

Article

Capturing Phytoplankton Diversity in the Central Adriatic Sea: Complementary Insights from DNA Metabarcoding and Morphological Identification

S. Skejić , B. Milić Roje * , J. Arapov , Ž. Ninčević Gladan , M. Straka , A. Bakrač , T. Bonačić ,
D. Rabadan , T. Tomašević , M. Bužančić  and O. Vidjak 

Institute for Oceanography and Fisheries, Split-Dalmatia County, 21000 Split, Croatia; sanda@izor.hr (S.S.); arapov@izor.hr (J.A.); nincevic@izor.hr (Ž.N.G.); straka@izor.hr (M.S.); bakrac@izor.hr (A.B.); bonacic@izor.hr (T.B.); rabadan@izor.hr (D.R.); pavelin@izor.hr (T.T.); buzancic@izor.hr (M.B.); vidjak@izor.hr (O.V.)

* Correspondence: broje@izor.hr

Abstract

Monitoring phytoplankton diversity is essential for the conservation of marine ecosystems and the protection of human health. This study compares light microscopy (LM) and DNA metabarcoding (MB) for analysing phytoplankton community at a long-term station in the Central Adriatic Sea, representing the first application of metabarcoding in this region. Sampling was conducted in June 2024 using Niskin bottles and a 53 µm plankton net. Four genetic markers (COI, 18S V4, 18S V9, *rbcL*) were applied to bulk plankton DNA to broaden taxonomic coverage and assess their detection performance relative to LM. Among the molecular markers, *rbcL* detected the highest taxonomic richness and was particularly effective for diatom species detection. In contrast, 18S V4 and 18S V9 captured a broader spectrum of phytoplankton diversity, especially outperforming LM and the other two molecular markers in detecting a previously unrecognized but ecologically important phytoflagellate group. Species-level resolution within the taxonomically challenging genera *Chaetoceros* and *Pseudo-nitzschia* was considerably improved with metabarcoding. However, several taxa observed by LM, including certain coccolithophores and thecate dinoflagellates, were not detected by any of the molecular markers. Overall, the results show that methodological choices strongly influence biodiversity estimates and highlight the value of a complementary integrative approach in assessing phytoplankton diversity.

Keywords: phytoplankton community composition; environmental DNA; microscopy; Mediterranean Sea; *rbcL*; 18S; COI; amplicon sequencing

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1. Introduction

Plankton biodiversity is one of the fundamental indicators of the ecological status of marine environments, providing insight into ecosystem stability. As primary producers, phytoplankton play a crucial role in biogeochemical cycles and the overall functioning of ocean ecosystems [1]. Sudden alterations in plankton diversity such as rapid proliferation of specific taxa or the occurrence of harmful algal blooms can disrupt entire marine ecosystems [2].

In the context of marine protection, phytoplankton are recognized as biological quality elements under the EU Water Framework Directive [3] and as key indicators of

marine biodiversity under the Marine Strategy Framework Directive [4]. In this context, several phytoplankton-related indicators such as productivity shifts, life forms, species composition (including non-indigenous or toxic species), and changes in spatial and temporal diversity have been proposed for inclusion in environmental management policies [5–8]. Phytoplankton monitoring programs are fundamental for detecting changes in plankton dynamics and assessing long-term environmental trends [9]. Those programs often rely on long-term ecological research (LTER), where consistent time series of data are collected at fixed stations. In the Adriatic Sea, established LTER sites include those in the Northern Adriatic (e.g., refs. [10–14]) and in the Central Adriatic [15–17]. The role of these stations has proven to be of exceptional importance, comparable to similar long-term efforts in other European coastal systems [8].

The Adriatic Sea has a strong north-to-south gradient in its oceanographic characteristics. The shallow Northern Adriatic is strongly influenced by freshwater and nutrient input from the Po River, often having a more eutrophic state [18], while the southern part is deeper and more oligotrophic [19]. The Central Adriatic, the focus of our study, represents a distinct environment that exhibits intermediate hydrographic conditions and depths. Consequently, it combines features of both northern and southern sub-basins, with intermediate trophic conditions, water column stratification, and hydrodynamic regimes that support a highly dynamic phytoplankton community.

Kaštela Bay is a semi-enclosed coastal embayment in the Central Adriatic Sea, covering an area of 61 km² with an average depth of 23 m. The bay receives freshwater primarily from the Jadro River, as well as from smaller streams and submarine groundwater springs. Water circulation is mainly driven by local winds and shows strong seasonal variability, with weak summer circulation occasionally leading to partial isolation of the eastern part of the bay. Temperature ranges from 10 to 27 °C and salinity from 28.17 to 38.19 PSU, while sea surface temperature has exhibited a significant warming trend over recent decades [16,20]. Following improvements in wastewater treatment, the bay has experienced a gradual reduction in nutrient enrichment and an overall improvement in trophic status in recent decades.

Adriatic time series of phytoplankton composition and abundance are primarily based on light microscopy, following the Utermöhl method [21]. Although traditional approaches to plankton taxonomy have provided a solid foundation for ecological monitoring, they face methodological constraints and are time-consuming. Moreover, identification of cryptic species remains challenging. Over the past two decades, a major methodological advancement in plankton taxonomy has emerged through the application of molecular techniques. In the Adriatic Sea, the use of metabarcoding in plankton research represents a promising approach, conducted at several localities in the Northern Adriatic [22–27] and in the southern part [28,29], while significant progress has been achieved in the Central Adriatic targeting the genus *Pseudo-nitzschia* [30]. In 2024, we initiated the BioPlan project (NPOO 2021–2026), focused on the entire eukaryotic plankton of the Central Adriatic, aiming to include the molecular approach in biodiversity studies and plankton monitoring and to assess its performance versus traditional morphological identification.

Most molecular studies focus on the universal 18S eukaryotic domain, which enables comparisons across different taxonomic levels of eukaryotic groups [25,31,32]. However, several studies have shown that the *rbcL* marker is more suitable for barcoding than 18S, especially for smaller fragments [25,33,34], and is particularly useful for assessing diatom diversity in coastal waters. Although all photosynthetic phytoplankton possess a *rbcL* gene, the primer set used here targets diatoms only. *RbcL* therefore functions as a diatom-specific marker by design in the present study. The COI marker is gen-

erally considered ineffective for planktonic microalgae; nevertheless, including COI can improve species identification success in multimarker eukaryotic plankton studies [35] and contribute to the detection of single-celled eukaryotic heterotrophs [36], which are also targeted in project BioPlan.

The main objective of the present study is to elevate the baseline of diversity and composition of the phytoplankton community in the Central Adriatic Sea using an integrated methodological framework that combines classical morphotaxonomic identification through light microscopy, with molecular approaches based on multimarker gene sequencing. We hypothesized that DNA metabarcoding would detect a substantial fraction of phytoplankton taxa undetected before by light microscopy.

