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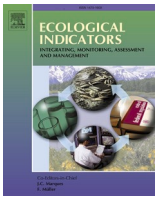

# Assessing ecological status in karstic lakes through the integration of phytoplankton functional groups, morphological approach and environmental DNA metabarcoding

Nikola Hanžek [a], Marija Gligora Udovič [b], Katarina Kajan [c,d], Gábor Borics [e], Gábor Várbíró [e], Thorsten Stoeck [f], Petar Žutinić [b], Sandi Orlić [c,d], Igor Stanković [a,*]

[a] Hrvatske Vode, Central Water Management Laboratory, Ulica grada Vukovara 220, HR-10000 Zagreb, Croatia
[b] University of Zagreb, Faculty of Science, Department of Biology, Rooseveltov trg 6, HR-10000 Zagreb, Croatia
[c] Division of Materials Chemistry, Ruđer Bošković Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia
[d] Center of Excellence for Science and Technology-Integration of Mediterranean Region (STIM), HR-10000 Zagreb, Croatia
[e] Centre for Ecological Research, Institute of Aquatic Ecology, Department of Tisza Research, Bem square 1, H-4026 Debrecen, Hungary
[f] University of Kaiserslautern, Ecology Group, Erwin-Schrödinger-Straße, D-67663 Kaiserslautern, Germany

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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

* Corresponding author.
*E-mail address:* igorstankovic1@gmail.com (I. Stanković).

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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.

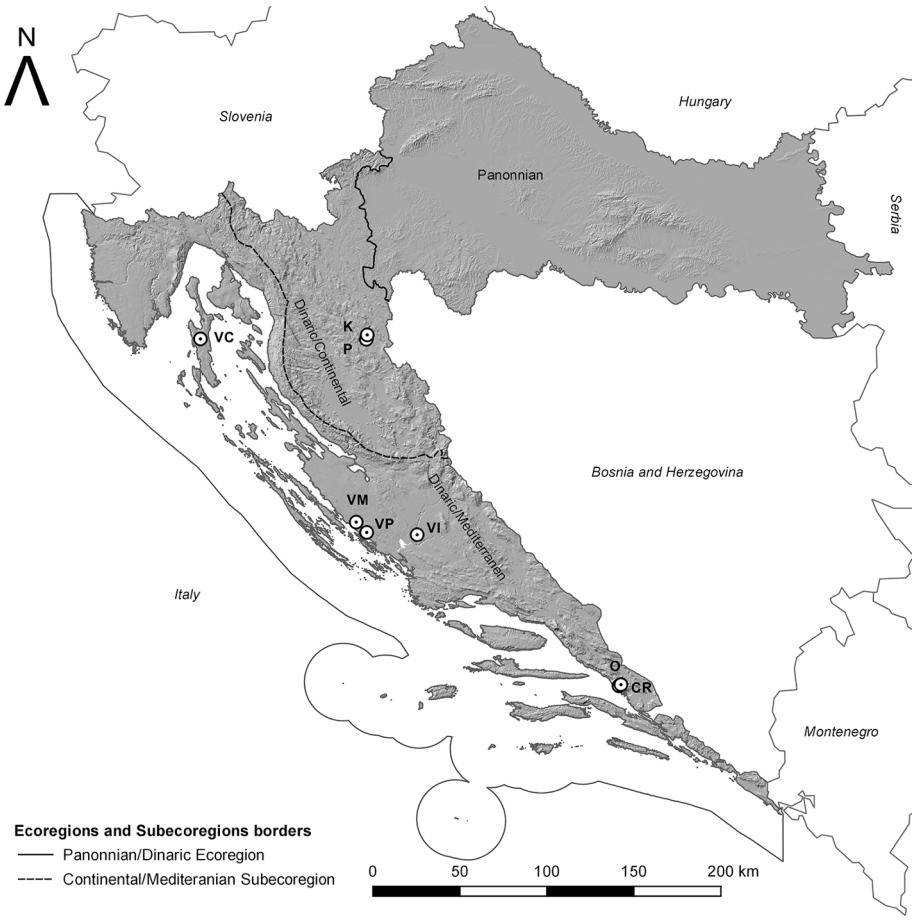


**Fig. 1.** Map of investigated lakes. Site codes are explained in Table 1.

## 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 ($m^3$) | $220.3 \times 10^6$ | $12.7 \times 10^6$ | $7.7 \times 10^6$ | $141.6 \times 10^6$ | | $103 \times 10^6$ | $7 \times 10^6$ | $7.3 \times 10^6$ |
| 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 $i^{th}$ 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:

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

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

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

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

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

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

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 (3) and Choanozoa (1). 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 Oćuša. 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 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

