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PRIMARY RESEARCH PAPER

Comparative analysis of phytoplankton diversity using microscopy and metabarcoding: insights from an eLTER station in the Northern Adriatic Sea

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Abstract The monitoring of phytoplankton is crucial to highlight changes in the marine ecosystems. In the present study, the phytoplankton community of an eLTER station in the Northern Adriatic Sea was analysed combining two approaches, i.e. microscopy and eDNA metabarcoding (targeting V4 and V9 regions of the 18S rRNA gene, and using PR2 and SILVA as reference databases), to highlight the strengths and weaknesses of these two methods. Metabarcoding revealed a so far unknown phytoplankton diversity (99 genera and 151 species), while microscopy detected 14 genera and 44 species not revealed by metabarcoding. Only a small percentage of genera and species were shared by the two methods (microscopy and metabarcoding), 18S regions (V4 and V9) and reference databases (PR2 and SILVA). Metabarcoding showed a community characterized by a higher number of phytoflagellate and dinoflagellate genera and species, in comparison with microscopy where diatom and dinoflagellate taxa were the most represented. Moreover, metabarcoding failed to

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reveal almost all the coccolithophores. The results confirmed metabarcoding as a powerful tool, but it should still be combined with microscopy to have a more detailed information on the community and to counteract the drawbacks of metabarcoding, such as gaps in the reference databases.

Keywords Microalgae · Diversity · Environmental DNA · Long-Term Ecological Research · Amplicon sequencing

Introduction

Phytoplankton communities play essential roles in the marine ecosystems, not only for their oxygen production and contribution to higher trophic level/food production, but also for their involvement in biogeochemical cycles, drawdown of CO_2 from the air, and climate regulation (Hays et al., 2005; Vallina & Simó, 2007; Blanchard et al., 2012; Falkowski, 2012; Araujo et al., 2022; Naselli-Flores & Padisák, 2023). Phytoplankton communities are characterized by high turnover, and they can reveal changes in the marine ecosystems due to their rapid response to environmental and oceanographic conditions.

In the Northern Adriatic Sea (NAS), one of the most productive areas of the Mediterranean Sea (D'Ortenzio & Ribera d'Alcalà, 2009), the long-term phytoplankton response to environmental and climatic changes, such as riverine runoff, temperature increases and nutrient availability, has been well documented in several European Long-Term Ecological Research (eLTER) sites (https://elter-ri.eu/) reporting changes in abundance, biomass and/or shifts in the seasonal rhythm of the major blooms (Mozetič et al., 2010; Bernardi Aubry et al., 2012; Marić et al., 2012; Cerino et al., 2019; Totti et al., 2019; Cozzi et al., 2020; Vascotto et al., 2021; Neri et al., 2022, 2023).

A reliable identification of phytoplankton up to the lower taxonomic level (i.e. species) is essential for several reasons, e.g. to detect toxic species for human and marine ecosystems (Pinto et al., 2023), to document allochthonous species that can alter the ecosystem equilibrium (Zenetos et al., 2010), and to assess biodiversity. The estimation of biodiversity in pelagic habitats has been included in the assessment of the Good Environmental Status (2008/56/EC), and many indicators related to phytoplankton (e.g. shifts in productivity, life forms, composition in terms of non-indigenous or toxic species, changes in the spatial and temporal diversity) have been proposed to be considered in the management policies (Wasmund et al., 2017; Tweddle et al., 2018; Varkitzi et al., 2018; McQuatters-Gollop et al., 2019; Rombouts et al., 2019; Francé et al., 2021).

Traditionally, the monitoring of phytoplankton communities has been conducted by expert taxonomists through inverted light microscopy, which provides detailed information on composition, abundances and biomass based on morphological criteria. However, this approach is time-consuming, and the less abundant/smaller species may be overlooked or misidentified. Moreover, the accurate identification can be further prevented by (i) several ultrastructural taxonomic details only visible by electron microscopy, (ii) presence of taxa difficult to distinguish morphologically, as they lack diagnostic morphology (cryptic species; Struck et al., 2017), and (iii) morphological diagnostic characters which vary under different environmental conditions.