2. Materials and Methods

2.1. Study Area and Sampling

Sampling was conducted in June 2024 at a long-term monitoring station in Kaštela Bay (Central Adriatic Sea) (Figure 1). Water samples for MB were collected from three depths (0, 10, and 30 m) using Niskin bottles (Altenholz, HYDRO-BIOS, Germany), together with duplicate vertical plankton net hauls (53 μ m mesh size). At each depth, 1 L of seawater was filtered in duplicate (technical replicates) through 0.2 μ m pore-size membrane filters for metabarcoding analysis. For light microscopy (LM), 0.3 L samples were collected from five depths (0, 5, 10, 20, and 30 m), complemented by an integrated plankton net sample representing the entire water column. As seasonal stratification is regularly observed in the study area, samples from multiple depths were collected to capture potential vertical heterogeneity in phytoplankton diversity.

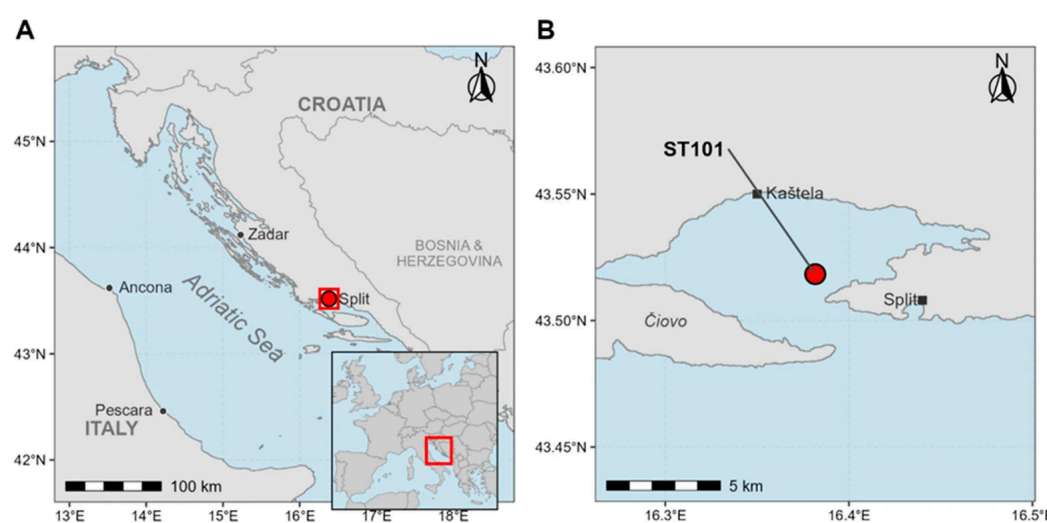


Figure 1. Map of the sampling site. (A) Sampling location shown in the Adriatic Sea; (B) location in the Kaštela Bay.

The filters were subsequently frozen for DNA metabarcoding (MB) targeting four genetic markers of the total eukaryotic plankton community. As the samples collected at 0 m using Niskin bottles failed during the molecular analysis, as well as one technical replicate of 10 m, they were excluded. Analysis was carried out on the remaining two depths (10 m and 30 m) and net tow samples, all in duplicates except the 10 m sample. The qualitative assessment included all species detected in both net samples and bottle samples by LM.

2.2. DNA Extraction and PCR Amplification

For DNA extractions, DNeasy PowerWater Kit for Microbial DNA from Water (Qiagen, Hilden, Germany) was used with some modifications. The filter was cut into two equal sections. One section was placed in a tube containing 10 MagNA Lyser Green beads (Roche Diagnostics, Basel, Switzerland) and 1 mL of PW1 solution, followed by incubation at 65 °C for 10 min. The sample was then homogenized using MagNA Lyser (Roche Diagnostics, Basel, Switzerland) homogenizer two times for 30 s at 7000 RPM. After this step, the manufacturer's instructions from the kit were followed. Final concentrations were checked using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

DNA Metabarcoding Library Preparation and Sequencing

Extracted DNA was sent to AllGenetics & Biology SL, Oleiros, Spain, where library preparation, sequencing, and bioinformatic analysis were carried out. The primers used for PCR amplification are shown in Table 1.

Table 1. Primers used for the metabarcoding library preparations.

Marker Name	Forward Primer Sequence (Author)	Reverse Primer Sequence (Author)	Amplicon Size
COI	5' GGWACWGGWTGAACWGTWTAYCCYCC 3' [37]	5' TAIACYTCIGGRTGICCRAARAAYCA 3' [38].	365 bp
18S V9	5' GTACACACCGCCCGTC 3' [39]	5' TGATCCTTCTGCAGGTTACCTAC 3' [40]	180 bp
18S V4	5' CCAGCASCYGC GGTAATTCC 3' [40]	5' ACTTTCGTTCTTGATYRATGA 3' [32]	430 bp
<i>rbcL</i>	5' AGGWGAAGYWAAAGGTTCTWTAYTTAAA 3' (modified from [25])	5' CCTTCTAAYTTACWACWACWG 3' (modified from [25])	312 bp

First and second PCR were performed using Supreme NZYTaQ 2x Green Master Mix (NZYTech, Lisboa, Portugal) with the conditions for each marker described in Supplementary Table S1. A negative control that contained no DNA was added to check for DNA contamination. After checking the products on 3% agarose, samples were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Bio-tek, Norcross, GA, USA) and equimolarly pooled according to DNA concentrations obtained by Qubit (Thermo Fisher Scientific, Waltham, MA, USA). The total pool was sequenced in a fraction of a NovaSeq PE250 flow cell (Illumina, San Diego, CA, USA).

2.3. Bioinformatic Analysis

Cutadapt v5 and v4.9 [41] software was used to remove remaining adapters, primers, and indices in sequences. The quality of the FASTQ files was assessed with FastQC software [42]. Reads were further processed in QIIME 2 [43] (release 2024.5). The DADA2 [44] algorithm was employed to filter and denoise reads, merge the forward and reverse reads with a minimal overlap of 12 bp, create amplicon sequence variants (ASVs), and remove chimeric sequences.

Taxonomic assignment of each ASV was conducted with a pre-trained classifier of the Diat.barcode database [45] (version 12) for *rbcL*, Protist Ribosomal Reference database (PR2) [46] (release 5.0.0) for 18S V4 and V9, and COI reference database [47] (version 3 May 2023 with additional alterations by AllGenetics) for COI. The algorithms

sklearn and vsearch for COI, both implemented in QIIME 2, were used for comparing sequences with the reference database. Singletons (ASVs with only one read); ASVs occurring at frequencies below 0.01%; unidentified sequences; ASVs present in the negative controls; and sequences assigned to non-target organism groups were discarded. For COI and V9, additional assignment of the unidentified ASVs was performed against the NCBI's Nucleotide-core database (last updated in 25 March 2025) using the algorithm BLASTn v2.16.0+ [48] with the following parameters: percent identity of 97%, e-value of 1×10^{-5} , and a minimum hit coverage of 80%. Only when all hits shared the same assignment at one taxon level was the ASV assigned to that taxon. Still, most unidentified ASVs remained unassigned after this step. ASV-level tables were then collapsed (agglomerated) to species level by summing the reads of all ASVs sharing the same species.

