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Original Research Article

Detection of charophyte algae (Characeae) in lakes using eDNA metabarcoding

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ABSTRACT

Detection of charophyte algae (Characeae) and identification to species level is important for biodiversity conservation and ecological status assessment, and environmental DNA (eDNA) might enable fast and replicable species detection. The aims of our study were to evaluate the performance of the *rbcL* gene marker in detecting eDNA of Characeae and to compare the eDNA method with the results from traditional sampling. Characeae were sampled from two freshwater and two saline lakes in Serbia during spring, summer, and autumn 2023. In total, 7 species within 3 genera (*Chara, Nitella* and *Nitellopsis*) were detected by the traditional method, and species identity was confirmed using barcoding of the *rbcL* gene. Metabarcoding of eDNA detected 7 charophyte taxa, 6 from the genus *Chara* and *Nitellopsis obtusa*. *C. canescens* was detected to the species level by eDNA, while other taxa could be assigned to species groups within the genus Chara only. The charophyte genus *Nitella* was not detected using eDNA metabarcoding, even though it was found by traditional methods. Our study confirms that it is possible to use eDNA metabarcoding to detect the presence of Characeae in lakes. The overall agreement on the presence and absence of taxa between traditional mapping and eDNA was 80,5%, across four different lakes and three seasons. The *rbcL* marker, however, cannot discriminate all Characeae to species level, limiting its use for biodiversity monitoring or ecological status assessment.

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1. Introduction

Charophyte algae (Characeae) are macroscopic benthic algae that commonly occur in standing and slowly flowing freshwater or brackish habitats. They deliver many important ecosystem services, such as providing food and habitat for other aquatic species, or purifying water (Schneider et al., 2015a, Schubert et al., 2018). Many charophyte species are rare and red-listed (Stewart, 2024). Charophyte algae may, however, be easily overlooked in the field because of their inconspicuousness and due to species-specific differences in life cycles. Some charophyte species develop in early spring and typically have completed their life cycle at a point in time when other species only start growing (Marković et al. 2023, Pukacz et al., 2024). To detect all species occurring in a water body, several field surveys may therefore be necessary throughout the growing season. In addition, charophyte algae may exhibit great phenotypic plasticity, which often makes morphological species determination difficult (Schubert, 2014, Schneider et al., 2015b, 2016). Despite these challenges, many species of charophyte algae are commonly used for ecological status assessment, e.g. according to the Water Framework Directive (WFD) (EC (European Commission 2000), Stelzer et al., 2005, Kolada, 2021).

Currently, the standard method for ecological status assessment involves manual collection of charophyte algae combined with morphological species determination. This process is time-consuming and therefore costly. In addition, rare species may be overlooked, and the phenotypic plasticity of charophyte algae may lead to uncertain species determination. It is, therefore, important to improve the effectiveness of charophyte species detection in aquatic ecosystems and enhance the accuracy of species determination. For this purpose, DNA barcoding and metabarcoding are important tools. Metabarcoding of environmental DNA (eDNA), i.e. the detection of a broad range of species simultaneously in an environmental sample, has recently become widely used for, e.g. biodiversity assessment, biomonitoring, invasive species control, and the discovery of rare, threatened, unknown, and cryptic species in different environments (Ruppert et al., 2019, Banerjee et al., 2022). Compared to traditional monitoring methods, eDNA metabarcoding may be a cost-effective and time-efficient approach, making it ideal for large-scale monitoring efforts (Smart et al., 2016, Sternhagen et al., 2024).

Many eDNA studies have been published on aquatic animals, such as fish (Thomsen et al., 2012, Xiong et al., 2022) and macroinvertebrates (Duarte et al., 2021, Vourka et al., 2023), or microscopic algae, such as diatoms (Mbao et al., 2023; Vidaković et al. 2024), or phytoplankton (Andersson et al., 2023, Tapolczai et al., 2025). In contrast, eDNA studies on aquatic plants have long been scarce. Recently, however, a considerable number of studies have emerged (Espinosa Prieto et al., 2023; Espinosa Prieto et al., 2024), focusing on various habitats, such as rivers (Coghlan et al., 2021, Espinosa Prieto et al., 2024), ponds (Robertson, 2024) or coastal areas (Bombin et al., 2024) and species groups, such as pondweeds (Kuzmina et al., 2018), invasive aquatic macrophytes (Fujiwara et al., 2016, Anglès d'Auriac et al., 2019) or threatened species (Tsukamoto et al., 2021). While DNA barcoding of charophytes has been advancing for over a decade (Schneider et al., 2015b, 2016, Trbojević et al. 2020, Mjelde et al., 2021, Romanov et al., 2023) improving charophyte sequence libraries in databases such as in the National Centre for Biotechnology Information (NCBI) and the Barcode of Life Data System (BOLD), metabarcoding is not yet an established method for charophyte algae.

In this study, we used eDNA metabarcoding to detect the presence of charophyte algae in lakes. The aims of our studies were: (1) to compare traditional methods, i.e. manual collection of charophyte algae combined with morphological species identification, with eDNA metabarcoding of water samples using *rbcL* as marker gene, (2) to verify the morphological species identification by barcoding selected samples of charophyte algae, (3) to assess the suitability of metabarcoding for the detection of charophyte species in freshwater and saline lakes.

2. Materials and Methods

2.1. Study sites

Sampling was done in two freshwater and two saline lakes in Serbia, in spring, summer and autumn 2023. The four study lakes are located in different regions in Serbia, all of which experience a pronounced continental climate (Fig. 1). The saline ponds Pečena Slatina (PS) and Plava banja (PB) are situated in the northern province of Vojvodina. The freshwater reservoirs, Sava Lake (SL) and Markovačko Lake (ML), are situated in central Serbia (Fig. 1). The general characteristics of the study lakes are given in Table 1. Sampling was done in spring, summer and autumn to detect the potential occurrence of short-lived charophyte algae, which might occur in early spring only, as well as to capture different life stages of longer-lived charophytes (e.g., the period of sexual reproduction). In each lake, samples were taken at four sites per lake (Fig. 1), using both traditional and eDNA methods.

2.2. Traditional charophyte sampling and identification

In each lake, charophytes were collected from three 10–15 m wide transects, perpendicular to the shore, up to the maximum vegetation limit. Sampling was done from a boat, using a rake, and by snorkeling. The abundance of each species in each transect was estimated according to a five-degree scale: 1 = very rare, 2 = infrequent, 3 = common, 4 = frequent, 5 = abundant, predominant. Additionally, charophytes were collected from the central point in each lake, using a rake. To estimate the overall abundance of each species in each lake, abundances from the three transects and the central point were combined. Charophyte species were identified using the keys by Krause (1997), Bailly and Schaefer (2010) and Mouronval et al. (2015). The nomenclature follows Schubert et al. (2024a). Samples were preserved in an alcohol solution (50% ethanol: glycerin, 1:1) and a dry herbarium and deposited in the BIOLACHC collection at the University of Belgrade, Institute of Chemistry, Technology and Metallurgy, National Institute of the

