018 and March 2018 ($\chi2$ (7) = 20.76, $p < 0.05$; Nemenyi post-hoc, $p < 0.05$).

With respect to phagotrophic protists, ciliates comprised almost 60% of taxa (Fig. 2) and 30% of abundance at most sampling sites (Fig. 3). The exceptions were sites N1, N2 and N3, where ciliates were slightly outnumbered by testacean taxa, i.e. members of Rhizaria (Cercozoa) (Fig. 4A). 41% of the total recorded ciliates were omnivorous, while 36% fed on bacteria, selectively or together with other food sources such as diatoms or algae. Bacterivorous and omnivorous taxa dominated at both - control and revitalized - sites. The remaining 21% of taxa were predators such as *Litonotus* sp., *Lacrymaria olor* (Müller, 1786) Bory, 1824; *Loxophyllum meleagris* (Müller, 1773) Dujardin, 1841; *Acineria uncinata* Tucolesco, 1962 or algivores (2%).

The mean values of abundance for testacean taxa recorded at revitalized sites was twice as high as those observed at control sites. The abundance of phagotrophic protists reached its maximum in December 2017 (334 ind. cm^{-2}) at site N2, when Trinematide abundance peaked. The most common ciliate was *Cyclidium* sp. (15% of total phagotrophic protist taxa), while the most common cercozoan taxon was *Trinema* sp. (8% of total phagotrophic protist taxa).

With regards to micro-metazoan fauna, nematodes were the most abundant group, reaching a maximum of 302 ind. cm^{-2} at C2 in October 2017 (Fig. 2). In average, control sites had higher abundance of nematodes then revitalized sites, but supported lower number of micro-metazoan groups, with five taxa in total: Gastrotricha, Hydrachnidia, Nematoda, Oligochaeta and Rotifera. Unlike nematods, rotifers were more abundant at revitalized sites (Fig. 4B).

The abundance of periphytic taxa was significantly positively correlated with orthophosphate concentration while positive correlations with pH, nitrites and COD were not significant. A negative correlation between flow velocity and abundance was observed (Table 3). The ANOSIM for periphyton revealed significant differences between control and revitalized sites ($R = 0.202$, $p < 0.05$). Fitting the significant environmental variables ($p < 0.05$) into the NMDS analysis based on Bray-Curtis similarity (Fig. 5A), it was observed that greater abundance at revitalized sites correlated with increasing levels of temperature, alkalinity, nitrites, and orhophosphates, whereas abundance at control sites decreased with higher dissolved oxygen concentration (mainly during winter dates). Abundances of 5 taxa were found significantly correlated to the gradients of taxa assemblage composition represented by the NMDS space ($p < 0.01$; Fig. 5B). Samples from revitalized sites were correlated with greater abundances of Bdelloidea, Rotifera, Oligochaeta and phagotrophic protist taxa *Trinema* sp. Significant negative correlations with NMDS2 axis were observed for Nematoda which in fact were more abundant at control sites.

3.3. Organic and inorganic matter

Inorganic matter (i.e., tufa) content ranged from 15% (of dry sample) in autumn to a maximum of 100% in spring. The mean values of tufa content per season are shown in Fig. 6A. Friedman's test indicated no significant differences in tufa content among sampling sites ($p > 0.05$), but a trend towards lower values at revitalized sites was observed. Significant seasonal differences were recorded ($\chi2$ (7) = 36.81, $p < 0.001$) with December 2017 and January 2018 measuring significantly higher tufa content compared to spring (March 2018, May 2018) and autumn dates (October 2017).

Organic matter content in samples ranged from 1% in spring to a maximum of 100% of sample recorded in autumn. The mean seasonal values of organic matter in samples are shown in Fig. 6A. No significant differences were observed between sampling sites ($p > 0.05$), although revitalized sites had slightly higher amounts of organic matter (Fig. 6B). Sampling dates significantly differed concerning organic matter content ($\chi2$ (7) = 36.81, $p < 0.001$). Nemenyi post-hoc test revealed that, compared to March 2018 and October 2017, winter dates (i.e., December, January) accumulated significantly ($p < 0.05$) lower amounts of organic matter.

Tufa content was significantly positively correlated with flow velocity and significantly negatively correlated with temperature and nitrites (Table 3). Organic matter content showed significant positive correlations with temperature and nitrites, while it was significantly negatively correlated with flow velocity (Table 3).

3.4. Grain size and mineralogical analyses

Sediment samples were categorized in the following textural groups: gravelly muddy sand, muddy sandy gravel, sandy gravel and gravel. Gravel fractions in all samples were predominantly composed of broken, lithified tufa pieces. The highest percentages of gravel fractions were present in samples collected in autumn (November 2018) and winter (December 2017), with striking 96.1%. Accordingly, the ratios of sand and mud fractions were lowest in these two months. Sand and mud fractions were highest in spring (May) (sand 27.9–58.5%; mud 5.1–24.1%) and summer (June) (sand 20.2–78.5%; mud 5.6–21.4%). All samples were poorly sorted, and only a few collected in November and December were moderately and well sorted. The mean proportions of fractions are shown in Fig. 7. No significant differences were observed among sites or seasons. Calcite was the predominant mineral phase in all bulk samples and samples smaller than 0.063 mm. The predominant phase of an insoluble residual of all samples was quartz, with micas as an

Table 2
Periphyton taxa at all sampling sites expressed as mean number of individuals per cm^2 recorded during the entire study period (including all three replicates per site).

		Site						
		C		N				
Group	Taxa	C1	C2	N1	N2	N3	N4	N5
Amoebozoa								
	Arcella dentata Ehrenberg, 1830				47.7			
	Arcella sp.	45.7					59.3	
	Centropyxis sp.		45.3					
	Chaos sp.		43.8		231.5		31.7	48.5
	Difflugia acuminata Ehrenberg, 1838	144.4		153.8			81.1	49.7
	Difflugia sp.	42.8		57.1	85.1	31.8	41.8	54.6
	Filamoeba sp.	37.8	28.6	132.5			77.9	
	Mayorella sp.		28.6	88.1	42.3	37.0	41.8	44.9
	Polychaos annulatum (Penard) Smirnov & Goodkov, 1998		113.4					
	Polychaos sp.				53.7	41.8	34.6	35.8
	Thecamoeba sp.		43.8		77.2			
	Thecamoeba striata (Penard 1890) Schaeffer 1926	49.3			49.7		38.6	
	Vannella sp.			90.3		36.2	58.9	
Ciliophora								
	Acineria uncinata Tucolesco, 1962			44.2			41.8	41.4
	Aspidisca cicada O.F. Müller, 1786	35.8	89.1	67.3	40.7	38.9	43.0	70.7
	Aspidisca lynceus O.F. Müller, 1773	41.8	45.3	49.7	65.4		23.5	
	Cinetochilum margaritaceum Ehrenberg, 1838					43.8		
	Cyclidium sp.	69.6	79.3	44.9	49.7		102.6	40.6
	Euplotes affinis Dujardin, 1842						88.3	
	Euplotes sp.	43.6	40.6			23.9	81.1	
	Frontonia angusta Kahl, 1931							33.0
	Holosticha kessleri Wrześniowski, 1877							38.8
	Holosticha pullaster Müller, 1773				35.0		48.1	66.0
	Holosticha sp.		41.0		38.6	43.8	31.4	39.8
	Lacrymaria olor (Müller, 1786) Bory, 1824		47.7					
	Litonotus cygnus (O.F. Müller, 1776) Wrzesniowski, 1870			57.9	49.7		43.4	48.5
	Litonotus sp.	47.3		44.2				38.6
	Loxophyllum meleagris (Müller, 1773) Dujardin, 1841		19.5		25.5			
	Loxophyllum utricularie (Penard, 1922) Kahl, 1931			43.8				
	Oxytricha chlorelligera Kahl, 1932		55.7					33.0
	Oxytricha ferruginea Stein, 1859			46.5				
	Oxytricha sp.	44.4						
	Oxytrichidae non det.	40.8		44.2	65.9	37.7	40.6	52.2
	Stentor coeruleus Ehrenberg, 1830	99.4						
	Stentor multiformis (Müller 1786) Ehrenberg 1838			28.2				
	Stentor niger Müller, 1773			90.7				
	Stentor polymorphus (Müller 1773) Ehrenberg, 1830				49.7			
	Stentor sp.	49.3	48.5					
	Stylonychia mytilus (Müller, 1773) Ehrenberg, 1830	49.3						
	Stylonychia sp.				45.7			
	Trachelius ovum Ehrenberg, 1831						49.3	34.2
	Uroleptus musculus Kahl, 1932						37.4	
	Uroleptus sp.			46.1			41.4	
	Urosomoida agiliformis Foissner, 1982		19.5					
	Urostyla grandis Ehrenberg, 1830							48.5
	Urotricha sp.			28.2		47.7	37.4	
	Vorticella campanula Ehrenberg, 1831		48.5					
	Vorticella convallaria Linnaeus, 1758				49.7			
Dinoflagellata								
	Ceratium hirudinella (O.F.Müller) Dujardin 1841	60.1	83.5	43.8				47.3
Excavata								
	Vahlkampfia limax (Vahlkampf, 1905) Chatton & Lalung-Bonnaire, 1912		28.1	46.5	25.5		54.7	194.1
	Vahlkampfia sp.	39.6	103.1	42.4	36.8	43.4	41.6	36.7
Heliozoa								
	Acanthocystis sp.					37.4		
	Actinophyrs sol Ehrenberg, 1830	44.9		143.2	49.7	93.1	49.2	
	Actinospherium sp.				46.1	49.7		
Rhizaria								
	Chlamydophrys minor Belar, 1921							35.8
	Corythion dubium Taranek, 1871	37.8	44.4					
	Euglypha sp.	70.2	66.7	69.5	71.0	83.1	78.0	48.4
	Gromia sp.		188.9		35.0			
	Trinema lineare Penard, 1890	39.8		46.1	138.0	79.6		
	Trinema sp.	40.8	31.6	109.2	78.9	111.5	64.0	51.8
Crustacea								
	Copepoda				29.4			
Gastrotricha								
		48.5	23.5	45.3	41.8	93.1	53.4	39.4
Hydrachnidia								

(*continued on next page*)

Table 2 (*continued*)

		Site						
		C		N				
Group	Taxa	C1	C2	N1	N2	N3	N4	N5
				28.5	54.5			30.6
Insecta								
	Chironomidae			51.2	60.1	21.4	45.5	50.9
	Ephemeroptera							36.6
Nematoda								
		54.4	81.5	82.2	62.8	60.4	80.2	65.3
Oligochaeta								
		37.8						49.7
Rotifera								
	Bdelloidea non det.	40.8	47.2	65.6	69.8	57.7	48.2	61.4
	Cephalodella elegans Myers, 1924					47.7		
	Colurella uncinata Müller, 1773		37.8	53.0	88.7	118.7	44.6	51.4
	Dicranophorus sp.		28.6	35.8			41.2	
	Rotifera non det.	60.3			45.7		73.2	41.8
Tardigrada								
				45.3				
Turbellaria								
	Rhabdocoela				38.6			

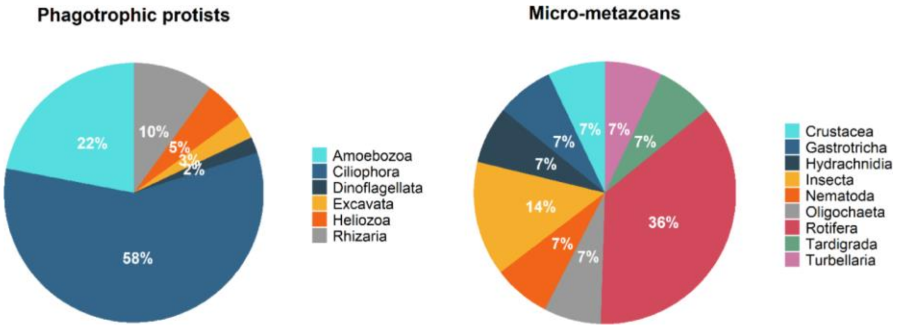

Fig. 2. Taxonomical diversity of periphytic phagotrophic protists and micro-metazoans during the study period. The relative proportions of individual taxa were calculated with regards to the total number of taxa observed during the entire study period (including all three replicates per site).

additional probable phase; however, due to the small amount present, this could not be determined with greater certainty.

4. Discussion

4.1. Environmental conditions

This study examined the differences in the in-stream environmental and periphyton community conditions between control (located within permanent streams) and revitalized (located within reactivated streams) sites following the removal of the invasive plant species *A. altissima*. While only measurements of total water hardness showed significant differences between control and revitalized sites, the remaining environmental parameters displayed interesting trends that reflected seasonality, but also effects of the landscape and hydrogeomorphological changes on the in-stream conditions within the experimental plot.

The Krka River exhibits several Mediterranean climate features, such as dry, hot summers and mild, rainy winters (Filipčić and Šegota, 2003; Bonacci et al., 2017). Measured water temperature values followed the usual local climate patterns with maximum values in summer and minimum values in winter. Relatively high oxygen values recorded during the entire study period are typical for karst rivers and in accordance with values obtained by Primc-Habdija et al. (2005) and Cukrov et al. (2008). The wide range of flow velocity during the study period can be attributed to the complex geomorphological features of the experiment plot, comprised of numerous tufa cascades, riffles, pools and dry tufa patches intersected with newly emerging streams, with a total surface of nearly 1 ha. Reactivated streams produced a strong water current, which typically weakened downstream. However, at the lowermost site (i.e., C2), peak flow velocity values were recorded during summer. This site represents an occasional waterfall and therefore acts as a barrier and generates a strong current, especially after heavy rains, such as those occurring in September 2018. Variations of flow velocity are quite common for karst rivers as previously reported by Pitois (2001) and Ridanović (1994), who occasionally recorded flow velocity and water level oscillations greater than 50 cm in the Plitvice Lakes hydrosystem (Croatia), which is hydrogeomorphologically similar to this study area.

Streams emerging following the removal of *A. altissima* generated a drastic change in the landscape, not just by creating an intersection of numerous water pathways, but also by draining the disturbed forest soil. These alterations affected most measured environmental parameters, at both the spatial and temporal scales. The higher nitrite and orthophosphate values observed in late summer and autumn (especially in autumn 2017) at revitalized sites can be associated with the soil drainage due to increased autumn rainfall and/or reduced oxygen levels that favour reduced ion forms (Sertić Perić et al., 2018). Regardless of the fact that dissolved nitrogen and phosphorus forms can be anthropogenic (WHO,

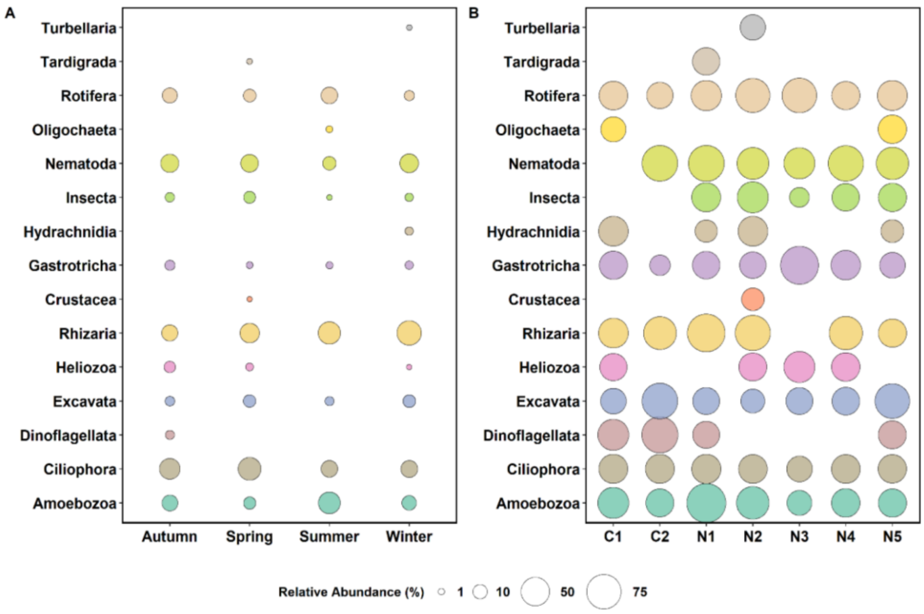

Fig. 3. Abundance of different phagotrophic protists and micro-metazoans over the entire study period: (A) seasonally; (B) across sampling sites. The proportions were calculated from abundance values recorded during the entire study period (including all three replicates per site).

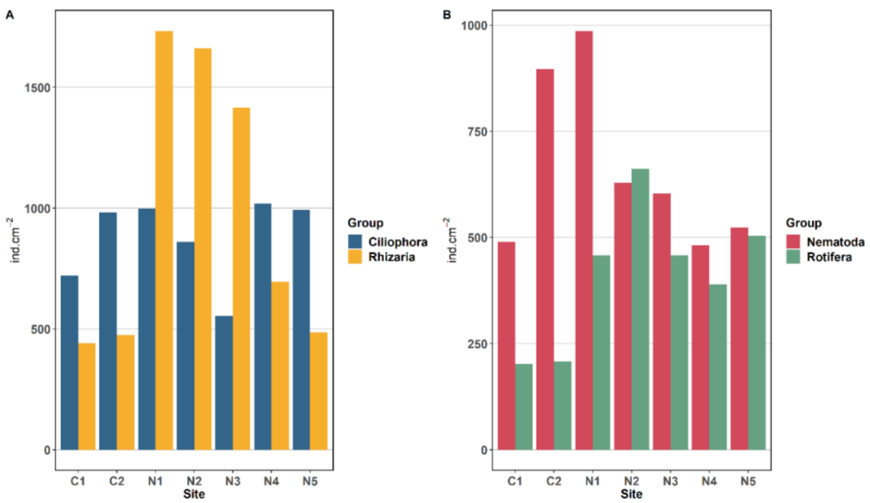

Fig. 4. Total abundance of main phagotrophic protist and micro-metazoan groups over the entire study period across sampling sites. The values were calculated from abundance values recorded during the entire study period (including all three replicates per site).

2004, 2011), in this case they most likely originate from river-born material (Meybeck, 1993) since only a small portion of the experimental plot is exposed to tourism activities (walking trails, viewpoints, rest areas). Despite the fact that nitrates likely represent the dominant form in the total nitrogen fluxes values (Sertić Perić et al., 2018), they displayed no clear pattern. Slightly higher nitrate values were observed after rainy periods, though without significant temporal differences. In comparison to values recorded by Primc-Habdija et al. (2005) in the same hydrosystem, we observed a slight increase in nutrient concentrations, though within the range of values characteristic for an

Table 3
Spearman's correlation coefficients (Rs) between the measured environmental and periphyton parameters. Values significant at $p < 0.05$ are in bold.

Variable pair		Rs	p
Flow velocity	Tufa	**0.595**	**<0.05**
	Organic matter	**−0.595**	**<0.05**
	No. taxa	−0.247	>0.05
	Abundance	−0.197	>0.05
pH	Tufa	−0.234	>0.05
	Organic matter	0.234	>0.05
	No. taxa	0.141	>0.05
	Abundance	0.207	>0.05
Orthophosphates	Tufa	0.100	>0.05
	Organic matter	−0.100	>0.05
	No. taxa	0.106	>0.05
	Abundance	**0.352**	**<0.05**
Temperature	Tufa	**−0.289**	**<0.05**
	Organic matter	**0.289**	**<0.05**
	No. taxa	0.046	>0.05
	Abundance	0.138	>0.05
Nitrites	Tufa	−0.345	**<0.05**
	Organic matter	**0.345**	**<0.05**
	No. Taxa	−0.033	>0.05
	Abundance	0.054	>0.05
COD	Tufa	−0.155	>0.05
	Organic matter	0.155	>0.05
	No. Taxa	0.107	>0.05
	Abundance	0.185	>0.05

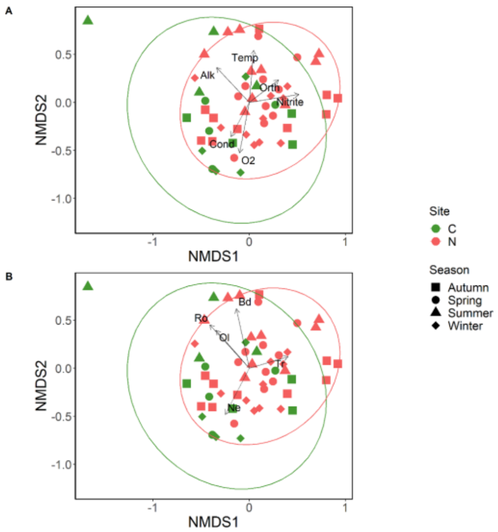

Fig. 5. Position of sampling sites in the nonmetric multidimensional scaling diagram based on Bray-Curtis similarity index for periphyton considering: (A) significant environmental parameters (Alk = alkalinity, Temp = temperature, Orth = orthophosphate, O_2 = dissolved oxygen concentration) and (B) significant taxa (Bd = Bdelloida, Ne = Nematoda, Ol = Oligochaeta, Ro = Rotifera, Tr = Trinema sp.); stress value = 0.21.

oligotrophic karst environment. Although significant differences were observed only for total water hardness, revitalized sites also had lower alkalinity, oxygen and pH values in comparison to control sites. This is likely due to a presence of soil-derived organic material containing a high amount of humic carbohydrates (Hadžija et al., 1985), which could lower these values. Accordingly, COD levels measured at revitalized sites were higher, indicating the possibility of intensive soil drainage.

4.2. Driving forces shaping periphyton assemblages and tufa deposition

In general, the number of phagotrophic protist and micro-metazoan taxa was low at all sites - in comparison to previous studies within another Croatian tufa-depositing hydrosystem (i.e. Plitvice Lakes National Park) (Matoničkin Kepčija et al., 2011; Gulin and Matoničkin Kepčija, 2012), where the number of periphytic taxa was twice as high as here. The reason for the low number of taxa in these samples may be due to the sampling methodology (corer), which could be too destructive for sampling delicate communities such as periphyton. In addition, homogenization by vigorous shaking may not have been strong enough to detach and suspend some organisms, as some phagotrophic protists tend to attach strongly to surfaces (Risse-Buhl et al., 2009). Artificial substrates seem to provide better insights into periphyton assemblages as they allow direct microscopic observation (Wu, 2016). However, in this study, artificial substrates might not have given such a good understanding of tufa and organic matter content as provided by natural substrate sampled by corer.

Regarding the observed seasonal periphyton patterns, the trend of slightly higher number of taxa observed in late summer and autumn could be related to the lower flow velocity and consequently lower tufa deposition. According to the Köppen classification, Krka National Park has a moderately warm Mediterranean rainy climate (Csa) with dry and hot summers, and most precipitation in winter months (Šiljeg et al., 2020). Thus, average monthly water levels in winter are almost two times higher than those in summer (Marguš, 2011) and followed by intensified water flow. Although an increase in water velocity enhances particle movement and nutrient uptake (Saravia et al., 2001), flow velocity over 0.5 ms^{-1} (Horner and Welch, 1981) or 0.6 ms^{-1} (Horner et al., 1990) causes sloughing. When accompanied by intensive calcite precipitation, this can negatively affect the periphyton community, detaching or burying it (Pitois, 2001; Pitois et al., 2003). The higher flow velocity in the autumn and winter months likely washed away finer particles, leaving only gravel fractions (lithified tufa pieces) in the sediment samples. Presumably, grain size also influenced the periphyton differences between the control and revitalized sites. Although no significant differences were found, gravel fractions were more prevalent in samples from revitalized sites (particularly at sites N2 and N3) and could have influenced the periphyton colonization process. Pentecost (2005) recorded a positive relationship between substrate size and the degree of colonization, as larger substrates were less likely to be displaced by flood events. However, the higher number of taxa at revitalized sites is more likey the result of abundant food resources originating from the surrounding forest soil. In fact, quartz and mica minerals from the insoluble residual of stream substrate likely indicate soil drainage, thereby supporting this idea. A positive, though insignificant correlation between the number of taxa and COD was most evident for the amebozoan and cercozoan protists, particularly the testate amoebas. The maximum number of testacean taxa recorded at revitalized sites was twice as high as at control sites, which might be due to the differences in the surrounding soil composition and type, and nutrient (nitrites, orthophosphates) availability among sites. Thus, it is likely that the slightly higher COD, nitrite and orthophosphate values accompanied by slightly reduced dissolved oxygen concentrations enhanced algal and microbial community growth at revitalized sites, supporting higher number of taxa and abundance of testate amoebae, which are mostly phagotrophic organisms feeding on bacteria, algae and fungi (Todorov and Bankov, 2019). Another point to be considered is the importance of substrate moisture and pH, both identified as major abiotic predictors shaping microbial communities, especially cercozoans (Fiore-Donno et al., 2019). The record abundance of Euglyphidae observed at revitalized sites (maximum 334.13 ind cm^{-2}) is in accordance with the results obtained by Ehrmann et al. (2012) and Fiore-Donno et al. (2019), suggesting the preference of Euglyphidae towards slightly acidic soils, thus possibly explaining their colonization in the soil-like conditions present in the reactivated streams. The effect of soil drainage present at

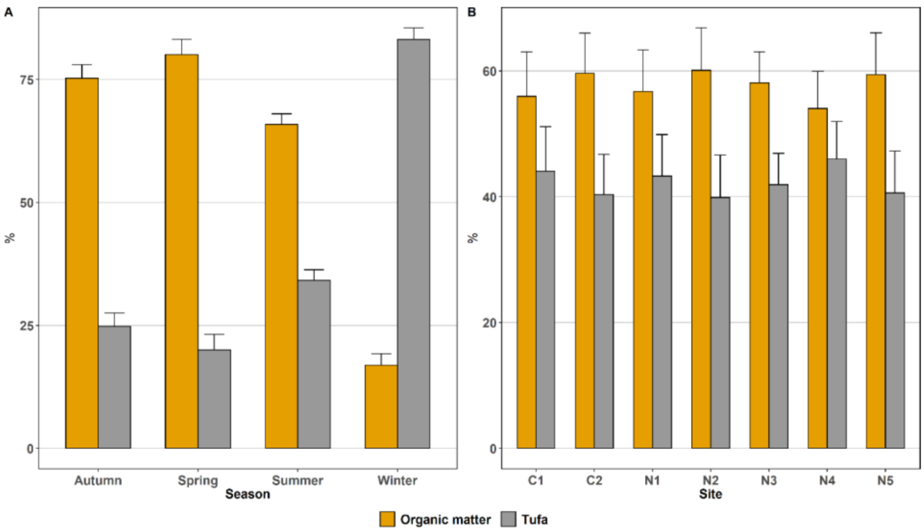

Fig. 6. Mean (±SE) tufa and organic matter content in samples: (A) over different seasons during the study period; (B) across control (C) and revitalized (N) sites within the experimental plot; Description of the sampling site codes is given in Methods.

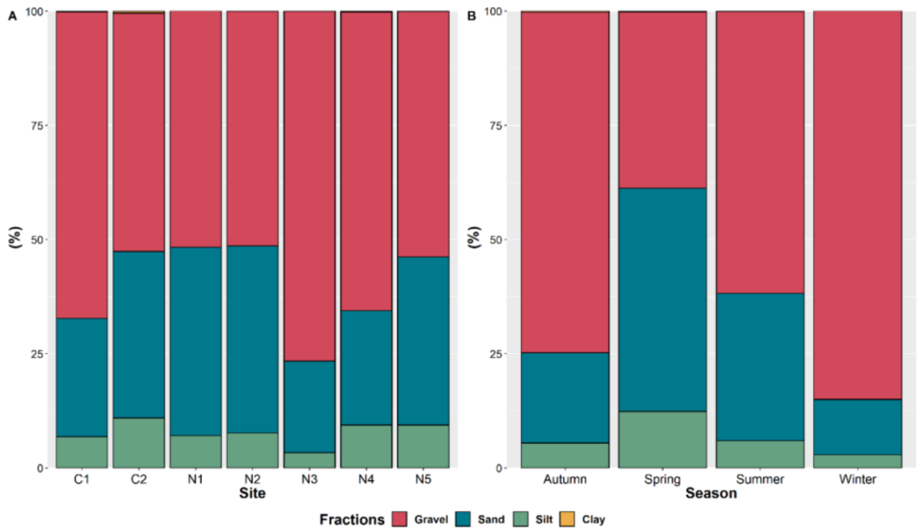

Fig. 7. Mean proportions of gravel fractions in samples with respect to sites (A) and seasons (B).

revitalized sites is also reflected in the composition and abundance of ciliate communities, dominated by bacteriovorous and omnivorous ciliate taxa such as scuticociliate *Cyclidium* sp., hypotrichs *Uroleptus* sp. and species from Oxytrichidae family but also predator species *A. uncinata* which feeds on heterotrophic flagellates and bacteria (unlike predator taxa occurring at control sites which feed predominantly on ciliates and smaller micro-metazoans) (Foissner et al., 1995; Foissner and Berger, 1996). On the other hand, control sites supported a striking number of nematodes, most likely due to the moss cover that provides shelter and has been recognized as a hot-spot for nematode abundance and diversity (Barbuto and Zullini, 2006). Nematode activity can influence periphyton structure through bioturbation and grazing (Farmer, 1992; Traunspurger, 2000) or even cause periphyton detachment (Gaudes et al., 2006), possibly leading to lower taxa number at control sites. Mostly, the observed micro-metazoan community was comprised of taxa frequently found in the permanent periphytic meiofauna (Kreuzinger-Janik et al., 2015). Meiofauna representatives, including rotifers and nematodes, are common at tufa barriers, often in high numbers (Dražina et al., 2013) as they exhibit a wide range of feeding strategies allowing them to exploit the diverse resources available in periphyton (Schroeder et al., 2013). These small meiofauna organisms often represent food for larger macroinvertebrates, such as larval caddisflies and stoneflies (Beier et al., 2004). However, members of the insect fauna were detected only at revitalized sites and were completely absent from control sites, likely due to a scarcity of food resources. Unlike the rather diverse meiofauna taxa recorded in periphyton samples, micro-crustaceans (exclusively copepods) at the revitalized site N2 likely appeared with seston from the upstream Visovac Lake, which can be a source of planktonic organisms along lotic stretches in the study area (Špoljar et al., 2005).

A strong negative correlation between tufa and temperature was observed in this study, with significantly higher values of deposited tufa in winter. Temperature is considered to be one of the crucial factors in the tufa deposition process, as it influences the chemical reaction rate (Zhang et al., 2001) and intensifies deposition rates during summer and autumn (Arp et al., 2001). Lower water temperatures might reduce tufa deposition (Drysdale et al., 2003). For instance, calcite deposition was almost negligible in the Plitvice Lakes hydrosystem during the winter, when mean temperatures were around 4 °C (Matoničkin Kepčija et al., 2005). However, some authors (e.g., Pedley and Rogerson, 2010) disregard the temperature role, and emphasize flow velocity as the major force driving tufa precipitation processes. Thus, we suggest that the higher tufa deposition observed during cooler seasons (e.g., autumn and winter) is mostly driven by faster currents that significantly enhance the CO_2 degassing process and calcite precipitation (cf. Zhang et al., 2001). Fast water currents might also explain the significantly lower organic matter content recorded during winter, as they could have affected the sedimentation rate and organic matter retention (Habdija et al., 2004). Another possible explanation relies on a combination of flow velocity and microbial community, i.e., in fast currents, higher deposition is driven by the degassing process, while in the slow currents, biofilm (periphyton) calcification plays the dominant role (Merz-Preiß and Riding, 1999). For this reason, precipitation at revitalized sites (especially N1), which exhibited the highest periphyton abundance, would be more affected by the community structure itself than by the water current. Still, significantly lower amount of tufa observed at revitalized sites is most likely the result of organic matter presence, particularly humic acids drained from the surrounding, forest soils. Pentecost (2005) reported that chemical analyses of wet tufa samples reveal that tufa often contains organic matter and mineral grains of allochthonous origin, most often from the surrounding soil or bedrock. However, the presence of simple organic compounds and humic substances (i.e., fulvic acid, humic acid and humin) can also cause carbonate growth inhibition (Inskeep and Bloom, 1986; Hoch et al., 2000; Reddy, 2012). These natural organic materials generally represent dissolved organic material (DOM), commonly found in terrestrial and aquatic environments (Aiken et al., 1985). Thus, higher COD values observed at revitalized sites might indicate the presence of humic substances from the surrounding soil that can cause reduced oxygen concentrations (Aoki et al., 2007). Higher tufa deposition at control sites could also be due to the dense moss cover (comprised of *Palustriella commutata*, *Rhynchostegium riparium* and *Agrostis stolonifera;* A. Alegro, unpublished data) occurring at these sites over the tufa rocks. Aquatic mosses are well-known tufa-forming organisms; they thrive in rapids, where intensive gas exchange occurs, enhancing calcite deposition and subsequent moss encrustation (Golubić et al., 2008). During this study, reactivated streams were not colonised by mosses (personal observation), likely because they require a longer time period for colonization.

5. Conclusion

Although the expected differences between control (C) and revitalized (N) sites for most environmental parameters were not significant, clear patterns were observed between the C and N sites, indicating substantial changes in the landscape after the removal of invasive species. Revitalized sites had lower values of flow velocity, conductivity, pH, alkalinity and total water hardness while exhibiting higher values of COD, nitrites and orthophosphates due to the presence of soil-derived organic material and intensive soil drainage. Organic matter content was generally higher at revitalized sites, but it did not inhibit tufa deposition, which was more intense at control sites probably due to higher flow velocity. The influence of the surrounding forest soil was reflected not only in the mineralogical analysis of tufa sediment, but also in the abundance and diversity of periphyton, which were higher at revitalized sites. It was likely due to the abundant food resources provided by the soil surrounding the revitalized sites. Although expected, strong fluctuations in flow velocity and lower microhabitat heterogeneity at revitalized sites did not hinder periphyton colonization process. Results of the present study indicate that ecosystem responses to hydromorphological changes generated by invasive species removal can be efficiently detected and monitored at the microscale (i.e., periphyton scale). The microscale- (periphyton-) research approach is likely to impose minimal disturbance in sensitive ecosystems, such as fragile tufa depositing hydrosystems. Considering significant responses of phagotrophic protists (particulary ciliates and cercozoans) to environmental conditions, we suggest that they should be included in further monitoring programmes of tufa-forming rivers and streams, especially in fast-changing, soil-engaging systems such as reactivated, intermittent and/or ephemeral channels and streams. Anticipating that only an interdisciplinary approach can shed considerable light on the complex and delicate process of tufa formation, we propose that the microscale- (periphyton-) approach should be included in tailoring sustainable management scenarios for karst tufa-precipitating aquatic habitats, including reactivated streams at tufa barriers.

CRediT authorship contribution statement

Vesna Gulin: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Renata Matoničkin Kepčija:** Conceptualization, Resources, Supervision, Funding acquisition, Writing - review & editing. **Mirela Sertić Perić:** Methodology, Investigation, Writing - review & editing. **Igor Felja:** Investigation, Writing - review & editing. **Hana Fajković:** Investigation, Writing - review & editing. **Kristina Křižnjak:** Investigation.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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Supplementary Table 1

Site	Date	Temperature	O2	Flow velocity	Conductivity	pH	COD	Alkalinity	Total water hardness	Nitrite	Nitrate	Orthophosphate
C1	30.10.2017.	15,1	9,82	0,07		8,02	1,3357478	130	218,94	0,0928	0,823529412	0,016853933
C1	4.12.2017.	10,1	11,11	1,48		7,89	1,4143212	130	213,6	0	0,552941176	0,019101124
C1	29.1.2018.	10,2	11,09	1,41	554	7,85	0,4714404	145	170,88	0,012	1,182352941	0
C1	27.3.2018.	11,2	10,57	0,4	505	8,06	0,785734	115	137,06	0,012	0	0
C1	14.5.2018.	18,1	9,53	0,85	543	8,05	0,3142936	210	145,96	0,0264	1,1	0,013483146
C1	26.6.2018.	20,6	9,02	0,41	388	8,42	1,2571744	180	183,34	0,0184	0,252941176	0
C1	5.9.2018.	23,8	8,43	0,5	382	8,26	1,4143212	145	192,24	0,02	1,058823529	0,071910112
C1	5.11.2018.	16,7	9,47	0,95	369	8,25	2,7893557	110	220,72	0,1328	0,352941176	0,042134831
C2	30.10.2017.	15,5	9,89	0,19		8,2	1,8071882	145	256,32	0,0816	1,088235294	0,011235955
C2	4.12.2017.	9,9	11,26	1,18		7,92	0,785734	95	247,42	0	0,535294118	0,045505618
C2	29.1.2018.	10,1	11,12	0,6	530	7,81	0,6285872	110	161,98	0,0064	0,894117647	0
C2	27.3.2018.	10,5	10,77	0,7	505	7,89	2,4357754	90	153,08	0,012	0	0
C2	14.5.2018.	19,4	9,04	0,26	515	9,04	0,785734	190	215,38	0,0136	0,982352941	0,009550562
C2	26.6.2018.	20,9	8,94	0,63	366	8,42	1,1393143	190	252,76	0,0048	0,4	0
C2	5.9.2018.	23,4	8,46	1,66	370	8,21	1,7286148	130	213,6	0,0392	1,211764706	0,098876404
C2	5.11.2018.	16,8	9,42	0,18	354	8,48	2,5143488	140	240,3	0,0616	0,329411765	0,007303371
N1	30.10.2017.	15,2	9,84	0,02		8,5	1,964335	125	242,08	0,0704	0,435294118	0,034269663
N1	4.12.2017.	9,9	11,22	0,69		7,91	8,5252139	125	238,52	0	0,517647059	0,050561798
N1	29.1.2018.	10,3	11,11	0,42	516	8,01	0,392867	120	163,76	0,0112	1,029411765	0
N1	27.3.2018.	11,6	10,78	0,35	513	8,01	1,7286148	95	147,74	0,0008	0	0
N1	14.5.2018.	18,6	9,22	0,38	533	8,14	0,392867	200	192,24	0,0216	0,958823529	0,008988764
N1	26.6.2018.	20,7	8,94	0,69	375	8,47	1,3357478	185	206,48	0,0056	0,411764706	0
N1	5.9.2018.	24,05	8,32	0,55	375	8,31	2,9857892	155	179,78	0,0288	1,035294118	0,108988764
N1	5.11.2018.	16,7	9,46	0,48	367	9,02	2,6714956	90	229,62	0,1384	0,370588235	0,082022472
N2	30.10.2017.	15,1	9,9	0,35		7,99	2,5143488	175	224,28	0,1424	1,252941176	0,003932584
N2	4.12.2017.	10	11,15	0,61		7,93	0,7071606	100	227,84	0	0,064705882	0,029775281
N2	29.1.2018.	10,5	11,09	0,42	521	7,96	0,8643074	145	186,9	0,012	1,223529412	0
N2	27.3.2018.	11,4	10,89	0,4	502	8,04	2,4357754	70	153,08	0,0104	0	0
N2	14.5.2018.	19	9,16	0,4	531	7,66	0,392867	195	211,82	0,02	1,017647059	0,011235955
N2	26.6.2018.	20,8	8,91	0,53	383	8,39	1,3750345	180	234,96	0,0088	0,370588235	0
N2	5.9.2018.	24,05	8,28	0,33	374	8,23	1,1000276	175	186,9	0,1024	0,811764706	0,107303371
N2	5.11.2018.	16,5	9,33	0,95	366	8,21	2,6322089	125	222,5	0,1312	0,3	0,048876404
N3	30.10.2017.	15	9,97	0,24		8,06	0,9428808	150	222,5	0,0912	0,511764706	0,002808989
N3	4.12.2017.	10	11,14	1,17		7,83	0,6285872	105	215,38	0	0,311764706	0,032022472
N3	29.1.2018.	10,8	10,8	0,47	533	7,97	0,9428808	112,5	158,42	0,008	0,894117647	0
N3	27.3.2018.	10,9	10,76	0,21	513	7,35	1,1000276	115	172,66	0,0088	0	0
N3	14.5.2018.	20,8	8,59	1,21	530	8,06	0,7071606	195	183,34	0,0176	1	0,01011236
N3	26.6.2018.	20,5	8,97	0,19	383	8,46	1,4143212	175	178	0,0088	0,370588235	0
N3	5.9.2018.	23,7	8,28	0,45	378	8,13	1,4143212	125	181,56	0,0568	1,182352941	0,099438202
N3	5.11.2018.	18,4	9,12	1,09	362	8,22	2,750069	100	211,82	0,1352	0,288235294	0,010674157
N4	30.10.2017.	15,8	9,96	0,03		8,03	1,4928946	150	236,74	0,0416	0,705882353	0,006179775
N4	4.12.2017.	10,1	11,17	1,24		7,84	0,6285872	120	233,18	0	0,752941176	0,025280899
N4	29.1.2018.	10,3	11,11	1,19	515	7,92	0,8643074	115	160,2	0,012	1,064705882	0
N4	27.3.2018.	10,6	10,88	0,24	513	8,06	1,0214542	75	142,4	0,0136	0	0
N4	14.5.2018.	19,5	8,99	1,36	522	7,96	0,4714404	200	183,34	0,016	0,935294118	0,008426966
N4	26.6.2018.	20,5	8,98	0,64	378	8,47	1,1393143	190	190,46	0,004	0,382352941	0
N4	5.9.2018.	23,4	8,38	0,66	374	8,14	3,3000828	125	165,54	0,0744	1,052941176	0,1
N4	5.11.2018.	18,1	9,29	1,07	360	8,22	2,5929222	80	227,84	0,1432	0,335294118	0,02247191
N5	30.10.2017.	15,2	10,12	0		8,1	2,0429084	130	149,52	0,032	0,329411765	0,023033708
N5	4.12.2017.	10,2	11,11	1,04		7,73	1,3750345	115	197,58	0	0,382352941	0,035393258
N5	29.1.2018.	10,5	11,04	0,39	512	7,98	0,5107271	135	176,22	0,0352	0,958823529	0
N5	27.3.2018.	12,2	10,35	0,15	505	7,78	1,178601	120	89	0,0104	0	0
N5	14.5.2018.	19,2	9,08	1,07	535	8,03	0,3142936	205	169,1	0,024	1,070588235	0,011797753
N5	26.6.2018.	20,5	8,98	0,65	384	8,46	2,7893557	210	112,14	0,0168	0,358823529	0
N5	5.9.2018.	24,15	8,28	0,98	376	8,31	1,2571744	105	197,58	0,052	1,105882353	0,073033708
N5	5.11.2018.	18,5	9,17	0,7	357	8,19	2,4357754	125	229,62	0,144	0,341176471	0,021910112

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Taxonomic and Functional Metrics of Ciliates and Amoeboid Protists in Response to Stream Revitalization

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Tufa-depositing streams provide great microhabitat complexity and are therefore inhabited by various periphytic phagotrophic organisms such as ciliates and amoeboid protists. Recent removal of invasive plant species *Ailanthus altissima* (Mill.) Swinge from the Skradinski buk tufa barrier (Krka National Park, Croatia) resulted in changes in the barrier hydromorphology including the reactivation (revitalization) of dry streams. The objective of this study was to investigate: (1) the taxonomic and functional response of periphytic ciliates and amoeboid protists to stream revitalization by comparing taxonomic (i.e., abundance, species richness and diversity) and functional (i.e., functional diversity) metrics between revitalized (N) and control sites (C) during 1 and 2-months immersion period; (2) which environmental and (3) periphyton-associated factors shape the taxonomic and functional metrics and to what extent; (4) how duration of immersion affects taxonomic and functional metrics at revitalized sites. Our results showed that taxonomic and functional metrics of ciliates and amoeboid protists responded to the prevailing conditions characteristic of revitalized tufa-depositing streams: changing hydrology (occasional high flow or drought), soil drainage, and extensive inorganic matter, i.e., tufa deposition, although their responses were somewhat different. The two assemblages also showed different responses of taxonomic and functional metrics with respect to immersion duration: while the taxonomic and functional diversity of ciliates at N sites increased with longer immersion, indicating niche diversification, those of amoeboid protists hardly changed with time. Our results suggest that a comprehensive analysis of taxonomic and functional metrics of ciliates and amoeboid protists could be a good proxy for assessing revitalization of tufa-depositing streams. However, the temporal component should always be considered when conducting such studies, as the colonization processes of ciliates and amoeboid protists are quite complex, especially in tufa-depositing streams.

Keywords: eukaryotic, single-celled, freshwater, soil, phagotrophic, functional traits, protozoa

INTRODUCTION

Protists are the most diverse and widespread eukaryotes (de Vargas et al., 2015; Adl et al., 2019; Sieber et al., 2020). They perform important ecosystem functions and play a fundamental part in aquatic food webs (Weisse et al., 2016). Phagotrophic protists (those that utilize food through phagocytosis) have recently been shown to be even more diverse than their phototrophic counterparts (Singer et al., 2021). As the most important microbial predators in aquatic and soil ecosystems, phagotrophic protists transfer carbon and energy between micro- and macrofauna, significantly influencing ecosystem-level processes (Sherr and Sherr, 1984, 2002; Risse-Buhl et al., 2015; Geisen et al., 2017).