The taxonomic inventory obtained through metabarcoding was carefully curated to retain only marine phytoplankton taxa, while non-target groups such as Metazoa, Fungi, terrestrial plants, ciliates, and other obvious contaminants were excluded. Taxonomic assignments were verified and updated according to current nomenclatural standards using AlgaeBase [49] and evaluated against current knowledge of Adriatic phytoplankton diversity, including published Adriatic checklists [26,50] and our laboratory species checklists. Particular attention was given to taxa representing potentially new records for the Adriatic Sea, which were manually verified through database searches and literature review. Synonyms of newly recorded taxa were also checked. Ambiguous assignments, unlikely records, and taxa inconsistent with their known ecological distribution were removed. Analyses at the genus and species levels included only records assigned to the corresponding taxonomic rank, while sequences identified exclusively at higher taxonomic levels were excluded. The numbers of ASVs and reads remaining after each processing step are reported in Supplementary Table S2.

Resulting ASV tables were imported into R, version 4.5.2 [51], where all the following analyses were performed. Visualizations and data handling were performed using the tidyverse package (v2.0.0) [52], ggpubr (v0.6.2) [53], and patchwork (v1.3.2) [54]. The VennDiagram package (v1.7.3) [55] was employed for visualizations of species and genera overlap among markers/methods. For relative abundance bar plots of separate phytoplankton functional groups, taxa contributing less than 2% of the total reads of that group within each marker were grouped as "Others" to improve readability. Relative abundance was calculated by pooling all reads from all samples of the same genus/functional group and dividing by the total number of reads. For the LM, the same pooling was performed with cell counts instead of reads. Correction factors (CFs) described in Martin et al. [56] were used to generate relative abundance plots for the 18S markers (bars labelled CF). For the 18S markers, relative abundance was analysed both with and without the application of CF. These correction factors were applied to account for the inflation of read counts caused by the high copy number of 18S genes present in some species. There were 166 CFs used for diatoms, 4919 for dinoflagellates, and 5.23 for phytoflagellates and coccolithophores.

Bootstrapped rarefaction (100 iterations) was used before calculation of alpha diversity indices to account for differences in sequencing depth among samples using the rrarefy function in vegan package v2.7.2 [57]. The rarefaction depth was set to the minimum library size across samples (separate rarefactions per marker) after the exclusion of samples with an exceptionally small number of reads (COI sample ST101-10m) with only 198 reads and 4 ASVs. Including this sample in the diversity calculation would lead to discarding over 95% of other COI samples. Diversity indices, Shannon diversity, and inverse Simpson metrics were calculated using only ASVs determined to species or

genus level. Pearson correlation (999 permutations) was calculated to assess agreement between markers in diversity indices.

For the generation of the map of the study area, coastlines were obtained from the GADM database [58] (v4.1) and Natural Earth [59]. The map was drawn with sf [60] and ggspatial packages [61] in R. Coordinates are in WGS 84 (EPSG:4326). The metabarcoding-derived species inventory was thoroughly validated against the current knowledge of Adriatic phytoplankton assemblages and manually curated to exclude unlikely taxonomic assignments and potentially spurious records [49].

2.4. Light Microscopy Phytoplankton Identification

Phytoplankton was analyzed with inverted microscopes (Olympus IX50 (Olympus Corporation, Tokyo, Japan), Olympus IX51 (Olympus Corporation, Tokyo, Japan) and Leica MB40008 (Leica Microsystems CMS, Wetzlar, Germany)) following the Utermöhl method [21]. Identification and counting were performed at 400× magnification based on a count of at least 400 cells. Phytoplankton cells were identified at the lowest possible taxonomic level and subsequently categorized into the main groups of diatoms, dinoflagellates (both autotrophic and heterotrophic species), coccolithophores, and phytoflagellates. The classification of identified taxa according to ecological group was performed up to the genus level. HAB species were determined based on the IOC-UNESCO Taxonomic Reference List of Toxic Microalgae [62] and the global overview of HAB [63].

3. Results

The number of sequencing reads and ASVs obtained with different markers is shown in Supplementary Table S2. Phytoplankton reads include reads remaining after quality control and manual curation to retain only phytoplankton sequences. COI was the least suitable marker, yielding the lowest portion of phytoplankton reads relative to the total number of reads (1.3%) and the fewest phytoplankton ASVs detected (27). The molecular marker 18S V4 had the most ASVs in total (560), while *rbcL*, being a diatom-only marker, had the highest phytoplankton reads portion (67.2%), as expected.

Qualitative assessment of phytoplankton diversity with metabarcoding revealed substantially higher taxonomic richness with a total of 177 genera and 312 species compared to LM diversity, which detected 57 genera and 99 species. The number of MB detected species and genera classified into four functional phytoplankton groups (diatoms, dinoflagellates, coccolithophores, phytoflagellates) is shown in Figure 2. The full species list, with the methods and markers that detected each taxon, is given in Supplementary Table S4.