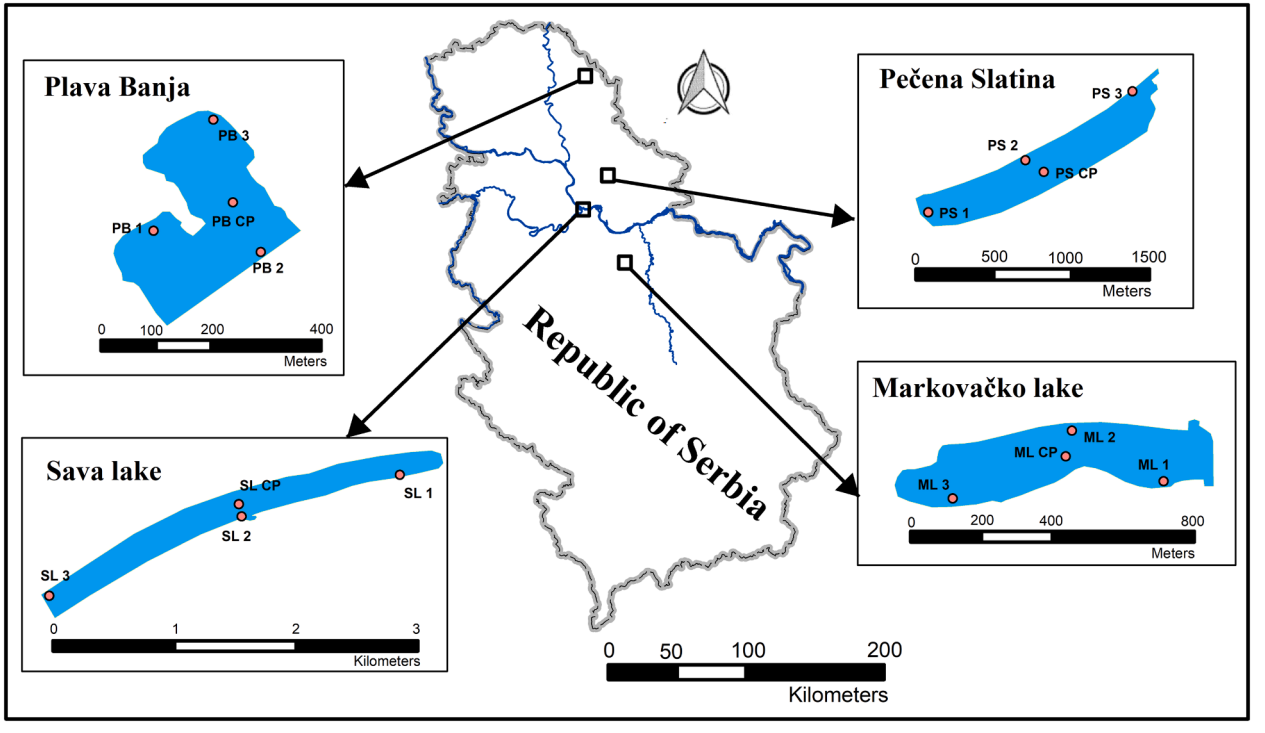

Fig. 1. Location of lakes and sampling sites. In each lake, samples were taken from three sites along the shore, and from the central point (marked CP in the maps) (map background based on open access data provided by the European Space Agency [ESA], 2023).

Republic of Serbia.

2.3. Barcoding of Characeae samples

Species identity of the morphological determination was checked by barcoding selected charophyte samples. We barcoded the *rbcL* region, i.e. the same marker used in the eDNA metabarcoding approach described below. The *rbcL* gene was chosen because it is a relatively unspecific marker which is easily amplified, and sequences are available for many aquatic and land plants (Kress and Erickson, 2007, Schneider et al., 2015b, Revéret et al., 2023). Total genomic DNA was extracted from silica-dried material of eight Charophyte samples. After adding 180 μl ATL buffer to each sample, they were heated up to 100 °C for 5 min. The samples were then transferred to tubes with approx. 0.3 g of Zirconium beads (autoclaved; 0.7 mm). After bead beating (2 ×6400 rpm for 30 s) in a Precellys 24 bead beater (Bertin, Technologies, Saint-Quentin, France the samples were further processed using the DNeasy® Blood & Tissue Kit Spin-Column Protocol: Purification of Total DNA from Animal Tissues (Qiagen, Oslo, Norway). The DNA extracted was then stored at −20 °C until further analysis.

The primers rbcLanew-F (TGTCACCACARACAGARACTAAARC), modified after Levin et al. (2003), and rbcLanew-R GTAAART-CAAGYCCACCRCG, modified after Kress and Erickson (2007), were selected for *rbcL*. PCRs for *rbcL* were performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System or CFX Opus Real-Time PCR Systems (Bio-Rad Laboratories, Oslo, Norway) using the Ssofast EvagreenTM Supermix (Bio-Rad Laboratories, Oslo, Norway). The reaction mixture (30 μl) contained 15 μl SsoFast™ EvaGreen® Supermix, 300 nM forward primer, 300 nM reverse primer, 2 μl template DNA and RNAse/DNAse free water. The cycling conditions were as follows: one cycle of 30 s at 95 °C; and then 35 cycles each consisting of 10 s at 95 °C, 20 s at 62 °C, and 20 s at 72 °C, followed by a melt curve analysis in the temperature range from 60 to 95 °C. The amplified PCR products were purified using the Monarch® Spin PCR & DNA Cleanup Kit (5 μg) (NEB #T1130) (New England Biolabs, Ipswich, MA, USA).

Sequencing of the purified amplicons was performed using the same primers as for PCR. For each PCR product, both strands were sequenced on an ABI 3730 Avant genetic analyser using the BigDyeterminator V.3.1 cycle sequencing kit (Applied Biosystems, Thermo Fisher Scientific Oslo, Norway) according to the manufacturer's instructions.

Sequences were aligned using Align (version 03/2007) MS Windows-based manual sequence alignment editor (SequentiX – Digital DNA Processing, Klein Raden, Germany) to obtain a DNA sequence alignment, which was then corrected manually to a sequence length of 520 bp. *Tolypella glomerata* (MH424120) was employed as outgroup, seven Serbian Charophyte samples from this study and 35 additional Charophyte sequences derived from GenBank (NCBI 2025) and Bold v4 (Ratnasingham et al., 2024) were included in the analyses. Phylogenetic trees for *rbcL* genes were constructed using the ML algorithm in Mega v. 12 (Kumar et al., 2024). In the ML analyses, evolutionary substitution models were evaluated using Mega v. 12 (Kumar et al., 2024). The T92 + I evolutionary model was found to be the best-fitting evolutionary model for the *rbcL* gene tree. ML analysis of the *rbcL* tree was performed with 1000 bootstrap replicates using Mega v. 12 (Kumar et al., 2024). The sequence data were submitted to the NCBI database under the accession numbers PV594400-PV594406 (Fig. 2).

2.4. eDNA metabarcoding of water samples

Water samples for eDNA were taken at the same sites where traditional sampling was conducted. At each transect, water was sampled into three 1.5 L sterile plastic bottles, one at each third of the transect, and then mixed to form an integrated sample covering the entire transect. In addition, one integrated 1.5 L water sample was taken at the central point (Fig. 1). In total, 48 eDNA samples (4 in spring, 4 in summer and 4 in autumn in each of the four study lakes, respectively) were collected. Immediately after sampling, i.e. while still at the lake, the water samples were filtered through Sterivex filters, using the Pressure Assisted Filtration system (PAF; NIRAS A/S, Allerød, Denmark). For preservation, 700 μl ATL buffer (Qiagen, Oslo, Norway) was added immediately after filtration to the Sterivex filters, which were then stored at 4 °C until transport to Norway. In Norway the samples were then stored at 4 °C until further analysis, which was performed after 3–10 months, depending on when the samples were taken. No field blanks were included in the filtration and analysis.