Due to their ubiquity, abundance and sensitivity, phagotrophic protists have been recognized as excellent bioindicators in a variety of environments (Payne, 2013). Ciliates (Ciliophora) and testate amoebae (Amoebozoa and Rhizaria) are very common groups of phagotrophic protists in freshwater and soil ecosystems (Singer et al., 2021). They are also the best-studied protists, partly because they are easy to isolate and have a distinctive morphology (Foissner, 1999; Todorov and Bankov, 2019). Naked amoebae, on the other hand, are understudied due to their smaller size and a lack of knowledge on their morphological features, although they dominate many protist communities, particularly in periphyton and benthos, as well as in soil (Coleman and Wall, 2015; Geisen, 2016; Singer et al., 2021).

Different protist groups may play opposing or complementary roles in ecosystem functioning. Thus, analyzing their functional diversity (FD) may enhance our understanding of various protist traits and their role in the ecosystem (Dumack et al., 2020). Functional diversity analysis is based on linking ecosystem processes to species' functional traits, i.e., organisms' characteristics that influence not only their fitness, but also their interactions with the environment (Nock et al., 2016). Trait-based approaches offer an opportunity to understand the relationship between microbial diversity and ecosystem functioning better than taxonomy-based approaches, especially since taxonomic and functional diversity are not always linearly correlated (Laliberté and Legendre, 2010). However, the trait-based approach has rarely been applied to protists (Weisse, 2017; Fiore-Donno et al., 2019).

Phagotrophic protists have already been recognized as good bioindicators of the karst environments' (e.g., tufa-depositing lakes, rivers, and streams) quality (Primc-Habdija et al., 2001; Matoničkin Kepčija et al., 2011; Kulaš et al., 2021). A recent study by Gulin et al. (2021) showed that periphyton responds to revitalization of dry tufa-depositing streams, opening new possibilities for the application of periphytic organisms as proxies of the stream revitalization success. The study focused on evaluating the response of periphyton based on sampling natural substrates, i.e., stream substrate containing tufa particles, which allowed analysis not only of the abundance and diversity of periphytic taxa, but also of the granulometric and mineral constituents of the substrate itself. However, the method of sampling with a corer proved too aggressive for periphytic organisms, resulting in low abundance and diversity of periphyton. In contrast, the present study is an improvement, because it is based on evaluating the response by monitoring periphyton development on artificial substrates over time so that the periphyton can be studied directly without damaging it. It focuses on phagotrophic protists (ciliates and amoeboid protists) and analyses both their taxonomic and functional diversity. Since periphyton exhibits complex spatio-temporal colonization trends (Franco et al., 1998; Risse-Buhl and Küsel, 2009), 1 and 2-months immersion periods were covered.

Our first objective was to assess whether taxonomic (abundance, species richness and diversity) and functional (i.e., functional diversity) metrics of ciliates and amoeboid protists differed between control (C) sites (located within permanent streams) and revitalized (N) sites (located within reactivated streams). We expected higher abundance and diversity (taxonomic and functional) of ciliates and amoeboid protists at revitalized sites than at control sites. This was based on the fact that conditions that prevail at reactivated streams due to newly created waterways and soil drainage, represent a positive disturbance, which can improve the diversity of habitats, species and their survival strategies (Timoner et al., 2014). A better understanding of the effects of disturbance on functional diversity is critical because functional diversity is likely related to ecosystem resilience, i.e., the capacity of a system to absorb shocks, reorganize, and maintain the same structure and function (Walker, 1995).

The second objective was to determine the influence of several environmental factors (flow velocity, temperature, dissolved oxygen concentration, pH, conductivity, alkalinity, total water hardness, concentrations of nitrites, nitrates and orthophosphates, and chemical oxygen demand) on the taxonomic and functional metrics of ciliates and amoeboid protists. Some of these factors have already shown a significant effect on the assemblage metrics of periphytic taxa in revitalized tufa streams (Gulin et al., 2021). Based on previous findings, we expected several organic matter associated factors (e.g., chemical oxygen demand, orthophosphates) to increase the abundance and taxonomic diversity of phagotrophic protists while others (e.g., nitrites) were expected to yield the opposite (decreasing) effect (Gulin et al., 2021).

Our third objective was to estimate how periphyton-associated factors (organic and inorganic matter (tufa) content, chlorophyll *a* concentration) affect the taxonomic and functional metrics of ciliates and amoeboid protists. Based on the findings of Gulin et al. (2021), we expected that increased organic matter content (and subsequently chlorophyll *a* concentration) would lead to increased abundance of protists. On the other hand, we expected that increased inorganic matter (i.e., tufa) content would decrease the taxonomic and functional diversity of protists as intense tufa deposition can lead to detachment and sloughing (Pitois et al., 2001, 2003).

Finally, our fourth objective was to examine the extent to which duration of immersion in water influences changes in taxonomic and functional metrics of ciliates and amoeboid protists at revitalized sites. We expected to observe a trend of increasing values of taxonomic and functional metrics with longer immersion duration, for both ciliates and amoeboid protists from revitalized sites.

The links between ecosystem functioning and the taxonomic and functional diversity of phagotrophic protists could be fundamental to resource management and conservation planning, particularly in protected and sensitive areas such as national parks. The results of this research would make an important contribution to understanding fragile and complex tufa-depositing ecosystems.

MATERIALS AND METHODS

Study Area

The study was conducted on a 1 ha experimental plot at the Skradinski buk tufa barrier-the longest and final tufa barrier in the watercourse of the Krka River, a karst river in the Dinaric region of Croatia, protected as a national park since 1985. Following an extensive aerial survey (Phantom 4) and detailed vegetation mapping, which determined that the invasive plant *Ailanthus altissima* (Mill.) Swinge (tree of heaven) dominated the area and was causing the dryness of the barrier due to its strong root system, individual specimens of the invasive tree were mechanically removed in August 2017 with permission from the Croatian Agency for Environment and Nature. Within 2 months of the removal, five streams in the experimental plot, that had previously dried up completely, were reactivated. The sampling design included seven sampling sites selected after removal of an invasive plant species: two control sites (C) in permanent streams where water was present before and after plant removal, C1 (15.966381, 43.805752)-control site where water had been present before and after the removal of *A. altissima* and displaying well-developed moss cover; C2 (15.966279, 43.805772)-control site without moss cover and five revitalized (N) sites: N1 (15.965449, 43.806438), N2 (15.965324, 43.806624), N3 (15.965246, 43.806541), N4 (15.965186, 43.806489), N5 (15.965538, 43.806225), representing newly reactivated streams (**Figure 1**).

Sampling Design and Identification

On May 10, 2019, 14 3D-printed Plexiglass carriers were placed, two per each sampling site, each containing three glass slides (7.6 × 2.6 cm) to serve as artificial substrates for periphyton development (**Figure 1**). Prior to immersion into the stream, the slides were washed in detergent, 1 M hydrochloric acid and distilled water. The total effective surface area of each glass slide was 17.18 cm^2 because it was partially covered by Plexiglass. Sampling was designed to immerse slides in the water for a period of 1 or 2 months, with three slides collected per sampling event at each site. Due to the high seasonality of the Krka River (Schöll et al., 2012), four sampling events were chosen for each immersion period, covering four different seasons. The dates on which slides were placed in the water and collected for the 1-month period were May 10–June 10, 2019 (spring), June 10–July 15, 2019 (summer), September 30–November 3, 2019 (autumn), February 2–March 8, 2020 (winter). The dates on which the slides were immersed for the 2-months period were placed and collected were June 10–August 15, 2019 (summer), September 30–December 2, 2019 (autumn), December 2, 2019–February 2, 2020 (winter), March 8–June 5, 2020 (spring, immersion period prolonged due to COVID-19 pandemic). A total of 168 slides were placed, 84 for the 1-month immersion period and another 84 for the 2-months immersion period. However, due to unpredictable events such as disturbance by visitors (sampling sites were located in close proximity to visitor trails and probably aroused curiosity) and seasonal drying of streams, only 55 slides were collected for the 1-month immersion period and 52 for the 2-months immersion period. More slides were collected from N sites due to the higher number of sampling sites in the first place. After collection, the slides were transferred to plastic containers filled with a small amount of ambient water and stored at 4°C in the dark. They were then examined directly under the microscope using Zeiss Axioimager A2 with DIC objectives and Axiocam 305 digital camera, within 24 h of sampling, except for few slides that were examined within 48 h. Ciliates and amoeboid protists were identified at species level using Zen 2.4 imaging software and relevant literature (Kahl, 1930; Page, 1976, 1991; Foissner et al., 1991, 1992, 1994, 1995; Foissner and Berger, 1996; Smirnov and Brown, 2004; Smirnov et al., 2011; Todorov and Bankov, 2019). For each taxon, 10–15 photomicrographs were taken and subjected to morphometric measurement (**Figure 2**). Additional video clips were used to record movements and distinguishing features.

Environmental Factors

The following environmental factors were measured at each sampling site using the respective portable field meters: temperature (T) and dissolved oxygen concentration (DO) (oximeter OXI 96, WTW GmbH, Weilheim, Germany), pH (pH meter 330i, WTW GmbH, Weilheim, Germany), conductivity (Cond) (conductometer Sension 5, Hach, Loveland, Colorado, United States), and flow velocity (FV) (flow velocity meter P600, Dostmann electronic GmbH, Wertheim- Reicholzheim, Germany). An additional 1 L water sample was collected and stored at 4°C for subsequent laboratory analysis of the water. This included the analysis of alkalinity (Alk), total water hardness (TWH), concentrations of nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$) and orthophosphate ($P\text{-}PO_4^{3-}$) (according to APHA, 1985), and total chemical oxygen demand (COD) using the standardized acidic potassium permanganate titrimetric method (Deutsches Institut für Normung, 1986).

Periphyton-Associated Factors (Organic/Inorganic Matter Content and Chlorophyll *a* Concentration)

After microscopic examination, periphyton from each slide was divided into two equal parts-one was used to determine the organic/inorganic content and the other was scraped to measure the chlorophyll *a* concentration (a proxy of primary productivity) (Falkowski and Raven, 1997). For the determination of organic matter content as ash-free dry mass, the samples were dried at 104°C to constant weight, then ashed at 400°C for 4 h and reweighed. The mass difference

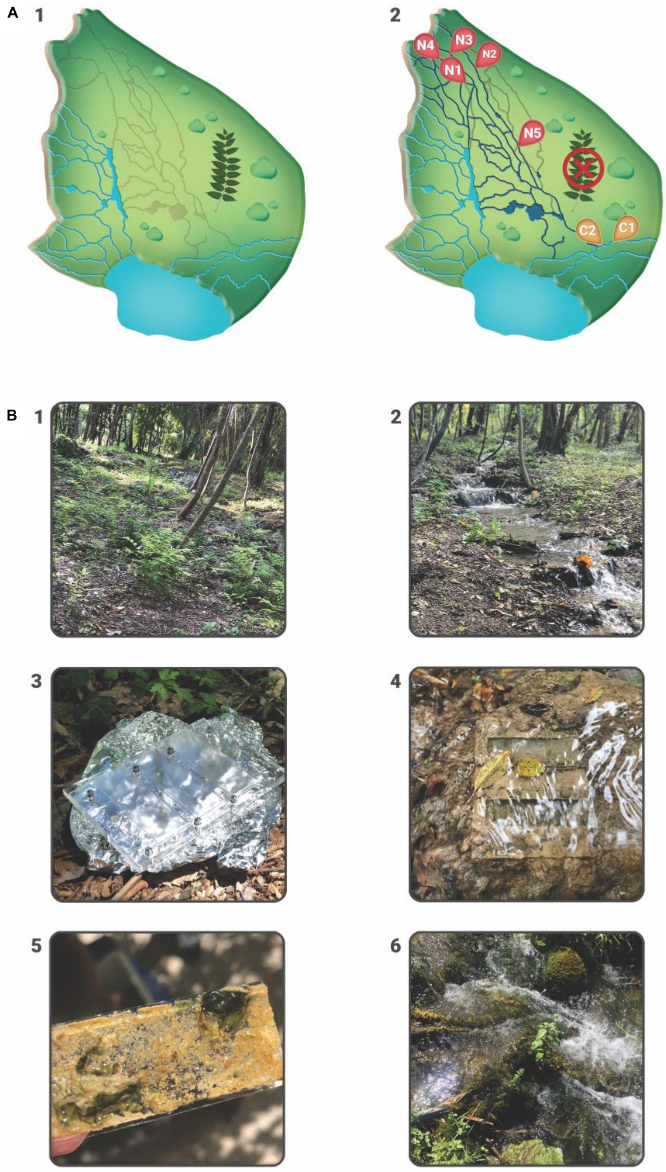

FIGURE 1 | (A) Experimental plot at Skradinski buk barrier with graphical presentation before and after the removal of *A. altissima*. Gray lines in A1 represent dry streams which were revitalized upon invasive plant removal. A2 shows seven sampling sites: two control sites located within permanent streams, C1 and C2, and five revitalized sites N1–N5. **(B)** Details from the experimental plot: 1—dry streams overgrown with *A. altissima*, 2—revitalized streams upon the removal of *A. altissima*, 3—3D printed Plexiglass carriers, 4—Plexiglass carriers with glass slides placed in stream bed, 5—a detail of a glass slide from N site with deposited inorganic matter, i.e., tufa, 6—well-developed moss cover from C1 site.

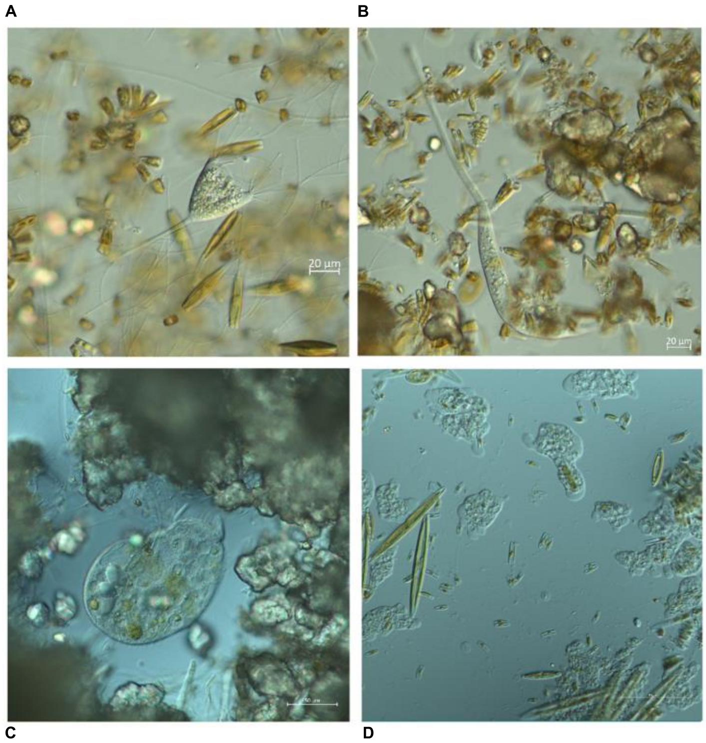

FIGURE 2 | Photomicrographs of different organisms observed during the study period. The organisms were examined directly under the microscope using Zeiss Axioimager A2 with DIC objectives and captured using Axiocam 305 digital camera. **(A)** Ciliate *Acineta* sp., **(B)** ciliate *Lacrymaria* sp., **(C)** ciliate *Stentor* sp., **(D)** various naked amoebas.

between the dried sample and the ashed sample was used to express the amount of organic matter while the mass difference between the ashed sample and the glass slide was used to express the amount of inorganic matter, i.e., deposited tufa. Values were expressed as the mass of organic/inorganic matter (mg) content per cm^2 surface area. Chlorophyll *a* concentration was determined by the ethanol extraction method (Nusch, 1980). Values were expressed as mass of chlorophyll *a* (µg) per 8.59 cm^2 surface area.

Statistical Analyses

Data Exploration and Visualization

The data were summarized and displayed using standard statistical measures (mean and standard deviation) and graphically presented to illustrate possible trends using ggplot2 package v. 3.3.5 (Wickham, 2016) in R v. 4.1.1 (R Core Team, 2021).

Species richness, Shannon and Simpson diversity indices were calculated independently for ciliates and amoeboid protists as a measure of alpha diversity using R vegan package v. 2.5.7 (Oksanen et al., 2020). Diversity indices were subsequently converted to the effective number of species (true diversity) following a procedure proposed by Jost (2006, 2007). Abundance was calculated independently for ciliates and amoeboid protists as the number of individuals per cm^2 surface area for 1 and 2-months immersion periods.

Functional traits were assigned to each species examined in this study based on the relevant literature for ciliates and amoeboid protists (Foissner and Berger, 1996; Foissner et al., 2002; Adl et al., 2019; Fiore-Donno et al., 2019). Ciliates were classified into categories (functional groups) based on the following functional traits: food source, feeding strategy, ecosystem preference, habitat preference, motility, mode of locomotion and life form. Likewise, the amoeboid protists were assigned to the following functional categories (groups): food source, habitat, and morphology (the presence or absence of shell). A detailed overview of the criteria for classification can be found in **Supplementary Tables 1**, **2**.

To minimize the effects of qualitative and quantitative selection of functional traits on the results of functional diversity measures that are shown to be potentially significant (Pakeman, 2014; Zihao et al., 2021), we focused on the robust measures of functional dispersion (FDis) and RaoQ quadratic diversity (RaoQ) to quantify the functional diversity of ciliates and amoeboid protists. FDis is defined as the weighted mean distance in multidimensional trait space of individual species to the centroid of all species, where the weights are the relative abundances of the species (Laliberté and Legendre, 2010; Cappelatti et al., 2020). RaoQ is defined as the sum of pairwise distances between species in multidimensional trait space weighted by their relative abundance (Ricotta and Moretti, 2011). By construction, the two functional dispersion indices are not influenced by species richness (Teittinen and Virta, 2021). FDis and RaoQ values were calculated in R using the FD package v. 1.0–12 (Laliberté and Legendre, 2010; Laliberté et al., 2014). Indices of functional diversity were calculated separately for ciliate and amoeboid protist assemblages at C and N sites for the 1 and 2-months immersion periods.

Data Analysis

Each of the four data sets (1 and 2-months immersion periods for ciliates and amoeboid protists), environmental data and data regarding periphyton-associated factors were tested for normality using Shapiro-Wilk's test in R prior to further analysis. Since the data were not normally distributed (Shapiro-Wilk's test, $p < 0.05$) and sphericity was violated (Mauchly's test, $p < 0.05$), differences in environmental and periphyton-associated factors between C and N sites were tested using the analysis of similarity (ANOSIM), a non-parametric test for evaluating a dissimilarity matrix instead of raw data (Clarke and Warwick, 1994) from the R package vegan v. 2.5.7 (Oksanen et al., 2020).

Generalized linear mixed models (GLMMs) were constructed using SPSS Statistics ver. 28.0 (IBM Corp, 2021) to test for differences in ciliate and amoeboid protist assemblages between C and N sites for the 1 and 2-months immersion periods. The taxonomic metrics considered were: abundance, species richness, taxonomic (True) diversity derived from the Shannon and Simpson indices while the functional diversity indices included FDis and RaoQ. Only those environmental factors that showed significant differences (ANOSIM) between C and N sites were selected as inputs to the models. GLMMs with the variables "site," "COD," "TWH" and "nitrites" as fixed effects were constructed to evaluate the relationships between 1-month assemblages of ciliates and amoeboid protists and environmental factors. For the 2-months immersion period, the variables "site," "DO," "conductivity," "pH," "COD," "alkalinity," "TWH" and "orthophosphates" were selected as fixed effects. The variables "replicate" and "season" were included as random effects for both model variations, by recommendation of Jost (2007).

Additional GLMMs with periphyton-associated factors (organic and inorganic matter content, chlorophyll *a* concentration) as fixed effects were constructed for both ciliate and amoeboid protist assemblages for the 1 and 2-months immersion periods. Due to repeated measures sampling setup, first-order autoregressive (AR1) covariance structure of random effects was assumed in all models constructed within this study (Field, 2009).

RESULTS

Environmental Factors

Values of all environmental factors measured in this research can be found in **Supplementary Table 3**. The COD values for the 1-month period at N sites were significantly lower (R = 0.45, p = 0.015) in comparison to C sites while the opposite was found for nitrites (R = 0.64, p = 0.007) (**Figure 3**).

For the 2-months immersion period, N sites had significantly lower values of COD (R = 0.52, p = 0.004), pH (R = 0.69, p = 0.015) and orthophosphates (R = 0.70, p = 0.001), while higher values of conductivity (R = 0.91, p = 0.004) in comparison to C sites (**Figure 3**).

Periphyton-Associated Factors

All periphyton-associated factors differed significantly between C and N sites for both immersion periods (R = 1.00, $p < 0.001$). Organic matter content was lower at N sites for both periods (**Figure 4**), but it could be noticed that N sites occasionally had extremely high values (30.83 mg per cm^2 during the 1-month period and 7.22 mg per cm^2 during the 2-months period), while C sites had no such extreme outliers. Inorganic matter content was lower at N sites than at the C sites during the 1-month period, but showed the opposite during the 2-months period, reaching record values of 143.60 mg per cm^2. Chlorophyll *a* concentration at N sites was higher compared to C sites during the 1-month period and lower during the 2-months period. However, it could be noticed that N sites, unlike C sites, had some extremely high chlorophyll *a* concentrations during both periods.

Taxonomic and Functional Metrics of Ciliate Assemblages

In this study, a total of 78 ciliate species were identified, most of them (48) at N sites. Ciliate taxonomic metrics (abundance, species richness, Shannon and Simpson derived True diversity indices) were higher at N sites than at C sites for both immersion periods (**Figure 5**). Although no significant differences were confirmed by GLMMs with environmental and periphyton-associated factors for the 1-month period, all taxonomic metrics showed significant differences between N sites and C sites for the 2-months period (**Tables 1**, **2**). As for correlations between taxonomic metrics and environmental factors, no significant correlations were found for the 1-month period, but several were observed for the 2-months period: abundance was significantly positively correlated with COD, species richness was significantly negatively correlated with conductivity, while True diversity values (Shannon and Simpson) were significantly positively correlated with pH and orthophosphates but significantly negatively correlated with conductivity (**Table 3**). Periphyton-associated factors were found to have significant correlations with taxonomic metrics for both 1 and 2-months periods (**Table 4**). All taxonomic metrics except abundance were positively correlated with inorganic matter and negatively correlated with organic matter and chlorophyll *a* for the 1-month period. The opposite was found for the 2-months period when all taxonomic metrics were positively correlated with organic matter and chlorophyll *a*, while negatively correlated with inorganic matter. Significant correlations were found for species richness and True diversity (Shannon) values for both periods and abundance for the 2-months period.

FDis values for ciliates were lower at N sites compared to C sites for both immersion periods, whereas RaoQ values were higher at N sites for the 1-month period but lower for the 2-months period (**Figure 5**). When tested with GLMMs including environmental and periphyton-associated factors, significant differences were confirmed only for FDis values for the 1-month period and RaoQ values for the 2-months period (**Tables 1**, **2**). Both functional metrics for the 2-months period were negatively correlated with conductivity, while they were positively correlated with pH, COD, and orthophosphates, although the correlations were significant only for RaoQ (**Table 3**). As for periphyton-associated factors, FDis and RaoQ showed quite different correlations for the 1-month period: FDis was significantly positively correlated with organic matter and negatively correlated with inorganic matter, whereas RaoQ was significantly negatively correlated with organic matter and positively correlated with inorganic matter. Both functional metrics were then negatively correlated with chlorophyll *a*, while a significant correlation was found only for RaoQ. With regards to the 2-months period, both

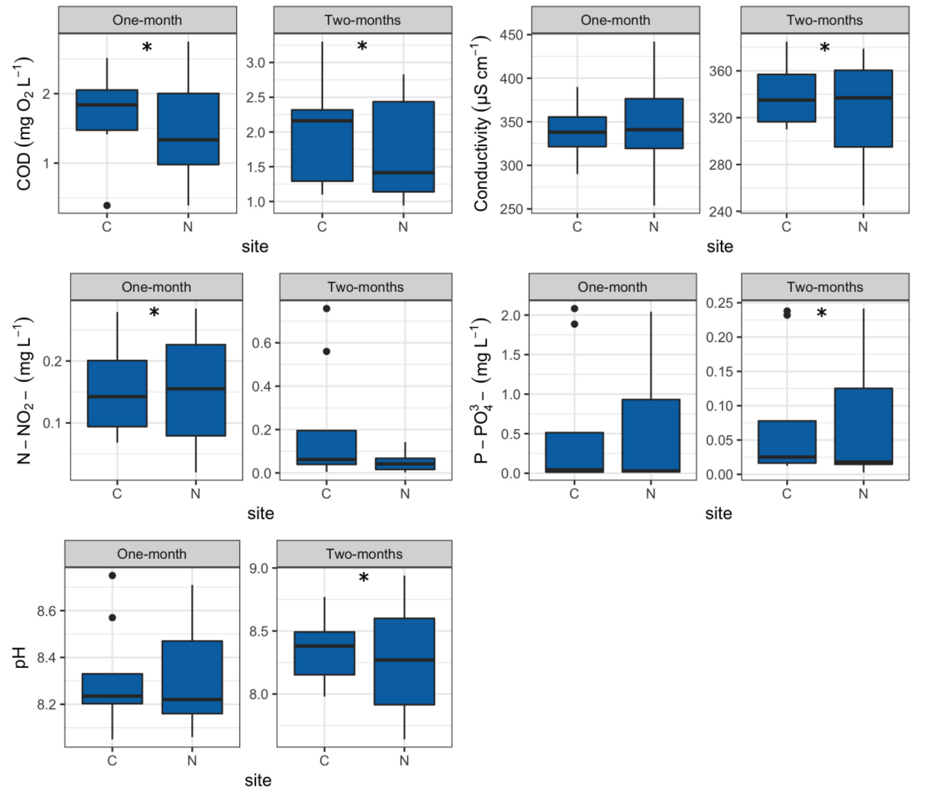

FIGURE 3 | Box plots showing selected environmental factors at control (C) and revitalized sites (N) for the 1 and 2-months immersion periods. Asterisk symbol (*) indicates statistically significant differences among estimated means (ANOSIM, $p < 0.05$). Upper and lower edges of the boxes are the first and third quartiles; the line inside the box represents the median; individual dots are outliers. Abbreviations: COD—total chemical oxygen demand, N-NO_2^-—nitrites, P-PO_4^{3-}—orthophosphates.

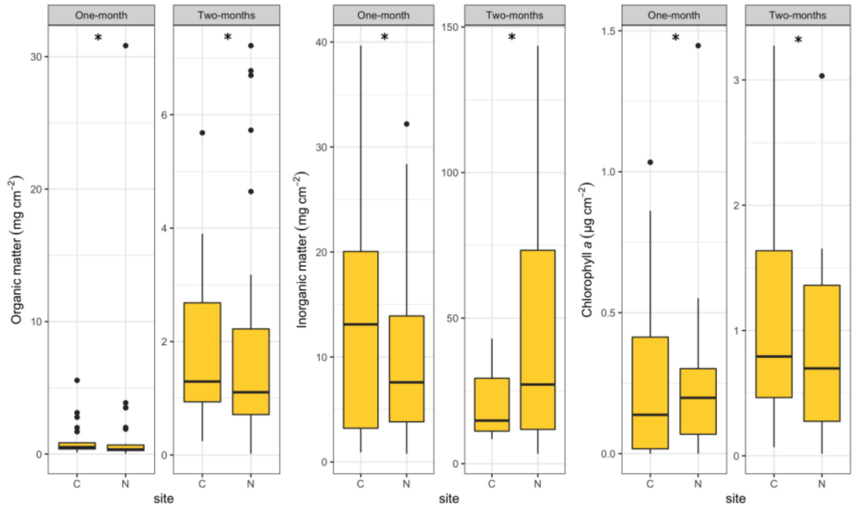

FIGURE 4 | Box plots showing periphyton-associated factors at control (C) and revitalized sites (N) for the 1 and 2-months immersion periods. Asterisk symbol (*) indicates statistically significant differences among estimated means (ANOSIM, $p < 0.05$). Upper and lower edges of the boxes are the first and third quartiles; the line inside the box represents the median; individual dots are outliers.

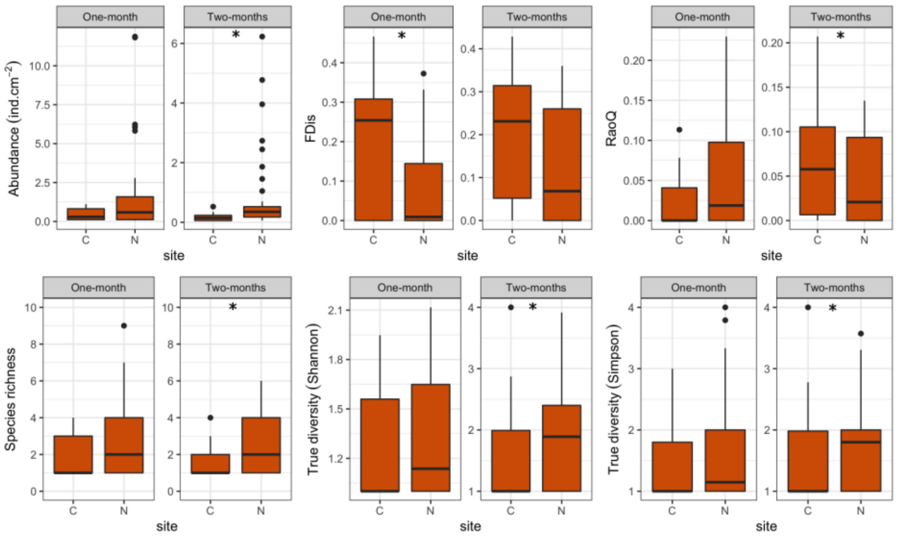

FIGURE 5 | Box plots showing ciliate taxonomic and functional metrics at control (C) and revitalized sites (N) for the 1 and 2-months immersion periods. Asterisk symbol (*) indicates statistically significant differences among estimated means (GLMM, $p < 0.05$). Upper and lower edges of the boxes are the first and third quartiles; the line inside the box represents the median; individual dots are outliers.

TABLE 1 | GLMM (full model) output showing differences in ciliate assemblage metrics between control and revitalized sites (1 and 2-months immersion periods) with sites and environmental factors as fixed effects, and season and replicate as random effects.

	Assemblage parameter	*F*	*p*	d.f	d.f. corrected	p C-N
One-month	Abundance	1.092	0.301	1	50	0.282
	Species richness	0.982	0.327	1	50	0.309
	True diversity (Shannon)	0.064	0.801	1	50	0.801
	True diversity (Simpson)	0.194	0.661	1	50	0.657
	FDis	6.054	**0.017**	1	50	0.287
	RaoQ	0.186	0.668	1	50	0.655
Two-months	Abundance	10.583	**0.001**	1	43	**0.001**
	Species richness	5.314	**0.026**	1	43	**0.028**
	True diversity (Shannon)	5.988	**0.019**	1	43	**0.0014**
	True diversity (Simpson)	4.349	**0.043**	1	43	**0.035**
	FDis	0.261	0.612	1	43	0.754
	RaoQ	9.931	**0.003**	1	43	**0.035**

Statistically significant effects ($p < 0.05$) are reported in bold. F, F statistic; d.f., degrees of freedom.

TABLE 2 | GLMM (full model) output showing differences in ciliate assemblage metrics between control and revitalized sites (1 and 2-months immersion periods) with sites and periphyton-associated factors as fixed effects, and season and replicate as random effects.

	Assemblage parameter	*F*	*p*	d.f	d.f. corrected	p C-N
One-month	Abundance	3.038	0.087	1	50	**0.046**
	Species richness	0.126	0.724	1	50	0.725
	True diversity (Shannon)	0.124	0.726	1	50	0.727
	True diversity (Simpson)	0.520	0.474	1	50	0.477
	FDis	26.586	**< 0.001**	1	50	**< 0.001**
	RaoQ	1.939	0.184	1	50	0.242
Two-months	Abundance	23.467	**< 0.001**	1	47	**< 0.001**
	Species richness	22.315	**< 0.001**	1	47	**< 0.001**
	True diversity (Shannon)	14.877	**< 0.001**	1	47	**< 0.001**
	True diversity (Simpson)	8.796	**0.005**	1	47	**0.004**
	FDis	0.172	0.680	1	47	0.690
	RaoQ	0.002	0.994	1	47	0.994

Statistically significant effects ($p < 0.05$) are reported in bold. F, F statistic; d.f., degrees of freedom.

FDis and RaoQ were positively correlated with chlorophyll *a* and negatively correlated with organic and inorganic matter. Correlations with inorganic matter were the only found significant (**Table 4**).

The detailed GLMM report with all correlations between environmental factors and the 1 and 2-months taxonomic and functional metrics of ciliates can be found in **Supplementary Table 4**.

In terms of immersion duration, it could be noticed that all ciliate taxonomic metrics at N sites increased with longer immersion. The same could be observed for FDis while RaoQ values remained the same.

Taxonomic and Functional Metrics of Amoeboid Protists

A total of 52 species of amoeboid protists were identified in this study, most of which (41) belonged to N sites. Almost no differences were observed between N and C sites with respect to most taxonomic metrics for both immersion periods (**Figure 6**). Abundance was slightly lower at N sites for the 1-month period while True diversity Shannon values were significantly lower at N sites for the 2-months period, when tested with GLMMs that included environmental and periphyton-associated factors (**Tables 5**, **6**). As for correlations with environmental factors, all taxonomic metrics for the 1-month period were positively correlated with COD, while the correlation with abundance was found significant. With regards to the 2-months period, all taxonomic metrics negatively correlated with COD and orthophosphates, while a positive correlation was found with pH and conductivity. Most of these correlations were significant (**Table 7**). As for correlations with periphyton-associated factors, all taxonomic metrics for the 1-month period were positively correlated with all three factors, while significant correlations were found between species richness and inorganic matter. For the 2-months period, there was a significant positive correlation between abundance and organic matter, while all taxonomic metrics were significantly negatively correlated with chlorophyll *a* (**Table 8**).

FDis and RaoQ values were higher at N sites compared with C sites for the 1-month period, whereas they were lower for the 2-months period (**Figure 6**). The only significant difference was found for the 1-month period. Regarding correlations with environmental factors, both functional indices were negatively correlated with COD for both immersion periods, while significant correlations were found only for the 2-months period. In addition, FDis was significantly positively correlated with pH and orthophosphates (**Table 7**). As for periphyton-associated factors, both functional indices were positively correlated with all periphyton-associated factors for the 1-month period, whereas they were negatively correlated with chlorophyll *a* for the 2-months period, although not significantly (**Table 8**).

The detailed GLMM report with all correlations between environmental factors and the 1 and 2-months taxonomic and functional metrics of amoeboid protists can be found in **Supplementary Table 5**.

With respect to immersion duration, species richness and True diversity (Shannon and Simpson) values at N sites were found to increase with longer immersion, while abundance decreased slightly. The functional indices remained more or less the same with longer immersion duration.

TABLE 3 | GLMM (full model) output showing main effects of environmental factors (fixed effects) on taxonomic and functional metrics of ciliate assemblages, with season and replicate as random effects.

	Assemblage parameter	Environmental parameter	*F*	*p*	d.f.	d.f corrected	Coefficient
One-month	Abundance	COD	0.137	0.713	1	50	−0.149
		Nitrites	3.079	0.085	1	50	3.947
	Species richness	COD	1.117	0.296	1	50	0.234
		Nitrites	0.013	0.910	1	50	−0.089
	True diversity (Shannon)	COD	0.469	0.497	1	50	0.068
		Nitrites	2.542	0.117	1	50	−0.592
	True diversity (Simpson)	COD	0.141	0.709	1	50	−0.063
		Nitrites	0.952	0.334	1	50	−0.608
	FDis	COD	1.166	0.285	1	50	−0.430
		Nitrites	0.064	0.801	1	50	0.429
	RaoQ	COD	0.732	0.396	1	50	−0.511
		Nitrites	4.497	**0.039**	1	50	−4.838
Two-months	Abundance	COD	5.844	**0.009**	1	43	−0.776
		Conductivity	4.059	0.050	1	43	−0.010
		pH	0.719	0.395	1	43	1.604
		Ortho	0.971	0.348	1	43	5.925
	Species richness	COD	0.547	0.464	1	43	0.173
		Conductivity	8.348	**0.006**	1	43	−0.010
		pH	3.594	0.065	1	43	1.276
		Ortho	1.223	0.275	1	43	3.340
	True diversity (Shannon)	COD	1.811	0.185	1	43	0.264
		Conductivity	8.976	**0.005**	1	43	−0.009
		pH	9.489	**0.004**	1	43	1.929
		Ortho	4.022	0.051	1	43	5.522
	True diversity (Simpson)	COD	1.714	0.197	1	43	0.234
		Conductivity	9.639	**0.003**	1	43	−0.009
		pH	12.055	**0.001**	1	43	2.021
		Ortho	4.810	**0.034**	1	43	5.685
	FDis	COD	1.462	0.233	1	43	1.693
		Conductivity	0.659	0.465	1	43	−0.019
		pH	2.076	0.071	1	43	2.954
		Ortho	1.252	0.273	1	43	13.017
	RaoQ	COD	7.844	**0.009**	1	43	3.018
		Conductivity	2.293	0.263	1	43	−0.012
		pH	3.193	0.071	1	43	4.823
		Ortho	1.496	0.107	1	43	6.979

Statistically significant effects ($p < 0.05$) are reported in bold. F, F statistic; d.f., degrees of freedom.

DISCUSSION

Our results revealed that ciliates and amoeboid protists respond differently to stream revitalization at the assemblage level. While ciliate assemblages at N sites had higher abundance and taxonomic diversity compared to those at C sites for both immersion periods, taxonomic metrics of amoeboid assemblages differed little between sites, even with longer immersion. The two protist assemblages also differed in their functional response: while the functional diversity of ciliates was lower at N sites compared to C sites for both immersion periods, that of amoeboid protists was higher at N sites for the 1-month period but lower for the 2-months period, in comparison to C sites.

The lower functional diversity of ciliates at N sites compared to C sites for both immersion periods suggests that the level of disturbance in reactivated streams, in the form of occasionally changing hydrologic conditions (high flow or drought), was either too high or too low to promote functional diversity, judging by the intermediate disturbance hypothesis (IDH). IDH rests on the assumption that a maximum level of diversity is achieved when the assemblage is exposed to an intermediate level of disturbance (Connell, 1978). It is likely that in our case, disturbance filtered out incompatible suites of traits, so that only a subset of species with disturbance-resistant traits could colonize a disturbed habitat (Lavorel et al., 2008; Biswas and Mallik, 2010). Thus, compared to C sites, ciliate assemblages at N sites could be expected to consist mainly of prospective colonizers, with disturbance-intolerant species (r-strategists) absent or their share in the assemblage minimized, leading to increased similarity and functional redundancy within the

TABLE 4 | GLMM (full model) output showing main effects of periphyton-associated factors (fixed effects) on taxonomic and functional metrics of ciliate assemblages, with season and replicate as random effects.

	Assemblage parameter	Community parameter	F	p	d.f.	d.f. corrected	Coefficient
One-month	Abundance	Organic matter	0.007	0.935	1	50	0.010
		Inorganic matter	0.732	0.396	1	50	−0.014
		Chlorophyll *a*	0.918	0.343	1	50	−0.755
	Species richness	Organic matter	0.013	0.908	1	50	−0.002
		Inorganic matter	2.254	0.140	1	50	0.014
		Chlorophyll *a*	6.250	**0.016**	1	50	−0.540
	True diversity (Shannon)	Organic matter	2.701	0.107	1	50	−0.014
		Inorganic matter	2.266	0.139	1	50	0.007
		Chlorophyll *a*	20.578	**< 0.001**	1	50	−0.259
	True diversity (Simpson)	Organic matter	2.902	0.095	1	50	−0.019
		Inorganic matter	37.675	**< 0.001**	1	50	0.005
		Chlorophyll *a*	889.41	**< 0.001**	1	50	−0.337
	FDis	Organic matter	31.786	**< 0.001**	1	50	0.141
		Inorganic matter	0.195	0.709	1	50	−0.018
		Chlorophyll *a*	0.459	0.517	1	50	−0.568
	RaoQ	Organic matter	1.490	0.228	1	50	−0.054
		Inorganic matter	3.245	0.077	1	50	0.038
		Chlorophyll *a*	10.050	**0.003**	1	50	−2.469
Two months	Abundance	Organic matter	13.031	**< 0.001**	1	47	0.370
		Inorganic matter	29.264	**< 0.001**	1	47	−0.036
		Chlorophyll *a*	2.762	0.103	1	47	0.219
	Species richness	Organic matter	1.727	0.195	1	47	0.090
		Inorganic matter	7.652	**0.008**	1	47	−0.011
		Chlorophyll *a*	3.034	0.088	1	47	0.182
	True diversity (Shannon)	Organic matter	1.142	0.291	1	47	0.028
		Inorganic matter	5.258	**0.026**	1	47	−0.005
		Chlorophyll *a*	0.581	0.450	1	47	0.002
	True diversity (Simpson)	Organic matter	0.393	0.534	1	47	0.038
		Inorganic matter	2.907	0.095	1	47	−0.006
		Chlorophyll *a*	0.444	0.509	1	47	0.061
	FDis	Organic matter	0.477	0.493	1	47	−0.223
		Inorganic matter	13.003	**< 0.001**	1	47	−0.045
		Chlorophyll *a*	2.099	0.154	1	47	0.330
	RaoQ	Organic matter	0.517	0.476	1	47	−0.235
		Inorganic matter	8.579	**0.005**	1	47	−0.039
		Chlorophyll *a*	3.785	0.058	1	47	0.445

Statistically significant effects ($p < 0.05$) are reported in bold. Legend: F, F statistic; d.f., degrees of freedom.

assemblage (Segovia et al., 2016). On the other hand, it appears that amoeboid assemblages at N sites during the 1-month period consisted mainly of highly tolerant or even drought-resistant species (K-strategists) that would thrive under hydrologically extreme conditions (Carballeira and Pontevedra-Pombal, 2021), in contrast to the r-strategists that probably dominated the 2-months period, leading to differences in functional diversity between the two periods.

Our results suggested that the reasons for these different responses to stream revitalization in terms of taxonomic and functional metrics could also lie in the effects of environmental and periphyton-associated factors. While some factors had a similar effect on both assemblages, others affected the assemblages quite differently. The overall observed positive effect of COD, pH, and organic matter content on taxonomic metrics of both ciliates and amoeboid protists is consistent with previous findings by numerous authors who have worked with these protists (Cowling, 1994; Smith, 1996; Verhoeven, 2002; Bates et al., 2013; Bradford, 2016; Geisen, 2016; Fiore-Donno et al., 2019). The likely reason for this is the increased diversity and quantity of bacteria that are their main food source, as the deposited organic material supports attachment of bacteria that decompose both dissolved and particulate organic matter (Foissner, 2014). However, the dominance of bacteria as a food source could have led to functional homogeneity and explain why functional diversity of both ciliates and amoeboid protists was negatively affected by COD and/or organic matter.

Inorganic material, i.e., tufa, on the other hand, had a different effect. While it positively affected the taxonomic and functional metrics of both protist groups for the 1-month period, the same

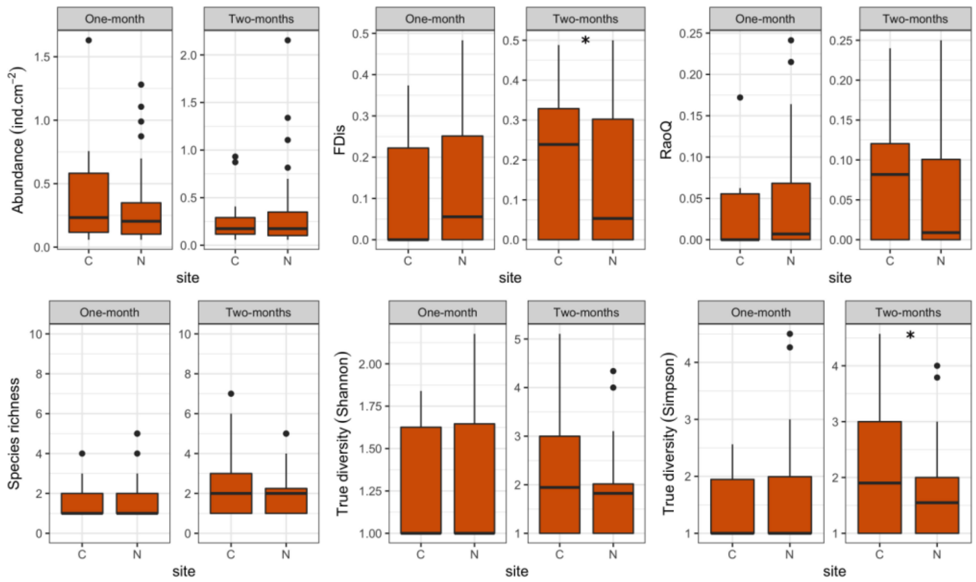

FIGURE 6 | Box plots showing taxonomic and functional metrics of amoeboid protists at control (C) and revitalized sites (N) for the 1 and 2-months immersion periods. Asterix symbol (*) indicates statistically significant differences among estimated means (GLMM, $p < 0.05$). Upper and lower edges of the boxes are the first and third quartiles; the line inside the box represents the median; individual dots are outliers.