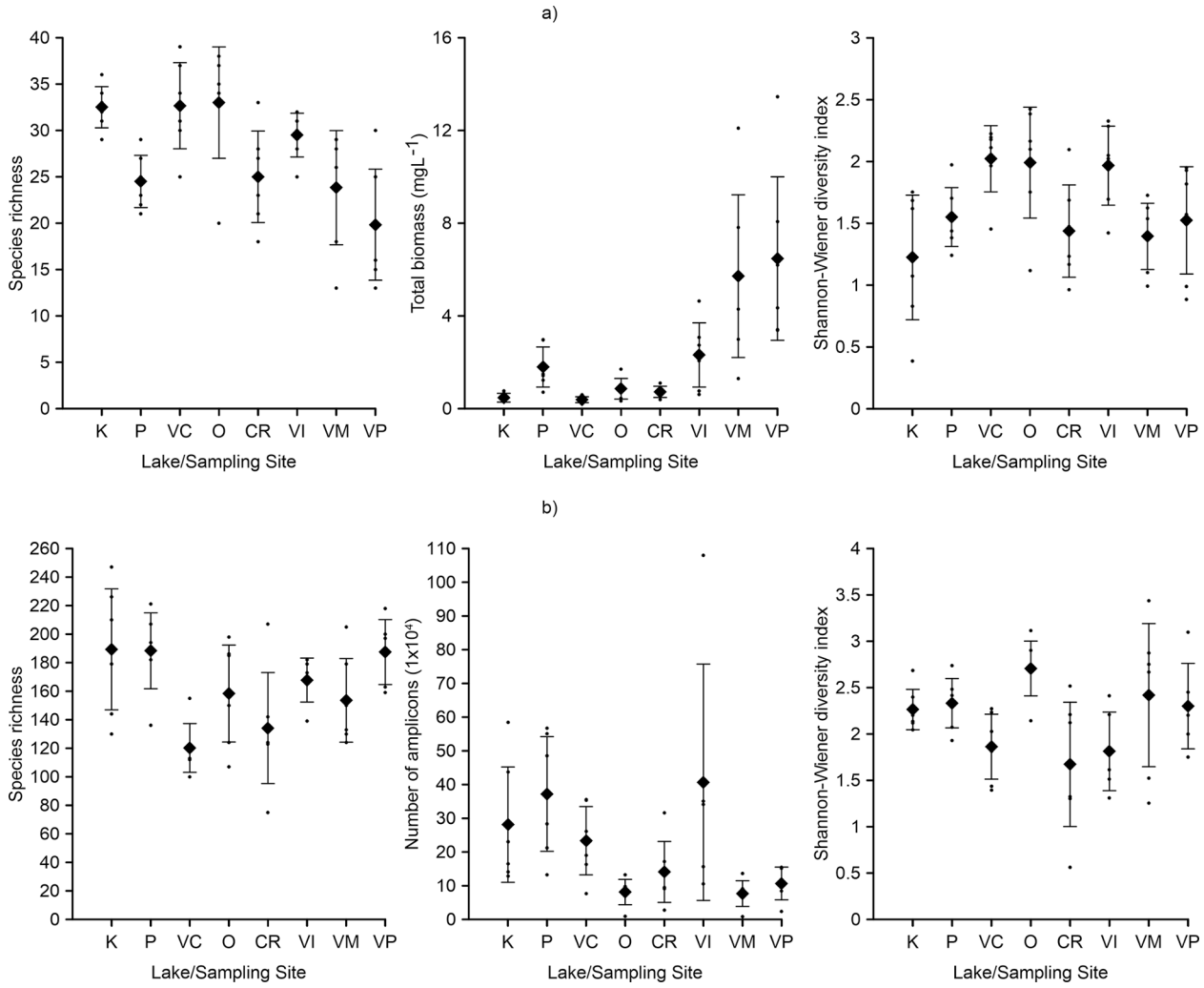


**Fig. 2.** Distribution of species richness, total biomass, number of amplicons, and Shannon-Wiener diversity index values provided by the morphological (a) and molecular (b) approaches. Rhomboids indicate the mean values. Vertical lines represent the upper and lower quartiles. Dots indicate values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

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 (3). 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,* 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

**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 | | | | | | | | |

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 **$L_O$** was the most frequent FG occurring in all five lakes. **$L_O$** 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 **$L_O$** and **F**. The most important FGs in lakes Visovac and Kozjak were **B** and **A**, respectively. The **$L_O$** 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**.