For all the following steps, DNA extraction was conducted according to Spens et al. (2017). As the final step, the DNeasy® Blood & Tissue kit (Qiagen, Oslo, Norway) was used according to the manufacturer`s protocol. The extracted DNA was stored at −20 °C until further analysis. No extraction blanks were included in the analysis.

The same primers, PCR protocols, and PCR machines were used as described above in the chapter on barcoding. The reaction mixture (30 μl) contained 15 μl SsoFast™ EvaGreen® Supermix, 300 nM forward and reverse primer and RNAse/DNAse free water. The cycling conditions were as follows: one cycle of 30 s at 95 °C; and then 35 cycles each consisting of 10 s at 95 °C, 20 s at 62 °C, and 20 s at 72 °C, followed by a melt curve analysis in the temperature range from 60 to 95 °C. The amplified PCR products were purified

Table 1
General characteristics of the study lakes.

Study Lake	Type of water body	Coordinates (WGS84)	Surface area	Average depth	Max depth
Pečena Slatina (PS)	natural saline pond	45°05'02.1"N 20°29'41.3"E	~ 26 ha	< 1 m	1.2 m
Plava banja (PB)	artificial saline pond	45°48'10.0"N 20°26'59.9"E	~ 7 ha	~ 5 m	7 m
Sava Lake (SL)	freshwater reservoir	44°47'03.5"N 20°23'32.2"E	~ 90 ha	~ 4–6 m	12 m
Markovačko Lake (ML)	freshwater reservoir	44°23'22.8"N 20°39'32.8"E	~ 17 ha	~ 6–7 m	8 m

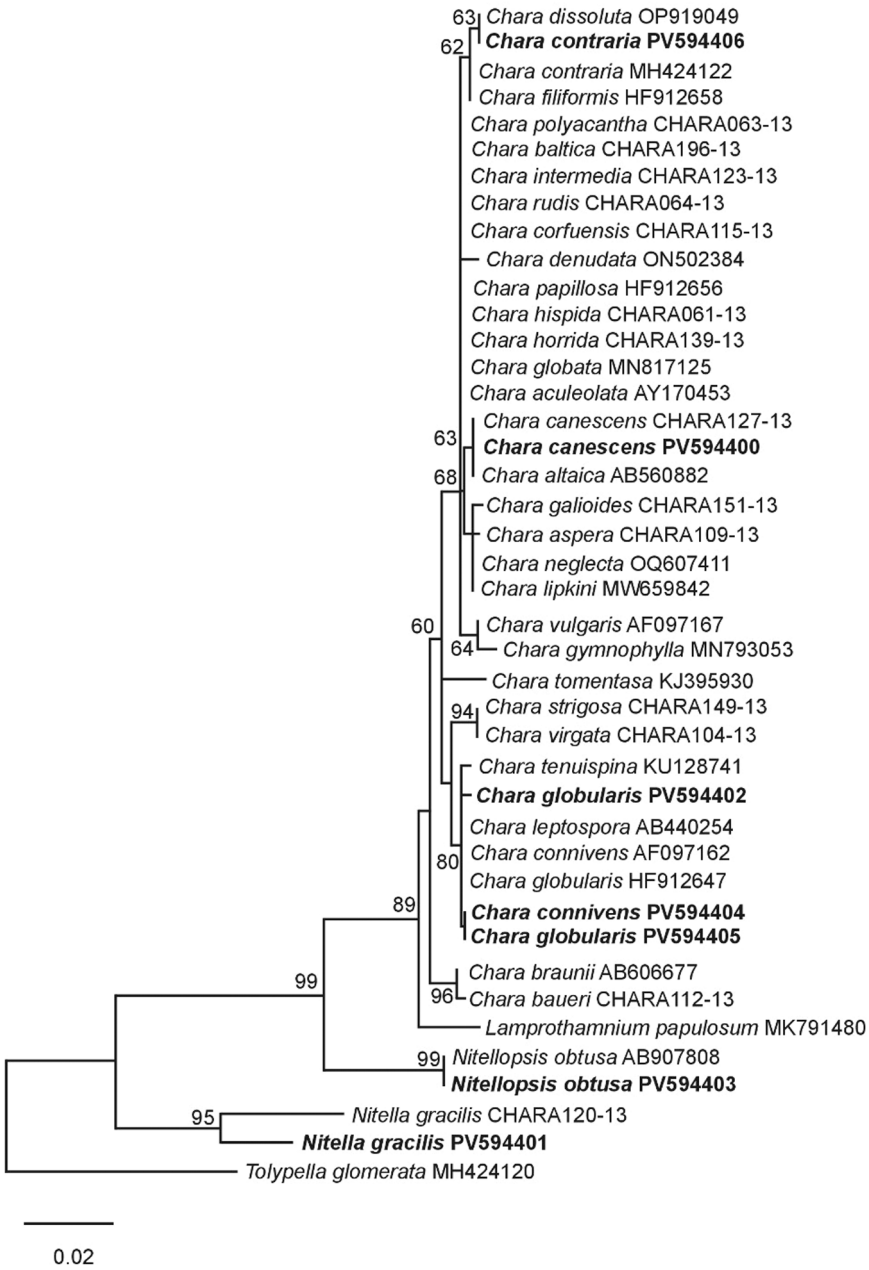

Fig. 2. Maximum likelihood (ML) tree of *rbcL* sequences of 42 Charophyte samples. Samples from this study are marked in bold. Bootstrap values above 50% are shown in the tree. The bar indicates 2% sequence divergence. *RbcL* is a conservative marker and therefore generally does not differentiate between closely related Characeae species. We therefore consider the sample "Chara contraria PV594406" (at the top of Fig. 2) as correctly identified, because it was aligned to the group to which *C. contraria*, *C. dissoluta* and *C. filiformis* belong. A similar argumentation is true for all other *Chara* samples in our study.

using the Monarch® Spin PCR & DNA Cleanup Kit (5 μg) (NEB #T1130) (New England Biolabs, Ipswich, MA, USA). Between 13 and 80 ng were used as template (Table 2).

Chara zeylanica M59 (Mjelde et al., 2021) was used as a positive control and RNA/DNA free water as a negative control. Two barcoding libraries were prepared, one including 12 amplicon samples from Sava Lake and 12 amplicon samples from Markovačko Lake and one including 12 amplicon samples from Plava banja and 12 amplicon samples from Pečena Slatina, each sample containing 200 fmol of amplicon DNA. The Ligation sequencing amplicons -Native Barcoding Kit 24 V14(SQK-NBD114.24) (Oxford Nanopore Technologies, Oxford, UK) was used according to the protocol of the manufacturer (https://nanoporetech.com/document/ligation-sequencing-gdna-native-barcoding-v14-sqk-nbd114-24). Sequencing was conducted using a MinION Flow Cell (R10.4.1) plugged

Table 2
eDNA amount (ng) used as template.

Lake	Sava lake				Pečena Slatina				Plava banja				Markovačko lake			
Transect	**SL1**	**SL2**	**SL3**	**SLCP**	**PS1**	**PS2**	**PS3**	**PSCP**	**PB1**	**PB2**	**PB3**	**PBCP**	**ML1**	**ML2**	**ML3**	**MLCP**
spring	17	15	21	30	15	14	39	13	44	82	26	28	29	20	27	47
summer	13	12	13	13	15	14	12	16	23	30	24	25	16	19	18	13
autumn	15	13	14	13	46	22	14	22	25	47	38	37	26	13	21	33

into a MINION device and MinKNOW v. 25.09.16 (Bream 8.8.3, Configuration 6.8.9, Dorado 7.11.2, MinKNOW Core 6.8.11) (Oxford Nanopore Technologies, Oxford, UK). Real-time basecalling was conducted during sequencing and the raw data were demultiplexed with Dorado 7.11.2 in MinKNOW into barcode separated FASTQ files with a QC 9.