TABLE 5 | GLMM (full model) output showing differences in amoeboid protist assemblage metrics between control and revitalized sites (1 and 2-months immersion periods) with sites and environmental factors as fixed effects, and season and replicate as random effects.

	Assemblage parameter	*F*	*p*	d.f	d.f. corrected	p C-N
One-month	Abundance	0.082	0.776	1	50	0.781
	Species richness	0.009	0.925	1	50	0.924
	True diversity (Shannon)	0.345	0.560	1	50	0.554
	True diversity (Simpson)	0.482	0.491	1	50	0.479
	FDis	0.566	0.455	1	50	0.458
	RaoQ	0.607	0.440	1	50	0.428
Two-months	Abundance	0.661	0.421	1	43	0.406
	Species richness	0.274	0.603	1	43	0.595
	True diversity (Shannon)	0.005	0.941	1	43	0.941
	True diversity (Simpson)	0.116	0.735	1	43	0.738
	FDis	5.246	**0.027**	1	43	0.121
	RaoQ	1.743	0.194	1	43	0.282

Statistically significant effects ($p < 0.05$) are reported in bold. F, F statistic; d.f., degrees of freedom.

TABLE 6 | GLMM (full model) output showing differences in amoeboid protist assemblage metrics between control and revitalized sites (1 and 2-months immersion periods) with sites and periphyton-associated factors as fixed effects, and season and replicate as random effects.

	Assemblage parameter	*F*	*p*	d.f	d.f. corrected	p C-N
One-month	Abundance	1.782	0.188	1	50	0.243
	Species richness	0.007	0.932	1	50	0.932
	True diversity (Shannon)	0.003	0.955	1	50	0.955
	True diversity (Simpson)	0.146	0.704	1	50	0.700
	FDis	1.555	0.218	1	50	0.244
	RaoQ	2.641	0.110	1	50	0.118
Two-months	Abundance	0.144	0.706	1	47	0.712
	Species richness	1.420	0.239	1	47	0.267
	True diversity (Shannon)	2.607	0.113	1	47	0.131
	True diversity (Simpson)	4.528	**0.039**	1	47	0.050
	FDis	1.698	0.199	1	47	0.256
	RaoQ	0.608	0.439	1	47	0.482

Statistically significant effects ($p < 0.05$) are reported in bold. F, F statistic; d.f., degrees of freedom.

TABLE 7 | GLMM (full model) output showing main effects of environmental factors (fixed effects) on taxonomic and functional metrics of amoeboid protist assemblages, with season and replicate as random effects.

	Assemblage parameter	Environmental parameter	*F*	*p*	d.f.		Coefficient
One-month	Abundance	COD	4.834	**0.033**	1	50	0.690
		Nitrites	2.463	0.123	1	50	2.164
	Species richness	COD	1.629	0.208	1	50	0.169
		Nitrites	8.021	**0.007**	1	50	−3.526
	True diversity (Shannon)	COD	0.379	0.541	1	50	0.050
		Nitrites	6.716	**0.012**	1	50	−1.404
	True diversity (Simpson)	COD	0.655	0.422	1	50	0.100
		Nitrites	5.413	**0.024**	1	50	−2.208
	FDis	COD	0.146	0.704	1	50	−0.318
		Nitrites	3.805	0.057	1	50	−17.316
	RaoQ	COD	0.370	0.546	1	50	−0.437
		Nitrites	4.466	**0.040**	1	50	−15.668
Two-months	Abundance	COD	3.861	0.056	1	43	−0.611
		Conductivity	5.605	**0.022**	1	43	0.013
		pH	5.347	**0.026**	1	43	2.548
		Ortho	1.893	0.176	1	43	−6.440
	Species richness	COD	0.149	0.701	1	43	−0.091
		Conductivity	13.119	**< 0.001**	1	43	0.022
		pH	1.949	0.170	1	43	0.988
		Ortho	5.915	**0.019**	1	43	−8.005
	True diversity (Shannon)	COD	0.598	0.444	1	43	−0.174
		Conductivity	12.899	**< 0.001**	1	43	0.016
		pH	1.774	0.190	1	43	0.891
		Ortho	5.127	**0.029**	1	43	−6.502
	True diversity (Simpson)	COD	1.051	0.311	1	43	−0.217
		Conductivity	13.285	**< 0.001**	1	43	0.016
		pH	1.276	0.265	1	43	0.707
		Ortho	5.754	**0.021**	1	43	−6.580
	FDis	COD	4.779	**0.034**	1	43	−1.534
		Conductivity	0.523	0.473	1	43	−0.009
		pH	11.541	**0.001**	1	43	7.378
		Ortho	6.533	**0.014**	1	43	23.133
	RaoQ	COD	0.522	0.474	1	43	−0.526
		Conductivity	0.042	0.839	1	43	−0.003
		pH	3.950	0.053	1	43	4.859
		Ortho	3.839	0.057	1	43	19.350

Statistically significant effects ($p < 0.05$) are reported in bold. F, F statistic; d.f., degrees of freedom.

was not observed for the 2-months period. It appears that the higher inorganic matter content during the 2-months period, whose deposition was facilitated by slightly higher temperature, conductivity, and flow velocity at N sites (**Supplementary Table 3**), negatively affected the ciliate taxonomic and functional metrics, but did not cause any negative effects on those of the amoeboid assemblages. Although the presence of inorganic matter can increase microhabitat heterogeneity (Beisel et al., 2000; Bednar et al., 2017; Singer et al., 2021) and thus serve as an "inoculum" for periphyton supporting high abundance and diversity (Primc and Habdija, 1987; Wörner et al., 2000; Zimmermann-Timm, 2002), there appears to be a critical point at which inorganic matter content no longer promotes high abundance and taxonomic diversity of ciliates. It could be that ciliates are much more sensitive to burial and sloughing than amoeboid protists because they do not predominantly glide along the substrate as amoebae do but exhibit various modes of locomotion: many of them are free-swimmers or even move by jumping and rotating (Foissner and Berger, 1996; Esteban and Fenchel, 2021). Amoeboid protists (especially naked amoebae) are also smaller in size compared to ciliates, which allows them to savor the microhabitats and be very closely associated with the substrate (Preston, 2003).

The two assemblages also responded differently with respect to immersion duration: while ciliate taxonomic and functional metrics at N sites increased with longer immersion, the same was observed only for some taxonomic metrics of amoeboid protists (species richness and True diversity values), while the abundance decreased, and functional metrics remained more or less the same. Since chloropyhll *a* concentration showed

TABLE 8 | GLMM (full model) output showing main effects of periphyton-associated factors (fixed effects) on taxonomic and functional metrics of amoeboid protist assemblages, with season and replicate as random effects.

One-month	Assemblage parameter	Community parameter	*F*	*p*	d.f.	d.f. corrected	Coefficient
	Abundance	Organic matter	0.001	0.981	1	50	0.001
		Inorganic matter	0.007	0.935	1	50	−0.001
		Chlorophyll *a*	2.170	0.147	1	50	0.558
	Species richness	Organic matter	2.530	0.118	1	50	0.030
		Inorganic matter	5.245	**0.026**	1	50	0.020
		Chlorophyll *a*	3.568	0.065	1	50	0.558
	True diversity (Shannon)	Organic matter	0.012	0.915	1	50	0.001
		Inorganic matter	0.001	0.969	1	50	0.001
		Chlorophyll *a*	0.001	0.972	1	50	0.001
	True diversity (Simpson)	Organic matter	1.707	0.197	1	50	0.028
		Inorganic matter	3.318	0.075	1	50	0.014
		Chlorophyll *a*	1.907	0.173	1	50	0.375
	FDis	Organic matter	0.733	0.396	1	50	0.061
		Inorganic matter	7.863	**0.007**	1	50	0.087
		Chlorophyll *a*	0.001	0.980	1	50	0.025
	RaoQ	Organic matter	1.612	0.210	1	50	0.080
		Inorganic matter	7.828	**0.007**	1	50	0.072
		Chlorophyll *a*	0.363	0.550	1	50	0.446
Two months	Abundance	Organic matter	6.653	**0.013**	1	47	0.185
		Inorganic matter	2.537	0.118	1	47	−0.006
		Chlorophyll *a*	17.245	**< 0.001**	1	47	−0.736
	Species richness	Organic matter	0.022	0.882	1	47	0.008
		Inorganic matter	3.885	0.055	1	47	0.005
		Chlorophyll *a*	21.774	**< 0.001**	1	47	−0.460
	True diversity (Shannon)	Organic matter	1.552	0.219	1	47	0.070
		Inorganic matter	0.390	0.535	1	47	0.002
		Chlorophyll *a*	12.681	**< 0.001**	1	47	−0.297
	True diversity (Simpson)	Organic matter	2.197	0.145	1	47	0.074
		Inorganic matter	0.412	0.524	1	47	0.002
		Chlorophyll *a*	11.839	**0.001**	1	47	−0.261
	FDis	Organic matter	0.051	0.822	1	47	0.037
		Inorganic matter	1.616	0.210	1	47	0.011
		Chlorophyll *a*	1.078	0.304	1	47	−0.282
	RaoQ	Organic matter	0.220	0.642	1	47	0.115
		Inorganic matter	0.640	0.428	1	47	0.010
		Chlorophyll *a*	1.096	0.300	1	47	−0.392

Statistically significant effects ($p < 0.05$) are reported in bold. F, F statistic; d.f., degrees of freedom.

significant correlations with most taxonomic and functional metrics of both assemblages and reached extreme values at times during the 2-months period at N sites, it is reasonable to assume that the different responses to immersion duration could be due to this. Although the observed orthophosphate levels during the 2-months period were still quite low and within the range characteristic of tufa-depositing streams (Primc-Habdija et al., 2005), they may have caused algal proliferation and subsequently higher chlorophyll *a* concentration. The dense algal coating of the substrate may have contributed to heterogeneity of food sources and microhabitats (Algarte et al., 2017) which together led to niche diversification, an increase in the share of disturbance-resistant ciliate species and subsequently an increase in taxonomic and functional diversity. Another possible explanation could be that changing hydrological conditions at N sites activate the dormant part of the microbial assemblages, leading to increased functional diversity over time (Velasco-González et al., 2020). On the other hand, the alginate covering the bacteria likely triggered a different response in the amoeboid assemblages, leading to reduced or even absent uptake of bacteria by amoeboid protists. This is likely due to the alginate coating increasing the overall size of the bacterial particles or altering their "flavor" (Heaton et al., 2001; Parry, 2004), resulting in reduced abundance of amoeboid protists during the 2-months immersion period.

The taxonomic and functional diversity of protists is a valuable indication of environmental conditions and ecosystem stability. Understanding the factors that structure protist assemblages is a prerequisite for using these organisms to predict environmental quality and future changes in ecosystem functioning. Protists have been used in environmental monitoring, but only to a limited extent because of the difficulties associated with sophisticated morphological identification methods. However, comprehensive studies that also consider their functional traits (Fournier et al., 2012) could make them more recognizable and increase the frequency of using protists as indicators of ecosystem functioning.

CONCLUSION

Linking taxonomic and functional metrics to environmental conditions can improve our understanding of biological processes, especially in ecosystems undergoing extensive changes, such as revitalized streams. Our results showed that taxonomic and functional metrics of ciliates and amoeboid protists responded to the prevailing conditions characteristic of revitalized tufa-depositing streams: changing hydrology (occasional high flow or drought), soil drainage, and extensive inorganic matter, i.e., tufa deposition, although their responses were somewhat different. While both protist assemblages benefited from COD and the presence of organic matter supported by favorable pH, as this provided their main food source–bacteria, ciliates were more sensitive to the presence of inorganic matter content than amoeboid protists. However, in terms of chlorophyll *a* concentration, ciliates appeared to benefit more from the algal coating as it resulted in heterogeneity of food sources and microhabitats, which increased their taxonomic and functional diversity, while it decreased the uptake of bacteria for amoeboid protists, negatively affecting their abundance. The two assemblages also showed different responses of taxonomic and functional metrics with respect to immersion duration: while the taxonomic and functional diversity of ciliates at N sites increased with longer immersion, indicating niche diversification, those of amoeboid protists hardly changed with time. The results presented suggest that a comprehensive analysis of taxonomic and functional metrics of ciliates and amoeboid protists could be a good proxy for assessing revitalization of tufa-depositing streams. However, the temporal component should always be considered when conducting such studies, as the colonization processes of ciliates and amoeboid protists are quite complex, especially in tufa-depositing streams.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

VG, MSP, and RMK contributed to conception and design of the study. VG and BV organized the trait database. VG and FR performed the statistical analysis. VG wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.842395/full#supplementary-material

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aSupplementary table S1. Functional traits of periphytic ciliate species sampled at Skradinski buk tufa barrier (Krka National Park, Croatia).

Taxa	Food source	Feeding strategy	Ecosystem preference[1]	Habitat preference[2]	Motility	Mode of locomotion	Life form
Acineria uncinata Tucolesco, 1962	phagotrophic protists, small metazoans	predation	active sludge	benthos, periphyton	motile	crawling	solitary
Acineta flava Kellicott 1885	phagotrophic protists, small metazoans	predation	lentic and lotic (freshwater)	periphyton	sessile	unknown	solitary
Acineta tuberosa Ehrenberg, 1833	phagotrophic protists, small metazoans	predation	active sludge	periphyton	sessile	unknown	solitary
Amphileptus sp.	phagotrophic protists, small metazoans	predation	active sludge	periphyton	motile	free-swimming	solitary
Aspidisca cicada O. F. Müller, 1786.	bacteria	filtration	active sludge	benthos, periphyton	motile	jumping, rotating	solitary
Aspidisca lynceus O.F. Müller, 1773	bacteria	filtration	active sludge	benthos, periphyton	motile	jumping, rotating	solitary
Aspidisca sp.	bacteria	filtration	active sludge	benthos, periphyton	motile	jumping, rotating	solitary
Chilodonella caudata Stokes, 1885	bacteria	filtration	active sludge	benthos, periphyton	motile	gliding	solitary
Chilodonella uncinata (Ehrenberg, 1838)	bacteria	filtration	active sludge	benthos, periphyton	motile	gliding	solitary
Chlamydonella alpestris Foissner, 1979	bacteria, diatoms	predation	soil	benthos, periphyton	motile	gliding	solitary
Chlamydonellopsis plurivacuolata Blatterer & Foissner, 1990	diatoms	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	crawling	solitary
Chlamydonellopsis sp.	diatoms	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	crawling	solitary
Cinetochilum margaritaceum Perty, 1852	algae, bacteria	filtration	active sludge	planktonic	motile	free-swimming	solitary
Colpoda cucullus O.F. Müller, 1786	omnivorous	filtration	soil	benthos	motile	free-swimming	solitary
Colpoda inflata Stokes, 1885	algae, bacteria	filtration	soil	benthos	motile	free-swimming	solitary
Colpoda steini Maupas, 1883	bacteria	filtration	soil	planktonic	motile	free-swimming	solitary
Cyclidium glaucoma O. F. Müller, 1773)	bacteria	filtration	active sludge	planktonic	motile	jumping, rotating	solitary
Cyclidium sp.	bacteria	filtration	active sludge	planktonic	motile	jumping, rotating	solitary
Dysteria fluviatilis (Stein, 1859) Blochmann, 1895	bacteria	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	crawling	solitary
Dysteria sp.	bacteria	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	crawling	solitary
Frontonia acuminata Ehrenberg, 1833	omnivorous	filtration	soil	planktonic	motile	free-swimming	solitary
Frontonia atra Ehrenberg, 1834	diatoms	filtration	lentic and lotic (freshwater)	benthos, periphyton	motile	free-swimming	solitary
Gastrostyla mystacea (Stein, 1859) Sterkl, 1878	omnivorous	filtration	soil	benthos	motile	gliding	solitary
Gastrostyla steini Hemberger, 1982	omnivorous	filtration	soil	benthos	motile	gliding	solitary
Halteria chlorelligera Kahl, 1932	algae	filtration	lentic and lotic (freshwater)	planktonic	motile	jumping	solitary
Holophrya discolor Ehrenberg, 1833	omnivorous	predation	active sludge	benthos, periphyton	motile	free-swimming	solitary
Holophrya ovum Ehrenberg, 1831	omnivorous	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	free-swimming	solitary
Holophrya sp.	omnivorous	predation	active sludge	planktonic	motile	free-swimming	solitary
Holophrya teres Ehrenberg, 1834	omnivorous	predation	lentic and lotic (freshwater)	planktonic	motile	free-swimming	solitary
Holosticha kessleri (Wrześniowski, 1877) Šrámek-Hušek, 1957	bacteria	filtration	lentic and lotic (freshwater)	benthos	motile	crawling	solitary
Holosticha multistilata Kahl, 1928	omnivorous	filtration	lentic and lotic (freshwater)	benthos	motile	free-swimming	solitary
Holosticha pullaster (Müller, 1773) Foissner, Blatterer, Berger & Kohmann, 1991	omnivorous	filtration	soil	benthos	motile	free-swimming	solitary
Lacrymaria olor O. F. Müller, 1776	phagotrophic protists, small metazoans	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	free-swimming	solitary
Lembadion bullinum (Müller, 1786) Perty, 1849	omnivorous	filtration	lentic and lotic (freshwater)	benthos	motile	free-swimming	solitary
Lembadion sp.	omnivorous	filtration	lentic and lotic (freshwater)	benthos	motile	free-swimming	solitary
Leptopharynx costatus Mermod, 1914	algae, bacteria	predation	soil	planktonic	motile	crawling	solitary
Litonotus cygnus (O.F. Müller, 1776) Wrzesniowski, 1870	phagotrophic protists, small metazoans	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	crawling	solitary
Litonotus lamella Schewiakoff, 1896	phagotrophic protists, small metazoans	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	crawling	solitary
Litonotus sp.	phagotrophic protists, small metazoans	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	crawling	solitary
Loxophyllum utriculariae Penard, 1922	phagotrophic protists, small metazoans	predation	lentic and lotic (freshwater)	periphyton	motile	crawling	solitary
Nassula picta Greeff, 1888	cyanobacteria	predation	soil	planktonic	motile	crawling	solitary
Oxytricha chlorelligera Kahl, 1932	omnivorous	filtration	lentic and lotic (freshwater)	benthos, periphyton	motile	free-swimming	solitary
Oxytricha ferruginea Stein, 1859	omnivorous	filtration	lentic and lotic (freshwater)	benthos	motile	free-swimming	solitary
Oxytricha setigera Stokes, 1891	bacteria	filtration	soil	benthos	motile	free-swimming	solitary
Oxytricha sp.	omnivorous	filtration	lentic and lotic (freshwater)	benthos	motile	crawling	solitary
Paraurostyla viridis (Stein, 1859) Borror, 1972	bacteria	filtration	lentic (freshwater)	benthos	motile	free-swimming	solitary

Paraurostyla weissei (Stein, 1859) Borror, 1972	omnivorous	filtration	lentic and lotic (freshwater)	benthos	motile	crawling	solitary
Phialina sp.	phagotrophic protists, small metazoans	predation	soil	benthos, periphyton	motile	free-swimming	solitary
Plagiocampa rouxi Kahl, 1926	algae, bacteria	predation	soil	planktonic	motile	free-swimming	solitary
Platyophrya vorax Kahl, 1926	omnivorous	predation	soil	benthos	motile	free-swimming	solitary
Pleurotricha grandis Stein, 1859	algae, diatoms	filtration	lentic and lotic (freshwater)	benthos	motile	crawling	solitary
Pseudochilodonopsis algivora (Kahl, 1931) Foissner, 1979	algae, bacteria	predation	lentic and lotic (freshwater)	planktonic	motile	unknown	solitary
Pseudochilodonopsis fluviatilis Foissner, 1988	diatoms	predation	active sludge	benthos, periphyton	motile	crawling	solitary
Pseudochilodonopsis sp.	algae	predation	lentic and lotic (freshwater)	planktonic	motile	crawling	solitary
Pseudocohnilembus pusillus (Quennerstedt, 1869) Foissner & Wilbert, 1981	bacteria	filtration	soil	benthos, periphyton	motile	crawling	solitary
Pseudovorticella sp.	algae, bacteria	filtration	soil	benthos, periphyton	semi-sessile	unknown	colonial
Rimostrombidium lacustris (Foissner, Skogstad & Pratt, 1988) Petz & Foissner, 1992	algae, bacteria	filtration	lentic (freshwater)	planktonic	motile	jumping	solitary
Rimostrombidium sp.	algae, bacteria	filtration	lentic (freshwater)	planktonic	motile	jumping	solitary
Spathidium sp.	cyanobacteria	predation	soil	planktonic	motile	free-swimming	solitary
Spirostomum ambiguum (Müller, 1786) Ehrenberg, 1835	omnivorous	filtration	lentic and lotic (freshwater)	benthos, periphyton	motile	free-swimming	solitary
Stentor roeselii Ehrenberg, 1835	omnivorous	filtration	lentic and lotic (freshwater)	benthos, periphyton	semi-sessile	unknown	solitary
Stentor sp.	omnivorous	filtration	lentic and lotic (freshwater)	benthos, periphyton	motile	free-swimming	solitary
Sterkiella histriomuscorum Foissner, Blatterer, Berger & Kohmann, 1991	omnivorous	predation	soil	benthos	motile	free-swimming	solitary
Tetrahymena pyriformis (Ehrenberg, 1830) Furgason, 1940	bacteria	filtration	active sludge	benthos	motile	free-swimming	solitary
Thigmogaster sp.	bacteria	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	gliding	solitary
Trithigmostoma cucullulus Jankowski, 1967	omnivorous	predation	soil	benthos, periphyton	motile	crawling	solitary
Trochilioides recta Kahl, 1928	bacteria	predation	lentic and lotic (freshwater)	benthos, periphyton	motile	free-swimming	solitary
Uroleptus musculus O. F. Muller, 1773	omnivorous	filtration	lentic and lotic (freshwater)	benthos, periphyton	motile	free-swimming	solitary
Uroleptus piscis (O. F. Müller, 1773) Ehrenberg, 1831	omnivorous	filtration	lentic and lotic (freshwater)	benthos, periphyton	motile	crawling	solitary
Uronema nigricans O. F. Müller, 1786) Florentin, 1901	omnivorous	filtration	lentic and lotic (freshwater)	planktonic	motile	rotating	solitary
Urostyla grandis Ehrenberg, 1830	omnivorous	predation	lentic and lotic (freshwater)	benthos	motile	free-swimming	solitary
Urotricha sp.	algae, bacteria	filtration	lentic and lotic (freshwater)	planktonic	motile	free-swimming	solitary
Vorticella aquadulcis, Stokes, 1887	algae, bacteria	filtration	active sludge	benthos, periphyton	semi-sessile	unknown	colonial
Vorticella convallaria-complex Linnaeus, 1758	bacteria	filtration	active sludge	benthos, periphyton	semi-sessile	unknown	colonial
Vorticella infusionum-complex Dujardin, 1841	bacteria	filtration	soil	benthos, periphyton	semi-sessile	unknown	colonial
Vorticella octava-complex Stokes, 1885	bacteria	filtration	active sludge	periphyton	semi-sessile	unknown	colonial
Vorticella sp.	omnivorous	filtration	active sludge	periphyton	semi-sessile	unknown	colonial

[1]Ecosystem preference refers to the type of ecosystem in which the species preferentially occurs. Species labelled "soil" or "active sludge" also occur in soil and active sludge in addition to freshwater ecosystems (lentic and lotic).

[2]Habitat preference refers to the type of habitat in which the species preferentially occurs. Species labelled "planktonic" also occur in plankton in addition to benthos and periphyton.

Supplementary table S2. Functional traits of periphytic amoeboid protist species sampled at Skradinski buk tufa barrier (Krka National Park, Croatia).

Taxa	Food source	Habitat	Morphology
Amoeba proteus (Pallas, 1766) Leidy, 1878	protists	freshwater	naked amoeba
Arcella sp.	protists	freshwater	naked amoeba
Chaos sp.	diatoms	mosses	naked amoeba
Chlamydophrys minor Belar, 1921	unknown	freshwater	testate amoeba
Cyclopyxis kahli Deflandre, 1929	bacteria	soil	testate amoeba
Dermamoeba sp.	cyanobacteria	soil	naked amoeba
Discamoeba sp.	bacteria	mosses	testate amoeba
Euglypha bryophila Brown, 1911	bacteria	mosses	testate amoeba
Euglypha cristata Leidy, 1874	bacteria	freshwater	naked amoeba
Euglypha laevis Perty, 1849	omnivorous	mosses	testate amoeba
Filamoeba nolandi Page, 1967	bacteria	freshwater	naked amoeba
Filamoeba sp.	bacteria	freshwater	naked amoeba
Flamella sp.	omnivorous	soil	testate amoeba
Hartmanella sp.	bacteria	freshwater	naked amoeba
Heterophrys sp.	bacteria	freshwater	naked amoeba
Korotnevella sp.	bacteria	soil	naked amoeba
Lecythium hyalinum (Ehrenberg 1838) Hertwig and Lesser 1874	bacteria	freshwater	naked amoeba
Lecythium terrestris	algae, fungi	soil	testate amoeba
Mayorella augusta Schaeffer, 1926	omnivorous	soil	naked amoeba
Mayorella penardi Page, 1972	omnivorous	soil	naked amoeba
Mayorella sp.	omnivorous	soil	naked amoeba
Mayorella viridis Leidy, 1874	omnivorous	mosses	naked amoeba
Nuclearia radians (Greeff 1869) Patterson 1984	bacteria	freshwater	naked amoeba
Nuclearia simplex Cienkowsky 1865	bacteria	freshwater	naked amoeba
Nuclearia sp.	bacteria	freshwater	naked amoeba
Parachaos sp.	protists	freshwater	naked amoeba
Paradermamoeba sp.	bacteria	freshwater	naked amoeba
Penardia mutabilis Cash, 1904	bacteria	soil	naked amoeba
Platyamoeba sp.	bacteria	soil	naked amoeba
Polychaos annulatum (Penard, 1902) Smirnov & Goodkov, 1997	omnivorous	soil	naked amoeba
Polychaos dubium (Schaeffer, 1916)	omnivorous	soil	naked amoeba
Polychaos fasciculatum (Penard, 1902) Schaeffer 1926	protists	soil	naked amoeba
Polychaos sp.	protists	soil	naked amoeba
Raphidiophrys sp.	bacteria	soil	naked amoeba
Reticulomyxa filosa Nauss 1949	bacteria	freshwater	naked amoeba
Saccamoeba limax Dujardin, 1841	bacteria	freshwater	naked amoeba
Saccamoeba lucens Frenzel, 1892	bacteria	freshwater	naked amoeba

Saccamoeba sp.	bacteria	freshwater	naked amoeba
Thecamoeba quadrilineata (Carter, 1856) Lepşi, 1960	bacteria	soil	naked amoeba
Thecamoeba similis (Greeff, 1891) Lepşi, 1960	bacteria	soil	naked amoeba
Thecamoeba sp.	bacteria	freshwater	naked amoeba
Thecamoeba terricola (Greeff,1866) Lepsi, 1960	omnivorous	mosses	testate amoeba
Trachelocorythion pulchellum Penard, 1890	bacteria	freshwater	naked amoeba
Trichamoeba sinuosa Siemensma & Page, 1986	bacteria	freshwater	naked amoeba
Trichamoeba sp.	bacteria	soil	testate amoeba
Trinema enchelys (Ehrenberg, 1938) Leidy, 1878	bacteria	soil	testate amoeba
Trinema lineare Penard, 1890	bacteria	soil	testate amoeba
Vahlkampfia sp.	bacteria	freshwater	naked amoeba
Vahlkampfia tachypodia Glaser, 1912	bacteria	freshwater	naked amoeba
Vannella simplex Bovee, 1965	bacteria	freshwater	naked amoeba
Vannella sp.	bacteria	freshwater	naked amoeba

Supplementary table S3. Values of all environmental factors measured during the research period.

One-month immersion period

Site	Season	Temp (°C)	O_2 (mg L^{-1})	Flow velocity (ms^{-1})	Conductivity (µS cm^{-1})	pH	COD $KMnO_4$ (mg O_2 L^{-1})	Alkalinity (mg $CaCO_3$ L-1)	Total water hardness (mg $CaCO_3$ L^{-1})	N-NO_2^- (mg L^{-1})	N-NO_3^- (mg L^{-1})	P-PO_4^{3-} (mg L^{-1})
C1	Spring	21.60	8.79	0.59	345.00	8.24	1.79	80.00	128.16	0.1752	6.9471	0.0545
C1	Summer	22.80	8.46	0.35	330.00	8.23	1.41	110.00	258.10	0.0712	0.5000	0.0112
C1	Autumn	21.00	8.51	0.37	296.00	8.18	2.04	125.00	320.40	0.1016	0.2647	0.0112
C1	Winter	10.80	10.84	0.53	387.00	8.57	2.08	110.00	382.70	0.2792	6.2118	2.0822
C2	Spring	21.90	8.71	1.23	343.00	8.21	0.39	75.00	165.54	0.1616	6.9118	0.0556
C2	Summer	22.80	8.48	0.98	333.00	8.25	1.49	115.00	158.42	0.0680	0.3000	0.0309
C2	Autumn	20.80	8.98	0.71	290.00	8.05	2.51	135.00	311.50	0.1232	0.3176	0.0146
C2	Winter	10.50	10.94	0.69	390.00	8.75	1.89	95.00	373.80	0.2776	6.6529	1.8858
N1	Spring	21.60	8.56	0.56	347.00	8.37	0.78	95.00	178.00	0.1616	5.0647	0.0382
N1	Summer	22.70	8.38	0.63	325.00	8.37	1.10	70.00	306.16	0.0632	0.3471	0.0197
N1	Autumn	20.70	9.04	0.78	283.00	8.20	2.12	145.00	391.60	0.1112	0.3412	0.0140
N1	Winter	10.80	10.79	0.92	383.00	8.57	1.81	100.00	356.00	0.2832	6.4100	1.8070
N2	Spring	21.40	8.59	0.39	335.00	8.33	0.86	110.00	160.20	0.1752	5.9647	0.0348
N2	Summer	22.60	8.39	0.41	319.00	8.08	1.26	85.00	169.10	0.0712	0.3588	0.0140
N2	Autumn	20.60	9.00	0.58	291.00	8.18	2.75	130.00	436.10	0.1176	0.2588	0.0180
N2	Winter	10.70	10.86	0.40	375.00	8.61	1.89	77.50	373.80	0.2848	6.9350	1.8850
N3	Spring	20.60	8.62	0.96	341.00	8.16	0.54	100.00	188.68	0.1640	6.5294	0.0556
N3	Summer	22.60	8.43	0.92	442.00	8.08	1.34	85.00	151.30	0.0200	0.3882	0.0185
N3	Autumn	20.60	8.74	0.68	254.00	8.13	2.04	125.00	293.70	0.1088	0.2882	0.0163
N3	Winter	10.60	10.90	1.73	378.00	8.61	1.96	95.00	338.20	0.2816	6.2940	1.9640
N4	Spring	20.60	8.82	1.35	349.00	8.22	0.39	80.00	211.82	0.1672	7.4235	0.0326
N4	Summer	22.60	8.51	0.99	320.00	8.06	1.10	110.00	249.20	0.0696	0.4471	0.0101
N4	Autumn	21.10	8.87	1.01	280.00	8.16	2.04	105.00	409.40	0.0872	0.2118	0.0146
N4	Winter	10.40	10.99	1.39	378.00	8.60	2.04	117.50	356.00	0.2776	6.7294	2.0429
N5	Spring	21.70	8.63	0.63	352.00	8.23	0.39	90.00	181.56	0.1552	6.9882	0.0287
N5	Summer	22.80	8.42	0.23	327.00	8.20	1.34	115.00	142.40	0.0712	0.3294	0.0202
N5*	Autumn											
N5	Winter	10.90	10.76	0.91	382.00	8.71	1.96	105.00	391.60	0.2808	6.7294	1.9643

*N5 Autumn - dry

Two-months immersion period

Site	Season	Temp (°C)	O_2 (mgL^{-1})	Flow velocity (ms^{-1})	Conductivity (µS cm^{-1})	pH	COD $KMnO_4$ (mg O_2 L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Total water hardness (mg $CaCO_3$ L^{-1})	N-NO_2^- (mg L^{-1})	N-NO_3^- (mg L-1)	P-PO_4^{3-} (mg L^{-1})
C1	Spring	21.30	8.65	0.45	350.00	7.98	1.10	100.00	391.60	0.0048	5.1800	0.2320
C1	Summer	26.60	7.78	0.55	323.00	8.34	3.30	70.00	176.22	0.7576	0.1706	0.0174
C1	Autumn	17.40	9.06	0.60	315.00	8.42	2.20	155.00	418.30	0.0512	0.3294	0.0264
C1	Winter	9.50	11.22	0.79	378.00	8.71	1.18	160.00	320.40	0.0736	0.8059	0.0253
C2	Spring	21.60	8.63	0.63	347.00	8.01	1.33	130.00	409.40	0.0056	5.2800	0.2380
C2	Summer	26.80	7.73	0.53	317.00	8.20	2.67	75.00	178.00	0.5600	0.1765	0.0124
C2	Autumn	17.30	9.17	0.42	310.00	8.42	2.12	125.00	311.50	0.0504	0.3471	0.0253
C2	Winter	9.70	11.13	0.75	385.00	8.77	2.20	145.00	338.20	0.0728	0.6000	0.0135
N1	Spring	21.30	8.49	0.62	345.00	7.69	0.94	135.00	356.00	0.0056	5.2118	0.2275
N1*	Summer											
N1	Autumn	17.70	9.07	0.64	297.00	8.27	1.73	275.00	510.86	0.0504	0.4471	0.0230
N1	Winter	9.80	11.09	0.97	376.00	8.90	1.81	125.00	348.88	0.0688	0.5941	0.0180
N2	Spring	22.90	7.90	0.39	337.00	7.64	1.02	120.00	391.60	0.0088	5.2176	0.2382
N2	Summer	26.40	7.56	0.29	324.00	8.00	2.59	70.00	105.02	0.0232	0.2059	0.0146
N2	Autumn	19.80	8.34	0.24	265.00	8.31	2.51	175.00	224.28	0.1424	1.2529	0.0039
N2	Winter	9.80	11.06	0.63	374.00	8.94	1.18	125.00	320.40	0.0680	0.8529	0.0163
N3	Spring	21.10	8.68	0.73	338.00	7.70	1.41	110.00	391.60	0.0072	5.2353	0.2416
N3	Summer	26.00	7.76	1.02	305.00	8.04	2.51	70.00	133.50	0.0408	0.1412	0.0202
N3	Autumn	20.00	8.86	0.66	289.00	8.32	0.94	150.00	222.50	0.0912	0.5118	0.0028
N3	Winter	9.70	11.04	1.35	375.00	8.91	1.10	155.00	339.98	0.0688	0.5765	0.0135
N4	Spring	20.90	8.72	0.82	347.00	7.74	1.10	100.00	391.60	0.0048	5.2300	0.2370
N4	Summer	25.80	7.88	0.91	305.00	8.04	2.51	70.00	124.60	0.0344	0.2176	0.0174
N4	Autumn	17.70	9.42	0.45	245.00	8.36	1.49	150.00	236.74	0.0416	0.7059	0.0062
N4	Winter	9.40	11.20	1.23	373.00	8.90	1.26	150.00	338.20	0.0664	0.6000	0.0180
N5	Spring	21.40	8,65	0.63	348.00	7.83	1.33	105.00	338.20	0.0024	5.2600	0.2320
N5	Summer	26.40	7.78	0.23	293.00	8.15	2.83	70.00	158.42	0.0368	0.1824	0.0146
N5	Autumn	17.50	9.10	0.28	292.00	8.38	2.36	75.00	421.86	0.0504	0.4529	0.0208
N5	Winter	9.70	11.12	0.84	379.00	8.82	1.18	170.00	309.72	0.0520	0.5118	0.0152

*N1 Summer - dry

Supplementary table S4. GLMM (full model) output showing main effects of environmental factors (fixed effects) on taxonomic and functional metrics of ciliate assemblages, with season and replicate as random effects. Statistically significant effects (p<0.05) are reported in bold. Legend: F – F statistic; d.f. – degrees of freedom.

	Assemblage parameter	Environmental parameter	F	p	d.f.	d.f corrected	Coefficient
One-month	Abundance	COD	0.137	0.713	1	50	-0.149
		Nitrites	3.079	0.085	1	50	3.947
		TWH	2.071	0.156	1	50	0.004
	Species richness	COD	1.117	0.296	1	50	0.234
		Nitrites	0.013	0.910	1	50	-0.089
		TWH	0.178	0.675	1	50	0.001
	True diversity (Shannon)	COD	0.469	0.497	1	50	0.068
		Nitrites	2.542	0.117	1	50	-0.592
		TWH	2.217	0.143	1	50	0.001
	True diversity (Simpson)	COD	0.141	0.709	1	50	-0.063
		Nitrites	0.952	0.334	1	50	-0.608
		TWH	0.717	0.334	1	50	0.001
	FDis	COD	1.166	0.285	1	50	-0.430
		Nitrites	0.064	0.801	1	50	0.429
		TWH	7.714	**0.008**	1	50	0.007
	RaoQ	COD	0.732	0.396	1	50	-0.511
		Nitrites	4.497	**0.039**	1	50	-4.838
		TWH	3.742	0.059	1	50	0.007
Two-months	Abundance	DO	1.162	0.312	1	43	0.476
		Conductivity	4.059	0.050	1	43	-0.010
		pH	0.719	0.395	1	43	1.604
		COD	5.844	**0.009**	1	43	-0.776
		Alkalinity	1.763	0.187	1	43	-0.006
		TWH	0.914	0.488	1	43	0.003
		Ortho	0.971	0.348	1	43	5.925
	Species richness	DO	0.484	0.490	1	43	0.126
		Conductivity	8.348	**0.006**	1	43	-0.010
		pH	3.594	0.065	1	43	1.276
		COD	0.547	0.464	1	43	0.173
		Alkalinity	1.672	0.203	1	43	-0.004
		TWH	0.223	0.639	1	43	0.001
		Ortho	1.223	0.275	1	43	3.340
	True diversity (Shannon)	DO	0.284	0.597	1	43	-0.085
		Conductivity	8.976	**0.005**	1	43	-0.009
		pH	9.489	**0.004**	1	43	1.929
		COD	1.811	0.185	1	43	0.264
		Alkalinity	0.186	0.668	1	43	0.001
		TWH	0.063	0.803	1	43	0.000
		Ortho	4.022	**0.051**	1	43	5.522
	True diversity (Simpson)	DO	0.746	0.393	1	43	-0.129
		Conductivity	9.639	**0.003**	1	43	-0.009
		pH	12.055	**0.001**	1	43	2.021
		COD	1.714	0.197	1	43	0.234

	Alkalinity	0.239	0.628	1	43	-0.001
	TWH	0.080	0.779	1	43	0.000
	Ortho	4.810	**0.034**	1	43	5.685
	DO	1.095	0.436	1	43	0.628
	Conductivity	0.659	0.465	1	43	-0.019
	pH	2.076	0.071	1	43	2.954
FDis	COD	1.462	0.233	1	43	1.693
	Alkalinity	2.035	0.137	1	43	0.010
	TWH	0.172	0.715	1	43	-0.002
	Ortho	1.252	0.273	1	43	13.017
	DO	0.761	0.133	1	43	0.635
	Conductivity	2.293	0.263	1	43	-0.012
	pH	3.193	0.071	1	43	4.823
RaoQ	COD	7.844	**0.009**	1	43	3.018
	Alkalinity	0.569	0.425	1	43	0.004
	TWH	0.170	0.717	1	43	-0.003
	Ortho	1.496	0.107	1	43	6.979

Supplementary table S5. GLMM (full model) output showing main effects of environmental factors (fixed effects) on taxonomic and functional metrics of amoeboid protist assemblages, with season and replicate as random effects. Statistically significant effects ($p<0.05$) are reported in bold. Legend: F – F statistic; d.f. – degrees of freedom.

	Assemblage parameter	Environmental parameter	F	p	d.f.		Coefficient
One-month	Abundance	TWH	3.330	0.074	1	50	-0.004
		Nitrites	2.463	0.123	1	50	2.164
		COD	4.834	**0.033**	1	50	0.690
	Species richness	TWH	2.685	0.108	1	50	0.002
		Nitrites	8.021	**0.007**	1	50	-3.526
		COD	1.629	0.208	1	50	0.169
	True diversity (Shannon)	TWH	3.283	0.076	1	50	0.001
		Nitrites	6.716	**0.012**	1	50	-1.404
		COD	0.379	0.541	1	50	0.050
	True diversity (Simpson)	TWH	3.888	0.054	1	50	0.002
		Nitrites	5.413	**0.024**	1	50	-2.208
		COD	0.655	0.422	1	50	0.100
	FDis	TWH	3.475	0.068	1	50	-0.011
		Nitrites	3.805	0.057	1	50	-17.316
		COD	0.146	0.704	1	50	-0.318
	RaoQ	TWH	2.742	0.104	1	50	-0.008
		Nitrites	4.466	**0.040**	1	50	-15.668
		COD	0.370	0.546	1	50	-0.437
Two-months	Abundance	DO	14.348	**<0.001**	1	43	-1.211
		Conductivity	5.605	**0.022**	1	43	0.013
		pH	5.347	**0.026**	1	43	2.548
		COD	3.861	0.056	1	43	-0.611
		Alkalinity	6.992	**0.011**	1	43	-0.013
		TWH	17.087	**<0.001**	1	43	0.009
		Ortho	1.893	0.176	1	43	-6.440
	Species richness	DO	29.918	**<0.001**	1	43	-1.257
		Conductivity	13.119	**<0.001**	1	43	0.022
		pH	1.949	0.170	1	43	0.988
		COD	0.149	0.701	1	43	-0.091
		Alkalinity	0.141	0.709	1	43	0.002
		TWH	17.196	**<0.001**	1	43	0.008
		Ortho	5.915	**0.019**	1	43	-8.005
	True diversity (Shannon)	DO	26.864	**<0.001**	1	43	-1.095
		Conductivity	12.899	**<0.001**	1	43	0.016
		pH	1.774	0.190	1	43	0.891
		COD	0.598	0.444	1	43	-0.174
		Alkalinity	0.661	0.421	1	43	0.003
		TWH	12.780	**<0.001**	1	43	0.006
		Ortho	5.127	**0.029**	1	43	-6.502
	True diversity (Simpson)	DO	26.470	**<0.001**	1	43	-1.028
		Conductivity	13.285	**<0.001**	1	43	0.016
		pH	1.276	0.265	1	43	0.707
		COD	1.051	0.311	1	43	-0.217

	Alkalinity	0.465	0.499	1	43	0.003
	TWH	11.517	**0.001**	1	43	0.006
	Ortho	5.754	**0.021**	1	43	-6.580
FDis	DO	4.951	**0.031**	1	43	-1.572
	Conductivity	0.523	0.473	1	43	-0.009
	pH	11.541	**0.001**	1	43	7.378
	COD	4.779	**0.034**	1	43	-1.534
	Alkalinity	1.424	0.239	1	43	0.014
	TWH	13.428	**<0.001**	1	43	-0.018
	Ortho	6.533	**0.014**	1	43	23.133
RaoQ	DO	0.953	0.334	1	43	-0.727
	Conductivity	0.042	0.839	1	43	-0.003
	pH	3.950	0.053	1	43	4.859
	COD	0.522	0.474	1	43	-0.526
	Alkalinity	0.017	0.896	1	43	0.002
	TWH	5.567	**0.023**	1	43	-0.012
	Ortho	3.839	0.057	1	43	19.350

Publikacija III

Restored tufa-depositing streams: a dynamic interface between terrestrial and aquatic ecosystems

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Abstract

Stream periphyton has implications for ecosystem processes, yet little is known about its function in response to restoration efforts. In this study, we compared the taxonomic and functional composition of periphytic ciliates between restored and unrestored (control) streams for two different immersion periods to identify species with indicator potential, identify ciliate functional traits that differ between the two stream types, and examine the effects of environmental parameters on species and functional trait composition. Our study showed that restored streams differed from control streams in terms of species and functional trait composition. In restored streams, better competitors, i.e., omnivorous and bacterivorous free-swimming ciliates predominated, utilizing a wider range of different niches created by the greater microhabitat complexity due to retention of allochthonous organic matter particles and precipitation of calcite crystals, i.e., tufa. One of these species was *Platyophrya vorax,* which was identified as a species with indicator potential for restored tufa-depositing streams. The relationship between habitat heterogeneity, ciliate functional traits, and organic matter dynamics suggests that restoration of tufa-depositing streams affects ecosystem functioning by influencing its functional components, highlighting the need to investigate such ecosystems through the prism of connected lotic and terrestrial ecosystems rather than isolated ecosystems.