### 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.

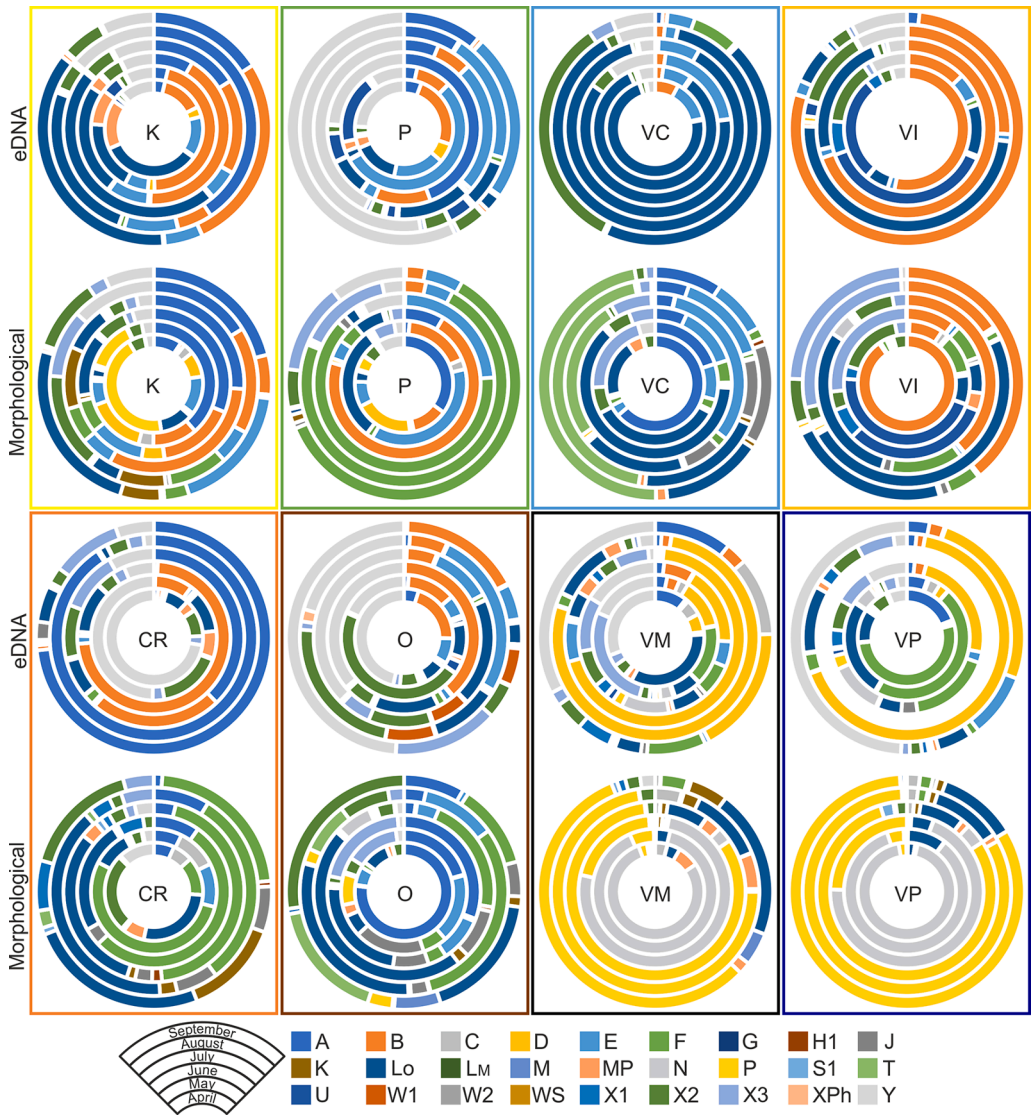


**Fig. 3.** Comparison of the proportion (%) of functional groups determined by morphological approach and eDNA metabarcoding between two samples in each lake for the period from April to September 2017. Concentric circles indicate the proportion (%) of a given functional group within a given month,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

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**, **L**$_{\mathbf{O}}$, 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. 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

