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Integration of Morphology-Based and Molecular Techniques to Study the Most Significant Diatom Order (Thalassiosirales) of Phytoplankton in the River Danube

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Keywords: centric diatoms | environmental variables | large river | metabarcoding | scanning electron microscopy

ABSTRACT

1. Metabarcoding is a rapidly developing field for studying aquatic life and provides a promising alternative to microscopy. However, accurate assessments require that database errors and species boundaries be addressed, yet the use of only a short gene sequences in metabarcoding, may be insufficient for accurate species identification. This study examines the potential of metabarcoding in replacing traditional microscopic methods in planktic diatom identification.
2. Phytoplankton samples were collected monthly from May 2021 to April 2022 at 13 sites on the Hungarian section of the River Danube. Environmental variables were measured, and electron microscopy and metabarcoding analyses were conducted.
3. Both morphological and DNA analysis methods were used to study the Thalassiosirales order. Although there was some overlap in the taxa identified by both methods, there were also discrepancies, with certain taxa detected exclusively by one method. P-distance analysis and BLAST search were used to correct misidentifications, revealing mismatches between database sequences and observed species.
4. Phytoplankton community exhibited varying temperature and nutrients optima and tolerance ranges, which influenced their distribution patterns. During spring, diatoms—particularly Thalassiosirales—dominated the phytoplankton, with proportions decreasing in summer. Algal biomass in the Danube was highest in March, decreasing sharply by the end of summer and remaining low until the end of the growing season, the decrease relating to changes in the TN:TP ratio, which was very low in the warm water period (mostly below 10), leading to nitrogen limitation.
5. Discrepancies between bioinformatic analysis and SEM observations revealed errors in the reference database. Our results clarify the functional group (FG) classification, which is important for both ecological status assessment and understanding of ecosystem functioning.

Éva Ács and János László Korponai should be considered joint first author.

6. Temperature is one of the most fundamental drivers of microbial nitrogen dynamics in rivers. Global warming is driving up the average temperature of the Danube, creating more favourable conditions for denitrification since the speed of microbial processes is higher in warm water. The increasingly common nitrogen limitation could potentially limit algal growth.
7. Environmental factors, especially temperature and nutrient concentrations, significantly influenced the Danube's phytoplankton communities, with implications for ecosystem health and water quality assessment. Integrated approaches combining molecular techniques with traditional morphological analysis are needed for comprehensive ecological assessments, but accurate species identification and ecological status assessment require completeness in reference databases and the correction of database errors.

1 | Introduction

The study of phytoplankton in Europe's second longest river, the Danube, began more than a century ago with Brunnthaler (1900), who found a dozen species of euplanktonic algae in the plankton of the main branch and tributaries of the Danube section at Vienna. Schallgruber (1944) made a qualitative analysis of the Danube plankton samples taken near Vienna and was the first to make a quantitative analysis. The study analysed an annual cycle, revealing that the Danube's phytoplankton is dominated by diatoms, similar to the phytoplankton found in other rivers such as the Volga, Warta and Elbe. Several summary studies of the results have been published (e.g., Szemes 1967a, 1967b; Kusel-Fetzmann 1998).

Alongside the International Association for Danube research (IAD), the International Commission for the Protection of the River Danube (ICPDR) has further strengthened Danube research, initiating and effectively supporting international Danube expeditions (Joint Danube Survey, JDS). Thus far, four expeditions (2001, 2007, 2013 and 2019) have studied phytoplankton along the length of the Danube and its main tributaries (Dokulil and Donabaum 2014), but the first comprehensive survey of phytoplankton was carried out during the JDS4 expedition in 2019, sampling throughout the growing season (April–September) and along the Danube (Stanković et al. 2023). In this study, centric diatoms of phytoplankton were enumerated in untreated samples using Utermöhl's method (CEN-EN 15204 2006) at the functional group level only.

Euplanktonic diatoms are one of the most significant primary producer groups in the River Danube and are present throughout the year. Seasonal variations in the abundance and species composition of centric diatoms in the Danube's phytoplankton community have been observed (Kiss 1984; Nausch and Kiss 1985; Kiss and Genkal 1993), with the highest abundance of centric diatoms occurring in the spring and early summer months. The identification of most species in this group using classical methods requires an electron microscope, which is slow, expensive and requires great expertise in taxonomy. However, they can also be found in the phytobenthos when settling out the plankton (Tapolczai et al. 2024), and their species-level identification is necessary for ecological status assessment. The European Union Water Framework Directive (European Commission 2000) made phytoplankton one of the biological quality elements to be assessed. There is an increasing demand to integrate molecular methods into organism-based water quality monitoring studies or even to replace traditional methods (e.g., slow and uncertain microscopic investigations that require extensive taxonomical knowledge with rapid, cost-effective DNA examinations).

There is a good similarity between the microscopic and metabarcoding methods for both running and standing waters in the case of phytobenthos (Duleba et al. 2021; Bíró et al. 2022; Ács et al. 2023). However, poor similarities have been observed in the case of phytoplankton, particularly in freshwater diatoms. This was primarily explained by the shortcomings in the reference databases (Salmaso et al. 2022; Hanžek et al. 2024). Most studies emphasise the significance of a reliable reference database with sufficient data.

Although electron microscopy has long been used to study planktonic centric diatoms in the Danube (Kiss 1986; Steinberg et al. 1987; Kiss and Nausch 1988; Schmid 1990), this is the first time electron microscopy results have been compared with molecular data on such a large river. The aims of our studies were:

(1) To investigate whether metabarcoding can replace (or at least integrate with) the microscopic method for the diatom order Thalassiosiraceae in the Danube, based on available sequence data in databases.

(2) To identify whether errors exist in the reference databases and to propose corrections.

(3) To investigate the centric diatoms (i.e., the order Thalassiosirales) at the species level during the growing season in the Danube's middle section, where the phytoplankton biomass is highest, to identify the key environmental factors shaping the centric community.

2 | Materials and Methods

2.1 | Sampling

Phytoplankton samples were collected monthly from May 2021 to April 2022 (Table 1) from 13 sampling points on the main arm of the Hungarian section of the River Danube, and one point on each of two side arms of the Danube, the Mosoni Danube and the Ráckevei-Soroksári Danube (RSD), in alignment with the JDS sampling points. The phytoplankton community was minimally impacted because we managed to take the sample during a medium water level period with a more or less balanced water discharge (Figure S1), avoiding significant changes in water discharge. We excluded November, December and January from our sampling as decades of experience had shown no presence of

TABLE 1 | Sampling sites and their abbreviations used in the figures.