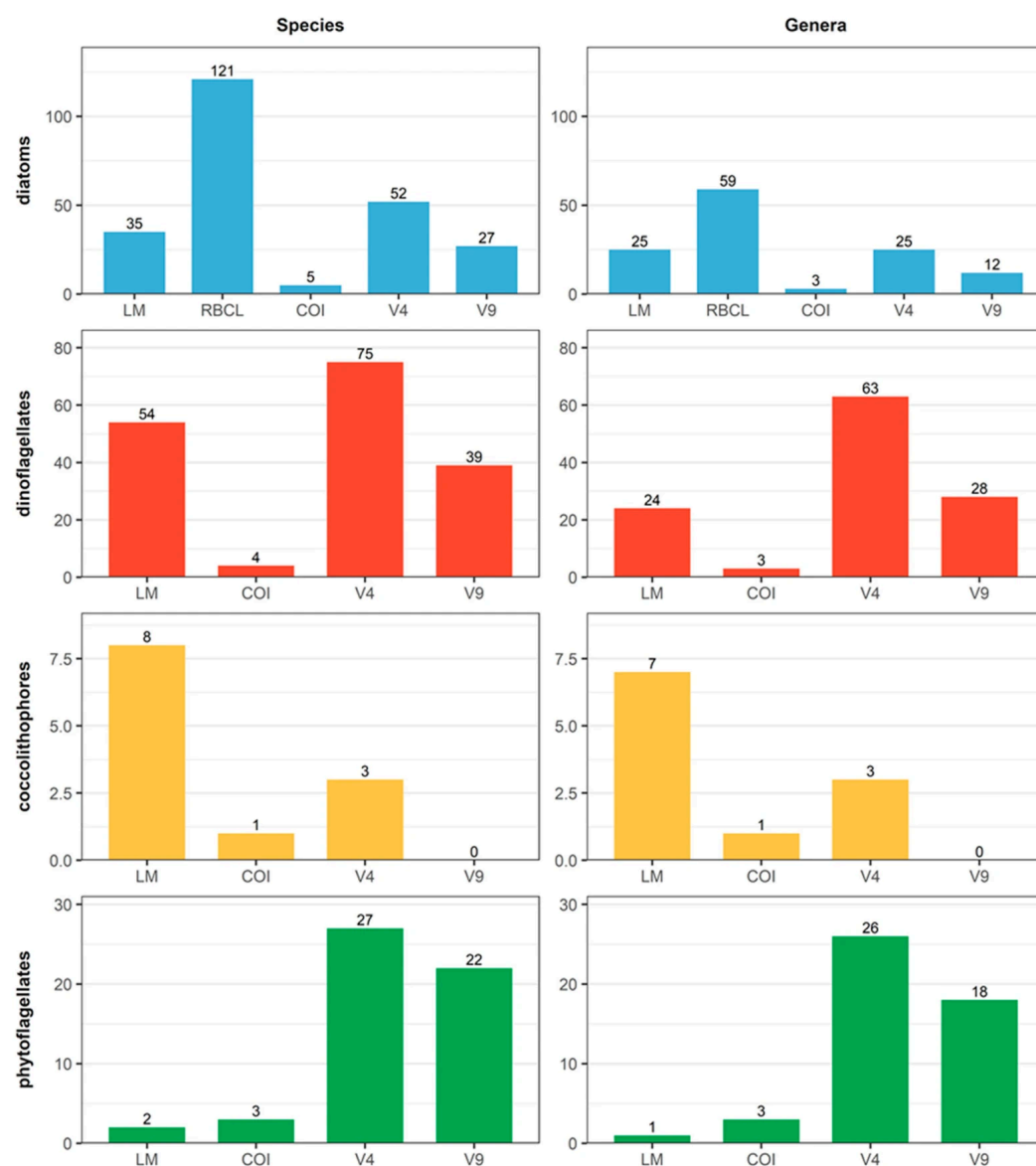


Figure 2. Barplots of species/genera counts of major phytoplankton groups obtained with LM and four different markers (note the different scales).

Among the molecular markers, 18S V4 detected the highest total species number (159 species, 119 genera), while 18S V9 detected 89 species and 59 genera. The 18S V4 marker was reliable in detecting species across the four major phytoplankton groups (Figure 2). COI recovered the smallest number, detecting only 13 species and 10 genera. As expected, *rbcL* proved to be the most effective marker for diatoms, with 121 species identified. For diatoms, the *rbcL* marker was followed by the 18S V4 marker (52 species) and LM (35 species). Dinoflagellate identifications showed better resolution with the 18S V4 marker, although a significant difference was not found between 18S V4 and LM. For coccolithophores, LM had the highest species count, with eight species detected, followed by 18S V4 with three species, whereas the contributions of 18S V9 and COI were negligible. Phytoflagellates were best characterized using both 18S V4 and V9 markers.

Species and genus detection showed limited overlap between MB and LM, or even when comparing individual MB markers (Figure 3). Not one species was detected by all methods or across all four MB markers, and the majority were unique to only one marker (Figure 3B). Only 23% of the 99 LM species were found with metabarcoding (Figure 3A). It is difficult to directly compare the *rbcL* marker with the other markers be-

cause it targets only diatoms; therefore, it was compared with the number of diatom species identified by LM, where it showed only a partial overlap (Figure 3C). The *rbcL* was detected in total 121 species, with 103 diatom species not recovered with LM and 91 diatom species not detected by other MB markers (Figure 3C,D).

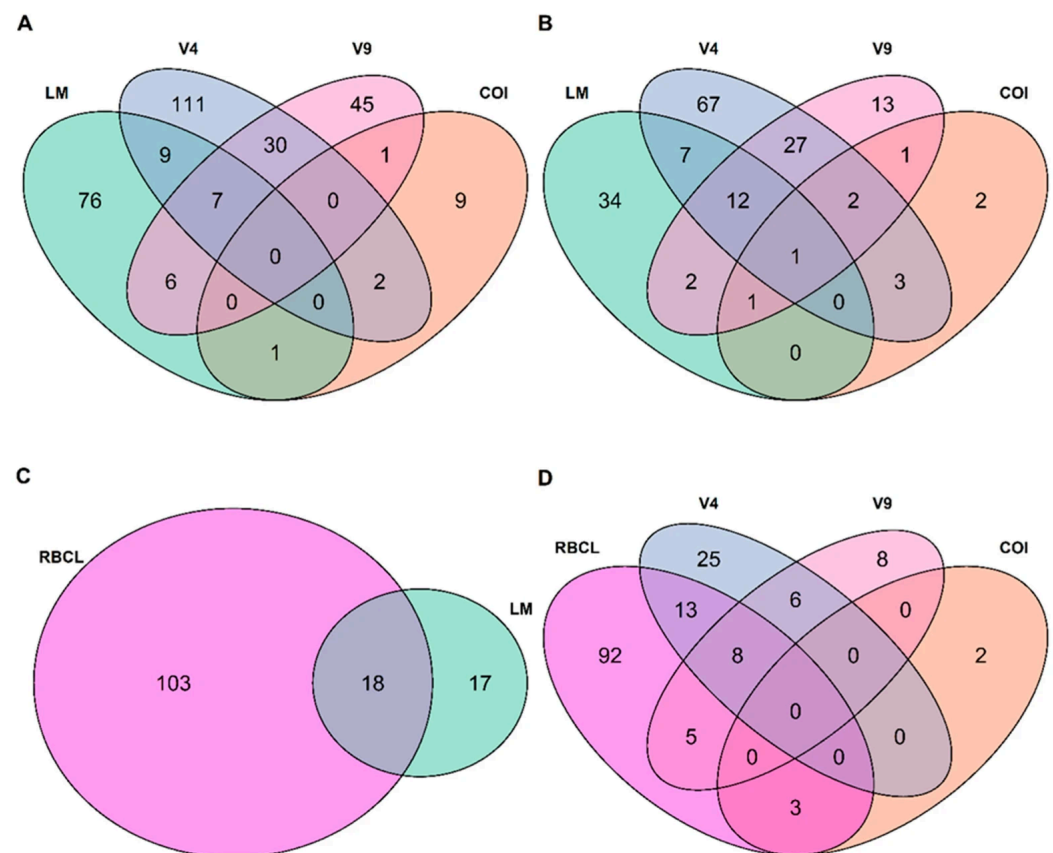


Figure 3. Venn diagrams of detected (A) species and (B) genera using LM and the three universal markers (18S V4, 18S V9, COI), (C) Venn diagram of detected diatom species using *rbcL* and LM, (D) Venn diagram of detected diatom species using all genetic markers.