Metagenomic analyses were conducted using the EPI2ME wf-metagenomics workflow (pysam,0.23.0, pandas,2.2.3, fastcat,0.20.0, minimap2,2.28-r1209, samtools,1.21, taxonkit, v0.19.0, Kraken,2.1.3) separately for each of the 48 samples (Oxford Nanopore Technologies, Oxford, UK) (The nf-core framework, Ewel et al., 2020). The workflow parameters were as follows: minimap 2 as classifier, minimum read length 300 bp, minimum read quality 10, minimum percent_identity 97%, minimum reference coverage 30. During the workflow, a fasta file with selected Charophyte sequences (520 bp length) from GenBank (National Center for Biotechnology Information (NCBI)[Internet], 2025) was used as reference (Table 3).

Bam files were generated during the workflow and visualized in the Integrative Genomic Viewer (IGV Version 2.18.4). Charophyta sequences (consensus or single sequences) aligned to the references were subsequently checked using BLAST against the NCBI nucleotide collection (nr/nt) database. Only BLAST results for each top hit, with E-values 0.0, more than 95% percent identity, and with more than 99% coverage were considered further. In the next step the verified sequences for each lake from the 4 spring, 4 summer and 4 autumn samples, respectively, were aligned with an expanded set of reference sequences (Charophyta sequences from Gen Bank (National Center for Biotechnology Information (NCBI)[Internet], 2025) and BARCODE OF LIFE DATA SYSTEMv4 (BOLD v4 database (Ratnasingham et al., 2024)) (Table S1). A phylogenetic tree was constructed for each lake using the maximum likelihood (ML) algorithm in MEGA version 12 (Kumar et al., 2024). In the ML analyses, evolutionary substitution models were evaluated in MEGA version 12 and T92 + I was selected as the best-fitting evolutionary model for *rbcL*. ML analyses were performed with 1000 bootstrap replicates in MEGA version 12 (Kumar et al., 2024). The position in the phylogenetic tree was then the basis for the assignment of the Charophyte taxa to a certain species or species group. The Charophyta sequences obtained by metabarcoding in each lake sample are shown in Table S2-S5.

3. Results

3.1. Traditional sampling and identification

Using the traditional method, we found a total of seven charophyte species in two of the four study lakes (Table 4). In Plava banja, only one charophyte species, *Chara canescens* Loisel., was found in summer and autumn, while in Sava Lake, six species belonging to three genera, *Chara*, *Nitella*, and *Nitellopsis*, were observed, three of them in all three study seasons (Table 4). No charophyte species were found in Pečena Slatina and Markovačko Lake. In Markovačko Lake, however, we found a very small amount of *Chara vulgaris* L. when visiting the lake for preparatory work before the summer field campaign, but we did not find any charophyte species during the summer field campaign. Similarly, *Nitella gracilis* (Sm.) C. Agardh was found very rarely in Sava Lake during preparatory work in spring, but not during the spring field campaign.

3.2. Barcoding of Characeae samples

Single specimens of all seven charophyte taxa found during the field campaigns (Table 2) were successfully barcoded for the *rbcL* gene. *RbcL* is a conservative marker and therefore often does not differentiate between closely related species but depicts groups of closely related species instead. Six out of the seven taxa were assigned to the species group matching the results from the morphological determination, thereby confirming the morphological species identification (Fig. 2). However, the sample morphologically determined as *C. virgata* Kütz. was identified as *C. globularis* by barcoding (accession no. PV 594402 in Fig. 2). We also attempted barcoding the small sample of *C. vulgaris* we collected from Markovačko Lake when visiting the lake for preparatory work in summer. Unfortunately, barcoding of this sample failed, most likely because the material available for barcoding only consisted of a very small fragment.

Table 3
Charophyta used as references for metabarcoding Taxa and NCBI accession numbers.

NCBI Acc. nr	taxon	NCBI Acc. nr	taxon
AY170453.1	*Chara aculeolata*	HF912656.1	*Chara intermedia*
AB560882.1	*Chara altaica*	MW659842.1	*Chara lipkinii*
AM710330.1	*Chara canescens*	MW987577.1	*Chara oryzae*
AF097161.1	*Chara connivens*	KU128741.1	*Chara tenuispina*
AF097162.1	*Chara connivens*	KJ395930.1	*Chara tomentosa*
MN793054.1	*Chara contraria*	HF912649.1	*Chara virgata*
MK770144.1	*Chara contraria*	AF097167.1	*Chara vulgaris*
AB359168.1	*Chara fibrosa*	HQ380481.1	*Chara zeylanica*
HQ380452.1	*Chara foliolosa*	LC772187.1	*Lamprothamnium succinctum*
OQ607412.1	*Chara galioides*	AF097170.1	*Lamprothamnium papulosum*
MN817125.1	*Chara globata*	ON184176.1	*Nitella gracilis*
AF097165.1	*Chara globularis*	AB907808.1	*Nitellopsis obtusa*
MN793053.1	Chara gymnophylla		

Table 4
Abundance of charophyte algae in four study lakes during three seasons, using traditional sampling.

	Plava banja			Pečena Slatina			Sava Lake			Markovačko Lake		
	spring	summer	autumn	spring	summer	autumn	spring	summer	autumn	spring	summer	autumn
Chara canescens		2	1									
C. connivens								3				
C. contraria							2	3	1			
C. globularis							1	2				
C. virgata							2	2	2			
Nitella gracilis								1				
Nitellopsis obtusa							1	3	2			

1 = very rare, 2 = infrequent, 3 = common, 4 = frequent, 5 = abundant, predominant

3.3. eDNA metabarcoding of water samples

Depending on the amount of particles in the water, between 70 and 1900 mL of water were filtered (Table 5). There was no correlation between the volume of filtered water and eDNA concentration in the samples (r^2=0,06) (Fig. S1).

Single reads were also included as a positive result when they covered at least 99% of the reference sequences with more than 95% similarity. Applying these constraints, eDNA metabarcoding detected 7 charophyte taxa in the four study lakes, six belonging to the genus *Chara* and one to the genus *Nitellopsis*, *N. obtusa* (Table 6, Fig. S2-5). In some instances, it was not possible to differentiate between *Chara* species using *rbcl*, but the sequences could be aligned to five species groups: *Chara connivens/globularis*-group, *Chara contraria*-group, *Chara aculeolata/globata*-group, *Chara lipkinii/galioides*-group, and *Chara vulgaris/gymnophylla*-group. In addition, some sequences were assigned to the species *Chara canescens* and *Nitellopsis obtusa*.

3.4. Comparison between the traditional method and eDNA metabarcoding

Assuming that the "*Chara contraria*-group" recorded by eDNA metabarcoding (Table 6) is identical with the "*Chara contraria* A.Br. ex Kütz." recorded by traditional sampling (Table 4), both traditional sampling and eDNA metabarcoding recorded a total of 9 charophyte taxa (Table 7).

