Sveučilište u Zagrebu

Prirodoslovno-matematički fakultet

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DOKTORSKI RAD

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Mentor:

Dr. sc. Igor Stanković

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APPLICATION OF eDNA METABARCODING AND MORPHOLOGICAL APPROACH IN THE PHYTOPLANKTON BASED ASSESSMENT OF ECOLOGICAL STATUS OF KARST LAKES

DOCTORAL THESIS

Supervisor:

Dr. sc. Igor Stanković

Zagreb, 2025.

Ovaj doktorski rad izrađen je u Glavnom laboratoriju za vode Instituta za vode „Josip Juraj Strossmayer“ te na Institutu „Ruđer Bošković“ pod vodstvom dr. sc. Igora Stankovića u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije na Biološkome odsjeku Prirodoslovno-matematičkoga fakulteta Sveučilišta u Zagrebu.

ŽIVOTOPIS MENTORA

Dr. sc. Igor Stanković rođen je 16. ožujka 1980. godine u Bjelovaru, gdje pohađa i završava osnovnu i srednju Medicinsku školu, smjer zdravstveno-laboratorijski tehničar. Diplomu za studij biologije (smjer: profesor biologije i kemije) stječe u siječnju 2007. godine. Potom upisuje doktorski studij te uspješno brani disertaciju u srpnju 2013. godine. Znanstveno zvanje znanstvenog suradnika stječe 2014. godine, a višeg znanstvenog suradnika 2022. godine.

Dr. sc. Igor Stanković je sudjelovao u nastavi Biološkog odsjeka, Prirodoslovno-matematičkog fakulteta kao naslovni asistent na kolegijima Ekologija životinja s biocenologijom, Ekologija životinja i zoogeografija, Opća ekologija te Opća zoologija za smjer inženjer molekularne biologije. Osim rada kroz praktičnu nastavu, više je puta bio kao pozvani predavač na kolegijima Ekologija protista i Višestruki stresori u okolišu: istraživanje i upravljanje.

2008. godine se zapošljava u Glavnom vodnogospodarskom laboratoriju Hrvatskih voda, gdje radi kao samostalni inženjer na uzorkovanju i analizi fitoplanktona i makrofita, a od 2012. godine obavlja i dužnost organizatora Službe za biološka ispitivanja. Glavni laboratorij za vode se 2022. godine izdvaja iz Hrvatskih voda u Institut za vode „Josip Juraj Strossmayer“, nakon čega 2024. godine dr. sc. Igor Stanković počinje raditi na mjestu voditelja Sektora za strateško planiranje.

Tijekom rada u Hrvatskim vodama i Institut za vode „Josip Juraj Strossmayer“ stekao je veliko međunarodno iskustvo te djeluje kao stručnjak od 2012. godine do danas u Potkomisiji za količinu i kakvoću voda Stalne hrvatsko–slovenske komisije za vodno gospodarstvo, kao hrvatski predstavnik od 2013. godine do danas u Radnoj skupini za ekološko stanje - ECOSTAT pri Europskoj komisiji te od 2016. godine do danas u Radnoj skupini za monitoring i ocjenu stanja pri Međunarodnoj komisiji za zaštitu rijeke Dunav. Sudjelovao je na više međunarodnih ekspedicija, od kojih se posebno ističu Zajednička istraživanja Dunava na kojima je 2013. godine bio član Glavnog tima za makrofite, 2019. godine član Glavnog tima za fitoplankton i makrofite te 2025. godine član Glavnog tima za fitoplankton i nacionalni koordinator. Na implementaciji Okvirne direktive o vodama sudjeluje od samih

početaka, a to uključuje razvoj metoda za ocjenu ekološkog stanja i potencijala na temelju osnovnih fizikalno-kemijskih pokazatelja i bioloških elemenata kakvoće, aktivno sudjelovanje u europskom interkalibracijskom procesu za biološke elemente kakvoće vode, sudjelovanje u pisanju nacionalnih legislativa i dokumenata, kreiranje nadzornog, operativnog i istraživačkog monitoringa, raspisivanje i praćenje razvojnih projekata iz područja ocjene ekološkog stanja i potencijala bioloških elemenata kakvoće vode i drugo.

Kontinuirano se znanstveno i stručno usavršava iz područja taksonomije i ekologije fitoplanktona, makroalgi i makrofita te je do sada boravio na Sveučilištu u Gironi (Španjolska), Sveučilištu u Potsdamu (Njemačka), Pannonia Sveučilištu u Veszpremu (Mađarska), Sveučilištu u Uppsali (Švedska), Sveučilištu South Bohemia u Českim Budějovicama (Češka), Sveučilištu prirodnih resursa i prirodnih znanosti – BOKU u Beču (Austrija), Sveučilištu Khon Kaen (Tajland). Intenzivno surađuje s kolegama iz drugih znanstvenih i obrazovnih institucija Hrvatske i svijeta.

Dr. sc. Igor Stanković do sada je u koautorstvu ukupno objavio 42 znanstvena i znanstveno stručna rada, od toga 32 citira baza Web of Science Core Collection, a 36 baza Scopus). Radovi su prema bazi Web of Science citirani ukupno 413 puta uz h-indeks 13, a prema Scopus bazi 473 puta, također uz h-indeks je 13. Na ukupno 13 publikacija je prvi i/ili dopisni autor od kojih je njih 11 originalnih znanstvenih radova. Čimbenik odjeka iznad medijana područja (Q1 i Q2) ima 24 rada, osam radova je u trećem kvartilu (Q3) te dva u četvrtom (Q4). Koautor je na četiri međunarodne znanstvene knjige te je urednik na jednoj knjizi sažetaka. Do danas je objavio 39 kongresnih priopćenja, od čega ih je 27 s međunarodnih znanstvenih skupova, a ostali su s domaćih znanstvenih skupova s međunarodnim sudjelovanjem. Recenzent je za brojne međunarodne časopise indeksirane u Web of Science i Scopus bazama te je član više znanstvenih i strukovnih organizacija

ZAHVALA

Zahvaljujem mentoru, dr. sc. Igoru Stankoviću s kojim je počelo istraživanje fitoplanktona i ideja doktorata. S kojim su se uvijek nalazila rješenja, iako se ponekad činilo kao da ih nema. Koji me strpljivo vodio i poticao kroz ovaj dug i izazovan put, dao mi neprocjenjive savjete i slobodu u tome da postanem bolji i samostalniji stručnjak. Na tome što je težio i inzistirao na detaljima i što je uvijek bio dostupan za sva pitanja i rasprave, znanstvene i životne.

Veliko hvala svim koautorima na doprinosu u pisanju i objavi znanstvenih radova, sastavnog dijela ove doktorske disertacije.

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Prirodoslovno-matematički fakultet

Biološki odsjek

PRIMJENA ANALIZE OKOLIŠNE DNA I MORFOLOŠKE ANALIZE FITOPLANKTONA U OCJENI EKOLOŠKOG STANJA KRŠKIH JEZERA

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Fitoplankton, jedan od pet bioloških elemenata kakvoće vode prema Okvirnoj direktivi o vodama, pouzdan je pokazatelj za praćenje ekološkog stanja vode zbog brzog odgovora na promjene u okolišu. Cilj ovog istraživanja bio je utvrditi usporedivosti mikroskopije (Utermöhl metoda) i analize okolišne DNA (18S rRNA V9) u ocjeni ekološkog stanje kakvoće vode za fitoplankton. Analizom i usporedbom taksonomskog i funkcionalnog sastava fitoplanktona te biomase, ocijenjeno je ekološko stanje krških jezera. Rezultati usporedbe zajednice fitoplanktona pokazali su slabu podudarnost i nisku usporedivost relativne biomase i brojnosti sekvenci, što zahtijeva potrebu za poboljšanjem referentnih baza podataka i standardizaciju kvantifikacije brojnosti i biomase. Unatoč razlikama u taksonomskom i funkcionalnom sastavu, usporedbom oba pristupa utvrđene su usporedive vrijednosti HLPI indeksa. Kao glavni čimbenici koji utječu na zajednicu fitoplanktona definirane su hranjive tvari, alkalitet, salinitet, svjetlost i temperature vode. Rezultati daju novu perspektivu u predviđanju promjena strukture i sukcesije fitoplanktona krških jezera u svrhu uspješnog upravljanja pod antropogenim utjecajem i klimatskim promjenama.

121 stranica, 156 literaturnih navoda, jezik izvornika: hrvatski

Ključne riječi: sastav fitoplanktona, 18S rRNA V9, Utermöhl metoda, Reynoldsove funkcionalne grupe, monitoring kakvoće vode, klorofil *a*

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Doctoral dissertation

APPLICATION OF eDNA METABARCODING AND MORPHOLOGICAL APPROACH IN THE PHYTOPLANKTON BASED ASSESSMENT OF ECOLOGICAL STATUS OF KARST LAKES

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Phytoplankton, one of the five biological quality elements of the Water Framework Directive, responds quickly to environmental changes and is therefore a reliable indicator for monitoring water quality. In this study, microscopy (Utermöhl method) was compared with eDNA metabarcoding (18S rRNA V9) to assess the ecological status of lakes by analysing phytoplankton composition, biomass, diversity and environmental responses in natural karst lakes. Results showed little overlap in the composition of taxonomic and functional groups and weak comparability between relative biomass and sequence abundance, highlighting the need to refine reference databases and standardize quantification. However, despite the differences in functional groups, the HLPI values indicated a comparable ecological status for both methods. Phytoplankton is primarily influenced by nutrients, alkalinity, salinity, light availability and water temperature. These results help to predict phytoplankton succession and support effective lake management under anthropogenic pressure and climate change.

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Keywords: Phytoplankton composition, 18S rRNA V9, Utermöhl method, Reynolds functional groups, water quality monitoring, Chlorophyll *a*

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SADRŽAJ

1. UVOD

1.1. Slatkovodni ekosustavi

Obilje vode je jedno od obilježja Zemlje, jer se prostire na čak 71% njezine površine. Najveći udio vode nalazi se u oceanima i morima, oko 97%, dok je preostalih 3% slatka voda. Najveći udio slatke vode, od oko 2%, sadržan je u obliku snijega i leda na polarnim krajevima i ledenjacima, dok je manje od 1% zastupljeno u obliku podzemnih voda, jezera i rijeka(Wetzel, 2001). Iako površinska slatka voda čini izrazito mali udio u ukupnoj vodi na Zemlji, njezina važnost iznimno je velika (Kundzewicz i sur., 2008).

Slatkovodni ekosustavi su među najraznolikijima na Zemlji, a uključuju površinske vode, podzemne vode, priobalne vode u ograničenim i poplavnim područjima te ekotone (npr. izvori), kao komponente u interakciji koje doprinose ukupnoj bioraznolikosti (Ward i Tockner, 2001). Na raznolikost organizama u slatkovodnim ekosustavima najviše utječu promjene okolišnih čimbenika kao što su temperatura, svjetlost, otopljene hranjive tvari, otopljenja organska tvar kao i geografsko područje te geologija staništa (Geist, 2011). Među slatkovodnim ekosustavima, jezera predstavljaju žarišta bioraznolikosti te imaju važnu ulogu u globalnim biogeokemijskim ciklusima, posebice u prijenosu, proizvodnji, transformaciji i skladištenju ugljika. Njihov nastanak proizlazi iz geoloških događaja poput tektonske, glacijalne i vulkanske aktivnosti rezultirajući velikim brojem tipova i podtipova, uključujući krška jezera (Håkanson, 2012).

Ključno obilježje krškog fenomena je djelovanje podzemnih i površinskih voda koje utječu na biološke procese, kako na površini tako i u podzemlju (Bonacci i sur., 2009). Zbog raznolikosti morfoloških, hidrogeoloških, hidroloških, ekoloških i drugih obilježja, potreban je interdisciplinarni pristup za istraživanje krških sustava, uključujući i jezera. Najveća prirodna jezera u Hrvatskoj smještena su u krškom području (Rubinić i Radišić, 2017). Krški reljef ima jedinstvene značajke koje ga izdvajaju od drugih tipova reljefa. Hidrologija u krškom reljefu, koji se odlikuju složenim odnosima površinskih i podzemnih voda te bogatstvom raznolikih krških oblika, izrazito je kompleksna (Bonacci, 1987). Kretanje vode u takvim područjima znatno se razlikuje od onoga u nekrškim područjima, što uvelike utječe na specifične osobine krških sustava (Bonacci i sur., 2009). Krška jezera rezultat su specifičnih krških pojava koje nastaju u područjima karbonatnih stijena, poznatih po svojoj slaboj sposobnosti zadržavanja vode, a nastaju morfološko-

erozijskim procesima ili formiranjem sedrenih barijera procesom stvaranja sedre. Zahvaljujući svojim geološkim, fizikalnim i kemijskim osobitostima, krška jezera predstavljaju jedinstvene slatkovodne ekosustave (Biondić i Biondić, 2014). Upravo te karakteristike dodatno naglašavaju važnost prirodnih jezera te potrebu za njihovim stalnim istraživanjem i praćenjem.

1.2. Fitoplankton

Fitoplankton je jedan od najvažnijih bioloških elemenata u slatkovodnim ekosustavima (Reynolds, 2006). Ova skupina organizama ima važnu ulogu u ekologiji krških jezera i stoga je bitna komponenta složenih interakcija u takvim okolišima (Reynolds, 2006, Gligora Udovič i sur., 2016).

Radi se o izrazito raznolikoj skupini jednostaničnih i kolonijalnih fotosintetskih prokariotskih cijanobakterija i eukariotskih algi prilagođenih slobodnoplutajućem životu u oceanima, morima i slatkovodnim ekosustavima te trenutno broji više od 30.000 vrsta (Guiry, 2012). U većini slatkovodnih i morskih ekosustava fitoplankton čini osnovu hranidbene mreže te je zbog svog nastanka prije više od 3,5 milijardi godina ostavio dubok utjecaj na biogeokemiju Zemlje. Jedan od najvažnijih procesa koji fitoplankton obavlja je fotosinteza. Fotosintetskom fiksacijom od oko 50 $x10^{15}$ g ugljika (C) godišnje fitoplankton je trenutno odgovoran za oko 50% primarne proizvodnje na Zemlji (Marañón, 2009).

1.3. Monitoring i zaštita slatkovodnih ekosustava

Iznimno velika važnost slatkovodnih ekosustava za život čovjeka kroz neprestanu potrebu korištenja prirodnih resursa osjetljivih na promjene iziskuje njihovu zaštitu i kontinuirano praćenje kakvoće vode. Stoga je u svrhu praćenja kakvoće vode i postizanja dobrog ekološkog i kemijskog stanja te zaštite površinskih i podzemnih voda, donesena Okvirna direktiva o vodama (ODV) (Europska komisija, 2000), jedan od najvažnijih pravnih dokumenata članica Europske unije u području upravljanja vodama. U sklopu ODV, ocjena ekološkog stanja površinskih voda temelji se na biološkim elementima kakvoće (BEK), fitoplanktonu, fitobentosu, makrofitima, makrozoobentosu i ribama te njima pratećim fizikalno-kemijskim, kemijskim i hidromorfološkim elementima. Najveći utjecaj na nemogućnost postizanja dobrog ekološkog stanja u 60% europskih

površinskih voda proizlazi iz preopterećenja hranjivim tvarima, kemijskog zagađenja te hidromorfoloških promjena na vodnim tijelima (EEA, 2018).

Pretjeran unos hranjivih tvari utječe na funkcioniranje ekosustava uz posljedicu ubrzane eutrofikacije, smanjenje bioraznolikosti te smanjenje ribljeg fonda (Alexander i sur., 2017), a odražava se kao prekomjeran rast primarnih proizvođača te dovodi do cvjetanja algi koje često mogu biti toksične. Jedna od posljedica je i smanjenje koncentracije kisika u donjim slojevima stratificiranih jezera, što značajno može utjecati na promjene u bioraznolikosti unutar sustava (Misra i Chaturvedi, 2016, Scholz i sur., 2017). Kemijsko onečišćenje slatkovodnih ekosustava ugrožava vodenu floru i faunu te negativno utječe na kvalitetu ljudskog života ugrožavanjem kvalitete pitke vode i ograničavanje korištenja rijeka i jezera u rekreacijske svrhe (Schmeller i sur., 2017). Na gubitak slatkovodnih staništa uvelike utječu i hidromorfološke promjene rijeka i jezera, mijenjajući njihov prirodan protok te dinamiku erozije i taloženja sedimenta (Poikane i sur., 2019).

Dinamika slatkovodnih ekosustava koja uključuje interakciju između kemijskih, fizikalnih i bioloških procesa dobro je istražena u jezerima (Bhateria i Jain, 2016, Woolway i sur., 2020). Interakcija i međuovisnost BEK i fizikalno-kemijskih pokazatelja uvelike doprinose kakvoći vode u svim slatkovodnim ekosustavima, uključujući i jezera. Stoga su spomenuti pokazatelji kakvoće vode direktno ovisni o okolišnim uvjetima na koje veliki utjecaj ima čovjek, što posljedično dovodi do fluktuacije hranjivih tvari koje su među glavnim pokretačima promjena u tim ekosustavima, a to je posebno izraženo u doba klimatskih promjena (Vasistha i Ganguly, 2020). Promjene fizikalno-kemijskih pokazatelja mogu biti nagle te njihova mjerenja često daju samo kratkoročne informacije o kakvoći vode, dok BEK reagiraju na promjene u okolišu kroz dulje vremensko razdoblje, zbog čega su u mogućnosti pružiti pouzdanije odgovore u praćenju i ocjeni ekološkog stanja voda, a samim time i bolje upravljanje (Lyche-Solheim i sur., 2013).

1.4. Utjecaj okolišnih čimbenika na zajednicu fitoplanktona u jezerima

Jezera imaju važnu ulogu u prirodi održavanjem ravnoteže ekosustava u cijelosti (Fluet-Chouinard i sur., 2017, Dodds i Whiles, 2020). Prirodna sukcesija jezera spor je proces dok ljudske aktivnosti brzo mijenjaju stanje jezerskih ekosustava, o čemu izravno ovisi i kakvoća vode (Søndergaard i sur., 2017, Vasistha i Ganguly, 2020). Osim izravnih ljudskih utjecaja, jezera su posljedično

osjetljiva i na klimatske promjene te je kontinuirano praćenje od iznimne važnosti za njihovu zaštitu i održivost (Angeler i sur., 2014, Woolway i sur., 2020). Jezera su staništa visoke raznolikosti s fluktuacijama hranjivih tvari kao jednim od glavnih pokretača promjena. Jedna od posljedica ovih čimbenika je eutrofikacija (Heino i sur., 2021). Jezerski ekosustavi, zbog svoje složenosti i povezanosti, pokazuju brze odgovore na promjene fizikalno-kemijskih i bioloških svojstava. Važnu ulogu u ekološkim svojostima vode dubokih jezera ima proces miješanja i stratifikacije. To je posebice izraženo kroz temperaturu vode, potrošnju otopljenog kisika i sadržaj hranjivih tvari te razmještaj fitoplanktona i njegovu raznolikost kroz slojeve vode (Wang i sur., 2022, Šarović i Klaić, 2023, Yue i sur., 2023). S druge strane, plitka jezera predstavljaju jedinstvene ekosustave u kojima hidrološki i okolišni čimbenici, kao što su vjetar, oborine, dotok vode i miješanje vodenog stupca, imaju snažan utjecaj na sastav vrsta i biomasu fitoplanktona (Weithoff i sur., 2000, Adrian i sur., 2009, Rühland i sur., 2015).

Dušik (N) i fosfor (P) esencijalni su makronutrijenti ključni u biokemijskim procesima fitoplanktona te kao ograničavajući čimbenici u omjeru N:P određuju dinamiku rasta fitoplanktona u vodenim ekosustavima (Frost i sur., 2023). Razumijevanje omjera N:P ključno je za objašnjenje i predviđanje dinamike fitoplanktona u različitim vodenim staništima i od velike je važnosti za učinkovito upravljanje i održavanje ekološke ravnoteže (Reeder, 2017, Reinl i sur., 2022, Redoglio i Sperfeld, 2024). Budući da oligotrofna jezera imaju nisku koncentraciju hranjivih tvari, osjetljivo reagiraju čak i na male promjene u dostupnosti N ili P. Nizak omjer N:P u oligotrofnim jezerima, koji je rezultat prirodno niske koncentracije N i P, može se znatno povećati visokim unosom N. Razvoj i rast fitoplanktona u tim jezerima mijenja se iz primarno ograničenog dušikom u primarno ograničen fosforom (Downing i McCauley, 1992, Bergström, 2010). S druge strane, visokim unosom P dolazi do promjene jezera iz primarno ograničeno fosforom u primarno ograničeno dušikom. Povećanim unosom N ili P dolazi do njihovog neuravnoteženog omjera što može dovesti do smanjene bioraznolikosti u hranidbenoj mreži jezera, niže kvalitete vode za ljudsku potrošnju i cvjetanja algi (Elser i sur., 2022, Wu i sur., 2022).

Uz primarne hranjive tvari N i P, silikati su također od velike važnosti za rast fitoplanktona u slatkovodnim ekosustavima, posebno algi kremenjašica (Zhang i sur., 2019). Uz navedeno i drugi pokazatelji određuju dinamiku fitoplanktona, uključujući temperaturu vode, salinitet, svjetlost, alkalitet, pH, suspendirane tvari, hidrološke karakteristike i ljudske aktivnosti (Maileht i sur., 2013,

Salmaso i Tolotti, 2021, Verspagen i sur., 2022, Stanković i sur., 2024). Primjerice, u bočatim ekosustavima salinitet djeluje kao pokretački mehanizam izmjene zajednice fitoplanktona iz primarno slatkovodne u zajednicu tolerantniju na porast saliniteta, bez promjena u koncentraciji hranjivih tvari, čime dolazi do razvoja pojedinih eurihalinih vrsta, što može rezultirati promjenama u bioraznolikosti (Moss, 1994).

BEK služe kao bioindikatori abiotičkog i biotičkog stanja okoliša u akumulaciji štetnih tvari ili u odgovoru na okolišni stres (Parmar i sur., 2016). Praćenje BEK zahtijeva standardizirane procese uzorkovanja, obrade uzoraka te identifikaciju prikupljenih organizama (Birk i sur., 2012). Ekološko stanje površinskih voda ocjenjuje se temeljem razvijenih nacionalnih metoda unutar država članica EU prema standardima definiranim u ODV-u (npr. brojnost, sastav zajednice). S ciljem usklađivanja metoda, Europska komisija organizirala je niz interkalibracija kako bi se omogućila usporedivost granica ekološkog stanja te metoda ocjenjivanja stanja između država članica EU (Poikane i sur., 2014).

1.5. Ocjena ekološkog stanja BEK fitoplankton

Prema ODV, fitoplankton je BEK za praćenje ekološkog stanja jezera i vrlo velikih rijeka. Tradicionalni biološki monitoring temeljem fitoplanktona bazira se na mikroskopskoj identifikaciji svojti putem Utermöhl metode (Utermöhl, 1958), a ocjena ekološkog stanja jezera i rijeka temelji se na taksonomskom sastavu, brojnosti, biomasi te učestalosti i intenzitetu cvjetanja algi (Europska komisija, 2011). Sukladno tome, razvijeni su indeksi za procjenu ekološkog stanja vodnih tijela temeljem fitoplanktona, primjerice Brettum Index (Brettum, 1989), Phyto-See-Index (Mischke i sur., 2008) i Indice Phytoplankton Lacustre (Laplace-Treyture i Feret, 2016). Padisák i sur. (2006) razvili su Q indeks za mađarska jezera na temelju koncepta funkcionalnih grupa (FG) kao sastava zajednice (Reynolds i sur., 2002). Naknadno su Borics i sur. (2007) unaprijedili Q indeks dodajući u formulu koncentraciju klorofila *a* pri čemu nastaje multimetrijski indeks naziva Hungarian lake phytoplankton indeks (HLPI) s Q indeksom kao jednom od glavnih komponenti. Q indeks izračunava se množenjem udjela pojedine FG u ukupnoj biomasi fitoplanktona s faktorom (F) određenim za svaku FG. Najvažniji dio u ocjeni ekološkog stanja je određivanje vrijednosti faktora (F), budući da on odražava vrijednosti FG u referentnim uvjetima za zadani tip jezera. Stabilna

teoretska osnova Q indeksa omogućuje njegovu primjenu u ocjeni ekološkog stanja bez geografskih ograničenja (Padisák i sur., 2006).

Jedan od temelja korištenja indeksa temeljenih na mikroskopskoj analizi jest taksonomska stručnost. Prednost mikroskopske analize je kvantifikacija, tj. mogućnost određivanja brojnosti i biovolumena potrebnih za izračun biomase pojedine svojte i ukupne biomase fitoplanktona kao osnova u izračunu indeksa za ocjenu ekološkog stanja temeljem fitoplanktona.

S druge strane, nedostatak mikroskopske analize očituje se u poteškoćama ili nemogućnosti identifikacije kriptičnih vrsta te razvojnih stadija svojti fitoplanktona (Hering i sur., 2018). Većinu pikofitoplanktonskih svojti (stanice veličine 0,2 do 2 μm), koje često predstavljaju važnu kariku u hranidbenim mrežama oligotrofnih i mezotrofnih jezera (Callieri, 2008) također nije moguće uočiti i identificirati svjetlosnim mikroskopom. Stručna i vremenska zahtjevnost mikroskopske analize potencijalno mogu dovesti do financijskog i prostornog ograničenja u praćenju stanja vodnih tijela (Gao i sur., 2018, Gelis i sur., 2024).

1.6. Analiza okolišne DNA

1.6.1. Biološki elementi kakvoće

Zbog sve jačeg antropogenog utjecaja i klimatskih promjena raste i potreba za proširivanjem postojećih programa monitoringa (Herrero i sur., 2018, Carvalho i sur., 2019), što uvjetuje primjenu novih pristupa poput okolišne DNA (eDNA). Okolišna DNA predstavlja sveukupni genetički materijal dobiven izravno iz okoliša bez izolacije određenog organizma (Seymour, 2019). Kao financijski i vremenski učinkovitiji molekularni pristup visoke reproduktivnosti, analiza okolišne DNA potencijalna je alternativa morfološkom pristupu te može iz temelja promijeniti tradicionalni pristup morfološke identifikacije organizama u praćenju kakvoće vode (Hering i sur., 2018, Pawlowski i sur., 2018, Ruppert i sur., 2019, Thomsen i sur., 2024). Međutim, protokoli primjene metode analize okolišne DNA još se uvijek razvijaju i usavršavaju, a standardizacija metode predstavlja veliki izazov prije implementacije u programe monitoringa (Hering i sur., 2018).

Testiranje metode analize okolišne DNA u identifikaciji vodenih organizama primjenjuje se kod gotovo svih BEK (Elbrecht i sur., 2017, Pawlowski i sur., 2018, Pont i sur., 2018, Pérez Burillo i sur., 2020). Istraživanja u kojima se koristi analiza okolišne DNA, detekcija svojti i bioraznolikost

izravno ovise o odabranim genetičkim markerima i početnicama, čiji pogrešan odabir može dovesti do izostanka detekcije organizama karakterističnih za istraživano stanište, posebice ako nisu uključene i morfološke metode identifikacije. Stoga je važno da genetički marker bude taksonomski informativan, tj. dobre razlučivosti u identifikaciji vrsta s obzirom da većina metrika korištenih u monitoringu zahtijeva identifikaciju do razine vrste. Jedan od glavnih izazova u istraživanjima koja uspoređuju tradicionalni morfološki pristup s analizom okolišne DNA jest odabir najprikladnijeg marker gena, radi prisutnosti prokariota i eukariota u izrazito raznolikoj zajednici fitoplanktona (Kezlya i sur., 2023, Gelis i sur., 2024).

1.6.2. Odabir marker gena u molekularnoj analizi zajednice fitoplanktona

Uspoređujući rezultate morfološke identifikacije s molekularnom analizom zajednice fitoplanktona, Eiler i sur. (2013) odabrali su 16S rRNA gen kao marker gen zbog svoje prisutnosti u prokariotskom i eukariotskom kloroplastu. Navedenim su istraživanjem utvrđeni različiti rezultati usporedbe relativnog broja sekvenci s brojnošću i biovolumenima fitoplanktonskih svojti, čime je utvrđena slabija podudarnost u odnosu na usporedbu biovolumena taksonomskih skupina (odjel) i relativnog broja sekvenci utvrđenih molekularnom analizom. Iako je u oba pristupa prisutna značajna podudarnost u sezonskoj dinamici fitoplanktona, primjenom 16S rRNA gena u 56% uzoraka određeno je manje od 100 sekvenci fitoplanktona te su prevladavale sekvence heterogenih bakterija (Eiler i sur., 2013). Stoga, Huo i sur. (2020) predlažu izbjegavanje korištenja 16S rRNA gena u određivanju raznolikosti eukariotskih zajednica fitoplanktona zbog pristranosti prema bakterijama, umjesto čega korištenjem gena 18S V7 rDNA dobivaju 3,5 puta veću raznolikost zajednice fitoplanktona u usporedbi s morfološkom identifikacijom. Unatoč tome što je usporedba metoda otkrila značajnu razliku u broju taksonomskih skupina te u brojnosti svojti fitoplanktona u odnosu na broj sekvenci unutar skupina, Huo i sur. (2020) zaključuju da se korištenjem morfološke i molekularne analize uvelike olakšava određivanje sastava i strukture zajednice fitoplanktona. Slični su rezultati prikazani u istraživanju usporedbe analize fitoplanktona gdje je korištenjem genetičkog markera 18S V4 rDNA također utvrđena oko 3,5 puta veća raznolikost zajednice u odnosu na morfološku identifikaciju (Groendahl i sur., 2017). S druge strane, analizom okolišne DNA korištenjem V4 regije 16S i 18S rRNA gena zabilježena je manja

raznolikost zajednice fitoplanktona u odnosu na morfološku identifikaciju (Malashenkov i sur., 2021).

Usporedba rezultata molekularnih analiza regija V4 i V9 18S rDNA gena u istraživanjima Tragin i sur. (2017) te Choi i Park (2020) ukazala je na velika odstupanja u preklapanju glavnih skupina organizama eukariotskih zajednica te na 20% veću brojnost OTU-ova identificiranih analizom V9 regije. U odnosu na V4 regiju 18S rDNA gena, analiza V9 regije omogućuje identifikaciju veće raznolikosti i na višoj taksonomskoj razini, posebice u zajednici dinoflagelata (Stoeck i sur., 2010, Stuart i sur., 2024). U istraživanju Stuart i sur. (2024), uz veću brojnost utvrđenih OTU-ova, V9 regija 18S rDNA gena pokazala je veću usklađenost s rezultatima utvrđenim mikroskopskom analizom. Usporedbom V4 i V9 regije, Choi i Park (2020) i Stuart i sur. (2024) preporučuju korištenje obje regije jer se međusobno nadopunjuju. Ipak, Stuart i sur. (2024) ističu da korištenje V9 regije omogućuje pouzdanu identifikaciju sastava zajednice fitoplanktona, odražavajući relativnu brojnost između skupina. Osim toga, naglašava da samostalna upotreba V9 regije smanjuje troškove sekvenciranja, a pritom pruža temeljne informacije o funkcioniranju istraživanih ekosustava.

Iako 18S rRNA gen marker daje bolju filogenetsku razlučivost fitoplanktona (Choi i Park, 2020, Malashenkov i sur., 2021), nedostatak mu je nemogućnost otkrivanja cijanobakterijskih svojti.

Općenito, gen 16S rRNA rijetko se samostalno koristi za analizu prokariota i eukariota (Kezlya i sur., 2023), a iako se troškovi zbog pripreme referentnih baza i količine podataka povećavaju, znanstvenici odvajaju te dvije komponente fitoplanktona. Prilikom analiza koriste se različite regije gena 16S i 18S rRNA (Malashenkov i sur., 2021) ili 16S i 23S rRNA (Gelis i sur., 2024) u usporedbi s morfološkom identifikacijom te 18S i 23S rRNA (Cahoon i sur., 2018) u usporedbi odabira gena povoljnijih za istraživanje fitoplanktona.

U analizi okolišne DNA korištenjem 16S i 18S rRNA gena za identifikaciju fitoplanktona jezera, veći broj svojti identificiran je pomoću gena 18S rRNA. Cijanobakterije roda *Microcystis*, *Prochlorococcus*, *Synechococcus* i *Cyanobium* prevladavale su u prokariotskoj komponenti fitoplanktona identificiranoj pomoću 16S rRNA gena, dok je vrsta *Ceratium hirundinella* bila najbrojnija komponenta eukariotske zajednice fitoplanktona u jezerima. Prema usporedbi rezultata okolišne DNA s morfološkom analizom, broj rodova i vrsta eukariotske komponente fitoplanktona utvrđenih analizom okolišne DNA manji je u odnosu na morfološku analizu. S druge strane,

raznolikost prokariotske komponente fitoplanktona na razini roda veća je analizom 16S rRNA gena u odnosu na morfološku analizu, čime se molekularna analiza pokazala kao osjetljiviji pristup za identifikaciju pikocijanobakterija teško vidljivih morfološkom analizom (Malashenkov i sur., 2021). Korištenjem marker gena 16S rRNA, Gelis i sur. (2024) utvrdili su manju raznolikost prokariotske komponente fitoplanktona na razini roda u odnosu na morfološki pristup, ali su također potvrdili dominaciju pikocijanobakterija koja se učestalo previđa morfološkom analizom.

Upotrebom marker gena u analizi prokariotske i eukariotske komponente fitoplanktona (16S i 23S rRNA) te usporedbom identificiranih svojti s morfološkom analizom utvrđene su veće i manje razlike u preklapanju svojti, ovisno o taksonomskim razinama. Preklapanje svojti u tri navedene analize postupno se smanjuje od razine odjela do razine vrste (58% preklapanja na razini odjela naprema 7,2% preklapanja na razini vrsta) te su slične vrijednosti zabilježene bez obzira na korišteni marker gen. Usporedbom dominantnih svojti identificiranih morfološkom analizom s oba molekularna pristupa (16S i 23S rRNA) utvrđeno je da ne dolazi do njihovog preklapanja te se dominantne vrste razlikuju ovisno o pristupu (Gelis i sur., 2024).

1.6.3. Genetske baze podataka

Kako bi se sekvencama utvrđenim sekvenciranjem ciljanog dijela marker gena dodijelilo ime, tj. identificiralo ih do vrste, potrebna je usporedba dobivenih sekvenci s referentnim sekvencama pohranjenim u bazama podataka u obliku DNA barkodova. Najčešće korištene baze podataka DNA barkodova su NCBI Genbank (Clark i sur., 2016), BOLD Systems (Ratnasingham i Hebert, 2007) i SILVA (Quast i sur., 2013), dok postoje i uže specijalizirane baze za pojedine skupine, primjerice Diat.barcode (Rimet i sur., 2019) za alge kremenjašice i Phytool (Canino i sur., 2021) za fitoplankton.

Znatno ograničenje u analizi okolišne DNA predstavlja nepopunjenost referentnih DNA baza, koje moraju biti dobro organizirane i sveobuhvatne u svrhu točnog dodjeljivanje sekvenci vrstama. Manjak popunjenosti baza rezultira velikim brojem nedodijeljenih operativnih taksonomskih jedinica (OTU) svojtama ili neidentifikaciji do razine vrste, što također ovisi o pripadnosti određenoj taksonomskoj skupini ili korištenoj regiji gena. Osim popunjenosti svojtama, glavne izazove u odabiru, razvoju i korištenju referentnih DNA baza podataka u taksonomskoj

identifikaciji predstavljaju: pogreške u taksonomskoj identifikaciji biološkog materijala pohranjenog u referentnoj bazi podataka, pogreške prilikom povezivanja ispravno identificiranog biološkog materijala s krivom sekvencom, dodijeljenost više različitih svojti istoj sekvenci, veći broj registriranih unosa iste svojte u bazu podataka (npr. sinonimi), niska taksonomska rezolucija unesenih svojti identificiranih na višim taksonomskim razinama, nepostojanje ili nedostatak podataka o varijacijama unutar pojedine vrste (Keck i sur., 2023).

Nedostaci DNA baza (SILVA, Greengenes i Phytool) u identifikaciji fitoplanktona utvrđeni su u istraživanjima, gdje je analizom okolišne DNA korištenjem marker gena 16S, 18S i 23S identificirano manje vrsta fitoplanktona u odnosu na morfološku analizu (Malashenkov i sur., 2021, Gelis i sur., 2024).

1.6.4. Morfološka identifikacija i analiza okolišne DNA

U preglednom radu Kezlya i sur. (2023) objedinjeni su razlozi koje treba uzeti u obzir prilikom interpretacije rezultata, s obzirom da objašnjavaju odstupanja u usporedbi analize okolišne DNA i morfološke identifikacije fitoplanktona. S molekularnog aspekta, pogreške koje se događaju tijekom analize okolišne DNA odražavaju se na rezultate u referentnoj bazi.

Također, tehničke pogreške do kojih može doći tijekom amplifikacije i sekvenciranja mogu dovesti do prividnog povećanja raznolikosti OTU-ova (Huo i sur., 2020, Gelis i sur., 2024). S druge strane, potpuni izostanak amplifikacije zbog neusklađenosti upotrebljenog seta početnica može dovesti do nedetektiranja pojedine vrste (Salmaso i sur., 2022).

Pogreške u identifikaciji koje mogu dati lažno pozitivne svojte, kao i razlike u razini i stupnju identifikacije predstavljaju glavne nedostatke morfološkog pristupa čime morfološki i molekularni pristupi ne moraju nužno pružiti isti uvid u zajednicu fitoplanktona (Salmaso i sur., 2022). U morfološkoj identifikaciji fitoplanktona koriste se manji volumeni uzorka te se identifikacija i brojanje fitoplanktona ograničava na probna polja i transekte u odnosu na okolišnu DNA utvrđenu filtriranjem većeg volumena vode zbog čega i usporedba takvog seta podataka nije idealna (MacKeigan i sur., 2022, Salmaso i sur., 2022).

Iako svaka metoda ima svoja ograničenja, s obzirom na standardizaciju i interkalibraciju morfoloških analiza, za bolje razumijevanje i tumačenje rezultata analize okolišne DNA bez obzira

radi li se o istraživanju bioraznolikosti ili ocjeni ekološkog stanja, još uvijek su relevantne studije koje koriste obje metode (Poikane i sur., 2014, Hering i sur., 2018, Pawlowski i sur., 2018).

Unatoč tome što analiza okolišne DNA i dalje nije metodološki uniformirana, ona omogućuje detekciju vrsta koje zbog svoje veličine i frekvencije pojavljivanja nisu utvrđene primjenom tradicionalnih metoda uzorkovanja i protokola biomonitoringa (Seymour i sur., 2020). S druge strane, ograničenja u implementaciji analize okolišne DNA za istraživanje i ocjenu ekološkog stanja slatkovodnih ekosustava očituju se u nepouzdanim procjenama strukture i veličine populacije te nedostacima sekvenci u bazama podataka (Weigand i sur., 2019).

U trenutku definiranja ciljeva i hipoteza ove doktorske disertacije, svega je nekoliko istraživanja uspoređivalo tradicionalni morfološki pristup i analize okolišne DNA, pri čemu u ocjeni ekološkog stanja za BEK fitoplankton nisu korišteni rezultati analize okolišne DNA (Eiler i sur., 2013, Pawlowski i sur., 2018). U okviru ove doktorske disertacije po prvi je puta u Hrvatskoj primijenjena analiza okolišne DNA za određivanje fitoplanktonske zajednice te je također po prvi puta ta zajednica korištena u okviru Okvirne direktive o vodama za ocjenu ekološkog stanja temeljem fitoplanktona.

Ubrzan razvoj metoda analiza okolišne DNA i njihova sve češća primjena u identifikaciji zajednice fitoplanktona (Malashenkov i sur., 2021, MacKeigan i sur., 2022, Sildever i sur., 2022, Canino i sur., 2023, Lv i sur., 2023) doprinosi sve pouzdanijem korištenju molekularnog pristupa u ocjeni ekološkog stanja temeljem fitoplanktona (Gelis i sur., 2024).

1.7. CILJEVI I HIPOTEZE ISTRAŽIVANJA

Ciljevi:

1. Utvrditi sastav zajednice fitoplanktona u prirodnim krškim jezerima površine veće od 0,5 km^2 u Republici Hrvatskoj pomoću morfološke identifikacije fitoplanktona (klasična mikroskopija) i pomoću analize okolišne DNA.
2. Usporediti sastav zajednice fitoplanktona morfološkim i molekularnim pristupom.
3. Definirati glavne okolišne čimbenike i jačinu njihovog utjecaja na zajednicu fitoplanktona identificiranu primjenom oba pristupa.
4. Sekvence dobivene analizom okolišne DNA grupirati u operativne taksonomske jedinice dodijeljene svojtama fitoplanktona i klasificirati ih prema Reynoldsovim funkcionalnim grupama.
5. Sastav zajednice fitoplanktona identificiran analizom okolišne DNA, primijeniti u ocjeni ekološkog stanja prirodnih krških jezera izračunom HLPI indeksa i omjera ekološke kakvoće.
6. Primijeniti koncentraciju klorofila *a* dobivenu spektrofotometrijski i pomoću visokoprotočne tekućinske kromatografije u izračunu HLPI indeksa i omjera ekološke kakvoće oba pristupa.
7. Ocjenu ekološkog stanja dobivenu standardiziranim morfološkim pristupom usporediti s rezultatima ocjene dobivene analizom okolišne DNA.
8. Definirati utjecaj okolišnih pritisaka na ocjenu ekološkog stanja dobivenu molekularnim pristupom u svrhu budućeg upravljanja vodnim tijelima.
9. Utvrditi primjenu Q indeksa i Reynoldsovog koncepta funkcionalnih grupa u ocjeni ekološkog stanja sastavom zajednice fitoplanktona identificiranog analizom okolišne DNA.

Hipoteze:

1. Morfološki pristup i pristup analize okolišne DNA su usporedivi u analizi zajednice fitoplanktona u krškim jezerima.
2. Reynoldsov koncept funkcionalnih grupa fitoplanktona se može primijeniti i na morfološki pristup te pristup analize okolišne DNA u krškim jezerima.

3. HLPI indeks izračunat temeljem okolišne DNA je primjenjiv u ocjeni ekološkog stanja prirodnih krških jezera u Hrvatskoj.

Ovom disertacijom obuhvaćene su četiri izvorne znanstvene publikacije (I-IV) koje ostvaruju zadane ciljeve i ispituju postavljene hipoteze. Publikacija I povezana je s prvim i drugim ciljem te ispituje prvu hipotezu. Publikacija II povezana je s četvrtim, petim, šestim, sedmim i devetim ciljem te ispituje drugu i treću hipotezu. Publikacije III i IV povezane su s trećim ciljem te ispituju drugu hipotezu.

U poglavlju ZNANSTVENI RADOVI prikazane su četiri publikacije dok je njihov objedinjeni doprinos sažet u poglavlju RASPRAVA.

2. PUBLIKACIJE

I. Hanžek N, Gligora Udovič M, Kajan K, Borics G, Várbíró G, Stoeck T, Orlić S i Stanković I, 2024. Comparative identification of phytoplankton taxonomic and functional group approach in karst lakes using classical microscopy and eDNA metabarcoding for ecological status assessment. Hydrobiologia 851(4):1015-1034.

II. Hanžek N, Gligora Udovič M, Kajan K, Borics G, Várbíró G, Stoeck T, Žutinić P, Orlić S i Stanković I, 2021. Assessing ecological status in karstic lakes through the integration of phytoplankton functional groups, morphological approach and environmental DNA metabarcoding. Ecological Indicators 131:108166.

III. Hanžek N, Šiljeg M, Šikić T i Stanković I, 2024. Phytoplankton in Deep Lakes of the Dinaric Karst: Functional Biodiversity and Main Ecological Features. Plants 13(16):2252.

IV. Stanković I, Gligora Udovič M, Žutinić P, Hanžek N i Plenković-Moraj A, 2024. Is salinity a driving factor for the phytoplankton community structure of a brackish shallow Mediterranean lake? Hydrobiologia 851(4):999-1013.

PUBLIKACIJA I.

I. Hanžek N, Gligora Udovič M, Kajan K, Borics G, Várbíró G, Stoeck T, Orlić S i Stanković I, 2024. Comparative identification of phytoplankton taxonomic and functional group approach in karst lakes using classical microscopy and eDNA metabarcoding for ecological status assessment. Hydrobiologia 851(4):1015-1034.