The biggest overlap in species detection, i.e., 30 species or 10% of the total number of species, was between the two 18S markers (Figure 3A). The non-overlapping portion was biggest for 18S V4. That marker alone detected 111 or 37.4% of the total number of species, while LM alone detected 76 (25.6%). Similar results were seen with genera, where 39.0% were detected by 18S V4 alone and 19.8% by LM alone (Figure 3B).

A particularly interesting result was that 188 of the 312 taxa detected by metabarcoding (approximately two-thirds of the total diversity) had never been recorded during more than 20 years of diligent phytoplankton investigations conducted in Kaštela Bay, when compared to internal LM laboratory datasets [64] (Supplementary Table S3). Of the 188 new taxa, 152 are new species or genera, and 36 represent sequences attributed to families, orders, or clades.

The relative abundance of the phytoplankton community revealed by MB was strongly marker-dependent (Figure 4). For the COI marker, phytoflagellates represented the most abundant functional group (51.4%), followed by diatoms (26.7%) and coccolithophores (18.8%). The 18S V4 marker was dominated by dinoflagellates (64.7%) and diatoms (32.6%), whereas 18S V9 reads were primarily assigned to dinoflagellates (48.8%) and diatoms (48.0%). After applying gene copy number correction factors to the 18S datasets in order to approximate cell number equivalents, both V4 and V9 datasets be-

came dominated by phytoflagellates (67.7% and 66.9%, respectively), followed by diatoms (26.9% and 32.0%). In contrast, dinoflagellates represented only 1.8% of the corrected 18S V4 dataset and 1.1% of the corrected V9 dataset. Conversely, the LM community was co-dominated by phytoflagellates (38.8%) and coccolithophores (30.6%), followed by diatoms (21.8%) and dinoflagellates (8.9%). Despite being a major component of the microscopically observed community, coccolithophores were virtually absent from both 18S datasets ($V4 \leq 0.1\%$, $V9 0\%$) and were recovered in substantial proportions only by COI (18.8%).

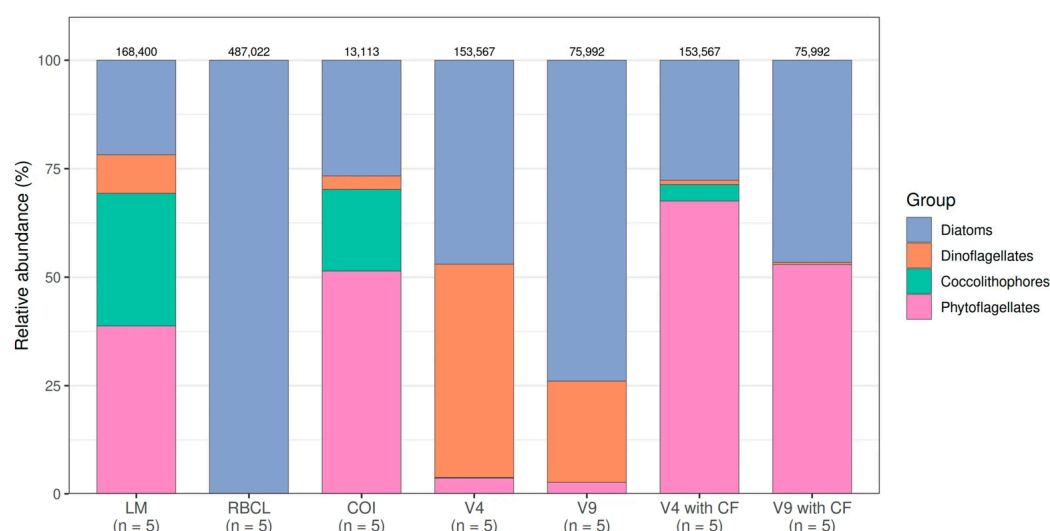


Figure 4. Relative abundances of the four functional groups as detected by LM and four MB markers. The 18S markers V4 and V9 data are shown with and without 18S copy-number correction factors (CF), as described in Martin et al. [56]. The total numbers of reads or cell counts are written above the bars. This figure displays only the four major phytoplankton functional groups; phytoplankton reads that could not be assigned to one of these groups are not represented.

The diatom community composition differed substantially among the methods (LM and MB) and markers (*rbcL*, COI, 18S V4 and V9), indicating strong methodological bias in the detected taxonomic structure (Figure 5A). The LM dataset was relatively heterogeneous, with notable contributions from the *Chaetoceros*, *Pseudo-nitzschia*, *Paralia*, *Thalassionema* and *Navicula* genera. The *rbcL* marker also detected a diverse assemblage, although with a higher relative abundance of *Chaetoceros* than LM and a similar contribution of *Pseudo-nitzschia*. In addition, some taxa such as *Paralia* and *Thalassionema*, which were relatively important in the LM dataset, showed no significant contribution. In contrast, the COI dataset was strongly dominated by only three genera: *Thalassiosira*, *Pseudo-nitzschia*, and *Chaetoceros*. The 18S V4 marker was characterized by a substantial dominance of *Chaetoceros* and the “Others” category but still retaining detectable proportions of *Pseudo-nitzschia* and *Skeletonema*. Finally, the 18S V9 dataset exhibited the highest dominance of *Chaetoceros* with only minor contributions from *Pseudo-nitzschia* and *Leptocylindrus*.

Relative abundance of dinoflagellates in LM consisted of diverse genera such as *Gyrodinium*, *Heterocapsa*, *Oxytoxum*, *Gymnodinium*, *Prorocentrum* and *Tripes*, together with moderate proportions of *Proto-peridinium* and *Alexandrium* (Figure 5B). In contrast, the COI dataset was strongly dominated by *Thoracosphaera*, *Azadinium* and *Scrippsiella*, which together accounted for almost the entire relative abundance. The 18S V4 marker revealed a relatively high proportion of Dino group1-clade 1. *Tripes*, *Gymnodinium*, and *Torodinium* were also detected in moderate abundances and large proportion of “Others”

category. Finally, the 18S V9 dataset was characterized by high relative abundances of Dino-Group-I-clade-1, *Triplos*, *Protoperidinium* together with moderate contributions from *Scrippsiella* and *Gonyaulax*.