For these reasons, increasing number of studies have been using DNA metabarcoding to investigate phytoplankton communities (Penna et al., 2017; Piredda et al., 2018; Gaonkar et al., 2020; Caracciolo et al., 2022; Gaonkar & Campbell, 2023; Grižančić et al., 2023; Matek et al., 2023; Specchia et al., 2023; Almandoz et al., 2024). This method, in addition to its capability to assess the whole biodiversity, including taxa (e.g. rare and cryptic ones) that are difficult to identify by light microscopy, allows higher comparability between studies compared to only relying on light microscopy which is heavily user-dependent (Turk Dermastia et al., 2023). On the other hand, metabarcoding provides only relative abundance values, and taxonomic assignment depends on reference databases, the incompleteness and errors of which can affect the biodiversity assessment (Weigand et al., 2019; Tzafesta et al., 2022).

In order to take advantage of the potential of both traditional and molecular approaches and to achieve the most reliable phytoplankton identification, an increasing number of studies have therefore coupled microscopy and metabarcoding, highlighting important differences, e.g. in the community composition and/or relative abundances (Abad et al., 2016; Piredda et al., 2017; Esenkulova et al., 2020; Santi et al., 2021; Andersson et al., 2023; Bilbao

et al., 2023; Pierce et al., 2023; Turk Dermastia et al., 2023), and emphasizing the importance of combining these methods for the monitoring of this planktonic fraction.

The aim of this study was to compare and integrate the biodiversity and taxonomic composition of eukaryotic phytoplankton determined by microscopy and DNA metabarcoding at the eLTER Senigallia coastal station, in the northern Adriatic Sea. DNA metabarcoding analysis was performed with different markers (18S rRNA V4 and V9) and using different reference databases, PR2 (Guillou et al., 2013) and SILVA (Quast et al., 2013), which allowed the evaluation of the different combinations in the assessment of phytoplankton communities. The analysis of the potential strengths and weaknesses of the microscopic and molecular methods, as well as of the main biases introduced by each approach allowed to highlight the best practice in the analysis and to achieve the most accurate and reliable representation of these important organisms in coastal environments.

Materials and methods

Study area and sampling

The study area represents the coastal station (SG01, 43.755° N, 13,2105° E, Fig. 1) of the eLTER Senigallia-Susak transect (DEIMS.iD: https://deims.org/be8971c2-c708-4d6e-a4c7-f49fcf1623c1), in the Northern Adriatic Sea, located at 1.2 nM from the western coast and with a bottom depth of 12 m.

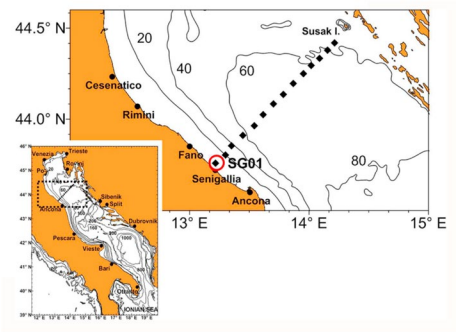

Fig. 1 Map of the study area. The dashed line represents the eLTER Senigallia-Susak transect, with the study station (SG01) highlighted by a red circle

The area is highly affected by the Western Adriatic Current, which brings southwards riverine waters (particularly from the Po River). The physical and chemical parameters of this station have been showed and widely discussed in previous papers (Totti et al., 2019; Neri et al., 2023). As in the whole Northern Adriatic basin, the study station shows the P-limiting condition typical of the Northern Adriatic Sea (Cozzi & Giani, 2011) and is characterized by a high seasonal and interannual variability, with a recent tendency towards oligotrophication (Totti et al., 2019; Grilli et al., 2020; Neri et al., 2023).

For both microscopy and metabarcoding, samples were collected monthly at the surface (0.5 m) using Niskin bottles from February to October 2019 on board of the Actea oceanographic vessel.

Phytoplankton samples for microscopy analyses were collected in 250-ml dark glass bottles and preserved by adding 0.8% prefiltered and neutralized formaldehyde (Throndsen, 1978). For metabarcoding, 2 L of seawater were filtered in cellulose nitrate filters (47 mm diameter, 1.2 µm pore-size, Sartorius), and preserved at -20 °C. The volume of seawater filtered for metabarcoding analyses was chosen based on what commonly reported in the literature in phytoplankton studies, where it typically ranges from 1 to 3 L (e.g. Piredda et al., 2017; Turk Dermastia et al., 2023).