1. Introduction

Ecological restoration refers to the process of managing and/or supporting the recovery of a degraded or destroyed ecosystem as a means of maintaining ecosystem resilience and conserving biodiversity (Convention on Biological Diversity, 2016). Restoration efforts (including river and stream restoration) are increasing daily worldwide (Palmer *et al.*, 2014). The year 2021 was designated as the beginning of the United Nations (UN) Decade on Ecosystem Restoration (2021-2030) - a global movement initiated by the UN General Assembly to support ecosystem restoration and reverse global ecosystem degradation. In these frames, the European Union (EU) Biodiversity Strategy aims to restore 25,000 km of European rivers by 2030 (at least 20% of land and water areas in the EU), and by 2050 to cover all ecosystems in need of restoration (Publications Office of the European Union, 2022).

The European Mediterranean region has been identified as one of the most climate-vulnerable regions and a climate change "hotspot" (Salvia *et al*., 2021). The increasing intensity of droughts, driven by longer dry periods and shorter wet periods have made this region one of the most susceptible to soil degradation and desertification in Europe (Ferreira *et al*., 2020). Carbonate formations and karst features characteristic of this area are subjected to the global problem of climate change, as many karst springs, rivers, and streams face significant hydrological changes such as increased flow intermittence characterised by seasonal loss of flow and surface drying (Frollini *et al*., 2022; Patekar *et al*., 2021; Sivelle *et al*., 2021; Stubbington *et al*., 2018).

Croatia hosts many karst features, as almost half of the country is karst territory (Patekar *et al*., 2021). Most recognized karst features are the tufa barriers, which are formed by interactions between precipitation of carbonate minerals and resident organisms at specific ambient conditions (Golubić *et al*., 2008). This process is largely determined by geochemical conditions/environmental parameters (i.e., oversaturation of water with calcium carbonate, slightly alkaline pH, low concentration of dissolved organic matter) and is therefore very sensitive to environmental changes (Pentecost, 2005). Many tufa-depositing forms are highly affected by local anthropogenic interference with the environment, the rapid expansion of tourism, and the increasing threat of climate change (Šiljeg *et al*., 2020).

Although not many restoration activities have been carried out so far in tufa-depositing systems in Croatia, there have been some successfully finished attempts. In the tufa depositing system of the Plitvice Lakes National Park, macrophyte overgrowth was identified as one of the main factors affecting the tufa-depositing process. Restoration methods that focused on macrophyte removal resulted in improved tufa deposition due to better aeration and reduced organic matter loading (Miliša *et al*., 2016; Pavlus and Novosel, 2004). In another Croatian national park (Krka National Park), parts of the Skradinski buk tufa barrier dried up due to uncontrolled growth of the invasive plant species *Ailanthus altissima* (Mill.) Swinge, whose strong root system caused overgrowth of the barrier, interruption of water flow, and eventual drying up of the streams (Gulin *et al*., 2021). Restoration efforts to remove the invasive plant from the Skradinski buk tufa barrier consisted of a comprehensive aerial survey (Phantom 4) and detailed vegetation mapping of a 1-ha experimental area at the barrier, which helped to locate the invasive plant specimens. Once the invasive plant individuals were identified, they were mechanically removed on several occasions during August 2017 with permission from the Croatian Agency for Environment and Nature. Within two months of removal, five streams in the experimental area, that had previously dried up, were reactivated (restored in terms of water connectivity).

The success of these restoration efforts was explored and confirmed by the results of Gulin *et al*., 2021, 2022), who used periphytic phagotrophic protists (e.g., ciliates) as bioindicators of restoration success. These results showed that the protist community structure and its functional metrics at restored tufa-depositing streams were responsive to the environmental conditions such as changing hydrology (i.e., occasional high runoff or drought), soil drainage, and extensive inorganic matter (i.e., tufa) deposition. The close connection between periphytic protists and the process of tufa deposition is an important element of the dynamics of the tufa-depositing ecosystems such as Krka National Park (Matoničkin and Pavletić, 1962; Primc-Habdija *et al*., 2001). The periphyton matrix retains and accumulates both organic matter particles and tufa particles, increasing microhabitat complexity/structural heterogeneity (Matoničkin Kepčija *et al*., 2011; Risse-Buhl *et al*., 2015; Dzubakova *et al*., 2018). Because periphyton is a substrate-bound community, it has shown promise in several studies for restoring lotic ecosystems. Timoner *et al*. (2012, 2014) have found changes in functional composition in response to disturbance and environmental changes such as desiccation, flooding, or eutrophication, and more recently Atristain *et al.* (2023) have shown that periphyton functional composition responds to restoration activities such as dam removal, opening up room for further implementation of periphyton in future studies. The usefulness of periphytic protists in detecting the ecological success of restoration efforts is due to the fact that they have several characteristics of reliable ecological indicators: (1) they are ubiquitously distributed throughout ecosystems, (2) they respond rapidly to environmental changes, (3) they are quantifiable at multiple levels of biological organization (species and community), and (4) have effects at levels above and below their position in the food web (Wu, 2016). Ciliates are commonly found in aquatic and terrestrial ecosystems (Esteban and Fenchel 2021; Singer *et al*., 2021) and, with over 8000 species described, play an essential role in microbial food webs as mediators of matter and energy transfer across trophic levels (Singer *et al*., 2021).

The results of the previous study by Gulin *et al*. (2022) showed that ciliate taxonomic metrics (abundance, species richness, Shannon and Simpson derived True diversity indices) were higher in restored *vs.* unrestored (i.e., control) streams for two different immersion periods (one- and two-months). On the other hand, functional metrics such as functional dispersion (FDis) in restored streams were lower during the one-month immersion period, but higher during the two-months period. Thus, we hypothesized that there should be differences in species and functional trait composition between the two (restored vs. unrestored/control) stream types. The first objective of the present study was (i) to examine the ciliate species occurrence in restored and control streams, and recommend species with indicator potential for restored streams. The second objective was (ii) to compare restored *vs.* control streams using community weighted means (CWM), to determine if there are differences in certain functional traits between the two stream types at the two different immersion periods (one- *vs.* two- months). We assumed that the lower functional diversity in restored streams as observed by Gulin *et al*. (2022) was a consequence of functional redundancy, i.e., that restored streams were dominated by species occupying the same functional niche. Furthermore, we hypothesized that ciliate communities of restored streams would consist primarily of bacterivorous motile species readily mobilized during occasional high flow or drought, and that these species are also characteristic representatives of terrestrial ecosystems as restored streams drain the local forest floor. Our third objective was (iii) to investigate the effects of environmental parameters (i.e., water physicochemical parameters, organic and inorganic (i.e., tufa) matter content, chlorophyll *a* concentration) on ciliate species composition and functional traits in restored streams. We assumed that organic matter content and nitrite concentration would affect ciliate occurrence and functional trait composition the most, as these parameters were identified as the most important for community-level metrics in the previous study (Gulin *et al*., 2022).

The results of the present study will help to understand how ciliates respond to stream restoration at the species and functional trait levels. Furthermore, these results could serve as guidance in identifying species that can serve as indicators of stream restoration processes, not only in Croatian tufa-depositing systems, but also at a larger spatial scale (e.g., regional/Mediterranean).

2. Materials and methods

2.1. Study area

The Krka River is a karst river in the Dinaric region of Croatia, located in the central part of the north-eastern Adriatic coast (Fig. 1). Its headwaters are located near the Dinara Mountain and consist of several independent springs from which the river enters a canyon and is characterized by several lotic and lentic (e.g., Lake Visovac) areas intersected by tufa barriers (e.g., Roški slap, Skradinski buk) before it flows into the Adriatic Sea near the town of Šibenik (Fig. 1). With a total catchment area of about 2427 km^2 (Bonacci and Ljubenkov, 2005) and an average annual discharge of 47.4 m^3s^{-1} (1990–2009), the Krka River is considered a medium-sized river in Croatia (Čanjevac and Orešić, 2015).

This study was conducted at the longest and last tufa barrier (Skradinski buk) in the Krka River watercourse (Fig. 1), part of which has been protected by the National Park category since 1985. A 1 ha trial area was delineated, from which the invasive plant *Ailanthus altissima* (Mill.) Swinge (tree of heaven) was mechanically removed in August 2017 with the permission of the Croatian Agency for Environment and Nature, after being classified as dominant by aerial survey and vegetation mapping. Within two months of removal, five streams in the experimental area, that had previously dried up, were completely reactivated. The sampling design included seven sampling sites: two located in unrestored, i.e., control streams (C sites) where water was present before and after plant removal, with the C1 site having typical, well-developed moss cover and the C2 site having no moss cover; and five located in restored streams, i.e., newly reactivated streams (N sites) selected after the removal of the invasive plant species. Growth of mosses on surfaces can provide substrates for calcite nucleation and trap detrital calcite, accelerating tufa deposition; thus, moss-covered substrates represent typical microhabitats of tufa barriers in Krka National Park (Matoničkin and Pavletić, 1962; Primc-Habdija *et al*., 2001). Aquatic mosses in lotic ecosystems are also a suitable substrate for colonization of numerous microscopic and macroscopic metazoans, as they provide protection from water flow and wash-off from the habitat and have a large capacity for retention and accumulation of organic matter, which is a food source for benthic organisms

(Traunspurger, 2002; Dražina *et al*., 2013, 2014). Details of the sampling sites and their descriptions are provided in Table 1.

2.2. Sampling design and ciliate identification

Due to the high seasonality of the Krka River (Schöll *et al*., 2012), the research included four sampling campaigns covering four different seasons, in period between May 2019 and June 2020. At the beginning of every sampling event, at each sampling site, two 3D-printed Plexiglass carriers with three glass slides (7.6 × 2.6 cm) in each one (Gulin *et al*., 2022), were immersed in the water **in the middle section of each stream**, one for a period of one month, and second for a period of two months (Table 2). Since glass slides were partially covered by Plexiglass, the total surface area of each slide available for periphyton development was 17.18 cm^2. Prior to immersion into the stream, the slides were cleaned with detergent, 1 M hydrochloric acid and distilled water. During each sampling event (after one-month and two-months immersion period in each season) three slides were collected per sampling site. In total, 168 slides were placed into the streams, 84 for the one-month immersion period and another 84 for the two-months immersion period. However, only 55 slides were collected for the one-month immersion period and 52 for the two-months immersion period, due to seasonal drying of streams and disturbance by visitors of the National Park (sampling sites were located in the close vicinity of visitor trails and probably aroused curiosity). Collected slides were placed in the plastic containers filled with a small amount of ambient water and stored at 4°C in the dark. The slides were examined within a maximum of 48 h from collection, using Zeiss Axioimager A2 with DIC objectives and Axiocam 305 digital camera. Ciliates were identified at species level using Zen 2.4 imaging software and relevant literature (Foissner *et al*., 1991, 1992, 1994, 1995; Foissner and Berger, 1996). For each taxon, 10-15 photomicrographs were taken and subjected to morphometric measurement. Additional video clips were used to record movements and distinguishing features.

2.3. Environmental parameters

On each sampling event, at all sampling sites (C1, C2, N1-N5), in situ measurements of the following environmental parameters were made using the appropriate portable field meters: temperature (T) and dissolved oxygen concentration (DO) (oximeter OXI 96, WTW GmbH, Weilheim, Germany), pH (pH meter 330i, WTW GmbH, Weilheim, Germany), conductivity (Cond) (conductometer Sension 5, Hach, Loveland, Colorado, United States), and flow velocity (FV) (flow velocity meter P600, Dostmann electronic GmbH, Wertheim- Reicholzheim, Germany). An additional water sample (1 L) was collected and transported at 4°C for subsequent laboratory analysis of the following parameters: alkalinity (Alk), total water hardness (TWH), and concentrations of nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO3^-$), and orthophosphate ($P\text{-}PO_4^{3-}$) (APHA, 1985), and total chemical oxygen demand (COD) using the standardized acidic potassium permanganate titrimetric method (Deutsches Institut für Normung, 1986).

After microscopic identification of ciliates, periphyton sample from each slide was separated into two equal parts. One part was used to determine the content of organic and inorganic matter in the sample. For this purpose, the samples were dried at 104°C to constant weight, then ashed at 400°C for 4 h and reweighed. The mass difference between the dried and the ashed sample represents the organic matter (OM) content, while the mass difference between the ashed sample and the glass slide represents the content of inorganic matter (IM), i.e., deposited tufa. The values were expressed as mass (mg) of organic or inorganic matter content per cm^2 of surface area. The other part of the sample was used to determine chlorophyll *a* concentration by ethanol extraction method (Nusch, 1980). Values were expressed as mass (µg) of chlorophyll *a* per cm^2 surface area.

2.4. Data preparation

The functional categorization of ciliates used for calculation of community weighted mean (CWM) values was conducted according to Gulin *et al*. (2022). Calculating CWM values is a useful way to calculate community trait values weighted by abundance of species in that community (Ricotta and Moretti, 2011). Ciliates were assigned to categories for the following functional traits: food source ("algae"; "algae, bacteria"; "algae, diatoms"; "diatoms"; "bacteria"; "cyanobacteria"; "omnivorous"; "phagotrophic protists, small metazoans"), feeding strategy ("filtration"; "predation"), ecosystem preference ("active sludge"; "lentic (freshwater)"; "lentic and lotic (freshwater)"; "soil"), habitat preference ("benthos"; "benthos, periphyton"; "periphyton"; "planktonic"), motility ("motile"; "semi sessile";" sessile"), mode of locomotion ("crawling"; "free-swimming"; "gliding"; "jumping", "rotating"; "unknown") and life form ("colonial"; "solitary"). The detailed table of allocated functional traits can be found in Gulin *et al*. (2022), and it has been used as a convenient reference in other recent studies concerning ciliates in the Krka River (e.g., Gulin *et al*., 2022; Gulin Beljak *et al*., 2022).

2.5. Data analysis

2.5.1. Indicator species analysis

Indicator species analysis (IndVal), which combines both abundance and frequency of a species's occurrence, was used to determine potential indicator species for C and N sites. IndVal values were calculated in R v. 4.2.2. (R Core Team, 2022) using the package indicspecies v. 1.7.12. Correlations between indicator species and environmental parameters were calculated with Spearman's correlations using the "rcorr" function from the R Hmisc package v. 4.7-2 and visualized with the "corrplot" function from the R corrplot package v. 0.92.

2.5.2. Functional composition of periphytic ciliates

The CWM values were calculated for each trait category at each site to quantify shifts in mean functional trait values within communities resulting from environmental selection for certain functional trait categories (Ricotta and Moretti, 2011). The CWMs were calculated using the "functcomp" function from the FD package v. 1.0-12.1. (Laliberté and Legendre, 2010; Laliberté *et al*., 2014). To determine if there were significant differences in CWM values for each trait category between C and N sites for both immersion periods, generalized linear mixed models (GLMMs) were constructed using SPSS Statistics v. 28.0. (IBM Corp, 2021). The variable "site" was considered a fixed effect, while the variables "replicate" and "season" were included as random effects in both model variations, as recommended by Jost (2007).

First-order autoregressive (AR1) covariance structure was assumed in all models, since data were collected repeatedly over time (in the course of several months) (Field, 2009). The differences in CWM values between C and N sites were visualized using R ggplot2 package v. 3.4.0. (Wickham, 2016).

2.5.3. Effects of environmental parameters on species and functional trait composition

The differences in the values of environmental parameters between C and N sites, were tested using the analysis of similarity (ANOSIM) and have already been reported in Gulin *et al*. (2022). Correlations between CWM values that differed significantly between C and N sites and environmental parameters, were calculated with Spearman's correlations using the "rcorr" function from the R Hmisc package v. 4.7-2 and visualized with the "corrplot" function from the R corrplot package v. 0.92.

Redundancy analysis (RDA) in R was used to assess the influence of environmental parameters on the spatial distribution of ciliate species (only those represented by more than 5% in total abundance per site) and CWM values (only those that differed significantly between C and N sites) at C and N sites. RDA is designed for correlated response variables (which is often the case for CWMs) and therefore allows many response variables to be analyzed simultaneously (by forming RDA axes, which are linear combinations of response variables) (Kleyer *et al*., 2012; Šmilauer and Lepš, 2014). Values for ciliate abundance and CWMs were transformed by the Hellinger's transformation prior to species-based RDA and CWM-based RDA (Legendre and Gallagher, 2001). Multicollinearity between environmental parameters was checked using the "vif.cca()" function from the R vegan package v. 2.6-4, and if the value was greater than 2, multicollinearity was considered high. The function "forward.sel()" from the R adespatial package v. 0.3-20. was used to select the best explanatory variables. Monte Carlo test with 999 unrestricted permutations was used to determine the significance of the model, axis, and explanatory variables (environmental parameters) at the $p<0.05$ level.

3. Results

3.1. Species occurrence and potential indicator species

Based on 1837 recorded ciliate individuals, 78 species were identified. The most abundant species at C sites during the one-month immersion period was *Pleurotricha grandis* (14.49% of the total abundance at C sites). Those with more than 10% of total abundance at C sites were *Trithigmostoma cucullulus* (12.08%,) and *Cyclidium* sp. (11.11%) followed by *Holophrya ovum* (9.18%), *Vorticella aquadulcis*-complex (8.21%), *Aspidisca cicada* (7.73%) and *Pseudochilodonopsis algivora* (7.25%). Other species were represented with less than 5%.

The most abundant species at N sites during the one-month immersion period was *Platyophrya vorax*, accounting for 72.58% of the total ciliate abundance at N sites, while additionaly only *Cinetochilum margaritaceum* had a proportion greater than 10% (10.27%). *Chilodonella uncinata* had a share of more than 5% in the total abundance (5.62%), while the other species were represented with less than 5%.

During the two-months immersion period, the species with highest proportion in total ciliate abundance at C sites were *Holophrya discolor* (35.71%) and *Holophrya* sp. (10.71%). The remaining species were represented by less than 5% in the total abundance. At N sites, *C. uncinata* (25.12%), *Holophrya* sp. (21.40%), *P. vorax* (16.98%) and *Chlamydonella alpestris* (6.28%) had the highest proportion in the total ciliate abundance, respectively. The remaining species were represented with less than 5%.

Indicator species analysis (IndVal) for the one-month period showed that *P. vorax* was the only species with indicator potential for N sites (stat = 0.275, p = 0.034). For C sites, the following species were detected as potential indicator species: *V. aquadulcis*-complex (stat = 0.318, p = 0.003), *H. ovum* (stat = 0.269, p = 0.011), and *A. cicada* (stat = 0.188, p = 0.037).

Indicator species analysis for the two-months period did not reveal any indicator species neither for N sites nor for C sites.

3.2. Functional trait composition

3.2.1. One-month immersion period

GLMMs for the one-month immersion period showed statistically significant differences between C and N sites for CWM values related to food source, ecosystem preference, habitat preference, and mode of locomotion, while CWM values related to motility, feeding strategy, and life form did not differ significantly between C and N sites. CWM values associated to feeding strategy, motility and life form can be found in Supplementary Fig. 1.

The CWM values of food source at N sites differed significantly from those at C sites only for the functional categories "bacteria", and "phagotrophic protists, small metazoans" (Table 3). N sites had significantly higher CWM values for the category "bacteria" and significantly lower values for the category "phagotrophic protists, small metazoans" than C sites (Table 3, Fig. 2).

The CWM values of ecosystem preference differed significantly between C and N sites for the functional categories of "lentic (freshwater)", "lentic and lotic (freshwater)", and "soil". In comparison to C sites, N sites had significantly higher CWM values for the category "soil", but significantly lower values for the categories "lentic (freshwater)" and "lentic and lotic (freshwater)" (Table 3, Fig. 3).

The CWM values of habitat preference differed significantly between C and N sites for the functional categories of "benthos, periphyton", "benthos" and "planktonic". The CWM value of category "benthos, periphyton" was significantly lower at N sites compared to C sites, whereas the opposite was found for the categories "benthos" and "planktonic" (Table 3, Fig. 4).

The CWM values of mode of locomotion differed significantly between C and N sites only for the functional categories "crawling" and "free-swimming". The CWM value of category "crawling" was significantly lower at N sites than at C sites, while the opposite was found for the category "free-swimming" (Table 3, Fig. 5).

3.2.2. Two-months immersion period

For the two-months immersion period, GLMMs showed statistically significant differences between C and N sites for CWM values of ecosystem and habitat preference, while the other CWM values did not differ significantly between restored and control streams. CWM values of food source, feeding strategy, motility, mode of locomotion and life form can be found in Supplementary Fig. 2.

The CWM values of ecosystem preference differed significantly between C and N sites only for the functional category "soil", whereby higher value was found at N than at C sites (Table 3, Fig. 6).

The CWM values of habitat preference differed significantly between C and N sites only for the functional categories of "benthos, periphyton" and "benthos". The CWM values of category "benthos, periphyton" was significantly higher at N than at C sites, whereas the opposite was found for the category "benthos" (Table 3, Fig. 7).

3.3. Environmental parameters

For the one-month immersion period, N sites had significantly lower COD values but significantly higher nitrite values than C sites. For the two-months immersion period, N sites had significantly lower COD, pH and orthophosphate values, while conductivity values were higher compared to C sites. A detailed overview of all environmental parameters has been reported in Gulin *et al*. (2022) and can be found in Supplementary Fig. 3.

Organic matter content was lower at the N sites than at C sites in both periods, but with occasional extremely high values, while there were no such extreme outliers at C sites. Inorganic matter content was lower at N sites than at the C sites during the one-month period, and the reverse was true during the two-months period. Chlorophyll *a* concentration was higher at N sites compared to C sites during the one-month period and lower during the two-months period, but with extremely high chlorophyll *a* concentrations during both periods in contrast to N sites. A detailed overview of organic, inorganic matter content and chlorophyll *a* concentration has been reported in Gulin *et al*. (2022) and can be found in Supplementary Fig. 4.

3.4. Effects of environmental parameters on species and functional trait composition

Spearman's correlation coefficient between ciliate species with indicator potential and environmental parameters was performed only for the one-month immersion period because IndVal analysis did not identify any indicator species for the two-months immersion period. *P. vorax* was found to be significantly positively correlated with nitrite concentration ($R = 0.409$, $p = 0.013$). Detailed Spearman's correlation results for the one-month immersion period can be found in Supplementary Fig. 5.

RDA ordination for the one-month immersion period was statistically significant ($F = 2.856$, $p = 0.001$). The first constrained axis (RDA1) described 13.6% of the variance ($F = 6.288$, $p =$

0.001), the second (RDA2) explained 11% of the variance (F = 5.069, p = 0.003), while the third axis (RDA3) explained 8.5% (F = 3.897, p = 0.028) and was therefore not visualized (Fig. 8). Three explanatory variables (environmental parameters) explained 33.1% of the variance in the data: nitrites (F = 5.041, p = 0.001), COD (F = 2.140, p = 0.030), inorganic matter (F = 2.358, p = 0.032). RDA showed that species representative for N sites, such as *P. vorax* and *C. margaritaceum*, were highly correlated with nitrites and organic matter. C sites exhibited stronger grouping compared to N sites. RDA ordination for the two-months immersion period was not statistically significant.

Spearman's correlation coefficient between CWM values and environmental parameters was calculated for both immersion periods. During the one-month immersion period, CWM food source values for the category "bacteria" were significantly negatively correlated with inorganic matter (R = -0.333, p = 0.013), while CWM mode of locomotion values for the category "crawling" were significantly negatively correlated with COD (R = -0.279, p = 0.039). The detailed Spearman correlation results for the one-month period can be found in Supplementary Fig. 6.

As for the two-month immersion period, the CWM ecosystem preference values for the category "soil" were found to be significantly negatively correlated with COD (R = -0.447, p = 0.001) and organic matter (R = -0.303, p = 0.029), while they were significantly positively correlated with pH (R= 0.314, p = 0.024). CWM habitat preference values for the category "benthos, periphyton" were significantly negatively correlated with pH (R = - 0.341, p = 0.013), while the opposite was found for the category "benthos" (R = 0.381, p = 0.005). A significant negative correlation between the category "benthos" and COD was also found (R = -0.276, p = 0.048). The detailed Spearman's correlation results for the two-months period can be found in Supplementary Fig. 7.

Only significantly different CWM values between C and N sites were used for RDA ordination for both immersion periods. RDA ordination of CWM values and environmental parameters for the one-month immersion period was found to be statistically significant (F = 2.067, p = 0.001). The first constrained axis (RDA1) described 11.4% of the variance (F = 6.891, p = 0.001). It was found that the first unconstrained axis (PC1) represented 23.4% of the total variance. None of the explanatory variables (environmental parameters) had a significant impact on the CWM values (Fig. 9).

The RDA ordination of CWM values and environmental parameters for the two-months immersion period proved to be statistically significant (F = 3.521, p = 0.006). The first constrained axis (RDA1) described 20.8% of the variance (F = 12.979, p = 0.004) while the first unconstrained axis (PC1) represented 56.8% of the total variance. Significant correlations were found between CWM habitat preference values for the category "benthos, periphyton" and COD (F = 3.776, p=0.041) (Fig. 10).

4. Discussion

4.1. Species with indicator potential for restored tufa-depositing streams

Previous study by Gulin *et al*. (2022) showed that several taxonomic metrics of ciliates were higher in restored streams than in control streams at the Skradinski buk tufa barrier for both immersion periods, while functional metrics such as functional dispersion (FDis) were lower during the one-month immersion period but higher during the two-months period. This study investigated further the possible reasons for these results by examining the differences in ciliate species and functional trait composition between restored and control streams; highlighting species that are characteristic and distinctive of restored and control streams (i.e. species with indicator potential); and understanding the environmental parameters that influence the composition of ciliate species and functional traits.

The results of this study revealed that although restored streams had higher species richness than the control streams during both immersion periods, their communities were strongly dominated by few species in terms of relative abundance. Contrary to that pattern, control streams had comparable share of 6 to 7 species. This was more pronounced during the one-month immersion period, when the colpodid *P. vorax* accounted for nearly 73% of the total ciliate abundance in restored streams, while the other species such as *C. margaritaceum* and *C. uncinata* accounted for only 5-10%. *P. vorax* was also identified as the only one with indicator potential for restored tufa-depositing streams, suggesting that it was not only abundant but also common in the periphyton of restored streams during the entire study period, which included repeated sampling. However, during the two-months immersion period, the community structure of the periphyton of restored streams showed less pronounced dominance of certain species, and proportions of species with the highest relative abundance, such as *P. vorax*, *C. uncinata*, *Holophrya* sp. and *C. alpestris*, were more or less equal.

4.2. Differences in ciliate functional traits between control and restored streams – restored tufa-depositing streams as a dynamic interface between terrestrial and aquatic ecosystems

The strong dominance of a few species in terms of relative abundance at restored streams was reflected in functional diversity during the one-month immersion period most likely because these species performed the same functional roles, resulting in higher competition between species and within species (Hooper *et al*., 2005). Restored streams favoured better competitors such as *P. vorax*. Species from the genus *Platyophrya* have several adaptations to highly competitive habitats such as being free-swimming (vs. periphyton crawling) and having bacteriophagous microstomes (Foissner and Wolf, 2009). This led to lower functional diversity at restored streams compared to control streams and was also evident regarding the species feeding on phagotrophic protists and small metazoans, which were significantly less abundant in restored streams than in the control streams as they are selective feeders. On the other hand, a longer immersion period, i.e. two-months immersion period, allowed the separation of functional niches, i.e., niche partitioning (Loreau and Hector, 2001) in restored streams, which facilitated species coexistence by reducing competition (DeLong and Vasseur, 2012). These results highlight the importance of study design in periphyton-based studies, as sampling periphyton that has only been immersed in water for a short period of time can lead to potentially incorrect conclusions about stream restoration outcomes and success evaluation.

The most abundant ciliate species of restored streams indicate a close link between terrestrial and aquatic ecosystems, both in terms of taxonomic and functional trait composition: *P. vorax*, *C. margaritaceum*, *C. uncinata*, and *C. alpestris* are frequently found not only in aquatic (freshwater) ecosystems, but also in soils and nutrient-rich man-made systems such as activated sludge (Foissner, 2016) in contrast to usually oligotrophic tufa-depositing systems. Restored streams in our study drained the forest floor that was previously completely dry, and it appears that this was the predominant factor affecting species and functional trait composition of ciliates in restored tufa depositing streams, suggesting that soil drainage in restored streams highly affects periphyton structure and ecosystem function.

Soil ciliate species are generally bacterivores, as was the case with the most species characteristic for restored streams in our study (*C. margaritaceum*, *C. uncinata* and *C. alpestris*), with the exception of *P. vorax*, which is an omnivorous species meaning that it feeds on other food sources additionally to bacteria (Esteban and Fenchel 2021). By grazing, ciliates control the size and composition of bacterial communities, indirectly regulating decomposition processes (Jia *et al*., 2021). The functional category of soil species showed a significant positive correlation with pH and a negative correlation with COD and organic matter in our study. Because restored streams often had higher nitrite concentrations than control streams due to increased drainage favouring reduced organic forms (e.g., nitrites), their concentration may have influenced the organic matter decomposition process and bacterial communities, indirectly affecting the abundance of soil ciliate species. Dissolved forms of nitrogen such as nitrites have the potential to affect organic matter decomposition depending on their concentration and pH (Riggs and Hobbie, 2016). Higher concentrations of dissolved forms of nitrogen can promote microbial growth and thus the decomposition process (Riggs and Hobbie, 2016), resulting in more food for ciliates, as found in the present study where the abundance of *P. vorax* was significantly positively correlated with nitrite concentration. However, when the concentration of dissolved nitrogen forms exceeds a threshold, it can lead to a reduction in microbial biomass (Jing *et al*., 2021) and consequently to a reduction in food for most soil ciliate species. This may have been the case in our study, but more data on carbon and nitrogen fluxes in restored streams are needed to better understand and verify these conclusions. *P. vorax* has recently been associated with the degradation of refractory carbon sources in terrestrial ecosystems (Pastorelli *et al*., 2022) and may therefore play an important role in carbon fluxes of restored streams as they seem to represent an interface between aquatic and terrestrial ecosystems.

Unlike control streams, restored streams in our study supported more motile, free-swimming ciliate species. These findings are consistent with the observed trends of Risse-Buhl and Kusel (2009), who showed that periphyton in streams with high flow rates (in our case restored streams) is dominated by highly motile, flattened, gulper feeding ciliates such as *P. vorax*, *C. uncinata*, and *C. alpestris* in our study. Occasional events of high flow in restored streams reported by Gulin *et al*. (2022) also seems to have benefited species characterized as planktonic such as *C. margaritaceum* but more during the one-month immersion period than the two-months immersion period. Planktonic species probably appeared with seston from the upstream Visovac Lake, which can be a source of planktonic organisms along lotic stretches in the study area as it has already been reported in Gulin *et al.* (2021). Risse-Buhl and Kusel (2009) also showed that flattened, filter-feeding ciliates dominate at slow flow rates, as was the case for species found in control streams, such as crawling *A. cicada* and semi-sessile *V. aquadulcis*-complex. Control sites favoured crawling species commonly found in stream benthos and periphyton of lentic and lotic parts along the Krka River (Kulaš *et al*., 2021; Gulin *et al*., 2021; Gulin Beljak *et al*., 2022).

Besides of flow velocity, the predominance of motile ciliates in restored streams could be a consequence of microhabitat complexity, a parameter we did not quantify in our study and which could be the missing explanatory variable in our RDA (evident from the higher representation of unconstrained axis in total variance compared to constrained). The combination of soil particles drained from the local soil, deposited tufa and retention of organic matter can increase periphyton heterogeneity in restored streams and provide a greater range of resource niches. These niches might be better exploited by motile, free-swimming than crawling ciliates, as they are likely better able to avoid being buried by the intense tufa deposition. The urostilids recorded in our study, such as the genera *Oxytricha*, *Tachysoma*, and *Urosomoida*, seem to support this idea. These ciliates possess morphological adaptations that allow them to hide or hunt among or within particle aggregates – most are short to long elliptical in outline, ventrally flattened, and dorsally distinctly vaulted (Berger, 1999). Although their relative abundance was not comparable to that of other species in the community and therefore they were not recognized as potential indicators of restored streams, they were more abundant in restored streams than in control streams (Gulin *et al*., 2022).

Heterogeneity, i.e., habitat complexity has often been considered in successful stream restoration efforts (Baattrup-Pedersen *et al*., 2022), but usually at the reach level (i.e., channel morphological features) rather than at the microhabitat level. Higher heterogeneity achieved through stream restoration has been associated with shifts in the functional composition of macroinvertebrate communities (Frainer *et al*., 2017; Hasselquist *et al*., 2018), but periphyton-level responses have been little studied (Huang *et al*., 2018) and require further attention. In their most recent comprehensive literature review, Baattrup-Pedersen *et al*. (2022) call for more studies that use functional parameters to evaluate stream restoration efforts of all ecosystem types at different spatial and temporal scales to allow for more robust comparisons between natural and restored ecosystems. Phagotrophic protists such as ciliates provide excellent model systems for studying some fundamental effects of ecological interactions on functional traits, that may also apply to larger organisms (Montagnes *et al.* 2012), so the results of this study may reveal potential responses of macroinvertebrates or fishes in the long term. Given the ubiquity of most ciliate species, as well as community structure in similar habitats (Fenchel and Finlay, 2004), a comparable functional response to restoration might be expected in freshwater ecosystems with high levels of sedimentation or in other karstic waters that have close links to terrestrial ecosystems.

5. Conclusions

Our results emphasize the potential value of functional traits as a tool for assessing the success of ecological restoration at the microscale. Future evaluation of the success of stream restoration in tufa-depositing environments should incorporate data on traditional taxonomic metrics, but also data on functional traits of substrate-related biota (periphyton, benthos) and quantifiable measures of microhabitat heterogeneity. In addition, it would be useful to include measures of organic matter decomposition (carbon and nitrogen fluxes) to enable quantification of microbially mediated decomposition for a comprehensive assessment of ecological responses to stream restoration.

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Tables

Table 1. Details of the sampling sites at Skradinski buk barrier and their descriptions.

Site	Coordinates	Details
C1	15.966381, 43.805752	site located in control stream where water had been present before and after the removal of *Ailanthus altissima* (Mill.) Swinge and displaying well-developed moss cover
C2	15.966279, 43.805772	site located in control stream without moss cover
N1	15.965449, 43.806438	site located in restored stream
N2	15.965324, 43.806624	site located in restored stream
N3	15.965246, 43.806541	site located in restored stream
N4	15.965186, 43.806489	site located in restored stream
N5	15.965538, 43.806225	site located in restored stream

Table 2. Details of the sampling events conducted between May 2019 and June 2020.

Immersion period	Season	Slides placed	Slides collected
One-month immersion period	Spring	May 10, 2019	June 10, 2019
	Summer	June 10, 2019	July 15, 2019
	Autumn	September 30, 2019	November 3, 2019
	Winter	February 2, 2019	March 8, 2020
Two-months immersion period	Spring	March 8, 2020	June 5, 2020*
	Summer	June 10, 2019	August 15, 2019
	Autumn	September 30, 2019	December 2, 2019
	Winter	December 2, 2019	February 2, 2020

*immersion period prolonged due to COVID-19 pandemic

Table 3. GLMM model output showing differences in community weighted mean (CWM) values of functional traits between control and restored streams (one-month and two-months immersion periods) with sites as fixed effects, and season and replicate as random effects. Only statistically significant fixed effects (p) and pairwise contrasts (P) obtained from the least significant difference post hoc test (p<0.05) are reported. Legend: F – F statistic; d.f. – degrees of freedom.

	Assemblage parameter	F	p	d.f.	d.f. corrected	P C-N
One-month	**CWM food source**					
	CWM bacteria	4.810	0.048	1	53	0.049
	CWM phagotrophic protists, small metazoans	16.031	<0.001	1	53	<0.001
	CWM Ecosystem preference					
	CWM lentic (freshwater)	10.186	0.001	1	53	0.001
	CWM lentic and lotic (freshwater)	4.314	0.043	1	53	0.045
	CWM soil	4.349	0.045	1	53	0.047
	CWM Habitat preference					
	CWM benthos, periphyton	21.676	<0.001	1	53	<0.001
	CWM benthos	5.066	0.029	1	53	0.031
	CWM planktonic	6.029	0.028	1	53	0.028
	CWM Mode of locomotion					
	CWM free-swimming	15.659	<0.001	1	53	<0.001
	CWM crawling	6.053	0.026	1	53	0.028
Two-months	**CWM Ecosystem preference**					
	CWM soil	14.468	<0.001	1	50	<0.001
	CWM Habitat preference					
	CWM benthos, periphyton	4.566	0.043	1	50	0.045
	CWM benthos	5.938	0.017	1	50	0.019

Captions of figures

Fig. 1. Location of Croatia within the Mediterranean region (A) and the Krka River catchment with the location of Skradinski buk barrier (B)

Fig. 2. Differences in community-weighted mean (CWM) values (± standard error) of food source functional trait categories in ciliate communities between control (C) and restored streams (N) for the one-month immersion period. Functional trait categories showing significant differences between C and N sites are indicated by asterisk (*) in the legend.

Fig. 3. Differences in community weighted mean (CWM) values (± standard error) of ecosystem preference functional trait categories in ciliate communities between control (C) and restored streams (N) during the one-month immersion period. Functional trait categories showing significant differences between C and N sites are indicated by asterisk (*) in the legend.

Fig. 4. Differences in community weighted mean (CWM) values (± standard error) of habitat preference functional trait categories in ciliate communities between control (C) and restored streams (N) for the one-month immersion period. Functional trait categories showing significant differences between C and N sites are indicated by asterisk (*) in the legend.

Fig. 5. Differences in community weighted mean (CWM) values (± standard error) of mode of locomotion functional trait categories in ciliate communities between control (C) and restored streams (N) for the one-month immersion period. Functional trait categories showing significant differences between C and N sites are indicated by asterisk (*) in the legend.

Fig. 6. Differences in community weighted mean (CWM) values (± standard error) of ecosystem preference functional trait categories in ciliate communities between control (C) and restored streams (N) during the two-months immersion period. Functional trait categories showing significant differences between C and N sites are indicated by asterisk (*) in the legend.

Fig. 7. Differences in community weighted mean (CWM) values (± standard error) of habitat preference functional trait categories in ciliate communities between control (C) and restored streams (N) for the two-months immersion period. Functional trait categories showing significant differences between C and N sites are indicated by asterisk (*) in the legend.

Fig. 8. Redundancy analysis (RDA) showing the relationships between ciliate species (red arrows) and environmental parameters (blue arrows) among control (C) and restored (N) sites for the one-month immersion period. Abbrevations: N = nitrites; COD = chemical oxygen demand; OM = organic matter; IM = inorganic matter; CHL = chlorophyll *a* concentration; *AC = Aspidisca cicada*; *CU = Chilodonella uncinata; CM = Cinetochilum margaritaceum; CYC = Cyclidium* sp.*; HO = Holophrya ovum; PV = Platyophrya vorax; PG = Pleurotricha grandis; PA = Pseudochilodonopsis algivora*; *TC = Trithigmostoma cucullulus*; *VAC = Vorticella aquadulcis*-complex.

Fig. 9. Redundancy analysis (RDA) showing the relationships between CWM values (red arrows) and environmental parameters (blue arrows) among control (C) and restored (N) sites for the one-month immersion period. Abbrevations: N = nitrites; COD = chemical oxygen demand; OM = organic matter; IM = inorganic matter; CHL = chlorophyll *a* concentration; FSB = food source "bacteria"; FSPPSM = food source "phagotrophic protists, small metazoans"; EPLF = ecosystem preference "lentic (freshwater)"; EPLLF = ecosystem preference "lentic and lotic (freshwater)"; EPS = ecosystem preference "soil"; HPBP = habitat preference "benthos, periphyton"; HPB = habitat preference "benthos"; HPP = habitat preference "planktonic"; MLFS = mode of locomotion "free-swimming"; MLC = mode of locomotion "crawling".

Fig. 10. Redundancy analysis (RDA) showing the relationships between CWM values (red arrows) and environmental parameters (blue arrows) among control (C) and restored (N) sites for the two-months immersion period. Abbrevations: COD = chemical oxygen demand; OM = organic matter; CHL = chlorophyll *a* concentration; EPS = ecosystem preference "soil"; HPBP = habitat preference "benthos, periphyton"; HPB = habitat preference "benthos".

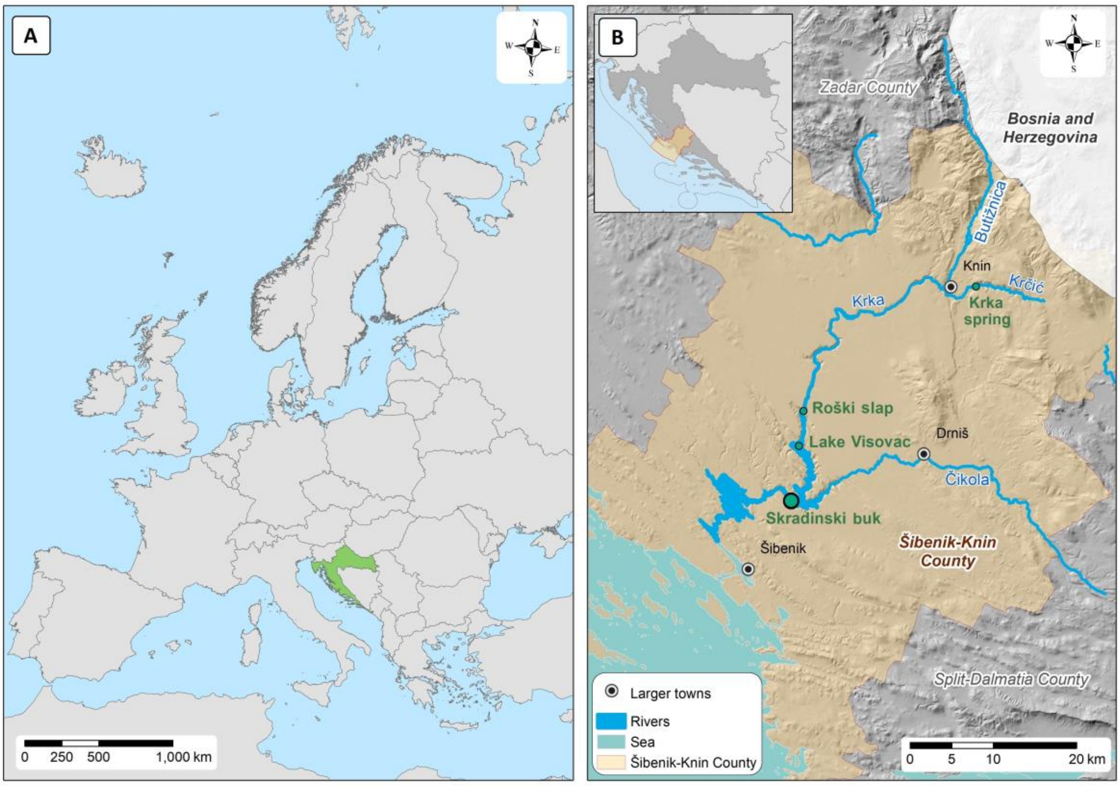

A
0 250 500 1,000 km
B
Zadar County
Bosnia and Herzegovina
Butižnica
Knin
Krčić
Krka
Krka spring
Roški slap
Lake Visovac
Drniš
Čikola
Skradinski buk
Šibenik
Šibenik-Knin County
Split-Dalmatia County
Larger towns
Rivers
Sea
Šibenik-Knin County
0 5 10 20 km

Fig. 1.