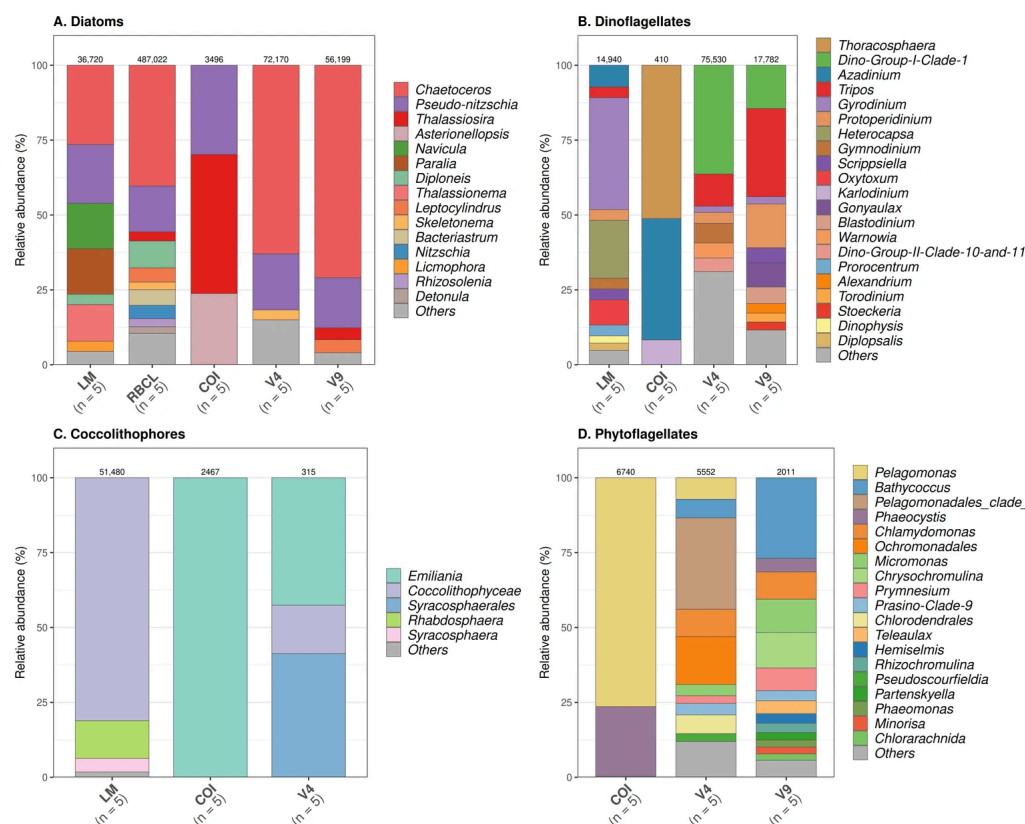


Figure 5. Relative abundances of genera as detected by LM and four MB markers. The functional groups are shown separately. Others are taxa that represent less than 2% of that functional group. Total number of reads or cell counts are written above the bars.

For phytoflagellates, the COI dataset was strongly dominated by *Pelagomonas* and *Phaeocystis* (Figure 5D). The 18S V4 marker revealed a substantially more heterogeneous assemblage characterized by the co-dominance of several phytoflagellate taxa—Pelagomonadales clade I, *Phaeocystis*, *Micromonas*, and members of the Pelagomonadales clade B4—while moderate contributions of *Chlamydomonas*, *Ochromonas*, *Chrysochromulina*, and *Prymnesiales* were also observed. Additionally, the relatively high proportion of the “Others” category suggests the detection of a broader diversity of low-abundance taxa compared with COI. For phytoflagellates, the 18S V9 dataset exhibited the highest apparent diversity and the most even distribution of taxa among all analyzed markers. Several taxa, including *Bathycoccus*, Pelagomonadales clade I, *Chlamydomonas*, *Micromonas*, *Phaeocystis*, *Pelagomonas*, *Chrysochromulina*, and *Hemiselmis*, contributed substantially to the total relative abundance.

Metabarcoding proved especially valuable for detecting small-sized species, such as the tiny colonial cells of the genus *Chaetoceros*, which are difficult to reliably identify using LM. In addition, LM detected only four *Chaetoceros* species, whereas the *rbcL* marker MB identified 27 species, making *Chaetoceros* the most diverse genus in the dataset.

The opposite was observed for coccolithophores, where LM was more efficient (Figure 5C). Furthermore, LM proved particularly effective for detecting certain thecate dinoflagellates (e.g., *Dinophysis*, *Oxytoxum*, *Diplopsalis*, *Ceratocorys*, and *Goniadoma*), which were entirely absent from the MB results.

When comparing alpha diversity indices obtained with different methods (bottle samples only), metabarcoding data produced higher values than light microscopy for both Shannon and inverse Simpson (Supplementary Figure S1). The 18S V4 marker had the highest Shannon diversity (4.00, after correction for 18S copy number 3.21), and V9 showed the highest inverse Simpson index (24.5, after correction for 18S copy number 21.4). The Shannon diversity and inverse Simpson indices calculated with data from different markers correlated best in the case of the two 18S markers (Pearson correlation, $r = 0.71$ and 0.75 respectively, Supplementary Figure S2). Per-sample correlation of MB with LM was not statistically meaningful, as there were only two shared samples.

Light microscopy (LM) detected six potentially harmful or toxic phytoplankton species, including *Pseudo-nitzschia delicatissima*, *Azadinium* sp., *Dinophysis caudata*, *Prorocentrum cordatum*, *Dinophysis tripos*, and *Gonyaulax fragilis*, with some taxa (e.g., *Dinophysis* spp. and *G. fragilis*) not detected by MB. Molecular approaches showed variable detection across markers, with most harmful species identified by only one or two markers (Table 2). The 18S V4 marker revealed eight harmful species, mainly small taxa and easily overlooked with LM, such as *Florenciella parvula*, *Vicicitus globosus*, *Phaeocystis globosa*, and *Prymnesium* sp. In contrast, the *rbcL* marker detected seven potentially toxic *Pseudo-nitzschia* species. Overall, MB recorded a broader HAB category with 23 potentially harmful species producing various toxin types (amnesic, paralytic, and yessotoxins), including ichthyotoxic (*Vicicitus globosus*) and high-biomass-forming species such as *Noctiluca scintillans*, showing complementary advantages using the two approaches.

Table 2. Potentially harmful phytoplankton species detected by metabarcoding (four markers).