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MICROALGAL FUNCTIONAL TRAITS

Comparative identification of phytoplankton taxonomic and functional group approach in karst lakes using classical microscopy and eDNA metabarcoding for ecological status assessment

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Abstract Phytoplankton is one of the five biological quality elements used to assess the ecological status of lakes within the Water Framework Directive. Classical morphological Utermöhl method and eDNA metabarcoding by Ilumina sequencing the hypervariable V9 region of the eukaryotic SSU rRNA gene were used to analyse the qualitative and quantitative composition of the phytoplankton and compared at the taxonomic and FG level to highlight advantages and disadvantages of eDNA metabarcoding method over classical microscopy. Samples were collected from April to September in seven Croatian natural karst lakes. Cluster analysis based on the Bray–Curtis similarity of taxa biomass (microscopy) and number of sequences (eDNA metabarcoding) clearly separated lakes showing that eDNA metabarcoding is sensitive to species change. Overlap at the species level between methods was found primarily in the taxa of Cryptophyta, Miozoa, and Ochrophyta, while some very common taxa of Bacillariophyta, Charophyta, and Chlorophyta identified by microscopy were not detected by eDNA metabarcoding, possibly due to incompleteness of the reference databases. At a higher organizational level, the results showed poor overlap of taxonomic and functional group composition and poor comparability of relative biomass to relative number of sequences, indicating the need to complete reference databases and standardize quantification to further develop eDNA metabarcoding for ecological status assessment.

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Introduction

Freshwater lakes are of fundamental importance to humans, especially as sources of drinking water. They also have important functions in nature, including ecosystem maintenance and balance (Fluet-Chouinard et al., 2017; Dodds & Whiles, 2020). The natural succession of lakes is a slow process, but the water quality of lakes today is directly dependent on human activities that rapidly change the state of the lake ecosystem (Søndergaard & Jeppesen, 2007; Vasistha & Ganguly, 2020). In addition to direct human impacts, lakes are also sensitive to climate change, which increases lake vulnerability, and continuous monitoring is crucial for lake sustainability (Angeler et al., 2014; Woolway et al., 2020). Therefore, the WFD (WFD, 2000) is one of the most important components of the European Union's water policy. Its main objective is to protect and improve the status of aquatic ecosystems by monitoring the ecological quality status of freshwater habitats and maintaining and promoting sustainable water use.

Physical and chemical properties can change rapidly and often provide short-term information about water quality. On the other hand, phytoplankton, as one of the five biological quality elements (BQEs) of the WFD, can provide more reliable responses in monitoring and assessing the ecological status of lakes because they respond to environmental changes over a longer period of time (Cordero et al., 2017; Ho et al., 2019).

Traditionally, the analysis of phytoplankton in freshwater ecosystems is based on microscopy, and ecological status assessment is based on taxonomic composition, abundance, biomass, frequency and intensity of algal blooms (EC, 2011). Because phytoplankton are a polyphyletic and extremely diverse group of organisms that change rapidly, identification and subsequent work with long taxa lists are often challenging. However, based on the common ecology and environmental preferences of species, a functional group (FG) approach was developed (Reynolds et al., 2002; Padisák et al., 2009). This approach facilitates not only to understand ecological processes within the pelagic realm, but also the understanding of phytoplankton response to environmental changes in lakes and rivers (Stanković et al., 2012; Gligora Udovič et al., 2017).

For the purposes of the WFD, ecological status is assessed using phytoplankton-based indices (Laplace-Treyture & Feret, 2016; Mischke et al., 2008; Padisák et al., 2006). The frequently used Q index developed by Padisák et al. (2006) is based on numerical evaluation of FGs as compared to the reference community in each lake type. This type of indices became officially accepted in ecological status assessment in several EU member states (Mischke et al., 2018; Borics et al., 2019; Hanžek et al., 2021). However, their reliable use, requires correct identification of phytoplankton species.

Microscopy and its difficulties in lengthy sample processing, the need for high taxonomic expertise, and the difficulty or impossibility of enumeration of cryptic species and identification of early-life phytoplankton stages (Hering et al., 2018), directly impact the temporal and spatial limitations of monitoring. Methods that are less time consuming but sufficiently reliable are needed to accelerate monitoring and detect the effects of human impact and climate change on lakes. Faster and less expensive methods would allow for quicker and better results and provide room to expand monitoring to a larger number of waterbodies. With this in mind, eDNA metabarcoding is a promising tool for monitoring BQEs, including phytoplankton (Hering et al., 2018; Pawlowski et al., 2018).

One of the major weaknesses of eDNA metabarcoding is the quantification of each group of organisms analysed. When using conventional analytical methods, quantification is standardised and expressed in various ways, e.g. individuals per m^2, biomass (mg L^{-1}), etc. So far, eDNA metabarcoding only provides quantification in terms of number of sequences. To improve the comparison between morphology and eDNA metabarcoding, a correction factor for diatom taxa has been developed (Vasselon et al., 2017). However,

this process is not always successful, as in the study by Pérez Burillo et al. (2020), who applied a correction factor to high throughput sequencing (HTS) reads to evaluate their effectiveness in improving the assessment of ecological status by diatom eDNA metabarcoding. Ecological status obtained by microscopy showed lower comparability (65.4%) compared to corrected HTS inventories than without applying the correction factor (69.8%). For phytoplankton, the correction factor is not yet developed, but in the study by Hanžek et al. (2021), the ecological status class was the same in 89.1% of the samples despite different taxonomic compositions and proportions of FGs between microscopy and eDNA metabarcoding. Further optimisation between cell numbers/biomass and DNA reads (number of sequences) is required (Santoferrara, 2019; Martin et al., 2022).

As an alternative to light microscopy, eDNA metabarcoding can be considered a promising tool for faster analysis (Xiao et al., 2014) and spatial expansion of monitoring. However, the use of environmental DNA is still in the research and development phase, and standardization of the method is a major challenge before implementation in official monitoring programs (Hering et al., 2018; Pawlowski et al., 2018).

The aim of this study is to i) compare taxonomic and FGs composition and both quantification methods of phytoplankton in seven Croatian natural karst lakes obtained by microscopy and eDNA metabarcoding, ii) highlight specific advantages and disadvantages of eDNA metabarcoding method (18S rRNA V9) in contrast to classical microscopy with the aim of ecological status assessment in karst lakes.

Materials and methods

Study site

The Dinaric and Pannonian Ecoregions are two ecologically and geographically defined areas covering Croatia. All seven Croatian natural karst lakes with an area of more than 0.5 km^2 are located in the Dinaric Ecoregion (Fig. 1). The location and physical characteristics of the lakes studied are listed in Table 1.

The Plitvice Lakes are the only ones located in the Continental Subecoregion and were formed by a combination of tectonic movements, the development of tuff-forming communities and travertine barriers. A total of 16 barrage lakes have formed in the region, of which Lake Kozjak is the deepest and largest, with Lake Prošće just behind it (Markowska, 2004). Both are dimictic mountain lakes under the influence of continental climate. All other lakes are located in the Mediterranean Subecoregion. Travertine barriers are also one of the key factors in the formation of lakes in the Mediterranean Subecoregion, such as a lentic dilatation on the Krka River, the Visovac karst barrier lake (Gligora Udovič et al., 2017). Lake Vransko on the island of Cres (also referred to as deep Vransko) is a cryptodepression, the deepest and the only one on the island, formed at the Pliocene–Pleistocene transition (Bonacci, 2014). Lake Vransko in the Vransko Lake Nature Park (also referred to as shallow Vransko; Motel and Prosika sampling sites) is a polymictic, shallow cryptodepression. It has the largest surface area of all natural karst lakes in Croatia (Šiljeg et al., 2015). Shallow Lake Vransko is slightly brackish (0.7–1.2‰), which is due to its location in the permeable karst area and to the artificially dug Prosika channel connecting the lake with the Adriatic Sea

Table 1 Position and physical properties of the investigated lakes

Lake/Sampling site	Vransko	Kozjak	Prošće	Motel	Prosika	Visovac	Crniševo	Očuša
Surface area (km^2)	5.75	0.82	0.68	30.2		5.72	0.43	0.55
Volume (m^3)	220.3×10^6	12.7×10^6	7.7×10^6	141.6×10^6		103×10^6	7×10^6	7.3×10^6
Max depth (m)	74.5	47	38	4.7		30	34	19.6
Longitude (WGS84)	14.39° E	15.61° E	15.60° E	15.55° E	15.62° E	15.98° E	17.41° E	17.42° E
Latitude (WGS84)	45.86° N	44.89° N	44.87° N	43.93° N	43.86° N	43.86° N	43.07° N	43.08° N
Elevation (a.s.l.) (m)	9	535	636	0.1		47	0.8	

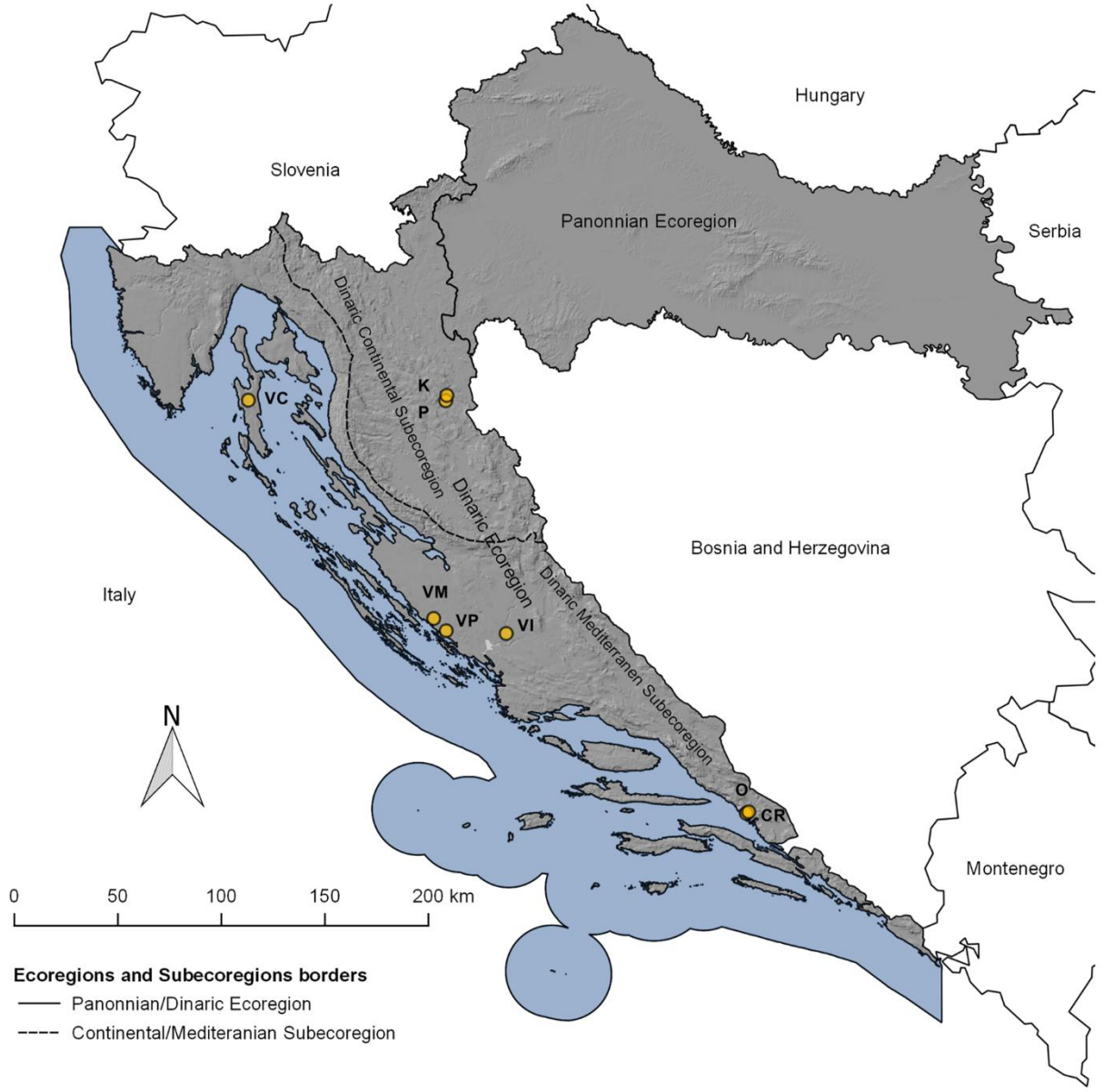


Fig. 1 Map of investigated lakes. Site codes: VC—deep Lake Vransko, K—Lake Kozjak, P—Lake Prošće, VM—shallow Lake Vransko, sampling site Motel, VP – shallow Lake Vransko, sampling site Prosika, VI—Lake Visovac, CR—Lake Crniševo, O—Lake Oćuša

(Gligora et al., 2007). The last two lakes are Crniševo and Oćuša lakes from the 5-lake complex of Baćina lakes. Lake Crniševo is also slightly brackish due to underground brackish water springs and saline water intrusion at the bottom due to its proximity to the sea. Lake Oćuša is the largest lake connected to Lake Crniševo, and there is no exchange of water between it and Lake Crniševo (Bonacci, 1984).

Sampling

Water samples were collected once a month from April to September in 2017 at the deepest point of each lake (CEN-EN, 2015a). Composite samples were collected with the Uwitec water sampler from the euphotic zone or epilimnion during thermal stratification or a maximum of up to 20 m during the

non-stratification period. Immediately after sampling, samples were stored in glass bottles (250 mL) for microscopy and preserved with alkaline Lugol's solution. For eDNA metabarcoding, immediately after sampling, the composite water samples were filtered through polycarbonate membrane filters (type GTTP; Whatman, UK) with 0.2 µm pore size using a peristaltic pump. The filters were stored in dry ice immediately after filtration was completed, where they were transported and stored at − 80 °C.

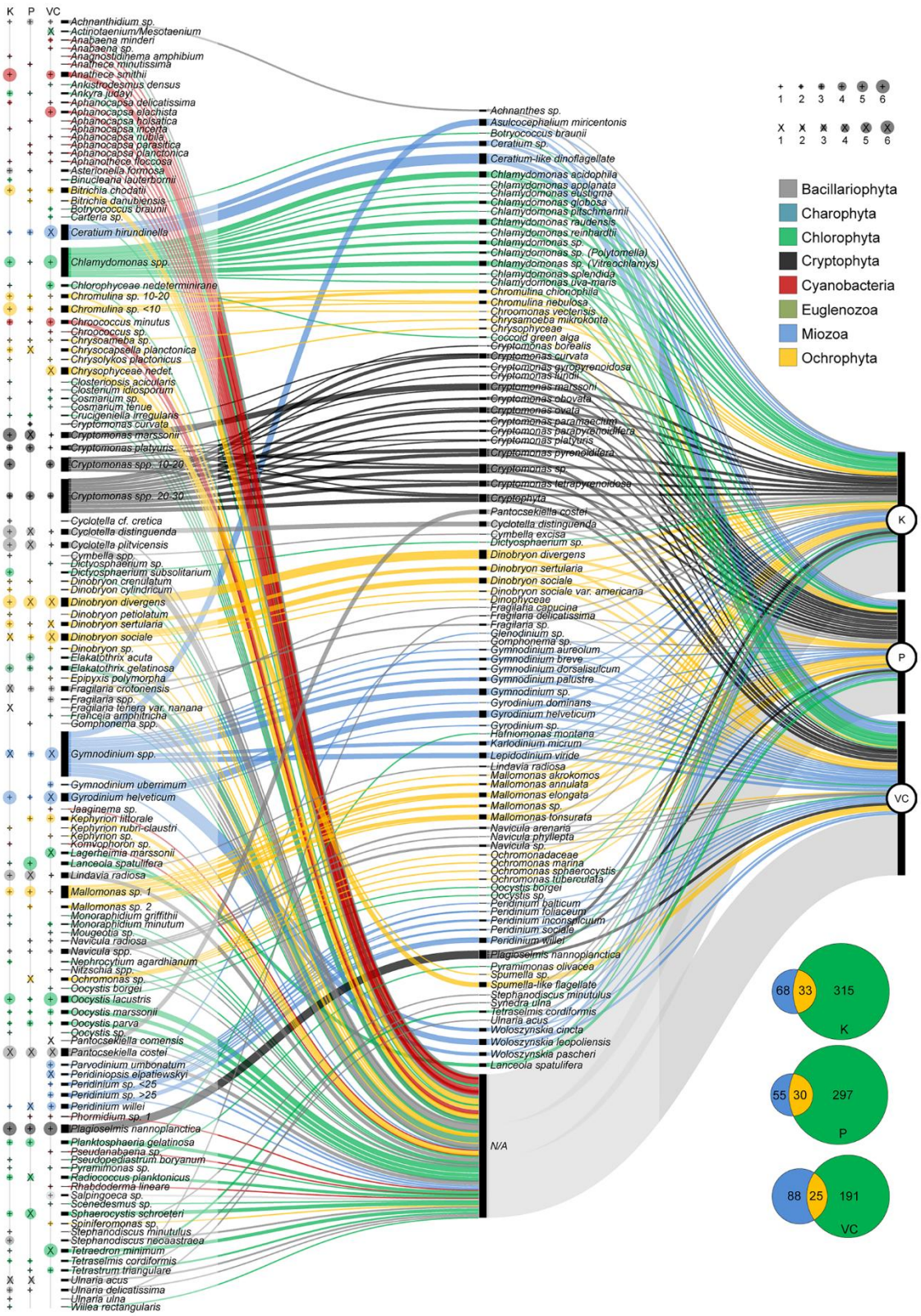
Sample analysis using light microscopy

Phytoplankton was identified and counted using inverted microscope Zeiss Axio Observer Z1 at 400×, 200×, and 100×magnification following Utermöhl (1958) method. Sedimentation units (unicellular, coenobium, filament, or colony) were counted in random counting fields or transects until 400 sedimentation units were reached at 400×magnification, providing a counting error of < 10% (Lund et al., 1958; CEN-EN, 2006). Once cells were assessed as having the closest regular geometric shape, their dimensions were used to calculate the biovolume of each measured cell. Biovolumes were calculated by determining the median individual size from max. 30 randomly chosen cells of each taxon and multiplied by the observed species abundance. Biomass (freshweight) was derived from biovolumes and used for further analyses, where 1 mm^3 L^{-1} = 1 mg L^{-1} (Rott, 1981; CEN-EN, 2015b). Additional identification of diatoms was done from permanent slides prepared by cleaning qualitative samples with warm hydrochloric acid and hydrogen peroxide and mounted using Naphrax solution (CEN-EN, 2009). Zeiss Axio Observer Z1 with DIC was used for diatoms identification at a 1000×magnification.

Sample analysis using eDNA metabarcoding

Total genomic DNA was extracted using the DNeasy PowerWater Kit (Qiagen GmbH Hilden, Germany) according to the manufacturer's instructions. A NanoDrop spectrophotometer (ND 2000, Thermo Scientific, Wilmington, DE, USA) was used to measure DNA concentration and purity. The DNA concentrations ranged from 1.5 to 11.5 ng μL^{-1} (average 3.85 ng μL^{-1}). The hypervariable V9 region of the eukaryotic SSU rRNA gene (approximately 150 bp long) was amplified using primer pair 1391F (5′-GTACACACCGCCCGTC-3′) and EukB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) following the protocol of Stoeck et al. (2010). To minimise PCR bias, three individual reactions were prepared per sample. Each primer pair was tagged with one of ten different barcodes, including a sample-specific tagging with 6-bp identifier fragments to allow demultiplexing in the downstream bioinformatics data processing. Triplicate PCR products per sampling point were pooled prior to sequencing library preparation. Samples were pooled into libraries that contained up to 10 different barcodes and sequencing libraries were prepared using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Libraries were sequenced on an Illumina NextSeq platform, generating 150-bp paired-end reads (SeqIT GmbH & Co. KG, Kaiserslautern, Germany). The generated sequences were deposited in the European Nucleotide Archive under project number PRJEB44080.

The base quality of raw sequence reads was checked using the FastQC software v0.11.5 (Andrews, 2017). For demultiplexing (removing barcodes and primers), raw paired-end Illumina reads were quality-filtered using the split_libraries.py script implemented in QIIME v.1.8.0 (Caporaso et al., 2010). Libraries were split into single sample according to the barcodes initially applied during the PCR. Filtering of reads with an expected barcode and removal of those was followed by matching the primer sequences at both 5′ and 3′-ends, as well. Reads were kept if they exactly matched the forward or reverse primers at their 5′-end and, at the same time, if they exactly matched the reverse or forward primers near to the 3′-end. Only reads with the same barcodes and primers, unambiguous nucleotides, and a minimum length of 90 base pairs were retained. The barcode- and primer-filtering produced 46 sample paired libraries. Additional quality trim was performed on paired-end reads using the bbduk function and their merging using the bbmerge function of the BBMap package (Bushnell, 2014). Finally, all sequences were de novo chimera-checked using UCHIME version 5.2.236 (Edgar et al., 2011). High-quality non-chimeric reads were clustered in Operational Taxonomic Units (OTUs) using SWARM v3.0.0 (Mahé et al., 2015) with default settings. Using a custom script, an OTU contingency table was created based on the output


K
P
VC
Achnanthidium sp.
Actinotaenium/Mesotaenium
Anabaena minderi
Anabaena sp.
Anagnostidinema amphibium
Anathece minutissima
Anathece smithii
Ankistrodesmus densus
Ankyra judayi
Aphanocapsa delicatissima
Aphanocapsa elachista
Aphanocapsa holsatica
Aphanocapsa incerta
Aphanocapsa nubila
Aphanocapsa parasitica
Aphanocapsa planctonica
Aphanothece floccosa
Asterionella formosa
Binucleana lauterbornii
Bitrichia chodatii
Bitrichia danubiensis
Botryococcus braunii
Carteria sp.
Ceratium hirundinella
Chlamydomonas spp.
Chlorophyceae nedeterminirane
Chromulina sp. 10-20
Chromulina sp. <10
Chroococcus minutus
Chroococcus sp.
Chrysoamoeba sp.
Chrysocapsella planctonica
Chrysolykos plactonicus
Chrysophyceae nedet.
Closteriopsis acicularis
Closterium idiosporum
Cosmarium sp.
Cosmarium tenue
Crucigeniella irregularis
Cryptomonas curvata
Cryptomonas marssonii
Cryptomonas platyuris
Cryptomonas spp. 10-20
Cryptomonas spp. 20-30
Cyclotella cf. cretica
Cyclotella distinguenda
Cyclotella pliticensis
Cymbella spp.
Dictyosphaerium sp.
Dictyosphaerium subsolitarium
Dinobryon crenulatum
Dinobryon cylindricum
Dinobryon divergens
Dinobryon petiolatum
Dinobryon sertularia
Dinobryon sociale
Dinobryon sp.
Elakatothrix acuta
Elakatothrix gelatinosa
Epipyxis polymorpha
Fragilaria crotonensis
Fragilaria spp.
Fragilaria tenera var. nanana
Franceia amphitricha
Gomphonema spp.
Gymnodinium spp.
Gymnodinium uberrimum
Gyrodinium helveticum
Jaaginema sp.
Kephyrion littorale
Kephyrion rubri-claustri
Kephyrion sp.
Komvophoron sp.
Lagerheimia marssonii
Lanceola spatulifera
Lindavia radiosa
Mallomonas sp. 1
Mallomonas sp. 2
Monoraphidium griffithii
Monoraphidium minutum
Mougeotia sp.
Navicula radiosa
Navicula spp.
Nephrocytium agardhianum
Nitzschia spp.
Ochromonas sp.
Oocystis borgei
Oocystis lacustris
Oocystis marssonii
Oocystis parva
Oocystis sp.
Pantocsekiella comensis
Pantocsekiella costei
Parvodinium umbonatum
Peridiniopsis alpatiewskyi
Peridinium sp. <25
Peridinium sp. >25
Peridinium willei
Phormidium sp. 1
Plagioselmis nannoplanctica
Planktosphaeria gelatinosa
Pseudanabaena sp.
Pseudopediastrum boryanum
Pyramimonas sp.
Radiococcus planktonicus
Rhabdoderma lineare
Salpingoeca sp.
Scenedesmus sp.
Sphaerocystis schroeteri
Spiniferomonas sp.
Stephanodiscus minutulus
Stephanodiscus neoastraea
Tetraedron minimum
Tetraselmis cordiformis
Tetrastrum triangulare
Ulnaria acus
Ulnaria delicatissima
Ulnaria ulna
Willea rectangularis
Achnanthes sp.
Asulcocephalium miricentonis
Botryococcus braunii
Ceratium sp.
Ceratium-like dinoflagellate
Chlamydomonas acidophila
Chlamydomonas applanata
Chlamydomonas eustigma
Chlamydomonas globosa
Chlamydomonas pitschmannii
Chlamydomonas raudensis
Chlamydomonas reinhardtii
Chlamydomonas sp.
Chlamydomonas sp. (Polytomella)
Chlamydomonas sp. (Vitreochlamys)
Chlamydomonas splendida
Chlamydomonas uva-maris
Chromulina chionophila
Chromulina nebulosa
Chroomonas vectensis
Chrysamoeba mikrokonta
Chrysophyceae
Coccoid green alga
Cryptomonas borealis
Cryptomonas curvata
Cryptomonas gyropyrenoidosa
Cryptomonas lundii
Cryptomonas marssonii
Cryptomonas obovata
Cryptomonas ovata
Cryptomonas paramaecium
Cryptomonas paramecium
Cryptomonas platyuris
Cryptomonas pyrenoidifera
Cryptomonas sp.
Cryptomonas tetrapyrenoidosa
Cryptophyta
Pantocsekiella costei
Cyclotella distinguenda
Cymbella excisa
Dictyosphaerium sp.
Dinobryon divergens
Dinobryon sertularia
Dinobryon sociale
Dinobryon sociale var. americana
Dinophyceae
Fragilaria capucina
Fragilaria delicatissima
Fragilaria sp.
Glenodinium sp.
Gomphonema sp.
Gymnodinium aureolum
Gymnodinium breve
Gymnodinium dorsalisulcum
Gymnodinium palustre
Gymnodinium sp.
Gyrodinium dominans
Gyrodinium helveticum
Gyrodinium sp.
Hafniomonas montana
Karlodinium micrum
Lepidodinium viride
Lindavia radiosa
Mallomonas akrokomos
Mallomonas annulata
Mallomonas elongata
Mallomonas sp.
Mallomonas tonsurata
Navicula arenaria
Navicula phyllepta
Navicula sp.
Ochromonadaceae
Ochromonas marina
Ochromonas sphaerocystis
Ochromonas tuberculata
Oocystis borgei
Oocystis sp.
Peridinium balticum
Peridinium foliaceum
Peridinium inconspicuum
Peridinium sociale
Peridinium willei
Plagioselmis nannoplanctica
Pyramimonas olivacea
Spumella sp.
Spumella-like flagellate
Stephanodiscus minutulus
Synedra ulna
Tetraselmis cordiformis
Ulnaria acus
Woloszynskia cincta
Woloszynskia leopoliensis
Woloszynskia pascheri
Lanceola spatulifera
N/A
1 2 3 4 5 6
1 2 3 4 5 6
Bacillariophyta
Charophyta
Chlorophyta
Cryptophyta
Cyanobacteria
Euglenozoa
Miozoa
Ochrophyta
K
P
VC
68 33 315
K
55 30 297
P
88 25 191
VC

◂**Fig. 2** Alluvial diagram showing the complete morphological taxa list (left) with the overlapping taxa identified by the eDNA metabarcoding list (right) and their occurrence in the first lake group (Kozjak (K), Prošće (P) and deep Lake Vransko (VC)). The signs + and X indicate the presence of taxa in the lake, where X denotes taxa contributing > 5% to the total biomass. The circles above the + and X signs represent the frequency of occurrence of taxa in six samples from April to September. The taxa are grouped and coloured according to the phylum to which they belong. Venn diagrams show the total number of taxa per lake identified by microscopy (blue) and eDNA metabarcoding (green) in the periods studied, and their combined number (yellow)

files of SWARM. Representative sequences from each operational taxonomic unit (OTU) were extracted and analyzed with BLASTn (BLAST v2.2.30, (Altschul et al., 1990) against NCBI's nucleotide reference database (NCBI-GenBank Flat File Release-GenBank 222.0). The criterion used is implemented in the default settings of BLAST. For placement of BLAST hits into higher taxonomic groups, we considered the BLAST hit with the highest sequence similarity at least 80% to any hit and e-value retaining sequences. The taxonomic information and the OTU contingency table were merged using a custom script. Non-protistan OTUs, reads associated with Bacteria, Archaea, Metazoa, Embryophyta, and ciliates as well as singletons, were excluded. Only OTUs related to the phytoplankton community at the family level, reaching ≥ 97% of identity, were used. Furthermore, according to the given NCBI ID, we have checked the genus or species level for OTUs with 100% identity. Prior to downstream analysis, OTUs with the same taxonomical assignment were merged.

Data analysis

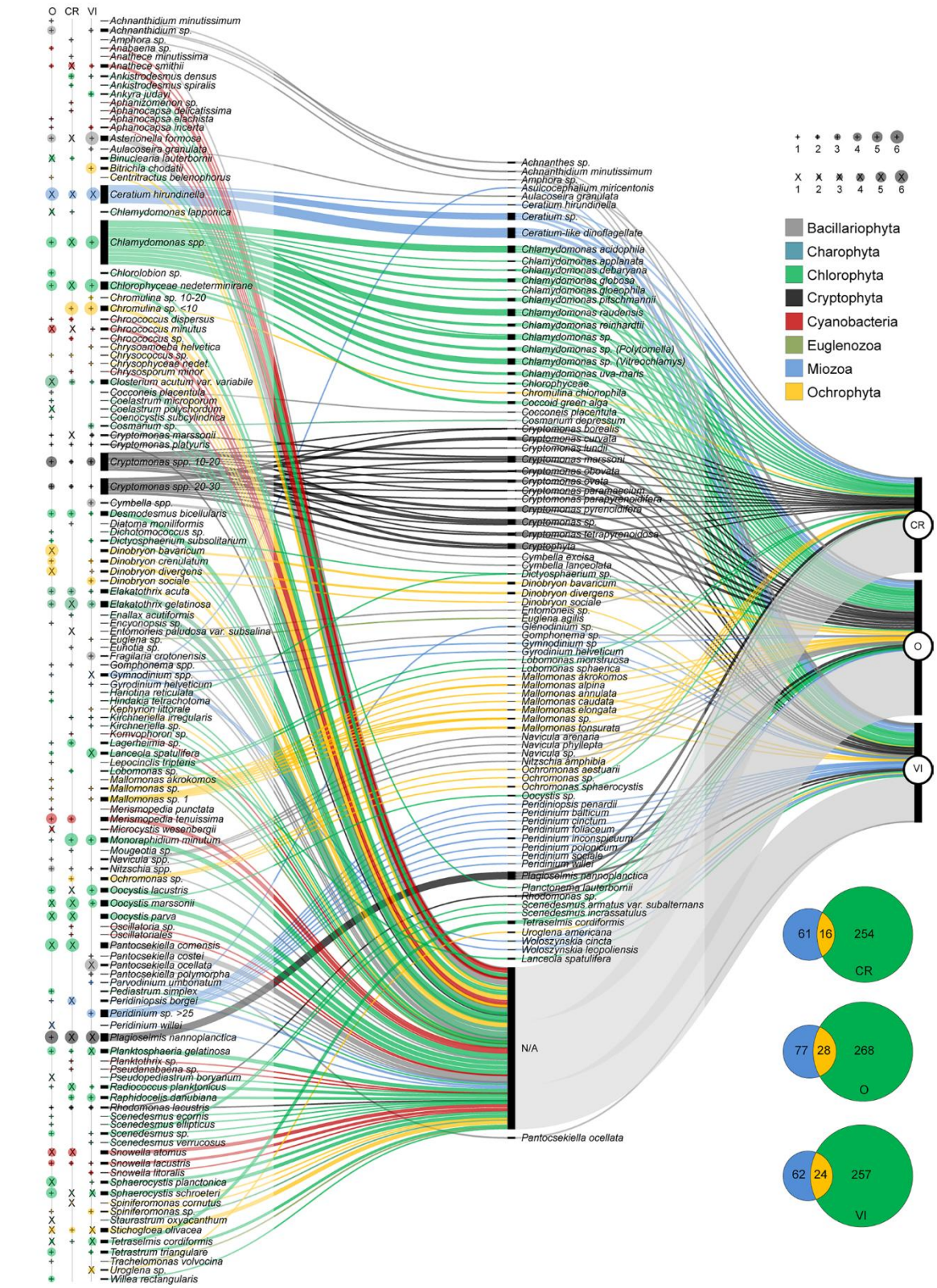
Using Primer 6 software (Clarke & Gorley, 2006), a cluster analysis based on Bray–Curtis similarity was performed for the taxonomic composition of phytoplankton determined separately by microscopy and eDNA metabarcoding to determine similarities within the months studied based on the biomass of phytoplankton taxa (microscopy) and the number of sequences of OTUs (eDNA metabarcoding). Prior to analysis, biomass and the number of sequences were square-root transformed. The composition and proportion of taxonomic groups and FGs were plotted using Grapher ™ (Golden Software, 2020). A box-whisker plot of microscopy-calculated relative total biomass and relative number of sequences (eDNA metabarcoding) for overlapping taxa only in all samples for individual lakes was plotted in Grapher ™. Alluvial diagrams showing the complete morphological taxa list with overlapping taxa obtained by eDNA metabarcoding were plotted using RAWGraphs 2.0 beta (Mauri et al., 2017). Prior to comparison of morphological and eDNA metabarcoding phytoplankton names were updated according to the Algaebase (Guiry & Guiry, 2022) and taxa were assigned to the FGs according to Borics et al. (2007), Padisák et al. (2009), and Reynolds (2002). OTUs belonging to the same taxon have been grouped under an updated phytoplankton name, now called the eDNA taxon, and the number of their sequences has been added up.

Results

Comparison of phytoplankton composition between microscopy and eDNA metabarcoding is presented as relative abundance of taxonomic groups, FGs, and Bray–Curtis similarity between samples/lakes. A full list of morphological taxa was also given as well as the frequency of occurrence in the samples and taxa contributing more than 5% to the total biomass. Only eDNA taxa that are overlapping with morphologically identified taxa are shown (Figs. 2, 3, 4). All eDNA taxa, including those that do not overlap with morphologically identified taxa, are shown in Supplementary table S1.

Taxonomic characterisation of the phytoplankton communities by microscopy and eDNA metabarcoding

In this study, a total of 217 taxa were identified microscopically, while 531 eDNA taxa of phytoplankton were taxonomically assigned using eDNA metabarcoding. The two cluster analyses based on Bray–Curtis similarity performed for phytoplankton communities, one by microscopy (Fig. 5) and the other by eDNA metabarcoding (Fig. 6), resulted in clear separation of lakes. The first group included lakes Kozjak, Prošće (both in Plitvice Lakes National Park) and deep Vransko, while the second group included Oćuša, Crniševo (both part of Baćina Lakes) and Lake Visovac. Shallow Lake Vransko with two


O CR VI
Achnanthidium minutissimum
Achnanthidium sp.
Amphora sp.
Anabaena sp.
Anathece minutissima
Anathece smithii
Ankistrodesmus densus
Ankistrodesmus spiralis
Ankyra judayi
Aphanizomenon sp.
Aphanocapsa delicatissima
Aphanocapsa elachista
Aphanocapsa incerta
Asterionella formosa
Aulacoseira granulata
Binuclearia lauterborni
Bitrichia chodatii
Centritractus belenophorus
Ceratium hirundinella
Chlamydomonas lapponica
Chlamydomonas spp.
Chlorolobion sp.
Chlorophyceae nedeterminirane
Chromulina sp. 10-20
Chromulina sp. <10
Chroococcus dispersus
Chroococcus minutus
Chroococcus sp.
Chrysoamoeba helvetica
Chrysococcus sp.
Chrysophyceae nedet.
Chrysosporum minor
Closterium acutum var. variabile
Cocconeis placentula
Coelastrum microporum
Coelastrum polychordum
Coenocystis subcylindrica
Cosmarium sp.
Cryptomonas marssonii
Cryptomonas platyuris
Cryptomonas spp. 10-20
Cryptomonas spp. 20-30
Cymbella spp.
Desmodesmus bicellularis
Diatoma moniliformis
Dichotomococcus sp.
Dictyosphaerium subsolitarium
Dinobryon bavaricum
Dinobryon crenulatum
Dinobryon divergens
Dinobryon sociale
Elakatothrix acuta
Elakatothrix gelatinosa
Enallax acutiformis
Encyonopsis sp.
Entomoneis paludosa var. subsalina
Euglena sp.
Eunotia sp.
Fragilaria crotonensis
Gomphonema spp.
Gymnodinium spp.
Gyrodinium helveticum
Handina reticulata
Hindakia tetrachotoma
Kephyrion littorale
Kirchneriella irregularis
Kirchneriella sp.
Komvophoron sp.
Lagerheimia sp.
Lanceola spatulifera
Lepocinclis triptens
Lobomonas sp.
Mallomonas akrokomos
Mallomonas sp.
Mallomonas sp. 1
Merismopedia punctata
Merismopedia tenuissima
Microcystis wesenbergii
Monoraphidium minutum
Mougeotia sp.
Navicula spp.
Nitzschia spp.
Ochromonas sp.
Oocystis lacustris
Oocystis marssonii
Oocystis parva
Oscillatoria sp.
Oscillatoriales
Pantocsekiella comensis
Pantocsekiella costei
Pantocsekiella ocellata
Pantocsekiella polymorpha
Parvodinium umbonatum
Pediastrum simplex
Peridiniopsis borgei
Peridinium sp. >25
Peridinium willei
Plagioselmis nannoplanctica
Planktosphaeria gelatinosa
Planktothrix sp.
Pseudanabaena sp.
Pseudopediastrum boryanum
Radiococcus planktonicus
Raphidocelis danubiana
Rhodomonas lacustris
Scenedesmus ecornis
Scenedesmus ellipticus
Scenedesmus sp.
Scenedesmus verrucosus
Snowella atomus
Snowella lacustris
Snowella litoralis
Sphaerocystis planctonica
Sphaerocystis schroeteri
Spiniferomonas cornutus
Spiniferomonas sp.
Staurastrum oxyacanthum
Stichogloea olivacea
Tetraselmis cordiformis
Tetrastrum triangulare
Trachelomonas volvocina
Uroglena sp.
Willea rectangularis
Achnanthes sp.
Achnanthidium minutissimum
Amphora sp.
Asulocephalium miricentonis
Aulacoseira granulata
Ceratium hirundinella
Ceratium sp.
Ceratium-like dinoflagellate
Chlamydomonas acidophila
Chlamydomonas applanata
Chlamydomonas debaryana
Chlamydomonas globosa
Chlamydomonas gloeophila
Chlamydomonas pitschmannii
Chlamydomonas raudensis
Chlamydomonas reinhardtii
Chlamydomonas sp.
Chlamydomonas sp. (Polytomella)
Chlamydomonas sp. (Vitreochlamys)
Chlamydomonas uva-maris
Chlorophyceae
Chromulina chionophila
Coccoid green alga
Cocconeis placentula
Cosmarium depressum
Cryptomonas borealis
Cryptomonas curvata
Cryptomonas lundii
Cryptomonas marssonii
Cryptomonas obovata
Cryptomonas ovata
Cryptomonas paramaecium
Cryptomonas parapyrenoidifera
Cryptomonas pyrenoidifera
Cryptomonas sp.
Cryptomonas tetrapyrenoidosa
Cryptophyta
Cymbella excisa
Cymbella lanceolata
Dictyosphaerium sp.
Dinobryon bavaricum
Dinobryon divergens
Dinobryon sociale
Entomoneis sp.
Euglena agilis
Glenodinium sp.
Gomphonema sp.
Gymnodinium sp.
Gyrodinium helveticum
Lobomonas monstruosa
Lobomonas sphaerica
Mallomonas akrokomos
Mallomonas alpina
Mallomonas annulata
Mallomonas caudata
Mallomonas elongata
Mallomonas sp.
Mallomonas tonsurata
Navicula arenaria
Navicula phyllepta
Navicula sp.
Nitzschia amphibia
Ochromonas aestuarii
Ochromonas sp.
Ochromonas sphaerocystis
Oocystis sp.
Peridiniopsis penardii
Peridinium balticum
Peridinium cinctum
Peridinium foliaceum
Peridinium inconspicuum
Peridinium polonicum
Peridinium sociale
Peridinium willei
Plagioselmis nannoplanctica
Planctonema lauterbornii
Rhodomonas sp.
Scenedesmus armatus var. subalternans
Scenedesmus incrassatulus
Tetraselmis cordiformis
Uroglena americana
Woloszynskia cincta
Woloszynskia leopoliensis
Lanceola spatulifera
N/A
Pantocsekiella ocellata
1 2 3 4 5 6
X X X X X X
1 2 3 4 5 6
Bacillariophyta
Charophyta
Chlorophyta
Cryptophyta
Cyanobacteria
Euglenozoa
Miozoa
Ochrophyta
CR
O
VI
61 16 254
CR
77 28 268
O
62 24 257
VI

◂**Fig. 3** Alluvial diagram showing the complete morphological taxa list (left) with the overlapping taxa identified by the eDNA metabarcoding list (right) and their occurrence in the second lake group (Oćuša (O), Crniševo (C) and Visovac (VI)). The signs + and X indicate the presence of taxa in the lake, where X denotes taxa contributing > 5% to the total biomass. The circles above the + and X signs represent the frequency of occurrence of taxa in six samples from April to September. The taxa are grouped and coloured according to the phylum to which they belong. Venn diagrams show the total number of taxa per lake identified by microscopy (blue) and eDNA metabarcoding (green) in the periods studied, and their combined number (yellow)

sampling sites, Motel and Prosika, was separated from all other lakes as the third sample group.

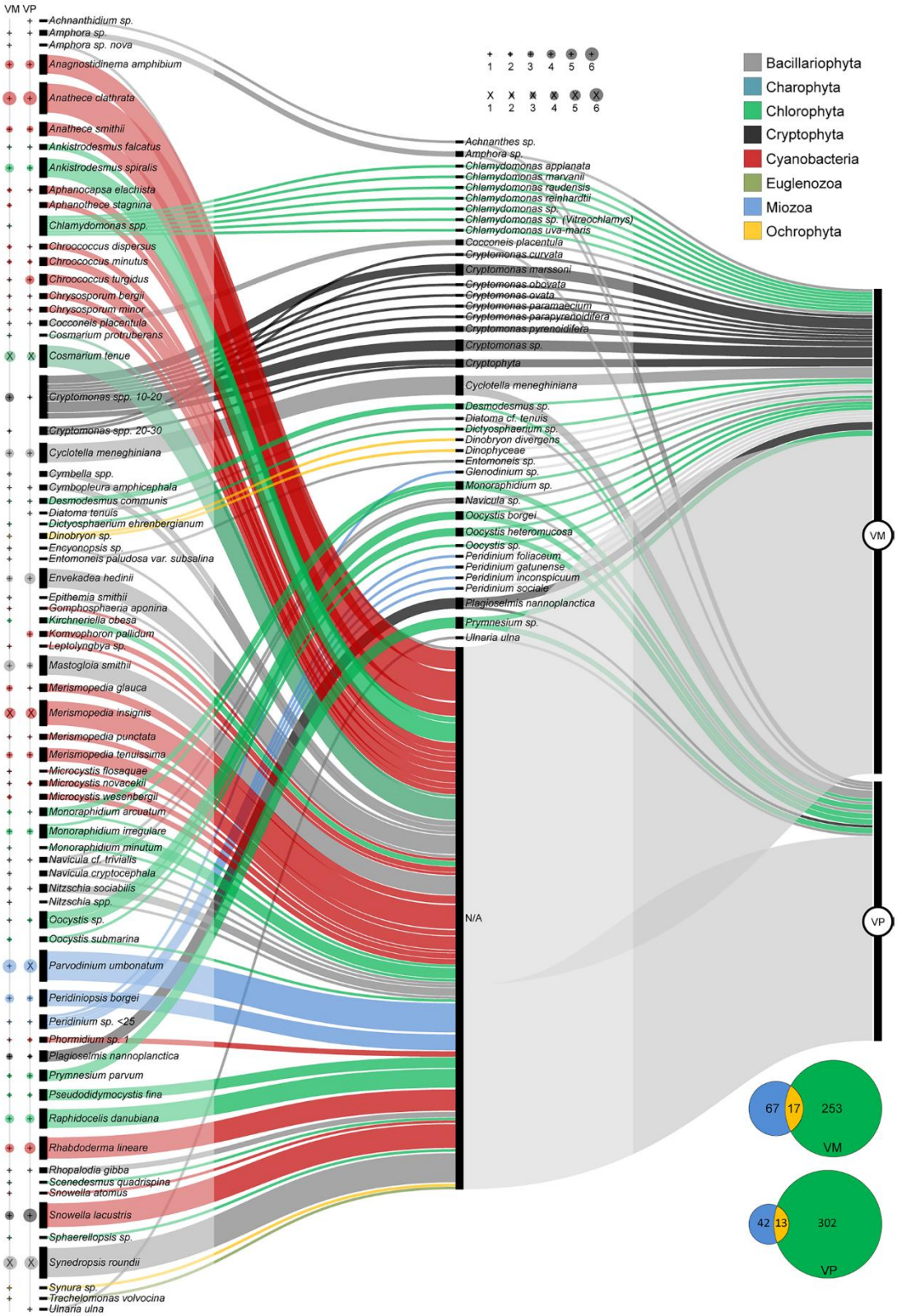
Species richness in lakes/sampling sites determined by microscopy and eDNA metabarcoding and the number of taxa that overlap are shown in Venn diagrams (Figs. 2, 3, 4). The highest species richness with 88 taxa was found by microscopy in deep Lake Vransko. The lake with the lowest number of taxa was Lake Vransko, sampling site Prosika, where only 42 taxa were identified. The most eDNA taxa that could be taxonomically assigned to phytoplankton taxa were identified in Lake Kozjak (315), while deep Lake Vransko had the lowest number of identified eDNA taxa (191). The lakes with the most overlapped taxa identified by both microscopy and eDNA metabarcoding were Kozjak (33) and Prošće (30), while shallow Lake Vransko (Prosika sampling site) had the least overlap of taxa identified by both methods (13).