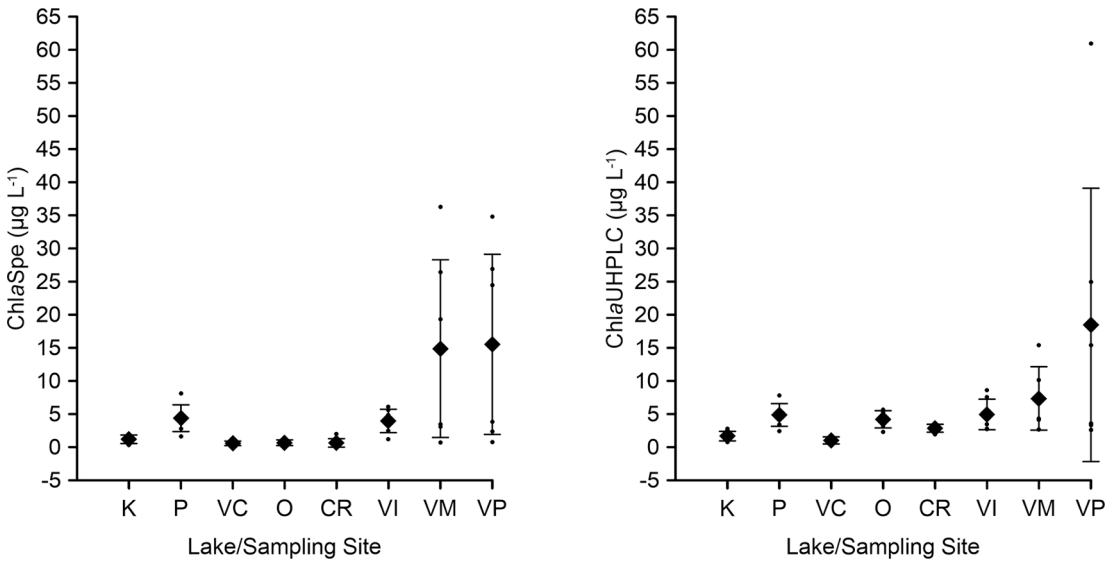


**Fig. 4.** Differences between Chl *a* concentrations measured spectrophotometrically (Chl*a*Spe) and using UHPLC (Chl*a*UHPLC). Rhomboids indicate the mean values. Vertical lines represent the upper and lower quartiles. Dots indicate values for each sample. Lake/Sampling Site codes are explained in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

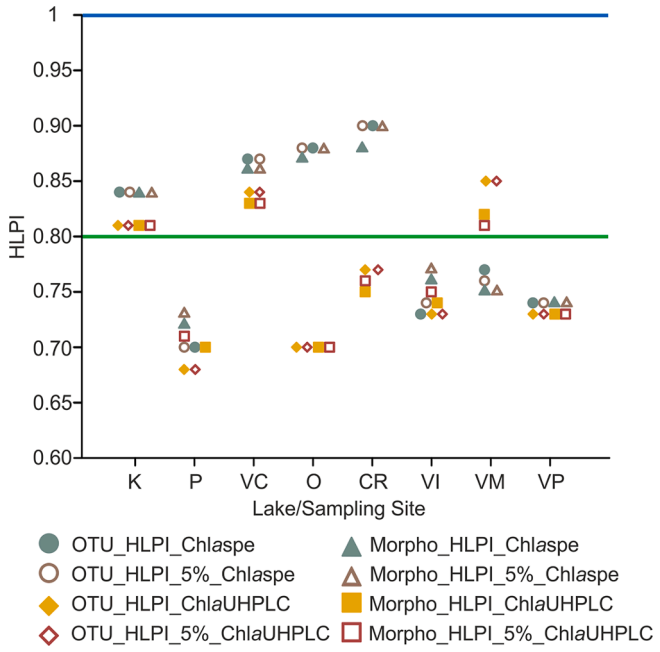


**Fig. 5.** Differences between the HLPI obtained by morphological approach (Morpho_HLPI, Morpho_HLPI_5%) and eDNA metabarcoding (OTU_HLPI, OTU_HLPI_5%) calculated with two Chl *a* measurements methods, spectrophotometry (ChlaSpe) and UHPLC (ChlaUHPLC). 5% in the code indicates taxa/OTUs that contributed 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.)

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* 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 ($p <$

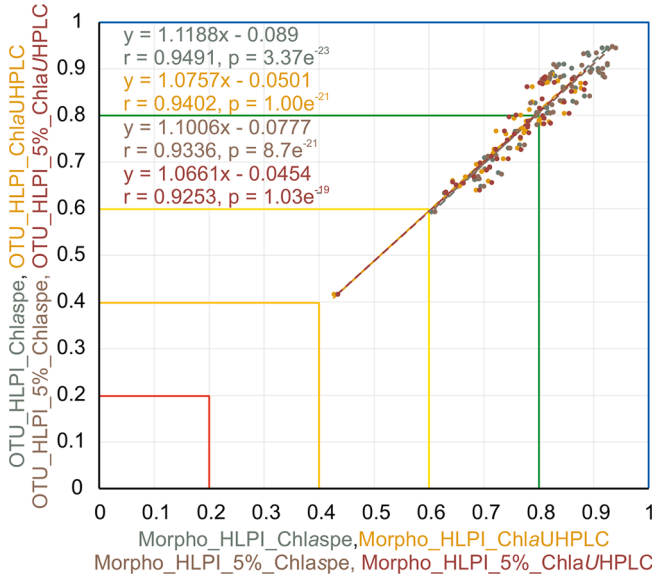


**Fig. 6.** Correlation of HLPI values between morphological approach (Morpho_HLPI, Morpho_HLPI_5%) and eDNA metabarcoding (OTU_HLPI, OTU_HLPI_5%) calculated with two Chl *a* measurements methods, spectrophotometry (ChlaSpe) and UHPLC (ChlaUHPLC). 5% in the code indicates taxa/OTUs that contributed more 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.)

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 **$L_O$**, 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 **$L_O$** 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 Oćuša 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 Udovič:** 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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## References

Abad, D., Albaina, A., Aguirre, M., Laza-Martínez, A., Uriarte, I., Iriarte, A., Villate, F., Estonba, A., 2016. Is metabarcoding suitable for estuarine plankton monitoring? A comparative study with microscopy. Mar. Biol. 163 https://doi.org/10.1007/s00227-016-2920-0.