Waterbody	Locality	EOV Y	EOV X	rkm	Abbr.
Duna	Medve	545,914	272,580	1805.6	Med
Mosoni-Duna	Vének	553,401	266,583	2.5	Vén
Duna	Gönyű	558,316	266,821	1791.3	Gön
Duna	Szőny	587,165	266,602	1761	Szö
Duna	1 km below Szőny	587,987	266,618	1760.2	Szö1
Duna	Dunaalmás	595,500	265,770	1752.4	Dal
Duna	Szob	635,882	274,105	1707	Szo
Duna	Dunakeszi	655,379	257,117	1666	Bpf
Duna	Budapest	650,793	237,407	1644.9	Zöl
RSD	Tass	644,938	188,194	0.5	RSD
Duna	Ercsi	639,554	211,305	1613.5	Erc
Duna	Dunaföldvár	641,211	162,667	1560.5	Dfö
Duna	Paks	636,869	138,163	1526.6	Pak
Duna	Baja	640,464	94,232	1480.1	Baj
Duna	Mohács	622,272	72,666	1446.3	Moh

algae in the Danube during this very low light period. Three litres of water samples were collected from each site at the thalweg, 10 cm below the water surface (Kiss, Schmidt, and Ács 1996) and stored in the dark at a temperature between 4°C and 8°C before analysis. Following accurate shaking in the laboratory, a 2 dL subsample was taken from the sample to measure the pigment concentrations and the remaining sample was filtered through a 3 μm Isopore polycarbonate membrane filter (Merck Millipore, $d = 5$ cm). Algal cells that remained on the filter were resuspended in a few millilitres of filtered water and preserved with a.r. absolute ethanol (> 70% final concentration) for DNA extraction for metabarcoding investigations. For samples collected in March, May and July, 1 mL of the slurry resuspended from the filter was fixed with formaldehyde (final concentration 4%) for scanning electron microscopy investigations.

2.2 | Environmental Variables

In situ, the water temperature (Temp), pH, dissolved oxygen (DO) content and specific electric conductivity (Cond.) were measured with a portable multiparameter digital meter (Multi 350i-WTW, Germany) and turbidity (NTU) with a 210 IR turbidimeter (Lovibond, UK).

In the laboratory, total organic carbon (TOC) and total nitrogen content (TN) were quantified by applying a MULTI N/C 3100 analyser (Analytik Jena, Germany). Anion (Cl^-, SO_4^{2-}, NO_3^-) and cation (Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}) concentrations were determined with a Dionex ICS 5000+ dual channel ion chromatograph (Thermo Fisher Scientific, USA). For quantification of phosphate ions (PO_4^{3-}), water samples were filtered (Whatman RC 55, 0.45 μm pore size), and then concentrations were determined following the standard spectrophotometric method (Eaton et al. 2005). The total phosphorous concentrations (TP) were determined by unfiltered samples with persulfate acidic digestion, and then the process followed the PO_4^{3-} measurement. Total chlorophyll *a* (Chl-a) content and the pigment concentration of main algal groups were measured fluorometrically in the laboratory with a BBE moldaenke AlgaeLabAnalyser instrument.

2.3 | Morphological Investigation

Diatom valves were cleaned using hydrogen peroxide and hydrochloric acid, washed with distilled water and filtered onto an Isopore polycarbonate membrane filter with a 3-μm pore diameter (Merck Millipore), fixed on metal stubs with double-sided carbon tape, painted in spots with conductive silver paint (NO-VOC Silver Paint, SPI Supplies) and coated with gold using a rotary-pumped spatter coater Quorum Q150R S. The prepared samples were investigated with a Zeiss EVO MA 10 scanning electron microscope (SEM) operated at 10 kV and 10 mm working distance using secondary electron detectors. From every sample, 100 diatom valves belonging to the Thalassiosirales order were identified at specific level. In this way, the counting gave the relative abundances of species as a percentage.

2.4 | Metabarcoding

DNA was extracted from subsamples preserved with ethanol, taking 2 mL from the subsample and centrifuging it at 5000×g for 10 min. The supernatant was discarded and 20 μL AP1 lysis buffer from DNEasy Plant Mini Kit (Qiagen) was added to the pellet that was resuspended in it. This slurry was mixed and incubated at 95°C for 20 min. Then a 100 μL AP1 buffer was

added. After cooling, 2 μL proteinase K (20 mg/mL, Thermo Scientific) was added, mixed and the mix incubated at 56°C for 3 h. Digestion was terminated by heating the mix above 90°C for 10 min. After that, another 100 μL AP1 buffer and 4 μL RNase A from the kit were added and the purification was continued according to the manufacturer's instructions. This method of DNA extraction was compared to the method by Vautier et al. (2020) based on NucleoSpin Soil kit (Macherey-Nagel) on benthic diatom samples in a ring test (Vasselon et al. 2021), providing similar results.

A partial (263 bp) region of *rbc*L gene (barcode marker) was amplified by polymerase chain reaction (PCR) and sequenced on an Illumina MiSeq platform. Duleba et al. (2021) describe the primer and adapter sequences, the conditions of PCR, library preparation and sequencing in detail, briefly: first PCR was performed with *rbc*L-specific primers designed by Vasselon et al. (2017) supplemented with Illumina overhang P5/P7 adapters. PCR products were purified with 1.0× AMPure XP magnetic beads (Beckman Coulter). Concentration was measured with Qubit 4 Fluorometer (Invitrogen) and the Qubit dsDNA HS Assay Kit. Index reactions with Nextera DNA CD Indices with P5/P7 adapters and P7/P5 tags attached were performed on purified PCR products that were diluted to equimolar concentrations. Products of index reactions were purified, quantified as previously and subjected to quality control performed with an Agilent TapeStation System 4150 (Agilent) using the Agilent High Sensitivity D1000 ScreenTape Assay Reagents. For the run on the Illumina MiSeq system, the final library pool was diluted to 4 nM concentration. Sequencing was performed using the Illumina MiSeq V2(500) Reagent Kit and a 2×250 bp read length. Polymerase chain reactions, library preparation and sequencing were performed by Biomi Ltd. (Hungary).

Raw sequence data were analysed using the Keck et al. (2019) method, that is the DADA2 pipeline (Callahan et al. 2016) applied to diatom *rbc*L metabarcoding. This workflow involved the removing of primer sequences, trimming and filtering of reads according to their quality, dereplicating, filtering with the core sample inference algorithm of DADA2, aligning and merging paired forward and reverse reads into one contiguous sequence, removing chimaeras and taxonomic assignment. For this last step, Diat.barcode 9.2 (Rimet et al. 2019) was used as reference database (see more: Duleba et al. 2021).

An average of 122,572 reads (ranging from 18,650 to 231,860) were acquired per sample, of which 87.1% on average (57.6%–98.8%; 17,184–213,448 reads, 107,460 on average) were identified as Thalassiosirales through bioinformatic analysis (Table S1). Read numbers were corrected using the biovolume correction factor developed by Vasselon et al. (2018). Relative abundance was calculated for each taxon in the samples dividing the corrected read number of a given taxon by the total corrected read number of the sample. Relative abundances of taxa ranked into Thalassiosirales according to the bioinformatic analysis described above were compared to the relative abundances of Thalassiosirales taxa observed under SEM. Only sequences ranked into Thalassiosirales were considered. This comparison indicated that taxonomic assignment of sequences required correction mainly regarding the species of the genera *Cyclotella*, *Cyclostephanos* and *Stephanodiscus*. Therefore, sequences of these genera were downloaded from Diat.barcode and National Center for Biotechnology Information (NCBI) GenBank databases and used for comparison with our sequences. Sequences were aligned with CLUSTALW (Thompson, Higgins, and Gibson 1994) implemented in BioEdit (Hall 1999). Pairwise uncorrected p-distance values were calculated with MEGA7 (Kumar, Stecher, and Tamura 2016). This software was used for performing maximum likelihood analysis for the *Cyclotella* genus. Corrected taxonomic assignment was performed: sequences were identified as the species that showed the least p-distance. In some of our sequences, the lowest p-distance was relatively high (>1%). In these cases, a Basic Local Alignment Tool (BLAST) (Altschul et al. 1990) search was performed in the Nucleotide collection database (nr/nt) using the Standard Nucleotide BLAST programme, megablast (highly similar sequences) algorithm with the default parameter settings. This algorithm found the most similar sequence even if the query sequence could not be fully aligned to those in the database (coverage is lower than 100%), probably due to sequencing errors. Corrected identifications were used in further analyses.