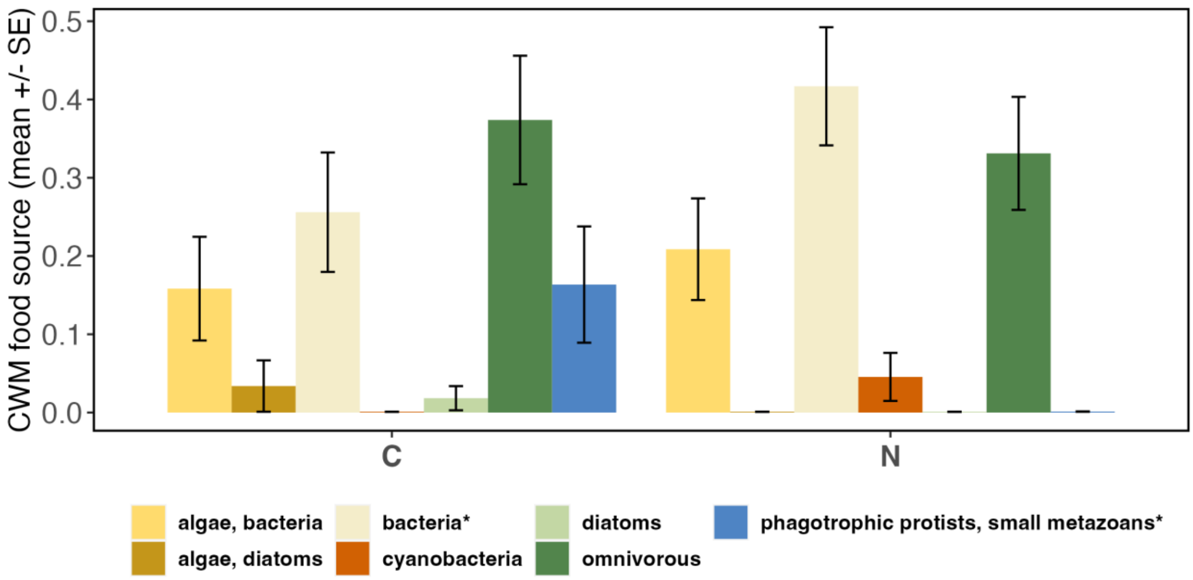
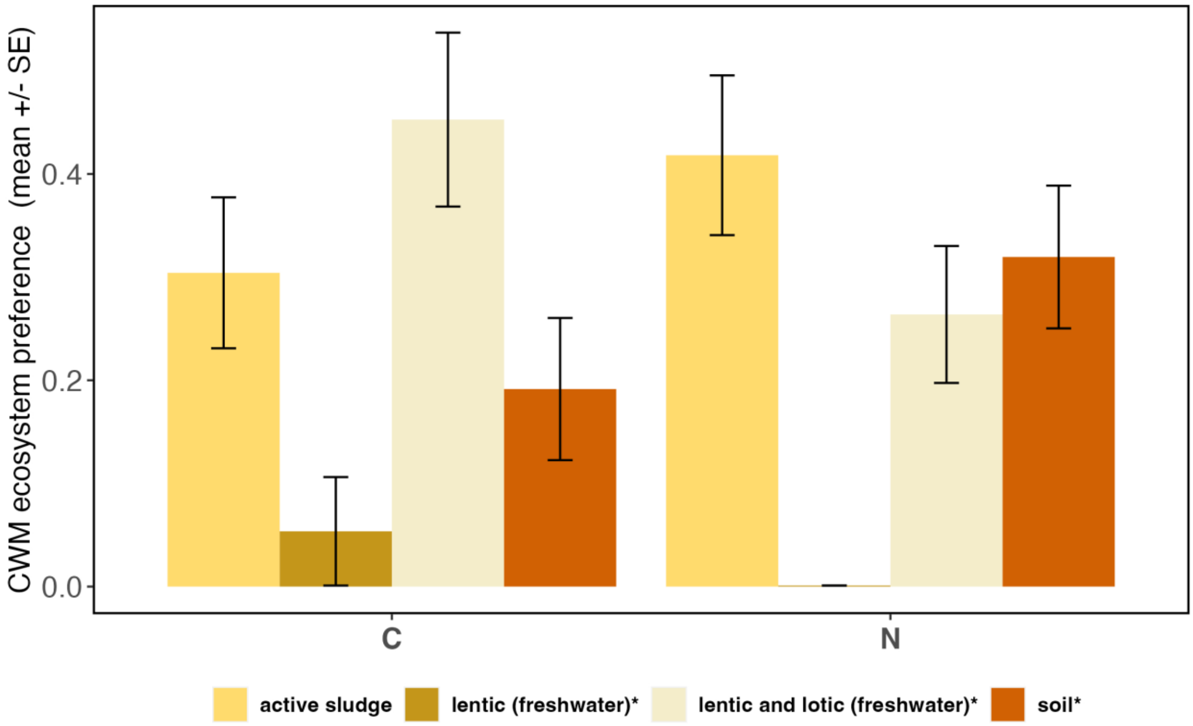
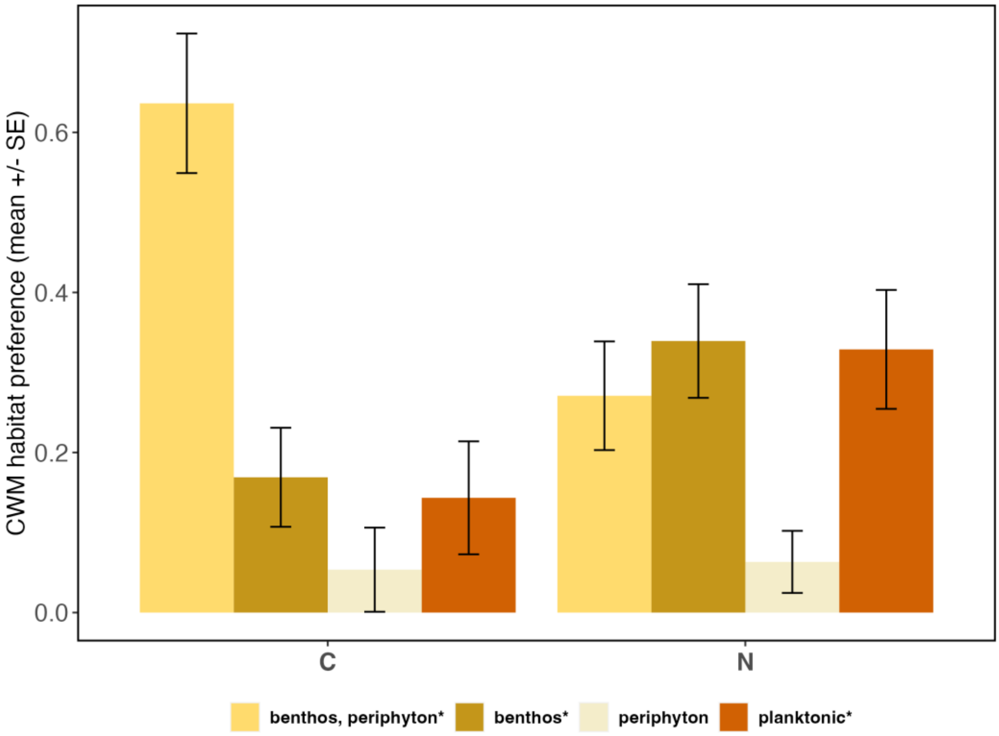

CWM food source (mean +/- SE)
0.0
0.1
0.2
0.3
0.4
0.5
C
N
algae, bacteria
bacteria*
diatoms
phagotrophic protists, small metazoans*
algae, diatoms
cyanobacteria
omnivorous

Fig. 2.



CWM ecosystem preference (mean +/- SE)
0.0
0.2
0.4
C
N
active sludge
lentic (freshwater)*
lentic and lotic (freshwater)*
soil*

Fig. 3.



CWM habitat preference (mean +/- SE)
0.0
0.2
0.4
0.6
C
N
benthos, periphyton*
benthos*
periphyton
planktonic*

Fig. 4.

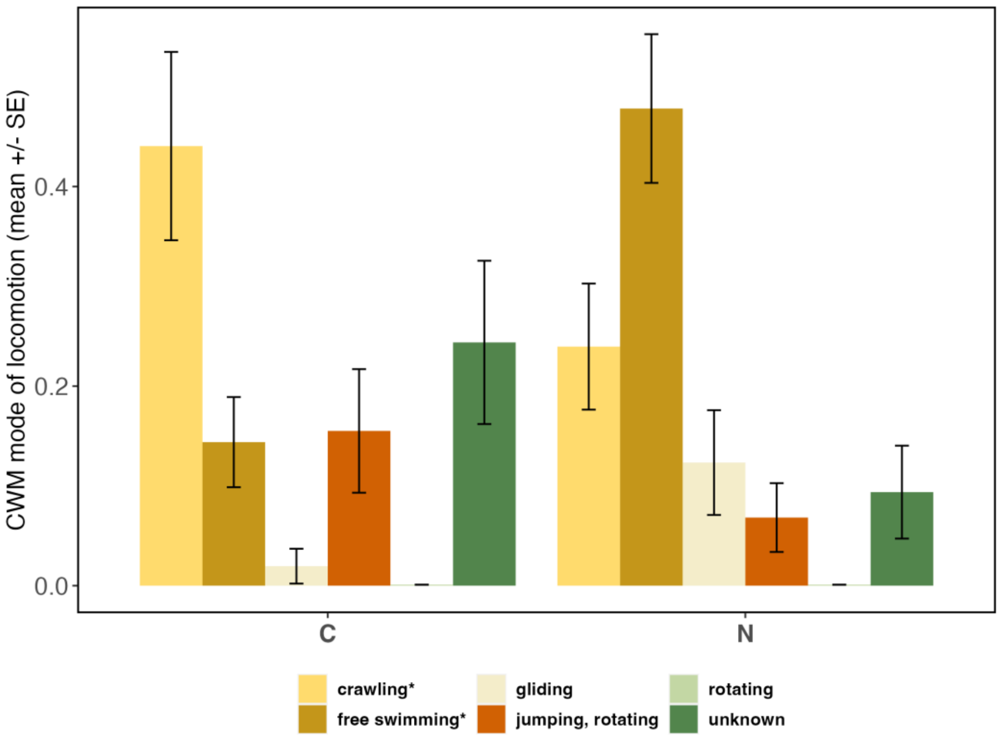

CWM mode of locomotion (mean +/- SE)
0.0
0.2
0.4
C
N
crawling*
free swimming*
gliding
jumping, rotating
rotating
unknown

Fig. 5.

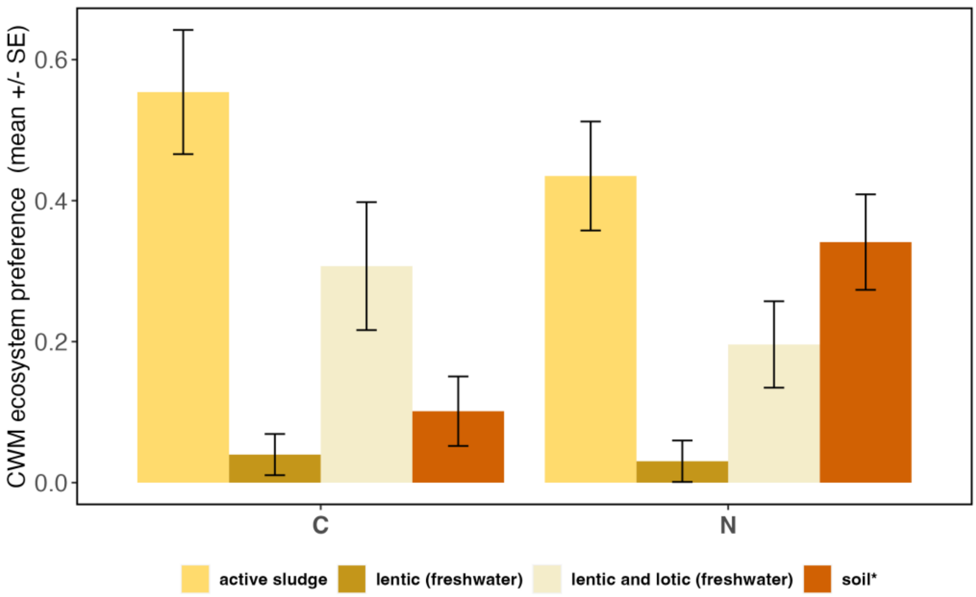

CWM ecosystem preference (mean +/- SE)
0.0
0.2
0.4
0.6
C
N
active sludge
lentic (freshwater)
lentic and lotic (freshwater)
soil*

Fig. 6.

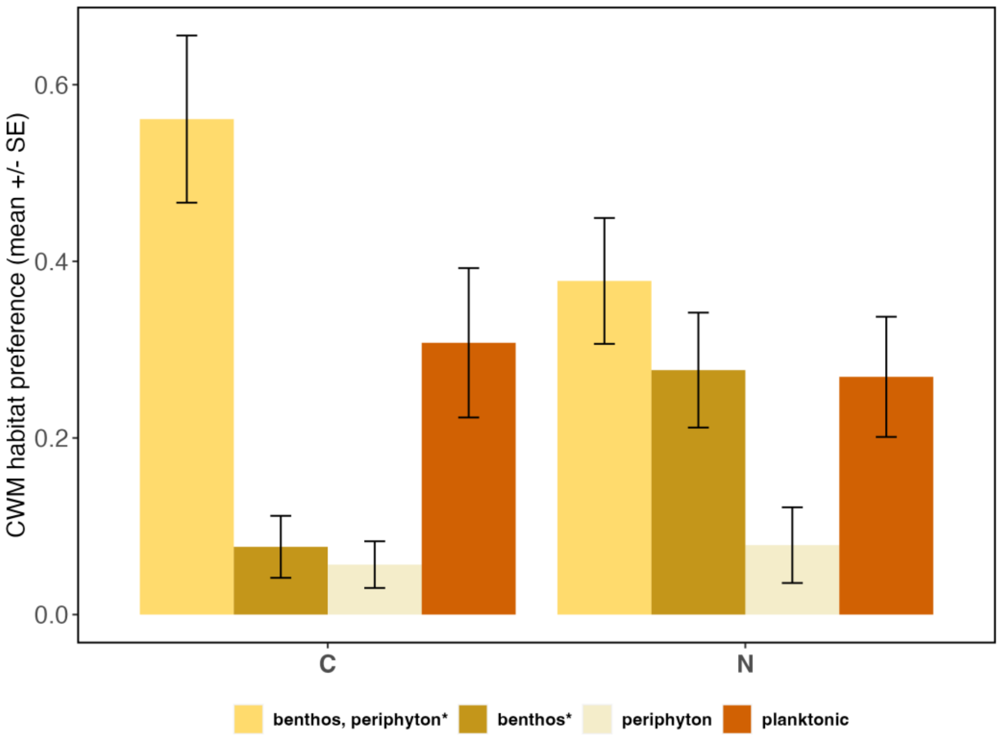

CWM habitat preference (mean +/- SE)
0.0
0.2
0.4
0.6
C
N
benthos, periphyton*
benthos*
periphyton
planktonic

Fig. 7.

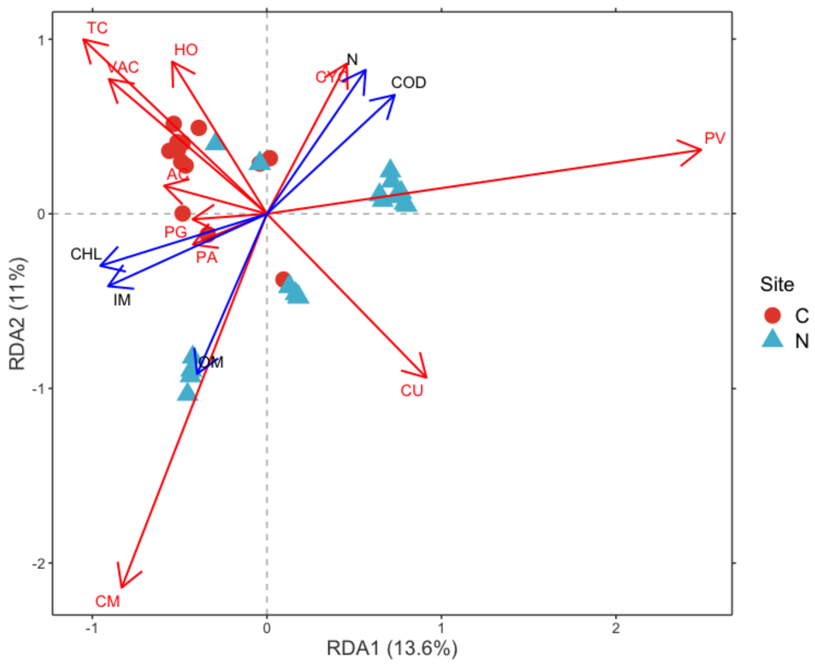

TC
VAC
HO
CY
N
COD
PV
AC
PG
PA
CHL
IM
OM
CU
CM
RDA1 (13.6%)
RDA2 (11%)
Site
C
N

Fig. 8.

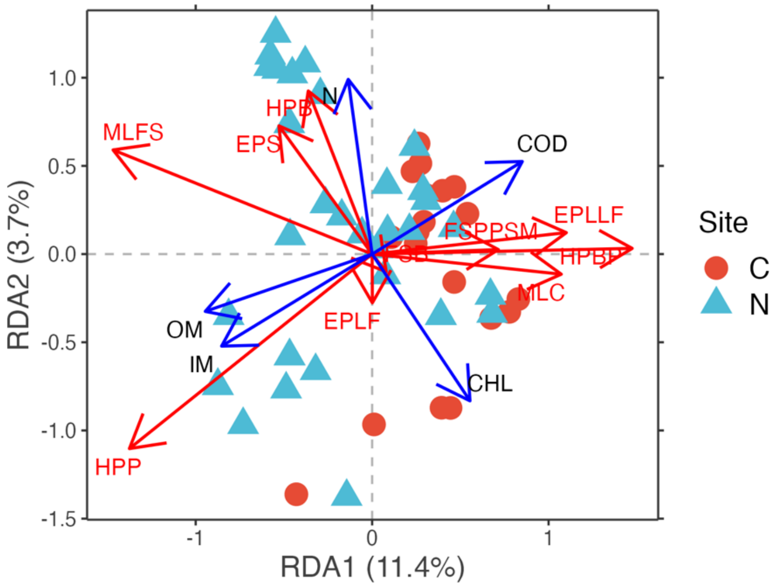

RDA2 (3.7%)
RDA1 (11.4%)
MLFS
HPB
EPS
N
COD
EPLLF
FSPPSM
HPBP
MLC
EPLF
OM
IM
CHL
HPP
Site
C
N

Fig. 9.

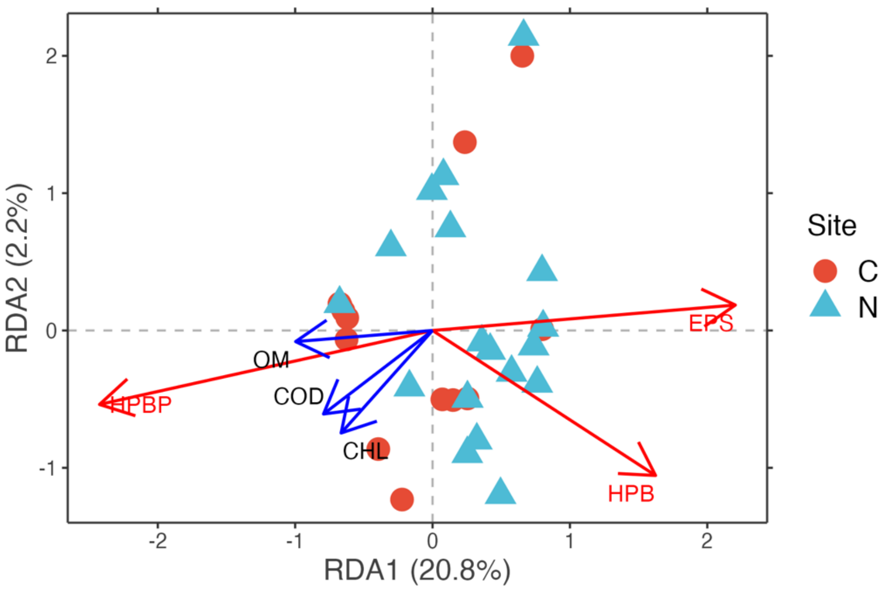
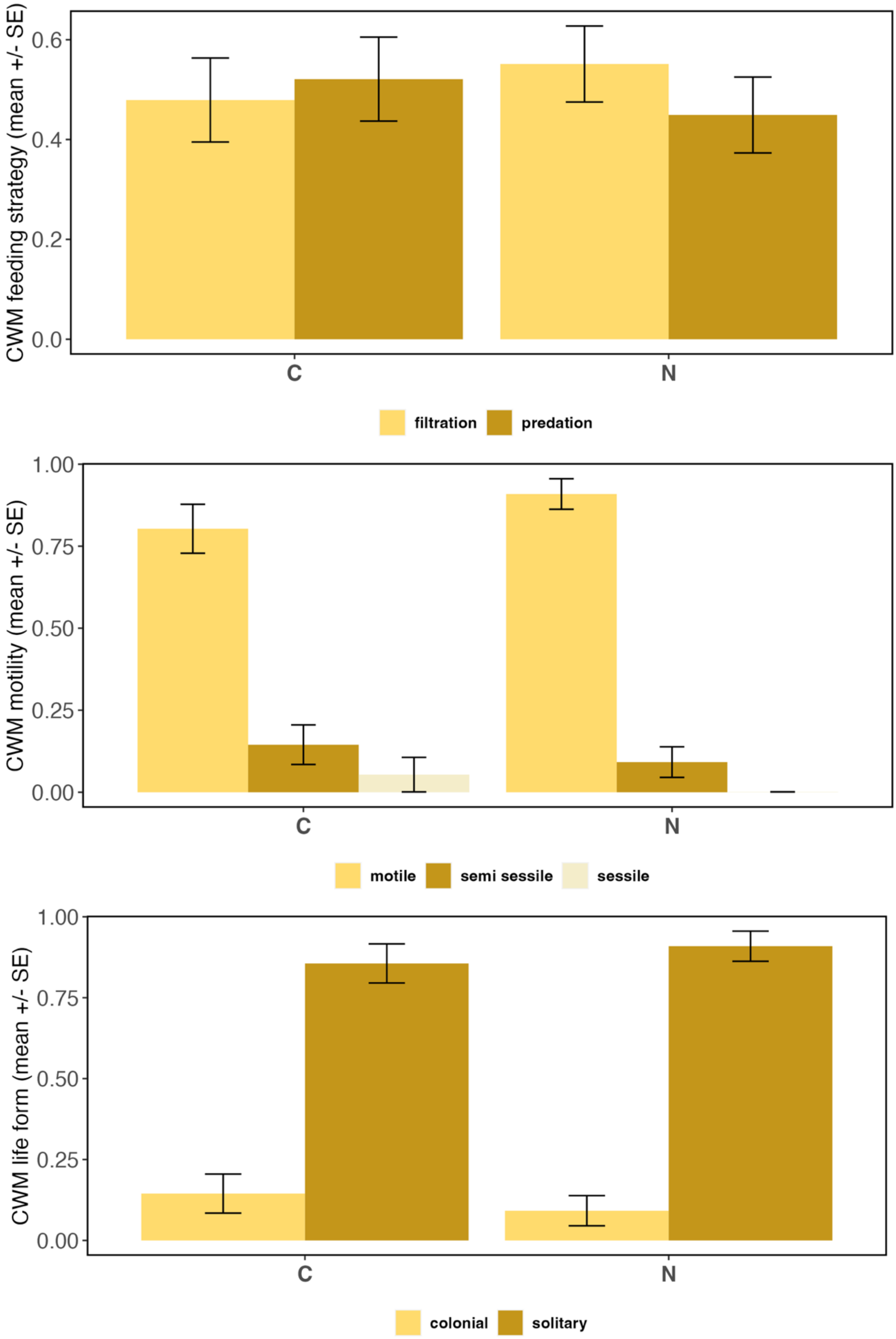
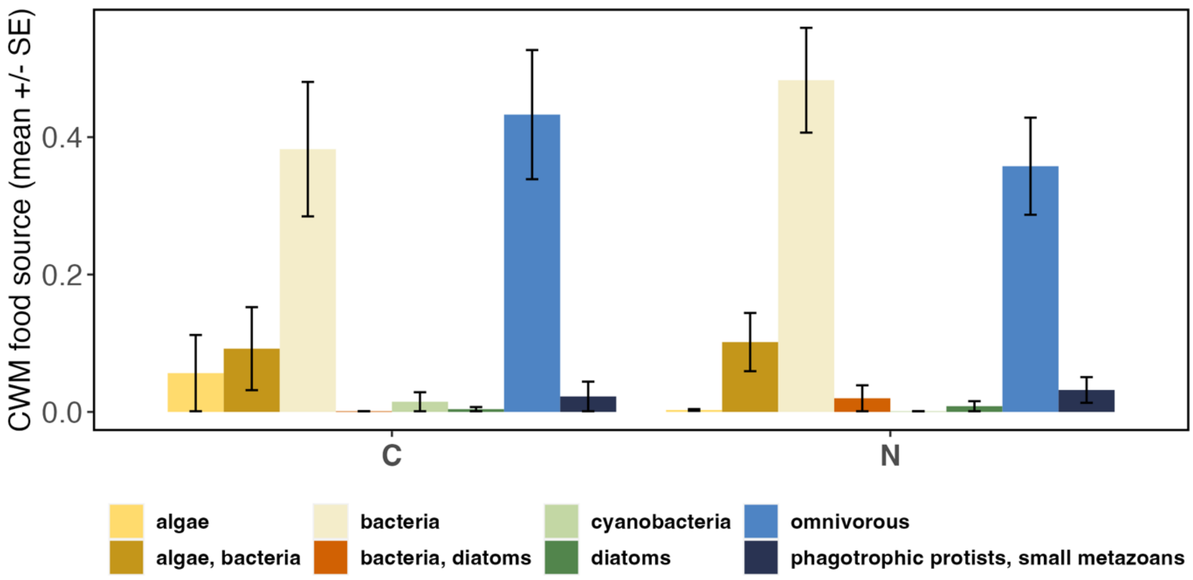
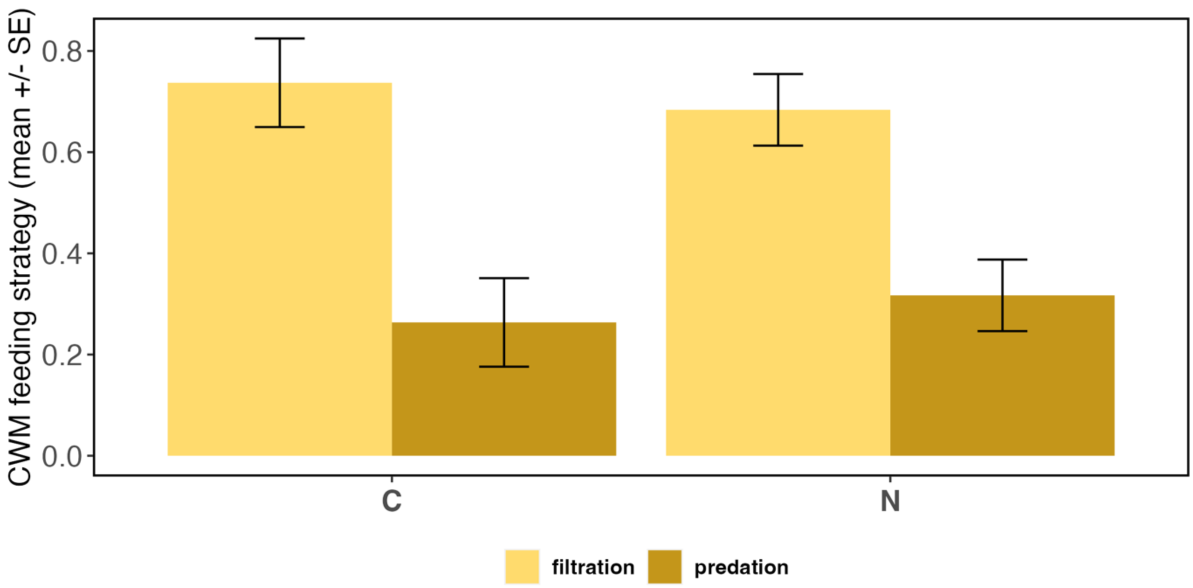

RDA2 (2.2%)
RDA1 (20.8%)
EPS
OM
HPBP
COD
CHL
HPB
Site
C
N

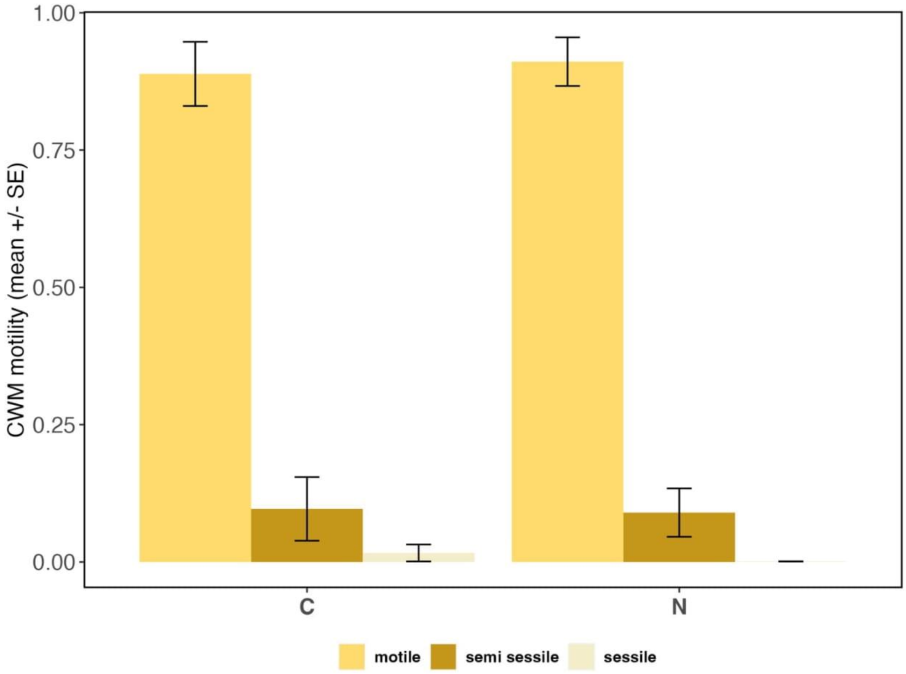
Fig. 10.

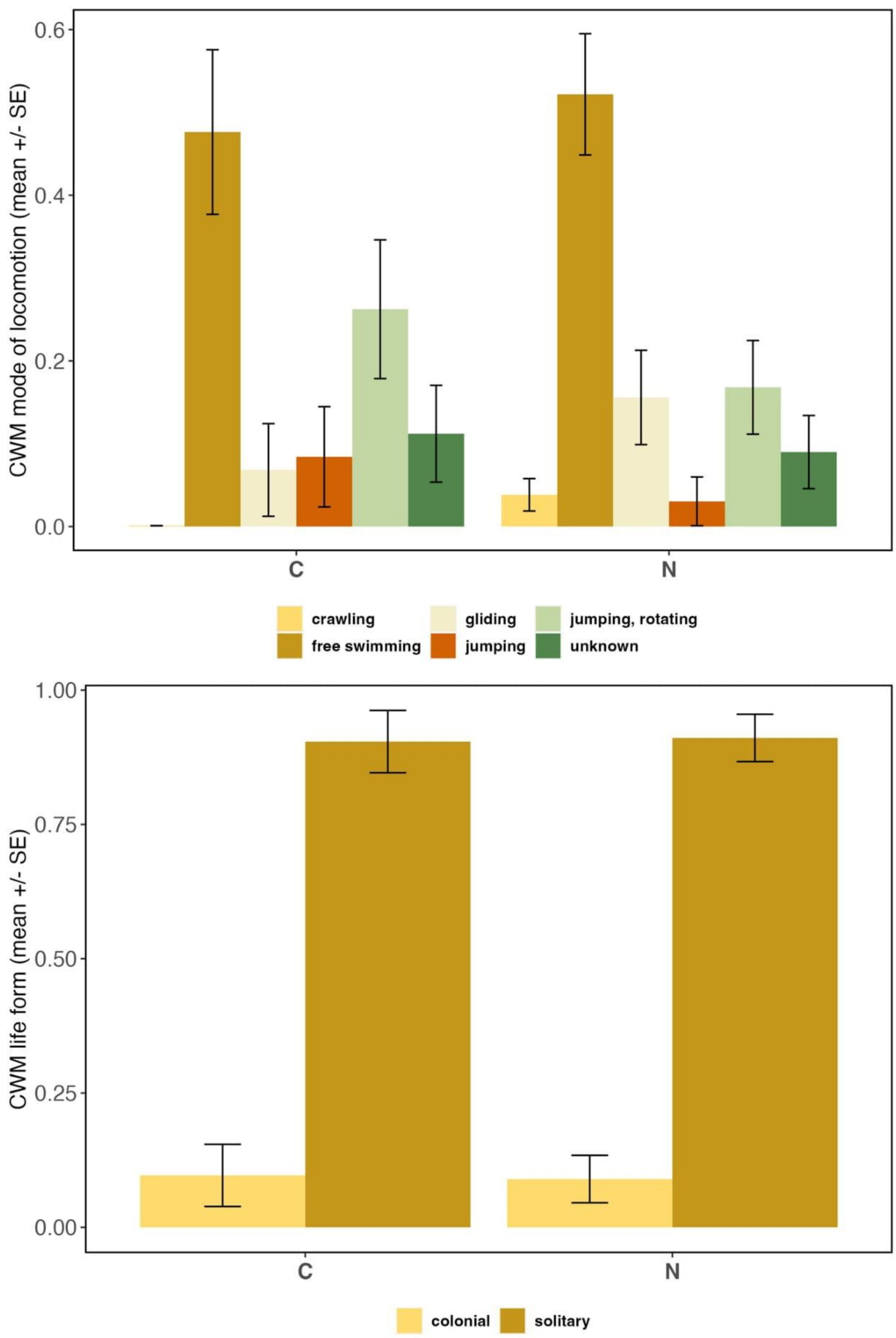


Supplementary Fig. 1. Differences in community-weighted mean (CWM) values (± standard error) of feeding strategy; motility; and life form functional trait categories in ciliate communities between control (C) and restored streams (N) for the one-month immersion period.

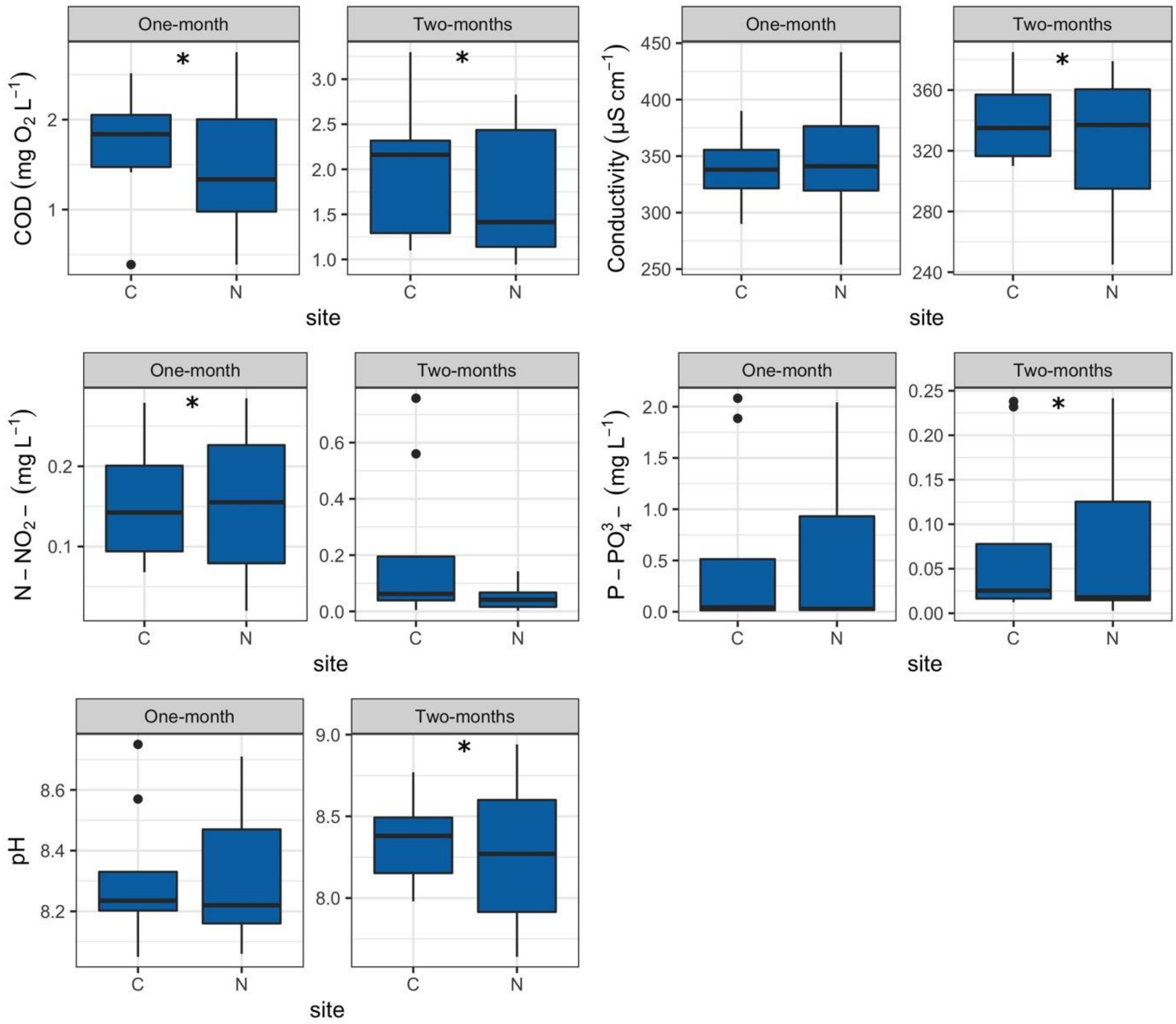

CWM food source (mean +/- SE)
0.0
0.2
0.4
C
N
algae
algae, bacteria
bacteria
bacteria, diatoms
cyanobacteria
diatoms
omnivorous
phagotrophic protists, small metazoans


CWM feeding strategy (mean +/- SE)
0.0
0.2
0.4
0.6
0.8
C
N
filtration
predation

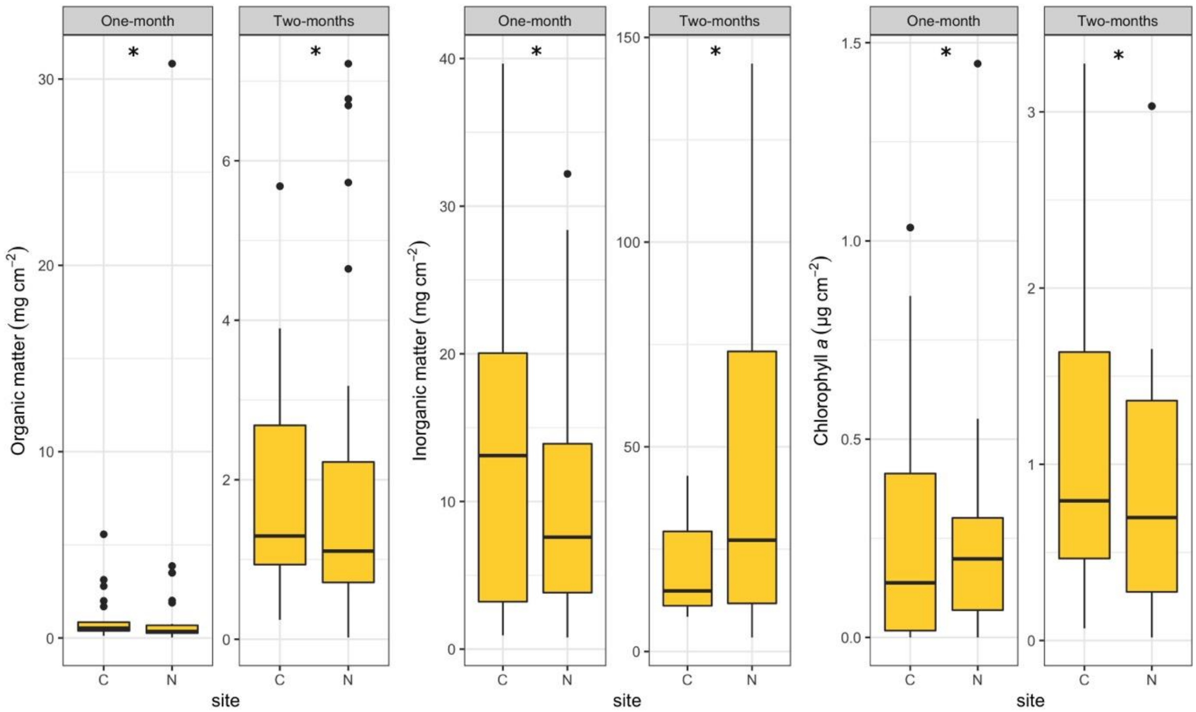

1.00
0.75
0.50
0.25
0.00
CWM motility (mean +/- SE)
C
N
motile
semi sessile
sessile



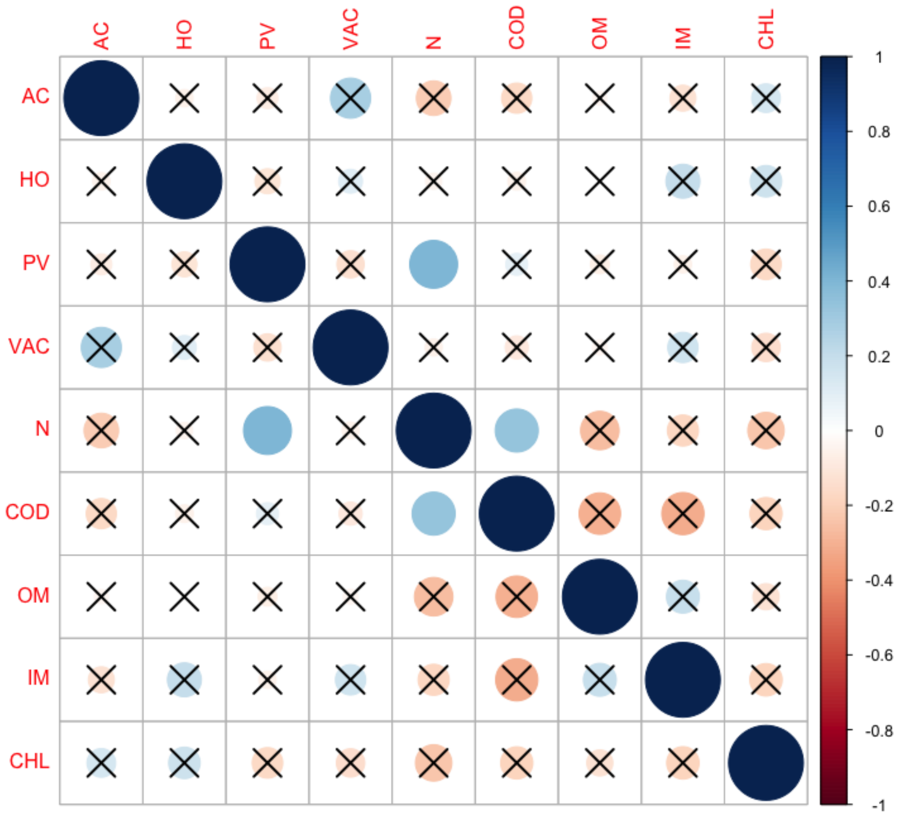
Supplementary Fig. 2. Differences in community weighted mean (CWM) values (± standard error) of food source; feeding strategy; motility; mode of locomotion; life form functional trait categories in ciliate communities between control (C) and restored streams (N) during the two-months immersion period.