Most microscopically identified taxa belonging to Cryptophyta, Miozoa, and Ochrophyta overlap with taxa identified by eDNA metabarcoding. Morphologically identified taxa belonging to the genus *Chlamydomonas* were well recognised by eDNA metabarcoding, whereas most other microscopically identified green algae (Chlorophyta) were not detected by eDNA metabarcoding. The high percentage of microscopically identified species of Bacillariophyta resulted in less overlap of this group with taxa detected by eDNA metabarcoding (Figs. 2, 3, 4).

The dominant species identified by microscopy and detected by eDNA metabarcoding were *Pantocsekiella costei* (J.C.Druart & F.Straub) K.T.Kiss & E.Ács, *Pantocsekiella ocellata* (Pantocsek) K.T.Kiss & Ács, *Cyclotella distinguenda* Hustedt, *Fragilaria crotonensis* Kitton, *Dinobryon divergens* O.E.Imhof, *Dinobryon sertularia* Ehrenberg, *Peridinium willei* Huitfeldt-Kaas, *Ceratium hirundinella* (O.F.Müller) Dujardin, *Gyrodinium helveticum* (Penard) Y.Takano & T.Horiguchi and taxa belonging to genus *Gymnodinium* and genus *Cryptomonas*. *Pantocsekiella costei* was identified by both methods in lakes Kozjak and Prošće, but surprisingly not in deep Lake Vransko, where one of the dominant taxa from the Bacillariophyta group also occurred. Besides *P. costei* in deep Lake Vransko, the dominant taxa identified by microscopy but not detected by eDNA metabarcoding were *Pantocsekiella comensis* (Grunow) K.T.Kiss & E.Ács, *Synedropsis roundii* Torgan, Menezes, & Melo, together with some representatives of Charophyta (*Cosmarium tenue* W.Archer, *Actinotaenium/Mesotaenium*) and Chlorophyta (*Radiococcus planktonicus* J.W.G.Lund, *Sphaerocystis planctonica* (Korshikov) Bourrelly, *Sphaerocystis schroeteri Chodat*). These are most represented in lakes Crniševo, Oćuša and shallow Lake Vransko, which resulted in lower eDNA metabarcoding detection.

When directly comparing the two methods, some taxa identified by microscopy at the genus level could be overlapped by more representatives identified by eDNA metabarcoding, as more species of the same genus were identified. The genera identified by eDNA metabarcoding were *Gymnodinium* spp., *Peridinium* spp., *Ceratium* spp. (Miozoa), *Cryptomonas* spp. (Cryptophyta), and *Chlamydomonas* spp. (Chlorophyta), which are shown in Figs. 2, 3, 4.

The quantification capacity of microscopy compared to eDNA metabarcoding, expressed as the proportion of biomass of microscopically identified taxa detected by eDNA metabarcoding was highest in lakes Prošće, Kozjak and Visovac (8.5–97.5%). Lakes deep Vransko and Oćuša followed with 17.6–70.7%, while brackish lakes Crniševo and shallow Vransko had the lowest proportion of biomass. The proportion in Lake Crniševo ranged from 13.3 to 25.2% with an outlier of 67.0%, while in shallow Lake Vransko a sample in which no taxa were detected by eDNA metabarcoding accounted for up to 8.9% of the relative biomass. The quantification capacity of eDNA metabarcoding compared to microscopy, expressed as the relative number of sequences of taxa detected by eDNA metabarcoding that were also detected by microscopy, showed a similar pattern in the lakes with minor differences. The highest proportion of sequences was in lakes deep Vransko and Visovac (76.4–94.0%), followed by lakes Kozjak, Prošće and Oćuša (6.6–83.3%). A lower proportion of the


VM VP
Achnanthidium sp.
Amphora sp.
Amphora sp. nova
Anagnostidinema amphibium
Anathece clathrata
Anathece smithii
Ankistrodesmus falcatus
Ankistrodesmus spiralis
Aphanocapsa elachista
Aphanothece stagnina
Chlamydomonas spp.
Chroococcus dispersus
Chroococcus minutus
Chroococcus turgidus
Chrysosporum bergii
Chrysosporum minor
Cocconeis placentula
Cosmarium protruberans
Cosmarium tenue
Cryptomonas spp. 10-20
Cryptomonas spp. 20-30
Cyclotella meneghiniana
Cymbella spp.
Cymbopleura amphicephala
Desmodesmus communis
Diatoma tenuis
Dictyosphaerium ehrenbergianum
Dinobryon sp.
Encyonopsis sp.
Entomoneis paludosa var. subsalina
Envekadea hedinii
Epithemia smithii
Gomphosphaeria aponina
Kirchneriella obesa
Komvophoron pallidum
Leptolyngbya sp.
Mastogloia smithii
Merismopedia glauca
Merismopedia insignis
Merismopedia punctata
Merismopedia tenuissima
Microcystis flosaquae
Microcystis novacekii
Microcystis wesenbergii
Monoraphidium arcuatum
Monoraphidium irregulare
Monoraphidium minutum
Navicula cf. trivialis
Navicula cryptocephala
Nitzschia sociabilis
Nitzschia spp.
Oocystis sp.
Oocystis submarina
Parvodinium umbonatum
Peridiniopsis borgei
Peridinium sp. <25
Phormidium sp. 1
Plagioselmis nannoplanctica
Prymnesium parvum
Pseudodidymocystis fina
Raphidocelis danubiana
Rhabdoderma lineare
Rhopalodia gibba
Scenedesmus quadrispina
Snowella atomus
Snowella lacustris
Sphaerellopsis sp.
Synedropsis roundii
Synura sp.
Trachelomonas volvocina
Ulnaria ulna
1 2 3 4 5 6
X X X X X X
1 2 3 4 5 6
Achnanthes sp.
Amphora sp.
Chlamydomonas applanata
Chlamydomonas marvanii
Chlamydomonas raudensis
Chlamydomonas reinhardtii
Chlamydomonas sp.
Chlamydomonas sp. (Vitreochlamys)
Chlamydomonas uva-maris
Cocconeis placentula
Cryptomonas curvata
Cryptomonas marssoni
Cryptomonas obovata
Cryptomonas ovata
Cryptomonas paramaecium
Cryptomonas parapyrenoidifera
Cryptomonas pyrenoidifera
Cryptomonas sp.
Cryptophyta
Cyclotella meneghiniana
Desmodesmus sp.
Diatoma cf. tenuis
Dictyosphaerium sp.
Dinobryon divergens
Dinophyceae
Entomoneis sp.
Glenodinium sp.
Monoraphidium sp.
Navicula sp.
Oocystis borgei
Oocystis heteromucosa
Oocystis sp.
Peridinium foliaceum
Peridinium gatunense
Peridinium inconspicuum
Peridinium sociale
Plagioselmis nannoplanctica
Prymnesium sp.
Ulnaria ulna
N/A
Bacillariophyta
Charophyta
Chlorophyta
Cryptophyta
Cyanobacteria
Euglenozoa
Miozoa
Ochrophyta
VM
VP
67 17 253
VM
42 13 302
VP

◂**Fig. 4** Alluvial diagram showing the complete morphological taxa list (left) with the overlapping taxa identified by the eDNA metabarcoding list (right) and their occurrence in the third lake group (shallow Lake Vransko, sampling site Motel (VM) and Prosika (VP)). The signs + and X indicate the presence of taxa in the lake, where X denotes taxa contributing > 5% to the total biomass. The circles above the + and X signs represent the frequency of occurrence of taxa in six samples from April to September. The taxa are grouped and coloured according to the phylum to which they belong. Venn diagrams show the total number of taxa per lake identified by microscopy (blue) and eDNA metabarcoding (green) in the periods studied, and their combined number (yellow)

relative number of sequences was observed in Lake Crniševo between 0.1 and 22.5% with an outlier of 63.7%, while in the shallow Lake Vransko it varied between 0.3 and 18.5% with an outlier of 44.7%. The results also showed that in the case of shallow Lake Vransko, with two sampling sites, both the proportion of biomass and the relative sequences were drastically lower at the Prosika sampling site (Fig. 7).

Composition of the taxonomic groups

Phytoplankton taxa identified by microscopy belonged to nine taxonomic groups, while those identified by eDNA metabarcoding belonged to ten taxonomic groups. Neither the taxonomic nor the FG composition based on relative number of sequences fully matched the composition based on relative biomass. In Lake Kozjak, Bacillariophyta was the dominant taxonomic group in both relative biomass and relative number of sequences. The Miozoa and Ochrophyta taxonomic groups were codominant in relative number of sequences but were represented in a smaller proportion of relative biomass. A similar proportion of relative biomass and relative number of sequences of Bacillaryophyta was present in April, May, and June in Lake Prošće. In July, August and September, morphological identification revealed a dominance of Chlorophyta, while in eDNA metabarcoding the taxonomic group of Cryptophyta dominated in the relative number of sequences. In deep Lake Vransko, the relative biomass of taxonomic groups identified by microscopy showed codomination of Miozoa, Ochrophyta, and Charophyta, while Charophyta were not detected and Miozoa dominated in the relative number of sequences. Unlike in most lakes, the taxonomic groups presented as relative phytoplankton biomass and relative number of sequences showed similar composition in Lake Visovac, but in different proportions. The taxonomic group Chlorophyta was dominant in lakes Crniševo and Oćuša according to the relative biomass, but according to the relative number of sequences, Bacillariophyta and Cryptophyta were the dominant groups. The representatives of Charophyta from April to June and Bacillariophyta from July to September showed a clear dominance in the relative biomass of the shallow Lake Vransko. On the other hand, the proportion of groups determined by metabarcoding did not correspond to the dominant groups determined by microscopy (Fig. 8).

Composition of the FGs

Phytoplankton taxa identified by microscopy belonged to 25 FGs, whereas those identified by eDNA metabarcoding belonged to 21 FGs. Phytoplankton FG composition calculated as relative biomass identified by microscopy and relative number of sequences obtained by eDNA metabarcoding mostly did not overlap (Fig. 9). The eDNA metabarcoding showed mainly the presence and dominance or codominance of codons **B**, $\mathbf{L_O}$, **Y** and **E**, especially in lakes Kozjak, Prošće, Visovac, Oćuša and Crniševo, while microscopy showed a greater diversity of FGs in these lakes, with the exception of June (codon **B**) and July, August and September (codon **F**) in Lake Prošće. Representatives assigned to FGs that dominated in deep Lake Vransko (codon **T**), Lake Crniševo (codon **F**), and shallow Lake Vransko (codons **N** and **P**) were not identified by eDNA metabarcoding. Similar proportions of some FGs were detected in Lake Prošće (codon **E**) in May, in deep Lake Vransko (codon $\mathbf{L_O}$) in June, in Lake Visovac (codon **U**) in May and June, and in July and August (codons $\mathbf{L_O}$ and **B**). Since no taxon of cyanobacteria was sequenced using this eDNA metabarcoding method, the FGs to which these taxa belong (codons **H1**, **K**, $\mathbf{L_M}$, **M**, and **S1**) are not shown as relative number of sequences (Fig. 9).

Discussion

Using qualitative and quantitative data of the phytoplankton community in karst lakes we compared classical microscopy and eDNA metabarcoding in

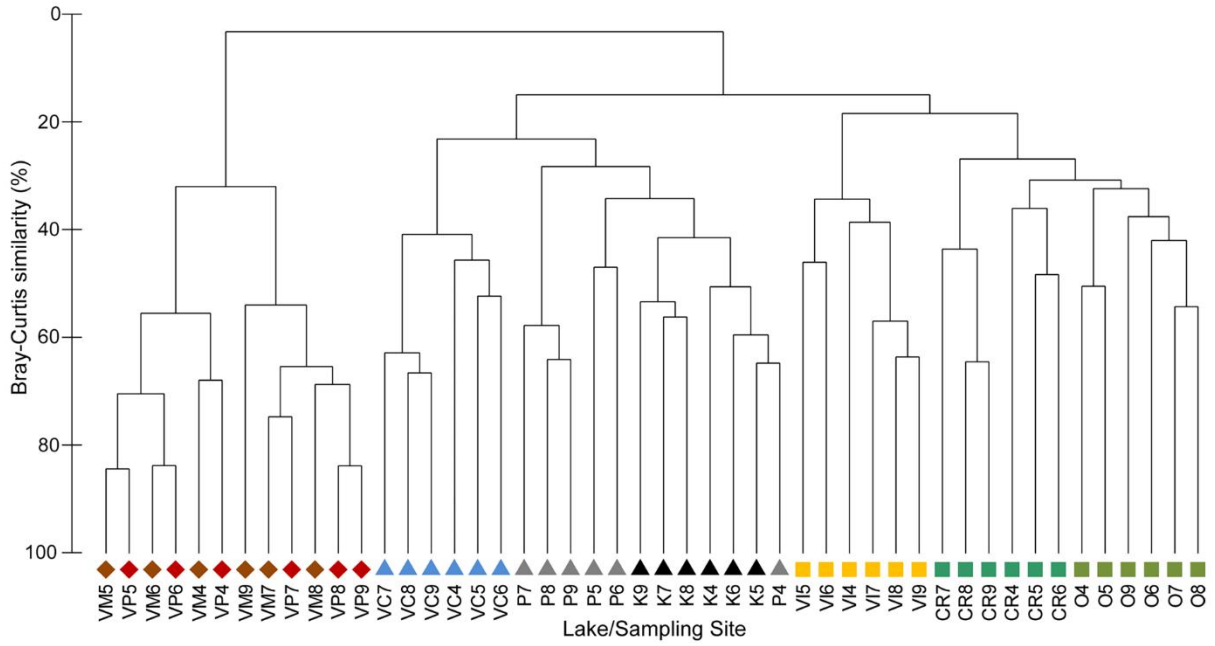


Fig. 5 Dendrogram of cluster analysis based on the Bray–Curtis similarity index of phytoplankton communities identified by microscopy and expressed as biomass between lakes from April to September. The lakes are coded with symbols in different colours, while the numbers represent the months. The location codes of the lakes are given in Fig. 1

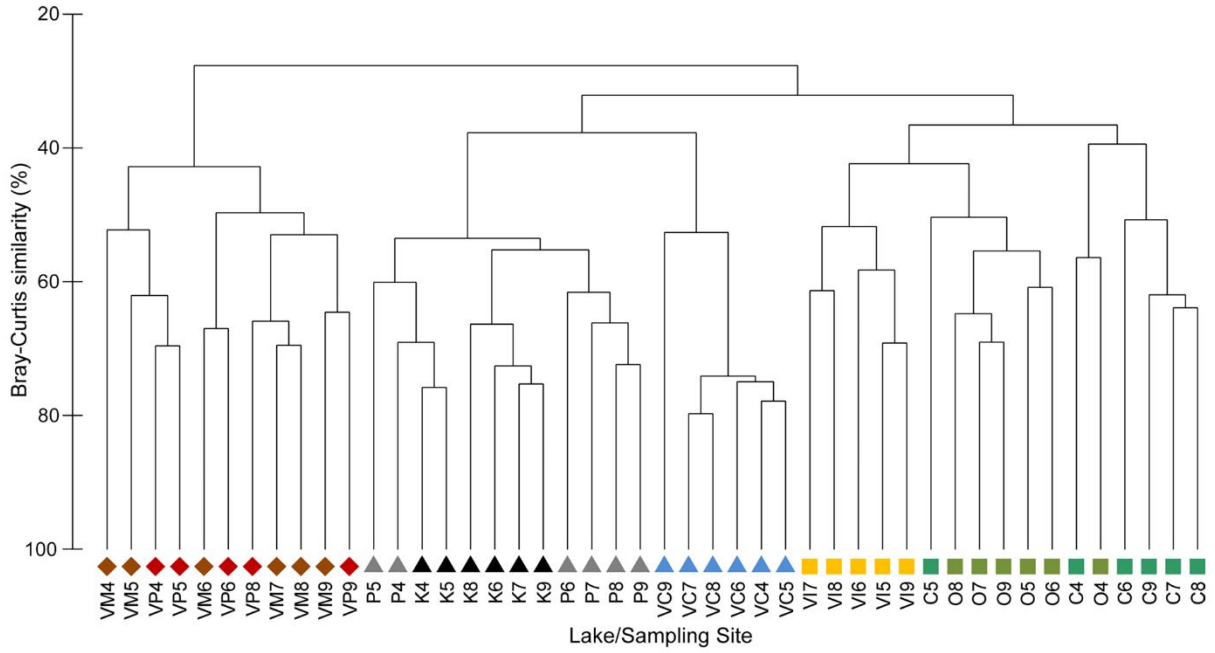


Fig. 6 Dendrogram of cluster analysis based on the Bray–Curtis similarity index of phytoplankton communities identified by eDNA metabarcoding and expressed as number of sequences between lakes from April to September. The lakes are coded with symbols in different colours, while the numbers represent the months. The location codes of the lakes are given in Fig. 1

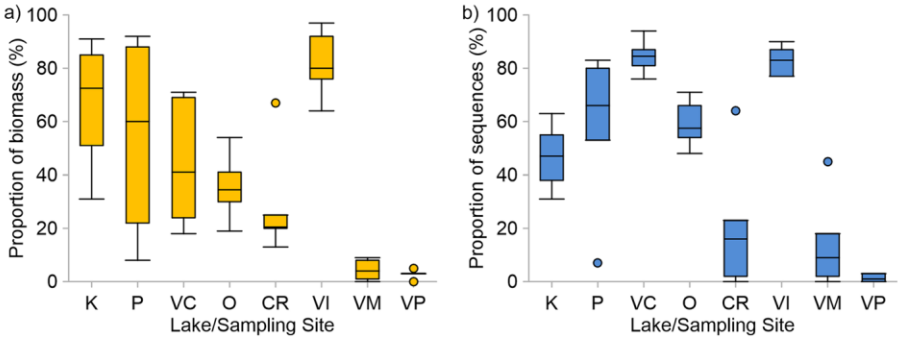


Fig. 7 Quantitative capacity for (a) Utermöhl method (relative biomass), and (b) eDNA metabarcoding method (relative number of sequences) for all mutually overlapping taxa presented as Box-Whiskers plot for all samples in individual lakes. Sample counts for all lakes is six ($n=6$), except for shallow Lake Vransko (VM) and Lake Visovac (VI) is five ($n=5$). The centre line indicates the median value, while outliers are indicated as dots. The location codes of all the lakes are given in Fig. 1

terms of taxonomic and FG composition for future ecological status assessment. Consequently, as there is little overlap in taxa between methods, only little overlap in FGs and relative abundances was found. The above results call into question the use of eDNA metabarcoding as a phytoplankton assessment method for natural karst lakes, which will be discussed below, but at the same time open new research opportunities.

In the study by Eiler et al. (2013), the 16S rRNA gene was used as a marker for eDNA metabarcoding because it is present in prokaryotes, including cyanobacteria, and in the chloroplasts of eukaryotic algae, but the taxonomic lists still showed low overlap with microscopy. Later, Huo et al. (2020) suggested that 16S rRNA might be a poor choice for detecting eukaryotic phytoplankton communities. When comparing 16S rRNA with other methods, such as 18S rRNA and their barcode coverage gaps in the reference library, 16S rRNA detected double less phytoplankton taxa in comparison to 18S rRNA (Tzafesta et al., 2022). When using eDNA metabarcoding, not only the method but also the type of habitat should be taken into account, because cyanobacteria are being more associated with eutrophic and hypereutrophic habitats (Almanza et al., 2018), therefore do not represent a significant gap if not properly detected in less productive habitats. Since four of the six lakes included in our study are predominantly oligotrophic and mesotrophic and eukaryotic algae dominate, the hypervariable V9 region of the 18S rRNA, which does not include cyanobacteria, was used according to this principle.

The results of this study showed clear separation of lakes based on cluster analysis of Bray–Curtis similarity of phytoplankton taxa biomass (microscopy) and number of sequences (eDNA metabarcoding) suggesting that eDNA metabarcoding is method sensitive to species change. This also suggests that the method has potential to identify different phytoplankton communities based on lakes trophic status consistent with the study by Eiler et al. (2013), as studied lakes differ in productivity (National Gazette, 20/23).

Traditional microscopy in phytoplankton analysis primarily requires high taxonomic expertise and is time-consuming in sample processing, which, among other things, directly affects the number of phytoplankton samples examined for WFD assessment. In contrast, eDNA metabarcoding could be a promising alternative in phytoplankton identification (Kim et al., 2019; Malashenkov et al., 2021), which is also shown in this study, as 40% more taxa were identified with eDNA metabarcoding than with microscopy.

In the study by Malashenkov et al. (2021), most overlapping taxa belonging to the taxonomic groups Bacillariophyta, Miozoa, Ochrophyta and Cryptophyta were identified between microscopy and eDNA metabarcoding using the 18S rRNA gene marker. Using the 18S rRNA gene, Bacillariophyta, Miozoa and Cryptophyta were also among the most represented groups identified by Muhammad et al. (2021). The 18S rRNA gene used in our study led to the identification of most taxa by both microscopy and eDNA metabarcoding in oligotrophic Lake Kozjak, deep Lake Vransko and mesotrophic lakes Prošće and Visovac. In the mentioned lakes, phytoplankton

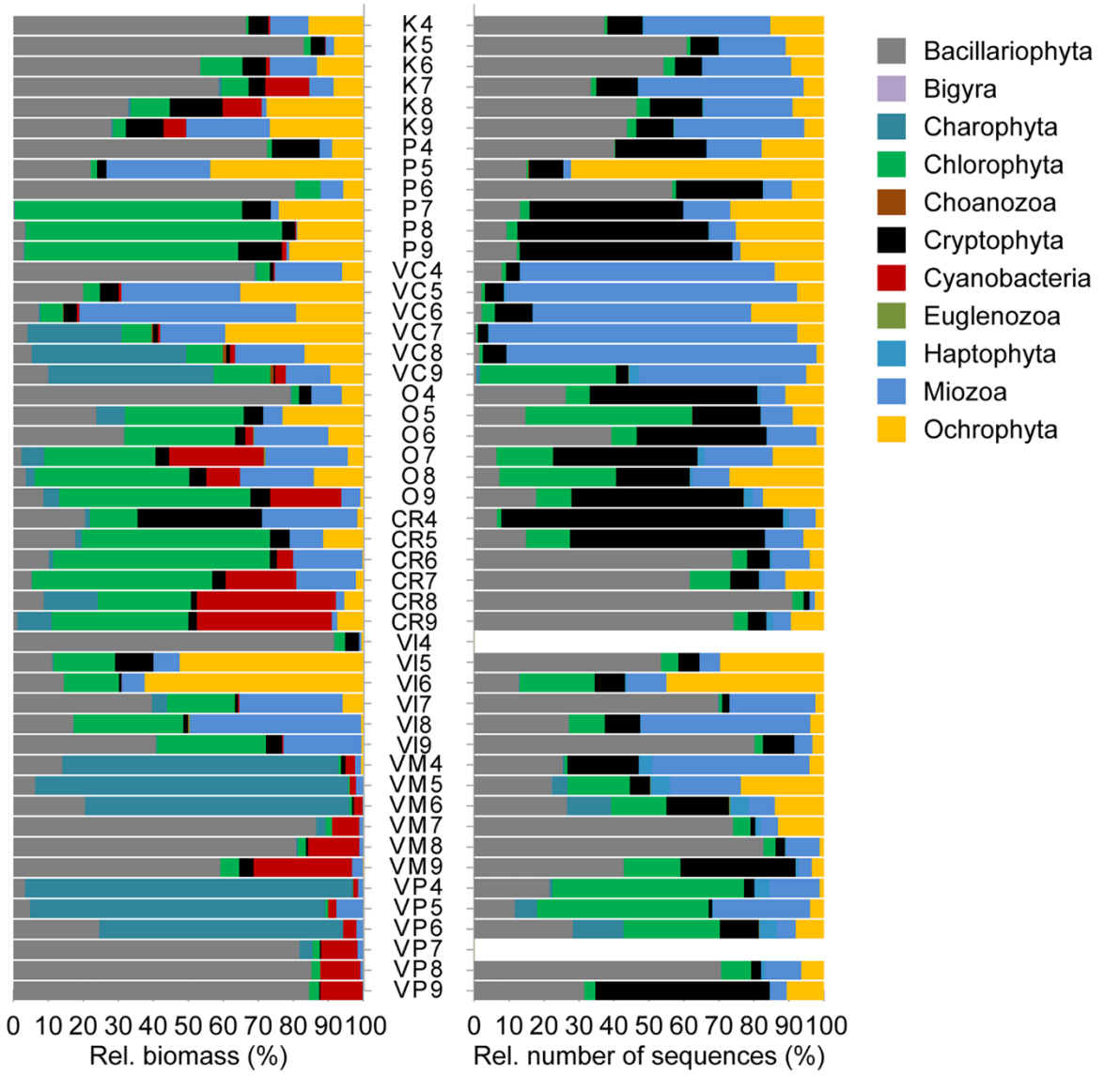


Fig. 8 Relative biomass (left) and relative number of sequences (right) of taxonomic groups of phytoplankton identified by microscopy and eDNA metabarcoding in natural karst lakes. Cyanobacteria are not included in the left panel, as this group cannot be detected with the target genes used (18S) (i.e. there is nothing to compare). The codes on the y-axis represent the locations (VC—deep Lake Vransko, K—Lake Kozjak, P—Lake Prošće, VM—shallow Lake Vransko, Motel sampling site, VP—shallow Lake Vransko, Prosika sampling site, VI—Visovac Lake, CR—Crniševo Lake, O—Oćuša Lake) and the months of sampling from April to September

communities were dominated by taxa belonging to Bacillariophyta, Miozoa, Ochrophyta and Cryptophyta, presenting 18 s rRNA as a potentially reliable gene marker for the detection of the mentioned groups.

Some very common taxa such as *Asterionella formosa* Hassall, *Pantocsekiella comensis*, *Synedropsis roundii*, *Cosmarium tenue*, *Elakatothrix gelatinosa* Wille, *Oocystis lacustris* Chodat, *Oocystis marssonii* Lemmermann, *Oocystis parva* West & G.S.West, *Radiococcus planctonicus*, *Sphaerocystis schroeteri*, *Sphaerocystis planctonica*, *Actinotaenium/Mesotaenium* etc. identified by microscopy were not detected by eDNA metabarcoding, possibly due to

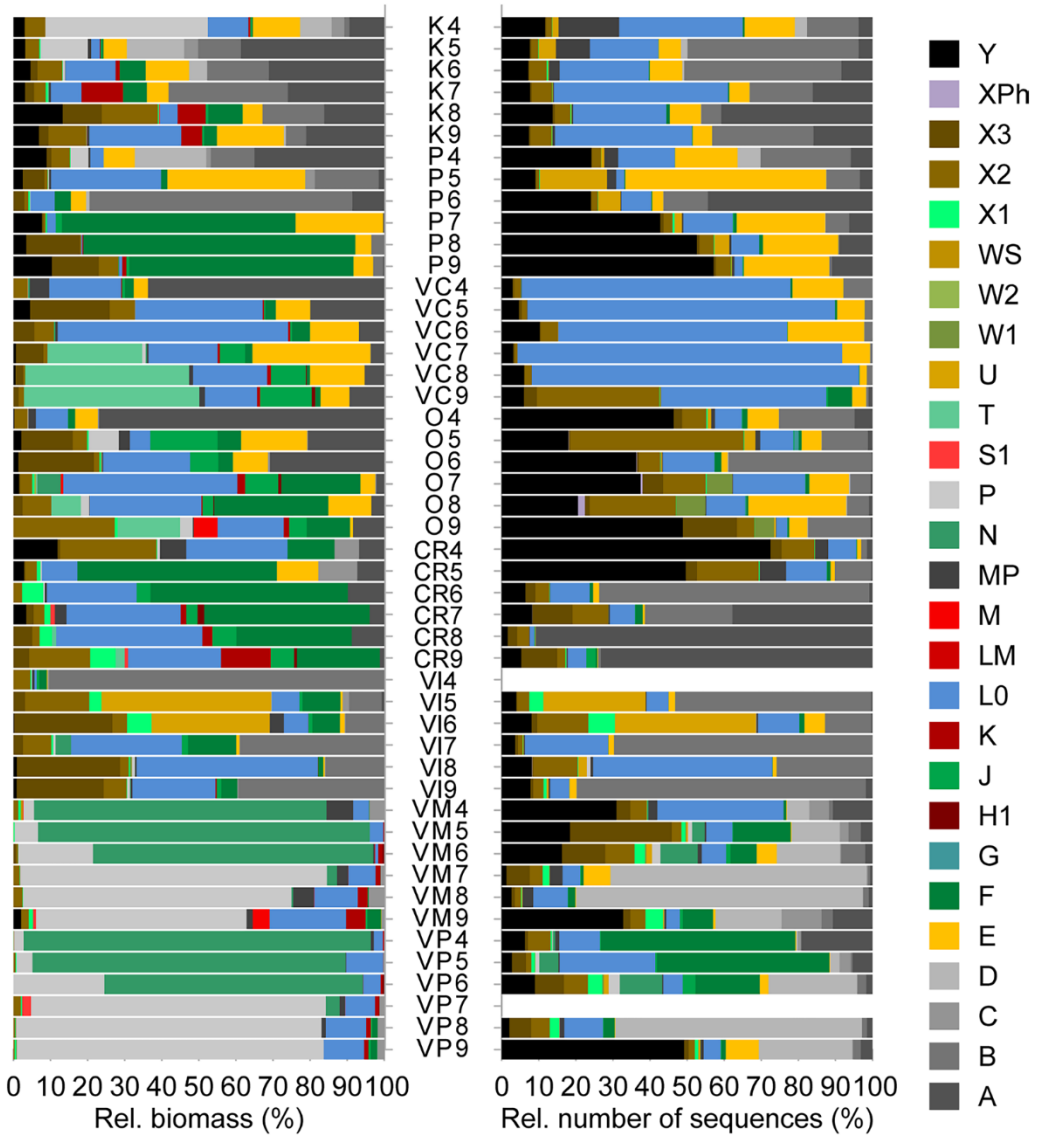


Fig. 9 Relative biomass (left) and relative number of sequences (right) of functional groups of phytoplankton identified by microscopy and eDNA metabarcoding in natural karst lakes. The codes on the y-axis represent the locations (VC—deep Lake Vransko, K—Lake Kozjak, P—Lake Prošće, VM—shallow Lake Vransko, Motel sampling site, VP—shallow Lake Vransko, Prosika sampling site, VI—Visovac Lake, CR—Crniševo Lake, O—Oćuša Lake) and the months of sampling from April to September

incompleteness and errors in databases of reference DNA sequences for identified taxa (Groendahl et al., 2017; Santi et al., 2021; Tzafesta et al., 2022). In order to have the possibility to use eDNA metabarcoding for monitoring purposes, the database urgently needs to be supplemented and completed, as suggested by Tzafesta et al. (2022). One of the shortcomings that may affect the comparison between methods is the short V9 region of the 18S rRNA gene, which provides limited phylogenetic information. On the other hand, the longer V4 region of the 18S rRNA gene provides better phylogenetic/taxonomic resolution, often down to species or genus level (Geisen et al., 2019). For future studies, the combination of the V4 and V9 regions of the 18S rRNA gene should be used in the analysis of metabarcoding in eukaryotic

phytoplankton, as they complement each other (Choi & Park, 2020). Another limitation could be the uncertainties related to amplification and Next-generation sequencing (Kim et al., 2019), which could explain the lack of detection of the dominant species *P. costei* in deep Lake Vransko, while it was detected in the lakes Kozjak and Prošće (Gligora Udovič et al., 2017). The results also suggest that the lower overlap of Bacillariophyta taxa between the two methods is due to the high percentage of species that could be identified by microscopy (*A. formosa, P. comensis, S. roundii*), whereas eDNA metabarcoding was not able to detect all of them. The use of rbcL markers in karst lakes, where planktonic diatoms often dominate, could be useful and allow a more confident taxonomic resolution of diatoms in the future. Another option would be a comparison at a higher taxonomic level, possibly contributing to a greater overlap between the methods (Groendahl et al., 2017; Weigand et al., 2019; Malashenkov et al., 2021; Santi et al., 2021). Salmaso et al. (2022) point out the strong impact of sampling effort on sequence coverage, implying that rare phytoplankton species have a lower chance of being sampled and analysed, so the sequence gap in the reference database might be larger in less studied geographical areas. In our study, the slightly brackish, shallow Lake Vransko and Lake Crniševo had the lowest number of overlapping taxa. The specificity of karst lakes, especially the slightly brackish shallow Lake Vransko, and their characteristic species such as *S. roundii* (Blanco et al., 2019) could be the main reason for the weaker overlap of taxa, as there are no sequenced taxa in the currently available databases (Weigand et al., 2019). A larger number of cyanobacterial taxa not covered by the V9 region of 18S rRNA also influenced the weakest overlap in shallow Lake Vransko and Lake Crniševo.

As the understanding of the relationship between cell number/biomass and DNA reads (number of sequences) is not yet fully understood, the use of eDNA metabarcoding to quantify phytoplankton, which is crucial for assessing ecological status, still raises questions (Pérez Burillo et al., 2020; Santi et al., 2021). The number of sequences per cell affects estimates of abundance of certain species for WFD assessment, which may be under- or over-represented due to cell size, chloroplast size or number of chloroplasts per cell (Vasselon et al., 2017; Kelly et al., 2018; Pérez Burillo et al., 2020; Santi et al., 2021). In our study, representatives of Miozoa and Cryptophyta were potentially overrepresented in eDNA metabarcoding. This can be explained by the microscopic identification of taxa with a larger biovolume (*C. hirundinella*, *Gymnodinium* spp., *G. helveticum*, *Cryptomonas platyuris* Skuja, *Cryptomonas* spp.), which potentially yield a larger number of sequences due to the size of their cells and chloroplasts, leading to weak comparability (Piredda et al., 2017; Pérez Burillo et al., 2020). The Bacillariophyta representative *P. ocellata* was only identified in Lake Visovac by microscopy, while *P. ocellata* was detected by eDNA metabarcoding in all lakes studied. In contrast, the dominant species *P. comensis* (Lake Oćuša and Lake Crniševo) and *S. roundii* (shallow Lake Vransko) were detected by microscopy and not by eDNA metabarcoding. Thus, our results suggest that the main disadvantages of eDNA metabarcoding may lie in the specific markers used in amplification of reads, primer bias and bioinformatics pipeline (Salmaso et al., 2022).

Despite the weak overlap of taxa in general between the two methods (microscopy and eDNA metabarcoding), taxa from the groups Bacillariophyta, Miozoa and Ochrophyta were identified by eDNA metabarcoding in our study in low productive lakes (lakes Kozjak, Prošće, deep Lake Vransko and Visovac) to a high proportion, as they are naturally the most diverse and abundant group in these lakes (Gligora Udovič et al., 2017). In these lakes, overlapping taxa had similar proportions of biomass and number of sequences in both methods, which could be due to the overrepresented taxa replacing the number of sequences of the underestimated taxa and being involved in the final similar proportion in both methods (Santi et al., 2021). The development and application of correction factors based on biovolumes (Vasselon et al., 2017; Mortágua et al., 2019) and the addition of the NCBI reference database would potentially provide more reliable results, considering the unexplored use of eDNA metabarcoding in specific karst lakes and lakes in general.

The results of this study also showed a different composition and proportion of FGs between microscopy and eDNA metabarcoding due to differences in taxonomic groups and their representatives. In the study by Hanžek et al. (2021), comparable assessments of ecological status were presented despite the non-overlapping compositions and proportions of

FGs determined by microscopy and eDNA metabarcoding. In this case, the factor weight assigned to FGs with similar habitat requirements played an important role in the final assessment, although FGs differed between microscopy and eDNA metabarcoding. Considering the differences in our study, it is primarily necessary to strive for accurate identification of phytoplankton species by eDNA metabarcoding in order to obtain a reliable assessment.

Conclusions

The phytoplankton communities of the studied karst lakes identified by microscopy and eDNA metabarcoding showed a weak overlap of taxa for the 18S rRNA V9 gene, possibly due to the shorter sequence reads, uncertainties related to amplification and Next-generation sequencing and the presence of gaps in the taxonomic completeness of the NCBI GenBank taxonomic reference database. Although eDNA metabarcoding is widely used in aquatic samples and has great potential for biodiversity monitoring and ecological studies, the coverage of taxonomic reference databases for phytoplankton needs to be increased. Currently, errors and incompleteness in reference databases are among the major drawbacks of eDNA metabarcoding, along with quantification by number of sequences rather than biomass. This led to differences in the proportions and compositions of FGs, which put eDNA metabarcoding on hold as a reliable tool for assessing the ecological status of Croatian karst lakes, but at the same time opened up new research opportunities. In order to bring the results of eDNA metabarcoding closer to the assessment of ecological status, further efforts are needed to solve the problem of quantification and to complement the reference databases, focusing on species in specific habitats.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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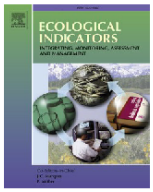

Assessing ecological status in karstic lakes through the integration of phytoplankton functional groups, morphological approach and environmental DNA metabarcoding

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ABSTRACT

Phytoplankton is one of the key Biological Quality Elements within the Water Framework Directive, used to assess the ecological status of surface water bodies. Water samples for phytoplankton identification were collected from April to September at a total of eight sampling sites in all six Croatian natural karstic lakes with an area greater than 0.5 km^2. The main objective was to show the comparability of environmental DNA metabarcoding (Illumina sequencing using the hypervariable V9 region of the eukaryotic SSU rRNA gene) with morphologically based assessment and its applicability in assessing the ecological status of lakes. The value of Hungarian lake phytoplankton index (HLPI) indicating the final ecological status was calculated for both datasets using biomass and composition metrics. Chlorophyll *a* concentration measured using Ultra-High-Performance Liquid Chromatography and Spectrophotometer giving two biomass metrics along with the functional group approach (FG) as the composition metric for the complete taxa/operational taxonomic units (OTUs) lists as well as for the taxa/OTUs that contributed more than 5% to the total biomass/number of amplicons gave four to four HLPI values per sample. HLPI values from both approaches were highly correlated (Pearson's $r > 0.92$) and classified into good or high ecological status, although different compositions and proportions of FGs were recorded, thus giving the important role to the equal or similar factors assignment to different FGs with similar ecological demands and favourable habitats. In 89% of the samples, HLPI values indicate an equal range of ecological status and most differences were found due to different methods of Chlorophyll *a* measurement. Different composition metrics between approaches showed significant differences ($p < 0.05$) only in lakes Prošće and Crniševo. This study showed the applicability of the V9 region of 18S rRNA in ecological status assessment for oligotrophic and mesotrophic lakes due to the comparable results between approaches, but further development and standardization of eDNA metabarcoding are needed for the implementation in routine monitoring programs.

1. Introduction

A large proportion (60%) of European surface water bodies fail to reach good ecological status. The main impacts on freshwater bodies arise from nutrient enrichment, chemical pollution and hydromorphological alterations (EEA, 2018). Nutrient enrichment results in eutrophication, which impairs ecosystem function and services, leading to a decline in aquatic biodiversity and a decline in fish stocks (Alexander et al., 2017). It also enhances plant growth and toxic algal blooms, both of which may cause oxygen depletion and loss of life in the bottom layer of water (Misra and Chaturvedi, 2016; Scholz et al., 2017). Chemical pollution of aquatic habitats threatens aquatic flora and fauna

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and affects the quality of human life by compromising drinking water quality or the use of rivers and lakes for recreational purposes (Schmeller et al., 2017). Hydromorphological changes to rivers and lakes often alter natural flow levels and sediment dynamics, which results in the loss of aquatic habitats (Poikane et al., 2019). Therefore, national and international regulations, such as the European Water Framework Directive (WFD, 2000) have been implemented in the EU Member States to monitor the ecological quality status of freshwater bodies and to maintain and protect the quality of surface waters.

Ecosystem dynamics that involve interactions between chemical, physical and biological processes have been well studied in lakes (Bhateria and Jain, 2016). In the WFD, Biological Quality Elements (BQEs), including phytoplankton, macrophytes, phytobenthos, benthic invertebrate fauna and fish in concerto with supporting physical, chemical and hydromorphological quality elements are crucial elements for assessing the ecological status of surface waterbodies.

Because physical and chemical parameters can change rapidly and their measurements often provide only short-term information on water quality, the biological component is the most informative backbone of lake monitoring. Biological communities respond to environmental changes over time, providing a more reliable and time-integrated ecological quality assessment (Lyche-Solheim et al., 2013).

BQEs serve as bioindicators of the abiotic and biotic state of the environment in the accumulation of toxic substances or the response to environmental stress. Bioindication requires standardized processes, including field sampling, sample processing, and identification of collected organisms (Birk et al., 2012). The ecological status of surface waterbodies is assessed by national assessment methods developed individually by EU Member States according to standards defined in the WFD (e.g. abundance, community composition). In order to bridge the methodological discrepancies, the European Commission organized a series of intercalibration exercises to ensure comparability of ecological status boundaries and national assessment methods between EU Member States. The results of the completed intercalibration indicated it to be a valid approach for comparison and harmonization of national assessment systems (Poikane et al., 2014).

Traditional biological monitoring methods that rely on microscopic identification of BQEs can lead to inaccurate assessments and biased results due to misidentification, low comparability, and limited taxonomic resolution (Elbrecht et al., 2017, Huo et al., 2020). Microscopy-based approaches require taxonomic expertise for accurate identification of taxa on which biotic metrics and indices are based. In addition, microscopic identification of individual taxa included in a monitoring sample is time-consuming, making monitoring of freshwater habitats a very expensive task and limiting monitoring to low spatial and temporal scales (Elbrecht et al., 2017). This is unsatisfying because anthropogenic and climate stress on surface waters is increasing and so is the demand for future monitoring program (Herrero et al., 2018). A more cost- and time-efficient alternative with high reproducibility could be environmental eDNA metabarcoding, a technology that has the potential to fundamentally change traditional biological assessments of environmental quality (Hering et al., 2018; Pawlowski et al., 2018). However, currently this molecular technology is still in development and presents a significant challenge as it needs to be standardized before implementation in routine monitoring programs (Hering et al., 2018).

A recent review indicated a relatively good correlation (on average, 70–80% congruence) between conventional (microscopy) and molecular indices obtained from the same macroinvertebrate communities across several studies (Pawlowski et al., 2018). Even more significant progress was obtained for a morpho-genetic comparison of benthic diatom communities (Apothéloz-Perret-Gentil et al., 2017). In addition, eDNA metabarcoding has shown promise as a tool for freshwater fish monitoring; eDNA metabarcoding has been used to detect higher numbers of species through a non-invasive sampling method with significantly less sampling effort compared to traditional morphology-based approaches (Pont et al., 2018). Current limitations of metabarcoding include the definition of the population structure and size, identification to species level, and shortcomings with databases (Valentini et al., 2015), but see Cordier et al. (2018) for taxonomy-free approaches. Difficulties have also been reported for the diagnosis of macrophytes and macroalgae using DNA-based methods (Hering et al., 2018).

According to the WFD, phytoplankton is a BQE of great importance for monitoring lakes and very large rivers. Quality assessment based on phytoplankton communities relies on taxonomic composition, abundance, biomass, and frequency and intensity of algal blooms (EC, 2011). Accordingly, phytoplankton-based indices have been developed for the estimation of the ecological status of water bodies. Such indices take into account the biomass, abundance and species composition of communities, e.g., the Phyto-See-Index (Mischke et al., 2008) and the Indice Phytoplankton Lacustre (Laplace-Treyture and Feret, 2016). Padisák et al. (2006) developed the Q assemblage index for Hungarian lakes based on the functional group (FG) concept (Reynolds et al., 2002). The index takes into account shares of FGs in the total biomass multiplied by a factor number (F) defined for each FG. The most important part of the assessment is the determination of the factor numbers (F), since they reflect the values of FG in the reference condition for a given lake type. The sufficiently solid theoretical basis of the Q assemblage index allows its application as an assessment tool for ecological status without geographical limitations (Padisák et al., 2006). Although the above methods were developed for data derived from traditional microscopic investigation of samples, phytoplankton data provided by eDNA metabarcoding could potentially be used for these purposes as well.