	Species	V4	V9	COI	<i>rbcL</i>
1	<i>Pseudo-nitzschia fraudulenta</i>		x		x
2	<i>Pseudo-nitzschia delicatissima</i>		x		x
3	<i>Pseudo-nitzschia hasleana</i>			x	x
4	<i>Pseudo-nitzschia subfraudulenta</i>			x	x
5	<i>Pseudo-nitzschia cuspidata</i>			x	
6	<i>Pseudo-nitzschia calliantha</i>				x
7	<i>Pseudo-nitzschia pseudodelicatissima</i>				x
8	<i>Alexandrium andersonii</i>		x		
9	<i>Alexandrium ostenfeldii</i>	x			
10	<i>Azadinium</i> sp.			x	
11	<i>Azadinium concinnum</i>		x		
12	<i>Lingulaulax polyedra</i>	x			
13	<i>Karlodinium veneficum</i>			x	
14	<i>Margalefidinium fulvescens</i>	x	x		
15	<i>Noctiluca scintillans</i>	x			
16	<i>Pseudochattonella farcimen</i>			x	
17	<i>Pseudochattonella verruculosa</i>		x		
18	<i>Protoceratium reticulatum</i>		x		
19	<i>Ostreopsis ovata</i>		x		
20	<i>Florenciella parvula</i>	x			
21	<i>Vicicitus globosus</i>	x			
22	<i>Phaeocystis globosa</i>	x		x	
23	<i>Prymnesium</i> sp.	x			

4. Discussion

In this study, light microscopy (LM) and DNA metabarcoding (MB) were successfully integrated for the first time in the Central Adriatic Sea, significantly extending current knowledge of local phytoplankton diversity at the long-term station in Kaštela Bay. Phytoplankton at this coastal station has been investigated using light microscopy (LM) since the 1970s, consistently revealing high biodiversity within the resident community. A recent assessment by Skejić et al. [16] documented 396 taxa identified by LM in Kaštela Bay over a 14-year period, underscoring both a high level of taxonomic expertise and sustained analytical effort invested in sample processing. The aim of the present study was to overcome the inherent limitations of traditional microscopy by applying a multi-marker molecular approach and to enhance the phytoplankton diversity baseline by integrating the complementary input of both methods.

Although the study is based on a single sampling event in June 2024, metabarcoding uncovered a remarkably high and previously unrecognised level of phytoplankton structural complexity, tripling LM detections. This unexpectedly high taxonomic richness across nearly all major phytoplankton groups demonstrates the capacity of metabarcoding to uncover a substantial fraction of diversity that may remain undetected using conventional approaches. Interestingly, of the 312 taxa detected by metabarcoding, approximately one-third represent taxa not previously recorded during 20 years of light microscopy (LM)-based phytoplankton monitoring in the study area. At the same time, the detection overlap between MB and LM was relatively small, with fewer than 25% of the species detected by LM also found with MB, demonstrating the complementarity rather than interchangeability of traditional and molecular approaches. Recent studies from the Northern Adriatic have similarly demonstrated the effectiveness of metabarcoding for detecting phytoflagellates and dinoflagellates, whereas microscopy has traditionally been more effective for larger phytoplankton such as diatoms and dinoflagellates [26,65].

Metabarcoding markers yielded higher Shannon and inverse Simpson alpha-diversity values than light microscopy. However, after copy number correction, the Shannon index was lower, indicating a more realistic state of community. We also found that the indices calculated from the two 18S markers were strongly positively correlated, suggesting that these markers capture broadly similar patterns of sample diversity.

Integrating and interpreting data generated from the four different molecular markers proved challenging, as each marker provided a distinct view of community composition and exhibited different taxonomic coverage and resolution. This issue was particularly relevant for toxic phytoplankton species, which are distributed across multiple phylogenetic groups. As a result, diversity estimates were highly marker-dependent and, in some cases, not directly comparable.

The pronounced differences in relative abundance among markers (Figures 4 and 5) could be explained by the variation in rRNA gene copy number, primer biases, and reference database limitations. Applying functional group 18S rRNA gene-copy number corrections brought the V4 and V9 relative abundance profiles closer to those of COI and LM, although substantial differences remained. The gene-copy number corrections should be interpreted as approximations rather than estimates of absolute cell abundance. Per-cell 18S copy number varies by several orders of magnitude among phytoplankton groups and also within groups and even among strains of a single species. Martin et al. [56] also found that the copy number correction does not improve cell abundance estimates for diatoms, probably because of large volume plasticity that cannot be captured by a single group-level factor. Recent work by Zhou et al. [66] has explored calibrating DNA-based abundance directly against cell counts and phytoplankton carbon

biomass, offering a potentially more ecologically meaningful route to quantitative metabarcoding.

Analysis of relative abundance by functional group (Figure 5) showed that markers detected largely different genera, as also illustrated by the Venn diagrams (Figure 3). Only V4 and V9 recovered broadly similar genus compositions, except for phytoflagellates. Their differences likely reflect amplicon length, as the longer V4 region provides higher taxonomic resolution needed for species detection than V9. COI showed a strong primer bias toward metazoans and recovered only 27 phytoplankton ASVs, representing just 10 genera (1 coccolithophore, 3 phytoflagellate, 3 diatom, and 3 dinoflagellate genera). Consequently, its relative abundance profile was strongly distorted.

Reference database limitations further contribute to these discrepancies. Thecate dinoflagellates remain underrepresented in 18S databases because many species are unculturable [67]. More dinoflagellate than diatom genera lack 18S reference sequences [68], and several have complex life cycles involving parasitic or long-lived cyst stages. In addition, the 18S region often does not have sufficient variability to resolve dinoflagellates to genus or species level, leaving many ASVs unassigned or assigned to Dinophyceae. For this group, LSU (28S) and ITS markers provide higher taxonomic resolution [69,70]. The relatively poor representation of coccolithophores in our metabarcoding results may be due to limitations in current reference databases [71]. The coccolithophores accounted for about 30% of the LM community but were negligible in the 18S data and had an appreciable COI read fraction (18.8%), but with only one species detected. Many coccolithophore species are difficult to isolate, establish, and maintain in culture, which has likely constrained the generation of reference sequences for these taxa. As a result, significant gaps may still exist in available sequence databases, potentially leading to lower taxonomic resolution and reduced detection of coccolithophores in metabarcoding studies. The near-total absence in the 18S marker communities points to marker-specific primer bias in addition to the reference–database gaps [71]. Additionally, the calcified coccosphere may reduce cell lysis and lower the DNA yield during extraction. These results suggest that LM remains essential for analyzing the coccolithophore community, which current metabarcoding markers underrepresent.

Similar conclusions have been reported in other multimarker studies, showing that methodological choices strongly influence biodiversity estimates and that light microscopy and metabarcoding should be regarded as complementary rather than interchangeable methods [65,72,73].

Overall marker performance (excluding *rbcL*) for phytoplankton species detections in our study can be summarised as 18S V4 > 18S V9 > COI, with both 18S markers showing the highest performance for dinoflagellates and diatoms, followed by phytoflagellates, and COI showing similarly low yields for all three groups. We conclude that COI, as applied here, is not a suitable marker for phytoplankton.