2.5 | Data Analysis

Site diversity was determined using Jaccard and Bray–Curtis similarity. To identify the taxa responsible for the main difference between morphology- and DNA-based indices, SIMPER analyses were applied. We conducted a partial redundancy analysis (RDA) revealing variables associated with phytoplankton community structure. The RDA was applied to DNA data and prior to the analysis, data were Hellinger transformed, whereas the environmental variables (Chl-a, Temp, DO, pH, Cond., NTU, TP, TN, TOC) were standardised for unit variance (*z*-score transformation). Removing the effects of sample sites, sites were introduced to the RDA as a conditional factor. The variance inflation factors (VIF) were calculated for each constraint, and the variable with a high VIF (20 < VIF) was removed from the constraints. Function inertcomp was used to decompose the variance into partial, constrained and unconstrained components for each site or species. The total variance of species compositional data can be regarded as total beta-diversity. Thus, the local contributions to beta-diversity (LCBD) and species contributions to beta-diversity (SCBD) can also be determined (Legendre and De Cáceres 2013). The analyses were conducted using the PAST software (Hammer, Harper, and Ryan 2001) and vegan package in R statistical environment (Oksanen et al. 2022; R Core Team 2023).

3 | Results

3.1 | Comparison of Results From Morphological and Metabarcoding Investigations

Altogether 35 taxa of the order Thalassiosirales were detected by DNA and morphological analyses (Table S2). Of these, 23 were identified by both methods (Figure S2), 7 were identified by morphological analysis only (Figure S3) and 5 were identified by DNA analysis alone. The taxa identified by morphological analysis alone were *Cyclotella atomus* var. *gracilis*, *C. meduanae*, *C. scaldensis*, *Stephanocostis chantaicus*, *Stephanodiscus*

lacustris, *S. parvus* and *S. rugosus*. *Cyclotella distinguenda*, *Stephanodiscus suzukii*, *Thalassiosira gessneri*, one *Cyclotella* sp. and one *Discostella* sp. were detected only by DNA analysis. Morphologically, we distinguished *S. hantzschii* f. *hantzscii* and *S. hantzschii* f. *tenuis*, but treated them together in all comparisons (both forms are shown in Figure S2) as no genetic difference was detectable.

Although both morphological and DNA analysis detected *Skeletonema potamos* and *S. subsalsum*, these two species were excluded from the comparisons because quantitative assessment of these species with an electron microscope is difficult due to the fragile nature of their frustules. On average, 47% (ranging from 21% to 65%) of Thalassiosirales species were detected using both morphology and DNA sequence analysis. In the case of dominant species (relative abundance > 5%), this proportion was 68% (ranging from 40% to 100%).

The Jaccard index, which measures the similarity of species based on their presence or absence, had a mean value of 0.50 (min. 0.31, max. 0.67). The Bray–Curtis index, which takes into account species abundance, had a mean value of 0.52 (min. 0.12, max. 0.80). The difference between the median and the mean for the Bray–Curtis index was much larger than for the Jaccard index (Figure 1).

SIMPER analysis showed that in March the differences between morphological and DNA-based community composition in the samples were mainly caused by *Stephanodiscus binatus*, *S. rugosus*, *S. neglectus* and *S. hantzschii* (each contributing more than 5%). In May, in addition to these four species, *Cyclostephanos invisitatus* and *Discostella pseudostelligera* also contributed more than 5%. In July, the differences were primarily caused by *Discostella nipponica*, *D. pseudostelligera*, *D. woltereckii* and *Cyclotella meduanae* (Table S3).

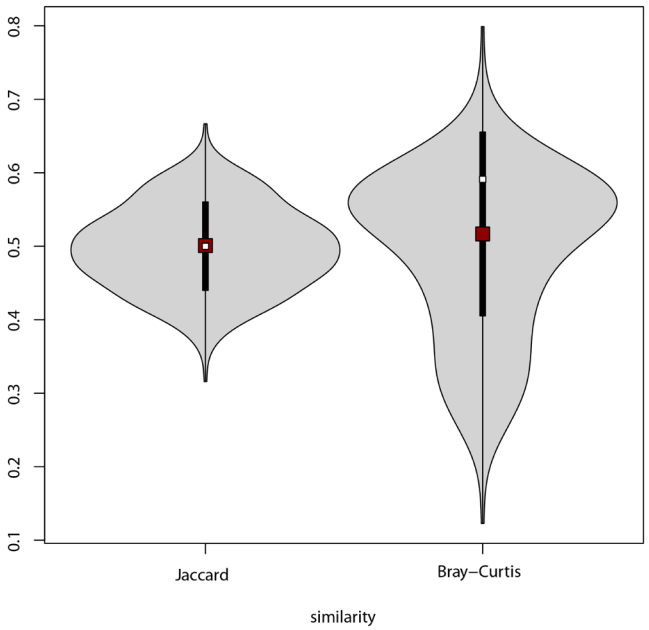

FIGURE 1 | Violin plot of the Jaccard and Bray–Curtis similarity indices of sample-pairs by morphology and DNA sequence analysis (dark red square: mean, white square: median).

Morphological characteristics used to differentiate the four similar *Stephanodiscus* species (*S. binatus*, *S. neglectus*, *S. parvus* and *S. rugosus*) and three similar *Discostella* species (*D. nipponica*, *D. pseudostelligera* and *D. woltereckii*) are given in Tables S4 and S5, respectively. We found no *S. minutulus* in SEM, and no ASVs could be identified as *S. minutulus* from the database during the DNA investigations.

3.2 | Errors and Absences in the Reference Database Used

Comparing the results of the first bioinformatic analysis of raw sequence data to SEM observations revealed discrepancies that could be reduced by additional sequence analysis involving sequences also from NCBI GenBank alongside sequences from the reference database used (Diat.barcode).

The p-distance analysis confirmed the original assignment for *Stephanodiscus*, *Cyclostephanos* and *Cyclotella* in most cases. However, additional analysis sometimes led to different identifications, helping to reveal reference database errors. Based on Diat.barcode, 57 amplicon sequence variants (ASVs) were ranked to the genus *Stephanodiscus* and most of them (42 ASVs) were assigned as *Stephanodiscus hantzschii*. However, no ASV was assigned to species in *Stephanodiscus minutulus* group (*S. minutulus*, *S. binatus*, *S. parvus*, *S. rugosus*, *S. neglectus*), albeit specimens of this complex were found under SEM.

Four ASVs were assigned as *Stephanodiscus* sp. with DADA2 and Diat.barcode and BLAST search indicated that they were closest to *Stephanodiscus* sp. KHR001. This strain was identified as *Praestephanos triporus* by Tuji et al. (2014); however, this correction was unfortunately not introduced into either GenBank or Diat.barcode. The assignment of these four ASVs was corrected to *P. triporus*, also found with SEM.