To date, however, few studies have compared eDNA metabarcoding datasets with morphology-based data for freshwater phytoplankton communities. The scarcely available information reports a low congruence of the spatiotemporal dynamics of phytoplankton inferred from microscopy data and metabarcoding data (Abad et al., 2016). This was explained by a lack of representative sequences in the current database for the targeted 18S rRNA gene, which could be potentially overridden by adding representative sequences of local species. A major challenge for phytoplankton community analyses using eDNA metabarcoding as a BQE is the choice of the most informative taxonomic gene marker. Eiler et al. (2013) proposed the 16S rRNA gene because of its presence in prokaryotes (including cyanobacteria) and the eukaryotic chloroplast. Thus, this gene would allow cross-domain analyses of phytoplankton. However, chloroplasts do not reflect cell size, and the number of chloroplasts varies per cell, which could explain the observed weak correspondence between the eDNA metabarcoding data and the microscopic biovolume estimation. The 18S rRNA gene as a taxonomic eDNA marker provided better phylogenetic resolution (Joo et al., 2010). Nevertheless, this marker is unable to detect Cyanobacteria as an important algal component of freshwater habitats. Regardless of the shortcomings reported from the few eDNA metabarcoding studies, Eiler et al. (2013) were able to discriminate lakes of different trophic status based on eDNA metabarcoding profiles of freshwater phytoplankton communities, thus indicating eDNA metabarcoding as a promising tool for water quality status assessments.

To further develop eDNA metabarcoding as a tool for future lake monitoring, the results of a comparative study are given where assessment results of the traditional microscopy-based method and that of the eDNA metabarcoding approach have been presented.

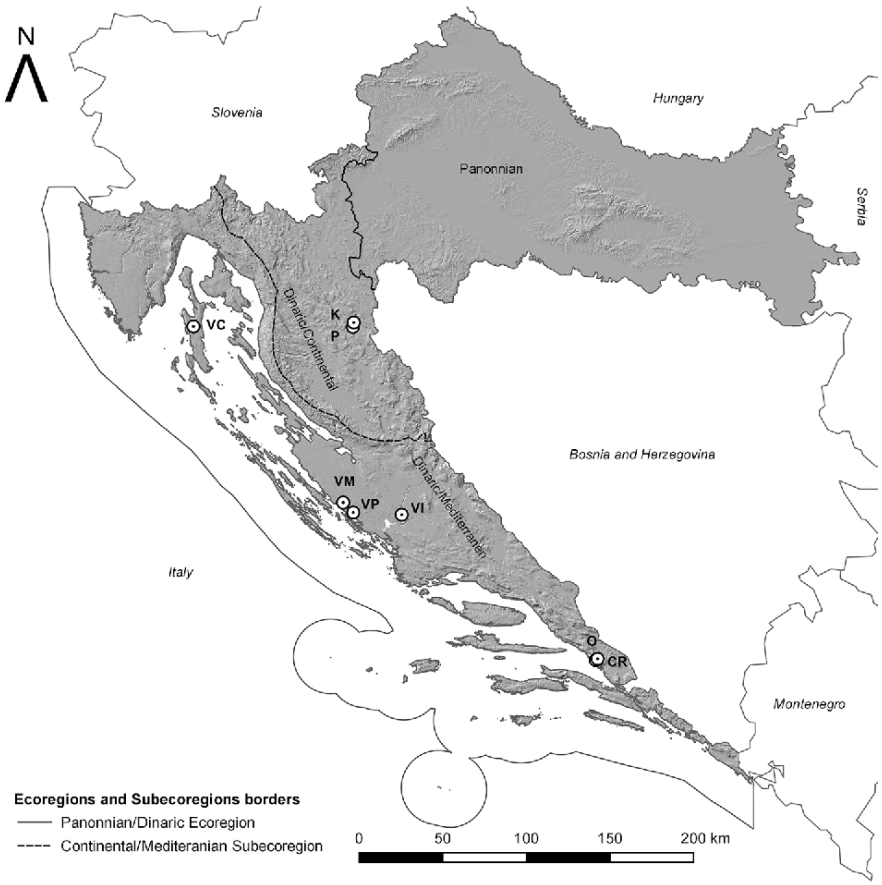

Fig. 1. Map of investigated lakes. Site codes are explained in Table 1.

2. Material and methods

2.1. Study site

Croatia is divided into two Ecoregions: Pannonian and Dinaric. Croatian natural lakes with an area greater than 0.5 km^2 are all karstic lakes located in the Dinaric Ecoregion (Fig. 1) and their detailed characteristics are given in Table 1. The origin of the Plitvice Lakes is complex due to the combination of tectonic movements and the formation of travertine barriers that contributed to the formation of 16 barrage lakes, out of which Lakes Kozjak and Prošće are the biggest (Markowska, 2004). Travertine barriers are also one of the fundamental features which lead to the formation of the karstic barrage Lake Visovac, a lentic dilatation on the Krka River (Gligora Udovič et al., 2016). Other lakes are cryptodepressions. Except for the shallow polymictic Lake Vransko (Vransko Lake Nature Park, further mentioned as shallow Vransko; sampling sites Motel and Prosika), all lakes are deep. The lake with the greatest depth is Lake Vransko on the Island of Cres (further mentioned as deep Vransko). Lakes Kozjak and Prošće are dimictic due to the influence of the continental climate, while all other lakes are monomictic influenced by the Mediterranean climate. Besides its shallow profile, the shallow Lake Vransko differs from the other lakes by being strongly influenced by the Adriatic Sea through underground and surface connections. Due to underground brackish springs, Lake Crniševo has slightly brackish water (Bonacci, 1984).

2.2. Description of the classical microscopic methods

Water samples were collected at the deepest part of each lake once a month from April to September by taking samples from the euphotic

Table 1
Location and physical properties of the investigated lakes: VC – deep Lake Vransko, K – Lake Kozjak, P – Lake Prošće, VM – shallow Lake Vransko, sampling site Motel, VP – shallow Lake Vransko, sampling site Prosika, VI – Lake Visovac, CR – Lake Crniševo, O – Lake Oćuša.

Lake (abbrv. on the map)		Plitvice Lakes		Lake Vransko			Baćina Lakes	
	Vransko (VC)	Kozjak (K)	Prošće (P)	Motel (VM)	Prosika (VP)	Visovac (VI)	Crniševo (CR)	Oćuša (O)
Surface area (km^2)	5.75	0.82	0.68	30.2		5.72	0.43	0.55
Volume (m^3)	220.3×10^6	12.7×10^6	7.7×10^6	141.6×10^6		103×10^6	7×10^6	7.3×10^6
Max depth (m)	74.5	47	38	4.7		30	34	19.6
Longitude (WGS84)	14.39° E	15.61° E	15.60° E	15.55° E	15.62° E	15.98° E	17.41° E	17.42° E
Latitude (WGS84)	45.86° N	44.89° N	44.87° N	43.93° N	43.86° N	43.86° N	43.07° N	43.08° N
Elevation (a.s.l.) (m)	9	535	636	0.1		47	0.8	
Ecoregion/Subecoregion	Dinaric/Mediterranean	Dinaric/Continental		Dinaric/Mediterranean		Dinaric/Mediterranean	Dinaric/Mediterranean	

zone at intervals of one or two meters (CEN - EN, 2015a) using the Uwitec water sampler (Uwitec, Austria). Samples were stored in 250 ml bottles and preserved with Lugol's solution. According to the Utermöhl (1958) method, phytoplankton was counted using the inverted microscopes (Zeiss Axio Observer Z1, Olympus IX 51) at 400×, 200× and 100× magnification (CEN - EN 15204, 2006). Sedimentation units (unicell, coenobium, filament, or colony) were counted until reaching at least 400 specimens in random counting fields (CEN - EN 15204, 2006; Lund et al., 1958). Individual cells were measured and after approximation to regular geometrical form (CEN - EN, 2015b) the biovolume of each measured cell was calculated. By multiplying the population size of each taxon by the median volume of its cells, the biovolume was calculated and converted to biomass, assuming the density of the cells to be 1 g ml ^{1} (CEN – EN 16695, 2015; Rott, 1981). Permanent slides for diatom identification were made by cleaning the net samples using warm hydrochloric acid and hydrogen peroxide and mounted in the Naphrax solution (CEN - EN 15708, 2009). The diatoms were identified at a magnification of 1000× under the microscope equipped with DIC. Current identification literature was used for taxa identification and names were assigned according to Algaebase (Guiry and Guiry, 2021).

2.3. Microeukaryotic phytoplankton characterization

Integrated epilimnion samples were filtered with a peristaltic pump on polycarbonate membrane filters (type GTTP; Whatman, UK) with 0.2 μm pore size. The filters were immediately stored on dry ice and transferred to −80 °C until further processing.

According to the manufacturer's guidelines, total genomic DNA was extracted with the DNeasy PowerWater kit (Qiagen GmbH Hilden, Germany). The DNA concentration and purity were measured spectrophotometrically using a NanoDrop (ND 2000, Thermo Scientific, Wilmington, DE, USA). The hypervariable V9 region (about 150 bp long) of the eukaryotic SSU rRNA gene was amplified using the primer pair 1391F (5′-GTACACACCGCCCGTC-3′) and EukB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) following the protocol of Stoeck et al. (2010). To minimize PCR bias, three individual reactions per sample were prepared. Samples were further processed and sequenced on Illumina NextSeq by the SeqIT GmbH & Co. KG (Kaiserslautern, Germany). The sequences generated for this study were deposited in the European Nucleotide Archive under project number PRJEB44080.

Quality trimming of paired-end reads was done using the bbduk function and merged using bbmerge function of the BBMap package (Bushnell, 2014). The merged reads were quality-filtered again using QIIME v1.8.0 (Caporaso et al., 2010). Reads with the exact barcodes and primers, unambiguous nucleotides, and a minimum length of 90 base pairs were retained. Chimera filtering was done by using UCHIME (Edgar et al., 2011). Non-chimeric reads were clustered using SWARM v3.0.0 (Mahé et al., 2015) with default settings into Operational Taxonomic Units (OTUs). The microeukaryotic reads were blasted against the NCBI's nucleotide reference database (NCBI-GenBank Flat File Release 220.0) using blastn (BLAST v2.2.30). Nontarget OTUs such as metazoans, embryophytes, ciliates, etc., as well as singletons and doubletons, were excluded. Only OTUs affiliated to the phytoplankton community on the family level were filtered by the quality of the blast result (≥98 % identity) and used in further analysis.

2.4. Determination of Chlorophyll a (Chl a) concentration

The spectrophotometric determination of the Chl *a* concentration was performed according to the international standard HRN ISO 10260 (2001). Water was filtered through Whatman GF/F glass filters, these were extracted in 96% ethanol and measured using a UV–VIS spectrophotometer (Perkin Elmer Lambda 25).

Ultra-High-Performance Liquid Chromatography (UHPLC) was used as a second method for Chl *a* analysis. Water filtration for pigment analysis was performed with Whatman GF/F glass filters which were immediately frozen and stored at −80 °C. Pigments were extracted using the mixture of acetone/methanol (7:2 v/v). Samples were sonicated in a cold-water bath for 3 min and centrifuged at 12 000 rpm for 3 min. The volume of 1 ml of supernatant was transferred into the dark cuvette and analyzed using Shimadzu Prominence LC – 2030C 3D I series plus with UV–VIS detector. Chromatographic separation of pigments was achieved using the modified method proposed by Pinckney et al. (2011) on 40 °C heated Phenomenex Luna 3μ C8(2) 100 Å column with binary solvent 0 min 100% A, 20 min 100% B, 25 min 100% B, 27 min 100% A, 30 min 100% A; A: 80% methanol + 28 mM ammonium acetate, B: methanol). The flow rate was 0.8 ml min ^{1}. Identification and quantification of the peaks were based on the absorbance spectra. Chl *a* was detected at 665 nm and 770 nm. Calibration of HPLC peaks was performed using commercial standards DHI Lab Products (Denmark) (Higgins et al., 2011).

2.5. Assignment of taxa and OTUs identified by a morphological approach and eDNA metabarcoding into the appropriate functional groups

To assess the ecological status of Croatian lakes two metrics, based on biomass and composition, were calculated. Chl *a* concentration is used as a biomass metric. Measured Chl *a* values were converted into the normalized scale with equal class widths and standardized class boundaries using the 3rd order polynomial regression equations Eqs. (1)–(4) (Gligora Udovič and Žutinić, 2020).

- Lakes: deep Vransko, Kozjak, Prošće, Oćuša, Crniševo, Visovac

$$\text{If Chla} < 5.3\,\mu\text{g L}^{-1};\ \text{EQR}_{\text{Chla}} = 0.0074x^2 - 0.1149x + 1 \quad (1)$$

$$\text{If Chla} > 5.3\,\mu\text{g L}^{-1};\ \text{EQR}_{\text{Chla}} = 0.00005x^2 - 0.0118x + 0.6617 \quad (2)$$

- Shallow Lake Vransko (sampling sites Motel and Prosika)

$$\text{If Chla} < 50\,\mu\text{g L}^{-1};\ \text{EQR}_{\text{Chla}} = -0.0161x + 0.9826 \quad (3)$$

$$\text{If Chla} > 50\,\mu\text{g L}^{-1};\ \text{EQR}_{\text{Chla}} = -0.004x + 0.4 \quad (4)$$

The composition metric is based on the functional group approach proposed by Padisák et al. (2006). This approach requires assigning species to the appropriate phytoplankton functional groups (FGs) based on the species autecology and habitat preferences (Padisák et al., 2009; Reynolds et al., 2002). After taxa and OTUs identified by morphological approach and eDNA metabarcoding were classified into FGs, factor numbers (F) were assigned to each (Table 2).

Table 2
Coda of the functional groups (FGs), and the proposed factor numbers (F).

FG	S1	S2	SN	XPh	H1	G	J	M	C	P	T	X1	LM	W1	W2	Q
F	1	1	1	1	1	3	3	3	5	5	5	5	5	5	5	5
FG	D	Y	E	K	L_O	WS	MP	A	B	N	Z	X3	X2	F	U	V
F	7	7	7	7	7	7	7	9	9	9	9	9	9	9	9	9

The value of the composition metric Q_k (Padisák et al., 2006) was calculated according to Eq. (5).

$$Q_k = \sum_{i\ 1}^{s} (p_i F) \quad (5)$$

where:

- p_i: is the relative contribution of the ith assemblage to the total biomass,
- F: is a factor number that evaluates the given functional group in the given lake type.

The calculated Q_k values of each phytoplankton sample are divided with the maximum value of the index (9) for the Q_k values standardization using Eq. (6).

$$Q_k\_stand = Q_k/9 \quad (6)$$

Eqs. (7)–(12) were used as type-specific 3rd order polynomial regression equations for composition metric (Q_k_stand) conversion into the normalized scale with equal widths and standardized class boundaries (Gligora Udovič and Žutinić, 2020). Those values are considered normalized EQR_Q values. Polynomial regression equations for composition metric (Q_k_stand) conversion to EQR_Q values for Croatian lakes (x: value of Qk_norm) are as follows:

$$\text{Deep Vransko}: y = -2e^{-13}x^3 - 8e^{-14}x^2 + 0.9302x - 2e^{-14} \quad (7)$$

$$\text{Kozjak}: y = 7e^{-13}x^3 - 9e^{-13}x^2 + 0.8696x - 2e^{-14} \quad (8)$$

$$\text{Prošće}: y = 0.8989x - 4e^{-15} \quad (9)$$

$$\text{Visovac}: y = -2e^{-13}x^3 - 9e^{-14}x^2 + 0.9756x - 8e^{-14} \quad (10)$$

$$\text{Shallow Vransko}: y = 7e^{-13}x^3 - 9e^{-13}x^2 + 0.9877x - 6e^{-14} \quad (11)$$

$$\text{Očuša and Crniševo}: y = 7e^{-13}x^2 + 0.9195x - 8e^{-15} \quad (12)$$

- Deep Vransko: $y = -2e^{13}x^3 - 8e^{14}x^2 + 0.9302x - 2e^{14}$ (7)
- Kozjak: $y = 7e^{13}x^3 - 9e^{13}x^2 + 0.8696x - 2e^{14}$ (8)
- Prošće: $y = 0.8989x - 4e^{15}$ (9)
- Visovac: $y = -2e^{13}x^3 - 9e^{14}x^2 + 0.9756x - 8e^{14}$ (10)
- Shallow Vransko: $y = 7e^{13}x^3 - 9e^{-13}x^2 + 0{,}9877x - 6e^{14}$ (11)
- Očuša and Crniševo: $y = 7e^{13}x^2 + 0.9195x - 8e^{15}$ (12)

The Hungarian lake phytoplankton index (HLPI) composed of the combination of the two metrics as the weighted average of the EQR values was proposed by Borics et al. (2018). HLPI in Eq. (13) represents the final ecological state of the lake:

$$HLPI = EQR_Q + 2xEQR_{Chla}/3 \quad (13)$$

HLPI: Hungarian lake phytoplankton index
EQR_Q: normalized EQR of the composition metric
$EQR_{Chl\ a}$: normalized EQR of the biomass (Chl *a* metric)

The Q index considered for the calculation of HLPI has been computed both for data gained by morphological approach and eDNA metabarcoding. For both data sets, the index was calculated for the complete taxa/OTUs list as well as for the taxa contributing with more than 5% in the total biomass/number of amplicons, giving four different Q values and corresponding EQRs of the HLPI. In addition, two values of biomass metric were used (Chl *a* obtained spectrophotometrically and using UHPLC), which altogether resulted in eight values of the HLPI index. The abbreviations of the eight ways of HLPI calculations are as follows:

HLPI calculations when all taxa and OTUs are considered:
1. Morpho_HLPI_Chl*a*Spe 3. OTU_HLPI_ Chl*a*Spe
2. Morpho_HLPI_Chl*a*HPLC 4. OTU_HLPI_Chl*a*HPLC

HLPI calculations when only taxa/OTUs contributed more than 5% in total biomass/number of amplicons were considered:
5. Morpho_HLPI_5%_Chl*a*Spe 7. OTU_HLPI_5%_Chl*a*Spe
6. Morpho_HLPI_5%_Chl*a*UHPLC 8. OTU_HLPI_5%_Chl*a*UHPLC

Morpho: composition is given by microscopic investigations
OTU: composition is given by eDNA metabarcoding
Chl*a*Spe: Chl *a* concentrations were obtained spectrophotometrically
Chl*a*UHPLC: Chl *a* concentrations were obtained using UHPLC

2.6. Ecological status class assignment

The ecological status class was assigned by applying the class boundaries based on the national methodology (Gligora Udovič and Žutinić, 2020). Boundary settings for five classes (High/Good, Good/Moderate, Moderate/Poor and Poor/Bad) were set as an equidistant division of the EQR gradient at 0.8, 0.6, 0.4 and 0.2 (WFD, 2000).

2.7. Data analysis

In Primer 6 software (Clarke and Gorley, 2006), a one-way SIMPER analysis based on Bray-Curtis similarity was performed on phytoplankton composition obtained by the morphological approach and eDNA metabarcoding for identification of characteristic taxa/OTUs and FGs describing the phytoplankton community. Shannon-Wiener diversity index and species richness were calculated for data obtained by the morphological approach and eDNA metabarcoding as measures of alpha diversity using Primer 6 software. Phytoplankton and FGs biomass and OTUs number of amplicons were transformed using the logarithm function (log(X + 1)) before statistical analyses. Pearson's correlation coefficients of HLPI values between eDNA metabarcoding and morphological approach were calculated using IBM SPSS Statistics (IBM, 2017). After checking the normal distribution with the Shapiro-Wilk test (Shapiro and Wilk, 1965), differences in the HLPI values between eight different types of index calculations were evaluated by a paired *t*-test with IBM SPSS Statistics. The value of $p < 0.05$ was considered significant. Correlations of HLPI between morphological approach and eDNA metabarcoding as well as comparison of share (%) of FG obtained by both approaches were shown using Microsoft Office Excel 365. Mean values and standard deviation were plotted using Grapher™ (Golden Software, 2020).

3. Results

3.1. Morphological approach

A total of 217 phytoplankton taxa were identified based on the morphological approach. These taxa were classified into nine major groups (Phyla): Chlorophyta (65), Bacillariophyta (45), Cyanobacteria (44), Ochrophyta (32), Charophyta (10), Miozoa (10), Cryptophyta (7), Euglenozoa and Choanozoa. The mean values of species richness varied from 18 to 35 taxa in the lakes. The lowest mean species richness was obtained at the sampling site Motel, while the highest was in Lake Očuša. The Shannon-Wiener diversity varied between 1.35 and 2.14 with the lowest value in Lake Kozjak and the highest in deep Lake Vransko (Fig. 2). In total 63 taxa contributed to the total biomass with >5%. SIMPER analysis singled out 24 taxa representatives for natural karstic lakes in Croatia. The dominant taxa in the lakes are presented in Table 3. In Lake Kozjak, seven taxa had the greatest contribution to the total biomass, with *Pantocsekiella costei* as the dominant species. In Lake Prošće, six taxa contributed the most to biomass, while *Sphaerocystis schroeteri* was the dominant species. The highest biomass contribution in the deep Lake Vransko was attributed to seven taxa, with co-dominance of the dinoflagellate *Ceratium hirundinella*, the diatom *P. costei*, desmid from the genus *Actinotaenium/Mesotaenium* and chrysophyte taxa from the genus *Dinobryon*. In Lake Visovac, three species contributed most to

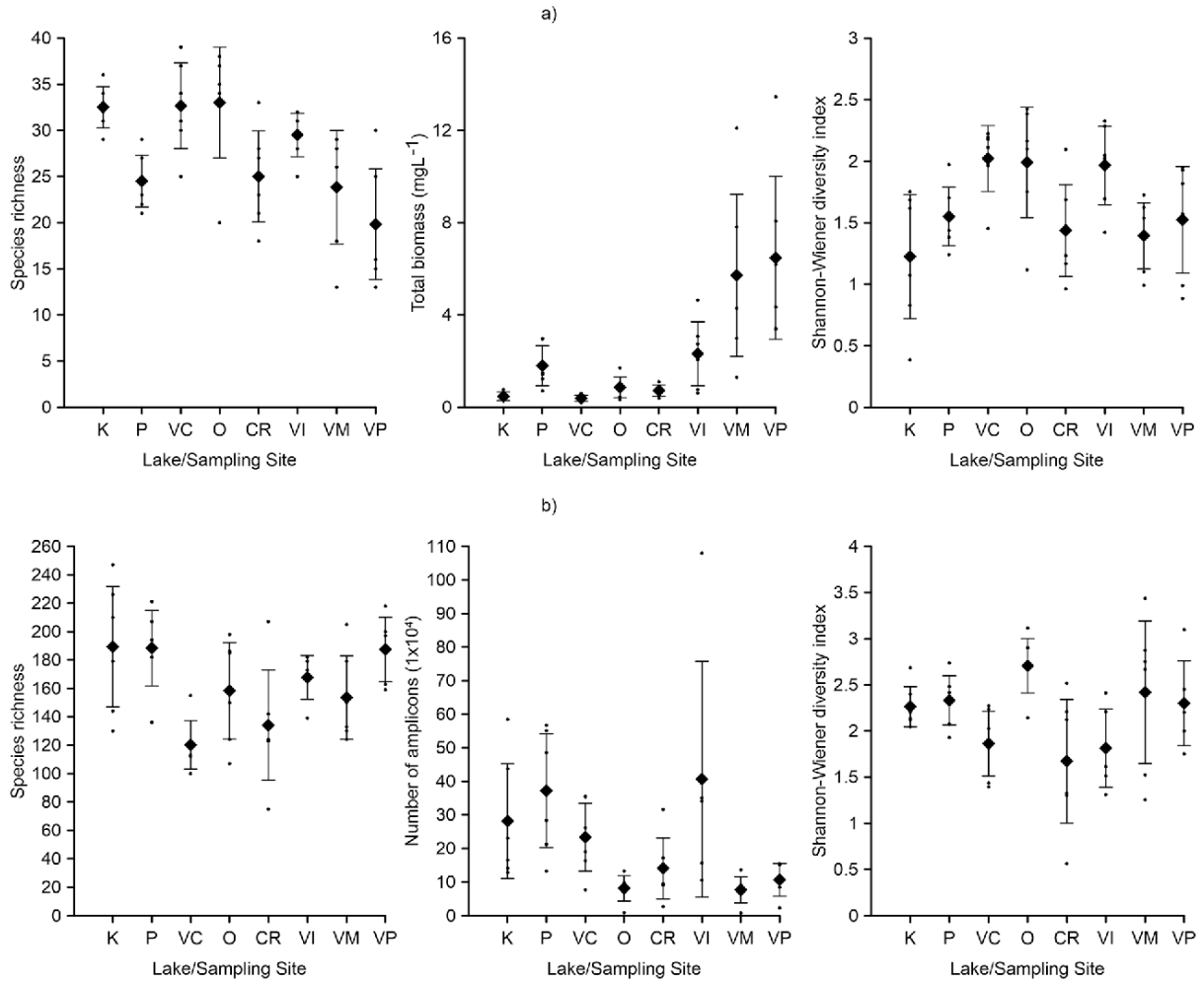

Fig. 2. Distribution of species richness, total biomass, number of amplicons, and Shannon-Wiener diversity index values provided by the morphological (a) and molecular (b) approaches. Rhomboids indicate the mean values. Vertical lines represent the upper and lower quartiles. Dots indicate values for each sample. Lake/Sampling Site codes are explained in Table 1.

the total biomass, with the domination of diatom *Pantocsekiella ocellata*. In Lake Crniševo, seven taxa contributed most to the biomass, while *C. hirundinella, Pantocsekiella comensis, Snowella atomus* and *Oocystis marssonii* co-dominated the assemblages. The dominant species that characterized the phytoplankton community in Lake Oćuša was *P. comensis*. Besides *P. comensis,* six additional taxa contributed most to the biomass. Shallow Lake Vransko was characterized by two dominant species, *Synedropsis roundii* and *Cosmarium tenue,* that had the largest share in biomass. According to the SIMPER analysis, the taxa with the greatest contributions to biomass were identical with those that typified the lakes. These taxa were also most responsible for distinctions between factor levels (Table 3).

3.2. Molecular approach

A total of 96,880,216 amplicons were obtained by Illumina sequencing on the 46 samples. After quality filtering steps, 9,508,838 amplicons were retained and clustered into 715 OTUs, taxonomically assigned to phytoplankton taxa. Of the 715 OTUs assigned to phytoplankton taxa, 484 OTUs were assigned to species level. The number of OTUs not classified to the species level was 231, of which 159 OTUs were classified at the genus level, while 72 OTUs fell into the higher classification categories. These OTUs were classified into 10 major groups (Phyla): Chlorophyta (219), Ochrophyta (145), Bacillariophyta (139), Miozoa (97), Cryptophyta (61), Euglenozoa (18), Charophyta (16), Haptophyta (10), Bigyra (7) and Choanozoa . Based on eDNA metabarcoding, species richness showed higher mean values ranging from a minimum of 116 in deep Lake Vransko to a maximum of 195 OTUs in Lake Kozjak. Shannon-Wiener diversity index obtained by eDNA metabarcoding showed higher values compared to morphological approach. The lowest mean value of Shannon-Wiener diversity index was obtained in Lake Visovac (1.62), while the highest value, 2.71, was in the shallow Lake Vransko at sampling site Motel (Fig. 2). The SIMPER analysis identified a total of 20 descriptive OTUs contributing with more than 5% in the total number of amplicons in all investigated lakes (Table 3).

In Lake Kozjak, four OTUs contributed most to the total number of amplicons, while two co-dominant OTUs were *Pantocsekiella ocellata* and *Gyrodinium helveticum*. In Lake Prošće, five OTUs had the greatest contribution to the total number of amplicons and *Cryptomonas marssonii* was the dominant species. A dominant OTU in the deep Lake Vransko was *Gymnodinium* sp., with *G. helveticum* and *Ceratium* sp. contributing highly to the total number of amplicons. In Lake Visovac, the dominant OTU was *P. ocellata*, while the species *Uroglenopsis americana* and *Biecheleria cincta* highly contributed to the total number of amplicons. In Lake Crniševo, six OTUs had the greatest contribution to the total number of amplicons, while *Thalassiosira* sp. was dominant. The co-dominant OTUs that characterized Lake Oćuša were *P. ocellata,*

Table 3
Descriptive phytoplankton taxa/OTUs obtained by the SIMPER analysis presented as a contribution to the similarity within all samples for each Lake/Sampling Site (C, %) and frequency of appearance in samples (F, %) through the whole study period from April till September 2017. Both approaches, morphological and eDNA metabarcoding are presented. Lake/Sampling Site codes are explained in Fig. 1.

Morphological approach	**K**		**P**		**VC**		**VI**		**CR**		**O**		**VM**		**VP**	
Taxa	**C**	**F**	**C**	**F**	**C**	**F**	**C**	**F**	**C**	**F**	**C**	**F**	**C**	**F**	**C**	**F**
Actinotaenium/Mesotaenium					17	50										
Ceratium hirundinella (O.F.Müller) Dujardin					19	100	16	100	20	67	17	100				
Chroococcus minutus (Kützing) Nägeli											3	66				
Chrysophyceae unindent.					6	83										
Cosmarium tenue W.Archer													29	83	26	66
Cryptomonas marssonii Skuja	8	100	9	83												
Cyclotella distinguenda Hustedt in Gams	4	100	6	66												
Cyclotella plitvicensis Hustedt	7	100														
Dinobryon divergens O.E.Imhof	8	100	15	100	6	100					11	83				
Dinobryon sociale (Ehrenberg) Ehrenberg					6	100										
Gyrodinium helveticum (Penard) Y.Takano & T.Horiguchi	4	100									4	100				
Lindavia radiosa (Grunow) De Toni & Forti			6	83												
Oocystis marssonii Lemmermann									11	100						
Oocystis parva West & G.S.West									5	83	4	83				
Pantocsekiella comensis (Grunow) K.T.Kiss & E.Ács									14	100	30	100				
Pantocsekiella costei (J.C.Druart & F.Straub) K.T.Kiss & E.Ács	37	100			12	83										
Pantocsekiella ocellata (Pantocsek) K.T.Kiss & E.Ács							52	100								
Parvodinium elpatiewskyi (Ostenfeld) Kretschmann, Zerdoner & Gottschling					4	66										
Plagioselmis nannoplanctica (H.Skuja) G.Novarino, I.A.N.Lucas & S.Morrall	5	100	6	100					4	100	6	100				
Radiococcus planctonicus J.W.G.Lund									5	67						
Snowella atomus Komárek & Hindák									13	67						
Sphaerocystis schroeteri Chodat			35	83												
Synedropsis roundii Torgan, Menezes & Melo													54	100	56	100
Tetraselmis cordiformis (H.J.Carter) F.Stein							4	67								
eDNA metabarcoding	**K**		**P**		**VC**		**VI**		**CR**		**O**		**VM**		**VP**	
OTUs	**C**	**F**	**C**	**F**	**C**	**F**	**C**	**F**	**C**	**F**	**C**	**F**	**C**	**F**	**C**	**F**
Biecheleria cincta (Siano, Montresor & Zingone) Siano							6	100								
Ceratium sp.					14	100			6	100	8	100				
Chlamydomonas raudensis Ettl											8	100				
Cryptomonas marssonii Skuja	7	100	20	100												
Cryptomonas curvata Ehrenberg			13	100					8	100	12	100	3	100		
Cryptomonas sp.			13	100					8	100	12	100	3	100		
Cryptophyta									6	100	13	100	3	100	7	100
Cyclotella cryptica Reimann, J.C.Lewin & Guillard	13	100	11	100									5	100	6	100
Cyclotella meneghiniana Kützing													3	100		
Dinobryon divergens O.E.Imhof			13	100												
Gymnodinium sp.					46	100										
Gyrodinium helveticum (Penard) Y.Takano & T.Horiguchi	26	100			15	100										
Monoraphidium pusillum (Printz) Komárková-Legnorová															3	100
Nephrochlamys subsolitaria (G.S.West) Korshikov															26	100
Pantocsekiella ocellata (Pantocsek) K.T.Kiss & E.Ács	25	100					56	100	16	100	19	100	4	100	3	100
Parvodinium elpatiewskyi (Ostenfeld) Kretschmann, Zerdoner & Gottschling																
Parvodinium inconspicuum (Lemmermann) Carty													4	100	3	100
Thalassionema bacillare (Heiden) Kolbe													47	100	24	100
Thalassiosira sp.									30	100						
Uroglenopsis americana (G.N.Calkins) Lemmermann							8	100								

Cryptophyta, *Cryptomonas curvata*, *Cryptomonas* sp., *Chlamydomonas raudensis* and *Ceratium* sp. In the shallow Lake Vransko, eight OTUs had the greatest contribution to the total number of amplicons. *Thalassionema bacillare* was most dominant at sampling site Motel, while the sampling site Prosika was co-dominated by *T. bacillare* and *Nephrochlamys subsolitaria*. The SIMPER analysis identified that the OTUs with the greatest number of amplicons were the same ones that typified lakes with a 100% frequency of occurrence. These OTUs were also most responsible for the distinctions between factor levels (Table 3).

3.3. *Reynolds' functional groups determined by the morphological approach*

According to the morphological approach and taxonomical enumeration of phytoplankton, the phytoplankton communities were classified into 25 coda of Reynolds' FGs. Representatives of 19 FGs contributed to more than 5% of the total biomass. The seasonal succession (from April to September) of the FGs based on the morphological approach is shown in Fig. 3.

A total of 11 FGs were identified as descriptive according to SIMPER analysis. The $\mathbf{L_O}$ was the most frequent FG occurring in all five lakes. $\mathbf{L_O}$ was dominant in deep Lake Vransko and Lake Oćuša. Other descriptive FGs included **E**, **T** and **A** in deep Lake Vransko, and **A**, **F** and **E** in Lake Oćuša. Lake Crniševo was characterized by the co-dominance of FGs $\mathbf{L_O}$ and **F**. The most important FGs in lakes Visovac and Kozjak were **B** and **A**, respectively. The $\mathbf{L_O}$ was a descriptive FG in the phytoplankton community of lakes Visovac and Kozjak, together with **X3** in Lake Visovac and **B**, **E** and **X2** in Lake Kozjak. The representatives of FG **F** showed dominance in Lake Prošće. Both sampling sites of the shallow Lake Vransko, Motel and Prosika, were characterized by FGs **P** and **N**.

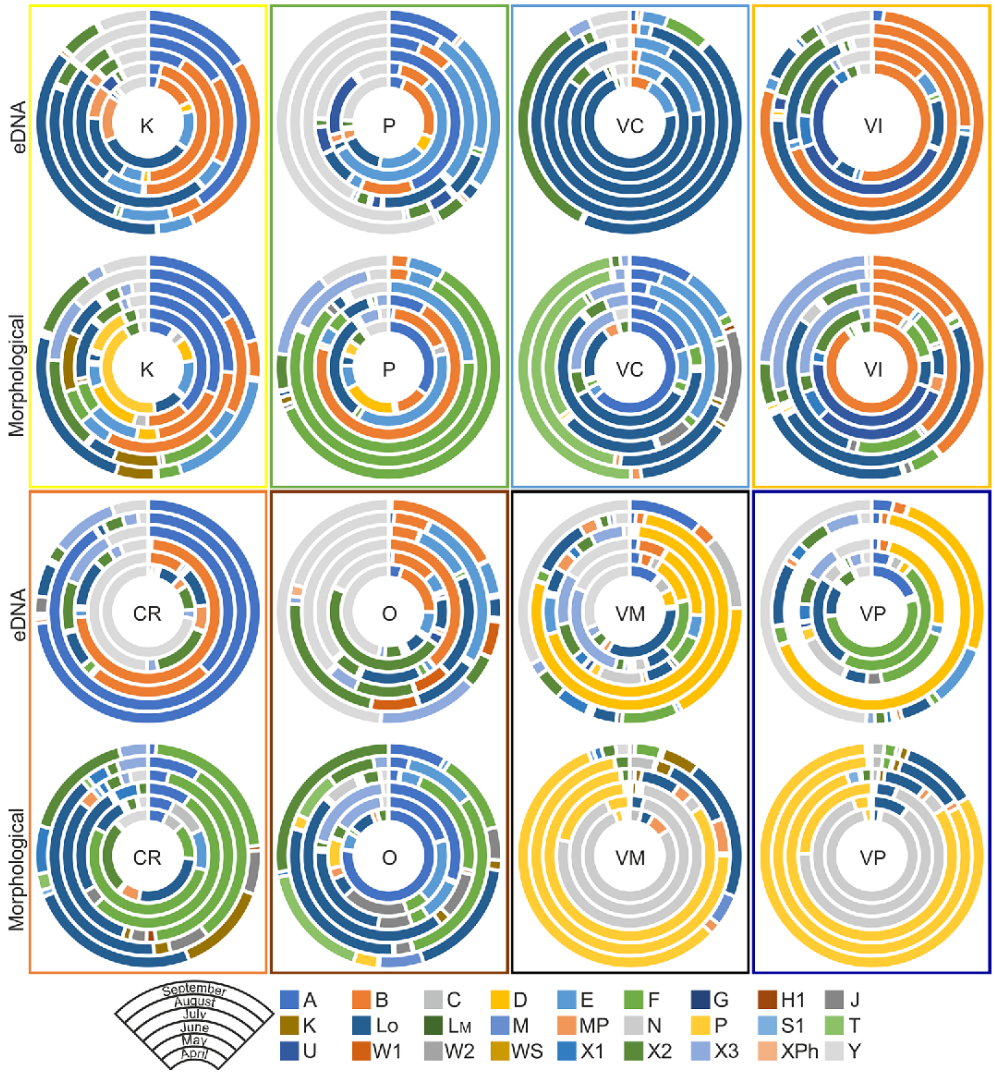

Fig. 3. Comparison of the proportion (%) of functional groups determined by morphological approach and eDNA metabarcoding between two samples in each lake for the period from April to September 2017. Concentric circles indicate the proportion (%) of a given functional group within a given month, from the inside (April, smallest diameter circle) to the outside (September, largest diameter circle). Lake/Sampling Site codes are explained in Table 1.

3.4. Reynolds' functional groups determined by eDNA metabarcoding

OTUs provided by eDNA metabarcoding and taxonomically assigned to phytoplankton taxa were classified into 21 FGs. Representatives of 14 FG contributed more than 5% to the total number of OTUs. The seasonal succession (from April to September) of FG based on eDNA metabarcoding is shown in Fig. 3.

The SIMPER analysis identified 10 descriptive FGs among those listed in more than 5% in total OTUs number of amplicons. FGs that were descriptive at all sampling sites were **B**, $\mathbf{L_O}$, and **Y**. In addition to the listed FGs, **E** was descriptive at five sampling sites (lakes Kozjak, Prošće, deep Vransko, Oćuša and Visovac) and representatives of FG **X3** were descriptive at four sampling sites (Motel and Prosika of the shallow Lake Vransko and Lakes Crniševo and Oćuša). FGs **D** and **F** were descriptive in the shallow Lake Vransko. Representatives of FG **U** were descriptive in Lake Visovac and in the shallow Lake Vransko at sampling site Motel. FG **X2** was descriptive in all lakes and sampling sites except sampling site Motel, while FG **A** was not descriptive only in the deep Lake Vransko and Lake Visovac.

3.5. Biomass metrics (Chl a concentrations)

The values of Chl *a* concentration measured spectrophotometrically varied between 0.2 and 36.3 μg L^{-1} (Fig. 4). The lowest values were measured in lakes Crniševo and Oćuša (0.2 μg L^{-1}) and the highest at sampling sites Motel (36.3 μg L^{-1}) and Prosika (34.8 μg L^{-1}) in shallow Lake Vransko. The highest Chl *a* values of deep karstic lakes were measured in Lakes Prošće (8.1 μg L^{-1}) and Visovac (6.1 μg L^{-1}). Values of Chl *a* concentration determined using UHPLC varied between 0.4 and 60.9 μg L^{-1}. The lowest value was measured in deep Lake Vransko (0.4 μg L^{-1}), while the highest was at sampling site Prosika (60.9 μg L^{-1}). The highest values for deep karstic lakes were measured in lakes Visovac (8.6 μg L^{-1}) and Prošće (7.8 μg L^{-1}).

3.6. Ecological status assessment

Mean HLPI values for the period studied, based on total biomass and total number of amplicons, as well as values calculated by taxa that contributed more than 5% to total biomass and by OTUs that contributed more than 5% to total number of amplicons, are shown in Fig. 5.

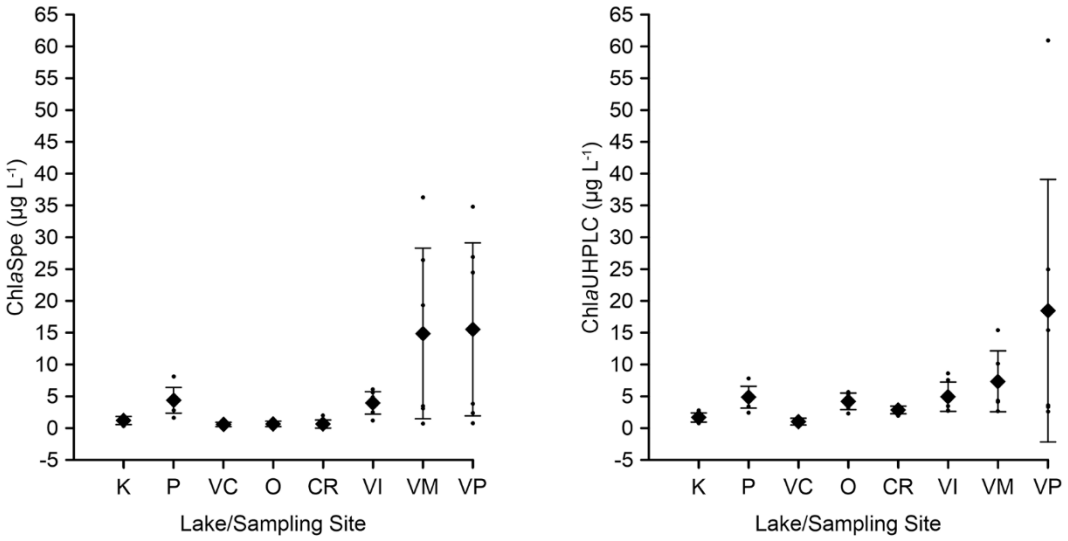

Fig. 4. Differences between Chl *a* concentrations measured spectrophotometrically (Chl*a*Spe) and using UHPLC (Chl*a*UHPLC). Rhomboids indicate the mean values. Vertical lines represent the upper and lower quartiles. Dots indicate values for each sample. Lake/Sampling Site codes are explained in Table 1.