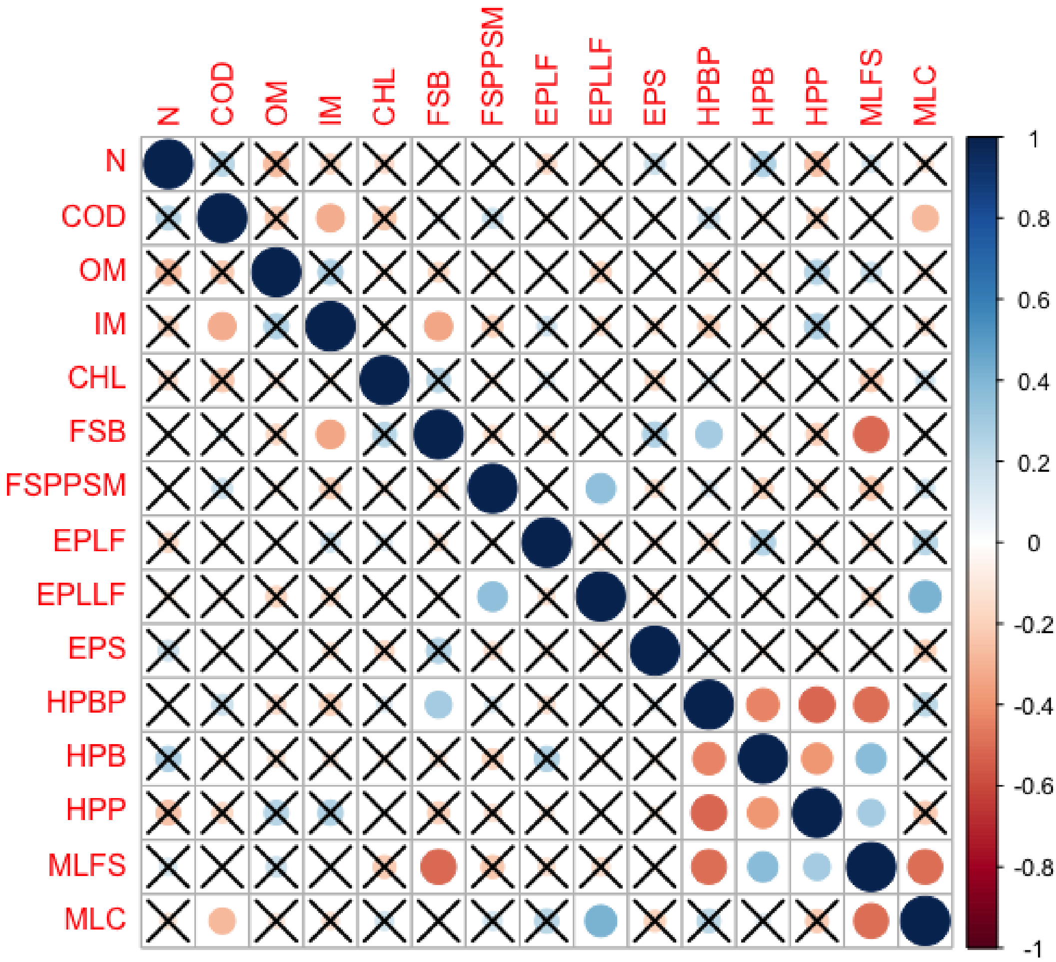
Supplementary Fig. 3. Box plots showing selected environmental parameters at control (C) and restored sites (N) for the one-month and two-months immersion periods. Asterisk symbol (*) indicates statistically significant differences among estimated means (ANOSIM, p<0.05). Upper and lower edges of the boxes are the first and third quartiles; the line inside the box represents the median; individual dots are outliers. Abbreviations: COD - total chemical oxygen demand, N-NO_2^- - nitrites, P-PO_4^{3-} - orthophosphates.



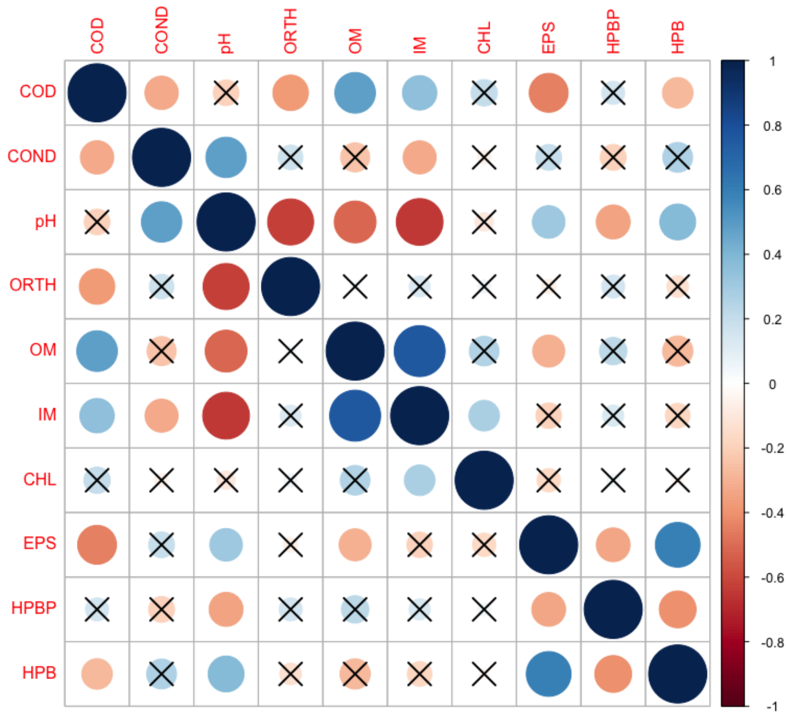
Supplementary Fig. 4. Box plots showing organic matter and inorganic matter content; and chlorophyll *a* concentrations at control (C) and restored sites (N) for the one-month and two-months immersion periods. Asterisk symbol (*) indicates statistically significant differences among estimated means (ANOSIM, p<0.05). Upper and lower edges of the boxes are the first and third quartiles; the line inside the box represents the median; individual dots are outliers.



Supplementary Fig. 5. Pairwise Spearman's rank correlation matrix of ciliate taxa abundance and environmental parameters for the one-month immersion period. Only taxa identified by Indicator species analysis (IndVal) were used for the purpose of correlation. Circle size reflects the magnitude of the correlation coefficient. Color represents the level of Spearman's correlations (blue means positive correlation and red means negative correlation). X represents statistically insignificant correlations (p>0.05). Abbrevations: *AC* = *Aspidisca cicada*, *HO* = *Holophrya ovum, PV* = *Platyophrya vorax, VAC* = *Vorticella aquadulcis*-complex*;* N = nitrites; COD = chemical oxygen demand; OM = organic matter; IM = inorganic matter; CHL = chlorophyll *a* concentration.



Supplementary Fig. 6. Pairwise Spearman's rank correlation matrix of community weighted mean (CWM) values of functional trait categories and environmental parameters for the one-month immersion period. Only categories that were significantly different according to GLMMs were used for correlation. Circle size reflects the magnitude of the correlation coefficient. Color represents the level of Spearman's correlations (blue means positive correlation and red means negative correlation). X represents statistically insignificant correlations ($p>0.05$). Abbrevations: N = nitrites; COD = chemical oxygen demand; OM = organic matter; IM = inorganic matter; CHL = chlorophyll *a* concentration; FSB = food source "bacteria"; FSPPSM = food source "phagotrophic protists, small metazoans"; EPLF = ecosystem preference "lentic (freshwater)"; EPLLF = ecosystem preference "lentic and lotic (freshwater)"; EPS = ecosystem preference "soil"; HPBP = habitat preference "benthos, periphyton"; HPB = habitat preference "benthos"; HPP = habitat preference "planktonic"; MLFS = mode of locomotion "free-swimming"; MLC = mode of locomotion "crawling".



Supplementary Fig. 7. Pairwise Spearman's rank correlation matrix of community weighted mean (CWM) values of functional trait categories and environmental parameters for the two-months immersion period. Only categories that were significantly different according to GLMMs were used for correlation. Circle size reflects the magnitude of the correlation coefficient. Color represents the level of Spearman's correlations (blue means positive correlation and red means negative correlation). X represents statistically insignificant correlations (p>0.05). Abbrevations: COD = chemical oxygen demand; COND= conductivity; ORTH= orthophosphates; OM = organic matter; IM = inorganic matter; CHL = chlorophyll *a* concentration; EPS = ecosystem preference "soil"; HPBP = habitat preference "benthos, periphyton"; HPB = habitat preference "benthos".

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Special Issue
Ciliates as Model Organisms: From 'omics' to Genetics, Ecology and Signaling

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Changes in Phylogenetic and Functional Diversity of Ciliates along the Course of a Mediterranean Karstic River

Vesna Gulin Beljak [1,*], Antonija Kulaš [1], Guillaume Lentendu [2], Barbara Vlaičević [3], Marija Gligora Udovič [1], Mirela Sertić Perić [1], Fran Rebrina [1], Petar Žutinić [1], Sandi Orlić [4] and Renata Matoničkin Kepčija [1]

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Abstract: Ciliates are a group of phagotrophic protists found in a wide variety of ecosystems. This study builds on recent studies of ciliates in the Krka river and investigates changes in the phylogenetic and functional diversity of ciliates in biofilm to predict the phylogenetic and functional structure of ciliates in other karstic rivers. Biofilm samples were collected from four representative locations: upstream (Krka spring), midstream (Marasovine), and downstream (Roški slap, Skradinski buk) of the Krka river to test for differences in phylogenetic and functional diversity of ciliates in relation to location and positioning on tufa stones (light/dark-exposed side of tufa stone). Our results showed that Krka spring had higher phylogenetic species variability, lower phylogenetic diversity, and lower functional richness than Skradinski buk, suggesting phylogenetic overdispersal at Krka spring. This could be due to environmental filtering, competitive exclusion, or a combination of these factors. As the first study of its kind in the Mediterranean, our results shed light on the phylogenetic and functional diversity of ciliates in karst ecosystems and provide a basis for future ecological and conservation efforts.

Keywords: eukaryotes; karst; environmental filtering; freshwater; phagotrophic; competitive exclusion

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1. Introduction

Ciliates (Ciliophora) are a widely distributed group of phagotrophic protists [1]. Because of the various food they consume and the different feeding mechanisms, ciliates are considered essential elements in the trophic webs of freshwater ecosystems [2]. Their ubiquity, abundance, and sensitivity make them excellent bioindicators in a number of different environments, including karst environments [3–5]. Not only are they widely distributed in various types of communities (benthos, biofilm, and plankton) in tufa-depositing ecosystems, but they also show a rapid response in terms of their taxonomic and functional diversity to changing conditions, such as revitalization of tufa barriers [6,7].

Environmental filtering and competitive exclusion have been recognized as main drivers of phylogenetic and functional diversity of protists in different environments [8–10]. While competitive exclusion is thought to be stronger at the local scale (alpha diversity), environmental filtering is considered more likely to influence regional community patterns (beta diversity) [11–13]. These two processes assumingly have opposing effects on community structure with interspecific competition suppressing niche overlap in co-occurring species on the one hand, and environmental filtering promoting ecological similarity on the other [14,15]. When ecological niches are phylogenetically conserved, these two processes are expected to lead to a distinct phylogenetic community structure: competition results in phylogenetic overdispersion (i.e., divergence), and environmental filtering results in phylogenetic clustering [16]. These effects are also reflected in functional aspects, with convergence or divergence in functional diversity [16].

Incorporating multiple diversity indices (beyond taxonomic diversity) through an integrative approach is now considered essential for a comprehensive understanding of ecosystems and their conservation [17]. While phylogenetic diversity can shed light on how evolutionary history has shaped present-day microbial communities, functional diversity allows linking environmental responses and ecosystem functions [18]. Phylogenetic diversity (usually expressed by Faith's PD—the sum of branch lengths of a phylogenetic tree [19]) is a critical component of modern ecology and is considered one of the best predictors of ecosystem function in different ecosystems [20]. Greater PD implies greater variation in functional traits because species in an assemblage are expected to encompass greater spans of independent evolutionary time, allowing them to accumulate greater variation in ecological traits and provide complementary functions [21]. In addition to PD, PSV (phylogenetic species variability index [22]) is one of the most commonly used metrics to illustrate phylogenetic diversity. It quantifies how phylogenetic relatedness reduces the variance of a hypothetical neutral trait shared by all species in a community, indicating phylogenetic dispersion [22].

In addition to phylogenetic alpha diversity, phylogenetic beta diversity measures such as UniFrac [23,24] also provide a valuable source of information as they use similarities and differences between species [25,26]. This additional information makes phylogenetic beta diversity measures more effective in representing ecological patterns, determining whether microbial protist communities (ciliates) are significantly different, identifying individual lineages that contribute to these differences, and revealing broad-scale patterns that span many environmental samples [24,27,28].

Functional diversity (FD) is generally based on species' functional traits, i.e., traits of organisms that influence not only their fitness but also their response to the environment and/or their impact on ecosystem properties community [29]. Higher functional diversity is related to improved ecosystem resilience, which is the ability of a system to absorb shocks, reorganize, and maintain the same structure and function community [30]. One of the key components of functional diversity is functional richness (FRic), defined as the amount of niche space occupied by species within the community [31]. The results of Mouchet et al. [32] show that FRic is highly informative in detecting both environmental filtering and competitive exclusion, and that it more accurately represents the change in dimensionality of functional space caused by community structure than other metrics of functional diversity because it is based on presence/absence rather than abundance data.

According to Weisse [33], the functional categorisation of aquatic ciliates needs to be as detailed as possible, as the use of broad categories and collective terms is not very useful for functional diversity-related analyses. For example, grouping ciliates into (too) broad categories of food source (algivorous, bacterivorous, omnivorous) may be one of the reasons why Van Wichelen et al. [34] did not detect differences in functional composition and diversity between 66 turbid and clear-water shallow lakes in north-western Europe. Therefore, functional traits in this study were assigned in as much detail as possible according to Foissner's classification [3,35] and the latest revision by Adl et al. [36]. Ciliates were assigned to categories for the following functional traits: motility (euplanktonic; motile; semi-sessile; sessile; unknown), life form (colonial; solitary; unknown), mode of locomotion (crawling/creeping; drifting; free-swimming; gliding; jumping; rotating; unknown), habitat preference (active sludge; active sludge, soil; anaerobic mud; brackish; lentic and lotic (freshwater); marine; soil; symbiotic), food source (algae; algae, bacteria; algae, cyanobacteria; algae, diatoms; astomata; bacteria; cyanobacteria; diatoms; fungi; histophagous; omnivorous; parasitic; phagotrophic protists, small metazoans; unknown), and feeding strategy (filtration; filtration, facultative predation; predation; unknown).

This set of traits has proven useful in several recent studies concerning ciliates [37,38] and in assessing the functional response of ciliates to stream revitalization at the Skradinski buk tufa barrier, Krka river [7]. Assignment of functional traits, i.e., categorisation, was based on coding of data obtained after careful review of the literature. For example, we found literature data relevant to habitat preference for 11 species of the genus *Vorticella*, and more than 50% of these species occurred in active sludge in addition to lotic and lentic habitats, so the genus *Vorticella* was assigned "active sludge". This simply means that the genus occurs in active sludge in addition to the predominant freshwater habitats. The reason for distinguishing "active sludge" as a separate trait was simply to highlight that some of the genera detected in the study also occur in nutrient-rich habitats and can reach high abundances (in contrast to usually oligotrophic tufa-depositing systems). This reasoning was also applied for the "soil" and "marine". The same coding principle was applied to all other categories (food source, feeding strategy, motility, mode of locomotion, and life form). If we found that more than 50% of the species within the genus fed on something other than just two types of food, they were classified as "omnivorous"; otherwise, their food source was differentiated into two dominant sources, e.g., "algae, bacteria".

This study builds on the results of Kulaš et al. [5] who showed that the taxonomic diversity of ciliates studied using a combined approach of traditional microscopic analysis and environmental DNA (eDNA) metabarcoding can be a good bioindicator of karstic environments such as the Krka river. It aims to investigate the changes in phylogenetic and functional diversity of ciliates in the Krka river biofilm to predict phylogenetic and functional structure of ciliates in other karstic environments. Since previous results showed significant differences in ciliate community structure between upstream and downstream sampling locations, our goal was to test whether the location effect was also reflected in phylogenetic and functional diversity. We hypothesised that both phylogenetic and functional diversity of ciliates would increase at downstream, leading to significant differences with regard to location but also in relation to positioning on tufa stone (light/dark-exposed side of tufa stone), as microhabitat complexity has already been recognised as one of the main factors driving taxonomic diversity of protists in tufa-depositing environments, including the Krka river [6,7]. In addition to the selected metrics, our study also provides insights into the composition of the ciliate community in terms of functional groups, thus providing a comprehensive and valuable database of assigned functional traits. Being the first to examine the phylogenetic and functional diversity of ciliates in a Mediterranean karstic river, our study lays the foundation for future research on this topic and opens a new opportunity to more frequently include ciliates in monitoring and conservation plans for various karst freshwater habitats, not only for tufa barriers.

2. Materials and Methods

2.1. Study Area

The Krka river is a 73 km long river in the Dinaric region of Dalmatia, Croatia. It is a specific karstic river with a strong connection of surface and groundwater, characterised by tufa barriers formed through deposition of $CaCO_3$ under specific physical and chemical conditions [39,40]. The headwaters of the Krka river are located near the Dinara mountain and consist of three permanents springs that flow through the Knin Karst Polje after the headwaters [41,42]. Along the composite valley of the Krka river, there are seven major tufa barriers forming waterfalls, in the downstream direction [43]. Some of them form lacustrine sections in the river, influencing the dynamics of the river by creating segments with alternating lotic and lentic parts. The length of the Krka river from the source to the last tufa barrier Skradinski buk is 49 km. Thereafter, the river flows into the Adriatic Sea near the town of Šibenik in a brackish estuary about 25 km long [43]. The four sampling locations (Krka spring, Marasovine, Roški slap, Skradinski buk) were chosen to represent the upstream (Figure 1), midstream, and downstream sections of the river. Sampling locations were adapted from [5], while Krka spring (I and II), Roški slap (I and II) and Skradinski buk (I and II) were sampled on two representative habitats.

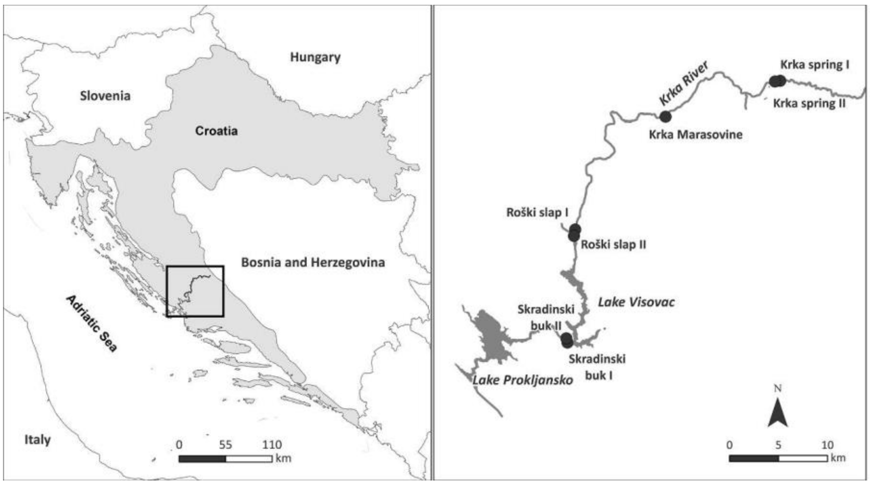

Figure 1. Map of the four sampling locations (Krka spring, Marasovine, Roški slap and Skradinski buk) situated at the Krka river, Croatia (Krka spring, Roški slap and Skradinski buk were sampled on two representative habitats—marked with "I" and "II").

2.2. Sampling Procedure

Sampling was conducted from 21 to 23 September 2017 along the course of the Krka river. At each sampling location, three individual sub-locations were chosen (at least 10 m apart), with each successive sub-location selected upstream of the previously sampled one. Transverse samples were exceptionally taken at those locations where longitudinal samples were not possible due to waterfalls. Pooled samples were collected at each sub-location, consisting of at least five randomly selected tufa stones which were scrubbed and/or brushed with a new toothbrush to collect biofilm from the light-exposed and dark-exposed sides of the tufa stones and rinsed with water. Samples of the biofilm were stored for molecular analysis in Falcon tubes (50 mL) without added preservatives, kept on ice during transport to the laboratory, and stored at −20 °C until further processing. For physicochemical analysis, in situ measurements of water temperature, pH, conductivity (EC), oxygen concentration and saturation were performed using a portable multimeter (Hach HQ40d, Germany). Samples for water chemistry analysis were collected simultaneously with the biological samples and stored at −20 °C until processing in the laboratory. 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. DNA Extraction and PCR Reaction

DNA extraction, PCR reaction, and bioinformatic processing were performed as previously described in [5]. DNA was isolated from the frozen epilithic biofilm. The first step was to remove excess water by centrifugation (4000× *g* for 1 min). The DNeasy PowerSoil Kit (Qiagen, Germany) was used for DNA isolation, following the manufacturer's instructions. In the final step of the isolation, a minor modification was made by adding 60 µL of sterile, DNA-free, PCR-grade water instead of the C6 solution from Qiagen. The yield and quality of extracted DNA was measured using a spectrophotometer (BioSpec Nano, Shimadzu, Japan). Environmental DNA was amplified using the universal hypervariable V9 region of the SSU rRNA gene (approximately 130 base pairs) with primer pairs 1391F

(5′-GTACACACCGCCCGTC-3′) and EukB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) designed by [44]. PCR products were assessed by visualisation on a 1% agarose gel according to the protocol of [45,46]. Sequencing libraries were prepared using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA). Libraries were sequenced on an Illumina NextSeq platform generating 150-bp paired-end reads (SeqIT GmbH & Co. KG, Kaiserslautern, Germany).

2.4. Bioinformatics and Statistics

Raw Illumina reads were demultiplexed using Cutadapt v1.18 [47], removing barcodes in the 5´ to 3´ combination, and then processed using the DeltaMP pipeline v0.3 (https://github.com/lentendu/DeltaMP, accessed on 30 October 2018.). In the final steps, sequences were grouped into OTU using SWARM v2 [48] 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 [49]. A consensus taxonomy with a 60% threshold was created for Operational Taxonomic Units (OTUs) with multiple best matches to different taxonomy in the database. To keep only protist OTUs, OTUs assigned to the following taxa were removed: Streptophyta, Metazoa, Fungi, unclassified Archaeplastida, unclassified Eukaryota, and unclassified Opisthokonta. Ciliophora was one of the dominant groups of protists in the dataset [5]. Using Vsearch (v. 2.17.0), only the sequences of Ciliophora were extracted for all subsequent statistical and phylogenetic analyses. OTUs of ciliates were also removed if they were not phylogenetically placed within the known eukaryotic clades using the evolutionary placement algorithm [50] reduced to the V9 region. Phylogenetic trees were inferred using multiple sequence alignments of OTU representatives generated with MAFFT v7.490 [51], and picking the highest likelihood tree inferred with IQtree v1.6.12 [52] using the GTR+FO+G model.

Statistical analyses were conducted in R v.4.1.2 [53]. To avoid bias in alpha and beta phylogenetic diversity indices due to unequal sequencing depth of the main taxa and subtaxa in the different samples, read counts were normalized using the center-log ratio transformation [54]. Phylogenetic species variability index—PSV [22] and PD- phylogenetic diversity [55]—were estimated with "picante" package v.1.8-2 [56]. PSV is statistically independent of species richness and reaches a value of 1 when all species are unrelated and zero as species become more related.

Beta diversity was estimated using the unweighted (based on presence/absence) and weighted (based on relative abundance) UniFrac distance [23] with "phyloseq" package v.1.36-0 [57]. To test significance and to detect individual and combined effects of locations and exposed sides, beta diversity was constrained by the PERMANOVA permutation test. Non-metric multidimensional scaling (NMDS) was used to visualise beta diversity with significant environmental variables fitted to the ordination using the *envfit* function of the "vegan" package v2.5-7.0 [58]. The fit (R^2) of each variable to the ordination was assessed with a Monte Carlo analysis of 10,000 permutations.

Functional diversity of ciliates was quantified using functional richness metric (FRic), which calculates the volume of the multidimensional convex hull encompassing all ordinated trait values in a set [59]. This metric captures the total amount of variation in trait values, making it conceptually analogous to Faith's PD [60]. FRic was calculated using the "FD" package v.1.0-12. [61,62]. A detailed overview of the criteria for classification can be found in Supplementary Table S1.

Proportions of ciliate functional groups at each sampling location were visualised using packages "ggrepel" v.0.9.1. [63] and "tidyverse" v.1.3.1. [64].

Tukey's HSD parametric test was applied to test location and side effect (for each location pair and side pair) for phylogenetic alpha diversity indices (PD and PSV) and functional richness metric (FRic), separately. The results were visualised as different letters above boxplots using "ggplot2" v.3.3.5 [65].

The demultiplexed raw reads were deposited in the Sequence Read Archive of ENA and are publicly available under project number PRJEB39359.

3. Results

3.1. Sequencing

A total of 32,361,941 sequencing reads were yielded from the remaining 39 samples into 394,492 OTUs [5]. After extraction of low abundance reads, a total of 31,907,533 high-quality reads were clustered into 82,918 OTUs. In the end, a total of 5,413,607 protist reads were found in 11,295 OTUs. Within the protist OTUs, only 466,344 reads were taxonomically assigned to Ciliophora with 3724 OTUs. The Ciliophora reads were extracted from the dataset and further analysed in detail. The number of families, genera, and species of ciliates identified were detailed in an earlier study [5].

3.2. Ciliate Phylogenetic and Functional Diversity Metrics

The results of Tukey's HSD parametric test confirmed significant differences in PD, PSV and FRic with regard to the location (Figure 2a,b), but not with regard to side effect (light/dark exposure). At the upstream location of Krka spring had significantly lower values of PD and FRic in comparison to the downstream-most location Skradinski buk while the opposite was true for PSV values. The detailed results of Tukey's HSD parametric test can be found in Supplementary Table S2.

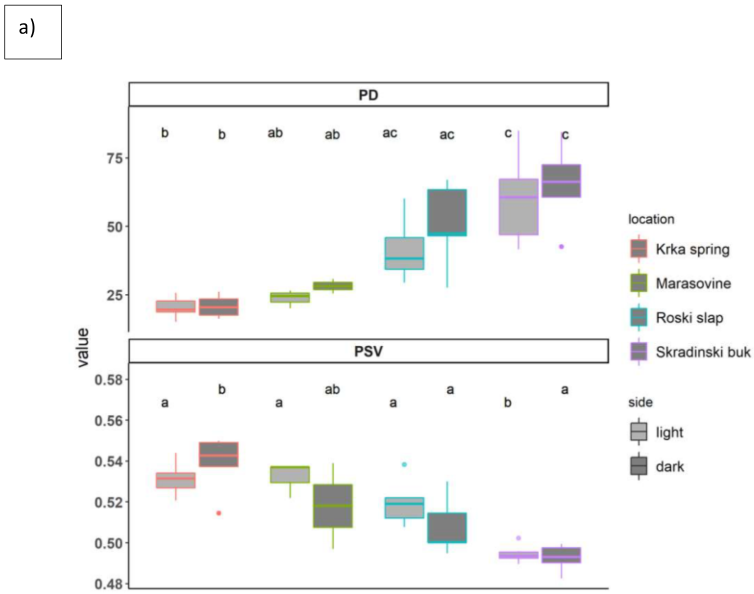

Figure 2. *Cont.*

b)

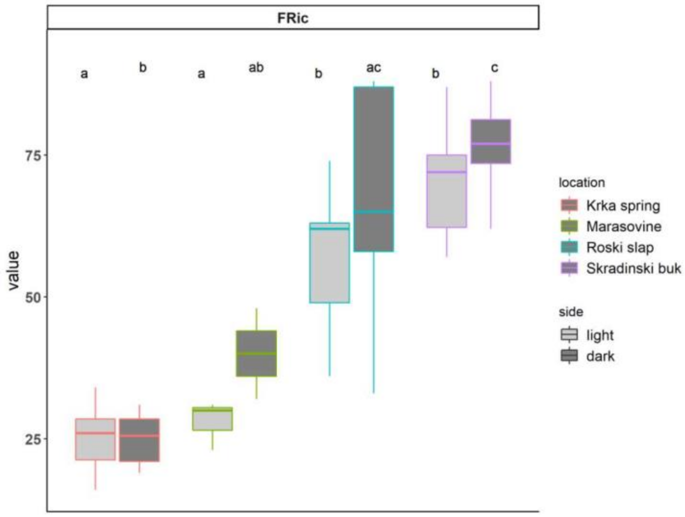

Figure 2. Changes in phylogenetic alpha diversity indices for PD—phylogenetic diversity and phylogenetic species variability index—PSV (**a**) and functional richness—FRic (**b**) visualised by boxplots for each sampling side and location; the horizontal thick black band represents the median values, and the boxplot margins indicate first and third quartiles, different letters above boxplots indicate significant differences among sampling side and location based on Tukey's HSD test. The lighter grey colour denotes light-exposed samples, whilst darker grey colour denotes dark-exposed samples.

The community composition at sampling side and location was analysed via NMDS of the Unifrac distance measure for ciliates (Figure 3). There was a separation of sampling location, specifically a separation of the upstream and downstream parts of the river section. This was also confirmed by the PERMANOVA test, where the location effect showed significance (p = 0.003), while side (p = 0.987) and combined (p = 0.403) effects were not significant. NMDS ordination showed that environmental parameters consistently influenced the beta diversity of the ciliate community, resulting in a clear separation of upstream and downstream samples along the NMDS1 axis. Environmental parameters at the upstream locations were characterised by higher phosphate values (Krka spring) and higher electrical conductivity (Marasovine). Roški slap samples were separated between the upstream and downstream river section. Samples of Roški slap associated with the upstream samples were characterised by higher phosphate values, while the downstream samples were characterised by higher nitrate values, DOC and DIC. Higher values of DIC also characterised the samples at Skradinski buk, the downstream part of the river (Supplementary Table S3 [5]). The main parameters that showed a significant (p < 0.01) positive correlation with the MDS1 axis were pH, temperature, oxygen saturation, nitrates, TN, and DOC, while DIC (p < 0.05) showed a significant negative correlation with both axes MDS1 and MDS2 (Supplementary Table S4).

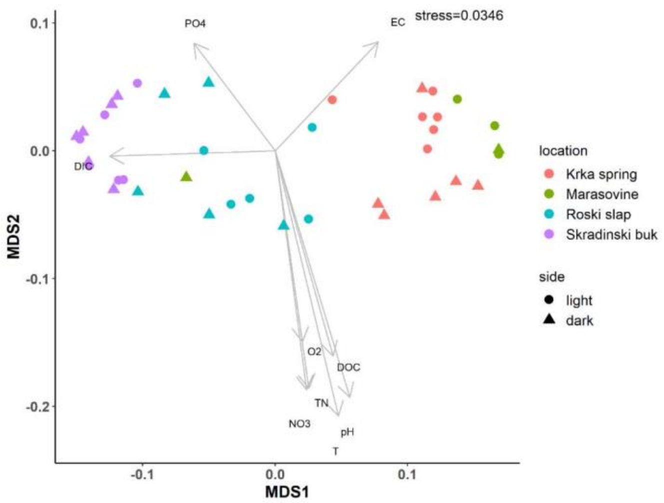

Figure 3. Non-metric multidimensional distance scaling (NMDS) analysis of the Unifrac distance indices of the ciliate community at OTU level with environmental parameters (DIC = dissolved inorganic carbon, DOC = dissolved organic carbon, EC = conductivity, N-NO_3, O_2 = oxygen saturation, pH, PO_4 = phosphates, T = temperature, TN = total nitrogen).

3.3. Community Composition with Regard to Ciliate Functional Groups

There were differences in the proportion of certain functional groups of ciliates upstream and downstream of the Krka river (Figure 4). In terms of motility and mode of locomotion, the upstream locations, i.e., Krka spring and Marasovine, had a higher proportion of semi-sessile and sessile species than the downstream location (Skradinski buk) (Figure 4a). Most of these sessile ciliates were colonial species, abundant only at the upstream locations (Figure 4b). As for the motile ciliates, most categories, in terms of mode of locomotion, were more or less evenly represented at the upstream locations, while the free-swimming ciliates dominated at Skradinski buk, where they reached a higher proportion than at the other sampling locations (Figure 4c). Skradinski buk was also the location with the highest proportion of euplanktonic ciliates.

In terms of habitat preference, the upstream locations were again more diverse than the downstream ones; however, Skradinski buk was the only location with brackish taxa and the one with the highest count of OTUs belonging to marine taxa (Figure 4d).

In terms of food source and feeding strategy, the same pattern emerged with a more diverse community at upstream locations. Skradinski buk had the highest proportion of omnivorous taxa in the community. These taxa also characterised the upstream locations; however, a substantial proportion of bacterivorous ciliates and those feeding on algae and diatoms was also found (Figure 4e). There were also differences in community composition in terms of feeding strategy. Krka spring was dominated by filter-feeders, while the community at Skradinski buk was dominated by taxa that were both filter-feeders and facultative predators (Figure 4f).

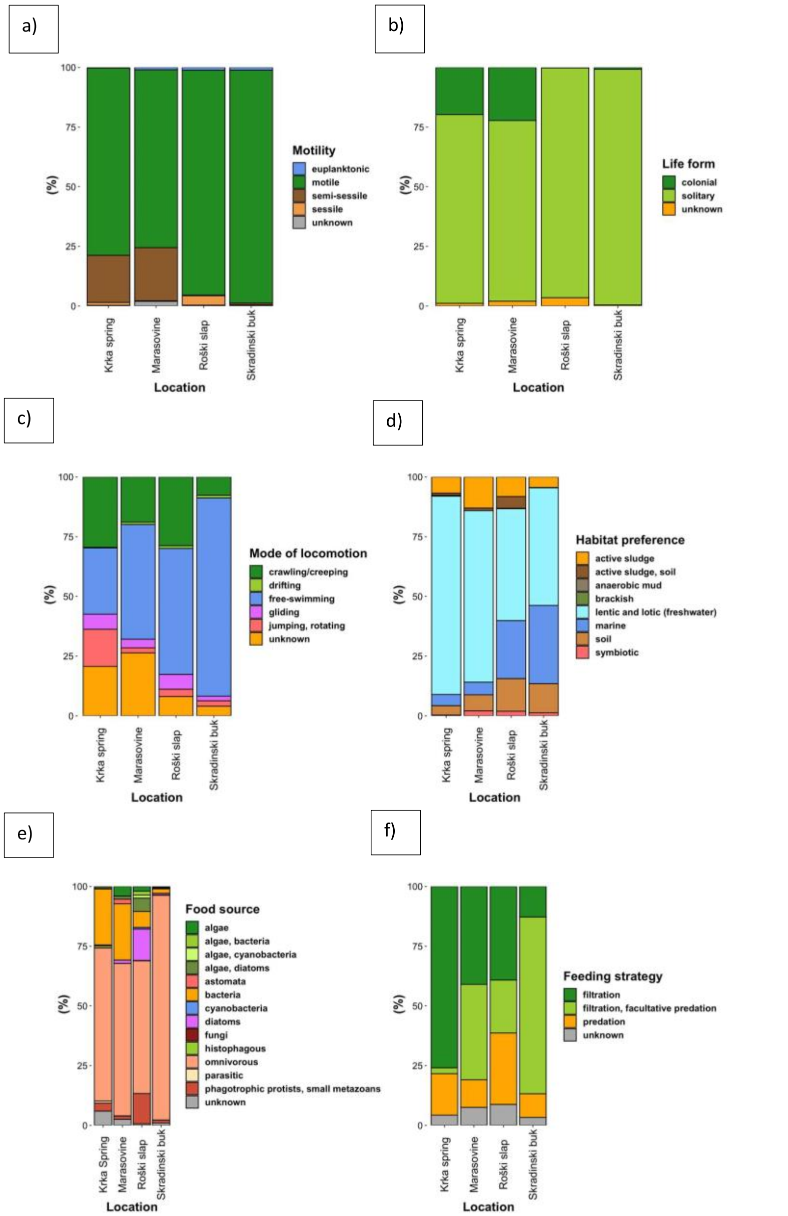

Figure 4. Proportion of functional groups with regard to (**a**) motility; (**b**) life form; (**c**) mode of locomotion; (**d**) habitat preference; (**e**) food source; (**f**) feeding strategy at each of the four sampling locations: Krka spring, Marasovine, Roški slap and Skradinski buk.

4. Discussion

The main finding of our study is that the upstream location, i.e., Krka spring, had the most divergent community and consisted of distantly related taxa. Under the assumption of phylogenetically conserved ecological niches, it could be hypothesised that this phylogenetic overdispersion resulted from competitive exclusion rather than environmental filtering. However, this hypothesis is plausible if environmental filtering and competitive exclusion have mutually exclusive effects on the community. Recent studies have instead suggested that environmental filtering and competitive exclusion are not strictly opposing processes but may act in parallel or even interact [10,16]. Therefore, the complex relationship between these two processes should be considered.

We hypothesise that the environmental conditions at Krka spring, e.g., lower nutrient concentrations (except for phosphates) and lower discharge rates, favoured better competitors, such as semi-sessile and sessile taxa, that were predominantly colonial, bacterivorous filter feeders. Some studies [66,67] have already shown that sessile and semi-sessile ciliates (as many heterotrichs) were much better competitors than free-swimming ciliates because of their lower weight-specific respiratory rates, which allow them to better reduce their metabolism upon starvation. Most of these sessile ciliates belonged to peritrichs, which have higher growth rates and are less likely to be washed away [33,68]. Peritrichs are also very successful filter feeders and feed primarily on bacteria. Kusuoka and Watanabe [68] reported increased competition within the community when peritrich abundance increases, as large colonies generate greater currents and can potentially filter more bacteria. Significantly lower discharge values at Krka spring than at the downstream locations Roški slap and Skradinski buk (detailed discharge values can be found in [5]) confirm this. The lower discharge appears to be favourable for sessile and semi-sessile ciliates, which tend to be more firmly anchored in the substrate and therefore more unlikely to detach. Semi-sessile filter feeders proliferate in patches that provide shelter but also allow circulation of water and food [69].

It is already known that phosphate concentration influences the biofilm functional trait composition [70–72]. Of all sampled locations, Krka spring had the highest phosphate concentration (Supplementary Table S3), which likely originated from groundwater. Karst springs in the Krka river basin are often associated with inflow of groundwater originating from the mountain Dinara [73]. Groundwaters in the Croatian and Slovenian karst areas rarely contain more than 0.1 mg $L{-}1$ of phosphates unless they are polluted [74]. Although the various forms of dissolved phosphorus are mostly derived from natural N and P cycles from river-born material, they can also be a result of anthropogenic activities [75,76]. Phosphates, along with nitrates, are the predominant forms in total N and P fluxes. It is likely that the high phosphate concentration at Krka spring originated from groundwater and promoted algal and microbial growth supporting colonial, bacterivorous, filter-feeding ciliates. Baković et al. [77] already found that karst underground habitats of Croatia and Bosnia and Herzegovina are a hotspot of protist biodiversity; therefore, it is likely that the influx of groundwater served as a mechanism of ciliate colonization at Krka spring. Interestingly, Kulaš et al [78] recorded diatom species (Aulacoseira granulata (Ehrenberg) Simonsen and Navicula cryptotenella Lange-Bertalot) at Krka spring that tolerate higher phosphate concentrations. This supports the idea that phosphate concentration, along with discharge rate, may be one of the main factors determining the ciliate functional community composition at Krka spring.

Although our results showed that the positioning on tufa stone (light/dark-exposed side of tufa stone) was not significant for the tested phylogenetic and functional diversity metrics, microhabitat conditions and complexity have still been recognized as one of the most important drivers of taxonomic diversity of protists (including ciliates) in tufa-depositing environments and in the Krka river basin itself [5–7]. Lentendu and Dunthorn [10] showed that local heterogeneity can influence competitive exclusion, and that competitive exclusion has a greater effect in less heterogenous habitats. Snyder and Chesson [79] also found that high local heterogeneity promotes coexistence of competing species.

Therefore, it is possible that Krka spring consisted of more homogeneous microhabitats and favoured more homogeneous environmental conditions than downstream locations. In fact, NMDS results showed that downstream locations were characterised by higher nitrate, DOC, and DIC values, suggesting that these locations may be considered nutrient-rich microenvironments that support the development of heterogeneous microhabitats where the strength of mutual exclusion is much lower [10]. Although the positioning of tufa stone may not have been the correct element to measure local heterogeneity because it did not show a significant effect, local heterogeneity may still be a factor influencing ciliate phylogenetic and functional diversity. Results of Gulin et al. [6,7] showed that the taxonomic and functional diversity of ciliates is highly dependent on methodology and varies significantly between natural and artificial substrates.

Local heterogeneity was also reflected in functional richness values. Higher FRic in the downstream locations of the Krka river are likely related to local microhabitat complexity, as the abundance of various tufa-depositing forms is much higher at the downstream locations, especially Skradinski buk [80]. Skradinski buk is the final tufa barrier located about 13 kilometres downstream from Roški slap and about 50 kilometres downstream from the source. Further downstream, the Krka river is under maritime influence, with brackish elements [80]. This is also evident from our results, which show that Skradinski buk was the only location supporting brackish taxa. On the other hand, the lowest FRic values at the Krka spring may indicate that some of the resources (alpha niches) potentially available to the community are not being utilized [31]. This also means that taxa which could take advantage of these conditions are not present, removing the buffer against environmental conditions and making the system more susceptible to invasion and disturbance, which is especially important since karst springs are considered sensitive, if not endangered [81].

Caution should always be exercised in interpreting the abundance of taxa recorded by amplicon sequences, as translating abundance from sequence data to biological abundance remains a problem. Variation in rDNA copy number among taxa could be one of the main reasons for incongruent results for Alveolata sequences (ciliates), as they make up the largest proportion of sequence data [31,82]. Therefore, the number of OTUs does not reflect the same number of different taxa recorded, as the accuracy of taxonomic assignment of short amplicon sequences at the species level is still problematic due to the incompleteness of the reference database. However, our results were confirmed and compared with the microscopic analysis in the previous study [5].

5. Conclusions

By showing that the phylogenetic and functional diversity of ciliates change along the Krka river course, our study provides a basis for predicting the phylogenetic and functional structure of ciliates in other karstic rivers. It is not clear whether phylogenetic overdispersion at the Krka spring resulted from environmental filtering, competitive exclusion, or a combination of these factors. The higher proportion of better competitors at Krka spring compared to the downstream-most location (Skradinski buk) may have resulted in the exclusion of less competitive taxa at the former location due to the specific environmental conditions. Therefore, it is necessary to implement other methods to address this question. Given that current knowledge of functional diversity is insufficient to determine the functional niche of most protists, linking phylogenetic diversity to community patterns under the assumption of phylogenetic niche conservatism is the most precise approach we can currently use to find evidence for large-scale and community-wide processes involved in the protist communities. As the first study of its kind in the Mediterranean, our results have undoubtedly shed light on the phylogenetic and functional diversity of ciliates in karst ecosystems and provided a foundation for future ecological and conservation efforts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10122493/s1, Supplementary Table S1. Functional traits of ciliates sampled at four locations (Krka spring, Marasovine, Roški slap, Skradinski buk) along the course of the Krka river, Croatia. Supplementary Table S2. Results of Tukey's HSD parametric test applied to test location and side effect (for each location pair and side pair) for phylogenetic alpha diversity indices (PD and PSV) and functional richness metric (FRic), separately. Supplementary Table S3. Environmental parameters at the four investigated locations (Krka spring, Marasovine, Roški slap, Skradinski buk) along the course of the Krka river, Croatia. Sampling locations Krka spring (I and II), Roški slap (I and II) and Skradinski buk (I and II) were sampled on two representative habitats. Supplementary Table S4. Results of non-metric multidimensional distance scaling (NMDS) analysis of the Unifrac distance indices of the ciliate community at OTU level with environmental parameters (DIC = dissolved inorganic carbon, DOC = dissolved organic carbon, EC = conductivity, N-NO_3^- = nitrates, O_2 = oxygen saturation, pH, P-PO_4^{3-} = phosphates, T = temperature, TN = total nitrogen). Statistically significant results ($p < 0.05$) are reported in bold.

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Data Availability Statement: The data presented in this study are openly available in Sequence Read Archive of ENA at https://www.ebi.ac.uk/ena/browser/home, reference number PRJEB39359.

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Supplementary Table S1. Functional traits of ciliates sampled at four locations (Krka spring, Marasovine, Roški slap, Skradinski buk) along the course of the Krka river, Croatia.