DNA extraction, sequencing and metabarcoding analysis

DNA was extracted using the DNeasy PowerWater Kit (QIAGEN, Germany) following the manufacturer's instructions. The filters were cut in half, each half was extracted separately and then pooled after elution. Quantity of the extracted DNA was assessed with Qubit Fluorimeter (Thermo Fisher Scientific). The hypervariable V4 and V9 regions of the universal 18S rRNA gene were targeted as they are taxonomically informative across a wide range of phytoplankton taxa, highly sequenced, well-represented in the reference databases, and they allow comparability with previous studies, being among the most commonly used regions for metabarcoding of phytoplankton communities (Amaral-Zettler et al., 2009; Stoeck et al., 2010; Pawlowski et al., 2012; Kezlya et al., 2023). Amplification was performed following the Illumina Sequencing Library Preparation protocol

(with 30 PCR cycles in the amplicon PCR) with V4 18S Next.For and V4 18S Next.Rev primers for V4 (Piredda et al., 2017), giving amplicons of ~470 bp, and V9 18S Next.For and V9 18S Next.Rev primers for V9 (Piredda et al., 2017), giving amplicons of ~270 bp. The sample of May failed to amplify for the V4 region, thus was not sequenced. Library preparation (including IDT for Illumina UD index set D (Illumina, San Diego, CA, USA), primers with sequence complementary to overhang adapter and sample specific barcodes), 2×250 bp paired end sequencing of equimolar ratios of the purified amplicon libraries on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) and demultiplexing were performed at the Department of Bioscience, Biotechnology and Biopharmaceutics at Università degli Studi di Bari.

For each raw read, quality and presence of adapters were inspected using FASTQC (v. 0.10.1) (Andrews & Braham Bioinformatics, 2010) and primers were trimmed using cutadapt (v. 4.5) (Martin, 2011). Sequences were then processed and analysed using QIIME2 (v. 2023.2) (Bolyen et al., 2019).

Quality filtering, denoising and pairing of the reads were performed with DADA2 (Callahan et al., 2016).

Taxonomy was assigned to amplicon sequence variants (ASVs) with a Naive Bayes classifier (Bokulich et al., 2018) trained for the specific 18S rRNA gene target region (V4, V9) against the PR2 v. 5.0.1 (Guillou et al., 2013) and SILVA 138 99% (Quast et al., 2013) reference databases.

Microscopy analysis

An inverted microscope (ZEISS Axiovert 135) equipped with phase contrast was used for the identification and counting of phytoplankton, following the Utermöhl method (Edler & Elbrachter, 2010). Counting was carried out at 400× magnification, along transects or in random visual fields, depending on cell abundance, to count a minimum of 200 cells. Moreover, a half of the Utermöhl chamber was analysed at 200× magnification to provide a more accurate estimation of the larger and rarer species that strongly influence the biomass value. Phytoplankton taxa were identified at the lowest possible taxonomical rank, and finally grouped in major groups, i.e. diatoms, dinoflagellates, coccolithophores, and phytoflagellates. Dinoflagellates were considered as a taxonomical group, with both autotrophic and heterotrophic species. Phytoflagellates include all groups that are not easily identifiable by light microscopy, often not even at the class level, e.g. haptophytes (except coccolithophores), cryptophytes, chrysophytes, dictyochophytes, raphidophytes, chlorophytes and euglenophytes.

Data analyses

Data analyses were performed using R software (R Core Team, 2021) on the presence/absence data obtained from the two applied methods, microscopy and metabarcoding. Within the metabarcoding method, analyses were further categorized based on different markers (V4 and V9) and databases (SILVA and PR2), for a total of five approaches: (i) microscopy, (ii) V4-SILVA, (iii) V9-SILVA, (iv) V4-PR2, and (v) V9-PR2.

To allow the comparison between microscopy and metabarcoding, phyla or classes not belonging to phytoplankton (e.g. ciliates, choanoflagellates, tintinnids, metazoans) were removed from metabarcoding datasets using R package phyloseq (McMurdie & Holmes, 2013). In the same way, organelle sequences (mitochondrial, nucleomorphic) were not considered. As in microscopy phytoflagellates are considered a unique artificial group (e.g. Neri et al., 2023), for the comparison between methods, we maintained the same group also in the metabarcoding analysis.

Species and genus names were checked on Algaebase (Guiry & Guiry, 2024) and updated to the current accepted nomenclature. In cases of uncertain taxonomy, the ASVs (amplicon sequence variant) were checked on NCBI, and mistakes were corrected or removed.