Two ASVs were identified as *Stephanodiscus suzukii* using the DADA2-based algorithm, with a *p*-distance of 0.4%–1.1% from the sequence of *S. suzukii* (previously identified as *Praestephanos suzukii* by Tuji et al. 2014). However, *S. suzukii* was not detected in any of our samples using SEM.

Cyclotella meduanae is a species that is frequently detected in the River Danube during warm periods. It was also found during the sampling series using SEM. However, it was not detected with metabarcoding as it is not currently recorded in either of the databases used. Some ASVs were found in our samples that were assigned to *Cyclotella* at the genus level only. Maximum likelihood tree illustrates the degree of sequence similarity between our *Cyclotella* sequences and those from the database (Figure S4).

The *Cyclotella atomus* var. *gracilis* found with SEM is not currently recorded in either Diat.barcode or NCBI GenBank under this name. The databases contain two rbcL sequences of *Cyclotella atomus* that are identical in their overlapping region. In Danube samples, three ASVs were assigned as *C. atomus*, which showed a *p*-distance of 0%–1.9% from those in the database. Two ASVs assigned only at the genus level showed a p-distance of 2.7%–3% from *C. atomus* sequences in the database.

Seven ASVs were identified as *Cyclostephanos tholiformis* using DADA2 based on Diat.barcode. These showed a *p*-distance of 0%–0.8% from *C. tholiformis* strain_FHTC15, which is recorded in Diat.barcode. However, the same distance was found for the three *Cyclostephanos delicatus* sequences recorded only in GenBank.

3.3 | Results of Environmental and Metabarcoding Investigations During the Growing Season

The Spearman correlation analysis revealed that chlorophyll *a* had a significant correlation with most measured environmental variables in the main arm (Figure 2A). It exhibited a negative correlation with temperature, soluble reactive phosphorus (SRP) and total phosphorus, while showing a positive correlation with dissolved oxygen (DO), total organic carbon (TOC) and nitrogen forms (mainly TN and nitrate), as well as with the TN:TP ratio. The total chlorophyll *a* concentration in the main arm exhibited significant seasonal and spatial variations (Figure 2B). The highest algal biomass was observed in March (average total chlorophyll *a* concentration of 59.7 $\mu g\,L^{-1}$), which decreased to 42.4 $\mu g\,L^{-1}$ by April. By August, phytoplankton had practically disappeared from the main arm, with an average total chlorophyll *a* concentration of only 7.3 and 4.8 $\mu g\,L^{-1}$ by September. During periods of higher total chlorophyll *a* concentrations, downstream sites showed a marked increase in biomass. The highest algal biomass (Table 2) was measured in March at Baja (90.68 $\mu g\,L^{-1}$) and Mohács (88.97 $\mu g\,L^{-1}$).

The proportion of diatoms in the phytoplankton, estimated from their pigment concentration, exhibited a seasonal variation (Figure 2C). They were most abundant in March, when on average 85% of the phytoplankton was composed of diatoms (basically euplanktonic diatoms, especially centric ones, investigated with light microscopy, but these results are not presented here for reasons of space). Until June, diatoms constituted more than 50% of the phytoplankton, that proportion decreasing significantly from July onwards, averaging only 22%–34%. During that period, total chlorophyll *a* concentrations were also very low, (effectively a pigment concentration of 1–2 $\mu g\,L^{-1}$).

In the two side arms, chlorophyll *a* showed a significantly positive correlation with DO and a negative correlation with SRP (Figure 3A). The highest chlorophyll *a* content recorded in the RSD was 85 $\mu g\,L^{-1}$ in September, while in the Mosoni Danube it was 43.32 $\mu g\,L^{-1}$ in March (Figure 3B). The maxima of diatoms were also observed in spring in both tributaries (Figure 3C) and the main arm of the River Danube. During the peak of Chl-a in the RSD, green algae had the highest biomass, representing 58% of the total.

The value of TN in the main arm was highest in February, then decreased markedly until the end of summer, increasing slowly again from September, but no marked trends could be observed in the TP values (data not shown). The TN:TP ratio was rather low on practically the entire Hungarian Danube section from July to October (Figure 4A), while the RSD was not below the limiting level (Figure 4B). The red colour on the figures indicates the samples where the ratio was below the potentially limiting level.

The results of the species of Thalassiosirales order and the environmental data for the RDA in the studied Danube section are shown in the ordination plot (Figure 5). Environmental variables explained the vast majority (66%), while sites as conditional variables explained just 9.6% of total variances. The unconstrained variance was 21% (Table 3). Moreover, the first four canonical axes explained 72.05% of the total variance, which contained a significant portion of the explained variances. Only the first two canonical components were considered since these explained more than 10% of the total variances (Table 4). The samples collected in the warmest water period (July–September) formed a discrete group, while samples collected in the coldest water period (February and March) formed another discrete group. All other samples were far from these groups (Figure 5).

The RDA 1 component had the highest significant correlation with T (−0.88), and the weakest was with TOC. Only TP was significantly correlated to the RDA 2 component (Table 5). *Discostella nipponica* and *Skeletonema potamos* showed a high negative correlation (−0.4 <), whereas *Stephanodiscus neglectus* showed a high positive correlation (0.4 <) with the RDA1 component. *Stephanodiscus binatus* presented a high positive correlation (0.4 <) with both canonical components. *Cyclostephanos invisitatus* and *Stephanodiscus hantzschii* correlated negatively with the RDA2 component.

The community (mainly *Cyclotella atomus*, *Cyclostephanos delicatus*, *Discostella nipponica*, *D. woltereckii*, *Skeletonema*

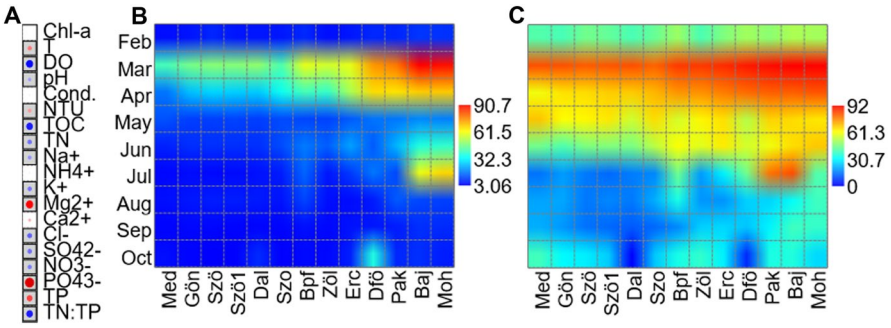

FIGURE 2 | (A) Spearman correlation plot between the chlorophyll *a* content of phytoplankton and the measured environmental variables (blue: positive, red: negative correlation. Significance < 0.05 are indicated with a box, and circle size corresponds to the degree of correlation) and (B) contour plot of seasonal and spatial changes of total chlorophyll *a* (data in $\mu g\,L^{-1}$) and (C) proportion of diatom pigment concentration in the River Danube at all sampling sites during our studies (see abbreviations in the text).

TABLE 2 | The minimum, maximum and average values of physico-chemical variables measured in the Hungarian section of the River Danube, as well as in the Mosoni Danube at Vének and Ráckevei-Soroksári Danube at Tass (see abbreviations in the text).