Genus	Food	Feeding strategy	Habitat preference	Motility	Mode of locomotion	Life form
Acineta	omnivorous	predation	lentic and lotic (freshwater)	sessile	unknown	solitary
Aegyriana	diatoms	predation	marine	motile	gliding	solitary
Amphisiella	omnivorous	predation	soil	motile	gliding	solitary
Agolohymena	histophagous	predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Ancistrum	bacteria	unknown	symbiotic	motile	jumping, rotating	solitary
Anigsteinia	bacteria	filtration	marine	motile	gliding	solitary
Anteholosticha	omnivorous	filtration	marine	motile	crawling/creeping	solitary
Apobryophyllum	phagotrophic protists, small metazoans	predation	soil	motile	gliding	solitary
Apodileptus	omnivorous	predation	soil	motile	gliding	solitary
Apogastrostyla	diatoms	filtration	marine	motile	crawling/creeping	solitary
Aristerostoma	bacteria	unknown	marine	motile	jumping, rotating	solitary
Askenasia	algae, diatoms	filtration	lentic and lotic (freshwater)	motile	jumping, rotating	solitary
Aspidisca	bacteria	filtration	active sludge	motile	crawling/creeping	solitary
Blepharisma	algae, bacteria	filtration	lentic and lotic (freshwater)	motile	free-swimming	solitary
Bresslaua	omnivorous	predation	soil	motile	free-swimming	solitary
Bryometopus	bacteria	filtration, facultative predation	soil	motile	free-swimming	solitary
Carchesium	bacteria	filtration	lentic and lotic (freshwater)	semi-sessile	unknown	colonial
Cardiostomatella	bacteria	filtration	marine	motile	free-swimming	solitary
Chaenea	bacteria	filtration	active sludge	motile	crawling/creeping	solitary
Chilodonella	bacteria	filtration	active sludge	motile	gliding	solitary
Chlamydodon	omnivorous	unknown	marine	motile	gliding	solitary
Chlamydonella	omnivorous	predation	soil	motile	gliding	solitary
Clevelandella	omnivorous	unknown	symbiotic	motile	free-swimming	solitary
Climacostomum	omnivorous	filtration, facultative predation	lentic and lotic (freshwater)	motile	crawling/creeping	solitary
Cohnilembus	omnivorous	unknown	symbiotic	motile	free-swimming	solitary
Coleps	omnivorous	predation	lentic and lotic (freshwater)	motile	free-swimming	solitary

Colpidium	omnivorous	predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Colpoda	omnivorous	predation	soil	motile	gliding	solitary
Colpodidium	bacteria	filtration	soil	motile	jumping, rotating	solitary
Conchophthirus	omnivorous	unknown	symbiotic	motile	crawling/creeping	solitary
Condylostoma	omnivorous	filtration	soil	motile	gliding	solitary
Cryptocaryon	omnivorous	unknown	symbiotic	motile	crawling/creeping	unknown
Cultellothrix	phagotrophic protists, small metazoans	predation	soil	motile	gliding	solitary
Cyclotrichium	omnivorous	unknown	lentic and lotic (freshwater)	motile	free-swimming	solitary
Cyrtohymena	omnivorous	predation	soil	unknown	unknown	solitary
Dexiotricha	bacteria	filtration	active sludge	motile	unknown	solitary
Diaxonella	omnivorous	unknown	marine	motile	unknown	solitary
Dileptus	omnivorous	predation	soil	motile	crawling/creeping	solitary
Diophrys	omnivorous	unknown	marine	motile	crawling/creeping	solitary
Enchelys	omnivorous	predation	soil	motile	free-swimming	solitary
Engelmanniella	bacteria	unknown	soil	unknown	free-swimming	solitary
Entorhipidium	omnivorous	unknown	symbiotic	motile	crawling/creeping	solitary
Epalxella	bacteria	filtration	anaerobic mud	motile	gliding	solitary
Ephelota	omnivorous	predation	symbiotic	sessile	unknown	solitary
Epiphyllum	phagotrophic protists, small metazoans	predation	brackish	motile	gliding	solitary
Etoschophrya	omnivorous	predation	soil	motile	jumping, rotating	solitary
Euplotes	omnivorous	filtration	active sludge	motile	crawling/creeping	solitary
Euplotidium	unknown	unknown	marine	motile	unknown	solitary
Exocolpoda	bacteria	unknown	soil	motile	unknown	solitary
Fabrea	algae, bacteria	filtration	marine	motile	free-swimming	solitary
Furgasonia	algae, cyanobacteria	predation	soil	unknown	unknown	solitary
Gastrostyla	omnivorous	filtration	active sludge, soil	motile	gliding	solitary
Gonostomum	omnivorous	filtration, facultative predation	soil	motile	free-swimming	solitary
Gymnodinioides	parasitic	unknown	symbiotic	sessile	free-swimming	solitary
Halteria	omnivorous	filtration	lentic and lotic (freshwater)	motile	jumping, rotating	solitary

Hartmannula	omnivorous	unknown	marine	motile	crawling/creeping	solitary
Hemicycliostyla	omnivorous	unknown	lentic and lotic (freshwater)	motile	unknown	solitary
Hemiophrys	unknown	predation	marine	motile	gliding	solitary
Hemiurosoma	bacteria	predation	soil	motile	gliding	solitary
Heterokeronopsis	unknown	unknown	marine	motile	crawling/creeping	solitary
Histiobalantium	omnivorous	unknown	lentic and lotic (freshwater)	euplanktonic	drifting	solitary
Holosticha	omnivorous	filtration	soil	motile	free-swimming	solitary
Isotricha	bacteria	filtration	symbiotic	motile	free-swimming	solitary
Kahliella	omnivorous	unknown	soil	motile	gliding	solitary
Kentrophoros	bacteria	filtration	marine	motile	gliding	unknown
Kuklikophrya	cyanobacteria	unknown	soil	motile	gliding	solitary
Lacrymaria	phagotrophic protists, small metazoans	predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Latteuria	bacteria	unknown	symbiotic	unknown	free-swimming	unknown
Lembadion	omnivorous	filtration, facultative predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Limnostrombidium	algae	unknown	lentic and lotic (freshwater)	motile	free-swimming	solitary
Litonotus	phagotrophic protists, small metazoans	predation	soil	motile	free-swimming	solitary
Loxodes	omnivorous	predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Loxophyllum	phagotrophic protists, small metazoans	predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Maryna	bacteria	unknown	lentic and lotic (freshwater)	motile	free-swimming	solitary
Mesanophrys	parasitic	unknown	symbiotic	motile	gliding	solitary
Meseres	omnivorous	unknown	soil	motile	jumping, rotating	solitary
Mesodinium	omnivorous	unknown	lentic and lotic (freshwater)	motile	jumping, rotating	solitary
Metaurostylopsis	algae, diatoms	unknown	marine	motile	crawling/creeping	solitary
Miamiensis	histophagous	unknown	symbiotic	motile	unknown	solitary
Microxysma	algae, bacteria	unknown	marine	unknown	unknown	solitary
Nassula	algae, cyanobacteria	predation	soil	motile	free-swimming	solitary
Novistrombidium	algae	unknown	marine	motile	free-swimming	solitary
Njinella	astomata	unknown	symbiotic	unknown	unknown	unknown

Obertrumia	algae, cyanobacteria	predation	soil	motile	free-swimming	solitary
Ophryoglena	histophagous	predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Oxytricha	omnivorous	filtration	lentic and lotic (freshwater)	motile	free-swimming	solitary
Paracineta	omnivorous	predation	soil	sessile	unknown	solitary
Parafurgasonia	bacteria	predation	soil	motile	crawling/creeping	solitary
Paramecium	omnivorous	filtration	lentic and lotic (freshwater)	motile	free-swimming	solitary
Paranassula	omnivorous	predation	marine	motile	free-swimming	unknown
Paraspathidium	unknown	predation	marine	motile	crawling/creeping	solitary
Paratetrahymena	bacteria	filtration	marine	motile	gliding	solitary
Paraurostyla	omnivorous	filtration	soil	motile	free-swimming	solitary
Paruroleptus	omnivorous	unknown	soil	motile	free-swimming	solitary
Perisincirra	omnivorous	unknown	soil	motile	gliding	solitary
Phacodinium	omnivorous	unknown	soil	motile	free-swimming	solitary
Phialina	phagotrophic protists, small metazoans	predation	active sludge	motile	free-swimming	solitary
Philasterides	histophagous	unknown	lentic and lotic (freshwater)	motile	free-swimming	solitary
Placus	omnivorous	predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Plagiocampa	omnivorous	predation	soil	motile	free-swimming	solitary
Plagiopyliella	unknown	unknown	symbiotic	unknown	unknown	solitary
Platyophrya	omnivorous	unknown	soil	motile	free-swimming	solitary
Pleuronema	omnivorous	filtration	lentic and lotic (freshwater)	motile	jumping, rotating	solitary
Polydiniella	bacteria	filtration	symbiotic	unknown	unknown	unknown
Porpostoma	diatoms	filtration	symbiotic	motile	free-swimming	solitary
Prorodon	omnivorous	predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Protospathidium	phagotrophic protists, small metazoans	predation	soil	motile	free-swimming	solitary
Pseudepistylis	unknown	unknown	lentic and lotic (freshwater)	semi-sessile	gliding	colonial
Pseudochilodonopsis	algae, diatoms	predation	lentic and lotic (freshwater)	motile	unknown	solitary
Pseudocyrtolophosis	bacteria	unknown	soil	motile	crawling/creeping	solitary
Pseudokeronopsis	omnivorous	unknown	marine	motile	free-swimming	solitary
Pseudomicrothorax	algae, cyanobacteria	predation	soil	motile	free-swimming	solitary
Pseudoplatyophrya	fungi	predation	soil	motile	jumping, rotating	solitary

Pseudotontonia	unknown	unknown	marine	motile	drifting	solitary
Pseudouroleptus	omnivorous	unknown	soil	motile	gliding	solitary
Pseudourostyla	omnivorous	predation	soil	motile	gliding	solitary
Pseudovorticella	algae, bacteria	filtration	soil	semi-sessile	unknown	colonial
Raabena	unknown	unknown	symbiotic	unknown	unknown	unknown
Remanella	unknown	unknown	marine	motile	gliding	solitary
Sandmanniella	omnivorous	unknown	soil	motile	free-swimming	solitary
Schizocaryum	omnivorous	unknown	symbiotic	motile	free-swimming	unknown
Spathidiopsis	phagotrophic protists, small metazoans	predation	symbiotic	motile	free-swimming	solitary
Spirostomum	algae, bacteria	filtration	lentic and lotic (freshwater)	motile	free-swimming	solitary
Stentor	omnivorous	filtration, facultative predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Sterkiella	omnivorous	predation	active sludge, soil	motile	free-swimming	solitary
Stokesia	omnivorous	predation	lentic and lotic (freshwater)	euplanktonic	drifting	solitary
Strombidinopsis	omnivorous	unknown	marine	euplanktonic	drifting	solitary
Strombidium	omnivorous	filtration	lentic and lotic (freshwater)	motile	jumping, rotating	solitary
Strongylidium	omnivorous	unknown	soil	motile	crawling/creeping	unknown
Stylonychia	omnivorous	filtration	lentic and lotic (freshwater)	motile	free-swimming	solitary
Tetrahymena	bacteria	filtration	active sludge	motile	free-swimming	solitary
Tiarina	algae	predation	marine	motile	free-swimming	solitary
Tintinnidium	omnivorous	filtration	lentic and lotic (freshwater)	euplanktonic	drifting	solitary
Tokophrya	omnivorous	predation	soil	sessile	unknown	solitary
Trachelius	omnivorous	predation	lentic and lotic (freshwater)	motile	unknown	solitary
Trachelocerca	omnivorous	unknown	marine	motile	gliding	solitary
Trichodina	omnivorous	filtration	symbiotic	motile	jumping, rotating	unknown
Trichodinella	omnivorous	unknown	symbiotic	motile	unknown	unknown
Trichopodiella	algae, bacteria	unknown	symbiotic	motile	free-swimming	solitary
Triplumaria	unknown	unknown	symbiotic	unknown	unknown	unknown
Trithigmostoma	algae, diatoms	predation	active sludge, soil	motile	crawling/creeping	solitary
Trochilia	omnivorous	predation	active sludge	motile	crawling/creeping	solitary

Trochochilodon	unknown	unknown	marine	motile	gliding	solitary
Tunicothrix	omnivorous	unknown	marine	motile	free-swimming	solitary
Urceolaria	omnivorous	filtration	symbiotic	motile	free-swimming	solitary
Urocentrum	omnivorous	filtration	lentic and lotic (freshwater)	motile	jumping, rotating	solitary
Uroleptus	omnivorous	filtration	lentic and lotic (freshwater)	motile	free-swimming	solitary
Uronema	omnivorous	filtration	lentic and lotic (freshwater)	motile	jumping, rotating	solitary
Uronemella	bacteria	unknown	marine	motile	jumping, rotating	unknown
Urostyla	omnivorous	predation	lentic and lotic (freshwater)	motile	free-swimming	solitary
Vampyrophrya	histophagous	unknown	lentic and lotic (freshwater)	motile	free-swimming	unknown
Vorticella	omnivorous	filtration	active sludge	semi-sessile	unknown	solitary
Vorticellides	bacteria	filtration	lentic and lotic (freshwater)	semi-sessile	unknown	colonial
Woodruffides	phagotrophic protists, small metazoans	predation	soil	motile	gliding	solitary
Zosterodasys	algae, diatoms	predation	lentic and lotic (freshwater)	motile	crawling/creeping	solitary

Supplementary Table S2. Results of Tukey's HSD parametric test applied to test location and side effect (for each location pair and side pair) for phylogenetic alpha diversity indices (PD and PSV) and functional richness metric (FRic), separately.

Metric	Side	Location pairs	diff	lwr	upr	*p*val
PD	light	Marasovine-Krka spring	3.370	-19.201	25.941	0.970
PD	light	Roski slap-Krka spring	21.200	1.873	40.531	0.029
PD	light	Skradinski buk-Krka spring	39.500	21.142	58.004	0.000
PD	light	Roski slap-Marasovine	17.800	-5.480	41.143	0.168
PD	light	Skradinski buk-Marasovine	36.200	13.630	58.772	0.002
PD	light	Skradinski buk-Roski slap	18.300	-0.959	37.698	0.066
PD	dark	Marasovine-Krka spring	7.460	-20.370	35.292	0.866
PD	dark	Roski slap-Krka spring	29.600	9.036	50.315	0.004
PD	dark	Skradinski buk-Krka spring	44.700	25.070	64.429	0.000
PD	dark	Roski slap-Marasovine	22.200	-6.303	50.732	0.155
PD	dark	Skradinski buk-Marasovine	37.200	9.458	65.119	0.008
PD	dark	Skradinski buk-Roski slap	15.070	-5.565	35.714	0.196
PSV	light	Roski slap-Skradinski buk	0.025	0.011	0.040	0.001
PSV	light	Krka spring-Skradinski buk	0.037	0.023	0.051	0.000
PSV	light	Marasovine-Skradinski buk	0.037	0.021	0.054	0.000
PSV	light	Krka spring-Roski slap	0.011	-0.003	0.026	0.149

PSV	light	Marasovine-Roski slap	0.012	-0.005	0.030	0.233
PSV	light	Marasovine-Krka spring	0.001	-0.016	0.018	0.990
PSV	dark	Roski slap-Skradinski buk	0.015	-0.009	0.039	0.294
PSV	dark	Marasovine-Skradinski buk	0.025	-0.007	0.058	0.151
PSV	dark	Krka spring-Skradinski buk	0.047	0.024	0.069	0.000
PSV	dark	Marasovine-Roski slap	0.010	-0.023	0.043	0.816
PSV	dark	Krka spring-Roski slap	0.031	0.008	0.055	0.009
PSV	dark	Krka spring-Marasovine	0.021	-0.011	0.054	0.263
Fric	light	Marasovine-Krka spring	2.833	-18.149	23.815	0.979
Fric	light	Roski slap-Krka spring	31.633	13.665	49.601	0.001
Fric	light	Skradinski buk-Krka spring	45.333	28.201	62.465	0.000
Fric	light	Roski slap-Marasovine	28.800	7.130	50.470	0.008
Fric	light	Skradinski buk-Marasovine	42.500	21.517	63.482	0.000
Fric	light	Skradinski buk-Roski slap	13.700	-4.268	31.668	0.171
Fric	dark	Marasovine-Krka spring	15.000	-16.679	46.667	0.538
Fric	dark	Roski slap-Krka spring	41.200	17.706	64.699	0.001
Fric	dark	Skradinski buk-Krka spring	51.500	29.099	73.901	0.000
Fric	dark	Roski slap-Marasovine	26.200	-6.261	58.662	0.136
Fric	dark	Skradinski buk-Marasovine	35.500	4.820	68.179	0.022

Fric	dark	Skradinski buk-Roski slap	10.300	-13.193	33.794	0.598

Supplementary Table 3. Environmental parameters at the four investigated locations (Krka spring, Marasovine, Roški slap, Skradinski buk) along the course of the Krka River, Croatia. Sampling locations Krka spring (I and II), Roški slap (I and II) and Skradinski buk (I and II) were sampled on two representative habitats.

	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-$NO2^-$ (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

Supplementary Table 4. Results of Non-metric multidimensional distance scaling (NMDS) analysis of the Unifrac distance indices of the ciliate community at OTU level with environmental parameters (DIC = dissolved inorganic carbon, DOC = dissolved organic carbon, EC = conductivity, $N\text{-}NO_3^-$ = nitrates, O_2 = oxygen saturation, pH, $P\text{-}PO_4^{3-}$ = phosphates, T = temperature, TN = total nitrogen). Statistically significant results ($p<0.05$) are reported in bold.

	NMDS1	NMDS2	R^2	*p*
pH	0.280	-0.960	0.363	**0.002**
EC	0.674	0.738	0.120	0.098
O_2	0.135	-0.991	0.204	**0.013**
T	0.224	-0.975	0.408	**0.001**
$N\text{-}NO_3^-$	0.125	-0.992	0.320	**0.003**
$P\text{-}PO_4^{3-}$	-0.590	0.808	0.098	0.146
TN	0.135	-0.991	0.317	**0.003**
DOC	0.263	-0.965	0.249	**0.006**
DIC	-0.999	-0.033	0.140	0.062

RASPRAVA

DINAMIKA FIZIKALNO-KEMIJSKIH PARAMETARA VODE I ORGANSKE TVARI U REVITALIZIRANIM TOKOVIMA

Ovim doktorskim radom primarno se pokušalo odgovoriti na pitanje imaju li perifiton i njegova heterotrofna komponenta, s naglaskom na fagotrofne protiste, bioindikatorski potencijal u praćenju restauracije, odnosno revitalizacije vodenih tokova sedrenih barijera. U svrhu analize bioindikatorskog potencijala perifitona u revitalizaciji, analiziran je niz okolišnih čimbenika poput fizikalno-kemijskih parametara vode, količine organske i anorganske tvari (sedre) u perifitonu te koncentracije klorofila *a* kao mjere primarne produkcije. Svi postavljeni ciljevi istraživanja ovog doktorskog rada obrađeni su u publikacijama **I, II, III i IV** kojima su testirane sve postavljene hipoteze. Publikacije **I** i **II,** uz analizu zajednice, pružaju temeljitu analizu okolišnih čimbenika čija dinamika upućuje na blisku povezanost revitaliziranih tokova s elementima bliskih kopnenih ekosustava. Publikacijom **I** obuhvaćeno je istraživanje unutar prve godine od uklanjanja invazivne vrste, pajasena, s testne plohe Skradinskog buka (2017./2018.), odnosno unutar prve godine ponovne aktivacije vodenih tokova. Ova publikacija pruža uvid u interdisciplinarno istraživanje revitalizacije vodenih tokova sedrene barijere Skradinski buk u kojem je uz sastav i brojnost perifitona uzorkovanog s prirodne podloge, fokus stavljen na mjeru heterogenosti staništa (prikazanu kroz granulometrijski i mineraloški sastav podloge te analizu udjela organske i anorganske tvari/sedre u perifitonu) te fizikalno-kemijske parametre vode. Cilj publikacije **II** ovog doktorskog rada bio je nadopuniti istraživanje prikazano u publikaciji **I** uzorkovanjem perifitona s umjetnih podloga tijekom dva različita razdoblja ekspozicije podloga (jednomjesečna i dvomjesečna) kroz iduće dvije godine istraživanja (2019. i 2020.). Stoga publikacija **II** pruža uvid u komplementarno istraživanje u kojem je prethodno istraživanje perifitona na prirodnoj podlozi prikazano publikacijom **I** nadopunjeno uzorkovanjem perifitona s umjetnih podloga, što je omogućilo usporedivost sastava perifitona te količine i dinamike organske i anorganske tvari (sedre) s prijašnjim rezultatima zabilježenim na Skradinskom buku (Matoničkin Kepčija i sur., 2017).

Sukladno pretpostavkama, pokazalo se da promjenjiva hidrologija i popratno ispiranje okolnog šumskog tla, koje karakterizira revitalizirane tokove, uvjetuje većinu okolišnih parametara tijekom prve (publikacija **I**) i druge godine istraživanja (publikacija **II**). Tijekom prve godine istraživanja u revitaliziranim tokovima izmjerene su prosječno veće vrijednosti koncentracije nitrita i ortofosfata te otopljene organske tvari (iskazane surogat mjerom kemijske potrošnje kisika), a nešto niže vrijednosti alkaliteta, otopljenog kisika i pH-vrijednosti od kontrolnih tokova. Tijekom druge godine istraživanja, revitalizirani tokovi zadržali su slične prosječne vrijednosti okolišnih parametara s višim vrijednostima nitrita i nižim pH-vrijednostima od kontrolnih tokova, dok su ostali parametri poput ortofosfata i otopljene organske tvari bili nešto niži u odnosu na kontrolne tokove, no isključivo zbog porasta tih parametara u kontrolnim tokovima tijekom jednomjesečne, odnosno dvomjesečne ekspozicije, u odnosu na prethodno istraživanje (publikacija **I**). U istraživanju obuhvaćenom publikacijom **I**, osim veće količine otopljene organske tvari u vodi, i u perifitonu revitaliziranih tokova zabilježena je nešto veća količina partikularne organske tvari u odnosu na perifiton kontrolnih tokova. Iako u drugoj godini istraživanja (publikacija **II**) količina otopljene i partikularne organske tvari u revitaliziranim tokovima nije bila značajno veća od one u kontrolnim tokovima, postaje u revitaliziranim tokovima bilježile su povremeno izrazito visoke vrijednosti (30,83 mg/cm^2 tijekom jednomjesečne ekspozicije i 7,22 mg/cm^2 tijekom dvomjesečne ekspozicije) dok postaje u kontrolnim tokovima nisu imale tako ekstremna odstupanja. Također, spomenute vrijednosti količine partikularne organske tvari zabilježene u perifitonu revitaliziranih tokova u publikaciji **II** predstavljaju maksimalnu količinu organske tvari zabilježenu tijekom istraživanja. U usporedbi s rezultatima istraživanja Matoničkin Kepčija i sur. (2017) koji tijekom jednomjesečne ekspozicije u perifitonu razvijenom na umjetnim podlogama Skradinskog buka bilježe prosječnu količinu organske tvari od 0,21 mg/cm^2, prosječna količina zabilježena u ovom istraživanju je gotovo sedam puta veća u revitaliziranim tokovima (1,42 mg/cm^2) tj. gotovo šest puta veća u kontrolnim tokovima (1,20 mg/cm^2) (publikacija **II**).

U lotičkim ekosustavima, organska tvar može biti autohtonog i alohtonog podrijetla (Webster i Benfield, 1986; Allan i Castillo, 2007; Romaní i sur., 2012). Autohtona organska tvar rezultat je fotosintetskih procesa unutar tekućica od strane cijanobakterija, algi i makrofita, ali je i posljedica odumiranja organizama (detritus). Alohtona organska tvar potječe iz okolnog krajolika npr. ispiranjem i procjeđivanjem vode iz okolnog tla ili je produkt okolne vegetacije (lišće/listinac i ostali biljni dijelovi). Za razliku od autohtone organske tvari koju čine većinom spojevi visoke bioraspoloživosti poput lipida, ugljikohidrata, aminokiselina i proteina, alohtona organska tvar je vrlo složena mješavina spojeva visoke molekularne mase koji se razlikuju po veličini i raspoloživosti za biološku razgradnju (Brandão i sur., 2018). Alohtona organska tvar također predstavlja značajan izvor organske tvari za heterotrofne organizme u lotičkim ekosustavima te uspješno nadoknađuje potencijalni gubitak brzo razgradive autohtone organske tvari nadopunjujući ju spojevima visoke molekularne mase kao što su fulvinske i huminske kiseline (Cunha i sur., 2010; Hansen i sur., 2016). Uslijed bliske povezanosti lotičkih ekosustava s elementima kopnenih ekosustava, promjene okolnog područja, vegetacije i tla utječu na promjene metabolizma unutar rijeka i potoka (Vannote i sur., 1980; Allan i Castillo, 2007; Romaní i sur., 2012). Promjene se mogu očitovati u kontroli dostupne svjetlosti/zasjenjenosti i dostupnosti hranjivih tvari za primarnu produkciju, odnosno količini autohtone organske tvari, ali i unosu listinca uslijed ispiranja okolnog tla, odnosno alohtone organske tvari (Fuß i sur., 2017). Prema konceptu riječnog kontinuiteta (engl. *river continuum concept*) (Vannote i sur., 1980), važnost izvora organske tvari unutar lotičkih ekosustava mijenja se od izvora prema ušću, odnosno u gornjim tokovima okolna vegetacija igra važnu ulogu predstavljajući izvor alohtone organske tvari poput otpalog lišća i komadića drveta dok je primarna produkcija ograničena uslijed zasjenjenosti tokova. Donje tokove pak karakterizira smanjena važnost okolne vegetacije, dok značajnu važnost dobiva primarna produkcija od strane perifitona. Međutim, koncept riječnog kontinuiteta ne vrijedi za sve tekućice, odnosno u rijekama koje odlikuje velika prostorna i vremenska dinamika ne dolazi do pravilne longitudinalne izmjene parametara vode i opisanih promjena u važnosti izvora organske tvari. Tako u krškim tekućicama na unos i dinamiku unosa organske tvari značajno utječu i prepreke u vidu sedrenih barijera te ujezerenja (Habdija i sur., 1994).

U lotičkim ekosustavima mediteranske regije alohtoni izvori organske tvari nadoknađuju nedostatak autohtone komponente uslijed isušivanja ili pojave bujičnih tokova stoga su od velike važnosti za perifiton (Romaní i sur., 2012; Gionchetta i sur., 2020). S obzirom na to da u fazi istraživanja revitalizacije tokova sedrene barijere Skradinski buk obuhvaćenoj publikacijom **I** nije mjerena primarna produkcija perifitona, ne može se sa sigurnošću tvrditi koji izvor organske tvari je od najveće važnosti za perifiton revitaliziranih tokova unutar prve godine od aktivacije, ali mjerenja i usporedba organske tvari (otopljene i partikularne) s kontrolnim tokovima upućuju na to da je u revitaliziranim tokovima i alohtona organska tvar od potencijalno velike važnosti te da se ta važnost sezonski mijenja. Tako je primarna produkcija vjerojatno glavni izvor organske tvari u proljetnim i ljetnim mjesecima dok u jesenskim i zimskim mjesecima alohtona organska tvar ima značajniju funkciju što zbog taloženja listinca tijekom jeseni, odnosno bujičnih tokova zimi. Sezonska dinamika organske tvari u istraživanju obuhvaćenom publikacijom **I** statistički je značajno ovisila o brzini strujanja vode. Iako su revitalizirani tokovi u prvoj godini od uklanjanja pajasena imali u prosjeku nešto manju brzinu strujanja od kontrolnih vodenih tokova, i dalje su pokazivali sezonsku dinamiku kao i kontrolni tokovi koja je karakteristična za podneblje i ne razlikuje se značajnije od prethodnih trendova zabilježenih na Skradinskom buku (Primc-Habdija i sur., 2001; Matoničkin Kepčija i sur., 2017). Ipak, neke od revitaliziranih tokova, za razliku od kontrolnih, tijekom jeseni i zime karakterizirala je pojava bujičnih tokova i više brzine strujanja (Dodatni materijal uz publikaciju **I**). U takvim uvjetima, uslijed ispiranja okolnog tla, alohtona organska tvar mogla bi biti od najveće važnosti za zajednicu jer veća brzina strujanja može biti limitirajućim faktorom za primarnu produkciju, premda su u literaturi zabilježeni i kontrastni učinci. Tako neka istraživanja učinka visoke brzine strujanja na primarnu produkciju perifitona ukazuju na smanjenje produkcije uslijed otplavljivanja algalnih filamenata (Biggs, 1996; Habdija i sur., 2000; Pérez-Calpe i sur., 2021), dok neka ukazuju na njen porast uslijed olakšane difuzije koja omogućava adsorpciju nutrijenata kroz perifitonski matriks pri većim brzinama strujanja vode (Pitois i sur., 2001; Matoničkin Kepčija, 2006; Matoničkin Kepčija i sur., 2017). U publikaciji **II** primarna produkcija u perifitonu revitaliziranih tokova bila je nešto veća od kontrolnih tokova tijekom jednomjesečne ekspozicije, odnosno niža tijekom dvomjesečne ekspozicije, uz povremeno ekstremno visoke vrijednosti koje nisu bile karakteristične za kontrolne tokove. U usporedbi s prethodno zabilježenim vrijednostima koncentracije klorofila *a* u istraživanju Matoničkin Kepčija i sur. (2017) koja je iznosila u prosjeku 0,99 $\mu g/cm^2$, primarna produkcija u publikaciji **II** i u revitaliziranim i kontrolnim tokovima niža je što se može objasniti različitim algalnim sastavom, odnosno moguće je da su u prethodnom istraživanju perifitonom dominirale reofilne algalne vrste dok su u publikaciji **II** dominirale labave i filamentozne vrste, sklone otplavljivanju (Biggs, 1996; Pérez-Calpe i sur., 2021). Razlike u vrijednostima konduktiviteta koji je bio gotovo dvostruko veći u istraživanju Matoničkin Kepčija i sur. (2017) nego u istraživanju obuhvaćenom publikacijom **II** te razlike u pripadajućim vrijednostima nutrijenata, odnosno spojevima dušika i fosfora, također bi mogle biti neki od razloga. Osim toga, na razlike u vrijednostima vjerojatno je utjecao i položaj umjetnih podloga koje su u istraživanju Matoničkin Kepčija i sur. (2017) bile postavljene na dijelu barijere u blizini utoka Čikole što je potencijalno utjecalo na unos nutrijenata, dok su u ovom istraživanju revitaliziranim tokovima prethodila ujezerenja u kojima se može istaložiti dio organske tvari. Primc-Habdija i sur. (1997) te Špoljar (1998) i Špoljar i sur. (2005) bilježe manji pronos sestona na Skradinskom buku te izraženiji retencijski utjecaj Visovačkog jezera na krupne čestice organske tvari koje zbog veće mase lakše sedimentiraju nego sitne, još jednom potvrđujući kompleksnost hidrologije krških tekućica koja utječe na dinamiku organske tvari. Iz rezultata publikacije **II** razvidno je da je u drugoj godini istraživanja najveća primarna produkcija (koncentracija klorofila *a*) bila u proljetnim, a najmanja u zimskim mjesecima sugerirajući da je uz dobro poznate sezonske razlike u temperaturi i insolaciji koje utječu na primarnu produkciju, pojava bujičnih tokova u revitaliziranim tokovima važan parametar koji ograničava primarnu produkciju i tako čini alohtonu organsku tvar dominantnim izvorom organske tvari u perifitonu tijekom zimskih mjeseci. Mnoga istraživanja pokazala su da raznolikost okolne vegetacije utječe na kvalitetu alohtone organske tvari i njenu dostupnost uvjetujući zajednice detritivora i bakteriovora koje se na njima razvijaju (Schindler i Gessner, 2009; Gessner, 2010). Na području testne plohe Skradinskog buka s koje je uklonjen pajasen, zabilježena su i brojna stabla poljskog jasena (*Fraxinus angustifolia* Vahl) i crnog graba (*Ostrya carpinifolia* Scop) (Hršak i sur., 2016), vrsta prepoznatih kao važan izvor alohtone organske tvari u mnogim istraživanjima dinamike organske tvari u lotičkim ekosustavima (Tank i sur., 2010).

Za razliku od kontrolnih tokova, neki od revitaliziranih tokova povremeno su presušili u ljetnim mjesecima zbog plitkog korita. Kako isušivanje uzrokuje značajno smanjenje autotrofne biomase jer direktno utječe na fotoautotrofe, za pretpostaviti je da je u razdobljima presušivanja korita zaostala alohtona organska tvar akumulirana u koritu imala veću važnost za perifiton revitaliziranih tokova. Timoner i sur. (2012) pokazali su da se u fazi presušivanja mediteranskih potoka algalna biomasa smanjila za 80% dok se bakterijska smanjila za samo 20% jer neke bakterije imaju sposobnost ostati aktivnima u uvjetima jako niske vlažnosti. Alohtona organska tvar akumulirana u koritu pospješuje procese respiracije od strane bakterija nakon ponovne uspostave vodenog toka (Datry i sur., 2011; von Schiller i sur., 2014; Sabater i sur., 2016). U revitaliziranim tokovima koji presušuju u ljetnim mjesecima metabolizam se vjerojatno mijenja od primata primarne produkcije koja je najvažnija u uvjetima prisustva vodenog toka do dominacije respiracije kao izvora energije u uvjetima presušivanja, zahvaljujući preostaloj alohtonoj organskoj tvari, a dijelom i huminskim spojevima koji imaju tendenciju zadržavanja u koritu (Catalán i sur., 2017). U novijem istraživanju dinamike organske tvari u povremenim potocima mediteranske regije Granados i sur. (2022) uočavaju progresivno smanjenje proteinskih spojeva autohtone organske tvari te povećanje huminskih spojeva alohtone organske tvari u perifitonu tijekom faze presušivanja, sugerirajući da povremeni potoci doživljavaju nagle promjene u sastavu organske tvari tijekom hidrološkog ciklusa, potencijalno utječući na mikrobnu aktivnost te posljedično raznolikost heterotrofnih organizama.

Pretvorba partikularne u otopljenu organsku tvar mineralizacijom u procesu razgradnje organske tvari od strane mikroorganizama ovisi o nizu okolišnih čimbenika, ali i sastavu iste (Romaní i sur., 2012). Tako se u procesu razgradnje listinca većina otpuštanja otopljene organske tvari (engl. *leaching*) događa unutar nekoliko dana pri čemu se čak 25% početne suhe mase svježe otkinutih listova gubi u prva 24 h (Allan i Castillo, 2007). Nakon toga slijedi kolonizacija od strane organizama koji pospješuju fragmentaciju i mineralizaciju. S obzirom na to da je alohtona organska tvar vrlo složena mješavina spojeva visoke molekularne mase slabije biološke iskoristivosti, značajne razlike u količini otopljene organske tvari koja je bila najveća u proljetnim mjesecima, a najmanja u jesenskim i zimskim mjesecima (publikacija **I**) potvrđuju ove pretpostavke o sezonskoj dinamici i promjenama u važnosti podrijetla organske tvari za perifiton revitaliziranih tokova. Pretvorba partikularne u otopljenu organsku tvar mineralizacijom i obrnuto - pretvorba otopljene organske tvari u partikularnu adsorpcijom na suspendirane čestice, dinamičan je proces koji ovisi o brojnim čimbenicima, a posljedično uvjetuje metaboličke procese unutar perifitona (Stedmon i Markager, 2005). Tako adsorpcija na suspendirane čestice/minerale ovisi o kemijskim obilježjima minerala pri čemu je kalcijev karbonat učinkovit „nositelj" partikularne organske tvari kao i mineralni dio detritusa (Remington i sur., 2007; De La Rocha i sur., 2008).

Otopljena organska tvar predstavlja najveći i najbolje raspoloživ izvor ugljika za bakterijsku zajednicu koja ima pionirsku važnost u prvim fazama kolonizacije podloge, odnosno prvim fazama razvoja perifitona (Bott i sur., 1984; Battin i sur., 2003). S druge strane, ugrađivanje slabo razgradive partikularne organske tvari (npr. huminskih spojeva) u bakterijski ekstracelularni matriks (EPS) i dalje rezultira niskim sadržajem dostupnih hranjivih tvari u ukupnoj masi perifitona (Weitere i sur., 2018). Kako bi uravnotežila svoj omjer hranjivih tvari i ugljika, heterotrofna komponenta perifitona (npr. fagotrofni protisti) mora nadoknaditi nizak sadržaj hranjivih tvari povećanjem stope hranjenja (ispaše) čime se mijenja sastav i raznolikost zajednice (Jochum i sur., 2017). Tako je relativni doprinos alohtone naspram autohtone organske tvari u prehrani fagotrofnih protista perifitona revitaliziranih tokova promjenjiv i ovisan ne samo o sezonskoj dinamici okolišnih čimbenika već i starosti i fazi razvoja perifitona, a heterotrofna komponenta perifitona se brzo prilagođava promjenama u dostupnosti resursa.

OSEDRAVANJE U REVITALIZIRANIM TOKOVIMA

Promjenjiva hidrologija (faze presušivanja i bujičnih tokova) revitaliziranih tokova reflektirala se i na količinu anorganske tvari, velik dio koje je činila sedra, u perifitonu revitaliziranih tokova tijekom prve godine istraživanja (publikacija **I**). Iako se udio sedre u perifitonu revitaliziranih tokova nije značajnije razlikovao od onog u kontrolnim tokovima, revitalizirani tokovi imali su povremeno niži udio sedre u perifitonu u odnosu na kontrolne tokove (publikacija **I**). Također, uočena je sezonska razlika u udjelu sedre u perifitonu s najvećim udjelom sedre u zimskim, a najmanjim u ljetnim mjesecima. Sezonska dinamika te utjecaj bujičnih tokova i presušivanja reflektirao se i u granulometrijskom/mineraloškom sastavu podloge. Rezultati granulometrijske analize pokazali su da je dominantna sastavnica podloge revitaliziranih i kontrolnih tokova šljunkoviti sediment koji se većinom sastojao od komada sedre. Iako nisu zabilježene značajne razlike u granulometrijskom sastavu između tokova, u revitaliziranim tokovima utvrđena je sezonska razlika s većim udjelom šljunkovite komponente tijekom jesenskih i zimskih mjeseci, odnosno većim udjelom sitnozrnate komponente u ljetnim mjesecima. Posljedica je to veće brzine strujanja i bujičnih tokova koji otplave sitnozrnatu komponentu u jesenskim i zimskim mjesecima. Također, rezultati granulometrijske analize potvrdili su i vrlo lošu sortiranost sitnozrnatog sedimenta. Svi su uzorci bili loše sortirani, a samo nekoliko prikupljenih u studenom i prosincu bilo je srednje i dobro sortirano. Stupanj sortiranja sedimenta funkcija je stabilnosti i postojanosti energetskih uvjeta taloženja (Tišljar, 2004). Slabo sortiranje tako ukazuje na kratak transport koji je najčešće posljedica bujičnih tokova (Folk, 1954). Kalcit je bio dominantna mineralna faza u svim skupnim uzorcima, dok je prevladavajuća faza netopljivog ostatka svih uzoraka bio kvarc čija prisutnost zajedno s tinjcima ukazuje na utjecaj ispiranja okolnog tla (Wilson, 2020).

S obzirom na to da je specifični cilj publikacije **I** bio pružiti uvid u granulometrijski sastav prirodne podloge, ova publikacija zbog odabrane metodologije nije dala uvid u količinu istaložene sedre nakon uklanjanja pajasena u revitaliziranim i kontrolnom tokovima, a koja bi mogla biti usporediva s prijašnjim istraživanjima na Skradinskom buku. Stoga publikacija **II** predstavlja nastavak istraživanja u kojem je osedravanje praćeno i kvantificirano na stakalcima u revitaliziranim i kontrolnim tokovima tijekom jednomjesečne i dvomjesečne ekspozicije. Količina anorganske tvari odnosno sedre istaložene tijekom jednomjesečne ekspozicije u revitaliziranim tokovima bila je značajno manja od one kontrolnih tokova, dok je za dvomjesečnu ekspoziciju uočeno obrnuto s ekstremno visokim vrijednostima zabilježenim upravo u revitaliziranim tokovima (npr. 143,60 mg/cm^2). U usporedbi s rezultatima Matoničkin Kepčija i sur. (2017) koji bilježe prosječnu količinu sedre od 1,64 mg/cm^2 tijekom jednomjesečne ekspozicije, prosječna količina sedre zabilježena tijekom jednomjesečne ekspozicije ovog istraživanja veća je deset puta u revitaliziranim, odnosno kontrolnim tokovima. Uočava se da je najveće osedravanje pa čak i s ekstremno visokim stopama zabilježeno tijekom ljetnih, a najmanje tijekom zimskih mjeseci. Ovi trendovi u skladu su s rezultatima Matoničkin Kepčija i sur. (2017) te Marić (2020) koji navode da je tijekom ljeta na Skradinskom buku zabilježena do pet puta veća količina sedre u odnosu na ostala godišnja doba, odnosno u skladu su s istraživanjima Srdoč i sur. (1985) te Matoničkin Kepčija (2006) provedenim na drugim sedrotvornim sustavima umjerenog pojasa.

Rezultati publikacije **II** upućuju na to da ljetna presušivanja revitaliziranih tokova nisu narušila proces osedravanja kao što se pokazalo u nekim sličnim istraživanjima. Tako Auqué i sur. (2013) bilježe vrlo niske ili čak negativne stope sedimentacije u sušnim razdobljima te uočavaju da je sedra tijekom sušnog razdoblja podložnija eroziji. U istraživanju prirasta sedre koje je simultano s ovim istraživanjem na istoj testnoj plohi Skradinskog buka proveo Marić (2020) metodom SfM fotogrametrije također je zabilježeno 1,5 puta veće osedravanje u ljetnom dijelu godine. Uzevši u obzir da su zabilježene količine sedre u revitaliziranim tokovima tijekom jednomjesečne ekspozicije bile gotovo deset puta veće od onih zabilježenih tijekom istraživanja Matoničkin Kepčija i sur. (2017), a količine organske tvari sedam puta veće, te činjenicu da isušivanje nije utjecalo na proces osedravanja, rezultati publikacije **II** sugeriraju da u procesu osedravanja u revitaliziranim tokovima količina organske tvari i posljedično zajednica organizama imaju veliku važnost. Pri tome značajnu ulogu ima razvoj opsežnog perifitonskog matriksa, koji zadržava i akumulira organsku tvar, ali služi i kao povoljna podloga za naseljavanje organizama. Također, bogato razvijena algalna zajednica vjerojatno je pospješila osedravanje s obzirom na to da alge uspješno vežu kristaliće kalcita pružajući podlogu za nukleaciju (Winsborough, 2000). Navedeno primjećuju i Matoničkin Kepčija i sur. (2017) koji utvrđuju snažnu pozitivnu korelaciju između količine organske tvari i sedre na umjetnim podlogama Skradinskog buka. U odnosu na količinu organske tvari na umjetnim podlogama revitaliziranih tokova, količina istaložene sedre bila je pet puta veća (uspoređujući maksimalne vrijednosti) što ukazuje da je anorganski materijal i dalje prevladavajući u perifitonu revitaliziranih tokova.

Iako s jedne strane prevelika količina sedimenta može dovesti do zatrpavanja organizama ili smanjenja prodiranja svjetlosti čime se ograničava primarna produkcija perifitona (Atristain i sur., 2023), s druge strane sediment može potaknuti prirast biomase perifitona djelujući kao izvor za ponovno naseljavanje nakon nepovoljnijih uvjeta (Dzubakova i sur., 2018), odnosno povećati heterogenost mikrostaništa (Bednar i sur., 2017; Singer i sur., 2021) i tako poslužiti kao „inokulum" za perifiton (Primc-Habdija i sur., 2001; Matoničkin Kepčija i sur., 2011, 2017). Ipak rezultati u publikaciji **II** sugeriraju na postojanje kritične točke u kojoj sadržaj anorganske tvari tj. sedre više ne potiče veliku brojnost i taksonomsku raznolikost trepetljikaša u revitaliziranim tokovima, već djeluje inhibitorno, sukladno prethodnim istraživanjima (Matoničkin Kepčija, 2006). S obzirom na to da je prosječna količina sedre zabilježena na umjetnim podlogama tijekom dvomjesečne ekspozicije bila značajno veća nego tijekom jednomjesečne ekspozicije, te da je tijekom jednomjesečne ekspozicije količina sedre pozitivno korelirala s taksonomskim metrikama trepetljikaša revitaliziranih tokova, a da je tijekom dvomjesečne ekspozicije uočeno suprotno, moguće je da je dio zajednice trepetljikaša bio zatrpan i otplavljen uslijed veće količine sedre, dok su ameboidni protisti puno manje osjetljivi zbog bliske povezanosti s podlogom (Preston, 2003). Tako ova publikacija osim kvantificiranju procesa osedravanja u perifitonu revitaliziranih tokova pridonosi i razumijevanju međuodnosa perifitona, heterogenosti mikrostaništa i procesa osedravanja koji je ključni fenomen NP Krka.