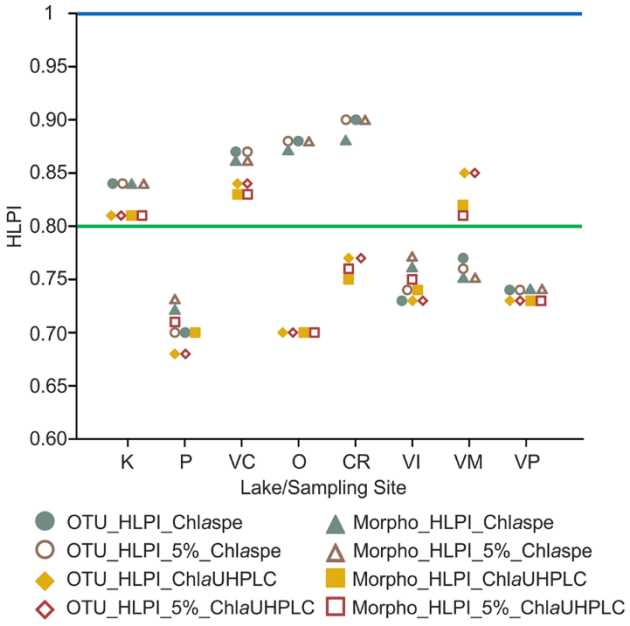

Fig. 5. Differences between the HLPI obtained by morphological approach (Morpho_HLPI, Morpho_HLPI_5%) and eDNA metabarcoding (OTU_HLPI, OTU_HLPI_5%) calculated with two Chl *a* measurements methods, spectrophotometry (ChlaSpe) and UHPLC (ChlaUHPLC). 5% in the code indicates taxa/OTUs that contributed more than 5% to the total biomass/number of amplicons. Symbols represent the mean values of the HLPI calculated for each Lake/Sampling Site during the investigated period between April and September 2017. The colour of the lines indicates ecological status class (High – Blue, Good – Green). Lake/Sampling Site codes are explained in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HLPI values in Lake Kozjak and deep Lake Vransko in all cases indicated High ecological status (0.83–0.87 and 0.81–0.84, respectively), while in lakes Prošće, Visovac and shallow Lake Vransko at the sampling site Prosika they indicated Good ecological status. In Lake Prošće the values ranged from 0.68 to 0.73, in Lake Crniševo from 0.73 to 0.77 and at sampling site Prosika of the shallow Lake Vransko they were between 0.73 and 0.74, indicating Good ecological status. HLPI obtained by morphological approach and eDNA metabarcoding for lakes Oćuša, Crniševo and sampling site Prosika from the shallow Lake Vransko showed a class differences, where different biomass metrics were used (Chl *a* measured by UHPLC in comparison with Chl *a* measured spectrophotometrically). In lakes Crniševo and Oćuša, HLPI values indicated Good ecological status (0.75–0.77 and 0.70, respectively) when they were calculated with Chl *a* concentration measured by UHPLC, and High ecological status when calculated with Chl *a* measured spectrophotometrically (0.88–0.90 and 0.87–0.88, respectively). At sampling site Motel of the shallow Lake Vransko, HLPI values calculated using Chl *a*

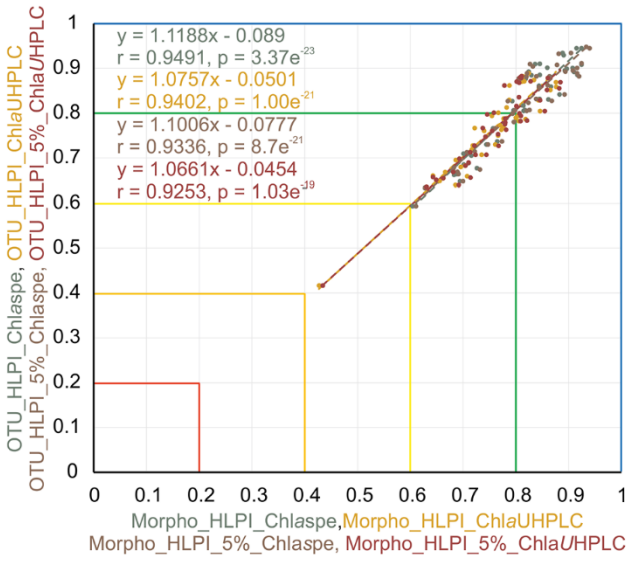

Fig. 6. Correlation of HLPI values between morphological approach (Morpho_HLPI, Morpho_HLPI_5%) and eDNA metabarcoding (OTU_HLPI, OTU_HLPI_5%) calculated with two Chl *a* measurements methods, spectrophotometry (ChlaSpe) and UHPLC (ChlaUHPLC). 5% in the code indicates taxa/OTUs that contributed more than 5% to the total biomass/number of amplicons. The colour of the lines indicates ecological status class (High – Blue, Good – Green, Moderate – Orange, Poor – Yellow, Bad – Red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

obtained by UHPLC indicated High ecological status (0.81–0.85) compared to the calculation using Chl *a* measured spectrophotometrically, indicating Good ecological status (0.75–0.77).

The four-four HLPI values calculated for the given samples using the morphological approach and eDNA metabarcoding and two biomass metrics (Fig. 4) showed a strong linear correlation ($p < 0.01$) (Fig. 6).

Differences among the eight types of HLPI index calculations ($p < 0.05$) evaluated by a paired *t*-test showed the following results:

a. There were no significant differences between the HLPI values in deep Lake Vransko and Lake Visovac.
b. In shallow Lake Vransko, only the sampling site Motel showed a significant difference between OTU_HLPI_Chl*a*UHPLC and OTU_HLPI_5%_Chl*a*UHPLC with Morpho_HLPI_Chl*a*Spe and Morpho_HLPI_5%_Chl*a*spe
c. Several others significant differences between the HLPI values in lakes Kozjak, Prošće, Oćuša and Crniševo were between the two different methods of Chl *a* analysis.
d. Significant differences in the HLPI values between different methods of phytoplankton determination (morphological approach vs. eDNA metabarcoding) were found only in Lake Prošće.
e. HLPI based on the morphological approach in lakes Prošće and Crniševo differ significantly between calculations from whole taxa and the taxa contributing more than 5% to the total biomass.

4. Discussion

In this study, different laboratory approaches were applied to reveal the phytoplankton taxonomic composition of lakes in Croatia and to assess their ecological status. Despite the substantial analytical differences, the approaches resulted in similar results.

Since the Shannon-Wiener diversity index combines richness and evenness into univariate vectors (Borics et al., 2020), its high value is given due to the presence of many species having well-balanced abundances. In this study, results obtained by eDNA metabarcoding provided higher mean values for both parameters compared to morphological data. The possibility of occurrence of similar key morphological features can result in difficulties in accurate species discrimination (Whitton and Potts, 2012; Wilmotte et al., 2017). Also, certain small-sized phytoplankton can be overlooked using light microscopy, thus reducing the diversity of species (Not et al., 2007; Xiao et al., 2014).

Compared to the number of OTUs, a much smaller number of taxa were identified by microscopy in this study. Even though variations in environmental conditions can affect different phenotypes among individuals of the same species (Luo et al., 2006; Soares et al., 2013) and phytoplankton richness may be overestimated due to the identification of different phenotypes and transition types of one certain species as separate taxa (Palińska and Surosz, 2008), eDNA metabarcoding showed substantially higher richness and diversity compared to traditional microscopy.

Morphological identification is especially difficult or even impossible for cryptic species (Huo et al., 2020) and the application of eDNA metabarcoding can complement the wide range of taxa that, due to their size and frequency, escaped detection using traditional sampling and biomonitoring protocols (Seymour et al., 2020). Analysis in this study showed that eDNA metabarcoding resulted in 2.5 times more phytoplankton OTUs compared to morphospecies. Although eDNA metabarcoding has proven to be a powerful tool for taxonomic identification, a comparison of eDNA metabarcoding and microscopy data has its limitations. The descriptive taxa *Actinotaenium/Mesotaenium* sp. and the species *Cosmarium tenue, Pantocsekiella comensis, Sphaerocystis schroeteri, Synedropsis roundii*, which were determined by microscopy and which contributed most to the biomass, were not identified by eDNA metabarcoding.

Species missing by the eDNA metabarcoding can appear due to mismatch of the primer set used. However, detection of the listed missing species in some samples explains the inapplicability for all taxa. Conversely, the absence of species identified by microscopy may be due to their non-existence in the reference library (Sun et al., 2019).

Indication of trophic status in temperate lakes can be provided by detecting and determining indicator algae in mid-summer (Bellinger and Sigee, 2015). According to Bellinger and Sigee (2015) cyanobacteria do not play an important role in oligotrophic and mesotrophic lakes. Investigating phytoplankton pigment composition compared to biomass in an oligotrophic lake, Buchaca et al. (2005) gave less importance to cyanobacteria due to their very low contribution. The 16S rRNA gene was used by Eiler et al. (2013) as a marker gene for the simultaneous detection of prokaryotic and eukaryotic phytoplankton due to its universality in cyanobacteria and presence in the chloroplast of eukaryotes. Based on the results in which less than 100 phytoplankton reads were detected in 56% of all samples tested and the prevalence of reads of heterotrophic bacteria, Huo et al. (2020) suggested that chloroplast 16S rRNA should be avoided for the detection of eukaryotic phytoplankton diversity.

Since the lakes included in this study are oligotrophic and mesotrophic and the descriptive species were eukaryotic algae, the hypervariable V9 region of 18S rRNA was used. The reason for choosing the V9 region in this study was based on a comparative analysis of V4 and V9 regions conducted by Choi and Park (2020) and Tragin et al. (2017), which resulted in a 20% higher eukaryotic OTUs abundance gained with V9 regions at a 97% identity threshold. In terms of taxonomy level, the V9 region revealed more diversity at a higher taxonomic level compared to the V4 region, especially for dinoflagellates (Stoeck et al., 2010). In the current study, FG $\mathbf{L_O}$, whose representatives are dinoflagellates detected with the V9 region, was descriptive in all investigated lakes. In the deep oligotrophic lakes Kozjak and Vransko, the dominance of OTUs assigned to $\mathbf{L_O}$ had the greatest contribution in assessing the ecological condition. The share of dinoflagellates in total biomass detected by microscopy also had a significant contribution to the assessment. As previously described in the study of Vasselon et al. (2017), a correlation between rbcL copy number and diatom biovolume was found, suggesting that high cell biovolume species can be overrepresented in eDNA metabarcoding data. As the average dinoflagellate cell is about 25–35 μm width × 30–45 μm length (Carty and Parrow, 2015), the number of amplicons of *Gymnodinium* sp., *G. helveticum,* and *Ceratium* sp. with high cell biovolume is potentially overrepresented by eDNA metabarcoding, thus resulting in a disagreement between methods. In mock communities, HTS data also confirmed that species with high cell biovolume are overrepresented and the ones with low values are underrepresented (Vasselon et al., 2017). *Sphaerocystis schroeteri*, a representative species of FG **F** and a descriptor with the highest share in biomass in Lake Prošće, and *Cosmarium tenue*, a representative species of FG **N** and one of two species with the highest share in biomass in the shallow Lake Vransko, were not even detected with the V9 region. As discussed above, the lack of detection could be due to a mismatch in the primer set used. Simultaneous application of V4 and V9 regions could provide a broader range of species, detect missing ones, and offer more reliable results for the analysis of eDNA metabarcoding in the eukaryotic community because the regions complement each other (Choi and Park, 2020).

Phytoplankton species can be classified into 38 Reynolds' FGs based on their ecological sensitivities and tolerances (Padisák et al., 2009; Reynolds et al., 2002). Factor numbers (F) are the most important part of the assessment and they are assigned to FGs considering phytoplankton distribution and stressor values (Gligora Udovič and Žutinić, 2020). Even though different compositions and shares of FGs were recorded when comparing morphological and eDNA metabarcoding results in this investigation, assessment of ecological status results fell in an equal range of quality classes in 89% of samples. Although the FGs that contributed most to the biomass and the OTUs number of amplicons differed among samples, results of this study showed that the factors assigned to FGs with similar ecological demands played an important role in the final assessment. Shallow Lake Vransko is a good example of

FGs **F** and **D** domination identified by eDNA metabarcoding and coda **N** and **P** determined by microscopy. FG **F** is characteristic for clear deeply mixed mesotrophic lakes, FG **D** for shallow turbid waters, while favorable habitats for FGs **P** and **N** are continuous or semi-continuous mixed layer (2–3 m thickness) in shallow lakes (Padisák et al., 2009; Reynolds et al., 2002). Due to similar ecological requirements and favorable habitats, FGs **F, D, P,** and **N** have similar or equal factor numbers, which, as previously stated, are the most crucial aspect for the Q index calculation. Based on different dominant FGs with similar or same factor numbers, the ecological status assessment for 10 out of 11 samples showed the same range in quality status for the shallow Lake Vransko.

While this study focused on oligotrophic and mesotrophic lakes, the representatives of FGs found by both methods had similar ecological demands. Even when the representative taxa were not congruent, similar ecological demands resulted in the assignment of similar or the same factor numbers, resulting in 41 of 46 samples with the same ecological status. The remaining five samples differed only in one quality class. In the study of Elbrecht et al. (2017) macroinvertebrate identification for stream monitoring showed a significant linear relationship comparing the number of morphologically identified and the number of sequencing reads taxonomically assigned to specimens. Significant correlations were also found in the study of Abad et al. (2016), where the relative abundance of morphologically identified taxa against the values given by the eDNA metabarcoding approach was compared. The study of Seymour et al. (2020) where biomonitoring assessment approaches for macroinvertebrates and diatoms were compared, showed the application of eDNA metabarcoding as a feasible replacement for traditional methods.

There were no significant differences between HLPI values based on the morphological approach and eDNA metabarcoding for two karstic lakes in Croatia, deep Lake Vransko and Lake Visovac, as there were no significant differences between the HLPI values based on two different methods of Chl *a* analyses. Significant differences in HLPI values between different methods of phytoplankton determination were found only in Lake Prošće. In the majority of the studied lakes, differences in HLPI values were found due to different methods of Chl *a* measurement. Peng et al. (2013) obtained accurate Chl *a* concentrations and compared them with values determined by the HPLC method. Due to the simplicity of the pretreatment procedure and low cost, in contrast to the need for relatively high purity reagents and higher determination costs, a spectrophotometric determination is preferred for routine laboratory determination of Chl *a* compared to HPLC. As the HPLC method is more precise and sensitive, especially when Chl *a* concentration is low, accurate values of lakes Očuša and Crniševo could have been missed with spectrophotometry, resulting in higher concentrations obtained with UHPLC, leading to a lower HLPI value, which deteriorated the ecological status. Higher Chl *a* concentrations determined by UHPLC at sampling site Motel resulted in a one-class difference between the morphological approach and eDNA metabarcoding. Lower HLPI values calculated using higher Chl *a* concentrations determined by UHPLC in lakes Kozjak, Prošće and deep Lake Vransko did not affect the change in ecological status class in these lakes.

Using the biomass/number of amplicons of the total taxa/OTUs list or taxa that contributed more than 5% to the total biomass (Teneva et al., 2020) and OTUs to the total number of amplicons in the calculation of the HLPI showed significant differences only in lakes Prošće and Crniševo.

5. Conclusions

The use of morphological approach and eDNA metabarcoding to assess the ecological status of Croatian natural karstic lakes resulted in a comparable ecological status. Lakes were classified into Good or High ecological status based on HLPI values obtained both for the total taxa/OTUs list and for taxa/OTUs contributing more than 5% to the total biomass/number of amplicons with very few exceptions. Differences in ecological status assessment values were mainly caused by differences in biomass estimation methods (spectrophotometric or UHPLC).

The V9 region of 18S rRNA has shown its applicability for assessing the ecological status of natural karstic lakes and further development of eDNA metabarcoding will contribute to a more accurate assessment of ecological status by providing more comparable taxa lists to morphological analyses and more comparable lists of FGs according to Reynolds' functional classification.

CRediT authorship contribution statement

Nikola Hanžek: Investigation, Writing - original draft, Visualization, Formal analysis. **Marija Gligora Udović:** Writing - review & editing, Formal analysis, Conceptualization. **Katarina Kajan:** Investigation, Data curation, Writing - original draft, Formal analysis. **Gábor Borics:** Supervision, Writing - original draft. **Gábor Várbíró:** Writing - review & editing. **Thorsten Stoeck:** Supervision, Writing - review & editing. **Petar Žutinić:** Investigation, Writing - review & editing. **Sandi Orlić:** Investigation, Resources, Project administration. **Igor Stanković:** Investigation, Formal analysis, Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

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

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Phytoplankton in Deep Lakes of the Dinaric Karst: Functional Biodiversity and Main Ecological Features

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Abstract: Phytoplankton is a polyphyletic group of organisms that responds rapidly to environmental conditions and provides a reliable response to changes, making it a good ecological indicator for water quality monitoring. However, a gradient is almost essential for a reliable relationship between pressure and impact. In a low-gradient environment, ingenuity is required to outsmart the limitations of the commonly used linear relationship. Here, we examine changes in biomass and functional biodiversity by analysing larger data sets (2013–2022) in six ecologically diverse, natural, deep Croatian karst lakes with low nutrient gradients using nonlinear correlation coefficients and multivariate analyses in 209 samples. We found that phytoplankton biomass was most strongly influenced by nutrients, salinity and alkalinity, while light availability and total nitrogen strongly influenced phytoplankton functional biodiversity. An additional analysis of the TN:TP ratio revealed that the oligotrophic Lake Vransko is nitrogen-limited, and lakes Kozjak and Prošće are phosphorus-limited. This further clarified the relationship of phytoplankton to nutrients despite the low gradient. The complex analysis in this study provides a new perspective for predicting changes in the structure and succession of phytoplankton in deep karst lakes for successful management under apparent anthropogenic pressure and climate change.

Keywords: Reynolds functional groups; chlorophyll *a*; salinity; nutrients; trophic status; water quality monitoring

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1. Introduction

Freshwater lakes, which are rich in biodiversity, are vulnerable to human impacts, with climate change and nutrient fluctuations being among the main drivers of change in these ecosystems. One of the consequences of these factors is eutrophication [1]. As lakes are complex ecosystems, they respond rapidly to environmental changes related to their physico-chemical and biological properties. The process of mixing and stratification also plays an important role in the ecological properties of lake water, in particular, water temperature, dissolved oxygen consumption and the nutrient content and distribution of phytoplankton and its diversity in the layers of the lake [2–4].

Nitrogen (N) and phosphorus (P) are essential macronutrients that are crucial for the biochemical processes of phytoplankton and, as limiting factors in the N:P ratio, determine the growth dynamics of phytoplankton in aquatic ecosystems, including lakes [5]. Understanding the N:P ratio is crucial for explaining and predicting phytoplankton dynamics in different aquatic habitats and is of great importance for the effective management and maintenance of ecological balance in lakes and other aquatic ecosystems [6–8]. Since oligotrophic lakes have low nutrient concentrations, they are sensitive to even small changes in the N or P supply. As a result of the low N and P concentration, the naturally low N:P ratio in oligotrophic lakes can increase considerably with high N inputs. The phytoplankton in these lakes can change from a primarily nitrogen-limited to a primarily phosphorus-limited form [9,10]. An increased N:P ratio can lead to reduced biodiversity in the lake's food

web, lower drinking water quality and algal blooms [11]. In addition to the main factors N and P, silicates are also of great importance for the growth of phytoplankton in freshwater ecosystems, especially for diatoms [12].

Not only nutrients are important for phytoplankton communities, but other factors also determine their dynamics, including water temperature, salinity, light availability, alkalinity, pH, suspended matter, hydrological characteristics and human activities [13–17]. Recognising that phytoplankton is an extremely diverse group of organisms, which makes it difficult to understand ecological processes and the influence of ecological indicators on phytoplankton, Reynolds developed the concept of functional groups based on the common ecology and environmental preferences of phytoplankton species [18–20].

Due to the great heterogeneity and variability in geological, morphological, hydrogeological, hydrological, hydraulic, ecological and other parameters, an interdisciplinary approach is required for the study of karst systems. A key feature of the karst phenomenon is the activity of groundwater and surface water, which influences biological processes both on the surface and underground [21]. Phytoplankton, one of the most important biological elements in freshwater ecosystems, plays an important role in the ecology of karst lakes and is thus an essential component of these complex interactions in such environments.

Stratified karst lakes are unique due to their geological, physical and chemical characteristics associated with karst landscapes. Such lakes in this study are mostly in an oligo- and mesotrophic state and are characterised by the presence of the phytoplankton groups Ochrophyta, Miozoa and Bacillariophyta, which are the most diverse and abundant [22,23]. Since they are mostly in a pristine state, they represent ecosystems that can be used to study changes in the phytoplankton community under the influence of humans and the resulting climate changes [24], as phytoplankton responds quickly and reliably to environmental changes and is a good ecological indicator of the state of nutrients and eutrophication [14,25].

2. Results

2.1. Environmental Characteristics of Lakes

The minimum, maximum and mean values for the physical and chemical properties of the water in all six lakes are shown in Table 1. The Secchi depth in the deep karst lakes ranged from 1.0 to 15.5 m, with the lowest values measured in Lake Crniševo and the highest in Lake Vransko, indicating an oligotrophic state for lakes Kozjak and Vransko, an oligo- to eutrophic state for Lake Crniševo and an oligo-mesotrophic state for all other lakes (Figure 1). The continental lakes Prošće and Kozjak were the coldest with a mean temperature of 12.8 and 13.3 °C, respectively, while the highest values were measured in the Mediterranean lakes with a mean temperature between 17.7 and 22.6 °C. The analysed lakes had a slightly alkaline character, with mean pH values of 8.1 to 8.3. Lake Prošće was the richest in dissolved oxygen, and Lake Visovac had the highest saturation, while Lake Oćuša was characterised by the lowest concentration of dissolved oxygen and the lowest saturation. The highest salinity and consequently the highest conductivity values were measured in the slightly brackish Lake Crniševo. In terms of nutrients, Lake Vransko had the lowest TN values (0.100-0.310 mg L^{-1}), while the highest TN values were measured in lakes Kozjak and Prošće with mean values of 0.685 and 0.740 mg L^{-1}, respectively (Figure 1). The TN values indicate an oligotrophic state of all lakes. The mean values of TP showed the lowest concentrations in lakes Oćuša and Crniševo (0.007 and 0.009 mg P L^{-1}), while the highest mean value was measured in Lake Prošće (0.018 mg P L^{-1}), indicating an oligotrophic state for all analysed lakes (Figure 1). The organic load, measured as the BOD and COD, was higher in the lakes Visovac, Crniševo and Oćuša (0.1–2.7 mg O_2 L^{-1}) than in the lakes Kozjak, Prošće and Vransko (0.3–1.8 mg O_2 L^{-1}). The TOC values measured in all lakes were between 0.65 and 3.70 mg C L^{-1} with the lowest values in the Kozjak, Prošće and Visovac barrage lakes and the highest in the Crniševo and Vransko lakes. The SiO_2 concentration in all lakes was between 0.13 and 4.96 mg L^{-1} with the lowest average

values in lakes Vransko and Crniševo (0.35 and 0.70 mg L^{-1}) and the highest in all other lakes (1.52–1.95 mg L^{-1}).

Table 1. Minimum, maximum and mean values of physical and chemical parameters for investigated lakes in the period from 2013 to 2022. Abbreviations used in text are in square brackets.

Parameter	Kozjak		Prošće		Vransko	
	Min-Max	**Mean**	**Min-Max**	**Mean**	**Min-Max**	**Mean**
Secchi visibility (m)	5.3–14.7	8.5	3.6–10.7	5.4	7.8–15.5	11.0
Temperature (°C)	7.6–18.8	13.3	8.4–18.1	12.8	9.9–21.7	17.7
pH	7.9–8.4	8.3	7.7–8.4	8.2	7.9–8.4	8.3
Alkalinity (mg $CaCO_3$ L^{-1})	202.0–236.0	214.0	214.0–249.0	230.0	102.0–139.0	112.0
Dissolved oxygen (mg L^{-1})	10.0–13.7	11.7	8.5–15.0	11.2	8.5–12.7	10.7
Oxygen saturation (%)	96.8–146.9	113.2	79.8–159.1	106.7	91.1–134.3	115.5
Conductivity (µS cm^{-1}) 25 °C	359.0–419.0	393.5	387.0–443.0	428.5	397.0–475.0	436.0
Salinity (‰)	0.10–0.14	0.13	0.00–0.15	0.13	0.11–0.15	0.13
Total nitrogen [TN] (mg N L^{-1})	0.420–0.990	0.685	0.520–0.940	0.740	0.100–0.310	0.100
Total phosphorus [TP] (mg P L^{-1})	0.002–0.059	0.013	0.002–0.038	0.018	0.002–0.044	0.014
Molar TN:TP ratio	10.5–493.7	62.5	19.4–523.2	45.4	2.5–228.4	14.8
Biological oxygen demand [BOD] (mg O_2 L^{-1})	0.3–1.8	0.8	0.3–1.7	1.0	0.3–1.5	0.6
Chemical oxygen demand [COD] (mg O_2 L^{-1})	0.4–2.2	1.2	0.4–3	1.4	0.4–2.4	1.4
Total organic carbon [TOC] (mg C L^{-1})	0.69–2.22	1.02	0.75–2.09	1.08	1.19–2.93	1.67
Silicates [SiO_2] (mg L^{-1})	0.57–3.41	1.60	0.35–4.96	1.58	0.13–1.39	0.35
Chlorophyll *a* [Chl-a] (µg L^{-1})	0.35–2.72	1.29	0.5–8.14	4.01	0.35–2.06	0.50
Total biomass (mg L^{-1})	0.12–2.05	0.46	0.35–4.65	1.49	0.21–1.21	0.38
Parameter	**Visovac**		**Crniševo**		**Oćuša**	
	Min-Max	**Mean**	**Min-Max**	**Mean**	**Min-Max**	**Mean**
Secchi visibility (m)	3.2–8.2	5.3	1.0–6.5	4.8	3.0–8.0	4.4
Temperature (°C)	13.2–23.0	18.0	11.8–27.3	21.4	14.2–27.0	22.6
pH	6.7–8.4	8.1	7.6–8.4	8.1	7.5–8.7	8.1
Alkalinity (mg $CaCO_3$ L^{-1})	171.0–245.0	210.1	139.0–368.8	171.0	112.0–201.0	150.0
Dissolved oxygen (mg L^{-1})	6.9–13.6	10.3	8.3–13.0	10.6	5.9–12.7	9.9
Oxygen saturation (%)	73.4–162.3	107.3	88.1–140.1	117.5	66.9–153.6	111.3
Conductivity (µS cm^{-1}) 25 °C	482.0–895.0	535.0	718.0–2930.0	1853.0	372.0–600.0	457.0
Salinity (‰)	0.16–0.31	0.20	0.29–1.50	0.88	0.11–0.30	0.22
Total nitrogen [TN] (mg N L^{-1})	0.220–0.753	0.350	0.240–1.010	0.385	0.100–0.909	0.429
Total phosphorus [TP] (mg P L^{-1})	0.003–0.033	0.012	0.002–0.045	0.009	0.002–0.036	0.007
Molar TN:TP ratio	11.7–150.3	33.9	11.5–694.1	48.6	11.4–316.1	39.6
Biological oxygen demand [BOD] (mg O_2 L^{-1})	0.3–2.4	0.8	0.1–2.4	1.2	0.1–2.7	0.9
Chemical oxygen demand [COD] (mg O_2 L^{-1})	0.3–3.5	1.1	0.3–5.4	2.6	0.3–2.8	1.4
Total organic carbon [TOC] (mg C L^{-1})	0.65–1.59	1.07	1.50–3.70	2.42	0.82–2.9	1.28
Silicates [SiO_2] (mg L^{-1})	0.40–4.52	1.95	0.17–2.11	0.70	0.28–3.54	1.52
Chlorophyll *a* [Chl-a] (µg L^{-1})	1.18–7.39	2.90	1.5–11.32	2.42	0.51–5.67	3.22
Total biomass (mg L^{-1})	0.38–4.70	1.27	0.22–6.87	0.82	0.22–3.81	0.86

The molar ratio TN:TP varied between the lakes (Figure 2). The lowest mean TN:TP ratio was calculated for Lake Vransko (14.8), which is thus categorised as a potentially N-limited lake. The highest ratios were determined in lakes Kozjak and Prošće (62.5 and 48.6), which categorises these lakes as potentially P-limited. The mean ratio values for lakes Visovac, Crniševo and Oćuša lie between the N- and P-limitation lines, indicating that these lakes are probably not nutrient-limited.

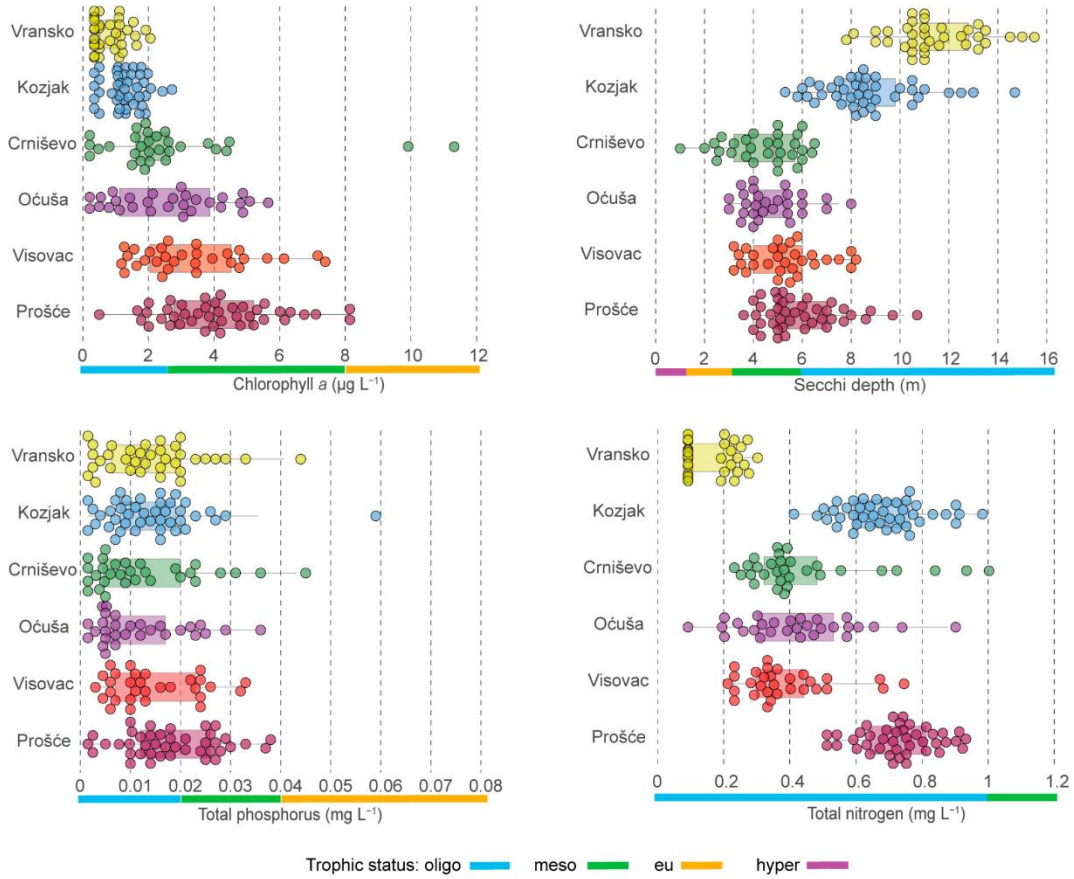

Figure 1. Classification of trophic state according to chlorophyll *a*, Secchi depth, total phosphorus and total nitrogen, shown as boxplots, with the values for each trophic state indicated by the colours below the x-axis. The centre line shows the median value, while outliers are shown as dots.

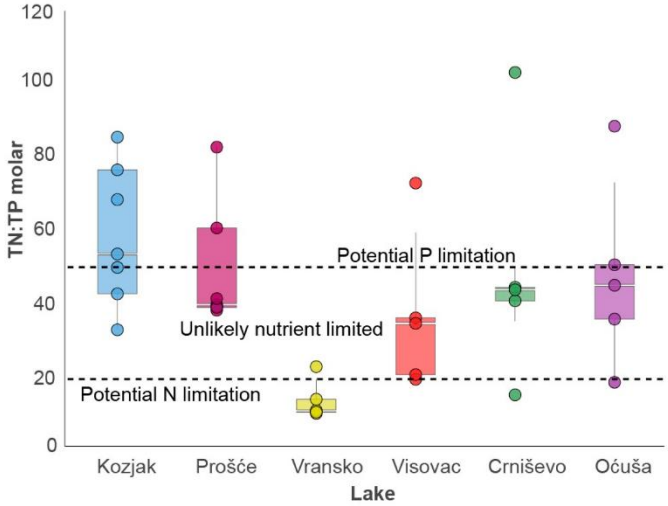

Figure 2. Boxplots of the average molar ratios TN:TP per lake. The lines show the TN:TP molar ratios at which N and P limitation can occur: <20 molar ratio N limitation; >50 molar ratio P limitation [26].

Cluster analysis of the Euclidean distance of water physical and chemical properties (including Secchi depth, alkalinity, conductivity, pH, salinity, temperature, BOD, COD, dissolved oxygen, saturation, nitrates, nitrites, TN, soluble reactive phosphorus, TP, TOC and SiO_2) was performed based on the average data for each lake. The analysis resulted in a clear grouping of lakes, although there were some exceptions (Figure 3). Lake Vransko formed a separate group. Lakes Kozjak and Prošće were grouped together. However, lakes Oćuša and Crniševo, both part of the Baćina Lakes complex, were not grouped together, with Lake Crniševo forming its own group for the majority of years. Lakes Oćuša and Visovac consistently formed a common group, also with minor exceptions.

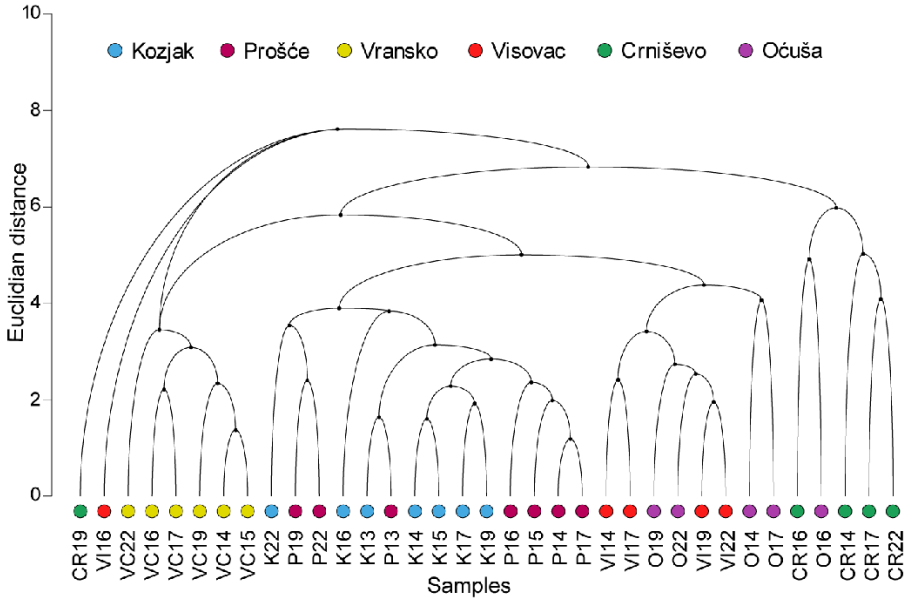

Figure 3. Dendrogram of the cluster analysis based on the Euclidean distance of the physical and chemical properties of the water in lakes. The lakes are coded with different coloured symbols, while the two attached numbers represent the years of the study from 2013 to 2022. The location codes of the lakes are shown in Figure 1.

2.2. *Phytoplankton Biomass and Its Relationship to Environmental Parameters*

Chl-a values showed that lakes Vransko and Kozjak were the least productive lakes with average values of 0.5 and 1.29 μg L^{-1}, respectively. The productivity of the other lakes was in the following order: Crniševo, Visovac, Oćuša and Prošće. The highest mean Chl-a was measured in Lake Prošće at 4.01 μg L^{-1}. Lakes Vransko, Kozjak and Crniševo had an oligotrophic status, while Oćuša, Visovac and Prošće had a mesotrophic status according to the mean Chl-a values (Figure 1).

Spearman correlation analysis of all 15 environmental parameters showed a similar relationship between the environmental variables and phytoplankton biomass, represented by Chl-a and total biomass (Table 2). The alkalinity, conductivity, salinity, TN, SiO_2 and presence of organic matter, represented by BOD, showed a significant positive relationship with both Chl-a and total biomass, while the Secchi depth and pH showed a significant negative relationship for both factors. TP showed a significant positive correlation, while TOC showed a significant negative correlation with phytoplankton biomass. COD and TN:TP showed a significant positive correlation with Chl-a, while dissolved oxygen and saturation showed a significant negative correlation with Chl-a.

Table 2. Spearman's Rho correlations (two-tailed) for relationships between phytoplankton biomass (including chlorophyll *a* concentration and total biomass) and environmental variables in all lakes. Correlation is significant at * $p \leq 0.05$ level and ** $p \leq 0.01$ level in bold; n.s. not significant; total number of samples = 209.

Environmental Variables	Lakes (n = 209)	
	Chlorophyll *a*	Total Biomass
Light availability	**−0.741 ****	**−0.571 ****
Alkalinity	**0.307 ****	**0.368 ****
Conductivity	**0.207 ****	**0.178 ****
pH	**−0.276 ****	**−0.212 ****
Salinity	**0.204 ****	0.150 *
Temperature	n.s.	n.s.
Biological oxygen demand	**0.331 ****	**0.256 ****
Chemical oxygen demand	0.165 *	n.s.
Dissolved oxygen	−0.152 *	n.s.
Oxygen saturation	−0.159 *	n.s.
Total nitrogen	**0.222 ****	**0.305 ****
Total phosphorus	n.s.	**0.180 ****
Total organic carbon	n.s.	−0.150 *
Silicon dioxide	**0.252 ****	**0.224 ****
TN:TP (mol)	0.147 *	n.s.

2.3. The Functional Composition of Phytoplankton

A total of 341 phytoplankton taxa were identified in 209 samples from six deep karst lakes. These taxa were categorised into ten main groups (Phyla), Chlorophyta (115), Bacillariophyta (66), Cyanobacteria (60), Ochrophyta (46), Charophyta (16), Miozoa (13), Cryptophyta (9), Euglenozoa (11), Haptophyta (3) and Choanozoa (2), and classified into 25 coda of Reynolds' FGs. The complete taxa list for each lake is included in the Supplementary Table S1.

The one-way SIMPER analysis based on the Bray–Curtis similarity of phytoplankton FGs showed that 12 FGs contributed to more than 5% similarity depending on the lake and were thus classified as descriptive FG coda (Table 3). The most common descriptive coda found in all six lakes were **L0**, **X2** and **F**. Representatives of these FGs were codominant in the phytoplankton communities in all lakes, with the exception of the dominance of **L0** in lakes Crniševo and Vransko. The most common representatives of centric diatoms, grouped in codon **A**, were descriptive in all lakes except Lake Visovac, with the highest similarity in lakes Kozjak, Vransko and Oćuša. Centric diatoms belonging to coda B and D together contributed the most to the similarity of samples in lakes Kozjak and Prošće. In addition, codon **B** was also descriptive in lakes Visovac and Vransko, while it dominated in lakes Prošće and Visovac. Coda E and **Y** were codominant in lakes Kozjak, Prošće and Oćuša. The FG coda that were specific to a single lake were **T** (Vransko), **X3** (Visovac), **X1** (Crniševo) and **J** (Oćuša).

The cluster analysis of the Bray–Curtis similarity of the composition of the functional groups based on the average biomass data of the lakes revealed a clear grouping according to the indicated trophic status of the lakes, with minor exceptions (Figure 4). The least productive lakes Vransko and Kozjak were grouped in a cluster with a similarity of over 40%. Both lakes were in a larger cluster with the slightly more productive lakes Oćuša and Crniševo, while the most productive lakes Prošće and Visovac were grouped separately with minor exceptions.

Table 3. Descriptive phytoplankton functional groups coda determined by SIMPER analysis are presented as a contribution to the similarity of all samples for each lake (SIMPER Ctb./%) over the entire study period from 2013 to 2022.

	Kozjak (*n* = 42)	**Prošće (*n* = 42)**	**Vransko (*n* = 36)**	**Visovac (*n* = 30)**	**Crniševo (*n* = 30)**	**Oćuša (*n* = 29)**
FG's	%	%	%	%	%	%
A	28.58	6.49	24.31	-	17.04	31.88
B	14.77	24.3	3.87	44.04	-	-
D	6.42	3.66	-	-	-	-
T	-	-	8.22	-	-	-
X3	-	-	-	8.85	-	-
X2	12.95	12.18	7.52	15.27	12.22	23.09
X1	-	-	-	-	6.59	-
E	9.37	16.38	10.25	-	-	10.73
Y	8.21	14.47	-	-	-	3
F	3.63	9.53	5.13	9.01	15.46	8.54
J	-	-	-	-	-	5.49
L0	7.19	4.46	32.6	13.13	39.16	9.92

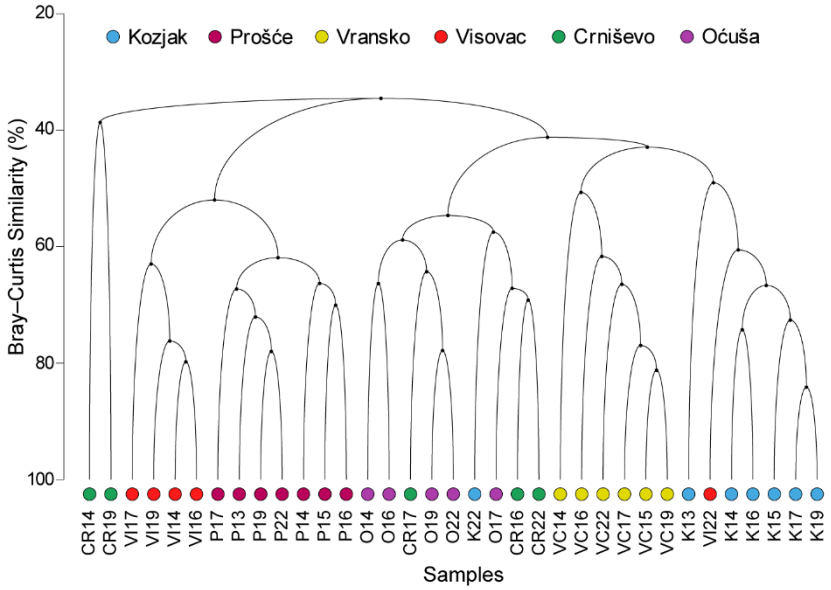

Figure 4. Dendrogram of the cluster analysis based on the Bray–Curtis similarity index of phytoplankton FG composition. The lakes are coded with different coloured symbols, while the two attached numbers represent the years of the study from 2013 to 2022. The location codes of the lakes are shown in Figure 1.

2.4. Relationship between Environmental Parameters and the Composition of Phytoplankton

The ordination diagram of the redundancy analysis (RDA) of the FG composition of the phytoplankton and the environmental variables is shown in Figure 5. The environmental variables with a significant influence on the phytoplankton composition for deep karst lakes were the TN, alkalinity, SiO_2, salinity, temperature, TOC and Secchi depth. The eigenvalues of the first two axes were 0.163 and 0.058, respectively, explaining 76.1% of the relationship between FGs and environmental data (Table 4). Axis 1 had the highest correlation with TN, while axis 2 had the highest correlation with Secchi depth. Codon **A** favoured conditions with more light, especially in lakes Vransko and Kozjak, while in lakes Visovac and Oćuša, coda **X3** and **X2** favoured less light. In lakes Kozjak and Prošće, codons **B**, **C**, **D** and **P** favoured conditions with higher TN, alkalinity and SiO_2. The highest

salinity and temperature characterised Lake Crniševo, which favoured coda **H1**, **L0** and **J**. However, TOC also characterised Lake Crniševo, together with Lake Vransko, which mostly favoured coda **K**, **N** and **T**. Codon **F** favoured conditions with low light and low TN at higher temperature and salinity.

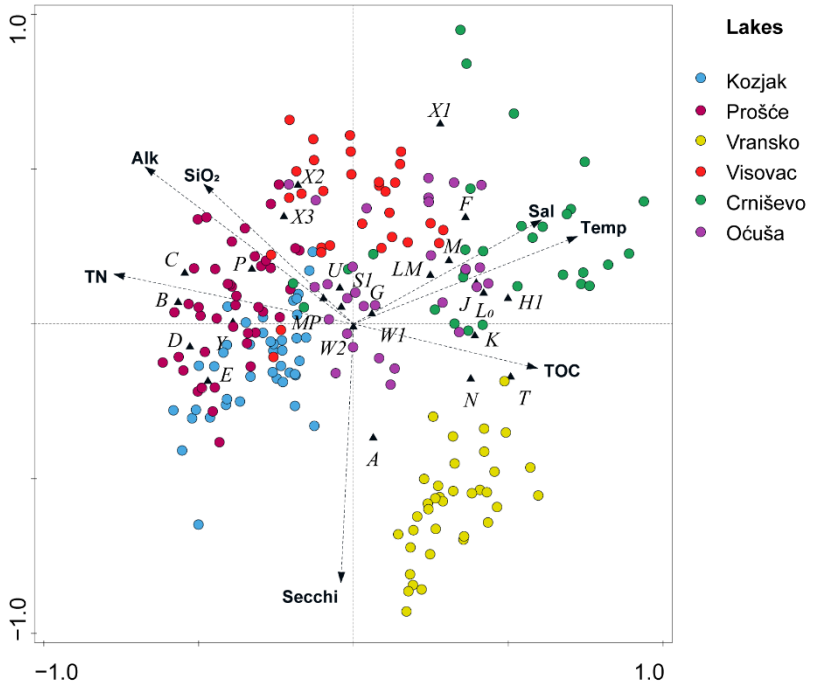

Figure 5. Redundancy analysis (RDA) between FGs and environmental variables for lakes throughout the study period. Codes of the variables: T—water temperature; Sal—salinity; Secchi—Secchi depth; Alk—alkalinity; TN—total nitrogen; SiO_2—silicates; and TOC—total organic carbon.

Table 4. Results of the redundancy analysis (RDA) between FGs and environmental parameters for deep karst lakes. [a] Axis summary statistics of the two extracted canonical axes and the percentage of variance explained by the RDA ordination; [b] correlation of the environmental variables with the ordination axes; explanatory variables at $p \leq 0.05$ significance level (999 permutations) in the forward selection are in bold with *p*-value. Codes of variables: T—water temperature; Sal—salinity; Secchi—Secchi depth; Alk—alkalinity; TN—total nitrogen; SiO_2—silicates; and TOC—total organic carbon.