For the results presented in Tables 6 and 7, taxa were counted as "present" if we detected at least one read with > 99% coverage and > 95% similarity using NCBI BLAST (Table S6). Using a threshold of > 1 read, no charophytes were detected by metabarcoding in Pečena Slatina, while only the *C. vulgaris/gymnophylla* group was detected in Markovačko Lake (Table S7-10). With a threshold of > 10 reads, no charophytes were detected in Markovačko Lake either. In Plava banja and Sava Lake, most charophyte groups were detected as present even with a threshold of > 10 reads (Table S7-S10). Table S11 gives a per sample summary which includes total raw reads, reads after QC, reads assigned to Characeae, and reads assigned to each detected group.

The presence of *C. canescens* was confirmed by eDNA metabarcoding in Plava banja in one summer sample (Table 7, Table S7-10). Using traditional mapping this species was found in Plava banja in summer and autumn. In Pečena Slatina, *C. canescens* sequences were detected with metabarcoding even though they were not detected with traditional sampling. However, we only found a few sequences (1 read in each of four samples with 96–98.5% identity to *C. canescens*). The absence of *C. canescens* in Sava and Markovačko Lakes, as well as in Plava banja in spring, was consistent between traditional mapping and eDNA metabarcoding (Table 7).

Two species, C. *connivens* Salzm. ex A. Br. and *C. globularis* Thuill. were detected by traditional sampling and eDNA metabarcoding in spring and summer in Sava Lake. However, the use of *rbcl* as a marker gene did not differentiate between these two species. In addition, eDNA metabarcoding detected the *C. connivens/globularis* group also in summer in Plava banja and in autumn in Markovačko Lake with only one read (Table 6, S11, Fig. S2, S5), when neither of these species was recorded by traditional sampling.

Both traditional mapping and metabarcoding consistently showed that *C. contraria* does not occur in Plava banja and Pečena Slatina. Metabarcoding and traditional mapping detected *C. contraria* in Sava Lake in spring and autumn (Table 6, Fig. S4). Interestingly, no *C. contraria* sequences were found in the summer samples when traditional mapping recorded the highest abundance (Table 4). *C. contraria* was recorded by metabarcoding in Markovačko Lake in spring, but only with one read, even though the species was not recorded in this lake by traditional sampling (Table 4, S11, Fig. S5).

One species, *C. virgata*, was consistently not detected by eDNA metabarcoding, even though it was recorded in all three seasons in Sava Lake by traditional mapping. Species identity of a morphologically determined *C. virgata* specimen, was identified as *C. globularis* by barcoding instead. Consequently, there are two ways how to interpret the results for *C. virgata:* (i) if we assume that all *C. virgata* recorded by traditional mapping in Sava lake in fact is *C. globularis*, the results must be recorded as "consistently not detected by eDNA metabarcoding nor traditional mapping after barcoding correction" (Table 4); (ii) if we, however, assume that only the barcoded sample was morphologically misidentified, while other specimen in Sava Lake in fact were *C. virgata*, the results must be recorded as "not detected by eDNA metabarcoding, even though it was recorded in all three seasons in Sava lake by traditional mapping".

Two taxa, the "*C. aculeolata/globata* group" and the "*C. lipkinii/galioides* group", were detected by eDNA metabarcoding although they had never been recorded by traditional mapping. Both groups were detected in some spring and autumn samples from Sava Lake and the "*C. aculeolata/globata* group in one spring sample from Plava banja (Table 6; Fig. S2, S4). *C. vulgaris/gymnophylla* was recorded with eDNA metabarcoding in Sava Lake in one spring and one autumn sample and in Plava Banja in two summer samples (Table 6; Fig. S2, S4). In Markovačko Lake metabarcoding revealed a few *C. vulgaris* sequences only in two spring samples (Table 6; Fig. S5). Interestingly, Markovačko Lake is the only one of the four lakes where *C. vulgaris* was morphologically detected, during a preparatory visit before the summer field campaign.

For *Nitellopsis obtusa* (Desv.) J. Groves, its absence was consistently confirmed by both methods in Plava banja and Pečena Slatina, as well as in summer in Markovačko Lake, while its presence was confirmed by both methods in spring and autumn in Sava Lake. It was detected by eDNA metabarcoding with one sequence in one spring and one autumn sample in Markovačko Lake, and only by traditional sampling in summer in Sava Lake (Table 6, Fig S2-S5).

Nitella gracilis was consistently not detected by eDNA metabarcoding, even though it was recorded in Sava Lake in low abundance in summer by traditional mapping, and species identity was confirmed by barcoding (Fig. 2).

In summary, out of a total of 108 records (9 taxa, 4 lakes in 3 seasons), 7 records (6,5%), belonging to four taxa, were unanimously recorded as present by both traditional mapping and eDNA metabarcoding. 77 records (71%) were unanimously recorded as absent by both traditional mapping and eDNA metabarcoding. In addition, if we assume that all *C. virgata* in Sava Lake in fact is *C. globularis* (as identified by barcoding), the absence of *C. virgata* in the eDNA of Sava Lake in all 3 seasons may also be carefully interpreted as "absent with both eDNA and traditional mapping after barcoding correction". Taking this into account, a total of 80 records (74%) were neither

Table 5
Water volumes (mL) filtered at the sampling locations at the study lakes.

Lake	Sava lake				Pečena Slatina				Plava banja				Markovačko lake			
Transect	**SL1**	**SL2**	**SL3**	**SLCP**	**PS1**	**PS2**	**PS3**	**PSCP**	**PB1**	**PB2**	**PB3**	**PBCP**	**ML1**	**ML2**	**ML3**	**MLCP**
spring	1300	900	700	1200	100	100	100	100	125	130	150	130	250	265	220	240
summer	1900	1000	1000	1200	195	190	500	90	80	70	70	75	350	300	230	270
autumn	1000	1280	1000	900	100	100	130	90	500	550	570	530	320	110	270	220

Table 6
Presence (+) and absence (-) of charophyte species or species groups in the four study lakes in three seasons, using eDNA metabarcoding of the *rbcL* marker from water samples. Taxa were counted as "present" when there occurred one or more reads with > 99% coverage and > 95% similarity using NCBI BLAST.

	Plava banja			**Pečena Slatina**			**Sava Lake**			**Markovačko Lake**		
	spring	summer	autumn	spring	summer	autumn	spring	summer	autumn	spring	summer	autumn
Chara canescens	-	+	-	+	+	+	-	-	-	-	-	-
Chara connivens/globularis group	-	+	-	-	-	-	+	+	-	-	-	+
C. contraria group	-	-	-	-	-	-	+	-	+	+	-	-
C. aculeolata/globata group	+	-	-	-	-	-	+	-	-	-	-	-
C. lipkinii/galioides group	-	-	-	-	-	-	+	-	+	-	-	-
C. vulgaris/gymnophylla group	-	+	-	-	-	-	+	-	+	+	-	-
Nitellopsis obtusa	-	-	-	-	-	-	+	-	+	+	-	+

Table 7
Comparison between results obtained by traditional sampling and eDNA metabarcoding. Taxa were counted as "present" when there occurred one or more reads with > 99% coverage and > 95% similarity using NCBI BLAST.