Statistical analyses were performed separately at both genus and species levels. Therefore, the ASVs whose taxonomy ended at higher levels, e.g. classes or higher (for genera analysis) and genus or higher (for species analysis) were removed. Venn diagrams were used to represent the number of genera and species found by the five different approaches and were built using the VennDiagram (Chen & Boutros, 2011) and eulerr (Larsson, 2022) R packages.

When the Venn diagrams highlighted taxa detected only by microscopy, Primer-BLAST (Ye et al., 2012) was used to exclude primer unsuitability as the reason for undetectability by verifying their specificity with

available sequences deposited in GenBank for those taxa.

A non-metric multidimensional scaling (NMDS) was performed on the richness (i.e. number of genera and species in the different phytoplankton groups and on the presence–absence of taxa obtained from the five distinct approaches). The metaMDS function from the vegan package (Oksanen et al., 2022) was used, setting the distance as Bray and Jaccard, when considering richness and presence–absence data, respectively. Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences among the groups that were highlighted by the NMDS, using the adonis function in the vegan package (Oksanen et al., 2022).

Results

Phytoplankton groups

The proportions of genera and species in the different phytoplankton groups (diatoms, dinoflagellates, coccolithophores and phytoflagellates) found by the five distinct approaches based on microscopy and metabarcoding (V4-SILVA, V9-SILVA, V4-PR2 and V9-PR2) are shown in Fig. 2a, b, for genera and species, respectively.

Considering the identification at genus level throughout the study period, using microscopy, dinoflagellate and diatom genera represented the highest percentage (40 and 38% respectively), followed by coccolithophores (13%) and phytoflagellates (10%).

As regards metabarcoding, the phytoflagellates represented the highest percentage (41 and 49% for V4-PR2 and V9-PR2, respectively, 43% for both V4-SILVA and V9-SILVA, respectively), followed by dinoflagellates (38 and 39% for V4-PR2 and V9-SILVA, respectively, 34% for both V9-PR2, V4-SILVA) and diatoms (19 and 22% for V4-PR2 and V4-SILVA, respectively, 18% for both V9-PR2 and V9-SILVA). Coccolithophore genera comprised 2% and 1% with V4-PR2 and V4-SILVA, respectively, while no coccolithophores were observed when using the V9 marker with either the SILVA or PR2 databases.

As regards the percentage of identified species, using microscopy, the highest percentages of species were represented by diatoms and dinoflagellates (42 and 41%, respectively), followed by coccolithophores

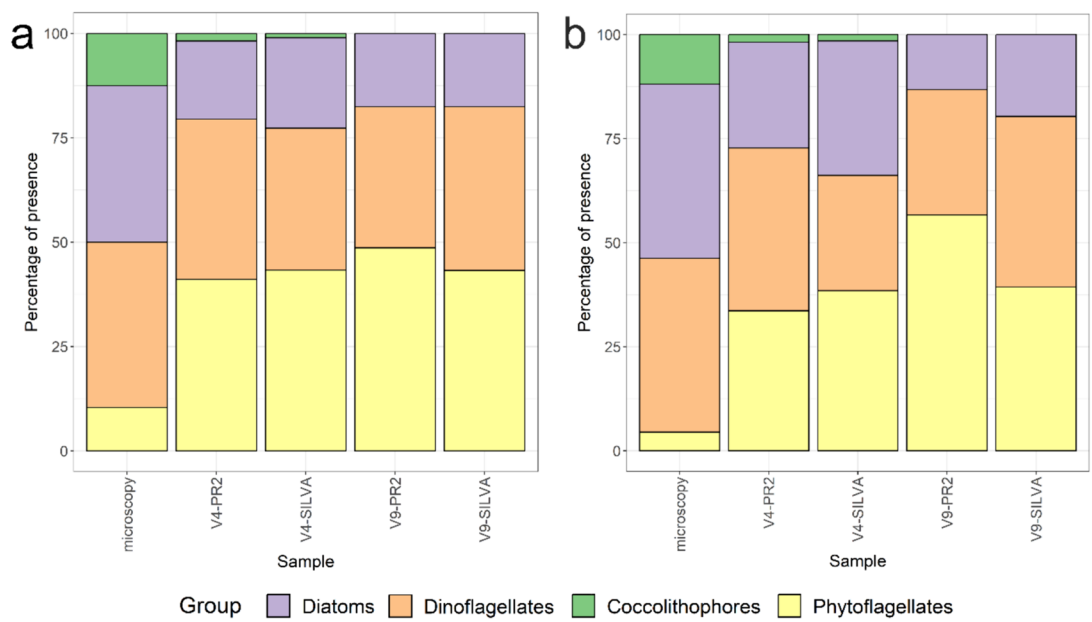

Fig. 2 Bar plots representing the percentages of presence of genera (**a**) and species (**b**) belonging to diatoms (violet), dinoflagellates (orange), coccolithophores (green) and phytoflagellates (yellow) obtained using the five applied approaches: microscopy, V4-PR2, V4-SILVA, V9-PR2 and V9-SILVA