	Axis 1	**Axis 2**
Axis summary statistics and variance in species data [a]		
Eigenvalues	0.163	0.058
FG–environment correlations	0.844	0.714
Cumulative percentage variance		
Of FG data	16.3	22.1
Of FG–environment relation	56.1	76.1
Correlations of environmental variables and redundancy axes [b]		
Temp	0.611	0.202
Sal	0.512	0.24
Secchi	−0.034	−0.597
Alk	−0.568	0.363
TN	−0.653	0.115
SiO_2	−0.408	0.323
TOC	0.502	−0.102

3. Discussion

In this study, we have found that the response of phytoplankton biomass and composition to environmental variables is different for each lake, as all lakes are unique habitats. However, it can be generalised that the environmental variables with the greatest influence on phytoplankton are nutrients, alkalinity, salinity, light availability and water temperature. These results suggest that there is a close relationship between phytoplankton and environmental variables, making phytoplankton a reliable biological indicator of the response to nutrients and eutrophication in freshwater lakes, especially on the threshold of climate change.

3.1. Physical and Chemical Properties of the Analysed Lakes and Indicators of Trophic Status

The higher TN concentrations in the Kozjak and Prošće lakes compared to the other studied lakes can be explained by higher concentrations of these variables in the spring water feeding the lakes and indicate the influence of the natural environment, e.g., seepage of groundwater through humus and transport by surface water from the forested surroundings [27]. The higher water temperatures in lakes Vransko, Visovac, Oćuša and Crniševo, which are located in the Mediterranean region, are directly related to the higher air temperatures characteristic of the Mediterranean climate [28]. In contrast, lakes Kozjak and Prošće, which are located in the mountainous continental region with its colder winters and milder summers, have lower water temperatures due to the continental climate [4,29].

3.2. Relationship between Environmental Variables and Phytoplankton Biomass

Light availability, as a crucial resource for autotrophic organisms, which is measured indirectly via the Secchi depth, has a direct influence on phytoplankton biomass [30]. However, the Secchi depth is higher in less productive lakes because the algae themselves contribute to lake turbidity [3,31,32]. This pattern was also found in the lakes studied, where low productivity means higher Secchi depth, making this parameter a good indicator of eutrophication [25]. The positive correlation of the BOD with Chl-a and total phytoplankton biomass in this study also proves that phytoplankton is a good indicator of anthropogenic influences, as the BOD is always elevated when phytoplankton blooms occur in organically enriched water from domestic or other sources [33].

The analysed lakes are in a mesotrophic and oligotrophic state. Nevertheless, Chl-a showed a negative correlation with dissolved oxygen and saturation, possibly due to the occurrence of stratification [34], where oxygen production takes place in the upper layers of the lake and at the thermocline, where more light and nutrients are available, while phytoplankton decomposition consumes oxygen in the lower layers, which could link the average oxygen values of the composite sample from the euphotic zone to the negative Chl-a. As phytoplankton biomass increases, the nocturnal periods when respiration replaces photosynthesis may also reduce dissolved oxygen and saturation. This event can also be explained by aquatic respiration and the oxidative degradation of organic compounds [35].

The importance of nutrients is critical for the growth and maintenance of phytoplankton communities [36], and nutrients are influenced by many factors, including salinity, which affects their availability, which in turn influences phytoplankton growth. N and P are the most important nutrients for growth in brackish water and estuarine ecosystems, and their concentrations, which are determined by the direct interaction of nutrients and salinity, consequently have a direct positive or negative influence on Chl-a and phytoplankton biomass [3,37,38]. This is important for our study because conductivity and salinity had a positive influence on phytoplankton growth. Their values were highest in lakes Crniševo and Oćuša, where Chl-a and total biomass were also high and showed a positive correlation with Chl-a.

An increase in alkalinity has a positive effect on Chl-a and the biomass of phytoplankton. As bicarbonate is the predominant form of carbon in freshwaters with a similar pH range to the lakes studied and its concentration often exceeds that of CO_2, it is much more accessible to photosynthetic organisms [39]. The alkalinity values were highest in the tufa-

dominated Prošće and Kozjak lakes, where the dominance of Fragilaria-like diatoms, which efficiently utilise HCO_3- as an inorganic carbon source, was noted [40]. Regarding the direct effects of nutrients, the availability and proportion of SiO_2 relative to dissolved inorganic nutrients are important in regulating competition between phytoplankton species [41]. In this study, the increase in phytoplankton biomass and Chl-a was accompanied by an increase in SiO_2. This observation is consistent with the findings of Fetahi et al. [30] and Dubourg et al. [42], as SiO_2 is particularly crucial for diatoms, one of the most numerous and abundant phytoplankton groups addressed in this work.

In the lakes studied, the increase in phytoplankton biomass together with Chl-a is accompanied by an increase in TN concentrations, while an increase in TP only contributes to phytoplankton biomass, confirming the importance of both N and P for phytoplankton growth and primary production in lakes [43,44]. P is widely considered to be the most important factor influencing phytoplankton growth [45], so N is second in controlling the eutrophication process by limiting N import into freshwater ecosystems. This is mainly due to the ability of some cyanobacteria to fix atmospheric nitrogen (N2) to fulfil their N requirements [46]. The positive correlation between the TN:TP ratio and Chl-a in our study emphasises that it is not sufficient to focus only on P limitation; both N and P play a crucial role in limiting phytoplankton biomass. Natural N and P concentrations in oligotrophic lakes are low [9], and this is also true for oligotrophic Lake Vransko with the lowest TN:TP ratios, which is in agreement with Elser et al. [47] and Bergström et al. [48]. Lake Vransko is thus N-limited, and the uptake of N can strongly increase the TN:TP ratio, so phytoplankton growth in such lakes can quickly change from a mainly N-limited to a mainly P-limited form [9]. While P often limits phytoplankton biovolume in many lakes, especially deep lakes, N is a better predictor of phytoplankton biomass than P when the N:P ratio is low, as shown by Dolman et al. [49] and Dolman and Wiedner [50]. Jiang and Nakano [51] also suggested that nitrogen plays a greater role than P in freshwater habitats characterised by a low nutrient supply, which is consistent with the nitrogen-limited oligotrophic Lake Vransko. In contrast to the findings of Bergström [9], according to which a low TN:TP ratio is characteristic of lakes with low productivity, Zhou et al. [52] argue that eutrophic lakes are generally characterised by low TN:TP ratios and that higher TN:TP ratios occur more frequently in mesotrophic and oligotrophic lakes. Since the lakes in the above study are highly eutrophic and have high N and P concentrations, their ratio is low, while Lake Vransko, with a low concentration of both nutrients, also has a low ratio. Therefore, with the exception of the above-mentioned Lake Vransko, these results are consistent with the potentially P-limited oligotrophic Lake Kozjak and the predominantly N- and P-limited oligo-mesotrophic lakes Visovac and Oćuša, as well as the mesotrophic lakes Crniševo and Prošće.

According to nitrogen and phosphorus as the main nutrients for phytoplankton growth, the lakes studied are nutrient-poor lakes and are described as oligotrophic. As the nutrient concentrations are low, the phytoplankton's need for their uptake is crucial. Jiang and Nakano [51] assumed that phytoplankton has a constant N requirement due to its importance for photosynthesis, while the P requirement can be more flexible due to adaptation and acclimatisation, as the cellular abundance of N in phytoplankton is less plastic than the P content according to Galbraith and Martiny [53]. Phytoplankton subject to dual N and P limitation would therefore have a higher requirement for N than for P, suggesting a greater importance of N for phytoplankton productivity in oligotrophic environments with a low nutrient supply [51]. This is consistent with the importance of TN as one of the determining factors for phytoplankton composition in low-nutrient lakes investigated in our study.

3.3. Composition of Phytoplankton FGs and Influence of Environmental Variables

As the results of this study show, the composition of phytoplankton reflects the effects of eutrophication, which is shown graphically in the cluster analysis, in which lakes with similar productivity are grouped together despite different grouping based on environ-

mental parameters. This confirms the role of phytoplankton as one of the most important biological elements in assessing the eutrophication gradient [14,54] and the importance of applying the concept of Reynold's functional groups in studies of phytoplankton in the environment [18–20]. The functional diversity was different for each lake and varied over time, but the coexisting functional groups are characteristic of natural oligotrophic to mesotrophic deep karst lake systems [22], which was also confirmed by the SIMPER analysis of FG composition in this study. The summarised results of this research provide an excellent basis and reference data for future observations of anthropogenic influence and climate change for the purpose of water management of the investigated lakes.

In this study, a comprehensive data set was used to investigate the response of phytoplankton to environmental changes, which will serve as a basis for further observation of changes due to anthropogenic influences and climate change in the lakes studied. The results showed that nutrients and water temperature have a significant influence on the phytoplankton community. These ecological indicators are directly influenced by climate change, which consequently affects the phytoplankton community and its biomass. Dory et al. [55] found that phytoplankton biovolume is more strongly influenced by the effects of temperature than by nutrient availability and also showed that the relative importance of temperature and nutrients for phytoplankton biovolume depends on the trophic status of lakes, with nutrients possibly playing a greater role in oligotrophic lakes, while temperature is more important in mesotrophic lakes. In nutrient-poor lakes, the lack of nutrients may prevent phytoplankton from responding to increasing water temperatures. In contrast, in more nutrient-rich environments, the removal of nutrient limitations increases the sensitivity of phytoplankton to warming. These findings and the results of our study provide a good basis for the further monitoring and investigation of phytoplankton composition and abundance in oligotrophic and mesotrophic lakes.

Although the study was conducted in karst lakes, the results may be generally applicable as the lakes studied are deep and stratified, just like many other lakes around the world with a cosmopolitan phytoplankton community and nitrogen and phosphorus as the main variables [56] to whose concentration changes the phytoplankton responds. The concentrations in the lakes studied are low, so the results obtained are very valuable for research and comparison with meso-oligotrophic lakes, which also have low nutrient levels. In addition, monitoring the composition and abundance of phytoplankton in relation to changing nutrient concentrations is essential for the functioning of freshwater ecosystems. The lakes studied represent largely intact ecosystems that can be used to study changes in the phytoplankton community under the influence of humans and the resulting climate changes.

Bray–Curtis similarity and clustering is a method for analysing beta diversity, and SIMPER analysis reveals characteristic and dominant taxa or functional groups in the categories/lakes studied. Both lack explanatory variables for a deeper understanding of environmental processes. Therefore, an RDA analysis was performed to explain the influence of environmental data on the phytoplankton composition. Regarding the specific nutrients in the two oligotrophic lakes, nitrogen and silicates, their higher concentrations in Lake Kozjak compared to Lake Vransko influenced the differences in phytoplankton community development. The FG composition of Lake Kozjak with codons **B**, **C**, **D** and **P** was more similar to Lake Prošće than to Lake Vransko, as these codons are more tolerant to light deficiency in both lakes than in Lake Vransko. Sensitivity to silicate depletion [18] could also influence the absence of the above-mentioned coda, as the silicate concentration is lowest in Lake Vransko. These factors, together with the higher alkalinity, determine other driving factors for lakes Kozjak and Prošće than for Lake Vransko. Desmids, which belong to codons **T** and **N** and favour environments with low alkalinity and nutrient content [57], are therefore one of the main components of Lake Vransko.

Cryptophytes in codon **X2** and their mesotrophic character is consistent with the occurrence in lakes Visovac and Oćuša and their tolerance to lower light conditions. The occasional oligo-mesotrophic character of these lakes is also consistent with the chloro-

phytes and ochrophytes of codon **X3**, which are characteristic of well-mixed oligotrophic environments [19] and occur in lakes Visovac and Oćuša. Both coda have a wide range of tolerance to changes in environmental conditions in these (oligo)mesotrophic lakes, confirming previous results [58,59]. The highest mean SiO_2 concentrations and the lack of light, especially in summer, most likely contributed to the high biomass and dominance of codon **B** in Lake Visovac, as this codon tolerates less light and is sensitive to Si depletion [18]. Codon **A**, specific for clear, deep lakes with low nutrients [18,19], favoured conditions with more light, especially in the less productive lakes Vransko and Kozjak.

Dinoflagellates in codon **L0** codominant with cholorophytes in coda **F** and **J**, both specific to mesotrophic lakes with clear epilimnion, favoured the higher water temperature and salinity in the warmest southernmost Mediterranean lakes Oćuša and Crniševo. Nitrogen-fixing cyanobacteria in codon **H1**, which are tolerant to low nitrogen, were found in Lake Crniševo according to their occurrence. Our results are in agreement with the findings of Li et al. [60], where chlorophytes were abundant in slightly brackish lakes (0.8–1.1 salinity). According to Maberly et al. [61], high temperatures are also favourable for the development of chlorophytes (codon **F**) and dinoflagellates (codon **L0**), which is consistent with the occurrence of this coda in our study. Codon **F** was frequently found codominant in lakes with clear epilimnion, but differences between Prošće, Vransko, Oćuša and Crniševo lakes in terms of light availability, temperature and nutrient availability are evident, confirming a wide range of tolerances and sensitivities for the species grouped in codon **F** [18,19,62]. The adaptability of codon **Y** to a wide range of habitats was consistent with the high frequency of occurrence in all lakes studied. Although the driving factor for its growth was the availability of light, its tolerance to low-light conditions with mixotrophic representatives enabled its occurrence during periods of low light [18,19,62,63]. Codon **E** also showed a high occurrence in the studied lakes; most likely, their mixotrophic character played an important role for the high biomass in Lake Oćuša, where the mean Secchi depth is the lowest, and in Lake Prošće, where light availability decreases strongly in summer.

4. Materials and Methods

4.1. Study Area

The Dinaric and Pannonian ecoregions represent two distinct ecological and geographical areas in Croatia (Figure 6). All six natural deep karst lakes, each covering an area of more than 0.5 km^2, are located in the Dinaric ecoregion. The Plitvice Lakes, located in the Dinaric Continental Subecoregion, were formed by a combination of tectonic shifts, the development of tufa formations and the presence of travertine barriers. A total of 16 barrage lakes have formed in the region, with Lake Kozjak being the deepest and largest, closely followed by Lake Prošće [4]. Both lakes have dimictic features typical of mountain lakes influenced by a continental climate. The other lakes are located in the Dinaric Mediterranean Subecoregion. The formation of Lake Visovac is a remarkable example of the lenticular dilation of the Krka River, which is also a lake formed by tufa formation [22]. The last three lakes are cryptodepressions on the Adriatic coast. The deepest, Lake Vransko on the island of Cres, was formed during the transition from the Pliocene to the Pleistocene [64]. Lakes Crniševo and Oćuša are part of the connected Baćina Lakes complex. Lake Crniševo has slightly brackish characteristics due to underground brackish water springs and saltwater intrusion due to its proximity to the sea. Lake Oćuša, the largest lake within the complex, although connected to Lake Crniševo, is a freshwater lake due to freshwater springs and as there is no water exchange between them [65]. The geographical coordinates and physical characteristics of these lakes are described in detail in a previously published article [23].

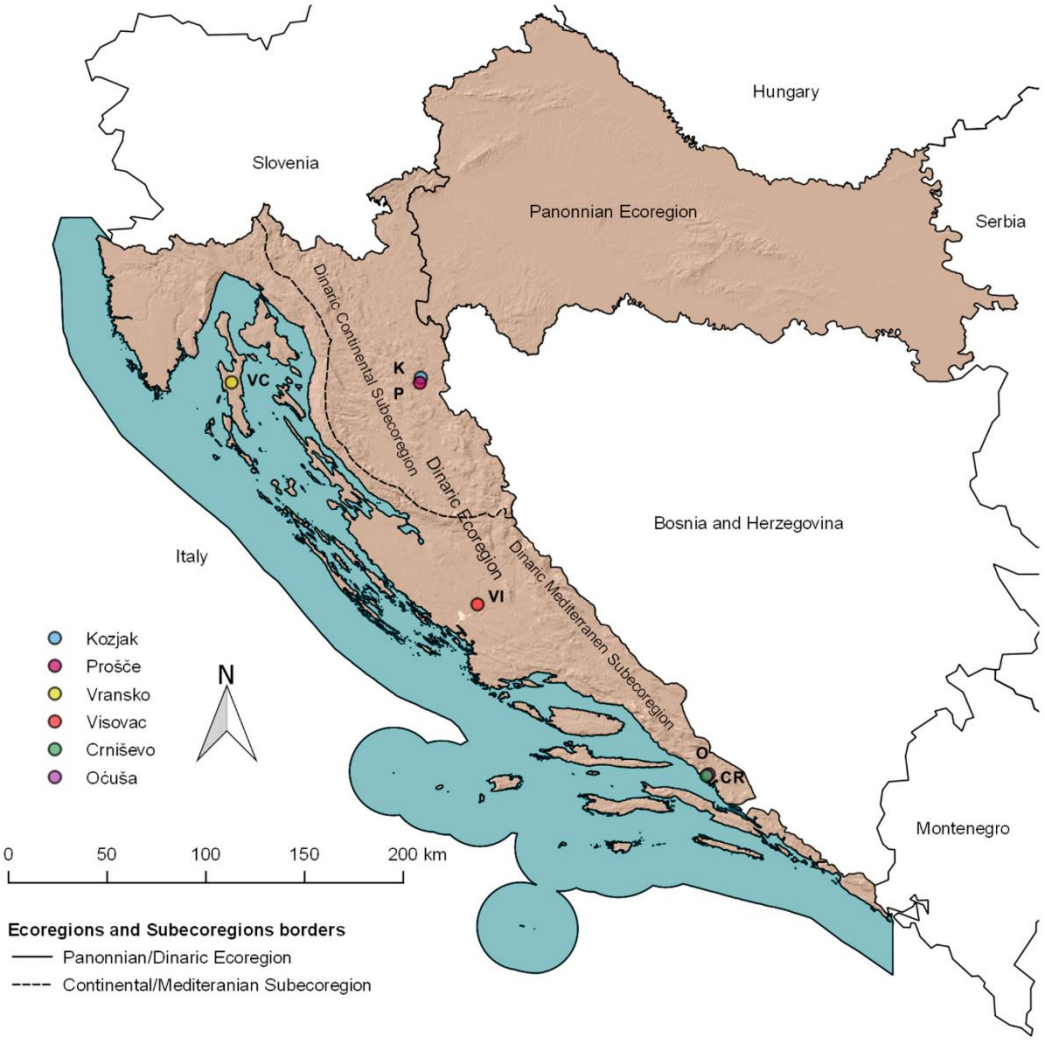

Figure 6. Map of investigated lakes. Lake codes: K—Lake Kozjak; P—Lake Prošće; VC—Lake Vransko; VI—Lake Visovac; CR—Lake Crniševo; and O—Lake Oćuša.

4.2. Sampling and Sample Analysis

The water samples were taken monthly during the growing season (April to September) in five to seven years between 2013 and 2022, with different dynamics depending on the water quality monitoring plan. A total of 209 phytoplankton samples were collected at the deepest point of each lake [66]. Both the phytoplankton samples and the samples for analysing the environmental parameters were collected as composite samples using a Uwitec water sampler. During thermal stratification, the composite samples were taken from the euphotic zone or the epilimnion, whichever was deepest, during the non-stratification period to a maximum depth of 20 metres. Immediately after collection, the phytoplankton samples were stored in 250 mL glass bottles and preserved with acidic Lugol's solution for further microscopic analysis. The phytoplankton was counted and identified according to the Utermöhl [67] method using an inverted microscope (Zeiss Axio Observer Z1 or Olympus IX 51 with DIC) at 400×, 200× and 100× magnification. The sedimentation units (unicellular, coenobium, filament or colony) were counted in random counting fields or transects until 400 sedimentation units were counted at 400× magnification, ensuring a counting error of less than 10% [68]. The individual cells were measured and their biovolume approximated to the nearest regular geometric shape. Biovolumes

were then calculated by determining the median size of up to 30 randomly selected cells within each taxon and multiplying this value by the observed taxon abundance. Biomass (fresh weight) was obtained from the biovolumes and used for subsequent analyses, with a conversion rate of 1 mm^3 L^{-1} equalling 1 mg L^{-1} [69,70]. Additional identification of diatoms was performed using permanent slides prepared by cleaning the samples with warm hydrochloric acid and hydrogen peroxide and then mounting them using Naphrax solution [71]. Diatoms were identified at 1000× magnification using an upright microscope (Zeiss Axio Observer Z1 or Olympus BX51 with DIC). After analysis, the names were revised in accordance with Algaebase [72], and the taxa were categorised into functional groups [18–20].

The measurement of environmental parameters is described in Stanković et al. [13].

4.3. Data Analysis

The map of the study area was created with QGIS 3.34 [73]. The cluster analysis of the environmental variables in the lakes based on Euclidean distance was carried out using Primer 7 software [74]. The chlorophyll a concentration (Chl-a), Secchi depth, total phosphorus, total nitrogen concentration and molar TN:TP ratio were displayed as boxplots in SCImago Graphica [75]. The Secchi depth, Chl-a, TP and TN were categorised into trophic status according to Miliša et al. [76], who modified OECD [77] boundaries to local conditions.

The Spearman correlation coefficient was used in this study to examine the relationships between phytoplankton biomass (including Chl-a and total biomass) and environmental variables in the lakes, using IBM SPSS Statistics [78]. Primer 7 software was also used for the cluster analysis of FG composition based on Bray–Curtis similarity. Prior to analysis, biomass was square-root-transformed.

Canonical redundancy analysis (RDA) was performed to evaluate the relationship between phytoplankton FG composition and environmental parameters in each lake. The analysis was performed using CANOCO 5.15 software [79]. All FGs, 209 samples and all environmental variables were included in the analysis. The ordination results were presented using correlation triplots. Phytoplankton biomass data were log-transformed, while environmental data were normalised prior to analysis. A draftman's plot was used to identify and remove variables with significant autocorrelation. Forward selection was then applied to data sets with response variables and environmental descriptors as explanatory variables. Only variables that showed significance at the level of $p \leq 0.05$ (999 permutations) were selected for further analysis.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/plants13162252/s1, Table S1: Complete list of phytoplankton taxa in the investigated lakes.

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MICROALGAL FUNCTIONAL TRAITS

Is salinity a driving factor for the phytoplankton community structure of a brackish shallow Mediterranean lake?

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Abstract Phytoplankton is a well-studied group of organisms that can change rapidly with environmental conditions, providing a reliable response to these changes. We analysed phytoplankton biomass, diversity, and its response to environmental conditions in the shallow brackish Mediterranean Lake Vransko. Although protected as Nature Park, the Lake's succession is enhanced by intensive agricultural activity and an artificial connection to the sea. Analysis of phytoplankton reveals a specific community composition strongly influenced not only by nutrients but also by salinity gradient, with species composition shifting from freshwater to brackish. Conditions of higher salinity support the dominance of brackish species, often with low biomass, while periods of low salinity are characterised by dominance of cyanobacteria or other freshwater species capable of rapidly taking up nutrients and forming algal blooms. Changes in water transparency caused by phytoplankton dynamics strongly influence the overall lake system through the availability of macrophyte growth and sediment fixation. These findings are critical for the future lake management, particularly its hydrological regime and maintenance of natural oligohaline and mesotrophic conditions. Understanding the response of phytoplankton to environmental conditions, exacerbated by anthropogenic influence and recent climate change, contributes to the protection of Mediterranean shallow lakes at local and global scales.

Keywords *Cosmarium tenue* · Cryptodepression · Phytoplankton functional groups · Polymictic lake · *Synedropsis roundii*

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Introduction

Phytoplankton play a critical role in aquatic ecosystems, contributing to carbon fixation from the atmosphere and producing the organic matter needed for food webs to function (Basu & Mackey, 2018). They

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are characterized by high diversity and rapid successional shifts in species composition that occur in response to changes in environmental factors. Identifying these factors and main processes that determine the seasonal succession of species in phytoplankton and community structure is one of the central aims in ecology (Masmoudi et al., 2015).

Shallow lakes represent unique ecosystems where hydrological and environmental factors (such as wind, precipitation or water inflow) have a strong influence on plankton development (Adrian et al., 2009; Rühland et al., 2015). Species composition and phytoplankton biomass in shallow lakes are mainly influenced by recurrent mixing of the water column (Weithoff et al., 2000). Shallow brackish lakes are unique in that they tend to have a variable species community that includes representatives from both freshwater and coastal marine environments. In such ecosystems, salinity acts as a switching mechanism that can shift the phytoplankton community from freshwater to more salt-tolerant during intermediate states of nutrient loading. Fluctuating salinity levels can induce changes in light intensity (Rijstenbil, 1987; Lionard et al., 2005) and nutrient availability, such as phosphorus release from sediments (Mohleji & Verhoff, 1980; Jordan et al., 2008; Hintz & Relyea, 2019) or presence of sulfur compounds (Cole et al., 1986; Liu et al., 2019) and ammonia (Rijstenbil, 1988; Seitzinger et al., 1991), which promote proliferation of selected species and may result in decrease in overall biodiversity (Moss, 1994; Flöder & Burns, 2004; Larson & Belovsky, 2013; Velthuis et al., 2023).

Phytoplankton covers a wide range of sizes, shapes, and taxonomic affiliations with precise ecological functions and roles in aquatic ecosystem processes. The morphological adaptations together with the functionality of the species gathered in the community lead to a specific set of traits. The assignment of functional traits of phytoplankton species allowed some classifications (Salmaso et al., 2015) that can be used in the interpretation of ecological processes. One of them is the highly acclaimed Reynolds' classification (Reynolds et al., 2002), which classifies species/taxa into robustly constructed groups based on their ecological characteristics and habitat properties. It has proven to be a powerful descriptor of phytoplankton succession in a wide range of freshwater lentic (Caroni et al., 2012; Žutinić et al., 2014; Allende et al., 2019) and lotic systems (Stanković et al., 2012; Wang et al., 2021), as well as a practical tool in bioassessment and application of phytoplankton as a biological quality element in ecological water quality assessment (Salmaso et al., 2015; Kruk et al., 2017).

In this study, we hypothesized that the phytoplankton community structure of a shallow brackish Mediterranean lake is primarily conditioned by changes in salinity. Our aim was to determine the effects of environmental variables on the phytoplankton community through a detailed analysis of phytoplankton composition and its relationship with different conditions of nutrients and salinity in a variable habitat, such as a shallow Mediterranean lake.

Materials and methods

Study area

Lake Vransko (syn. Lake Vrana) is located in the eastern Adriatic karst coastal area (Fig. 1). It is the largest natural freshwater lake in Croatia with an area of 30.02 km^2. The lake is located on the territory of Vransko jezero Nature Park, where it occupies more than half of the area. It is a polymictic,

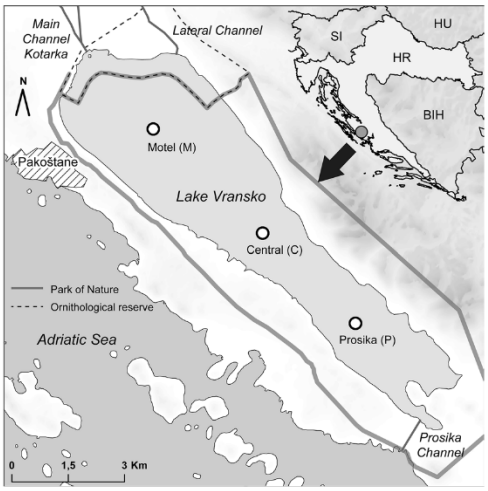

Fig. 1 Location of Lake Vransko in Croatia with main topographic features and designated sampling sites Motel (M), Prosika (P) and Central (C)

shallow cryptodepression with an average water depth between 2 and 2.5 m (Šiljeg et al., 2015). The catchment consists mainly of Cretaceous and Eocene limestones. A 0.8 to 2.5 km wide limestone ridge extends between the lake and the Adriatic Sea (Fajković et al., 2012). The lake is supplied with freshwater by several permanent freshwater springs and two artificial channels, Main (Kotarka) and Lateral channel which were built at the beginning of the twentieth century. The 800 m long Prosika artificial channel on the southwestern part of the ridge connects the lake with Adriatic Sea. Due to its location in the permeable karst area, Lake Vransko is usually slightly brackish, but in years with low freshwater inflow, higher salinity values can be measured (Rubinić & Katalinić, 2014). According to the EU Water Framework Directive (WFD 2000), Lake Vransko can be classified as a brackish surface water. Ornithological Reserve in the northwestern part and especially increased agricultural activity in the catchment contribute to external nutrient loads. In the warm summer months, macrophyte vegetation consisting predominantly of *Chara papillosa* Kützing with *Chara contraria* A.Braun ex Kützing and *Stuckenia* (*Potamogeton*) *pectinata* (L.) Börner dominate the bottom of Lake uniformly with a coverage of more than 50% (Gligora et al., 2007), presumably being responsible for high nitrogen uptake and triggering nitrogen limitation for phytoplankton (van Donk & Hessen, 1993; Jeppesen et al., 1997).

Sampling and analysis

Composite samples for phytoplankton and chemical analyses of water were collected monthly from April to September during 6 years (2004, 2009, 2014, 2016, 2017 and 2019) at two stations: Vransko Motel (M) and Vransko Prosika (P), apart from 2009 when the middle of the lake (C) was sampled, and 2019 when only station Vransko Motel was sampled. Each composite sample consisted of a mixture of equal volume discrete sample aliquots taken from subsurface (0.5 m), 1 m and 2 m depths.

Phytoplankton was counted using an Olympus BX51 inverted microscope equipped with DIC (differential interference contrast), following the Utermöhl (1958) method as described in (CEN - EN 15204, 2006). A minimum of 400 settling units were counted in a transect at 400 × magnification, yielding a counting error of < 10% (Lund et al., 1958; CEN - EN 15204, 2006). Biovolumes were calculated by determining an average individual size from up to 30 randomly selected cells of each taxon and then multiplying by the observed species abundance (Rott, 1981). Biomass (fresh weight) was derived from biovolumes and used for further analyses, where 1 mm^3 l^{-1} = 1 mg l^{-1} (CEN - EN 16695, 2015). Diatoms were identified from the permanent slides made by cleaning the net samples with warm hydrochloric acid and hydrogen peroxide and mounted in the Naphrax solution (CEN-EN 15708, 2009). They were examined under Olympus BX51 microscope at ×1000 magnification equipped with DIC. Taxa were identified to the lowest possible taxonomic level (species, genus) using relevant identification keys and names were assigned according to Algaebase (Guiry & Guiry, 2021). Phytoplankton species were classified into Reynolds' functional groups (Reynolds et al., 2002; Padisák et al., 2009).

Water transparency was estimated using a Secchi disc. Temperature, pH, conductivity, salinity, and dissolved oxygen were measured on site using a WTW Multiline P4 or HACH HQ40D Portable Multi Meter by measuring vertical profiles (0.5 m, 1 m, 2 m) which were then averaged. In years when salinity was not measured directly, it was calculated from conductivity values based on the linear equation obtained from samples when both parameters were measured in years 2014, 2017, and 2019 ($y = 0.0006x - 0.1291$; $R^2 = 0.9956$). Alkalinity was measured according to APHA (2005). Ammonia (NH_4^+-N), nitrites (NO_2^--N) and nitrates (NO_3^--N) were analysed using a Dionex 3000 ion chromatograph. Total nitrogen was analysed using a Shimadzu TOC-VCPH equipped with an analyser for TN (EN 12260:2003). Soluble reactive phosphorus (SRP) and total phosphorus (TP) were determined spectrophotometrically using a Perkin Elmer Lambda UV–VIS spectrometer (EN ISO 6878:2004; EN ISO 17294-2:2016).

Data analysis

To check for significance of changes in salinity on all stations sampled, PERMANOVA (Permutational multivariate analysis of variance) in Primer-E statistical package software (Anderson et al., 2015) was utilized with sampling site as a factor. The Shannon index (H') characterising community species diversity and the list of taxa that contributed more than 1% to

the total phytoplankton biomass in Lake Vransko during the study period were calculated based on species biomass in Primer v6 (Clarke & Gorley, 2006). To test the response of phytoplankton functional groups, total biomass, species number, and Shannon diversity to environmental conditions, Spearman's rho correlation coefficient was used in IBM SPSS Statistics 22 (IBM Corp. Released 2013). Canonical correspondence analysis (CCA) was performed in Canoco 5 (ter Braak & Šmilauer, 2012) to explore the relationships between Reynolds' FG assemblages and all environmental variables. Monte Carlo permutation test was applied to test the statistical significance of all axes, and forward selection was used to assess the importance of each variable. Environmental variables were normalised, and phytoplankton functional group biomass was transformed using the logarithm function prior to statistical analyses. Graphical plots were created using Grapher 15 (Grapher™ 2019).

Results

Physical and chemical variables of water

The description of the physical and chemical variables of the water ($n = 60$) with ranges, average values and standard deviation during the study period is given in Table 1. The salinity, total phosphorus (TP) and total nitrogen (TN) concentrations are presented in detail on Fig. 2a, b. Following the EU WFD categorization of transitional waters (WFD 2000), the 0.5‰ salinity level was set as a threshold between freshwater and brackish water (Fig. 2b). According to the physical and chemical properties of the water, Lake Vransko is a brackish lake with mesohaline, oxygen and nutrient rich water with meso- to eutrophic character.

During the study period salinity ranged from 0.7‰ (in 2016) to 3.8‰ (in 2009). Periods with low recorded salinity included years 2004, 2014, 2016, and 2019. These years were characterized by a low fluctuation of salt water spanning between 0.6 and 1.5‰, apart from the September sample of 2016 when a salinity pulse from the Prosika channel was recorded on station P (a threefold increase, from 0.65‰ up to 2‰). The year 2009 was distinguished with the highest salinity (over 3‰) measured in the middle of the Lake (station C) which lasted throughout the sampled months. For the year 2017, a very slow gradual increase of salinity levels was documented during spring on both stations (from 0.7‰ to 0.9‰, respectively), followed by a strong salinity pulse in summer with twofold and threefold salinity increase recorded on stations M and P, respectively.

Table 1 Range, average (Avg) and standard deviation (SD) values of physical and chemical variables of water in Lake Vransko over the study period from 2004 to 2019 for sampling stations Motel, Central and Prosika

Variable	Motel			Central			Prosika		
	Range	Avg	SD	Range	Avg	SD	Range	Avg	SD
Secchi depth (m)	0.6–2.5	1.5	0.5	1.0–1.5	1.2	0.5	0.6–3.1	1.8	0.5
Temperature (°C)	14.0–28.3	22.1	4.3	16.0–28.0	24.1	4.3	13.5–27.5	21.4	4.3
pH	7.1–9.3	8.4	0.5	7.6–8.7	8.2	0.5	7.0–9.3	8.3	0.5
Conductivity (µS cm^{-1})	1336–3480	2091	473	5400–6460	5873	473	1407–5300	2443	473
Salinity (‰)	0.7–1.8	1.1	0.2	3.1–3.7	3.4	0.2	0.7–2.9	1.3	0.2
Alkalinity (mg $CaCO_3$ l^{-1})	40.0–233.0	110.1	51.6	81.0–235.5	139.1	51.6	40.0–211.0	110.3	51.6
Dissolved oxygen (mg O_2 l^{-1})	8.2–14.8	10.4	1.6	8.7–12.0	10.1	1.6	7.4–12.4	9.7	1.6
Saturation (%)	95.0–179.0	119.5	20.7	92.4–149.7	120.6	20.7	89.5–143.7	110.3	20.7
Ammonia (µg N l^{-1})	0.004–0.394	0.068	0.106	0.020–0.240	0.093	0.106	0.004–0.574	0.113	0.106
Nitrites (µg N l^{-1})	0.001–1.070	0.120	0.258	0.002–0.032	0.015	0.258	0.001–1.290	0.144	0.258
Nitrates (µg N l^{-1})	0.010–2.31	0.375	0.591	0.55–1.913	0.950	0.591	0.010–2.040	0.390	0.591
Total nitrogen (TN) (µg N l^{-1})	0.510–2.360	1.064	0.462	0.646–2.158	1.204	0.462	0.570–2.320	1.076	0.462
Soluble reactive phosphorus (SRP) (µg P l^{-1})	0.002–0.024	0.007	0.006	0.003–0.020	0.006	0.006	0.001–0.024	0.006	0.006
Total phosphorus (TP) (µg P l^{-1})	0.007–0.055	0.021	0.011	0.008–0.030	0.021	0.011	0.003–0.059	0.023	0.011

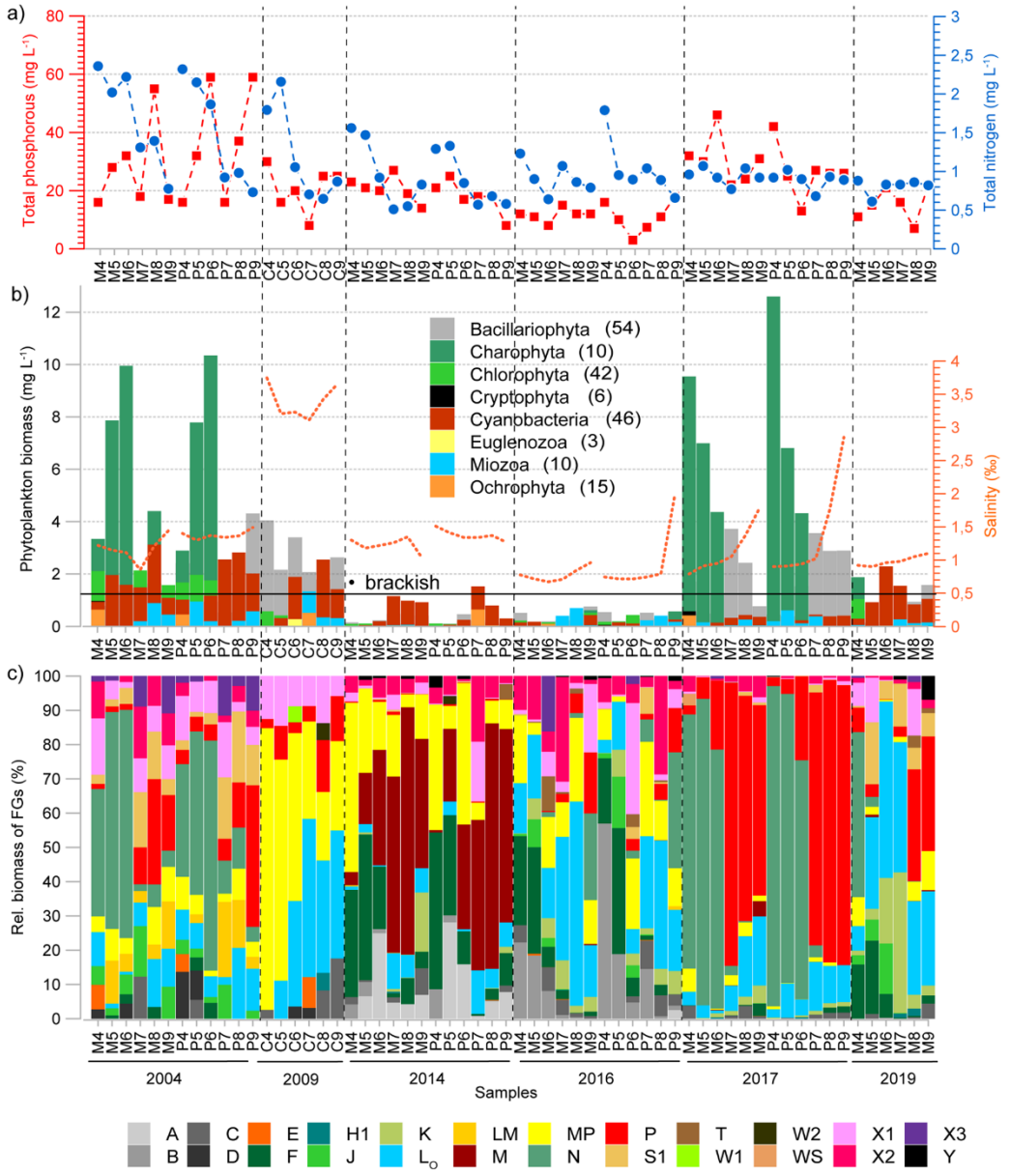

Fig. 2 Relative abundance of functional groups, biomass of phytoplankton taxonomic groups, salinity, total phosphorus, and total nitrogen at sampling sites Motel (M), Prosika (P) and Central (C) in Lake Vransko throughout the study period. The number of taxa in each taxonomic group is given in parentheses. Samples are labelled with the sampling site code and a month

The results of PERMANOVA confirmed a significant influence of spatial changes in salinity when sampling sites were used as a factor ($P = 0.0001$; $P < 0.05$), showing that salinity was significantly different on all three sampling sites. Regarding nutrient concentrations, both TP and TN have shown annual and interannual variability (Fig. 2a). The concentration of TP ranged from 2 µg P l^{-1} (in 2016) up to 60 µg P l^{-1} (in 2004), with years 2009, 2014, 2016, and 2019 characterized by lower values (> 30 µg P l^{-1}). In the year 2004 TP concentration on both stations gradually increased towards late summer (September), while during 2017 the highest TP concentrations were recorded at the beginning of summer (June). The highest concentration of TN was measured in 2004 (2.3 mg N l^{-1}) and the lowest in 2014 (0.4 mg N l^{-1}). Years with a higher variation of TN were 2004 and 2009, in which a spring concentration of TN decreased by a threefold in summer (from 2.3 mg N l^{-1} to 0.7 mg N l^{-1}, respectively). Similarly, years 2014 and 2016 were characterized by a twofold decrease of TN concentration. Finally, the years 2017 and 2019 were marked by a nearly steady concentration of TN (around 0.8 mg N l^{-1} in both years).

Phytoplankton composition and diversity

Phytoplankton dynamics expressed as biomass of taxonomic groups, relative abundance of functional groups together with salinity, TP and TN are shown in Fig. 2. Among the 186 taxa identified throughout the study period, taxonomic groups with the highest number of taxa were Bacillariophyta (54), Cyanobacteria (46) and Chlorophyta (42). A high number of taxa (118) appeared in at least one sample, accounting for > 1% of the total biomass (Supplementary Table 1). Nevertheless, only 18 taxa were dominant or subdominant and contributed > 20% biomass (mg of wet weight) in individual samples.

Total phytoplankton biomass varied from 0.25 mg l^{-1} to 15.09 mg l^{-1} in 2017 and 2004, respectively (Fig. 2b). In 2004, which was characterized by low salinity and high TP, Charophyta contributed the most to the total biomass, with a bloom of *Cosmarium tenue*, which belongs to the functional group **N** (Fig. 2a–c). Several cyanobacterial representatives were also present with high biomass, especially *Komvophoron pallidum* and *Planktolyngbya contorta* from functional group **S1**, and *Gomphosphaeria aponina* from functional group $\mathbf{L_M}$.

The bloom of *C. tenue* also occurred in 2017 in similar conditions of low salinity and high TP, but once salinity began to increase and TP decreased (Fig. 2a, b), diatom *Synedropsis roundii* became dominant as the representative of functional group **P** (Fig. 2c). In 2009, when the highest salinity values were present at moderate to low TP values, Bacillariophyta and Cyanobacteria alternated in dominance. The first group was represented by the species *Tetramphora croatica* and *Navicula trivialis*, which belong to the functional group **MP**, while functional group $\mathbf{L_O}$ included mainly the cyanobacterial representatives *Chroococcus minutus*, *Chroococcus turgidus*, *Merismopedia tranquilla* and *Snowella lacustris*.

The years 2014, 2016 and 2019 were characterized by low salinity, low nutrient concentration and low phytoplankton biomass. In 2014, Cyanobacteria were dominant or co-dominant with Chlorophyta. Functional group **M** included most cyanobacterial species with *Microcystis aeruginosa* and *Microcystis novacekii* as typical representatives, while Chlorophyta were represented with functional group **F** including *Dictyosphaerium subsolitarium* and *Raphidocelis danubiana* as the most dominant. The year 2016 was the most diverse in terms of changes in the dominance of different taxonomic and functional groups. Nevertheless, it was dominated by benthic diatoms from functional group **MP**, *Peridiniopsis borgei* representing Miozoa (dinoflagellates) and functional group $\mathbf{L_O}$, chlorophytes represented by *Crucigenia tetrapedia* and *R. danubiana* from functional group **F**, and cryptophyte species *Plagioselmis nannoplanctica* belonging to functional group **X2**. The year 2019 was characterized by a brief spring dominance of *C. tenue*, which was displaced by cyanobacterial species *M. punctata*, *Rhabdoderma lineare*, *Snowella atomus* and *Woronichinia compacta* from functional group $\mathbf{L_O}$ and *Anathece smithii* from functional group **K**. Towards the end of the sampling season with a slow increase in salinity, they were replaced by diatom *S. roundii* from codon **P**.