Alexander, T.J., Vonlanthen, P., Seehausen, O., 2017. Does eutrophication-driven evolution change aquatic ecosystems? Philos. Trans. R. Society B: Biol. Sci. 372, 20160041. https://doi.org/10.1098/rstb.2016.0041.

Apothéloz-Perret-Gentil, L., Cordonier, A., Straub, F., Iseli, J., Esling, P., Pawlowski, J., 2017. Taxonomy-free molecular diatom index for high-throughput eDNA biomonitoring. Mol. Ecol. Resour. 17, 1231–1242. https://doi.org/10.1111/1755-0998.12668.

Bellinger, E., Sigee, D.C., 2015. In: Freshwater Algae: identification, enumeration and use as bioindicators, Second edition. Wiley-Blackwell. https://doi.org/10.1002/9781118917152.

Bhateria, R., Jain, D., 2016. Water quality assessment of lake water: a review. Sustainable Water Resour. Manage. 2, 161–173. https://doi.org/10.1007/s40899-015-0014-7.

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

Bonacci, O., 1984. Promjene vodnog režima Bačinskih jezera - the Bačina Lakes water regime changes. Građevinar 36, 53–58.

Borics, G., Abonyi, A., Salmaso, N., Ptacnik, R., 2020. Freshwater phytoplankton diversity: models, drivers and implications for ecosystem properties. Hydrobiologia 848 (1), 53–75. https://doi.org/10.1007/s10750-020-04332-9.

Borics, G., Wolfram, G., Chiriac, G., Belkinova, D., Donabaum, K., Poikane, S., 2018, Intercalibration of the national classifications of ecological status for Eastern