	Plava banja			Pečena slatina			Sava Lake			Markovačko Lake		
	spring	summer	autumn	spring	summer	autumn	spring	summer	autumn	spring	summer	autumn
Chara canescens	-	+	-	+	+	+	-	-	-	-	-	-
C. connivens/globularis group	-	+	-	-	-	-	+	+	-	-	-	+
***C. contraria* group**	-	-	-	-	-	-	+	-	+	+	-	-
C. virgata	-	-	-	-	-	-	- (***)	- (***)	- (***)	-	-	-
C. aculeolata/globata group	+	-	-	-	-	-	+	-	-	-	-	-
C. lipkinii/galioides group	-	-	-	-	-	-	+	-	+	-	-	-
C. vulgaris/gymnophylla group	-	+	-	-	-	-	+	-	+	+	-(*)	-
Nitellopsis obtusa	-	-	-	-	-	-	+	-	+	+	-	+
Nitella gracilis	-	-	-	-	-	-	-	- (**)	-	-	-	-

(*) A small amount of *C. vulgaris* was found during a preparatory visit in Markovačko Lake, but it was not recorded during the field campaign. (**) A small amount of *N. gracilis* was found during preparatory work, but not during the spring field campaign. (***) *C. virgata* was morphologically identified in Sava Lake, but a respective sample was assigned to *C. globularis* by barcoding. It is therefore possible that *C. virgata* is truly absent from Sava Lake.

+	detected with both eDNA and traditional sampling
-	not detected by either traditional mapping or eDNA
+	detected with eDNA but not with traditional sampling
-	detected with traditional sampling, but not eDNA

detected with traditional mapping nor with eDNA (Table 7). Three records were recorded with traditional mapping, but not with eDNA. In addition, *N. gracilis* was detected during preparatory work in spring in Sava Lake, although it was not detected during the spring field campaign. Nevertheless, we know that the species was present in the lake in spring, so this record should also be noted as "recorded with traditional mapping but not with metabarcoding". One record, *C. vulgaris* in summer in Markovačko Lake, can also be carefully interpreted as "detected with traditional mapping but not with eDNA", since we know that the species was present in the lake, since we found it during preparatory work in Markovačko Lake, even though we did not register it in the transects during field work. Hence, five records can be interpreted as "detected with traditional mapping, but not with eDNA metabarcoding" (4,6%). 16 records (14,8%) were detected by eDNA but not with traditional mapping (Table 7). Consequently, the results regarding the presence and absence of taxa obtained through traditional mapping and eDNA metabarcoding were consistent in 80.5%, while they disagreed with each other in 19,5% of the records.

4. Discussion

Our study confirms that it is possible to use eDNA metabarcoding to detect the presence of Characeae in lakes. The overall agreement on presence and absence of taxa between traditional mapping and eDNA was 80,5%, across four different lakes and three seasons. We used *rbcL* both for metabarcoding of eDNA and barcoding of collected Characeae samples, although its discriminatory power for Characeae is lower than that of other markers, e.g., matK or ITS (Schneider et al., 2015b). We did this because *rbcL* is relatively unspecific, easily amplified, and sequences are available for many aquatic and land plants (Kress and Erickson, 2007, Schneider et al., 2015b, Revéret et al., 2023). We expected that using *rbcL* would enable us to detect at least those taxa across different genera of Characeae which have been detected in the studied lakes with traditional methods. We experienced, however, difficulties in discriminating among a number of different *Chara* species (Schneider et al., 2015b). Furthermore, another challenge is the filtration of enough lake water, especially in those lakes with a high amount of particles, like the saline lakes Pečena Slatina and Plava banja. Our results show that the concentration of harvested DNA was unrelated to the volume of filtered water, probably because eDNA is distributed patchy in lake water. Sava Lake had relatively clear water, and larger volumes could be filtered. However, eDNA concentrations differed considerably among the samples taken in Sava Lake and were not noticeably higher than in samples from other lakes, from where much smaller water volumes could be filtered. DNA can be attached to particles which most likely influences the eDNA yield from turbid lakes (Barnes et al., 2021).

The absence of *C. canescens* in Sava and Markovačko Lake was consistent between eDNA metabarcoding and traditional sampling, as well as its absence in spring and presence in water samples collected in Plava banja in summer, when the species was recorded as "infrequent" by the traditional method. In the autumn samples, however, which were taken at a time when *C. canescens* only occurred "very rare", we did not detect *C. canescens* by metabarcoding, possibly because the amount of eDNA in the water samples was too low and the filtered water volume was too small. *C. canescens* was not recorded by either metabarcoding or traditional sampling in spring, likely because the species is mostly annual and germinates from oospores in spring (Schubert et al., 2024b), rendering the occurrence of significant amounts of free DNA from damaged or fructifying plants unlikely. We found a few sequences of *C. canescens* in Pečena Slatina using eDNA metabarcoding, although it was not detected by traditional sampling. As these were single reads (Table 6, S11), the results must be interpreted with caution even though a single read occurred in each of 4 samples. However, a query cover of 99–100%, and a similarity above 95% combined with their clear clustering in the *C. canescens* group in the phylogenetic tree seem to indicate that the species or its DNA is present. The presence of sequences of *C. canescens* is also supported by findings of *C. canescens* in Pečena Slatina in both 2021 and 2022 (Sabovljević et al. 2022). *C. canescens* is regarded as an opportunist with low competitive ability (Schubert et al., 2024b). This means that its abundance is subject to annual changes. Our results carefully suggest that *C. canescens* occurred in low abundances in Pečena Slatina in 2023 but was overlooked by traditional sampling. This suggests that the DNA of *C. canescens* can be detected in water samples, even when the species only occurs in low abundances.

Four more taxa, *C. connivens*, *C. globularis*, *Chara contraria* and *Nitellopsis obtusa,* were detected by both, traditional sampling and eDNA metabarcoding in the study lakes. *C. globularis* and *C. connivens* cannot clearly be distinguished from each other using *rbcl* (Fig. 2) (Urbaniak and Combik, 2017) and the reads were assigned to a "*C. connivens/globularis* group". *C. contraria* could only be assigned to a "*C. contraria* group". Nevertheless, eDNA metabarcoding confirmed almost all traditionally sampled records of these taxa. The exceptions were the summer records of *C. contraria* and *N. obtusa* in Sava Lake, when species abundances, as recorded by traditional sampling, in fact were highest. It is possible that the concentration of eDNA of these taxa in the water samples from Sava Lake was too low. Various abiotic and biotic factors, e.g. water volume, depth and currents in a lake, exposure to UV light, pH, water temperature and bacterial composition, can lead to dilution and degradation of eDNA and thus influence eDNA concentration in water (Sahu et al., 2025). Generally, summer in Serbia in 2023 was very hot, with temperatures reaching almost 40°C, hence enhancing DNA degradation. Another factor is water exchange in the regulated Sava Lake. Every now and then, Sava Lake is flushed with water from the Sava River, potentially diluting eDNA. Consequently, the amount of eDNA in the water might have been too low in our water samples. However, *C. connivens/globularis* was successfully detected in summer in Sava Lake in three of the four samples taken, even though it was recorded with one read only in one of these samples (Table S11). This indicates that eDNA from some charophyte species indeed was present and detectable in summer in Sava Lake. The presence of other organisms, such as phytoplankton, could aggravate the filtering of the water and reduce the amount of characean eDNA collected on the filter. However, that is unlikely since the volume of filtered water in summer from Sava Lake was higher than from the other lakes (Table 3). It is difficult to assess how exactly these factors affected eDNA concentrations in the four study lakes.