(12%) and phytoflagellates (5%). When employing V4-PR2, 39% of species were dinoflagellates, 34% were phytoflagellates, 25% were diatoms and 2% were coccolithophores. With V4-SILVA, 38% of species belonged to phytoflagellates, 32% to diatoms, 28% to dinoflagellates and 2% to coccolithophores. Using V9-PR2, the majority of species were phytoflagellates (57%), followed by dinoflagellates (30%) and diatoms (13%), while with V9-SILVA, 41% of species were dinoflagellates, 39% were phytoflagellates and 20% were diatoms. The V9 marker did not detect any coccolithophores in either database.

The percentages of genera and species in the different phytoplankton groups in each month are shown in Fig. S1a and b, respectively. It can be observed that the per cent values show a high variability in each month and for each considered approach.

Genus identification

By combining microscopy and metabarcoding results, a total of 171 genera (Table S1) were recorded. Microscopy identified 48 genera, while metabarcoding identified 112 genera with V4-PR2, 97 with V4-SILVA, 75 with V9-PR2 and 75 with V9-SILVA.

The combination of microscopy, V4-PR2 and V4-SILVA resulted in a total of 151 genera. Among these, the following 24 genera (representing 16% of the total) were detected by all approaches (Fig. 3a): 13 diatoms (*Amphora*, *Asteromphalus*, *Cerataulina*, *Chaetoceros*, *Cyclotella*, *Leptocylindrus*, *Nitzschia*, *Pleurosigma*, *Proboscia*, *Pseudo-nitzschia*, *Skeletonema*, *Thalassionema*, *Thalassiosira*), ten dinoflagellates (*Alexandrium*, *Akashiwo*, *Gonyaulax*, *Gymnodinium*, *Noctiluca*, *Phalacroma*, *Protoceratium*, *Protoperidinium*, *Scrippsiella*, *Tripos*), and one dictyochophycean (*Dictyocha*). Coccolithophores were not identified by all three approaches together. The number of genera identified by both microscopy and V4 metabarcoding was six (4%) and two (1%) with PR2 and SILVA, respectively, while V4-PR2 and V4-SILVA shared 50 genera (33%). Considering genera observed uniquely by one approach, 32 (21%) were detected only by V4-PR2, 21 (14%) by V4-SILVA, and 16 (11%) by microscopy.

Regarding microscopy, V9-PR2 and V9-SILVA, their combination led to a total of 125 genera. Among these, the following 17 genera (representing 14% of the total) were common among all approaches (Fig. 3b): seven diatoms (*Chaetoceros*, *Cyclotella*, *Leptocylindrus*, *Pleurosigma*, *Pseudo-nitzschia*, *Skeletonema*, *Thalassiosira*), nine dinoflagellates (*Alexandrium*, *Gonyaulax*, *Gymnodinium*, *Karenia*, *Noctiluca*, *Prorocentrum*, *Protoceratium*, *Protoperidinium*, *Tripos*), and one dictyochophycean (*Dictyocha*). No coccolithophore was identified by the three approaches together. No genera were identified by both microscopy and V9-PR2, while microscopy and V9-SILVA shared four genera (3%). V9-PR2 and V9-SILVA shared 35 genera (28%). Considering the genera that were observed uniquely by each approach, 27 genera (22%) was detected only by microscopy, 23 (18%) by V9-PR2 and 19 (15%) by V9-SILVA.