Continental lakes: Biological quality Element: Phytoplankton.: Publications Office of the European Union, JRC Technical Reports, 2018, doi:10.2760/651989.
Buchaca, T., Felip, M., Catalan, J., 2005. A comparison of HPLC pigment analyses and biovolume estimates of phytoplankton groups in an oligotrophic lake. J. Plankton Res. 27, 91–101. https://doi.org/10.1093/plankt/fbh154.
Bushnell, B., 2014, BBMap: A Fast, Accurate, Splice-Aware Aligner. 9th Annual Genomics of Energy & Environment Meeting, Walnut Creek, USA.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303.
Carty, S., Parrow, M., 2015, Dinoflagellates. Freshwater Algae of North America: Ecology and Classification: 773-807, doi:10.1016/B978-0-12-385876-4.00017-7.
CEN - EN 15204, 2006. Water quality - Guidance standard on the enumeration of phytoplankton using inverted microscopy (Utermohl technique) (EN 15204:2006). European Committee for Standardization (CEN) Brussels, Belgium, 2006.
CEN - EN 15708, 2009. Water quality - Guidance standard for the surveying, sampling and laboratory analysis of phytobenthos in shallow running water (EN 15708:2009). European Committee for Standardization (CEN) Brussels, Belgium, 2009.
CEN – EN 16698, 2015. Water quality - Guidance on quantitative and qualitative sampling of phytoplankton from inland waters (EN 16698:2015). European Committee for Standardization (CEN) Brussels, Belgium.
CEN – EN 16695, 2015. Water quality - Guidance on the estimation of phytoplankton biovolume (EN 16695:2015). European Committee for Standardization, Brussels, Belgium.
Choi, J., Park, J.S., 2020. Comparative analyses of the V4 and V9 regions of 18S rDNA for the extant eukaryotic community using the Illumina platform. Sci. Rep. 10, 6519. https://doi.org/10.1038/s41598-020-63561-z.
Clarke, R. K., Gorley, R. N. Primer v6: User Manual/Tutorial. Plymouth, 2006.
Cordier, T., Lanzén, A., Apothéloz-Perret-Gentil, L., Stoeck, T., Pawlowski, J., 2018. Embracing environmental genomics and machine learning for routine biomonitoring. Trends Microbiol. 27, 387–397. https://doi.org/10.1016/j.tim.2018.10.012.
EC. Common Implementation Strategy for WFD 2000/60/EC, Guidance Document No. 14 - Guidance document on the intercalibration process 2008-2011. Official Journal of the European Communities 2011: 102, doi:10.2779/99432.
Edgar, R., Haas, B., Clemente, J., Quince, C., Knight, R., 2011. UCHIIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200. https://doi.org/10.1093/bioinformatics/btr381.
EEA. European waters - Assessment of status and pressures 2018. Publications Office of the European Union, Luxembourg 2018; No 7/2018: 85, doi:10.2800/303664.
Eiler, A., Drakare, S., Bertilsson, S., Pernthaler, J., Peura, S., Rofner, C., Simek, K., Yang, Y., Znachor, P., Lindström, E.S., De Smet, I., 2013. Unveiling distribution patterns of freshwater phytoplankton by a next generation sequencing based approach. PLoS ONE 8, e53516. https://doi.org/10.1371/journal.pone.0053516.
Elbrecht, V., Vamos, E.E., Meissner, K., Aroviita, J., Leese, F., Yu, D., 2017. Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. Methods Ecol. Evol. 8, 1265–1275. https://doi.org/10.1111/2041-210X.12789.
Gligora Udovič, M., Žutinić, P., 2020, Report on lake phytoplankton classification method in case where the Intercalibration exercise is not possible (Gap 3). 16.
Gligora Udovič, M., Cvetkoska, A., Žutinić, P., Bosak, S., Stanković, I., Špoljarić, I., Mršić, G., Kralj Borojević, K., Ćukurin, A., Plenković-Moraj, A., 2016. Defining centric diatoms of most relevant phytoplankton functional groups in deep karst lakes. Hydrobiologia 788, 169–191. https://doi.org/10.1007/s10750-016-2996-z.
Golden Software I. Grapher TM . Golden Software, Inc, 809 14th Street, Golden, Colorado 80401, 2020.
Guiry MD, Guiry GM. AlgaeBase. In: World-wide electronic publication, National University of Ireland, Galway, 2021, http://www.algaebase.org Accessed 03 June 2021.
Hering, D., Borja, A., Jones, J.I., Pont, D., Boets, P., Bouchez, A., Bruce, K., Drakare, S., Hänfling, B., Kahlert, M., Leese, F., Meissner, K., Mergen, P., Reyjol, Y., Segurado, P., Vogler, A., Kelly, M., 2018. Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. Water Res. 138, 192–205. https://doi.org/10.1016/j.watres.2018.03.003.
Herrero, A., Gutiérrez-Cánovas, C., Vigiak, O., Lutz, S., Kumar, R., Gampe, D., Huber-García, V., Ludwig, R., Batalla, R., Sabater, S., 2018. Multiple stressor effects on biological quality elements in the Ebro River: present diagnosis and predicted responses. Sci. Total Environ. 630, 1608–1618. https://doi.org/10.1016/j.scitotenv.2018.02.032.
Higgins, H.W., Wright, S.W., Schlüter, L., 2011, Quantitative interpretation of chemotaxonomic pigment data. in: Llewellyn, C.A., Egeland, E.S., Johnsen, G., Roy, S., (eds.) Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography. Cambridge University Press, Cambridge, 2011, pp. 257-313, doi: 10.1017/CBO9780511732263.010.
Huo, S., Li, X., Xi, B., Zhang, H., Ma, C., He, Z., 2020. Combining morphological and metabarcoding approaches reveals the freshwater eukaryotic phytoplankton community. Environ. Sci. Eur. 32, 37. https://doi.org/10.1186/s12302-020-00321-w.
HRN ISO 10260, 2001. Water quality - Measurment of biochemical parameters - Spectrophotometric determination of the chlorophyll-a concentration (ISO 10260: 1992). International Organization for Standardization.
IBM C. IBM SPSS Statistics for Windows, Version 25.0. 2017.
Joo, S., Lee, S.-R., Park, S., 2010. Monitoring of phytoplankton community structure using terminal restriction fragment length polymorphism (T-RFLP). J. Microbiol. Methods 81, 61–68. https://doi.org/10.1016/j.mimet.2010.01.025.