Besides being successfully detected in Sava Lake, both *Nitellopsis obtusa* and *Chara contraria* were detected by metabarcoding with single reads only in Markovačko Lake, *N. obtusa* in spring and autumn, and *Chara contraria* only in spring, with query cover 99–100% and similarity 95–98.5% (Table 6, S11). Using a threshold > 1 read, neither *Chara contraria* nor *N. obtusa* would be recorded as "present" by metabarcoding in Markovačko Lake (Table S7-10). Generally, Markovačko Lake could be a suitable habitat for these species, which tolerate a wide range of nutrient conditions (Bernhardt & Gregor 2024, Pall et al., 2024). Records of *C. contraria* and *N. obtusa* have also been described from other locations in Serbia (Vesić et al. 2016), but not in any of our study lakes except Sava Lake. Both species might potentially have been overlooked. The absence of *C. contraria* in Markovačko Lake in summer and autumn might be a consequence of the high floods that occurred during the late spring and early summer, reducing light availability and potentially negatively affecting the growth of Characeae. Pall et al. (2024) claim *N. obtusa* to be dominant only in oligo-mesotrophic to mesotrophic clear water lakes. In contrast, Markovačko Lake is turbid, hence explaining why *N. obtusa* is most likely rare, if present in the lake. In general, charophyte algae often grow patchy. For this reason, they can easily be overlooked in traditional sampling. The same is true, however, for eDNA, because DNA of infrequent and patchy populations might not be homogeneously distributed in the lake water. High flood events in Markovačko Lake during late spring and early summer might explain why no charophyte species were detected in this lake by traditional sampling.

C. connivens and *C. globularis* are two closely related species and cannot be distinguished based on *rbcL* sequences (Fig. 2). Nevertheless, eDNA metabarcoding successfully detected the species group, since their presence was confirmed for spring and summer in Sava Lake, when they were found by traditional sampling. Their absence in autumn in Sava Lake was also confirmed by eDNA. However, *C. connivens/globularis* was also detected by metabarcoding in summer in Plava banja and in autumn in Markovačko Lake, but with single reads only (Table 6, S11). We consider the presence of *C. connivens/globularis* in Plava banja unlikely, because Plava banja is a saline lake, and both *C. connivens* and *C. globularis* do not tolerate enhanced salinity (Torn and Nat, 2024, Zviedre and Schubert, 2024). Its detection by metabarcoding could, however, be explained by DNA transport by birds (Bohmann et al., 2014, Merkes et al., 2014) since these species were found in other locations in Serbia, especially *C. globularis* (Vesić et al. 2016, Trbojević et al. 2020). Markovačko Lake could be a suitable habitat for both species, but it is puzzling to explain why they were detected in autumn and not in spring and summer seasons. Possible reasons could be low abundance or absence in spring, followed by high floods, creating unsuitable conditions for charophyte growth. Furthermore, high summer temperatures could potentially enhance DNA degradation during summer, as previously discussed. Favourable conditions might have come back in late summer or early autumn, so these species might have returned to the site, but in small amounts, so they were overlooked by traditional sampling.

C. virgata is a taxon that was not detected by metabarcoding, but it was morphologically recorded as "infrequent" in all three seasons in Sava Lake. However, the specimen that was analysed by barcoding was clearly identified as *C. globularis*. *C. virgata* and

C. globularis are morphologically very similar to each other, and intermediate forms are commonly recorded (Nat, 2024). In previous years, *C. virgata* was recorded in Sava Lake (Schneider et al., 2020). However, genetic analyses by Trbojević et al. (2020) placed a sample from Sava Lake that morphologically resembled *C. virgata* into the *C. contraria* cluster, showing that morphological traits commonly used to determine *C. virgata* are misleading. Hence, *C. virgata* detected by the traditional method in 2023 might have been misidentified. In that case, the absence of *C. virgata* eDNA in all study lakes would be correct.

N. gracilis was detected by traditional sampling in summer in Sava Lake, but not with metabarcoding. This species is very graceful, as its name implies, fragile, small and very rare in the Sava Lake. Only a small amount of *N. gracilis* was found during preparatory work, but not during the summer field campaign. Hence, the amount of DNA present in the water was probably too low to be detected by metabarcoding.

An obvious discrepancy between traditional mapping and eDNA metabarcoding in our study is the occurrence of DNA of the *C. aculeolata/globata* group, in one Sava Lake and one Plava banja sample, respectively, with a relatively high number of reads (Table S11). The *C. aculeolata/globata* group was not recorded with traditional sampling in any of our sampling sites. Ten different *Chara* species share similar *rbcL* sequences and are grouped into one cluster, the *C. aculeolata/globata* group: *C. aculeolata*, *baltica*, *polyacantha*, *intermedia*, *rudis*, *corfuensis*, *papillosa*, *hispida, horrida*, and *globata* (Fig. 2). Of these, only *C. hispida* L. and *C. papillosa* Kütz. were recorded in Serbia before, but not in any of our four study lakes (Blaženčić 2014, Vesić et al. 2016, Tomović et al. 2022, Sabovljević et al. 2023, Marković, A., unpubl. data). Both species have previously also been found in other areas of the Balkans, as well as *C. aculeolata* Kütz. (Blaženčić et al. 2006). Therefore, in Sava Lake, the flushing of the lake with water from the Sava River might potentially have introduced eDNA from the *C. aculeolata/globata* group. DNA from the *C. aculeolata/globata* group may also have been transported by birds. Transport by birds has been confirmed for other organisms in other locations (Bohmann et al., 2014, Merkes et al., 2014).

Another group of Characeae is the *C. vulgaris/gymnophylla* group which has been detected with metabarcoding but not with traditional sampling during the study campaign. It is composed of two *Chara* species, *C. vulgaris* and *C. gymnophylla* (A. Braun) A. Braun (Fig. 2). Both taxa are described from Serbia (Trbojevic et al., 2024). Although the metabarcoding results suggest a presence of these taxa in three out of four study lakes, only at Markovačko Lake, a small fragment of *C. vulgaris* was found in the pre-study period, while it was never detected in any of the other study lakes. We nevertheless cannot exclude that this species occurs in all study lakes, since it can thrive in fresh to brackish waters and has a wide tolerance to other environmental parameters (Denys et al., 2024). DNA from these taxa could also have been transported by birds to all four study lakes (Bohmann et al., 2014, Merkes et al., 2014).

In Sava Lake *Chara* sequences belonging to the *C. lipkinii/galioides* group were found with metabarcoding in a few spring and autumn samples but not with traditional sampling. Using a threshold > 10 reads, the *C. lipkinii/galioides* group would, however, not be recorded as "present" by metabarcoding (Table S7-10, S11). Four different *Chara* species belong to the *C. lipkinii/galioides* group: *C. galioides* DC., *C. neglecta* Hollerb., *C. aspera* Willd., and *C. lipkinii* R. E. Romanov et al. *Chara galioides* is restricted to saline habitats, from slightly brackish to hypersaline conditions. It is distributed in Europe and North Africa, and Albania, Italy and Greece are the countries closest to Serbia where it was recorded (Lambert and Schubert, 2024, Blaženčić et al. 2006). However, *C. galioides* has never been recorded in Serbia. According to Schubert et al. (2024b), *C. galioides* often co-occurs with *C. canescens* in the Mediterranean region, so it might be possible for the two species to co-occur in inland saline habitats, like Pečena Slatina. Nevertheless, Sava Lake is not a suitable habitat for this species. *C. neglecta* is also a species inhabiting brackish habitats, but its distribution in Europe is limited to the south-east part around the Black and Azov Sea, and it has never been found in Serbia, nor the Balkans (Romanov, 2024). Again, Sava Lake is not a suitable habitat for this species either. *Chara lipkinii* was designated as a new species by Romanov et al. (2022), with various records found in different habitats, ranging from freshwater to brackish, but in localities only in Israel. However, Romanov et al. (2022) suggest that the presence of this species in areas north of Israel might be possible. Morphologically, *C. lipkinii* is similar to *C. squamosa* Desf., a species which has been recorded in Serbia many times (Trbojević et al. 2024, Romanov et al., 2024). Fertile specimens are easily distinguished since *C. squamosa* is monoecious, while *C. lipkinii* is dioecious. However, sterile specimens are almost impossible to distinguish (Romanov et al., 2024). No *rbcL* sequences are published for *C. squamosa*, but the *rbcL* sequence of *C. squamosa* and *C. lipkinii* might very well be similar to each other, because other monoecious/dioecious *Chara* species pairs have previously been shown to have similar DNA sequences, albeit for the *matK* marker (Schneider et al., 2016). It is therefore possible that *C. lipkinii* has been overlooked in Serbia, but it is also possible that the sequences we detected in our eDNA samples belong to *C. squamosa*. *C. aspera* was never found in Serbia before (Blaženčić et al. 2014), but there are records of this species in almost all surrounding countries (Blaženčić et al. 2006). Forms or *C. aspera* without spince-cells (*Chara aspera* f. *subinermis*) can resemble *C. connivens*, or if infertile, *C. virgata* or *C. globularis* (Baastrup-Spohr, 2024), so there is a small possibility that "*C. lipkinii/galioides*" DNA found in our samples belongs to *C. aspera*, while some of the sterile specimens of *C. virgata or C. globularis* found by traditional sampling belong to this taxon too. There were no sterile specimens of *C. connivens* in our study, while sterile specimens of *C. virgata* and *C. globularis* were found in Sava Lake during spring and autumn. These samples could belong to *Chara aspera* f. *subinermis*, and eDNA of the "*C. lipkini/galioides*" group in Sava Lake. However, in autumn, we have also found one fertile specimen of *C. virgata* having both male and female gametangia.