	Danube			Mosoni Danube			RSD		
Variables (units)	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**
Chl-a ($\mu g\,L^{-1}$)	3.06	90.68	19.49	2.06	45.79	15.18	4.65	85.00	31.39
T (°C)	5.60	23.30	14.94	6.50	23.10	14.64	7.20	23.10	15.34
DO ($mg\,L^{-1}$)	7.70	16.70	10.18	6.00	13.90	9.10	7.70	18.70	12.79
pH	7.13	8.57	7.80	7.14	8.15	7.68	7.46	8.62	7.99
Cond. ($\mu S\,cm^{-1}$)	313.00	550.00	409.98	409.00	586.00	498.25	330.00	514.00	396.71
Turbidity (NTU)	2.27	43.70	14.87	4.55	24.00	15.01	3.16	9.00	5.94
TOC ($mg\,L^{-1}$)	1.23	3.58	1.94	1.42	2.63	2.11	1.60	3.71	2.22
TN ($mg\,L^{-1}$)	1.29	2.95	1.93	1.48	2.77	1.93	1.15	2.61	1.70
Na^+ ($mg\,L^{-1}$)	7.47	20.48	12.02	13.97	29.56	19.84	9.90	20.71	13.57
NH_4^+ ($mg\,L^{-1}$)	< 0.1	0.08	0.05	< 0.1	0.17	0.08	< 0.1	0.19	0.07
K^+ ($mg\,L^{-1}$)	0.54	15.59	5.81	2.07	16.81	7.81	1.56	14.59	7.09
Mg^{2+} ($mg\,L^{-1}$)	2.28	22.26	8.80	2.75	16.11	10.24	2.95	13.77	7.73
Ca^{2+} ($mg\,L^{-1}$)	44.97	58.96	53.13	54.43	62.07	57.26	40.32	56.17	50.15
Cl^- ($mg\,L^{-1}$)	9.34	30.18	17.79	15.78	39.60	26.09	11.03	31.78	19.03
SO_4^{2-} ($mg\,L^{-1}$)	18.43	40.35	27.10	25.47	51.72	37.52	20.46	34.50	27.36
NO_3^- ($mg\,L^{-1}$)	3.50	12.22	7.17	4.39	10.12	7.20	4.15	10.59	6.36
SRP (PO_4^{3-} in $mg\,L^{-1}$)	< 0.01	0.38	0.10	0.03	0.34	0.16	0.01	0.14	0.07
TP ($mg\,L^{-1}$)	< 0.005	0.75	0.21	0.11	0.96	0.34	0.02	0.20	0.10
TN:TP	2.20	776.82	21.50	2.07	23.60	11.32	10.21	55.88	23.71

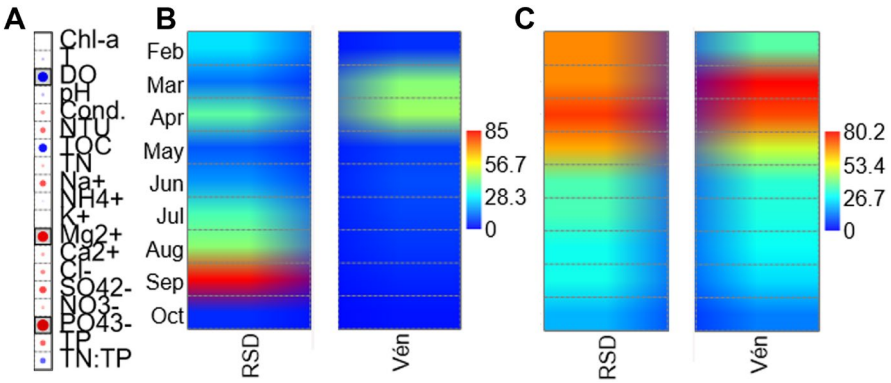

FIGURE 3 | (A) Spearman correlation plot between the chlorophyll *a* content of phytoplankton and the measured environmental variables (blue: positive, red: negative correlation. Significance < 0.05 are indicated with a box, and circle size corresponds to the degree of correlation) and (B) contour plot of seasonal and spatial changes of total chlorophyll *a* (data in $\mu g\,L^{-1}$) and (C) proportion of diatom pigment concentration in the Ráckevei-Soroksári Danube arm at Tass and Mosoni Danube at Vének during our studies (see abbreviations in the text).

potamos, *S. subsalsum* and *Thalassiosira pseudonana*) distribution of the first redundant component favoured higher T and lower TN and DO concentration, whereas *Stephanodiscus binatus* and *S. neglectus* preferred lower T and higher TN and DO, moreover *S. binatus* also preferred higher TP concentration. *Cyclostephanos invisitatus* and *Discostella pseudostelligera* preferred lower TP concentration (Figure 5).

4 | Discussion

4.1 | Comparison of Morphological and Metabarcoding Investigations

Metabarcoding has proved a promising tool for detecting algae species, particularly phytobenthic communities, in numerous

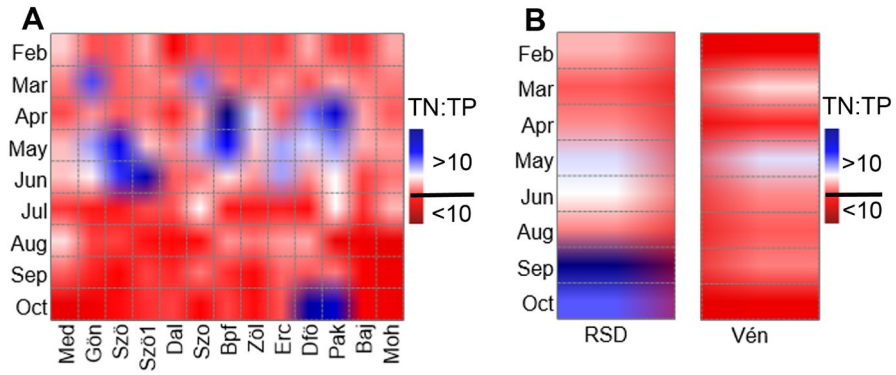

FIGURE 4 | Contour plot of seasonal and spatial changes in the TN:TP ratio (A) in the River Danube at all sampling sites and (B) in the Ráckevei-Soroksári Danube arm at Tass and Mosoni Danube at Vének during our studies (data in $\mu g L^{-1}$) (see abbreviations in the text).