Number of taxa (S) and Shannon diversity index (*H'*) are displayed on Fig. 3. The years 2004 and 2009 were characterized by high Shannon diversity index and low number of taxa. High values of both indices were recorded in 2014 and 2016, after which the number of taxa and diversity index dropped in 2017.

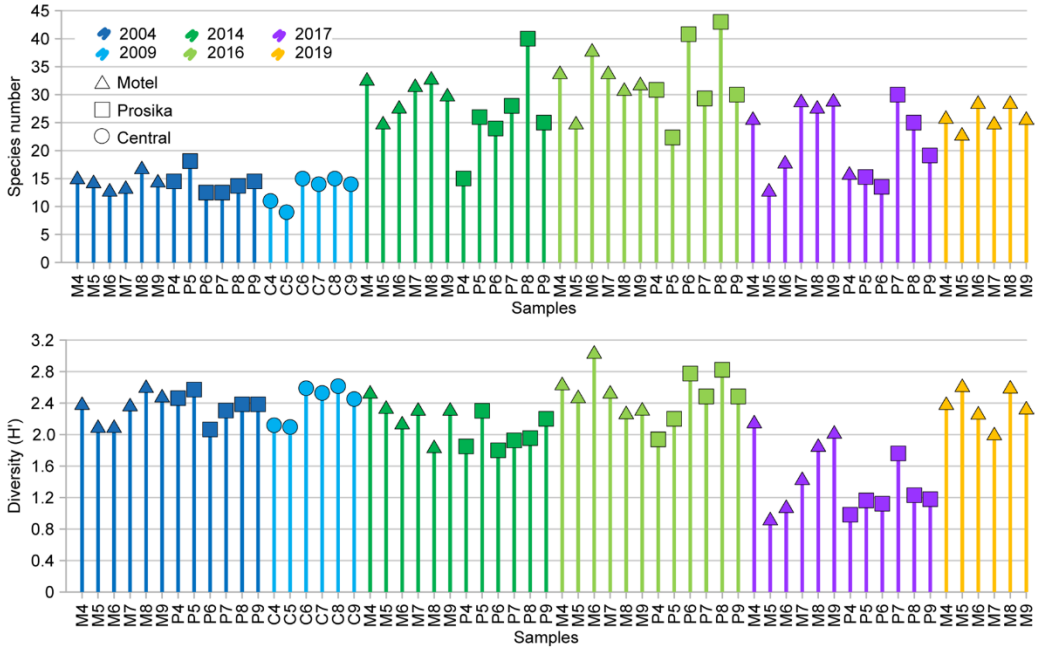

Fig. 3 Number of taxa (S) and Shannon diversity index (*H'*) of phytoplankton at sampling sites Motel (M), Prosika (P) and Central (C) in Lake Vransko throughout the study period. Samples are labelled with the sampling site code and a month. Years are marked with different colours, and sampling sites with different symbols

This was followed by a slow increase in both indices again in 2019.

Response of phytoplankton to environmental conditions

Biological descriptors such as functional groups, total biomass, species number and Shannon diversity index showed significant Spearman's rho correlations with environmental variables (tested levels of significance: $P \leq 0.05$ and $P \leq 0.01$; Table 2). Total phytoplankton biomass correlated positively with SRP, TP and nitrates, and negatively with Secchi depth and ammonium, but had no significant correlation with salinity. The relationship between phytoplankton functional groups and environmental variables indicated several main determining factors of the phytoplankton community dynamics. Higher biomass of the benthic functional group **MP** was measured during the period of high salinity and showed a positive correlation, while functional groups with typical freshwater representatives (**B**, **F**, **J**, **K**, **T**) showed a negative correlation with salinity. Functional group **N,** characterized by the highest measured biomass in 2017, showed a negative correlation with salinity and a positive correlation with TN, SRP and TP, while functional group **P**, represented mainly by a typical brackish water species *S. roundii*, correlated positively with salinity and TP. Phytoplankton richness, presented as species number, significantly decreased with higher salinity and higher TP, while phytoplankton diversity increased with high concentration of nitrogen ions, decreased with higher concentration of TP and showed no significant correlations with salinity.

The relationship between phytoplankton composition based on functional groups and environmental variables is summarized on the CCA ordination, reflecting the importance of salinity, water transparency and nutrients in shaping the phytoplankton community in Lake Vransko (Fig. 4; Table 3). The eigenvalues of the first two axes (0.147 and 0.103, respectively) explained 59.0% in constrained

Table 2 Spearman's rho correlations (2-tailed) between selected biological descriptors and environmental variables

	Secchi depth	Temperature	pH	Conductivity	Salinity	Alkalinity	Oxygen concentration	Saturation	Ammonia	Nitrites	Nitrates	Total nitrogen	Soluble reactive phosphorus	Total phosphorus
A	**0.338****	n.s	n.s	0.281*	n.s	0.288*	**− 0.367****	− 0.308*	**0.413****	n.s	n.s	**− 0.344****	n.s	n.s
B	0.288*	n.s	− 0.285*	**− 0.409****	**− 0.433****	0.309*	n.s	n.s	**0.505****	**0.711****	n.s	n.s	n.s	**− 0.431****
C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
D	n.s	n.s	n.s	n.s	0.276*	n.s	n.s	n.s	n.s	n.s	**0.372****	0.315*	n.s	n.s
E	n.s	n.s	n.s	n.s	n.s	n.s	0.266*	n.s	n.s	n.s	n.s	n.s	n.s	− 0.317*
F	n.s	n.s	n.s	− 0.256*	− 0.290*	**0.364****	n.s	− 0.311*	n.s	0.262*	n.s	n.s	n.s	n.s
H1	n.s	**0.435****	**0.376****	n.s	n.s	− 0.297*	− 0.272*	n.s	n.s	**− 0.362****	**− 0.396****	**− 0.355****	n.s	n.s
J	n.s	n.s	n.s	**− 0.365****	− 0.284*	n.s	n.s	n.s	n.s	**0.376****	0.272*	n.s	0.278*	n.s
K	n.s	n.s	n.s	**− 0.435****	**− 0.474****	**− 0.443****	n.s	n.s	**− 0.349****	n.s	**− 0.682****	**− 0.410****	n.s	− 0.296*
L_O	− 0.258*	n.s	n.s	n.s	n.s	**− 0.406****	n.s	0.320*	**− 0.428****	n.s	n.s	n.s	n.s	n.s
LM	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	0.322*	n.s	n.s	**0.355****
M	0.283*	n.s	n.s	n.s	n.s	n.s	**− 0.394****	− 0.275*	n.s	**− 0.394****	− 0.281*	**− 0.425****	n.s	n.s
MP	n.s	n.s	n.s	0.312*	**0.360****	n.s	n.s	n.s	n.s	n.s	**0.554****	n.s	n.s	n.s
N	**− 0.405****	n.s	n.s	**− 0.358****	− 0.268*	− 0.303*	0.320*	n.s	**− 0.391****	n.s	n.s	0.277*	**0.506****	0.279*
P	− 0.327*	n.s	**0.352****	n.s	0.293*	**− 0.640****	n.s	0.271*	**− 0.646****	**− 0.441****	n.s	n.s	n.s	**0.475****
S1	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	**− 0.434****	n.s	n.s	n.s	0.257*	n.s
T	n.s	n.s	n.s	− 0.318*	**− 0.340****	n.s	n.s	n.s	n.s	n.s	**− 0.408****	− 0.321*	n.s	**− 0.464****
W1	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
W2	n.s	0.256*	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
WS	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
X1	n.s	n.s	n.s	n.s	0.258*	n.s	n.s	n.s	n.s	n.s	**0.548****	n.s	n.s	n.s
X2	0.291*	n.s	n.s	− 0.255*	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
X3	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	**0.308***	n.s	n.s	n.s
Y	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TB	**− 0.478****	n.s	0.311*	n.s	n.s	− 0.311*	n.s	n.s	**− 0.522****	− 0.308*	0.282*	n.s	**0.413****	**0.528****
Spec. Num	0.271*	n.s	n.s	**− 0.337****	**− 0.412****	n.s	n.s	n.s	n.s	n.s	**− 0.526****	**− 0.407****	− 0.313*	**− 0.379****
Shannon	n.s	n.s	− 0.294*	n.s	n.s	n.s	n.s	n.s	0.309*	**0.497****	0.313*	n.s	n.s	**− 0.403****

n.s.: not significant; total number of samples for all variables is 60

*Correlation is significant at the $P \leq 0.05$ level (in bold)

**Correlation is significant at the $P \leq 0.01$ level (in bold)

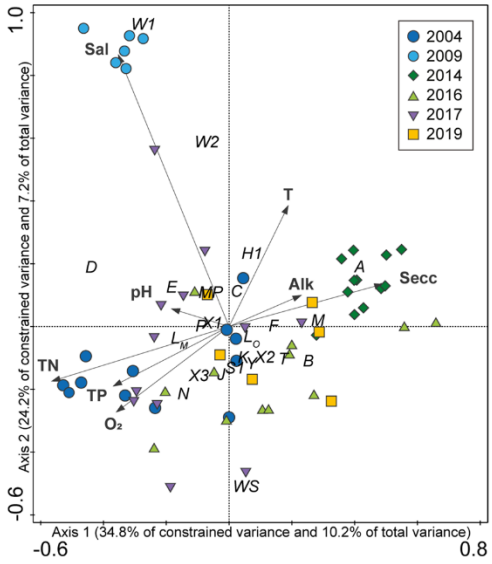

Fig. 4 Canonical correspondence analysis (CCA) triplot of phytoplankton functional groups, environmental variables and samples from all years studied. Environmental variables are abbreviated as: *Secc* Secchi depth, *T* temperature, O_2 Dissolved oxygen, *Sal* Salinity, *Alk* Alkalinity, *TP* Total phosphorus, *TN* Total nitrogen

variance of the functional group composition and environmental data. Pearson correlation analysis between functional groups and environmental variables (0.779 and 0.791, respectively) calculated for the first 2 axes indicated significant positive correlation between abiotic values and the phytoplankton functional group variables. According to the Monte Carlo permutation test, the ordination of the two axes was statistically significant ($P < 0.05$). Axis 1 correlated strongly with TN ($R = -0.440$), Secchi depth ($R = 0.382$), TP ($R = -0.287$) and O_2 ($R = -0.280$), while axis 2 had a high correlation with salinity ($R = 0.685$) and temperature ($R = 0.305$). The highest positive correlation with salinity and temperature were shown by euglenophytes from the coda **W1** and **W2,** together with pennate diatoms belonging to group **MP,** *Cyclotella meneghiniana* from group **C** and heterocytous cyanobacterial taxon *Anabaena* from association **H1**. Colonial chrysophyte genus *Dinobryon* from codon **E** correlated positively with salinity and

Table 3 Results of the canonical correspondence analysis (CCA) between FGs and environmental variables

	Axis 1	Axis 2
Axis summary statistics and variance in species data[a]		
Eigenvalues	0.147	0.103
FGs-environment correlations	0.779	0.791
Cumulative percentage variance		
Of FGs data	10.2	17.4
Of FGs-environment relation	34.8	59.0
Correlations of environmental variables and redundancy axes[b]		
Variable		
Secchi depth	0.3818	0.1071
Temperature	0.1467	0.3047
pH	− 0.143	0.0449
Salinity	− 0.2748	0.6847
Alkalinity	0.1792	0.0789
Dissolved oxygen	− 0.2798	− 0.2156
Total nitrogen	− 0.4399	− 0.1377
Total phosphorus	− 0.2873	− 0.1501

[a]Axis summary statistics of the two canonical axes extracted and the percentage of variance explained by CCA ordination

[b]Correlation of environmental variables with ordination axes

pH. Coda **P** (*Synedropsis roundii*) and **X1** (mainly chlorococcales from the genera *Ankistrodesmus* and *Monoraphidium*) were positioned close to the centroid with equal influence of all variables plotted in the biplot, while functional groups **N** (*Cosmarium tenue*), $\mathbf{L_M}$ (*Gomphosphaeria aponina*), **X3** (*Koliella longiseta* f. *tenuis*) correlated positively with nutrients (TP, TN) and dissolved oxygen concentration, and negatively with salinity. Centric diatoms from functional groups **A** (*Thalassiosira* spp.) and **B** (*Pantocsekiella ocellata*) colonial cyanobacteria from codon **M** (*Microcystis* spp.), and chlorophytes belonging to groups **F** and **T** were negatively associated with pH, salinity and nutrient concentration and positively associated with alkalinity and water transparency. Typical freshwater representatives, such as filamentous cyanobacteria from group **K** (*Aphanocapsa* spp.), colonial small-celled cyanoprokaryotes from group **S1** (*Planktolyngbya* spp.), flagellated cryptophytes from coda **X2** and **Y** (mainly *Plagioselmis nannoplanctica* and *Cryptomonas* spp.), with codon **J** coenobial chlorophytes (*Lemmermania tetrapedia*), correlated negatively with salinity.

Discussion

Phytoplankton biomass in shallow brackish lakes is determined by the strong interaction of nutrients and salinity (Meerhoff et al., 2012). Such systems are usually characterized by periodic alternations between freshwater and brackish phases, depending on the environmental stresses exerted on them (Obolewski et al., 2018). Although the salinity threshold below 0.5‰ (WFD, 2000) was not noted in Lake Vransko during the study period, the years with very low intrusion of seawater were regarded as the lake's freshwater phase, whilst the years with pulses of salinity were characterized as brackish phase (Fig. 2b). During the freshwater phase, the phytoplankton biomass of a subsequent brackish Lake Vransko is mainly controlled by the availability of nutrients (TP and TN) and water transparency. The years 2004 and 2017, when the highest biomass was reached, were both characterized by the spring bloom of *Cosmarium tenue*. This small-sized unicellular charophyte species is an indicator of mesotrophic conditions (Coesel & Meesters, 2007) included in codon **N**, which is characteristic of spring and early summer periods in temperate, shallow lakes with a mixed layer. The combination of both R- and SR-strategies allows this desmid to exploit its high affinity for phosphorus at low concentrations (Reynolds, 2006) and successfully dominate the phytoplankton community. Gligora Udovič and Plenković-Moraj (2003) highlighted species of the genus *Cosmarium* as important members of the spring–summer phytoplankton community of Njivice and Ponikve lakes, two shallow Mediterranean lakes in the northern region of Adriatic Sea (Croatia). Gligora et al. (2007) reported that the phytoplankton community of Lake Vransko in 2004 was clearly determined by the winter-spring prevalence of *Cosmarium tenue*, which was controlled by the availability of nutrients, mainly nitrogen compounds. In the summer and autumn of 2004, *C. tenue* was replaced by *Synedropsis roundii* (described as *Synedra* sp.) after the concentration of nutrients decreased (Gligora et al., 2007). This coincides with two summer-related events, the occurrence of high macrophyte cover and the drop in freshwater levels with consequent saltwater intrusion through the Prosika channel. The high cover of aquatic macrophytes in Lake Vransko in summer is probably responsible for high nitrogen uptake, triggering nitrogen limitation for phytoplankton (van Donk & Hessen, 1993; Jeppesen et al., 1997; Gligora et al., 2007). This influence on phytoplankton biomass and recorded changes in species dominance (Gligora et al., 2007) was also confirmed here, with high (Shannon) species diversity and low numbers of taxa present in 2004, 2009 and 2017, both showing a negative correlation with nitrate, TN, SRP and TP. On the other hand, long-term saltwater intrusion can cause fundamental changes in natural biogeochemical cycles and ecosystem structure and function (Widney et al., 2019), which include soil salinization, increased ammonium release, decreased plant productivity, declining species richness, and decreased N retention (Herbert et al., 2015; White Jr. & Kaplan, 2017). Increased ammonium release can lead to further eutrophication (Howarth & Paerl, 2008; Widney et al., 2019), which is more prevalent in oligotrophic systems, as the nitrification process usually starts earlier in lakes of higher trophic status and has a faster ammonium turnover (Leoni et al., 2018).

As shown by statistical analyses, the salinity below 2‰ and moderate availability of both TP and TN still support development of *Cosmarium tenue*. The summer dominance of the codon **P** representative *Synedropsis roundii* is associated with the depletion of nitrogen from the water column (Gligora et al., 2007), since phosphorus limitation during the summer period only affects *C. tenue* (Sommer, 1987). Indeed, *Synedropsis roundii* becomes competitive for phosphorus even in environments with low TP and TN concentrations and therefore displaces *Cosmarium* during summer periods when nitrogen is depleted from the water column. Lake Vransko as a shallow, temperate, continuously mixed lake with an average depth of 2–3 m represents an optimal habitat for groups **N** and **P** (Padisák et al., 2009), both of which indicate meso- to eutrophic conditions. Both coda **N** and **P** generally respond poorly to increased salinity, indicating that in years when these functional groups dominated the community (2004 and 2017), salinity was in the lower range.

In addition to *C. tenue*, several cyanobacterial representatives with high biomass were also present, especially the solitary, filamentous, non-nitrogen-fixing species *Komvophoron pallidum* and *Planktolyngbya contorta* from the **S1** functional group, and *Gomphosphaeria aponina* from the $\mathbf{L_M}$ functional group. Both **S1** species are shade-adapted cyanoprokaryotes

characteristic of turbid mixing environments and sensitive to flushing (Reynolds et al., 2002; Padisák et al., 2009), which may be common in the mixing period. On the other hand, the species of codon $\mathbf{L_M}$ are typical of small- to medium-sized lakes of higher trophic levels and usually occur in a highly irradiated part of the water column. Their representative, the small cyanobacterium *G. aponina*, is a freshwater species (Komárek & Hindák, 1988; Komárek & Anagnostidis, 1999) that achieves the favoured conditions of high light availability by forming spherical colonies in which each cell is connected to mucilaginous stalks and by buoyancy-regulating gas vesicles (Reynolds, 2006). In a series of mesocosm bioassays with varying N and P concentrations, Ma et al. (2015) showed that *Cosmarium* growth is strongly promoted under the influence of moderate N and P supply. These findings are congruent with our results, as the biomass of *C. tenue* significantly exceeded that of colonial and filamentous cyanobacteria.

The highest salinity levels (above 3‰) with moderate to low nutrients in 2009 negatively impacted total phytoplankton biomass and richness (number of species). However, these conditions were not limiting for the functional group **MP,** which is tolerant to frequent mixing and turbidity and is mainly composed of benthic diatoms (*Tetramphora croatica* and *Navicula trivialis*) and cyanobacterial representatives of the codon $\mathbf{L_O}$ (*Chroococcus minutus*, *Chroococcus turgidus*, *Merismopedia tranquila* and *Snowella lacustris*), which are assigned to shallow, medium to large lakes with a wide trophic range (Reynolds et al., 2002; Padisák et al., 2009). Both coda showed higher biomass and a positive correlation with salinity. *Navicula trivialis* is a benthic freshwater pennate diatom that is widely distributed in lakes and tolerates a wide range of pH, conductivity, and alkalinity (Lange-Bertalot, 2001; Rushforth & Spaulding, 2010). *Tetramphora croatica* is a benthic pennate diatom that has only recently been described and previously only detected from sediment core and diatom mat samples from Lake Vransko (Caput Mihalić et al., 2019). Nanoplanktonic species from the genus *Chroococcus* (*C. minutus*, *C. turgidus*), which occur as small spherical or compact colonies enveloped in mucus, are common in mesotrophic and eutrophic alkaline lakes with higher alkalinity (Reynolds, 2006; Komárek & Johansen, 2015). The sheet-forming colonial *M. tranquila* and the gas-vacuolate, stalk-forming colonial *S. lacustris* are also common in freshwater ecosystems (Komárek & Anagnostidis, 2000; Komárek & Johansen, 2015), as indicators of eutrophic conditions (Fernández et al., 2012; Bukowska et al., 2017). All of the above cyanobacterial taxa are known to form loose associations with emergent plants in shallow lakes and are known members of planktonic and metaphytic assemblages in shallow lakes (Vincent, 2009; Komárek & Johansen, 2015). However, high correlation of codon W1 with salinity during conditions of higher salinity in 2009 (Fig. 4) was not corroborated by further statistics (Table 2), most likely as result of a sole observation. *Euglena obtusa*, a representative of this association, is a cosmopolitan, widespread species which can be found in both freshwater and brackish systems and estuaries, usually occurring in habitats rich in organic matter, and is an indicator of moderate organic pollution (Ernest & Pringsheim, 1949; Steffensen, 1974; Reynolds et al., 2002; Wolowski, 2003).

Low concentrations of TP and TN, coupled with low salinity, in 2014, 2016, and 2019 constrained total phytoplankton biomass, confirming the strong causal relationship between primary production, water transparency and nutrient availability in Lake Vransko. These years were characterized by a strongly expressed macrophyte vegetation consisting predominantly of *Chara papillosa* Kützing with *Chara contraria* A.Braun ex Kützing and *Stuckenia* (*Potamogeton*) *pectinata* (L.) Börner as subdominant species, covering 60–90% of the total lake bottom (Alegro et al., 2019; Vuković et al., 2020). Both characeans are subcosmopolitan algae that have been described from a range of meso- and eutrophic shallow habitats and regularly form associations with *Stuckenia* (Van den Berg et al., 1999; Schneider et al., 2016). Although primarily a freshwater species, *C. contraria* is reported to be sensitive to reduced light and increased salinity (Steinhardt & Selig, 2011) whereas *C. papillosa* is known for its intermediate ecophysiological characteristics and salinity-specific adaptation to different habitats (Boegle et al., 2010; Nowak & Schubert, 2019). *Stuckenia pectinata* is a rhizomatous, vascular aquatic plant characteristic of eutrophic or brackish waters where it can form dense stands in lakes, is tolerant of disturbance, and has been found in highly calcareous, nutrient-poor lakes (Hill et al., 2004).

Although conditions of low nutrients, high light availability, and low salinity favoured stable macrophyte dominance over the 3-year period (2014, 2016 and 2019, respectively), several phytoplankton taxa from the Cyanobacteria and Chlorophyta groups were nevertheless successful in exploiting the remaining gaps, possibly through allelochemical competition (Mulderij et al., 2007; Mohamed, 2017) or via seasonal dynamics (Muylaert et al., 2010; Sayer et al., 2010; They et al., 2014). In 2014 the main representatives included cyanobacteria from codon **M** (*Microcystis aeruginosa*, *M. novacekii*), which co-dominated with chlorophytes from functional group **F** (*Dictyosphaerium subsolitarium* and *Raphidocelis danubiana*). The phytoplankton assemblage of 2019 was even more clearly dominated by cyanobacteria, namely by the groups $\mathbf{L_O}$ (*Merismopedia tranquila, Rhabdoderma lineare*, *Snowella atomus* and *Woronichinia compacta*) and **K** (*Anathece smithii*). The habitat template of these associations, which includes clear, shallow, medium-sized lakes covering a wide trophic status (Reynolds et al., 2002; Padisák et al., 2009), was consistent with the ecological and morphological characteristics of Lake Vransko and was also confirmed by statistical analyses (Spearman's rho correlation and CCA, Table 2; Fig. 4).

The lowest measured salinity and TP were the defining factors for the highest recorded diversity and species richness in 2016. Despite being well adapted to these variables (Reynolds et al., 2002; Padisák et al., 2009), several functional groups, such as benthic diatoms from functional group **MP** (*Mastogloia smithii*, *Envekadea hedinii*), functional group $\mathbf{L_O}$ (*Peridiniopsis borgei*), chlorophytes from functional group **F** (*Crucigenia tetrapedia*, *Raphidocelis danubiana*) and functional group **X2** (*Plagioselmis nannoplanctica*), alternated with an overall low biomass. However, neither group was able to occupy a permanently dominant position, reflecting the conditions under which the effects of shading, nutrient limitation, resuspension of sediment particles and excretion of allelopathic substances from macrophytes severely limited phytoplankton growth (Gligora et al., 2007; Mulderij et al., 2007).

Analysis of phytoplankton over several years in the Mediterranean shallow Lake Vransko reveals a specific community composition that is strongly influenced not only by nutrients but also by the salinity gradient, with species composition shifting from freshwater to brackish water, confirming our hypothesis. Conditions of higher salinity support the dominance of brackish benthic species, often of low biomass, with the dominance of *Tetramphora croatica*. Periods of low salinity are characterised by freshwater composition, with a shift in dominance between *Cosmarium tenue/Synedropsis roundii*, cyanobacteria or colonial green algae capable of rapidly uptake nutrients and form algal blooms. The results suggest that changes in lake transparency due to changes in algal community structure strongly influence the overall lake system through the availability of macrophyte growth and sediment fixation. A highly dynamic and specific environment, such as that present in Lake Vransko, supports high levels of diversity and the discovery of new species. The results of this study are valuable for future management of the lake, particularly with respect to maintaining the hydrologic regime and natural oligohaline and mesotrophic conditions. Understanding the responses of phytoplankton to environmental conditions, exacerbated by anthropogenic influence and current climate change, contributes to the protection of Mediterranean shallow lakes at local and global scales.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest All authors declare they have no financial or non-financial interests to disclose.

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3. RASPRAVA

Istraživanje obuhvaćeno publikacijom **I** u sklopu ove doktorske disertacije uspoređuje taksonomski i funkcionalni sastav zajednice fitoplanktona identificiran morfološkim pristupom i analizom okolišne DNA. U sklopu publikacije **II**, korištenjem kvalitativnih i kvantitativnih podataka ocijenjeno je ekološko stanje (HLPI indeks) temeljem fitoplanktona identificiranog morfološkim pristupom i analizom okolišne DNA. Primjenom Reynoldsovog koncepta funkcionalnih grupa i koncentracije klorofila *a* u publikaciji **II**, cilj je bio utvrditi mogućnosti primjene sastava zajednice fitoplanktona identificirane molekularnim pristupom te utvrditi prednosti i nedostatke analize okolišne DNA u svrhu ocjene ekološkog stanja. Ista kategorija ocjene ekološkog stanja utvrđena je u 89% uzoraka usporedbom korištenih metoda, bez obzira na zabilježene razlike u taksonomskom i funkcionalnom sastavu zajednice fitoplanktona identificiranog morfološkim i molekularnim pristupom.

Publikacije **III** i **IV** ispituju odgovor biomase taksonomskog i funkcionalnog sastava zajednice fitoplanktona na okolišne čimbenike u plitkom i dubokim prirodnim krškim jezerima. Okolišni čimbenici s najvećim utjecajem na zajednicu fitoplanktona istraživanih jezera su hranjive tvari, alkalitet, salinitet, svjetlost i temperatura.

Korištenjem višegodišnjeg seta podataka obuhvaćenog publikacijama **III** i **IV**, u plitkom (2004. – 2017.) i dubokim (2013. – 2022.) prirodnim krškim jezerima izvršene su statističke analize s ciljem dobivanja boljeg uvida utjecaja okolišnih čimbenika na zajednicu fitoplanktona u jezerima s malim gradijentom okolišnih čimbenika.

Višegodišnji set podataka morfološke analize zajednice fitoplanktona (publikacije **III** i **IV**) predstavlja pouzdaniju osnovu u odnosu na jednogodišnji set podataka zajednice fitoplanktona utvrđene morfološkim i molekularnim pristupom (publikacije **I** i **II**), zbog čega je analiza utjecaja okolišnih čimbenika provedena samo na temelju višegodišnjeg morfološkog seta podataka (publikacije **III** i **IV**).

Objavljena istraživanja u sklopu ove doktorske disertacije potvrđuju da je fitoplankton identificiran morfološkim pristupom pouzdani biološki element kakvoće u ocjeni ekološkog stanja te pouzdani pokazatelj odgovora na dostupnost hranjivih tvari i eutrofikaciju jezera. S druge strane, razlike u taksonomskom i funkcionalnom sastavu zajednice fitoplanktona, utvrđene usporedbom

morfološkog i molekularnog pristupa, ukazuju na potrebu za daljnjim istraživanjima analize okolišne DNA s ciljem unaprjeđenja i usavršavanja ove metode za identifikaciju fitoplanktona i njezinu primjenu u ocjeni ekološkog stanja.

3.1. Utvrđivanje sastava zajednice fitoplanktona morfološkim i molekularnim pristupom te usporedba pristupa u analizi zajednice fitoplanktona

Prvi cilj doktorske disertacije bio je *utvrditi sastav zajednice fitoplanktona u prirodnim krškim jezerima površine veće od 0,5 km^2 u Republici Hrvatskoj pomoću morfološke identifikacije fitoplanktona (klasična mikroskopija) i pomoću analize okolišne DNA* dok je drugi cilj bio *usporediti sastav zajednice fitoplanktona morfološkim i molekularnim pristupom*. Za potrebe prva dva cilja postavljena je hipoteza *morfološki pristup i pristup analize okolišne DNA su usporedivi u analizi zajednice fitoplanktona u krškim jezerima*.

Morfološki pristup analize fitoplanktona je vremenski i stručno vrlo zahtjevan, što između ostalog, izravno utječe na broj obrađenih uzoraka u svrhu monitoringa i ocjene ekološkog stanja u okviru Okvirne direktive o vodama (Europska komisija, 2000). S druge strane, analiza okolišne DNA je obećavajuća metoda u identifikaciji fitoplanktona (Kim i sur., 2019, Malashenkov i sur., 2021), što je prikazano i u Publikaciji **I**, gdje je molekularnim pristupom identificirano 40% više svojti u odnosu na morfološki pristup.

Korištenjem analize okolišne DNA u svrhu identifikacije zajednice fitoplanktona u obzir je potrebno uzeti ne samo metodu koja će se koristiti, nego i tip staništa koji se istražuje, s obzirom da zajednica fitoplanktona uključuje cijanobakterije i eukariotske alge. Prokariotske cijanobakterije češće se povezuju s eutrofnim i hipereutrofnim staništima (Almanza i sur., 2018). Budući da su četiri od šest jezera obuhvaćena našim istraživanjem pretežno oligotrofna i mezotrofna, a dominiraju eukariotske alge, u istraživanju je korištena hipervarijabilna V9 regija 18S rRNA, čijom detekcijom i identifikacijom nisu uključene cijanobakterije (Choi i Park, 2020).

Rezultati usporedbe biomase svojti fitoplanktona utvrđene morfološkim pristupom i broja sekvenci molekularnim pristupom pokazuju slično i jasno razdvajanje jezera temeljem klaster analize Bray-Curtis-ovog indeksa sličnosti. Navedeni rezultati u publikaciji **I** ukazuju da je metoda analize okolišne DNA osjetljiva na promjenu sastava svojti, iz čega također proizlazi potencijal metode u

identifikaciji različitih zajednica fitoplanktona na temelju trofičkog statusa jezera. To je u skladu s istraživanjem Eiler i sur. (2013), budući da se istraživana jezera razlikuju po produktivnosti, što je prikazano u publikaciji **III**.

Rezultati u publikaciji **I** prikazuju najveći broj podudarnosti morfološkog i molekularnog pristupa (gen 18S rRNA) u oligotrofnom jezeru Kozjak, dubokom Vranskom jezeru i mezotrofnim jezerima Prošće i Visovac, gdje u zajednici fitoplanktona dominiraju svojte iz koljena Bacillariophyta, Miozoa, Ochrophyta i Cryptophyta. Rezultati preklapanja svojti u skladu su s istraživanjima Malashenkov i sur. (2021), gdje većina svojti koje se preklapaju usporedbom oba pristupa pripadaju koljenima Bacillariophyta, Miozoa, Ochrophyta i Cryptophyta, a također je korišten marker gen 18S rRNA. Koristeći gen 18S rRNA, Bacillariophyta, Miozoa i Cryptophyta također su bile među najzastupljenijim skupinama koje su analizom okolišne DNA identificirali Muhammad i sur. (2021). Stoga, rezultati u publikaciji **I** predstavljaju 18s rRNA kao potencijalno pouzdan genski marker za detekciju spomenutih skupina.

U publikaciji **I**, neke vrlo česte svojte kao što su *Asterionella formosa* Hassall, *Pantocsekiella comensis* (Grunow) K.T.Kiss & E.Ács, *Synedropsis roundii* Torgan, Menezes, & Melo, *Cosmarium tenue* W.Archer, *Elakatothrix gelatinosa* Wille, *Oocystis lacustris* Chodat, *Oocystis marssonii* Lemmermann, *Oocystis parva* West & G.S.West, *Radiococcus planktonicus* J.W.G.Lund, *Sphaerocystis planctonica* (Korshikov) Bourrelly, *Sphaerocystis schroeteri* Chodat, *Actinotaenium/Mesotaenium* itd., identificirane morfološkim pristupom, nisu identificirane analizom okolišne DNA, potencijalno zbog nepotpunosti i greškama u bazama podataka DNA barkodova za uspoređene svojte (Groendahl i sur., 2017, Santi i sur., 2021, Tzafesta i sur., 2022). Iz tog razloga, za mogućnost pouzdanije analize fitoplanktona molekularnim pristupom, potrebna je nadopuna referentnih baza gena (Tzafesta i sur., 2022). Jedan od nedostataka koji može utjecati na usporedbu morfološkog i molekularnog pristupa u publikaciji **I** je kratka V9 regija 18S rRNA gena, koja u odnosu na V4 regiju pokazuje slabiju učinkovitost u identifikaciji svojti na nižim taksonomskim razinama (Stoeck i sur., 2010, Geisen i sur., 2019, Stuart i sur., 2024). Duža V4 regija 18S rRNA gena omogućuje identifikaciju svojti na nižim taksonomskim razinama, često do roda ili vrste (Geisen i sur., 2019). Stoga bi sekvenciranje V4 i V9 regija 18S rRNA gena doprinijelo boljem preklapanju eukariotske zajednice fitoplanktona na nižim taksonomskim razinama, s obzirom na njihovo međusobno nadopunjavanje (Stuart i sur., 2024). Još jedno ograničenje mogu

biti nepravilnosti povezane s amplifikacijom i sekvenciranjem sljedeće generacije (Kim i sur., 2019), što bi moglo objasniti izostanak detekcije dominantne vrste *Pantocsekiella. costei* u dubokom Vranskom jezeru, dok je detektirana u jezerima Kozjak i Prošće (Gligora Udovič i sur., 2016). Temeljem rezultata se može pretpostaviti da je manje preklapanje svojti Bacillariophyta posljedica visokog postotka vrsta identificiranih morfološkim pristupom (*A. formosa*, *P. comensis*, *S. roundii*), gdje molekularnim pristupom nije došlo do njihove identifikacije. Boljem preklapanju morfološkog i molekularnog pristupa u identifikaciji fitoplanktona mogla bi doprinijeti i usporedba svojti na višoj taksonomskoj razini (Groendahl i sur., 2017, Weigand i sur., 2019, Malashenkov i sur., 2021, Santi i sur., 2021). Na slabiju pokrivenost svojti iz slabije istraženih geografskih područja i specifičnih staništa u referentnim bazama upućuju i rezultati iz publikacije **I**, u kojoj je najmanji broj preklapajućih svojti zabilježen u blago bočatim jezerima pod utjecajem mediteranske klime, poput plitkog Vranskog jezera, gdje je prisutna karakteristična vrsta *S. roundii* (Blanco i sur., 2019), što je doprinijelo slabijem preklapanju morfološkog i molekularnog pristupa. Pokrivenost svojti u referentnim bazama može predstavljati ograničenje u identifikaciji rijetkih vrsta u takvim staništima (Salmaso i sur., 2022; Weigand i sur., 2019). Jedan od nedostataka primjene analize okolišne DNA u procjeni zajednica fitoplanktona je ograničena usporedivost brojnosti pojedinih skupina s rezultatima morfoloških metoda (Santi i sur., 2021, Gelis i sur., 2024, Mikhailov i sur., 2025). Iako su istraživanja Bilbao i sur. (2023) i Gelis i sur. (2024) pokazala određenu korelaciju u relativnoj brojnosti svojti unutar pojedinih skupina fitoplanktona, istovremeno su istaknute i značajne razlike u brojnosti usporedbom morfološkog i molekularnog pristupa, što je potvrđeno i u istraživanjima Piredda i sur. (2017) i Santi i sur. (2021). Razlike u brojnosti najčešće se pripisuju varijacijama u broju kopija gena među vrstama, što utječe na broj sekvenci te može dovesti do premale ili prevelike zastupljenosti pojedinih vrsta, razlikama u afinitetu i učinkovitosti početnica tijekom PCR amplifikacije, učinkovitosti ekstrakcije DNA te pogrešaka u procesu sekvenciranja, pripremi baza i bioinformatičkoj obradi (Tan i sur., 2015, Vasselon i sur., 2017, Shelton i sur., 2023, Mikhailov i sur., 2025). U publikaciji **I** i **II**, dominacija predstavnika koljena Miozoa i Cryptophyta utvrđena analizom okolišne DNA potencijalno je rezultat njihovog većeg biovolumena. Naime, biovolumenom veći predstavnici navedenih koljena identificirani morfološkim pristupom (*C. hirundinella*, *Gymnodinium spp.*, *G. helveticum*, *Cryptomonas platyuris* Skuja, *Cryptomonas spp.*) daju veći broj sekvenci zbog veličine svojih stanica i genoma (Martin i sur., 2022), što naposljetku

dovodi do razlika u usporedbi brojnosti (Piredda i sur., 2017, Pérez Burillo i sur., 2020) prikazanoj u publikacijama **I** i **II**.

Vrsta *P. ocellata* iz odjela Bacillariophyta morfološkim je pristupom identificirana samo u jezeru Visovac, a molekularnim u svim istraživanim jezerima obuhvaćenim publikacijama **I** i **II**. Visoka genetska sličnost od 99% između 18S rDNA sekvenci vrste *P. ocellata* i drugih vrsta iz istog roda, poput *P. costei*, kao i predstavnika rodova *Stephanodiscus* i *Cyclostephanos* (Acs i sur., 2016) otežava identifikaciju na nižoj taksonomskoj razini te utječe na zamjenu s drugim svojtama. S druge strane, dominantne vrste *P. comensis* (jezero Oćuša i Crniševo) i *S. roundii* (plitko Vransko jezero) identificirane su mikroskopiranjem, a nisu analizom okolišne DNA.

Izračun i primjena korekcijskih faktora na osnovi biovolumena (Vasselon i sur., 2017, Mortágua i sur., 2019) i nadopunjavanje NCBI baze omogućili bi pouzdanije rezultate, posebice slabo istražene zajednice fitoplanktona u krškim jezerima i općenito jezerima. U publikacijama **I** i **II** rezultati ukazuju na razlike u sastavu i udjelu funkcionalnih grupa utvrđenih primjenom oba pristupa. U publikaciji **II** prikazane su usporedive ocjene ekološkog stanja unatoč nepreklapajućim sastavima i udjelima FG. Usporedivosti ocjena doprinijele su vrijednosti faktora dodijeljene različitim FG sa sličnim ekološkim zahtjevima. S obzirom na razlike usporedbe pristupa u publikacijama **I** i **II**, primarno je potrebno težiti točnoj identifikaciji fitoplanktona analizom okolišne DNA s ciljem pouzdanije ocjene ekološkog stanja.

Rezultati djelomično potvrđuju postavljenu hipotezu s obzirom da je morfološkim pristupom i analizom okolišne DNA zabilježeno preklapanje pojedinih svojti fitoplanktona. Naime, iako je zabilježeno slabo preklapanje, predstavnici skupina Bacillariophyta, Miozoa, Ochrophyta i Cryptophyta pokazali su značajnije preklapanje u istraživanim oligotrofnim i mezotrofnim jezerima za koja su karakteristični te je njihova dominacija zabilježena morfološkom identifikacijom. Veći problem predstavljaju vrlo česte dominantne i koodominantne deskriptivne svojte utvrđene morfološkom identifikacijom, jer do njihovog utvrđivanja i identifikacije nije došlo analizom okolišne DNA, zbog čega dolazi do razlika u sastavima zajednice fitoplanktona. S druge strane, analizom okolišne DNA utvrđeno je 2,5 puta više operativnih taksonomskih jedinica pridruženih svojtama fitoplanktona u usporedbi s brojem svojti identificiranih morfološkim pristupom. Molekularni pristup pritom je omogućio bolju taksonomsku razlučivost u odnosu na morfološki pristup.

3.2. Ocjena i usporedba ekološkog stanja krških jezera temeljem zajednice fitoplanktona identificirane morfološkom analizom i analizom okolišne DNA kroz integraciju Reynoldsovog koncepta funkcionalnih grupa

Ciljevi doktorske disertacije koji daju odgovor na drugu (*Reynoldsov koncept funkcionalnih grupa fitoplanktona se može primijeniti i na morfološki pristup te pristup analize okolišne DNA u krškim jezerima*) i treću hipotezu (*HLPI indeks izračunat temeljem okolišne DNA je primjenjiv u ocjeni ekološkog stanja prirodnih krških jezera u Hrvatskoj*) uključuju *grupiranje sekvenci dobivenih analizom okolišne DNA u operativne taksonomske jedinice dodijeljene svojtama fitoplanktona i njihovu klasifikaciju prema Reynoldsovim funkcionalnim grupama*. Također je cilj *utvrđivanje primjene Q indeksa i Reynoldsovog koncepta funkcionalnih grupa u ocjeni ekološkog stanja sastavom zajednice fitoplanktona identificiranog analizom okolišne DNA*. Potrebno je *primijeniti koncentracije klorofila a dobivene spektrofotometrijski i pomoću visokoprotočne tekućinske kromatografije u izračunu HLPI indeksa i omjera ekološke kakvoće oba pristupa*. Naposljetku, *ocjenu ekološkog stanja dobivenu standardiziranim morfološkim pristupom potrebno je usporediti s rezultatima ocjene dobivene analizom okolišne DNA*.

U publikaciji **II** korištenjem morfološkog i molekularnog pristupa ocijenjeno je i uspoređeno ekološko stanje krških jezera. Bez obzira na taksonomske te posljedično i funkcionalne razlike, pristupi su rezultirali sličnom ocjenom ekološkog stanja. U publikaciji **II** sekvence utvrđene analizom okolišne DNA grupirane su u operativne taksonomske jedinice (OTU) i taksonomski pridružene odgovarajućim svojtama fitoplanktona, koje su potom svrstane u 38 Reynoldsovih funkcionalnih grupa na temelju njihovih fizioloških, morfoloških i ekološkim obilježja, kao i osjetljivosti i tolerancije na promjene u okolišu (Reynolds i sur., 2002, Padisák i sur., 2009). Faktori (F) su najvažniji dio u izračunu ocjene ekološkog stanja s obzirom da se njihove vrijednosti dodjeljuju funkcionalnim grupama uzimajući u obzir rasprostranjenost fitoplanktona i jačinu stresora (Gligora Udovič i Žutinić, 2020). U publikaciji **II**, dinoflagelati identificirani analizom okolišne DNA i svrstani u kodon **Lo** predstavljali su deskriptivnu FG u svim istraživanim jezerima.. U dubokim oligotrofnim jezerima Kozjak i Vransko, najveći doprinos u ocjeni ekološkog stanja molekularnim pristupom imala je dominacija svojti svrstanih u kodon **Lo**, dok je značajan doprinos usporedivoj ocjeni ekološkog stanja morfološkim pristupom imao udio dinoflagelata u ukupnoj

biomasi navedenih jezera. . *Sphaerocystis schroeteri*, reprezentativna vrsta za kodon **F** i deskriptivna vrsta s najvećim udjelom u biomasi jezera Prošće te *Cosmarium tenue*, reprezentativna vrsta za kodon **N** i jedna od dvije vrste s najvećim udjelom u biomasi plitkog Vranskog jezera, u publikaciji **II** nisu identificirane molekularnim pristupom. S druge strane, bez obzira što su usporedbom morfološkog i molekularnog istraživanja u publikaciji **II** zabilježene razlike u sastavu i udjelu FG, ocjena ekološkog stanja nalazila se u istoj kategoriji ekološkog stanja u 89% uspoređenih uzoraka. Navedeni rezultati ukazali su na utjecaj faktora dodijeljenih pojedinim FG. Naime, iako su utvrđene razlike u identifikaciji dominantnih svojti primjenom molekularnog i morfološkog pristupa u publikaciji **II**, svojte su, zbog sličnih ili istih ekoloških zahtjeva, svrstane u različite FG sličnih ili istih faktora relevantnih u ocjeni ekološkog stanja HLPI indeksom. Takva podudarnost rezultirala je visokom razinom slaganja u ocjeni ekološkog stanja, utvrđenoj primjenom oba pristupa. U plitkom Vranskom jezeru molekularnim pristupom zabilježena je dominacija predstavnika kodona **F** i **D** dok je morfološkim pristupom zabilježena dominacija predstavnika kodona **N** i **P**. Predstavnici kodona F karakteristični su za bistra mezotrofna jezera s dubokim miješanjem vodenog stupca, predstavnici kodona **D** za plitka mutna vodna tijela, dok su povoljna staništa za predstavnike kodona **N** i **P** kontinuirano ili povremeno izmiješani vodeni sloj (2-3 m dubine) plitkih jezera (Reynolds i sur., 2002, Padisák i sur., 2009). Zbog sličnih ekoloških zahtjeva i životnih staništa koja preferiraju, predstavnici kodona **F**, **D**, **N** i **P** imaju slične ili jednake brojeve faktora, koji su, kao što je prethodno navedeno, najvažniji dio u izračunu Q indeksa. Na temelju različitih dominantnih FG sa sličnim ili istim vrijednostima faktora, ocjena ekološkog stanja za 10 od 11 uzoraka rezultirala je istom kategorijom ekološkog stanja usporedbom morfološkog i molekularnog pristupa za plitko Vransko jezero. Predstavnici FG u istraživanim oligotrofnim i mezotrofnim jezerima, klasificirani objema metodama, sličnih su ekoloških zahtjeva. U slučajevima gdje se razlikuju reprezentativne svojte, slični ekološki zahtjevi rezultirali su dodjelom sličnih ili istih faktora dajući istu kategoriju ekološkog stanja za 41 od 46 istraživanih uzoraka u publikaciji **II**. Preostalih pet uzoraka razlikuje se u jednoj klasifikacijskoj kategoriji.