Metabarcoding-based approaches (V4-PR2, V4-SILVA, V9-PR2, V9-SILVA) detected a high number of genera never revealed by microscopy analysis (Table S2), including naked dinoflagellates (e.g. *Grammatodinium*, *Gyrodiniellum*, *Karlodinium*,

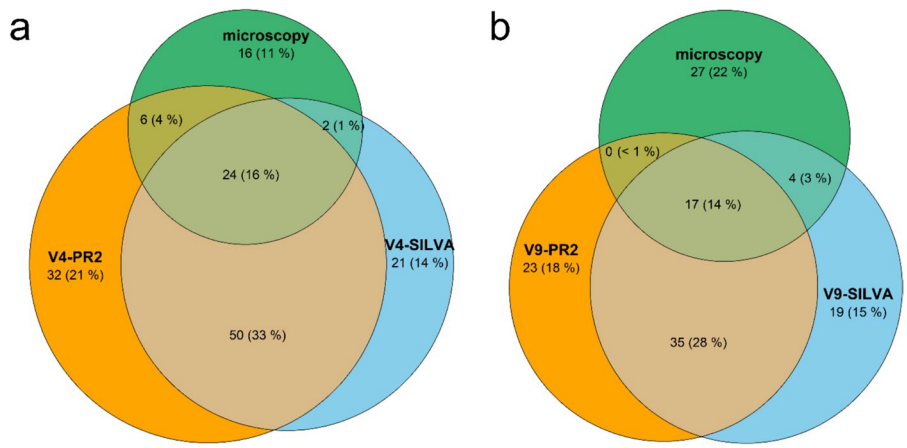

Fig. 3 Venn diagrams representing the number (and percentage) of shared and unique genera considering microscopy (green) and metabarcoding, using V4 (**a**) and V9 (**b**) 18S rRNA regions and PR2 (orange) and SILVA (blue) as reference databases for the taxonomic assignment

Lebouridinium, *Lepidodinium*, *Levanderina*, *Margalefidinium*, *Paragymnodinium*, *Proterythropsis*, *Polykrikos*, *Syltodinium*, *Torodinium*) and thecate dinoflagellates (e.g. *Archaeperidinium*, *Fragilidium*, *Heterodinium*, *Triadinium*). Moreover, metabarcoding provided the best resolution for the phytoflagellate group, in particular seven chrysophyceans (10% of total phytoflagellate diversity, mostly revealed by V4-SILVA), seven dictyochophyceans (10%, mostly revealed by V4-PR2 and V4-SILVA), seven mamiellophyceans (10%, mostly in V9-PR2), six chlorophyceans (9%, mostly in V4-PR2) and six cryptophyceans (9%, mostly found by V9-SILVA and V4-SILVA).

For those genera revealed microscopy but not by metabarcoding, primers for V4 and V9 regions of the 18S rRNA gene were checked and found to be suitable for them (i.e. no mismatches were observed or more rarely just single mismatches in the middle of the primers were detected).

Species identification

A total of 238 species (Table S2) were recorded combining microscopy and metabarcoding results. Microscopy identified 66 species, while metabarcoding identified 110 species with V4-PR2, 65 with V4-SILVA, 85 with V9-PR2 and 62 with V9-SILVA.

The combination of microscopy, V4-PR2 and V4-SILVA resulted in a total of 186 species, as depicted in the associated Venn diagram (Fig. 4a). The three approaches shared seven species (4%): three diatoms (*Cerataulina pelagica* (Cleve) Hendey, *Chaetoceros affinis* Lauder, *Pseudo-nitzschia pseudodelicatissima* (Hasle) Hasle) and four dinoflagellates (*Akashiwo sanguinea* (K.Hirasaka) Gert Hansen & Moestrup, *Noctiluca scintillans* (Macartney) Kofoid & Swezy, *Protoceratium reticulatum* (Claparède & Lachmann) Bütschli, *Protoperidinium bipes* (Paulsen) Balech). Neither coccolithophores nor phytoflagellates were identified by all three approaches together. The number of species identified by both microscopy and V4 metabarcoding was eight (4%) and three (2%) with PR2 and SILVA, respectively, while V4-PR2 and V4-SILVA shared 31 species (17%). Considering the species revealed uniquely by each approach, 48 (26%) were observed only using microscopy, 64 (35%) with V4-PR2 and 24 (13%) using V4-SILVA.