Compared to the internal LM laboratory dataset, the combined metabarcoding detections yielded 188 new species records for Kaštela Bay, summarized in Supplementary Table S3. Notably, the 18S markers (V4 and V9) revealed previously undocumented phytoflagellate diversity, with 40 taxa recorded for the first time in the area. In small and fragile cells such as phytoflagellates, fixation frequently distorts cell morphology and reduces LM detection performance [74–76], contributing to metabarcoding detection success in these groups [72]. Moreover, sample splitting or the use of small aliquots in LM sample processing (typically 25–50 mL) is bypassed in metabarcoding workflows, which commonly analyse larger sample volumes (e.g., 1 L), thereby increasing the likelihood of rare taxa detection [26,32,73]. MB also identified 70 previously unrecorded dinoflagellate species and 77 new diatom species, with 18S V4 showing highest

performance for detecting novel dinoflagellates (53 species) and *rbcL* for diatoms (56 species). Differences between metabarcoding and LM may also be due to DNA extraction efficiency influencing the recovered community, and some taxa may be underrepresented in MB because of incomplete cell lysis. Since all metabarcoding markers used the same DNA extraction protocol, this bias does not affect comparisons among markers but should be considered when comparing metabarcoding with LM.

Species-level resolution of several taxonomically challenging genera within the resident phytoplankton community of Kaštela Bay was effectively achieved with metabarcoding. Among diatoms, genera *Chaetoceros* and *Pseudo-nitzschia* emerged as the most diverse, with 27 and 11 species, respectively, detected primarily using the *rbcL* marker. Previous studies have shown that *Chaetoceros* frequently dominates both the diatom assemblage and the entire phytoplankton community in Kaštela Bay [16], yet species identification is constrained by the extremely small cell size. Metabarcoding surveys have reported 60 *Chaetoceros* species off Barcelona over a 15-year period [77] and 76 terminal taxa in the Gulf of Naples [78], where three-year time series delineated 73 taxa of *Chaetoceros* and *Bacteriastrum* combined [79]. Genus *Pseudo-nitzschia* presents a comparable taxonomic challenge, as LM identification rarely progresses beyond assignment to the *delicatissima* and *seriata* groups. This genus comprises numerous cryptic species that cannot be reliably distinguished morphologically, and several members are known toxin producers [30,80]. Among the 11 *Pseudo-nitzschia* species, all but 1 had been previously recorded from the study area, though exclusively through scanning electron microscopy (SEM). Similarly to our study, 16 species have been documented in the Gulf of Naples [81] and 14 across the entire Adriatic Sea [82]. The strong recovery by *rbcL* confirms its value as a high-resolution marker for diatoms, consistent with earlier studies from the Mediterranean Sea [33,83]. Even though we captured a substantial diversity of *Pseudo-nitzschia* using *rbcL*, other studies have shown that the ITS marker may offer an even higher resolution for species detection for this genus than the four markers we used [30,84].

Metabarcoding further revealed the presence of 23 potentially harmful species in Kaštela Bay, most of which had been overlooked by conventional LM. The presence of their DNA in the water column does not indicate bloom-forming populations, toxin production, or any environmental risk. It only shows that these taxa form part of the local species pool and may warrant closer attention in future monitoring. Although the trophic state of Kaštela Bay has gradually shifted towards oligotrophy in recent decades, and harmful algal blooms (HABs) have not been documented [85], the detection of low-abundance harmful taxa remains important for bloom surveillance and for elucidating the ecology of toxin-producing species [86].

Despite its undeniable advantages, metabarcoding also demonstrated limitations. Metabarcoding captures not only living cells but also dead cells and extracellular DNA. Therefore, some detected sequences may come from small, extracellular debris that cannot be detected by LM. Among the four functional groups examined, coccolithophores were the most difficult to detect using the MB (COI, 18S V9 and V4), likely due to incomplete reference databases and difficulties in culturing these organisms for genetic characterization [71]. Only four taxa were detected with 18S V4 and one with COI, with only one representing a new record for the study area.

Although metabarcoding detects a much broader range of taxa, alpha diversity may be overestimated due to variable 18S copy numbers or PCR biases [87], which pose challenges to the reliable estimates of actual cell counts.

As this study is based on a limited pilot survey conducted in a single season, conclusions regarding temporal variability remain preliminary. Future research should in-

clude year-round sampling to better characterize phytoplankton diversity and seasonal succession, while also identifying the most suitable genetic marker for reliable and cost-effective long-term monitoring.

5. Conclusions

Light microscopy (LM) and DNA metabarcoding (MB) using a multimarker approach were successfully integrated for the first time at a long-term station in the Central Adriatic Sea, significantly expanding current knowledge of local phytoplankton diversity. Our findings show that LTER Kaštela Bay harbours considerable hidden biodiversity, which is in line with our initial hypothesis that a considerable fraction of the phytoplankton diversity in the Central Adriatic Sea is overlooked when using a light microscopy approach alone. Compared with Utermöhl-based microscopy, MB added 188 new taxa to the existing local inventory, including 23 potentially harmful taxa, mostly overlooked by LM. Diversity estimates derived from MB were strongly dependent on the molecular marker used, highlighting the necessity of a multimarker approach in phytoplankton metabarcoding to overcome the inherent limitations of single-marker detection range and taxonomic resolution. Overlap between MB and LM was limited, with fewer than 25% of the 99 LM species also detected through metabarcoding. This confirms that microscopy remains the essential tool for phytoplankton monitoring, while demonstrating that metabarcoding serves as a powerful complement that continues to improve with methodological advances.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/d18070417/s1>. Supplementary Table S1. PCR conditions for the four metabarcoding markers; Supplementary Table S2. Number of amplicon sequence variants (ASVs) and sequencing reads remaining per marker after each bioinformatic processing step (denoising with DADA2, quality filtering, and phytoplankton taxonomic filtering). The aggregate numbers across all samples are shown; Supplementary Table S3. Phytoplankton taxa newly recorded by DNA metabarcoding (188 taxa) that were not listed in the existing regional LM checklists. For each taxon the taxonomic confidence/consensus of the assignment (QIIME2 classifier confidence for *rbcl*, 18S V4 and 18S V9, and consensus for COI, for taxa recovered by more than one marker, values are given per marker in the order listed). The columns MON and VIL indicate whether the taxon is reported in Montali et al. (2026) (MON) and in the historical Adriatic checklist of Viličić et al. (2002) (VIL). Rows shaded in grey indicate taxa resolved only to a clade or higher rank. ‘SEM’ denotes taxa were identified previously in area but only by scanning electron microscopy; Supplementary Table S4. Consensus list of all species detected with MB and LM; Supplementary Figure S1. Alpha diversity indices Shannon and Inverse Simpson calculated from data obtained by 5 different methods. Only bottle samples are shown as LM diversity indices can only be calculated with bottle samples; Supplementary Figure S2. Correlation of alpha diversity indices Shannon and Inverse Simpson for the same sample calculated with 18S V4 and V9 data (bottle and net).

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