ODGOVOR PERIFITONA NA REVITALIZACIJU

Pivotalne mjere restauracije lotičkih ekosustava većinom se odnose na uklanjanje brana i/ili vraćanje kanaliziranih rijeka i potoka u njihove prirodne tokove pri čemu je najveći fokus na obnovi hidrogeomorfoloških elemenata i povećanju heterogenosti staništa (Roje-Bonacci i Bonacci, 2016). Ukoliko je u procjenu uspješnosti mjera restauracije uključena i biološka komponenta, ona se najčešće bazira na makrobeskralježnjacima i ribama, a metrike koje se pritom koriste ograničene su na taksonomske metrike, rijetko uključujući i funkcionalni odgovor. Moderne metode istraživanja koje primjenjuju okolišno metabarkodiranje pokazale su da je globalna raznolikost protista u lotičkim ekosustavima podcijenjena, a njihova biomasa potencijalno dvostruko veća od biomase svih životinja (Geisen i sur., 2017). Uzevši u obzir spomenuto te činjenicu da protisti, a ponajprije fagotrofni protisti imaju ključno mjesto u hranidbenim mrežama lotičkih ekosustava, za očekivati je da je njihova primjena kao bioindikatora u detekciji različitih okolišnih promjena, pa tako i promjena nastalih uslijed mjera restauracije, sve češća. Ipak, za uspješnu implementaciju fagotrofnih protista u restauracijsku praksu, potrebno je istražiti i identificirati taksonomske i funkcionalne metrike čiji odgovor je vidljiv i mjerljiv, te razumjeti prostorno-vremensku dinamiku zajednice i pripadajućih okolišnih čimbenika.

Perifiton revitaliziranih tokova u ovom istraživanju imao je veći broj svojti fagotrofnih protista (dominantno okućenih ameba i trepetljikaša), ali isto tako i veći broj svojti meiofaune, odnosno mikroskopskih metazoa, u odnosu na kontrolne tokove, unutar prve godine od aktivacije vodenih tokova (publikacija **I**). U perifitonu revitaliziranih tokova najveću abundanciju i raznolikost postizale su okućene amebe i trepetljikaši čiji je broj svojti bio gotovo dvostruko veći na revitaliziranim tokovima u odnosu na kontrolne, te kolnjaci (Rotifera) od pripadnika meiofaune. S druge strane, perifiton kontrolnih tokova obilježavala je velika abundancija slobodnoživućih oblića (Nematoda) i manja abundancija kolnjaka u odnosu na revitalizirane tokove. Zabilježene razlike reflektiraju dominante okolišne uvjete. Kolnjaci uslijed brzog razvoja, partenogenetskog razmnožavanja i relativno kratkog životnog ciklusa mogu razviti populacije velike brojnosti, stoga uspješno toleriraju i uspijevaju u nestabilnoj okolini promjenjive dinamike (Traunspurger, 2002) kao što su revitalizirani tokovi sedrene barijere. Također, bentoske vrste kolnjaka, osim što stružu ili sakupljaju čestice hrane s podloge, su i predatori nad fagotrofnim protistima (dominantno trepetljikašima) i drugim mikroskopskim predstavnicima metazoa te konzumirajući perifiton pozitivno utječu na cjelokupnu zajednicu održavajući je u stanju logaritamskog rasta (Traunspurger, 2002). U perifitonu revitaliziranih tokova utvrđene su i ličinke mnogih kukaca, npr. porodice Chironomidae čiji veliki dio ishrane čine upravo kolnjaci (Schmid i Schmid-Araya, 1997) potvrđujući ulogu perifitona u prijenosu energije s nižih u više trofičke razine. S druge strane, mahovinski pokrov u kontrolnim tokovima pružio je dobro skrovište i potencijalni izvor hrane slobodnoživućim oblićima. Vodene mahovine lotičkih ekosustava pogodna su podloga za kolonizaciju brojnih mikroskopskih i makroskopskih metazoa, jer predstavljaju zaklon od strujanja vode i otplavljivanja iz staništa te imaju veliki kapacitet retencije i akumulacije organske tvari, koja je izvor hrane bentoskim organizmima (Traunspurger, 2002). Dražina i sur. (2013, 2014) u mahovinama reotopa sedrenih barijera NP Plitvička jezera bilježe dominantnost oblića iz reda Dorylaimida koji pripadaju isisavačima, odnosno konzumiraju biljno tkivo te perifiton i njegove komponente bušeći bodežićem i isisavajući citoplazmu mišićnim ždrijelom. S obzirom na to da u publikaciji **I** mikroskopski metazoa nisu determinirani do razine reda, ostaje za pretpostaviti da su i u perifitonu kontrolnih tokova dominirali oblići spomenutog načina ishrane, a njihov izostanak s revitaliziranih tokova uvjetovan je izostankom mahovinskog pokrova, koji se nije stigao razviti tj. za čiji razvoj je vjerojatno potrebno duže razdoblje. Laasonen i sur. (1998) te Haapala i sur. (2003) bilježe da je tendencija povećanju raznolikosti meiofaune paralelna s ponovnim razvojem vodenih mahovina u revitaliziranim potocima borealne regije. Uzevši u obzir činjenicu da unutar prve godine od provođenja restauracijskih mjera na plohi Skradinskog buka nije došlo do razvoja mahovinskog pokrova u revitaliziranim tokovima, rezultati publikacije **I** sugeriraju da bi mahovine mogle igrati važnu ulogu u oporavku skupina mikroskopskih metazoa u revitaliziranim tokovima sedrene barijere Skradinski buk.

Cilj publikacije **II** ovog doktorskog rada bio je nadopuniti istraživanje prikazano u publikaciji **I** praćenjem perifitona, njegovog sastava i dinamike tijekom iduće dvije godine istraživanja. Specifičan cilj ove publikacije bio je usporediti različite metode uzorkovanja perifitona na sedrenim barijerama i optimizirati korištenje umjetnih podloga u svrhu primjene istih u restauracijskoj praksi. Stoga publikacija **II** pruža uvid u komplementarno istraživanje u kojem je prethodna metodologija uzorkovanja prirodne podloge prikazana publikacijom **I** nadopunjena uzorkovanjem perifitona s umjetnih podloga. U tu svrhu, kao umjetne podloge korištena su predmetna stakalca umetnuta u pločice od pleksiglasa, dizajnirane i 3D printane za potrebe ovog istraživanja. S obzirom na to da su revitalizirani tokovi plitki tokovi koji ispiru meku podlogu (šumsko tlo), postojala je potreba za dizajnom specifičnim i prilagođenim za ovu svrhu, odnosno pločicama koje bi se mogle učvrstiti direktno u tlo, pritom izbjegavajući preveliku debljinu podloge kako ista ne bi ostala na suhome u slučaju niskog protoka i oskudice vodom. Najčešće vrijeme ekspozicije umjetnih podloga koje se koristilo u dosadašnjim istraživanjima perifitona u sedrotvornom ekosustavu NP Krka podrazumijevalo je period između dva do šest tjedana, tj. najčešće 30 dana temeljeno na MacArthur-Wilson modelu prema kojem bi zajednica trebala dostići ravnotežno stanje u 30 dana ekspozicije (Primc-Habdija i sur., 1997; Matoničkin Kepčija i sur., 2017). Sukladno pretpostavci da će primjena ovakve metodologije biti manje invazivna za zajednicu i omogućiti direktno pregledavanje predmetnih stakalaca pod mikroskopom, drugi specifičan cilj ove publikacije bio je istražiti taksonomske i funkcionalne metrike fagotrofnih protista, primarno ameboidnih protista i trepetljikaša u perifitonu revitaliziranih i kontrolnih tokova te utvrditi postoje li razlike među njima. Kombinacija različitih metrika može olakšati postizanje ciljeva u projektima restauracije te pružiti sveobuhvatniju procjenu uspješnosti mjera. Ameboidni protisti i trepetljikaši nerijetko su najbrojnija i najraznolikija komponenta perifitona sedrotvornih ekosustava RH pa tako i unutar lotičkih dijelova NP Krka i sedrene barijere Skradinski buk (Primc-Habdija i Matoničkin, 2005; Matoničkin Kepčija i sur., 2017; Kulaš i sur., 2021). Primjenom metode okolišnog metabarkodiranja i regije V9 Kulaš i sur. (2023) bilježe da su upravo ameboidni protisti i trepetljikaši među najzastupljenijim protistima Skradinskog buka.

S obzirom na to da se pokazalo kako i u izostanku učinka restauracije na taksonomske metrike promjene izazvane restauracijom mogu utjecati na sastav funkcionalnih značajki vrsta, što ima implikacije i za funkcioniranje ekosustava (Frainer i McKie, 2015), u istraživanje su uključene i funkcionalne metrike, odnosno mjere funkcionalne raznolikosti temeljene na prethodno dodijeljenim funkcionalnim značajkama. Rezultati publikacije **II** pokazuju da se taksonomske i funkcionalne metrike ameboidnih protista nisu značajno razlikovale između revitaliziranih i kontrolnih tokova, niti tijekom jednomjesečne ni dvomjesečne ekspozicije podloga uz iznimku da su revitalizirani tokovi imali nižu abundanciju tijekom jednomjesečne ekspozicije. Niža abundancija vjerojatno je posljedica veće primarne produkcije u perifitonu revitaliziranih tokova tijekom jednomjesečne ekspozicije. Iako su koncentracije ortofosfata tijekom istraživanja bile prilično niske i unutar raspona karakterističnog za područje Skradinskog buka (Primc-Habdija i Matoničkin, 2005; Matoničkin Kepčija i sur., 2017), mogle su pospješiti proliferaciju algi i posljedično veću koncentraciju klorofila *a*. Gust i opsežno razvijen algalni pokrov potencijalno je doveo do smanjenja ili čak prekida konzumacije bakterija od strane ameboidnih protista na što upućuje i značajno negativna korelacija s vrijednostima koncentracije klorofila *a*. Algalna prevlaka povećava ukupnu veličinu bakterijskih čestica ili mijenja njihov „okus“ (Heaton i sur., 2001), a osim toga, smanjenje konzumacije bakterija može biti i posljedica kombinacije različitih strategija „obrane“ plijena i interakcije predator-plijen. Neke bakterije npr. sintetiziraju spojeve za odbijanje predatora koji su većinom hlapljivi organski spojevi, a na temelju kojih trepetljikaši mogu razlikovati vrstu plijena (Jousset, 2012; Schulz-Bohm i sur., 2017). S obzirom na to da se znanje o interakciji predator-plijen temelji na tek nekoliko modelnih istraživanja te činjenicu da u publikaciji **II** nije istraživan sastav bakterijskih zajednica, ostaje samo za pretpostaviti da je ovaj tip interakcije također mogao utjecati na abundanciju ameboidnih protista. Funkcionalna raznolikost ameboidnih protista nije se značajnije mijenjala s promjenom ekspozicije podloga. Veća funkcionalna raznolikost ameboidnih protista zabilježena je u perifitonu revitaliziranih tokova tijekom jednomjesečne ekspozicije, dok je tijekom dvomjesečne ekspozicije bila niža u odnosu na kontrolne tokove, ali razlog tomu je nagli porast u funkcionalnoj raznolikosti kontrolnih tokova, a ne promjena u funkcionalnoj raznolikosti ameboidnih protista u revitaliziranim tokovima. S obzirom na pozitivnu korelaciju funkcionalnih metrika s količinom partikularne organske tvari, sedre i klorofila *a* tijekom jednomjesečne ekspozicije, evidentno je da je veći broj ekoloških niša za ameboidne protiste ponovno posljedica strukturne složenosti i velikog broja mikrostaništa. Različita mikrostaništa nastala uslijed taloženja kristalića sedre te retencije organske tvari omogućila su odvajanje funkcionalnih niša i suživot ameboidnih protista različitih funkcionalnih značajki, poput bakteriovornih te omnivornih svojti, ali i onih koje se hrane algama i cijanobakterijama kao rezultat razvoja obilnog algalnog pokrova. Kako daljnja analiza zastupljenosti funkcionalnih značajki ameboidnih protista u zajednici nije bila među specifičnim ciljevima ovog doktorskog rada već se isti koncentrirao na funkcionalne metrike na razini zajednice, ostaje za istražiti sastav funkcionalnih značajki u odnosu na dominantne okolišne uvjete.

Za razliku od ameboidnih protista, zajednice trepetljikaša perifitona revitaliziranih tokova značajno se razlikuju od onih unutar kontrolnih tokova u taksonomskim i funkcionalnim metrikama (publikacija **II**). Taksonomske metrike trepetljikaša revitaliziranih tokova više su od onih u kontrolnim tokovima za oba perioda ekspozicije (jednomjesečni i dvomjesečni). Za razliku od publikacije **I**, u ovom istraživanju zabilježen je dvostruko veći broj vrsta trepetljikaša (78) koji je usporediv s onim zabilježenim u prethodnom istraživanju perifitona Skradinskog buka (72) (Matoničkin Kepčija i sur., 2017). Ako se uzme u obzir da su osim broja vrsta trepetljikaša korištenjem umjetnih podloga utvrđene i usporedive količine organske i anorganske tvari diskutirane u prethodnim poglavljima, može se zaključiti da je metodologija korištenja umjetnih podloga povoljnija za istraživanja perifitonskih trepetljikaša u revitaliziranim tokovima Skradinskog buka čime se pruža odgovor na jedno od specifičnih pitanja ovog doktorskog rada. S druge strane, funkcionalne metrike trepetljikaša revitaliziranih tokova niže su u odnosu na kontrolne tokove za oba perioda ekspozicije što ukazuje na nižu funkcionalnu raznolikost. Ipak, razvidno je da se ta razlika smanjuje s dužinom ekspozicije sugerirajući da zajednica postaje sličnijom onoj kontrolnih tokova.

Kako se utjecaj dinamičnih i promjenjivih hidroloških uvjeta u vidu povremenih presušivanja i bujičnih tokova odrazio na većinu okolišnih parametara u revitaliziranim tokovima, izgledno je da isti djeluju nepovoljno i na funkcionalnu raznolikost trepetljikaša, sudeći prema IDH hipotezi (engl. *Intermediate Disturbance Hypothesis*) prema kojoj se najveća raznolikost ostvaruje pri srednjoj razini poremećaja (Bell, 2000). Odnos funkcionalne raznolikosti i poremećaja nije uvijek unimodalan te ovisi i o produktivnosti sustava, odnosno udio hranjivih tvari i produktivnost mogu povećati ili smanjiti kompeticiju između vrsta, ovisno o kontekstu te nizu biotičkih i abiotičkih čimbenika (Bell, 2000; Kadmon i Benjamini, 2006). Tako visoki sadržaj hranjivih tvari i visoka produktivnost mogu povećati kompeticiju jer osiguravaju više resursa za rast i reprodukciju, što može dati prednost određenim vrstama/funkcionalnim značajkama u odnosu na druge. Kada su resursi u izobilju, neke vrste mogu brže rasti i stvarati više potomaka, što može rezultirati kompeticijskom prednošću u odnosu na druge vrste. Nadalje, u sustavima s visokim udjelom hranjivih tvari i visokom produktivnošću, vrste koje su učinkovitije u korištenju resursa favorizirane su u odnosu na one koje su manje učinkovite (Cardinale i sur., 2006). S druge strane, i nizak udio hranjivih tvari, kao i niska produktivnost, također mogu dovesti do smanjenja funkcionalne raznolikosti uslijed kompetitivnog isključivanja određenih vrsta (kompetitivna ekskluzija, engl. *competitive exclusion*) (Leduc i sur., 2015). U uvjetima ograničenih resursa povećava se kompeticija za istim i favorizirane su samo one vrste koje su najkonkurentnije. Osim toga, u uvjetima niskog sadržaja hranjivih tvari kao i niske produktivnosti, sustav je osjetljiviji na poremećaje što može dodatno smanjiti funkcionalnu raznolikost isključivanjem nekih vrsta koje nisu dobro prilagođene takvim uvjetima (Cardinale i sur., 2006).

I u ovoj publikaciji kao i u prethodnoj (**I**), strukturna složenost i heterogenost mikrostaništa u vidu akumulirane partikularne organske tvari alohtonog ili autohtonog podrijetla (iskazane mjerenjem koncentracije klorofila *a*) te anorganske tvari tj. sedre pokazala se dominantnim faktorom koji uvjetuje zajednicu. S obzirom na to da je količina partikularne organske tvari u perifitonu dosezala maksimum tijekom jednomjesečne ekspozicije, a koncentracija klorofila *a* u perifitonu revitaliziranih tokova bila veća nego u kontrolnim tokovima, izgledno je da je veća produktivnost tijekom jednomjesečne ekspozicije favorizirala određene funkcionalne značajke. Takva favorizacija mogla je otežati uspostavu i razvoj drugih, te tako dovesti do snažnog kompetitivnog isključivanja koje je rezultiralo smanjenom funkcionalnom raznolikošću. U perifitonu revitaliziranih tokova uspijevaju se održati samo vrste čije funkcionalne značajke omogućavaju bolju toleranciju na promjenjive hidrološke uvjete, odnosno poremećaje. Premda publikacija **II** nije pružila uvid u sastav zajednice trepetljikaša već se primarno fokusirala na metrike zajednice, iz publikacije **III** evidentno je da se snažna dominacija nekoliko vrsta u smislu relativne abundancije u revitaliziranim tokovima odrazila na funkcionalnu raznolikost zato što su te vrste imale iste funkcionalne uloge, što je rezultiralo većim natjecanjem između vrsta (DeLong i Vasseur, 2012).

Cilj publikacije **III** bio je utvrditi koje funkcionalne kategorije trepetljikaša prevladavaju u revitaliziranim tokovima te ih usporediti s kontrolnim tokovima, a za uvid u sastav funkcionalnih značajki izračunate su ponderirane vrijednosti funkcionalnih značajki u zajednici (engl. *Community Weighted Means*, CWM). Time se mogu kvantificirati promjene u srednjim vrijednostima značajki unutar skupine, koje proizlaze iz odabira okoliša za određene kategorije funkcionalnih značajki (Ricotta i Moretti, 2011.) CWM vrijednosti potom su uspoređene korištenjem GLMM-ova te korelirane s okolišnim parametrima koji su pokazali značajne razlike između revitaliziranih i kontrolnih tokova kao što je već pokazano u publikaciji **II**. Revitalizirani vodeni tokovi dali su prednost oportunističkim, visoko tolerantnim generalistima kao što je vrsta *Platyophrya vorax* (Lüftenegger i sur., 1985) koja je činila i do 73% abundancije zajednice perifitona u revitaliziranim tokovima. Ova vrsta pokazala se i jedinom vrstom od potencijalne indikatorske važnosti za revitalizirane tokove sedrene barijere Skradinski buk pruživši tako odgovor na jedan od specifičnih ciljeva ove publikacije. Kao i većina trepetljikaša reda Colpodea, ova vrsta izuzetno je pokretljiva (naspram puzajućih vrsta) što je čini manje osjetljivom na fluktuacije okolišnih uvjeta i vrlo često dominira brojnošću u sustavima izloženim promjenama/poremećajima (Lüftenegger i sur., 1985; Foissner, 1999). Matoničkin Kepčija (2006) također bilježi veliku brojnost ove vrste u perifitonu Donjih jezera NP Plitvička jezera. Osim toga, omnivorni način ishrane ne ograničava je u izvoru hrane čineći ju fleksibilnijom u odnosu na specijaliste (npr. bakteriovore) (Lake i sur., 2007). Frainer i sur. (2017) također uočavaju povećani udio omnivornih konzumenata u revitaliziranim tokovima.

Trepetljikaši reda Colpodea većinom naseljavaju kopnene ekosustave i smatraju se r-stratezima u tlu, odnosno toleriraju snažne fluktuacije okolišnih uvjeta (Lüftenegger i sur., 1985). Za razliku od kontrolnih tokova, u perifitonu revitaliziranih tokova značajno je veći udio vrsta koje su osim u vodenim ekosustavima brojne i u tlu te sustavima bogatim hranjivim tvarima poput aktivnog mulja (Foissner, 2016). Upravo ove razlike u dominantnim funkcionalnim značajkama trepetljikaša perifitona revitaliziranih i kontrolnih vodenih tokova u publikaciji **III** sugeriraju blisku povezanost kopnenih i vodenih ekosustava unutar revitaliziranih tokova koja je već potvrđena uvidom u dinamiku organske tvari u publikacijama **I** i **II**. Vrsta *P. vorax,* prema recentnim istraživanjima, ima važnu ulogu u procesu degradacije ugljika u kopnenim ekosustavima (Pastorelli i sur., 2022) stoga ova vrsta ima implikacije i za važnost u kruženju ugljika i ciklusu organske tvari u revitaliziranim tokovima koji su okosnica kopnenih i vodenih ekosustava. Pozitivna korelacija abundancije *P. vorax* i koncentracije nitrita utvrđena tijekom jednomjesečne ekspozicije upućuje na to da otopljeni oblici dušika poput nitrita mogu stimulirati proces razgradnje organske tvari, a time i hrane za ovu vrstu. Uz spomenutu omnivornu vrstu *P. vorax*, većina trepetljikaša koji naseljavaju tlo su bakteriovori što je bio slučaj i u revitaliziranim tokovima unutar kojih su najveću abundanciju postizale vrste *Cinetochilum margaritaceum*, *Chilodonella uncinata* i *Chlamydonella alpestris*.

Osim razlika u izvoru hrane i preferenciji ekosustava, uočene su i razlike u pokretljivosti trepetljikaša između revitaliziranih i kontrolnih tokova, odnosno u perifitonu revitaliziranih tokova uočena je dominacija pokretnih slobodnoplivajućih trepetljikaša, dok je u kontrolnim tokovima veći udio sjedilačkih i polusjedilačkih vrsta, a od pokretnih trepetljikaša veći je udio puzajućih. Ovi su nalazi u skladu s opaženim trendovima Risse-Buhl i Küsel (2009), koji su pokazali da perifitonom u potocima s višom brzinom strujanja i bujičnim tokovima dominiraju dobro pokretni, spljošteni trepetljikaši kao što su vrste *P. vorax*, *C. uncinata* i *C. alpestris* utvrđene u ovoj publikaciji. Zabilježene vrste su dorzoventralno spljoštene, a spljoštenost im omogućava iskorištavanje smanjenog graničnog sloja vode tijekom veće brzine strujanja, pružajući im otpornost na fluktuacije hidroloških uvjeta (Saravia i sur., 2001). Matoničkin Kepčija i sur. (2017) također uočavaju dominaciju pokretnih vrsta trepetljikaša u uvjetima hidrološkog stresa, a Matoničkin Kepčija (2006) bilježi baš vrstu *C. uncinata* kao jednu od najotpornijih na otplavljivanje. Također, pokretne vrste iskorištavaju uvjete smanjene kompeticije s obzirom na otplavljene sesilne i polusesilne trepetljikaše stoga se razvijaju u velikom broju u revitaliziranim tokovima. Tullos i sur. (2009) smatraju da neke mjere restauracije poput rekonfiguracije kanala djeluju kao filter „protiv“ organizama koji zahtijevaju stabilna staništa i specifične izvore hrane, odnosno uočavaju da restaurirane tokove naseljavaju vrlo pokretljive vrste, oportunističke sklonosti staništu i hrani. Prevlast pokretnih trepetljikaša u revitaliziranim tokovima može se povezati i sa većom složenosti mikrostaništa uslijed ispiranja okolnog tla, nataloženih kristalića kalcita tj. sedre i zadržavanja čestica autohtone i alohtone organske tvari. Veći raspon različitih niša bolje iskorištavaju pokretni, slobodno plivajući nego puzajući trepetljikaši budući da su vjerojatno sposobniji izbjeći zatrpavanje intenzivnim taloženjem sedre. Takvi trepetljikaši su i pripadnici rodova *Oxytricha*, *Tachysoma* i *Urosomoida* zabilježeni u revitaliziranim tokovima. Ovi trepetljikaši posjeduju morfološke prilagodbe koje im omogućuju skrivanje ili lov među ili unutar nakupina čestica - većina je kratkih do dugih eliptičnih obrisa, ventralno spljoštenog tijela (Berger, 1999). Iako njihova relativna brojnost nije bila usporediva s onom drugih vrsta u zajednici i stoga nisu prepoznati kao potencijalno indikatorske vrste revitaliziranih vodenih tokova, vrste ovih rodova bile su brojnije u revitaliziranim nego u kontrolnim tokovima (publikacija **III**).

S druge strane, u perifitonu kontrolnih tokova uočena je dominacija sjedilačkih kružnotrepetljikaša među kojima je najzastupljenija bila vrsta *Vorticella aquadulcis*-complex koja se pokazala vrstom od indikatorskog potencijala za kontrolne tokove zajedno s pokretnim, ali puzajućim vrstama poput vrste *Aspidisca cicada*. Ove vrste već su prepoznate kao važna komponenta perifitona lotičkih dijelova rijeke Krke i Skradinskog buka, a Matoničkin Kepčija i sur. (2017) bilježe dominaciju sjedilačkih kružnotrepetljikaša (Peritrichia) u perifitonu Skradinskog buka za razliku od barijere Roškog slapa na kojem, kao i u revitaliziranim tokovima ovog istraživanja, bilježe dominaciju pokretnih trepetljikaša. Peritrihni trepetljikaši uglavnom su sjedilački bakteriovori koji žive trajno ili privremeno pričvršćeni za podlogu (Foissner i Berger, 1996; Arndt i sur., 2003). Prisutnost peritrihnih trepetljikaša kao najbrojnije komponente zajednice u perifitonu Skradinskog buka potvrđena je i recentnim istraživanjem Kulaš i sur. (2023) uz razliku roda *Carchesium* utvrđenog kao najbrojnijeg. Osim peritrihnih trepetljikaša, u publikaciji **III** u kontrolnim tokovima zabilježene su i vrste roda *Stentor* koje se nisu pojavljivale u perifitonu revitaliziranih tokova. Vrste ovog roda žive sjedilački ili polusjedilački izlučujući sluz kojom se pričvršćuju za podlogu (Arndt i sur., 2003), a njihova dominacija u uvjetima niske brzine strujanja zabilježena je i u NP Plitvička jezera (Primc-Habdija i sur., 2000). Istraživanje Kulaš i sur. (2023) potvrdilo je ova morfološka opažanja komplementarnim molekularnim metodama kojima je upravo rod *Stentor* bio najzastupljeniji među rodovima zabilježenim na Skradinskom buku. Dominacija sjedilačkih i polusjedilačkih trepetljikaša te pokretnih puzajućih trepetljikaša u kontrolnim tokovima, odnosno njihovo odsustvo u revitaliziranim tokovima, ukazuje još jednom na važnost hidrološke promjenjivosti i heterogenosti mikrostaništa u strukturiranju funkcionalnog sastava perifitonskih trepetljikaša revitaliziranih tokova.

FILOGENETSKA I FUNKCIONALNA RAZNOLIKOST TREPETLJIKAŠA RIJEKE KRKE

Uz niz abiotičkih faktora, važnost interspecijske kompeticije odnosno kompetitivnog isključivanja pokazala se jednim od ključnih čimbenika u oblikovanju sastava i raznolikosti perifitonskih trepetljikaša revitaliziranih tokova sedrene barijere Skradinski buk (publikacije **II** i **III**). S obzirom na navedeno, uloga kompetitivnog isključivanja dodatno je istražena u publikaciji **IV** ovog doktorskog rada, koja premda nije direktno vezana uz revitalizirane tokove Skradinskog buka, istražuje perifitonske trepetljikaše na četiri lokacije od izvora prema ušću rijeke Krke (izvor Krke, Marasovine, Roški slap, Skradinski buk) pruživši sveobuhvatan uvid u filogenetsku i funkcionalnu raznolikost trepetljikaša duž rijeke Krke. Dodatno, u istraživanje je uključena i komponenta raznolikosti mikrostaništa budući da je perifiton uzorkovan s ploha s gornje i donje strane supstrata (svjetlo/tamno izložena strana sedre). Riječni ekosustavi pružaju gotovo idealan empirijski kontekst za procjenu filogenetske strukture zajednice na različitim razinama (Lowe i sur., 2006; Múrria i sur., 2012). Okolišni čimbenici kao što su protok, brzina strujanja, temperatura te količina nutrijenata mijenjaju se longitudinalno od izvora do ušća rijeke, ali i unutar mikrostaništa omogućavajući okolišnom odabiru (engl. *environmental filtering*) ulogu u strukturiranju zajednica, uz kompeticiju (Vannote i sur., 1980; Lowe i sur., 2006; Allan i Castillo, 2007). Heterogenost staništa također se može odraziti na morfološke, fiziološke i trofičke značajke što rezultira ekološkom divergencijom filogenetskih linija (Múrria i sur., 2012).

Publikacija **IV** nastavak je istraživanja Kulaš i sur. (2021) u kojem je primjenom komplementarnih molekularnih metoda (DNA metabarkodiranja okoliša) i tradicionalne mikroskopske identifikacije pružen uvid u taksonomsku raznolikost trepetljikaša rijeke Krke. S obzirom na to da okolišni metabarkoding generira ogromnu količinu podataka o protistima, a analiza takvih podataka je zahtjevna, integrativni pristup nužan je u suvremenim istraživanjima fagotrofnih protista i protista uopće (Clamp i Lynn, 2017). U publikaciji **IV** funkcionalne značajke trepetljikaša dodijeljene su što je detaljnije moguće kako bi uspješno detektirale razlike u funkcionalnoj raznolikosti trepetljikaša među lokacijama koje predstavljaju hidrološki različite dijelove rijeke Krke. Trepetljikaši su svrstani u iste funkcionalne kategorije kao i u prethodne tri publikacije: izvor hrane, način/strategija hranjenja, preferencija ekosustava/staništa, pokretljivost, način kretanja (za pokretne vrste) i životni oblik, no broj funkcionalnih značajki unutar svake od kategorija bio je mnogo veći

(Dodatni materijal uz publikaciju **IV**). Filogenetska raznolikost (divergentnost) kvantificirana je metrikom filogenetske raznolikosti (engl. *phylogenetic diversity*, PD) i metrikom filogenetske varijabilnosti vrste (engl. *phylogenetic species variability*, PSV), a funkcionalna raznolikost metrikom funkcionalnog bogatstva (engl. *functional richness*, FRic). Ova kombinacija metrika pokazala se uspješnom u nizu prijašnjih istraživanja slične tematike (Ashford i sur., 2018).

Rezultati publikacije **IV** pokazali su da izvorišno područje rijeke Krke ima nižu filogenetsku i funkcionalnu raznolikost od nizvodno smještenih sedrenih barijera (uključujući Skradinski buk). Filogenetska divergentnost zajednice trepetljikaša u izvorišnom području rijeke Krke može biti rezultat kompetitivnog isključivanja ili okolišnog odabira ili pak izglednije kombinacije ovih čimbenika. U zajednicama koje su filogenetski udaljenije (karakterizira ih filogenetska disperzija/filogenetski divergentna zajednica), često sastavom vrsta u zajednici upravljaju interakcije vrsta (Cavender-Bares i sur., 2004; Emerson i Gillespie, 2008). Za razliku od barijere Skradinski buk, izvorišno područje karakterizirala je manja koncentracija otopljenih hranjivih tvari i niži protok stoga je moguće da su upravo ovi okolišni uvjeti favorizirali uspješnije kompetitore poput sjedilačkih i polusjedilačkih kolonijalnih trepetljikaša koji su uspješni bakteriovorni filtratori, rezultirajući filogenetski divergentnom zajednicom u izvorišnom području rijeke Krke. Nizak sadržaj hranjivih tvari karakterističan je za izvore krških rijeka (Bonacci, 1985), a rezultati ove publikacije ukazuju da su možda upravo takvi uvjeti u izvorišnom području rijeke Krke doveli do opadanja funkcionalne raznolikosti uslijed povećanja kompeticije koja vodi kompetitivnom isključivanju i eliminaciji onih funkcionalnih značajki koje ne čine dobrog kompetitora. Iako rezultati publikacije **IV** nisu pokazali da raznolikost mikrostaništa (svjetlo/tamno izložena strana kamena) ima značajan učinak na testirane metrike filogenetske i funkcionalne raznolikosti trepetljikaša, uvjeti i složenost mikrostaništa također mogu imati važnu ulogu u kompeticiji. Lentendu i Dunthorn (2021) pokazali su da lokalna heterogenost može utjecati na kompetitivno isključivanje, te da ono ima veći učinak u manje heterogenim staništima. Snyder i Chesson (2004) također su otkrili da visoka lokalna heterogenost potiče suživot konkurentnih vrsta. Izvorišno područje rijeke Krke obilježavaju homogeniji okolišni uvjeti i manja heterogenost mikrostaništa u odnosu na nizvodnu barijeru Skradinski buk koju pak karakterizira pregršt različitih geomorfoloških oblika sedre (Bonacci i sur., 2017), ali i maritimni utjecaj (što se reflektiralo u prisutnosti rodova koji su karakteristični za bočate vode), rezultirajući većom funkcionalnom raznolikošću u odnosu na izvorišno područje.

Od velike je važnosti utvrditi uvjetuje li interspecijska kompeticija zajednicu ili to dominantno čini okolišni odabir jer takva spoznaja poboljšava razumijevanje osjetljivosti ekosustava na promjene/poremećaje i olakšava predvidjeti kako će se pojedini ekosustavi mijenjati u svjetlu sve intenzivnijih klimatskih promjena. Naime, zajednice koje su dominantno strukturirane interspecijskom kompeticijom obično su stabilnije i manje podložne poremećajima od onih u kojima najveću važnost ima okolišni odabir (Didham i sur., 2005; Ashford i sur., 2018). U slučaju da je filogenetska i funkcionalna raznolikost trepetljikaša izvorišnog područja rijeke Krke dominantno uvjetovana kompetitivnim isključivanjem, perifiton izvorišnog područja mogao bi biti manje podložan poremećajima. Ipak, niža filogenetska i funkcionalna raznolikost izvorišnog područja također može ukazivati na to da su neke niše neiskorištene dalje sugerirajući odsustvo vrsta koje bi inače mogle iskoristiti takve uvjete predstavljajući zaštitu od poremećaja. Na taj način sustav postaje osjetljivijim na poremećaje u vidu invazivnih vrsta i raznolikih antropogenih poremećaja što je zabrinjavajuće s obzirom na to da se krški izvori prema ekološkoj mreži Natura 2000 smatraju prioritetnim među ugroženim stanišnim tipovima u RH (Radović i sur., 2006).

Pokazavši da se filogenetska i funkcionalna raznolikost trepetljikaša mijenja duž toka rijeke Krke, publikacija **IV** pruža svojevrsnu podlogu za daljnja istraživanja ove tematike u drugim krškim rijekama mediteranske regije. Iako nije dovoljno jasno je li filogenetska divergentnost zajednice trepetljikaša u izvorišnom području rijeke Krke rezultat kompetitivnog isključivanja ili okolišnog odabira ili pak kombinacije ovih čimbenika, ova publikacija postavila je temelje za buduća istraživanja ove skupine u drugim krškim lotičkim ekosustavima mediteranske regije. S obzirom na to da trenutno znanje o funkcionalnoj raznolikosti nije dostatno za određivanje funkcionalnih niša većine protista, povezivanje filogenetske raznolikosti sa funkcionalnim metrikama zajednice je trenutno najprecizniji pristup koji se može koristiti u ovakvim istraživanjima.

Usporedbom sastava funkcionalnih značajki perifitonskih trepetljikaša Skradinskog buka u publikaciji **IV** i onog u kontrolnim tokovima u publikacijama **II** i **III** uočava se preklapanje u velikom dijelu rezultata. Tako u oba istraživanja u zajednici prevladavaju tipične perifitonske i bentoske vrste/rodovi koji se pokreću puzanjem što potvrđuje već prepoznatu važnost hidroloških uvjeta u strukturiranju funkcionalnog sastava perifitonskih trepetljikaša. Prisutnost predatorskih trepetljikaša u perifitonu Skradinskog buka koja je zabilježena u oba istraživanja (publikacije **II**, **III** i **IV**) karakteristična je za stabilnu zajednicu u kojoj za razliku od kompeticije/kompetitivnog isključivanja koje je prepoznato kao potencijalno važan element strukturiranja sastava perifitona revitaliziranih vodenih tokova i izvorišnog područja Krke, važan utjecaj ima i predatorski pritisak (Arndt i sur., 2003). Uz navedeno, u publikaciji **IV** u perifitonu Skradinskog buka zabilježen je veliki udio euplanktonskih rodova te onih okarakteriziranih kao bočati i morski (odnosno koji se pojavljuju i u morskim ekosustavima) što nije zabilježeno istraživanjem prikazanim u publikacijama **II** i **III**. Time publikacija **IV** potvrđuje važnost komplementarnog istraživanja u kojemu su potencijalna ograničenja tradicionalne mikroskopske identifikacije nadopunjena metabarkodiranjem DNA čime je omogućen bolji uvid u funkcionalnu raznolikost perifitona sedrene barijere Skradinski buk.

Osim što se razlikuje duž longitudinalnog profila rijeke Krke (publikacija **IV**), funkcionalna raznolikost trepetljikaša razlikuje se i ovisi i o heterogenosti mikrostaništa, odnosno ovisi i o lateralnim elementima okolnih kopnenih ekosustava poput ispiranja tla i unosa alohtone organske tvari (publikacije **I**, **II**, **III**). Pokazavši da su u odnosu na ameboidne protiste mnogo osjetljiviji na okolišne promjene, odnosno revitalizaciju, trepetljikaši su potvrdili bioindikatorski potencijal i u praćenju revitalizacije vodenih tokova sedrenih barijera. Njihov odgovor na promjene okolišnih parametara poput koncentracije otopljenih tvari, pH-vrijednosti, brzine strujanja i protoka uslijed promjena u dinamici revitaliziranih tokova i ispiranja okolnog tla, mjerljiv je i dovoljno specifičan, čime ovaj doktorski rad pruža temelje za buduća istraživanja revitalizacije vodenih tokova sedrenih barijera krških ekosustava koja primjenjuju trepetljikaše kao bioindikatore istih.

ZAKLJUČAK

Glavni zaključci ovog doktorskog rada su:

- Perifiton kao zajednica brzo odgovara na revitalizaciju vodenih tokova sedrene barijere, a odgovor je mjerljiv i razvidan u razlici taksonomskih i funkcionalnih metrika heterotrofne komponente perifitona (fagotrofnih protista) između revitaliziranih i kontrolnih tokova.
- Uklanjanje invazivne vrste pajasena omogućilo je revitalizaciju sedrene barijere, koja se odvija postepeno s obzirom na hidrologiju, procese, fizikalno-kemijske čimbenike i biotu.
- Revitalizirani tokovi razlikuju se od kontrolnih u većini okolišnih parametara, posebno tijekom prve godine od aktivacije kada se uvjeti u revitaliziranim tokovima uslijed ispiranja okolnog šumskog tla, bujičnih tokova i povremenog isušivanja značajno razlikuju od onih u kontrolnim tokovima.
- U odnosu na ameboidne protiste, trepetljikaši su mnogo osjetljiviji na okolišne promjene što potvrđuje njihov bioindikatorski potencijal i u praćenju revitalizacije vodenih tokova sedrenih barijera.
- Funkcionalna raznolikost trepetljikaša mijenja se s dužinom ekspozicije, odnosno razlika između funkcionalne raznolikosti trepetljikaša perifitona revitaliziranih i kontrolnih tokova smanjuje se s dužinom ekspozicije sugerirajući da zajednica postaje sličnijom onoj kontrolnih tokova.
- Perifiton revitaliziranih tokova naseljavaju vrste trepetljikaša koje osim u kopnenim vodama, veliku brojnost postižu i u tlu poput vrste *Platyophrya vorax*, koja je identificirana kao potencijalno indikatorska vrsta trepetljikaša revitaliziranih tokova sedrene barijere Skradinski buk uz implikacije za važnost u kruženju ugljika i ciklusu organske tvari u revitaliziranim tokovima.
- Primjena umjetnih podloga manje je invazivna za heterotrofnu komponentu perifitona (ameboidne protiste i trepetljikaše) i predlaže se kao poželjnija metoda u praćenju učinaka revitalizacije.
- U revitaliziranim tokovima alohtona organska tvar je od potencijalno velike važnosti za zajednicu, pri čemu se važnost sezonski mijenja. Primarna produkcija vjerojatno je

glavni izvor organske tvari u proljetnim i ljetnim mjesecima, dok u jesenskim i zimskim mjesecima alohtona organska tvar ima značajniju funkciju, zbog taloženja listinca tijekom jeseni, odnosno bujičnih tokova zimi.

- Osedravanje je prisutno u revitaliziranim tokovima, a količina sedre istaložene tijekom jednomjesečne ekspozicije u revitaliziranim tokovima značajno je manja od one u kontrolnim tokovima. U usporedbi s rezultatima prijašnjih istraživanja, na postajama testne plohe Skradinskog buka zabilježeno je veće osedravanje.
- Osedravanje pridonosi trodimenzionalnoj strukturi i heterogenosti mikrostaništa revitaliziranih tokova, no taloženje sedre iznad granične vrijednosti uzrokuje zatrpavanje trepetljikaša koji su mnogo osjetljiviji na osedravanje u odnosu na ameboidne protiste.
- Filogenetska i funkcionalna raznolikost trepetljikaša mijenja se duž toka rijeke Krke, odnosno izvorišno područje Krke ima nižu funkcionalnu i filogenetsku raznolikost od nizvodne barijere Skradinski buk.
- Osim što se razlikuje duž longitudinalnog profila rijeke Krke, funkcionalna raznolikost trepetljikaša razlikuje se i ovisi i o heterogenosti mikrostaništa, odnosno ovisi i o lateralnim elementima okolnih kopnenih ekosustava poput ispiranja tla i unosa alohtone organske tvari.
- Komplementarnim korištenjem tradicionalne mikroskopske identifikacije i modernih metoda metabarkodiranja DNA nadilaze se potencijalna ograničenja tradicionalne mikroskopske identifikacije u detekciji pojedinih funkcionalnih značajki čime je omogućen bolji uvid u funkcionalnu raznolikost trepetljikaša perifitona sedrene barijere Skradinski buk.

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ŽIVOTOPIS

Vesna Gulin Beljak rođena je 22. ožujka 1990. godine u Šibeniku gdje završava osnovnu školu i gimnaziju. 2008. godine upisuje se na Biološki odsjek Prirodoslovno-matematičkog fakulteta (PMF) u Zagrebu, na preddiplomski smjer Znanosti o okolišu. Titulu sveučilišnog prvostupnika struke Znanosti o okolišu stječe 2011. godine te se iste godine upisuje na diplomski studij istog smjera. Tijekom diplomskog studija sudjeluje u programu IAESTE stručne prakse, u sklopu kojeg provodi dva mjeseca na Sveučilištu znanosti i tehnologije AGH u Krakovu, Poljska. Diplomski rad izrađuje u Laboratoriju za biološke učinke metala Instituta Ruđer Bošković te ga uspješno brani 2013. godine pod mentorstvom dr. sc. Vlatke Filipović Marijić. Na Biološkom odsjeku PMF-a u Zagrebu zapošljava se 2017. godine kao asistent gdje iste godine upisuje i prvu godinu poslijediplomskog doktorskog studija biologije. U sklopu poslijediplomskog studija sudjeluje u nizu radionica vezanih uz relevantno znanstveno područje slatkovodne ekologije i obrade podataka u R-u te svoje rezultate izlaže na 25 kongresa. Od 15 objavljenih znanstvenih publikacija, prvi je autor na četiri znanstvene publikacije. Od 2022. godine sudionica je dvogodišnjeg programa European Junior Water Programme u organizaciji institucije H2O people iz Nizozemske. Dobitnica je studentske nagrade Brdo za najbolju asistenticu u akademskoj godini 2020/21 te Erasmus+KA131 stipendije za mobilnost nastavnog i nenastavnog osoblja u akademskoj godini 2021/22. Sudjeluje u provođenju praktikumske nastave iz kolegija Protista, Beskralješnjaci, Urbana ekologija i Terenska nastava iz biološke raznolikosti protista i invertebrata te aktivno sudjeluje i pomaže u organizaciji niza popularno-znanstvenih događanja kao što su Noć Biologije, Smotra Sveučilišta u Zagrebu te provedbi projekta Djevojčice u STEM-u, Prve osnovne škole Ogulin. Mentorica je BIUSove sekcije za kopnene vode te članica Hrvatskog udruženja slatkovodnih ekologa (HUSEk) i European Fresh and Young Researchers (EFYR) za koje vodi društvene mreže.

CROSBI PROFIL: Vesna Gulin Beljak (CROSBI Profil: 34357, MBZ: 364170)

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