U publikaciji **II** koncentracija klorofila *a*, određena spektrofotometrijski i tekućinskom kromatografijom ultra visoke učinkovitosti (UHPLC), korištena je za izračun HLPI indeksa i omjera ekološke kakvoće u morfološkom i molekularnom pristupu. Razlike u izmjerenim koncentracijama klorofila *a* između metoda dovele su do odstupanja u kategorizaciji pojedinih ocjena ekološkog stanja. U većini istraživanih jezera razlike u vrijednostima HLPI indeksa bile su

posljedica primjene različitih metoda određivanja koncentracije klorofila *a*, a ne razlika u sastavu zajednica fitoplanktona utvrđenih morfološkim i molekularnim pristupom. U dubokom Vranskom jezeru i jezeru Visovac koncentracije klorofila *a*, određene spektrofotometrijski i UHPLC-om, nisu pokazale značajne razlike, što je rezultiralo i usporedivim HLPI indeksom. UHPLC-om, kao osjetljivijom i preciznijom metodom, osobito pri niskim koncentracijama, u jezerima Oćuša i Crniševo izmjerene su više koncentracije klorofila *a* u odnosu na spektrofotometriju. To je rezultiralo nižim HLPI indeksom čime je narušena ocjena ekološkog stanja u usporedbi s HLPI indeksom izračunatim koncentracijama izmjerenim spektrofotometrijom. Niže koncentracije klorofila *a* izmjerene UHPLC-om na postaji Motel rezultirale su razlikom u jednoj klasifikacijskoj kategoriji ekološkog stanja u odnosu na rezultat utvrđen spektrofotometrijom. Značajne razlike u HLPI vrijednostima koje proizlaze iz razlika u identifikaciji zajednice fitoplanktona, a ne iz koncentracije klorofila *a*, utvrđene su jedino u jezeru Prošće. Peng i sur. (2013) usporedili su koncentracije klorofila *a* izmjerene metodom spektrofotometrije i tekućinskom kromatografijom visoke učinkovitosti (HPLC) te zaključili da je spektrofotometrijska metoda pogodnija za rutinski monitoring zbog jednostavnosti i niske cijene.

Rezultati potvrđuju drugu postavljenu hipotezu za čiju potrebu su ostvareni ciljevi primjene Reynoldsovog koncepta funkcionalnih grupa fitoplanktona. Operativne taksonomske jedinice utvrđene analizom okolišne DNA dodijeljene su odgovarajućim svojtama fitoplanktona i svrstane u odgovarajuće FG, čime je utvrđen taksonomski i funkcionalni sastav zajednice fitoplanktona temeljen na molekularnom pristupu. Takva klasifikacija omogućila je primjenu Q indeksa, koji se, kao sastavni dio izračuna HLPI indeksa, koristio u ocjeni ekološkog stanja istraživanih jezera primjenom oba pristupa. Sastavni dio HLPI indeksa je i koncentracija klorofila *a* čije su vrijednosti, izmjerene spektrofotometrijski i UHPC-om, primijenjene u izračunu HLPI indeksa i omjera ekološke kakvoće za oba pristupa. Ciljevi zadovoljeni za potrebe ostvarivanja druge hipoteze preduvjet su za ispitivanje treće hipoteze. Usporedba ocjena ekološkog stanja utvrđenih standardiziranim morfološkim pristupom s ocjenama utvrđenim analizom okolišne DNA rezultirala je preklapanjem HLPI indeksa, čime je potvrđena treća hipoteza.

3.3. Utjecaj okolišnih čimbenika na biomasu te taksonomski i funkcionalni sastav zajednice fitoplanktona krških jezera

Treći cilj doktorske disertacije bio je *definirati glavne okolišne čimbenike i jačinu njihovog utjecaja na zajednicu fitoplanktona identificiranu primjenom oba pristupa* dok je *osmi cilj bio definirati utjecaj okolišnih pritisaka na ocjenu ekološkog stanja dobivenu molekularnim pristupom u svrhu budućeg upravljanja vodnim tijelima*. Za potrebe trećeg i osmog cilja postavljena je hipoteza *Reynoldsov koncept funkcionalnih grupa fitoplanktona se može primijeniti i na morfološki pristup te pristup analize okolišne DNA u krškim jezerima.*

3.3.1. Utjecaj okolišnih čimbenika na biomasu fitoplanktona u dubokim jezerima

U publikaciji **III** utvrđen je različit odgovor biomase i zajednice fitoplanktona na okolišne čimbenike pojedinačno za duboka jezera, s obzirom da je svako jezero jedinstveno stanište. Rezultati ovog istraživanja pokazuju kako su u krškim jezerima hranjive tvari, alkalitet, salinitet, svjetlost i temperatura vode čimbenici koji najviše utječu na zajednicu fitoplanktona. Rezultati publikacije **III** utvrđuju povezanost fitoplanktona i okolišnih čimbenika, potvrđujući fitoplankton pouzdanim biološkim indikatorom u odgovoru na dostupnost hranjivih tvari i pojavu eutrofikacije u slatkovodnim jezerima.

U publikaciji **III** potvrđen je obrazac viših vrijednosti Secchi dubine u niskoproduktivnim jezerima, što ovaj pokazatelj čini pouzdanim indikatorom stupnja eutrofikacije (Bellinger i Sigee, 2015). Svjetlost, kao ključni čimbenik za autotrofne organizme u procesu fotosinteze, pokazuje izravan utjecaj na biomasu fitoplanktona (Fetahi i sur., 2014), a dostupnost svjetlosti mjeri se upravo Secchi diskom. Veće Secchi dubine karakteristične su za jezera s niskom produktivnosti, budući da sam fitoplankton, doprinosi smanjenju prozirnosti vode (Wang i sur., 2022; Yang i sur., 2023). U publikaciji **III** zabilježena je pozitivna korelacija biološke potrošnje kisika (BPK_5) s koncentracijom klorofila *a* i ukupnom biomasom fitoplanktona. Povećane koncentracije hranjivih tvari potiču rast biomase fitoplanktona, što posljedično dovodi do veće produkcije organske tvari. Tijekom razgradnje organske tvari dolazi do potrošnje kisika, čime se povećava vrijednost BPK_5 (Wang i sur., 2007). Istovremeno, koncentracija klorofila *a* pokazuje negativnu korelaciju s koncentracijom otopljenog kisika i njegovim zasićenjem, potencijalno zbog pojave stratifikacije

(Sriyasak i sur., 2015), zbog čega se proizvodnja kisika odvija u gornjem vodenom stupcu jezera i na termoklini, gdje je veća dostupnost svjetlosti i hranjivih tvari. S druge strane, razgradnja organske tvari troši kisik u dubljim slojevima vodenog stupca (Dugener i sur., 2023). Kompozitni uzorak eufotičke zone obuhvaća i donje slojeve jezera, gdje dolazi do potrošnje kisika razgradnjom organske tvari, što je povezano s negativnom korelacijom koncentracije klorofila *a*. U publikaciji **III** utvrđeno je da su salinitet i vodljivost pozitivno korelirali s koncentracijom klorofila *a* u jezerima Crniševo i Oćuša, gdje su i koncentracije klorofila *a* i ukupne biomase bile visoke. Budući da su N i P ključne hranjive tvari za rast fitoplanktona u vodenim ekosustavima (Lui i Chen, 2012; Meerhoff i sur., 2012; Wang i sur., 2022; Maberly i sur., 2022b), njihova dostupnost i apsorpcija od strane fitoplanktona mogu varirati ovisno o salinitetu, što posljedično ima izravan pozitivan ili negativan utjecaj na koncentraciju klorofila *a* i biomasu fitoplanktona (Li i sur., 2021).

U publikaciji **III** prikazano je da povećanje alkaliteta pozitivno utječe na klorofil *a* i biomasu fitoplanktona. Budući da je bikarbonat prevladavajući oblik ugljika u kopnenim vodama sa sličnim pH rasponom, kao što je slučaj u istraživanim jezerima te je njegova koncentracija često viša od koncentracije CO_2, dostupniji je fotosintetskim organizmima (Maberly, 2014). Vrijednosti alkaliteta bile su najviše u jezerima bogatim sedrom (Prošće i Kozjak) u kojima je uočena dominacija penatnih algi kremenjašica poput roda *Fragilaria*, koje učinkovito iskorištavaju HCO_3^- kao anorganski izvor ugljika (Baattrup-Pedersen i sur., 2022).

Dostupnost i udio silikata u odnosu na otopljene anorganske hranjive tvari, važni su za reguliranje kompeticije između fitoplanktonskih vrsta (Ren i sur., 2020). To je vidljivo u publikaciji **III** gdje je povećanje koncentracije silikata popraćeno rastom biomase fitoplanktona i koncentracije klorofila *a*. Rezultati su u skladu s istraživanjima Fetahi i sur. (2014) i Dubourg i sur. (2015) s obzirom da su silikati posebno važni za alge kremenjašice, koje predstavljaju jednu od najbrojnijih i biomasom najzastupljenijih skupina istraživanih jezera obuhvaćenih publikacijom **III**.

3.3.2. Utjecaj omjera ukupnog dušika i ukupnog fosfora (TN:TP) na biomasu fitoplanktona u dubokim jezerima

U istraživanim jezerima publikacije **III**, povećanje koncentracije TP statistički značajno doprinosi rastu biomase fitoplanktona, dok povećanje TN statistički značajno prati povećanje biomase

fitoplanktona zajedno s koncentracijom klorofila *a*, čime se potvrđuje važnost dušika (N) i fosfora (P) za rast fitoplanktona i primarnu proizvodnju u jezerima (Filstrup i Downing, 2017, Yu i sur., 2022). P se smatra najvažnijim čimbenikom koji utječe na rast fitoplanktona (Schindler, 2012), stoga ograničavanje N uz već prisutan P pomaže u kontroli procesa eutrofikacije slatkovodnih ekosustava. Na stupanj eutrofikacije značajno utječe sposobnost pojedinih cijanobakterija da mogu fiksirati atmosferski dušik (N_2) (Schindler i sur., 2008). Pozitivna korelacija između omjera TN:TP i koncentracije klorofila *a* u publikaciji **III** ukazuje da biomasa fitoplanktona ovisi o uravnoteženoj dostupnosti N i P, pri čemu oba elementa mogu ograničavati rast dok su najveće vrijednosti biomase zabilježene kada su njihovi omjeri međusobno uravnoteženi što je u skladu s istraživanjem Filstrup i Downing (2017).

U publikaciji **III**, u dubokom oligotrofnom Vranskom jezeru potvrđeno je da su niske koncentracije N i P karakteristično obilježje takvih jezera (Bergström, 2010), pri čemu su zabilježene najniže pojedinačne koncentracije tih elemenata te najniži TN:TP omjer, što je u skladu s istraživanjima Bergström i sur. (2008) i Elser i sur. (2009). Prema rezultatima istraživanja, ograničavajući čimbenik u dubokom Vranskom jezeru je koncentracija N, čiji bi unos mogao snažno povećati omjer TN:TP, uslijed čega bi došlo do promjene u zajednici fitoplanktona u smjeru ravnoteže ili većinski P-ograničenih svojti (Bergström, 2010). Dok je biomasa fitoplanktona u mnogim, a posebice u dubokim jezerima često ograničena zbog nedostupnosti P, istraživanja Dolman i sur. (2016) i Dolman i Wiedner (2015) potvrđuju N kao bolji pokazatelj rasta fitoplanktona od P u slučaju niskog TN:TP omjera. Jiang i Nakano (2022) utvrdili su da N ima važniju ulogu od P u slatkovodnim staništima siromašnim hranjivim tvarima, što je utvrđeno u N ograničenom dubokom Vranskom jezeru istraženom u publikaciji **III**.

Za razliku od rezultata istraživanja Bergström (2010), prema kojem nizak omjer TN:TP karakterizira jezera niske produktivnosti, Zhou i sur. (2022) tvrde da niski TN:TP omjeri karakteriziraju eutrofna jezera te da do viših TN:TP omjera češće dolazi u mezotrofnim i oligotrofnim jezerima. U istraživanju Zhou i sur. (2022) jezera su eutrofna s visokim koncentracijama N i P što je rezultat niskog TN:TP omjera. Duboko Vransko jezero u publikaciji **III** također pokazuje nizak TN:TP omjer, ali u uvjetima niskih koncentracija N i P. Stoga su rezultati istraživanja u publikaciji **III** samo djelomično u skladu sa Zhou i sur. (2022) jer je oligotrofno duboko Vransko jezero ograničeno dostupnošću koncentracije N, oligotrofno jezero Kozjak

ograničeno dostupnošću koncentracije P, dok su oligo-mezotrofna jezera Visovac i Oćuša pretežno ograničena dostupnošću koncentracijama N i P, kao i mezotrofna jezera Crniševo i Prošće.

Temeljem niskih koncentracija N i P kao najvažnijih hranjivih tvari potrebnih za rast fitoplanktona, istraživana jezera u publikaciji **III** opisana su kao oligotrofna. S obzirom da je potreba fitoplanktona za navedenim hranjivim tvarima od esencijalne važnosti, Jiang i Nakano (2022) pretpostavili su da je N konstantno potreban zbog važnosti u procesu fotosinteze. S druge strane, zbog prilagodbe i aklimatizacije fitoplanktona, potreba za P može biti fleksibilnija. Između ostalog, prisustvo N u stanicama slabije je podložno varijacijama u usporedbi s P prema Galbraith i Martiny (2015). Stoga, zajednica fitoplanktona koja podliježe ograničenju N i P u oligotrofnim ekosustavima, gdje su koncentracije tih elemenata niske, pokazuje veću potrebu za N (Jiang i Nakano, 2022), što potvrđuje njegovu važnost i u skladu je s istraživanim jezerima siromašnim hranjivim tvarima u publikaciji **III**.

3.3.3. Utjecaj okolišnih čimbenika i eutrofikacije na funkcionalni sastav fitoplanktona dubokih jezera

Rezultati publikacije **III** prikazuju odgovor zajednice fitoplanktona na učinke eutrofikacije zbog čega su jezera slične produktivnosti grupirana zajedno, bez obzira na različito grupiranje temeljem okolišnih čimbenika. Ovime se potvrđuje uloga fitoplanktona kao jednog od najvažnijih bioloških elemenata u ocjeni stupnja trofije (Pasztaleniec, 2016, Salmaso i Tolotti, 2021) te važnosti primjene koncepta Reynoldsovih funkcionalnih grupa u istraživanju fitoplanktona (Reynolds i sur., 2002, Borics i sur., 2007, Padisák i sur., 2009). Unatoč prostornoj i vremenskoj raznolikosti FG u istraživanim jezerima prikazanoj u publikaciji **III**, fitoplanktonske su zajednice svih istraživanih jezera opisivale FG svojstvene prirodnim oligotrofnim i mezotrofnim dubokim jezerima (Gligora Udovič i sur., 2016), što je potvrđeno SIMPER analizom sastava FG. U istoj je publikaciji korišten opsežan skup podataka sa svrhom istraživanja odgovora zajednice fitoplanktona na promjene u okolišu, što je temelj za daljnje praćenje promjena uzrokovanih utjecajem čovjeka i klimatskim promjenama u istraživanim jezerima.

Iz rezultata je vidljivo da hranjive tvari i temperatura vode imaju značajan utjecaj na zajednicu fitoplanktona. Na ove okolišne čimbenike izravno utječu klimatske promjene, što posljedično

utječe na zajednicu fitoplanktona i njegovu biomasu (Cao i sur., 2023, Hao i sur., 2024, Paltsev i sur., 2024). Dory i sur. (2024) otkrili su da temperatura vode ima veći utjecaj na biovolumen (= biomasu) fitoplanktona u odnosu na dostupnost hranjivih tvari. Također su pokazali da relativna važnost temperature i hranjivih tvari ovisi o trofičkom stanju jezera, pri čemu pretpostavljaju da hranjive tvari imaju veću ulogu u oligotrofnim jezerima, dok je kod mezotrofnih jezera važnija temperatura vode. U jezerima siromašnim hranjivim tvarima, zbog njihove nedostupnosti može doći do izostanka reakcije fitoplanktona na povećanje temperature vode. Nasuprot tome, u staništima s dostupnim hranjivim tvarima povećava se osjetljivost fitoplanktona na povećanje temperature vode. Temeljem navedenih rezultata i rezultata publikacije **III**, postavljen je temelj za daljnje praćenje eutrofikacije i klimatskih promjena u oligotrofnim i mezotrofnim jezerima, kakva dominiraju na području Republike Hrvatske. Iako je istraživanje u publikaciji **III** provedeno u dubokim krškim jezerima, rezultati su široko primjenjivi zbog istih ili vrlo sličnih karakteristika brojnih jezera diljem svijeta (dubina i stratifikacija) sa sveprisutnom zajednicom fitoplanktona te N i P kao glavnim čimbenicima njegova rasta (Conley i sur., 2009).

Rezultati u publikaciji **III** pokazuju da usporedba dvaju oligotrofnih jezera sa sličnih koncentracija TP, veće koncentracije TN i silikata u jezeru Kozjak u odnosu na duboko Vransko jezero, utječu na razlike u zajednici fitoplanktona. Funkcionalni sastav jezera Kozjak s predstavnicima kodona **B**, **C**, **D** i **P** bio je sličniji jezeru Prošće nego dubokom Vranskom jezeru, s obzirom da se radi o predstavnicima kodona tolerantnijim na nedostatak svjetlosti u jezerima Kozjak i Prošće u odnosu na vrlo prozirno duboko Vransko jezero. Osjetljivost na nedostatak silikata (Reynolds i sur., 2002) također utječe na odsutnost predstavnika spomenutih kodona, jer je njihova koncentracija najniža u dubokom Vranskom jezeru. Navedeni čimbenici, uz viši alkalitet, definiraju zajednicu fitoplanktona u jezerima Kozjak i Prošće. S druge strane, alge jarmašice, koje pripadaju kodonima **T** i **N** preferiraju staništa s niskim alkalitetom i sadržajem hranjivih tvari (Coesel, 1983) te su jedni od dominantnih skupina u dubokom Vranskom jezeru. Prisutnost kriptofita, predstavnika kodona **X2**, s preferencijom za mezotrofna staništa i toleranciju na nedostupnost svjetlosti u skladu je s njihovom pojavnošću u jezerima Visovac i Oćuša. Povremeni oligomezotrofan karakter ovih jezera također je u skladu s pojavnošću zelenih i zlatnožutih algi iz kodona **X3**, karakterističnih za dobro izmiješana oligotrofna jezera (Padisák i sur., 2009). Predstavnici oba kodona imaju širok raspon tolerancije na promjene u okolišnim uvjetima (oligo)mezotrofnih jezera, što je potvrđeno u istraživanjima Viviane i sur. (2018) i Šimunović i sur. (2022).

Najviše srednje koncentracije silikata i nedostatak svjetlosti u jezeru Visovac, osobito ljeti, pridonijeli su visokoj biomasi i dominaciji predstavnika kodona **B**, karakterističnom na toleranciju na nedostatak svjetlosti i osjetljivost na nedostatak silikata (Reynolds i sur., 2002). Predstavnicima kodona **A**, specifičnim za bistra, duboka jezera s malo hranjivih tvari (Reynolds i sur., 2002, Padisák i sur., 2009), pogodovali su uvjeti s više svjetla, posebice u slabo produktivnim jezerima, dubokom Vranskom i jezeru Kozjak.

Dinoflagelati (kodon $\mathbf{L_O}$), u kodominaciji sa zelenim algama svrstanim u kodone **F** i **J**, specifičnim za mezotrofna jezera s epilimnijem, preferiraju višu temperaturu vode i viši salinitet u najjužnijim mediteranskim jezerima Oćuša i Crniševo, obuhvaćenim publikacijom **III**. Rezultati su također u skladu s istraživanjima gdje su zelene alge zastupljene u blago bočatim jezerima (salinitet 0,8 – 1,1) (Li i sur., 2021). Prema Maberly i sur. (2022a), visoka temperatura također pogoduje razvoju zelenih algi i dinoflagelata, što je u skladu sa zastupljenošću navedenih predstavnika kodona u publikaciji **III**. Predstavnici kodona **F** pokazali su čestu kodominantnost u jezerima unatoč izraženim razlikama između Prošća, Vranskog, Oćuše i Crniševa u temperaturi, dostupnosti svjetlosti i hranjivih tvari, što upućuje na širok raspon tolerancije i osjetljivosti vrsta svrstanih u navedeni kodon (Reynolds i sur., 2002, Padisák i sur., 2009, Becker i sur., 2010). Prilagodljivost predstavnika kodona **Y** širokom rasponu staništa bila je u skladu s njihovom visokom učestalošću pojavljivanja u svim istraživanim jezerima publikacije **III**. Iako je pokretački čimbenik za rast predstavnika navedenog kodona bila dostupnost svjetlosti, tolerancija na uvjete slabog osvjetljenja kod miksotrofnih predstavnika omogućuje njihovu zastupljenost upravo u takvim uvjetima (Reynolds i sur., 2002, Padisák i sur., 2009, Becker i sur., 2010, Salonen i sur., 2024). Predstavnici kodona **E** također su pokazali učestalu pojavnost u istraživanim jezerima obuhvaćenima publikacijom **III**. Jedan od razloga je miksotrofni karakter predstavnika (Reynolds, 2006), što je imalo važnu ulogu za visoku biomasu u jezeru Oćuša s najmanjom srednjom prozirnošću te u jezeru Prošće, gdje ljeti dostupnost svjetlosti snažno opada.

3.3.4. Utjecaj okolišnih čimbenika na biomasu, taksonomski i funkcionalni sastav fitoplanktona plitkog Vranskog jezera

S obzirom na ulazak morske vode u plitko Vransko jezero, u publikaciji **IV** istraživana razdoblja utjecaja okolišnih čimbenika na zajednicu fitoplanktona podijeljena su na slatkovodnu i bočatu

fazu. Tijekom slatkovodne faze, zajednica fitoplanktona prvenstveno je određena koncentracijama TN i TP te dostupnošću svjetlosti. Tijekom najvećih izmjerenih biomasa zajednice fitoplanktona, dominantna vrsta bila je *Cosmarium tenue* W.Archer svrstana u kodon **N**, karakterističan za proljetna i rana ljetna razdoblja u umjerenim plitkim jezerima izmiješanog stupca vode. Kombinacija R- i SR-strategija vrsti *C. tenue* omogućuje visoki afinitet prema fosforu tijekom njegovih niskih koncentracija (Reynolds, 2006), čime vrsta stvara uvjete za dominaciju u zajednici fitoplanktona. Tijekom ljeta i jeseni, smanjenjem dostupnosti hranjivih tvari, dominantna vrsta postaje *Synedropsis roundii* (Gligora i sur., 2007). Ljeti u plitkom Vranskom jezeru dolazi do razvoja makrofita koji pokrivaju većinu jezera, a uz to pad razine slatke vode omogućuje pojačan ulazak slane morske vode kroz kanal Prosika. Makrofiti pojačano asimiliraju N što dovodi do smanjenja njegove dostupnosti fitoplanktonu, što utječe na promjenu sastava fitoplanktona i smanjenje biomase (van Donk i Hessen, 1993, Jeppesen i sur., 1997, Gligora i sur., 2007). U publikaciji **IV** prikazano je da salinitet manji od 2‰, uz umjerenu koncentraciju TP i TN, predstavlja pogodne uvjete za razvoj vrste *C. tenue*. Ljetna dominacija predstavnika kodona **P**, vrste *S. roundii,* povezana je s potrošnjom N (Gligora i sur., 2007), budući da smanjenje koncentracije P tijekom ljetnih razdoblja negativno utječe isključivo na *C. tenue* (Sommer, 1987). Naime, *S. roundii* je prilagođen učinkovitijoj apsorpciji P u uvjetima niskih koncentracija TP i TN, što mu omogućuje istiskivanje *C. tenue* iz staništa tijekom ljetnih razdoblja s ograničenim koncentracijama N. Vrsta *S. roundii* ima izdužen (štapičast) oblik stanica, što joj omogućuje veću površinu za apsorpciju svjetlosti i asimilaciju hranjivih tvari, kao i bolju plovnost u vodenom stupcu. Nasuprot tome, *C. tenue* sa svojim kuglastim oblikom i manjom površinom ima smanjenu efikasnost u pristupu svjetlosti i hranjivim tvarima. Navedene morfološke prilagodbe utječu na njihove ekološke strategije i kompeticiju, pri čemu *S. roundii* ima prednost u uvjetima ograničenih resursa, dok *C. tenue* u uvjetima dostupnosti nutrijenata i svjetlosti ostvaruje brz rast (Reynolds i sur., 2006). Plitko, polimiktično Vransko jezero, prosječne dubine od 2-3 m optimalno je stanište za predstavnike kodona **N** i **P** (Padisák i sur., 2009), koji ukazuju na mezo- do eutrofni karakter. Predstavnici oba kodona osjetljivi su na povećanje saliniteta, što je u publikaciji **IV** vidljivo u godinama s nižim vrijednostima saliniteta, kada su predstavnici navedenih kodona dominirali zajednicom fitoplanktona.

U publikaciji **IV** na ukupnu biomasu i raznolikost fitoplanktona utjecale su najviše razine saliniteta izmjerene tijekom istraživanja (> 3‰), zajedno s niskim koncentracijama hranjivih tvari. Navedeni

uvjeti nisu bili ograničavajući za predstavnike kodona **MP**, tolerantne na česta miješanja i zamućenja vodenog stupca i predstavnike kodona **Lo**, čija je pojavnost karakteristična za plitka jezera srednje do velike površine, sa širokim rasponom trofičkog stanja (Reynolds i sur., 2002, Padisák i sur., 2009). Predstavnici navedenih kodona tijekom razdoblja s najvišim salinitetom zastupljeni su s većom biomasom te su pozitivno korelirali sa salinitetom. Niske koncentracije TP i TN, zajedno s niskim salinitetom, 2014., 2016. i 2019. ograničile su ukupnu biomasu fitoplanktona, potvrđujući snažnu uzročno-posljedičnu vezu između primarne proizvodnje, prozirnosti vode i dostupnosti hranjivih tvari u plitkom Vranskom jezeru. Najniži izmjereni salinitet i TP bili su ključni čimbenici za najveću zabilježenu raznolikost i bogatstvo vrsta u 2016. Unatoč dobroj prilagodbi navedenim pokazateljima (Reynolds i sur., 2002, Padisák i sur., 2009), pojedini predstavnici kodona **MP**, **Lo**, **F** i **X2**, iako prisutni s niskim biomasama, izmjenjivali su se unutar zajednice fitoplanktona. Međutim, nisu bili dominantni, pokazujući uvjete manjka svjetlosti, ograničenosti hranjivim tvarima, resuspendiranim česticama sedimenta i izlučivanja alelopatskih tvari iz makrofita odgovornim za ograničenje rasta fitoplanktona (Gligora i sur., 2007, Mulderij i sur., 2007).

Publikacijom **IV** se istraživanjem zajednice fitoplanktona u plitkom Vranskom jezeru otkriva da na specifičnost sastava zajednice, uz hranjive tvari, snažno utječe i gradijent saliniteta te do izmjene u sastavu dolazi i uslijed promjene slatke i bočate faze jezera. Uvjeti višeg saliniteta rezultiraju dominacijom bočatih bentičkih vrsta, često niske biomase, uz dominaciju vrste *Tetramphora croatica* Gligora Udovic, Caput Mihalic, Stankovic & Levkov. Razdoblja sniženog saliniteta obilježena su izmjenom dominacije između vrsta *C. tenue* i *S. roundii*, te cijanobakterija ili kolonijalnih zelenih algi, koje se odlikuju sposobnošću brze apsorpcije hranjivih tvari i potencijalom za uzrokovanje cvjetanja algi.

Predstavljeni rezultati publikacije **III** i **IV** djelomično potvrđuju treću postavljenu hipotezu, jer ne daju cjeloviti odgovor na treći i osmi cilj doktorske disertacije, s obzirom da za zajednicu fitoplanktona identificiranu analizom okolišne DNA nisu definirali glavne okolišne čimbenike i jačina njihovog utjecaja. S druge strane, korištenjem velikog seta podataka utvrđenih morfološkim pristupom (klasičnom mikroskopijom) u publikaciji **III** i **IV**, analiza biomase, taksonomskog i funkcionalnog sastava zajednice fitoplanktona klasificiranog Reynoldsovim konceptom u odnosu na prateće okolišne čimbenike, jasno je definirala čimbenike s najvećim utjecajem na zajednicu

fitoplanktona u krškim jezerima. Posebno se ističu koncentracije N i P te njihov omjer koji je po prvi puta analiziran u istraženim jezerima, ali i povezanost utjecaja saliniteta, svjetlosti i temperature na njihovu dostupnost. Od hranjivih tvari važnu ulogu imaju i silikati, što je vidljivo u jezerima bogatijim silikatima gdje dominiraju alge kremenjašice i FG kojima pripadaju (Gligora Udovič i sur., 2016). Također je bitno naglasiti da važnu ulogu u definiranju zajednice fitoplanktona ima kompetitivnost pojedinih svojti te njihove strategije u načinu apsorpcije hranjivih tvari, posebice N i P (Gligora i sur., 2007).

4. ZAKLJUČAK

Morfološkom identifikacijom i analizom okolišne DNA utvrđen je sastav zajednice fitoplanktona u prirodnih krškim jezerima u Republici Hrvatskoj. Zajednica fitoplanktona utvrđena je morfološkom identifikacijom i analizom okolišne DNA te je uspoređena.

Usporedbom taksonomskog i funkcionalnog sastava fitoplanktona identificiranog morfološkim i molekularnim pristupom zabilježeno je slabo preklapanje svojti utvrđenih mikroskopijom i analizom 18S rRNA V9 gena, potencijalno zbog korištenja kratke regije gena, nesigurnosti povezanih s amplifikacijom i sekvenciranjem nove generacije te nedovoljne popunjenosti NCBI referentne baze gena u analizi okolišne DNA.

Sekvence utvrđene analizom okolišne DNA grupirane su u taksonomske operativne jedinice kojima su dodijeljene svojte fitoplanktona te svrstane u Reynoldsove funkcionalne grupe. Time je omogućeno definiranje taksonomskog i funkcionalnog sastava zajednice fitoplanktona identificiranog analizom okolišnom DNA te ocjena ekološkog stanja.

Usporedba primjene morfološke analize i analize okolišne DNA u identifikaciji fitoplanktona za ocjenu ekološkog stanja prirodnih krških jezera rezultirala je usporedivim rezultatima. Jezera su temeljem vrijednosti HLPI indeksa izračunatog za obje metode kategorizirana u usporedivo dobro i vrlo dobro ekološko stanje. Odstupanja iste ocjene ekološkog stanja usporedbom metoda uglavnom su rezultat različitih metoda određivanja biomase fitoplanktona (spektrofotometrija i tekućinska kromatografija ultra visoke učinkovitosti). Većina identificiranih svojti značajno se razlikovala između dviju metoda zbog čega su klasificirane u različite funkcionalne grupe. Kako su bile sličnih ekoloških zahtjeva, dodijeljeni su im slični ili isti faktori, a primjena sličnih ili istih faktora u izračunu HLPI indeksa, bez obzira na različiti taksonomski i funkcionalni sastav zajednice fitoplanktona, rezultirao je usporedivom ocjenom ekološkog stanja. No kako izračunato isto i slično ekološko stanje ne počiva na usporedivom sastavu zajednice fitoplanktona, korištenje analize okolišne DNA u ocjeni ekološkog stanja u budućnosti zahtjeva značajno unaprjeđenje.

Kvantifikacija temeljena na broju sekvenci u odnosu na brojnost i biomasu fitoplanktona nije reprezentativna, što dovodi do razlika u udjelu i sastavu funkcionalnih grupa te otvara nova pitanja u svrhu pronalaska rješenja za problem kvantifikacije te upotpunjavanje referentnih baza gena s naglaskom na specifične svojte za pojedina staništa.

Korištenje V9 regije 18S rRNA u analizi okolišne DNA rezultiralo je različitim taksonomskim sastavom zajednice fitoplanktona što zahtijeva daljnji razvoj molekularnog pristupa za pouzdaniju identifikaciju fitoplanktona kroz korištenje odgovarajućih gena i nadopunu referentnih DNA baza. Nemogućnost kvantifikacije brojnosti i biovolumena fitoplanktona analizom okolišne DNA također predstavlja veliki izazov u primjeni molekularnog pristupa u ocjeni ekološkog stanja.

Okolišni čimbenici s najvećim utjecajem na zajednicu fitoplanktona identificiranu morfološkim pristupom u istraživanim jezerima su hranjive tvari (dušik, fosfor i silikati), alkalitet, salinitet, svjetlost i temperatura.

Rezultati utjecaja glavnih okolišnih čimbenika (hranjive tvari, alkalitet, salinitet, svjetlost i temperatura vode) na zajednicu fitoplanktona u dubokim krškim jezerima predstavljaju izvrsnu osnovu i referentne podatke za buduća praćenja antropogenog utjecaja i klimatskih promjena. Osim toga, praćenje sastava i brojnosti fitoplanktona s obzirom na promjenjive koncentracije hranjivih tvari važno je za funkcioniranje slatkovodnih ekosustava. Istraživana duboka krška jezera predstavljaju uglavnom netaknute ekosustave iznimno vrijedne za proučavanje promjena zajednice fitoplanktona pod utjecajem čovjeka i klimatskih promjena u svrhu upravljanja vodama istraživanih jezera.

Rezultati utjecaja okolišnih čimbenika, posebice hranjivih tvari, saliniteta te prozirnosti vode na zajednicu fitoplanktona vrijedni su za buduće upravljanje istraženim plitkim krškim jezerom, posebice u pogledu održavanja hidrološkog režima i prirodnih oligohalinih i mezotrofnih uvjeta.

Razumijevanje odgovora fitoplanktona na okolišne čimbenike, narušene antropogenim utjecajem i klimatskim promjenama, doprinosi zaštiti mediteranskih plitkih jezera na lokalnoj i globalnoj razini.

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6. ŽIVOTOPIS AUTORA

Nikola Hanžek rođen je 6. studenog 1989. godine u Varaždinu. Nakon završene Osnovne škole Vidovec i Gimnazije Varaždin, 2008. godine upisuje Preddiplomski sveučilišni studij biologije na Prirodoslovno-matematičkom fakultetu u Zagrebu. Završetkom Preddiplomskog studija 2011. godine, upisuje Diplomski sveučilišni studij ekologije i zaštite prirode (Modul: Kopnene vode). Tijekom diplomskog studija dobiva „Pohvalnicu Fakultetskog vijeća Prirodoslovno-matematičkog fakulteta za izuzetan uspjeh na diplomskom sveučilišnom studiju ekologije i zaštite prirode“.

Obranom diplomskog rada „Fiziološke prilagodbe jadranskog bračića (*Fucus virsoides* J. Agardh) na isušivanje i ultraljubičasto zračenje“ pod mentorstvom prof. dr. sc. Mirte Tkalec, u veljači 2014. godine stječe zvanje magistra ekologije i zaštite prirode (mag. oecol. et prot. nat.).

Nakon završetka studija zapošljava se u tvrtki Geonatura d.o.o. kao stručni suradnik u poslovima procjena utjecaja na prirodu i okoliš. Nakon tri godine rada u Geonaturi, 2017. godine zapošljava se u Hrvatskim vodama, gdje se počinje baviti uzorkovanjem i analizom biološkog elementa kakvoće fitoplankton u svrhu ocjene ekološkog stanja prema zahtjevima Okvirne direktive o vodama. Iste godine upisuje Poslijediplomski studij biologije na Prirodoslovno-matematičkom fakultetu. Godine 2022. zapošljava se u Institutu za vode „Josip Juraj Strossmayer“ gdje trenutno radi.

Tijekom rada u Hrvatskim vodama i Institutu za vode „Josip Juraj Strossmayer“ pohađa radionice u svrhu usavršavanja taksonomske i molekularne identifikacije fitoplanktona. Od 2024. godine član je stručne skupine za praćenje i ocjenu stanja pri Međunarodnoj komisiji za zaštitu rijeke Dunav. Objavio je 13 znanstvenih i stručnih radova, od čega je sedam znanstvenih radova citirano u bazama Web of Science i Scopus u časopisima s čimbenikom odjeka Q1. Radovi su prema bazi Web of Science citirani ukupno 50 puta uz h-indeks 5, a prema Scopus bazi 53 puta, također uz h-indeks 5. Do danas je objavio 13 kongresnih priopćenja, od kojih je usmeno izlagao na tri međunarodne konferencije.

7. PROŠIRENI SAŽETAK

Površinske slatke vode čine iznimno mali udio ukupne količine vode na Zemlji, no njihova važnost je izuzetna (Kundzewicz i sur., 2008). Među slatkovodnim ekosustavima, jezera se ističu kao žarišta bioraznolikosti te imaju važnu ulogu u globalnim biogeokemijskim ciklusima, osobito u prijenosu, proizvodnji, transformaciji i skladištenju ugljika (Wetzel, 2001, Geist, 2011). Fitoplankton, kao jedan od najvažnijih bioloških elemenata, ima bitnu ulogu u funkcioniranju vodenih ekosustava zahvaljujući sposobnosti fotosinteze, kojom pridonosi približno 50% ukupne primarne proizvodnje na Zemlji (Marañón, 2009). Zbog važnosti slatkovodnih ekosustava za čovječanstvo i potrebe za održivim korištenjem njihovih prirodnih resursa, zaštita i kontinuirano praćenje kakvoće vode od iznimne su važnosti. U cilju očuvanja i praćenja stanja voda, Europska unija donijela je Okvirnu direktivu o vodama (Europska komisija, 2000), kojom se uspostavlja sustav praćenja kakvoće vode i postizanja dobrog ekološkog i kemijskog stanja te zaštite površinskih i podzemnih voda. U tom kontekstu posebno su značajni biološki pokazatelji, jer za razliku od fizikalno-kemijskih, koji odražavaju trenutačno stanje, biološki elementi kakvoće reagiraju na promjene u okolišu kroz dulje vremensko razdoblje, pružajući pouzdaniju osnovu za ocjenu i upravljanje vodnim ekosustavima (Lyche-Solheim i sur., 2013). Na zajednicu fitoplanktona najveći utjecaj imaju hranjive tvari poput dušika, fosfora i silikata. Uz njih, na dinamiku fitoplanktona utječu i temperatura vode, salinitet, svjetlost, alkalitet, pH, suspendirane tvari, hidrološke karakteristike i ljudske aktivnosti (Maileht i sur., 2013, Salmaso i Tolotti, 2021, Verspagen i sur., 2022, Stanković i sur., 2024). Praćenje kakvoće vode putem fitoplanktona u jezerima i vrlo velikim rijekama temelji se na mikroskopskoj identifikaciji svojti, a ocjena ekološkog stanja uključuje analizu taksonomskog sastava, brojnosti, biomase te učestalosti i intenziteta cvjetanja algi (Europska komisija, 2011). S obzirom na sve jači antropogeni pritisak i klimatske promjene, raste potreba za proširivanjem postojećih programa monitoringa, a stručna i vremenska zahtjevnost mikroskopske analize potencijalno može dovesti do financijskog i prostornog ograničenja u praćenju stanja vodnih tijela (Gao i sur., 2018, Gelis i sur., 2024). Stoga se javlja potreba za primjenom novih pristupa, kao što je analiza okolišne DNA (Herrero i sur., 2018, Carvalho i sur., 2019). Kao financijski i vremenski učinkovitiji molekularni pristup visoke reproduktivnosti, analiza okolišne DNA predstavlja moguću alternativu morfološkoj identifikaciji u praćenju kakvoće vode (Hering i sur., 2018, Pawlowski i sur., 2018, Ruppert i sur., 2019,

Thomsen i sur., 2024). Međutim, još uvijek postoje ograničenja u korištenju okolišne DNA, osobito u procjeni strukture i veličine zajednice fitoplanktona te nedostatku referentnih sekvenci u bazama gena, što predstavlja ključne izazove u implementaciji ove metode u istraživanju i ocjeni ekološkog stanja slatkovodnih ekosustava (Weigand i sur., 2019).

Glavni ciljevi ove doktorske disertacije su utvrđivanje sastava zajednice fitoplanktona u krškim jezerima primjenom morfološke identifikacije i analize okolišne DNA, njihova međusobna usporedba te mogućnosti primjene analize okolišne DNA u ocjeni ekološkog stanja. Nadalje, cilj je definirati glavne okolišne čimbenike i jačinu njihovog utjecaja na zajednicu fitoplanktona, u svrhu poboljšanja budućeg upravljanja vodnim tijelima.

Disertacija obuhvaća četiri izvorne znanstvene publikacije kojima se ostvaruju postavljeni ciljevi. Publikacija I donosi usporedbu taksonomskog i funkcionalnog sastav zajednice fitoplanktona utvrđene morfološkim pristupom i analizom okolišne DNA. Rezultati su pokazali nisku podudarnost između sastava zajednice te nisku usporedivost relativne biomase i brojnosti sekvenci. Publikacija II bavi se ocjenom ekološkog stanja (HLPI indeks) temeljenom na oba pristupa. Iako su zabilježene razlike u taksonomskom i funkcionalnom sastavu fitoplanktona utvrđenom različitim pristupima, 89% uzoraka svrstano je u istu kategoriju ocjene ekološkog stanja. Ova se podudarnost pripisuje sličnim ili istim faktorima dodijeljenim različitim funkcionalnim grupama fitoplanktona i koncentraciji klorofila *a* korištenoj u izračunu ocjene. Rezultati analize okolišne DNA u svrhu ocjene ekološkog stanja otvaraju nove izazove za istraživanje i daljnji razvoj metode. Publikacije III i IV detaljno analiziraju odgovor biomase taksonomskog i funkcionalnog sastava fitoplanktona na okolišne čimbenike u plitkom i dubokim prirodnim krškim jezerima. Utvrđeno je da su hranjive tvari (dušik, fosfor i silikati), alkalitet, salinitet, svjetlost i temperatura najvažniji čimbenici koji oblikuju zajednicu fitoplanktona. Analize velikog skupa podataka iz istraživanih jezera omogućile su dublje razumijevanje utjecaja okolišnih čimbenika na zajednicu fitoplanktona u sustavima s malim gradijentom fizikalno-kemijskih pokazatelja. Višegodišnji set podataka morfološke analize zajednice fitoplanktona predstavlja pouzdaniju osnovu u odnosu na jednogodišnji set podataka zajednice fitoplanktona utvrđene morfološkim i molekularnim pristupom, zbog čega je analiza utjecaja okolišnih čimbenika provedena samo na temelju višegodišnjeg morfološkog seta podataka.

Morfološkom identifikacijom i analizom okolišne DNA utvrđen je sastav zajednice fitoplanktona u krškim jezerima. Molekularna analiza, temeljena na V9 regiji 18S rRNA otkrila je veći broj svojti, ali uz ograničeno preklapanje s rezultatima morfološke analize, što je posljedica tehničkih ograničenja i nepotpune baze referentnih gena. Unatoč razlikama u taksonomskom i funkcionalnom sastavu zajednice, obje metode dale su iste ili slične ocjene ekološkog stanja zahvaljujući sličnim ili istim faktorima dodijeljenim različitim funkcionalnim grupama. Ipak, kvantifikacija temeljena na broju sekvenci nije reprezentativna u odnosu na brojnost i biomasu fitoplanktona, što dovodi do razlika u udjelu i sastavu funkcionalnih grupa te postavljanja pitanja s ciljem rješavanja problema kvantifikacije i proširivanja referentnih baza gena. Rezultati usporedbe dvaju pristupa naglašavaju važnost daljnjeg razvoja analize okolišne DNA te ukazuju na vrijednost dubokih i plitkih krških jezera u praćenju utjecaja okolišnih čimbenika, antropogenih pritisaka i klimatskih promjena.