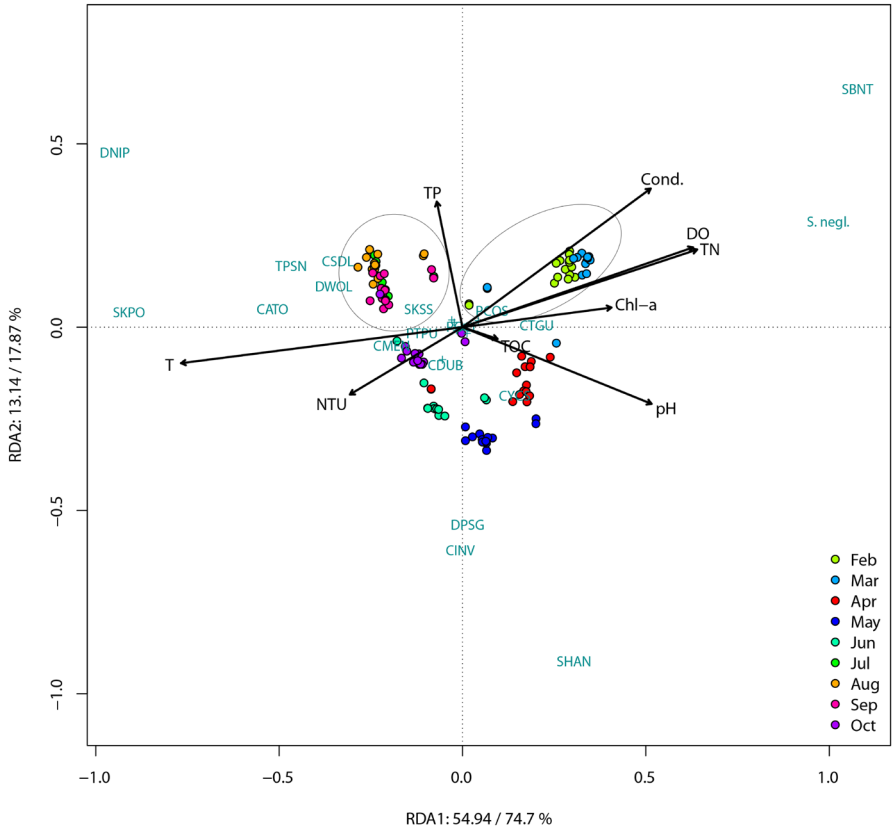

FIGURE 5 | The biplot of redundancy analysis (RDA) between the species of the Thalassiosirales order and abiotic parameters in the studied Danube section. See abbreviation of variables in the text and abbreviation of species in Table S2.

TABLE 3 | Result of the redundancy analysis. Partitioning of variance.

Partitioning of variance	Inertia	Proportion
Total	0.36595	1.0000
Conditioned	0.03521	0.0962
Constrained	0.24325	0.6647
Unconstrained	0.08749	0.2391

TABLE 4 | Summary table of explained variance on the significant RDA components.

	RDA1	RDA2	RDA3	RDA4
Eigenvalue	0.1817	0.0435	0.0077	0.0054
Proportion explained	0.5494	0.1314	0.0234	0.0163
Cumulative proportion	0.5494	0.6808	0.7042	0.7205
p	0.001	0.001	0.001	0.014

studies (Mora et al. 2019; Mortágua et al. 2019; Bíró et al. 2022; Ács et al. 2023); however, there are also habitats where the two approaches show relatively poor correspondence (Nistal-García et al. 2021). Most of the cited publications emphasise the significance of taxonomically reliable reference databases that include as many taxa as possible.

TABLE 5 | Variance explained by constrained variables in RDA (redundance analysis).

	RDA1	RDA2	Df	Inertia	*F*	Pr(>*F*)
Temp	**−0.88531**	−0.11337	1	0.160532	188.9849	0.001***
Cond	**0.59193**	0.43613	1	0.034479	40.5897	0.001***
Chl-a	**0.46873**	0.01686	1	0.013954	16.4267	0.001***
TN	**0.73822**	0.24418	1	0.011456	13.4865	0.001***
pH	**0.59488**	−0.24145	1	0.009517	11.2036	0.001***
TP	−0.08083	**0.39499**	1	0.003802	4.4760	0.010**
DO	**0.72523**	0.25090	1	0.003636	4.2806	0.016*
TOC	**0.11048**	−0.03724	1	0.002924	3.4426	0.025*
NTU	**−0.35428**	−0.21192	1	0.002952	3.4748	0.030*
Residual			103	0.87493		

Note: Numbers in bold represent the significant correlation with the RDA components. Signif. codes:
***< = 0.001.
**< = 0.001–0.05.
*< = 0.05.

In this study, phytoplankton species belonging to the order Thalassiosirales were examined using metabarcoding for samples collected monthly from the Hungarian Danube section. Additionally, samples collected in March, May and July were studied using both electron microscopy and DNA analysis. We selected these samples because decades of experience have shown the spring diatom peak of phytoplankton in the River Danube occurs in March and the abundance of phytoplankton significantly decreases by the end of summer (Abonyi et al. 2018).

Taxa that could only be identified morphologically were not included in the Diat.barcode 9.2 (Rimet et al. 2019) reference database for rbcL sequences, which explains why we could not identify them through DNA investigations.

Of the species found by DNA alone, only two could be identified at genus level in the database (*Cyclotella* sp. and *Discostella* sp.). *Cyclotella distinguenda* and *Thalassiosira gessneri* were found in 18% and 29% of the samples, respectively, but in very small read numbers, so the reason for the difference may be that they were not included in the 100 individuals counted by electron microscopy. During our previous investigations (Kiss et al. 2012), a few specimens of *Thalassiosira gessneri* had been found in the Danube and a few specimens of *Cyclotella distinguenda* were found in a Danube tributary (Rákos-stream) (Szabó et al. 2004).

In 84% of our samples, *Stephanodiscus suzuki* was identified on the basis of the reference database, although it was present in very small numbers. This species had not been found in Hungarian waters, despite its easily recognisable rimoportula: a stalked structure positioned at the valve face on the costa with a T-shaped elongated external opening. See Section 4.2 for a possible explanation for this discrepancy.

The morphological and DNA-based investigations revealed a similarity of over 50% in terms of species composition (Jaccard index) and quantitative conditions (Bray–Curtis index). Although this level of similarity cannot be considered high, it is encouraging and can be improved with the development of the reference database.

In March, the species of *Stephanodiscus minutulus* species complex (*S. binatus*, *S. parvus*, *S. rugosus*, *S. neglectus*) and *S. hantzschii* were primarily responsible for the differences between morphology- and DNA-based community composition in the samples. The taxonomic position of the *Stephanodiscus minutulus* species complex has been controversial and, morphologically, remains so in our opinion, despite a molecular study covering 4 markers (18S, LSU, *rbc*L and *cox*1) being carried out (Schultz et al. 2022), monoclonal cultures were only created from *S. binatus* and the previously unknown *Stephanodiscus* species. Based on their molecular results, the authors identified a new species of *Stephanodiscus*, which they named *S. neglectus* and provided its detailed morphological characteristics. They noted that *S. binatus* and *S. neglectus* share many morphological features. The valve surface of *S. neglectus* is flat and the valve face fultoportulae are usually located close to the centre. In contrast, the valve surface of *S. binatus* is slightly concave or convex, although it can also be flat, and the valve face fultoportulae are usually located in a more eccentric position. *Stephanodiscus neglectus* has 11–22 striae in 10 μm and has only one valve face fultoportula (Houk, Klee, and Tanaka 2014). *Stephanodiscus parvus* can be distinguished from *S. neglectus* by the number of areola rows in the fascicles. Although *S. neglectus* usually has three rows (rarely fewer or even four), *S. parvus* has two rows at the valve margin, with only one row in the middle. The valve surface of *S. parvus* can be flat or slightly concave/convex and the areolae are round, whereas the areolae of *S. neglectus* and *S. binatus* are rectangular. However, the other *S. minutulus* species complex were not found during the investigations, leaving taxonomic uncertainty in their case (both genetically and morphologically). This is described in detail in their article. The situation is complex because some authors do not consider *S. rugosus* an independent species. Instead, they interpret the slit-like areolae as a response to elevated silica concentration,

a phenomenon observed in many other centric diatom genera (Genkal and Håkansson 1990). However, in their book, Houk, Klee, and Tanaka (2014) consider it an independent species, despite only being distinguished from *S. parvus* by the slit-like areola. We observed this species in almost all of our samples, where it was often dominant, but it was never the only species from the *S. minutulus* species complex. We distinguished it morphologically, but the species sequence is not in the database, so the morphological distinction increases the difference between the two methods. Furthermore, the overlapping morphological characters described above make our morphological separation of the *S. minutulus* species complex uncertain.