In conclusion, the eDNA metabarcoding approach has confirmed that charophyte algae can be detected and determined to species/group level using *rbcL* as a marker gene. The overall match in presence/absence between traditional sampling and eDNA was 80,5%, which points to the conclusion that eDNA metabarcoding can be successfully used to detect charophyte algae in lake water with some accuracy, and as a very valuable tool to complement traditional sampling. It is, however, difficult to determine a general threshold for the number of reads above which a taxon should be counted as "present". For Pečena Slatina and for Markovačko Lake, no charophytes would count as "detected" if using a threshold of > 10 reads (Table S7-10, S11). This would match with the results from the traditional sampling in our project. However, *C. canescens* indeed was detected in Pečena Slatina in earlier years, so the single metabarcoding

reads in our study could indicate that the species, or its DNA, still is present in low amounts.

The most useful aspect of eDNA metabarcoding is indeed the detection of taxa that are not recorded by traditional sampling. Traditional macrophyte mapping usually records species in a number of transects. This method easily overlooks species which grow in patches and in areas between the transects. Our results show that eDNA could detect the presence of these rare or patchy taxa. One important example is the presence of *C. canescens* DNA in Pečena Slatina, where this species was not found in the 2023 survey but there are records of this species in previous years. However, as only one read was found in 4 samples each, those results have to be interpreted with caution (Table S6, S11).

Species detected by metabarcoding but not by traditional methods could have been wrongly determined. Misidentification is, however, unlikely for the *C. aculeolata/globata* and *C. vulgaris/gymnophylla* groups, because these groups are easily identified. Furthermore, no study has ever recorded significant amounts of these taxa in any of the study lakes (Blaženčić 1995, Schneider et al., 2020, Trbojević et al. 2020; Tomović et al. 2023, Sabovljević et al. 2021, 2024, Tomović et al. 2025).

Contaminated sample equipment could have caused false positive results. This could explain the presence of *C. connivens/globularis*, *C. vulgaris/gymnophylla*, *C. contraria* and *N. obtusa* sequences in the lakes, because these taxa do occur elsewhere in Serbia. Similarly, two members of the *C. aculeolata/globata* group, *C. hispida* and *C. papillosa*, were recorded elsewhere in Serbia before (Blaženčić 2014, Vesić et al. 2016, Tomović et al. 2022, Sabovljević et al. 2023, Marković, A., unpubl. data). Both species, as well as *C. aculeolata*, have previously also been found in other Balkan areas (Blaženčić et al. 2006). The equipment we used, however, to the best of our knowledge, has never been used in lakes where these species are present. We therefore consider contamination with DNA of the abovementioned taxa unlikely. The occurrence of DNA of the *C. aculeolata/globata* group, the *C. vulgaris/gymnophylla* group and the *C. lipkinii/galioides* group in the eDNA analyses, despite their absence or very rare detection by traditional methods, suggests that the detection of new or unexpected species in metabarcoding analyses should be interpreted with great caution. We suggest that such results should be supported by traditional methods before concluding that these species truly are present.

Alien DNA could have been introduced into the lakes by, e.g. water birds, other animals, fishing equipment, boats or events like lake flushing with river water, e.g. in Sava Lake. This could explain the presence of reads of the *C. aculeolata/globata* and *C. vulgaris/gymnophylla* groups in the metabarcoding analyses in some of the samples from Plava banja and Sava Lake, although the taxa are not present in the lake. Depending on the environmental conditions, DNA persists in lake water for days or weeks only (Strickler et al., 2015). This suggests that, after some time, it is unlikely to detect a species that is no longer physically present, both due to the rapid degradation rate of DNA and degradation due to UV radiation. Even though the presence of cells or particle-bound DNA may lead to a longer persistence of detectable DNA in water (Ficetola et al., 2008), we consider a large effect of alien eDNA in the study lakes unlikely.

The other type of discrepancy between traditional sampling and the eDNA approach is the taxa present at the site but not detected with eDNA metabarcoding. A possible explanation is that the eDNA concentration could have been too low, and consequently, the volume of filtered water too small to collect enough eDNA from species which were present in small amounts only. This could have been the case, e.g. for members of the genus *Nitella* despite its physical presence and the suitability of the selected marker gene and primers. Depending on the amount of inorganic and organic particles in the water, we managed to filter between 0.07 and 1.9 L of water before the filters clogged. In order to increase the volume of filtered water, more filters must be used per location in further studies. However, according to our results, even some species which were morphologically recorded as “common” were not detected in our eDNA samples. There can be many reasons for this discrepancy, and they are to be found in factors influencing DNA degradation or masking their detection by filtration. Therefore, we consider it important to study the factors that influence DNA degradation or mask its detection.

Finally, we conclude that the choice of *rbcL* as a marker gene has proven to enable the detection of Charophytes using metabarcoding and has the advantage of detecting a wider range of aquatic algae and plants, but it has lower discriminatory power at the species level than other marker genes, such as matK.

All relevant data supporting the findings of this study are deposited in Zenodo, and are available at the following link: Detection of charophyte algae (Characeae) in lakes using eDNA metabarcoding_DATA_FILES

CRediT authorship contribution statement

Aleksandra Marković: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review and editing. **Susanne C. Schneider**: Conceptualization, Formal analysis, Investigation, Project administration, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. **Danijela Vidaković**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Writing – review and editing. **Miloš Ćirić**: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review and editing. **Vladimir Petrović**: Formal analysis, Investigation, Visualization, Writing – review and editing. **Željka Milovanović**: Formal analysis, Investigation, Resources, Writing – review and editing. **Marc Anglès d'Auriac**: Methodology, Resources, Writing – review and editing. **Andreas Ballot**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing.

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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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2026.e04228.

Data availability

The data are available on Zenodo at the following link: Detection of charophyte algae (Characeae) in lakes using eDNA metabarcoding_DATA_FILES.

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