Laplace-Treyture, C., Feret, T., 2016. Performance of the Phytoplankton Index for Lakes (IPLAC): a multimetric phytoplankton index to assess the ecological status of water bodies in France. Ecol. Ind. 69, 686–698. https://doi.org/10.1016/j.ecolind.2016.05.025.
Lund, J.W.G., Kipling, C., Le Cren, E.D., 1958, The inverted microscope method of estimating algal numbers and the statistical basis of estimations by counting. Hydrobiologia 11: 143-170, doi:10.1007/BF00007865.
Luo, W., Pflugmacher, S., Pröschold, T., Walz, N., Krienitz, L., 2006. Genotype versus phenotype variability in Chlorella and Micractinium (Chlorophyta, Trebouxiophyceae). Protist 157, 315–333. https://doi.org/10.1016/j.protis.2006.05.006.
Lyche-Solheim, A., Feld, C.K., Birk, S., Phillips, G., Carvalho, L., Morabito, G., Mischke, U., Willby, N., Søndergaard, M., Hellsten, S., Kolada, A., Mjelde, M., Böhmer, Jürgen, Miler, O., Pusch, M.T., Argillier, C., Jeppesen, E., Lauridsen, T.L., Poikane, S., 2013. Ecological status assessment of European lakes: a comparison of metrics for phytoplankton, macrophytes, benthic invertebrates and fish. Hydrobiologia 704, 57–74. https://doi.org/10.1007/s10750-012-1436-y.
Mahé, F., Rognes, T., Quince, C., de Vargas, C., Dunthorn, M., 2015, Swarmv2: Highly-scalable and high-resolution amplicon clustering. PeerJ 2015; 3: e1420, doi: 10.7717/peerj.1420.
Markowska, J., 2004. The Origins of the Plitvice Lakes (Croatia). Miscellanea Geogr. 11, 93–99. https://doi.org/10.2478/mgrsd-2004-0012.
Mischke, U., Riedmüller, U., Hoehn, E., Schönfelder, I., Nixdorf, B., 2008, Description of the German system for phytoplankton-based assessment of lakes for implementation of the EU Water Framework Directive (WFD), pp. 117-146, doi:10.13140/2.1.3545.1847.
Misra, O., Chaturvedi, D., 2016. Fate of dissolved oxygen and survival of fish population in aquatic ecosystem with nutrient loading: a model. Modeling Earth Syst. Environ. 2, 112. https://doi.org/10.1007/s40808-016-0168-9.
Not, F., Zapata, M., Pazos, Y., Campaña, E., Doval, M., Rodríguez, F., 2007. Size-fractionated phytoplankton diversity in the NW Iberian coast: a combination of microscopic, pigment and molecular analyses. Aquat. Microb. Ecol. 49, 255–265. https://doi.org/10.3354/ame01144.
Padisák, J., Borics, Gábor, Grigorszky, I., Soróczki-Pintér, Éva, 2006. Use of phytoplankton assemblages for monitoring ecological status of lakes within the water framework directive: the assemblage index. Hydrobiologia 553, 1–14. https://doi.org/10.1007/s10750-005-1393-9.
Padisák, J., Crossetti, L.O., Naselli-Flores, L., 2009. Use and misuse in the application of the phytoplankton functional classification: a critical review with updates. Hydrobiologia 621, 1–19. https://doi.org/10.1007/s10750-008-9645-0.
Palińska, K.A., Surosz, W., 2008. Population of Aphanizomenon from the Gulf of Gdańsk (Southern Baltic Sea): differences in phenotypic and genotypic characteristics. Hydrobiologia 607, 163–173. https://doi.org/10.1007/s10750-008-9388-y.
Pawlowski, J., Kelly-Quinn, M., Altermatt, F., Apothéloz-Perret-Gentil, L., Beja, P., Boggero, A., Borja, A., Bouchez, A., Cordier, T., Domaizon, I., Feio, M.J., Filipe, A.F., Fornaroli, R., Graf, W., Herder, J., van der Hoorn, B., Iwan Jones, J., Sagova-Mareckova, M., Moritz, C., Barquín, J., Piggott, J.J., Pinna, M., Rimet, F., Rinkevich, B., Sousa-Santos, C., Specchia, V., Trobajo, R., Vasselon, V., Vitecek, S., Zimmerman, J., Weigand, A., Leese, F., Kahlert, M., 2018. The future of biotic indices in the ecogenomic era: integrating (e)DNA metabarcoding in biological assessment of aquatic ecosystems. Sci. Total Environ. 637-638, 1295–1310. https://doi.org/10.1016/j.scitotenv.2018.05.002.
Peng, F., Liu, S., Xu, H., Li, Z., 2013. A comparative study on the analysis methods for chlorophyll-a. Adv. Mater. Res. 726–731, 1411–1415. https://doi.org/10.4028/www.scientific.net/AMR.726-731.1411.
Pinckney, J.L., Millie, D.F., Heukelem, L.V., 2011, Update on filtration, storage and extraction solvents. in: Llewellyn, C.A., Egeland, E.S., Johnsen, G., Roy, S., (eds.). Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography. Cambridge University Press, Cambridge, 2011, pp. 627-635, doi: 10.1017/CBO9780511732263.024.
Poikane, S., Zampoukas, N., Borja, A., Davies, S., Bund, W., Birk, S., 2014. Intercalibration of aquatic ecological assessment methods in the European Union: lessons learned and way forward. Environ. Sci. Policy 44, 237–246. https://doi.org/10.1016/j.envsci.2014.08.006.
Poikane, S., Zohary, T., Cantonati, M., 2019. Assessing the ecological effects of hydromorphological pressures on European lakes. Inland Waters 10, 241–255. https://doi.org/10.1080/20442041.2019.1654800.
Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., Roset, N., Schabuss, M., Zornig, H., Dejean, T., 2018. Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. Sci. Rep. 8 https://doi.org/10.1038/s41598-018-28424-8.
Reynolds, C., Huszar, V., Kruk, C., Naselli-Flores, L., Melo, S., 2002. Towards a functional classification of the freshwater phytoplankton. J. Plankton Res. 24, 417–428. https://doi.org/10.1093/plankt/24.5.417.
Rott, E., 1981. Some results from phytoplankton counting intercalibrations. Schweiz. Z. Hydrol. 43, 34–62. https://doi.org/10.1007/BF02502471.
Schmeller, D.S., Loyau, A., Bao, K., Brack, W., Chatzinotas, A., De Vleeschouwer, F., Friesen, J., Gandois, L., Hansson, S.V., Haver, M., Le Roux, G., Shen, J., Teisserenc, R., Vredenburg, V.T., 2017. People, pollution and pathogens - global change impacts in mountain freshwater ecosystems. Sci. Total Environ. 622-623, 756–763. https://doi.org/10.1016/j.scitotenv.2017.12.006.
Scholz, S.N., Esterhuizen-Londt, M., Pflugmacher, S., 2017. Rise of toxic cyanobacterial blooms in temperate freshwater lakes: causes, correlations and possible