Stephanodiscus hantzschii is clearly distinct from the *S. minutulus* complex due to the absence of valve face fultoportula, making it easily distinguishable with an electron microscope. The differences are likely to be due to errors in the database, as many sequences in the database are based on light microscopy identification only.

In May, the difference in results between the two methods was caused by the species *Stephanodiscus hantzschii*, *Stephanodiscus neglectus*, *Cyclostephanos invisitatus*, *Discostella pseudostelligera*, *Stephanodiscus rugosus* and *Stephanodiscus binatus*. The reasons for the differences caused by *Stephanodiscus* species are explained above and by *Discostella* species below. One explanation for the observed differences attributed to *Cyclostephanos invisitus* is that the limitations of metabarcoding (Kezlya, Tseplik, and Kulikovskiy 2023) were encountered, and the sequenced rbcL section does not contain enough variation to distinguish *C. invisitatus* from other *Cyclostephanos* species. Schultz et al. (2024a) found a clear separation within the genus *Cyclostephanos* using three different loci. During our morphological studies, it was possible to rule out incorrect identification of this species, which can be clearly identified with an electron microscope. The valve surface is flat and there is a valve face fultoportula (sometimes 2) in the centric position. The fascicles consist of one row of areolae in the middle of the valve, becoming 2 or 3 towards its edge. The external opening of the rimoportula is a small circular or slit-like reinforced opening (Houk, Klee, and Tanaka 2014).

In July, the differences between morphology- and DNA-based community compositions in the samples were primarily due to *Discostella nipponica*, *Discostella pseudostelligera*, *Discostella woltereckii* and *Cyclotella meduanae*. The reason for the difference caused by *C. meduanae* is that it is a dominant species with an unknown sequence. At that time, the least similarity was observed. Uncertainties in morphological identification also explain the differences caused by the *Discostella* species. Houk, Klee, and Tanaka (2010) report an almost continuous morphological transition between *D. pseudostelligera* and *D. woltereckii*, with a high degree of overlap. The book does not mention *D. nipponica*. In 2006, Tuji and Williams transferred the species *Cyclotella glomerata* f. *nipponica*, (originally described in 1936 (Skvortsov 1936)), to the genus *Discostella* as *D. nipponica* (Tuji and Williams 2006). This was due to the presence of a valve face fultoportula in *C. glomerata*, which is absent in the genus *Discostella*. Unfortunately, the article provides no differential diagnosis from the very similar *D. pseudostelligera* and *D. woltereckii*. However, based on the description, the valve is slightly concave/convex, with 18/10 μm striae and a diameter of 3–4 μm. A short tube comprises the external opening of the marginal fultoportula. It should be noted that the marginal fultoportulae of *D. woltereckii* have a distinct external tubular projection (Houk, Klee, and Tanaka 2010). Most morphological features of the three species overlap. Therefore, our distinction is uncertain, increasing the difference between the two methods. When dealing with morphologically indistinguishable or barely distinguishable species, the molecular method can provide more precise results, but the question arises as to which method the specialist submitting the sequence to the database used to correctly identify the species. Also worth noting is that both Tuji et al. (2014) and our samples have shown genetic differences between the three species, whereas *D. pseudostelligera* and *D. woltereckii* belonged to the same clade and *D. nipponica* to a different clade in the study done by Schultz et al. (2024b).

4.2 | Errors and Absences in the Reference Database Used

Previous research has shown that when using the Diat.barcode database the DADA2 algorithm requires correction. Although the Diat.barcode database is curated for diatoms, sequences of certain taxa are either missing (Bíró et al. 2022) or recorded under incorrect names (Ács et al. 2023), although not as frequently as for GenBank. To identify more taxa, further analysis using BLAST and comparison of samples with microscopy results can be conducted (Bíró et al. 2022; Ács et al. 2023). In this case, we used a procedure that enabled us to detect additional errors in the reference database.

Based on Diat.barcode, several of our assignments as *Stephanodiscus hantzschii* appeared to be incorrect, probably because of the UTCC267 and UK876 strains being wrongly identified as *S. hantzschii*. The former sequence was identical to *S. binatus*, the latter to *S. neglectus*. (Schultz et al. 2022). A BLAST search found that 12 ASVs assigned as *S. hantzschii* showed closer similarity to *S. neglectus* (*p*-distance 0%–0.8%) than to *S. hantzschii* (p-distance 1.5%–2.3%). The *S. neglectus* species is not recorded on the Diat.barcode database, but is on the NCBI GenBank, which is used by BLAST. The assignment of these sequences as *S. hantzschii* was probably because of strain UK876.

Our results suggest that the sequence labelled as *Stephanodsicus suzukii* in the reference database is inaccurate. *S. suzukii* was first described at Lake Biwa, Japan in 2000 (Tuji and Kociolek 2000). Based on the analysis of four genes (18S and 28S rDNA, rbcL and psbC), it was classified as a new genus, *Praestephanos*, along with the species previously known as *Stephanodiscus triporus* (Tuji et al. 2014). The two species exhibited significant similarity and were grouped together on the phylogenetic tree based on all four genes. DNA was extracted from field specimens of *S. suzukii* collected from Lake Biwa. Six cells were selected under an inverted microscope for DNA extraction. It is worth noting that *S. suzukii* coexisted with *Praestephanos triporus* in Lake Biwa, but the attempt to culture a strain of *Stephanodiscus suzukii* was unsuccessful. The authors did not provide a detailed morphological presentation of *Praestephanos triporus* in the article. Only one external and one internal view of its valve are presented. It cannot then be excluded that *Stephanodiscus lacustris*, which can occur alongside *Praestephanos triporus*, was also found in

their sample. The two species can only be reliably separated by electron microscopy, and its DNA was amplified by PCR rather than *Stephanodiscus suzukii*. Thus, it is possible that the sequence entered in the reference database as *S. suzukii* actually belongs to the species *S. lacustris*, but further investigation is required. It is a fact that during morphological investigations *S. lacustris* was found in 40% of our samples and *Praestephanos triporus* in 74% of the samples, during DNA investigations in 98%. However, we only found a small number of individuals in each sample. The largest relative abundance was three *Stephanodiscus lacustris* and six *Praestephanos triporus*. In the DNA investigations, the highest relative read number of *P. triporus* was 4%. We did not find *Stephanodiscus suzukii* at all during the electron microscopic investigations of the 43 samples. The sequences in our samples that were assigned as *S. suzukii* but could belong to *S. lacustris* showed a low *p*-distance (0.4%–1.9%) from the *Praestephanos triporus* sequence, indicating that they belong to the same genus. If our assumption is correct and these sequences in our samples actually belong to *Stephanodiscus lacustris*, this species is also a member of the genus *Praestephanos*. Further investigations are required to confirm this. Another explanation could be that it is not possible to distinguish between *Stephanodiscus suzukii* and *S. lacustris* based on the sequenced barcode region.