countermeasures. Toxicol. Environ. Chem. 99 (4), 543–577. https://doi.org/10.1080/02772248.2016.1269332.
Seymour, M., Edwards, F.K., Cosby, B.J., Kelly, M.G., de Bruyn, M., Carvalho, G.R., Creer, S., 2020. Executing multi-taxa eDNA ecological assessment via traditional metrics and interactive networks. Sci. Total Environ. 729, 138801. https://doi.org/10.1016/j.scitotenv.2020.138801.
Shapiro, S.S., Wilk, M.B., 1965, An analysis of variance test for normality (complete samples)†. Biometrika 52: 591-611, doi:10.1093/biomet/52.3-4.591.
Soares, M., Lurling, M., Huszar, V., 2013, Growth and temperature-related phenotypic plasticity in the cyanobacterium Cylindrospermopsis raciborskii. Phycological Research 61: 61-67, doi:10.1111/pre.12001.
Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M., Breiner, H.-W., et al., 2010. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Mol. Ecol. 19 (Suppl 1), 21–31. https://doi.org/10.1111/j.1365-294X.2009.04480.x.
Sun, Z., Majaneva, M., Sokolova, E., Rauch, S., Meland, S., Ekrem, T., 2019. DNA metabarcoding adds valuable information for management of biodiversity in roadside stormwater ponds. Ecol. Evol. 9, 9712–9722. https://doi.org/10.1002/ece3.5503.
Teneva, I., Belkinova, D., Mladenov, R., Stoyanov, P., Moten, D., Basheva, D., et al., 2020, Phytoplankton composition with an emphasis of Cyanobacteria and their toxins as an indicator for the ecological status of Lake Vaya (Bulgaria) – part of the Via Pontica migration route. Biodiversity Data Journal 8: e57507, doi:10.3897/BDJ.8.e57507.
Tragin, M., Zingone, A., Vaulot, D., 2017. Comparison of coastal phytoplankton composition estimated from the V4 and V9 regions of 18S rRNA gene with a focus on photosynthetic groups and especially Chlorophyta. Environ. Microbiol. 20, 506–520. https://doi.org/10.1111/1462-2920.13952.
Utermöhl, H., 1958, Methods of collecting plankton for various purposes are discussed. SIL Communications, 1953-1996 9: 1-38, doi:10.1080/05384680.1958.11904091.
Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G.H., Geniez, P., Pont, D., Argillier, C., Baudoin, J.-M., Peroux, T., Crivelli, A. J., Olivier, A., Acqueberge, M., Le Brun, M., Møller, P.R., Willerslev, E., Dejean, T., 2015. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Mol. Ecol. 25, 929–942. https://doi.org/10.1111/mec.13428.
Vasselon, V., Bouchez, A., Rimet, F., Jacquet, S., Trobajo, R., Corniquel, Méline, Tapolczai, Kálmán, Domaizon, I., Mahon, A., 2017. Avoiding quantification bias in metabarcoding: application of a cell biovolume correction factor in diatom molecular biomonitoring. Methods Ecol. Evol. 9, 1060–1069. https://doi.org/10.1111/2041-210X.12960.
WFD. Directive 2000/60/ec of the European Parliament and of the Council 22.12.2000. Official Journal of the European Communities 2000; L327: 1-72.
Whitton, B.A., Potts, M., 2012, Introduction to the Cyanobacteria. Ecology of Cyanobacteria II: Their Diversity in Space and Time 1-13, doi:10.1007/978-94-007-3855-3_1.
Wilmotte, A., Laughinghouse, Iv H., Capelli, C., Rippka, R., Salmaso, N., 2017, Taxonomic Identification of Cyanobacteria by a Polyphasic Approach, pp. 79-134, doi:10.1002/9781119332169.ch4.
Xiao, X., Sogge, H., Lagesen, K., Tooming-Klunderud, A., Jakobsen, K.S., Rohrlack, T., Lovejoy, C., 2014. Use of high throughput sequencing and light microscopy show contrasting results in a study of phytoplankton occurrence in a freshwater environment. PLoS One 9, e106510. https://doi.org/10.1371/journal.pone.0106510.