Cyclotella meduanae is a species often found in the River Danube (Kiss et al. 2012; Genkal 2014); however, no DNA analysis of this species has been published, thus it is not recorded in databases. Rimet et al. (2018) demonstrated that high-throughput sequencing offers the potential of identifying sequences of dominant species in a sample, thereby improving the reference database. However, applying this approach to the *Cyclotella* genus is more challenging due to the fact that most of our *Cyclotella* sequences belong to one species, *C. meneghiniana*. As a result, assessing the average intraspecific variability is difficult. Additionally, *C. meneghiniana* itself exhibits high sequence variability, making it a species complex (Beszteri, John, and Medlin 2007). The situation is further complicated by the fact that Kulikovskiy et al. (2022) described the *Stephanocylus* genus based on the species *Stephanocyclus planum* and transferred *Cyclotella meneghiniana*, *C. cryptica* and *C. gamma* to it. In Diat.barcode, these species are still recorded as *Cyclotella*. The barcode region sequenced in our study does not overlap with the region available for *Stephanocyclus planum*, so we could not determine whether any of our sequences belonged to this genus. Additionally, this barcode region is too short to draw reliable conclusions about phylogenetic relationships; however, maximum likelihood tree could illustrate the degree of sequence similarity between our sequences and the database's. Overall, these data are insufficient to unambiguously identify the *Cyclotella meduanae* sequence.

Electron microscopy clearly identified *Cyclotella atomus* var. *gracilis* in several samples, but the available data is insufficient to determine which of the sequences in our samples belong to it or the expected distance from *C. atomus* var. *atomus*. It is also possible that these varieties cannot be distinguished on the basis of rbcL.

There were no differences between *Cylostephanos delicatus* and *C. tholiformis* sequences, making it impossible to differentiate between them based on this barcode region.

4.3 | Environmental and Metabarcoding Investigations During the Growing Season

The phytoplankton biomass revealed distinct seasonal and spatial patterns in the main arm. The downstream direction exhibited an increasing trend in its spatial change, a phenomenon familiar for the Hungarian Danube (Kiss, Schmidt, and Bartalis 1991; Kiss 1994) and other temperate lowland rivers (Piirsoo et al. 2008).

The highest algal biomass in the Danube was observed in March as it had been previously (Kiss and Genkal 1993; Dokulil 2015), but then decreased significantly by the end of summer, remaining low until the end of the growing season. The March peak is remarkable because, according to the Hungarian EU WFD protocol used for ecological status assessment, the first sampling month for phytoplankton in the Danube is April (Borics 2020). It is recommended that the first sampling month for the Danube be changed to March. The RSD exhibits lentic characteristics for most of the year due to its slow current, with a maximum water discharge of barely $50\,m^3\,s^{-1}$ and a minimum of $2\,m^3\,s^{-1}$. Its water level is regulated by sluices at either end of the 57 km long arm. The proposed protocol is, therefore, acceptable for the RSD.

Since the early 2000s, the Danube has become oligotrophic (Dokulil and Kaiblinger 2008; Dokulil and Donabaum 2014; Abonyi et al. 2018), due partly to the Danube's wastewater treatment programme, which has significantly reduced the amount of nutrients available to algae (Istvánovics and Honti 2012), and partly to global warming, as illustrated by the River Po (Gervasio et al. 2022). A TN:TP value of less than 10 is typically considered indicative of nitrogen limitation (Downing and McCauley 1992). On the other hand, when TN:TP > 50, phosphorus limits algal growth (Guildford and Hecky 2000). This occurred only five times during our study, but the TN:TP ratio was consistently low during the warm water period, mostly below 10, which could have potentially limited algal growth. Temperature is a fundamental factor that drives microbial nitrogen dynamics in rivers. Due to slower denitrification in winter, there is still enough nitrogen for algae growth at the end of winter and in spring. However, the proliferation of algae and acceleration of bacterial processes cause a sharp decrease in nitrogen levels in the water by the end of spring. This is because microbial processes occur faster in warm water, making conditions more favourable for denitrification (Frankenbach and Meyer 1999). Global warming means these phenomena are expected to intensify in rivers, particularly during periods of low water discharge (Shousha, Maranger, and Lapierre 2021; Gervasio et al. 2022).

As similar results were obtained with both the microscopic and metabarcoding assessments, we were able to examine the correlation between the Thalassiosirales species composition obtained through metabarcoding and the environmental variables. This gave us a better understanding of the autecological properties of the species, which in turn could help predict biological responses to global environmental changes. The study was conducted during a period of balanced water discharge, ensuring that changes in water flow had minimal impact on the relationship between species and environmental variables.

Of the measured variables, temperature, DO and TN had the most significant effect on the composition of the Thalassiosirales order, whereas pH, turbidity, conductivity and TP also impacted significantly. The Thalassiosirales order comprises four functional groups (FGs), namely A, B, C and D (Reynolds et al. 2002; Borics et al. 2007). Our results indicate that not all species within the same FG exhibit a correlation with the same environmental variables. For instance, *Discostella pseudostelligera* and *Skeletonema potamos* both belong to FG D, but *D. pseudostelligera* shows a correlation with TP, whereas *S. potamos* shows a correlation with TN. Therefore, we recommend refining the FG classifications to improve ecological status assessment. This is also important due to the increasing number of studies examining global warming and human impact on river phytoplankton at the functional group level (e.g., Abonyi et al. 2021). Autecological studies are crucial for accurate FG classifications and require good taxonomic knowledge, and further research is needed in several groups (Schultz et al. 2022).

The temperature, DO and TN concentrations were found to have an impact on the distribution of certain species, with *Cyclotella atomus*, *Cyclostephanos delicatus*, *Discostella nipponica*, *D. woltereckii*, *Skeletonema potamos* and *Thalassiosira pseudonana* species favouring higher temperature and lower TN and DO concentrations, whereas *Stephanodiscus binatus* and *S. neglectus* preferred lower temperature and higher TN and DO. *Cyclostephanos invisitatus* and *Discostella pseudostelligera* preferred a lower TP concentration. Alongside *Discostella nipponica*'s higher temperature preference, *D. woltereckii*, *Cyclostephanos delicatus* and *Thalassiosira pseudonana* additionally preferred a higher TP concentration. Although *Stephanodiscus hantzschii* and *S. neoastrea* preferred a higher TN concentration, their TP optimum was in the lower ranges, despite being considered a rather eutrophic species (Houk, Klee, and Tanaka 2014).

The *Cyclotella atomus*, *Discostella nipponica*, *D. woltereckii*, *Skeletonema potamos* and *S. subsalsum* species prefer higher temperatures, with an optima around 19-20°C. According to Houk, Klee, and Tanaka (2010), *Discostella woltereckii* is a common species mainly found in tropical areas, whereas *Cyclotella atomus* is common in warm, nutrient-rich rivers. The autecological properties of *Skeletonema potamos* and *S. subsalsum* are consistent with our experiences from previous decades (Duleba et al. 2014). The abovementioned species can be expected increasingly to dominate the Danube as average temperatures increase due to global warming (Dokulil 2014).

Author Contributions

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.