A total of 172 species were found combining microscopy, V9-PR2 and V9-SILVA. These three approaches shared four species (2%) (Fig. 4b), one

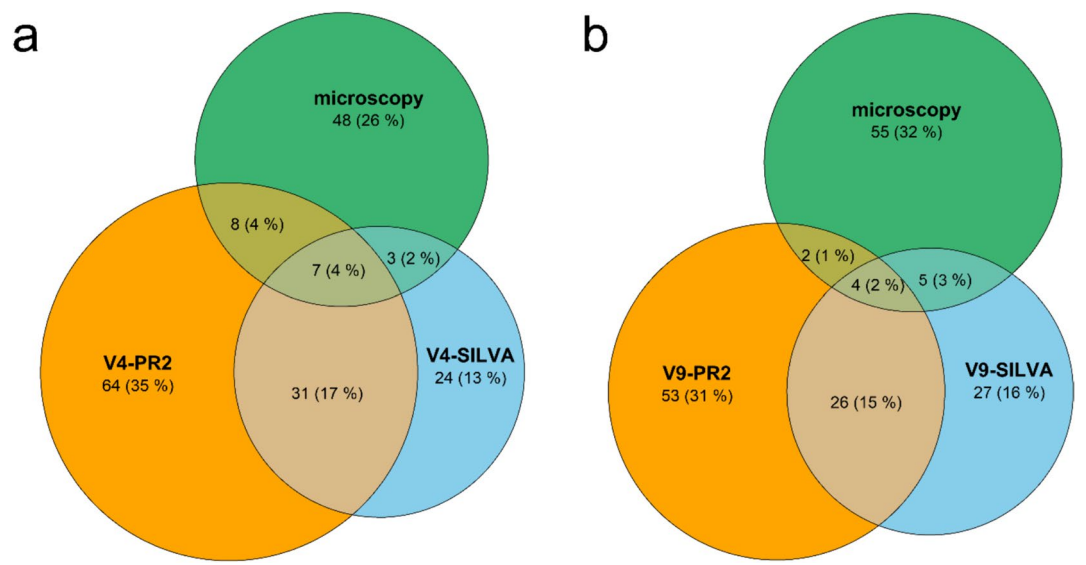

Fig. 4 Venn diagrams representing the number (and percentage) of shared and unique species considering microscopy (green) and metabarcoding, using V4 (**a**) and V9 (**b**) 18S rRNA regions and PR2 (orange) and SILVA (blue) as reference databases for the taxonomic assignment

diatom (*Chaetoceros affinis*) and three dinoflagellates (*Gonyaulax spinifera* (Claparède & Lachmann) Diesing, *Noctiluca scintillans* and *Protoceratium reticulatum*). Neither coccolithophores nor phytoflagellates were identified by all three approaches. The number of species shared by both microscopy and V9 metabarcoding was two (1%) and five (3%) with PR2 and SILVA, respectively, while V9-PR2 and V9-SILVA shared 26 (15%) species. Regarding the uniqueness of each approach, 55 species (32%) were revealed only by microscopy, 53 (31%) by V9-PR2 and 27 (16%) by V9-SILVA.

Metabarcoding based approaches (V4-PR2, V4-SILVA, V9-PR2, V9-SILVA) detected a high number of taxa not revealed by microscopy analysis (Table S2), including naked dinoflagellates (e.g. *Gymnodinium catenatum* H.W.Graham, *Gymnodinium dorsalisulcum* (Hulburt, J.J.A.McLaughlin & Zahl) Sh.Murray, Salas & Hallegraeff, *Gymnodinium impudicum* (S.Fraga & I.Bravo) Gert Hansen & Moestrup, *Gymnodinium smaydae* N.S.Kang, H.J.Jeong & Ø. Moestrup, *Gyrodiniellum shiwhaense* N.S.Kang, H.J.Jeong & Ø.Moestrup, *Gyrodinium dominans* Hulburt, *Gyrodinium fusiforme* Kofoid & Swezy, *Gyrodinium jinhaense* S.H.Jang & H.J.Jeong, *Karenia mikimotoi* (Miyake & Kominami ex Oda) Gert Hansen & Moestrup, *Paragymnodinium shiwhaense* N.S.Kang, H.J.Jeong, Moestrup & W.Shin, *Polykrikos geminatus* (F.Schütt) D.X.Qiu & Senjie Lin, *Polykrikos hartmannii* W.M.Zimmermann, *Polykrikos kofoidii* Chatton, *Polykrikos schwartzii* Bütschli), thecate dinoflagellates (e.g. *Alexandrium andersonii* Balech, *Alexandrium catenella* (Whedon & Kofoid) Balech, *Alexandrium hiranoi* T.Kita & Y.Fukuyo, *Alexandrium insuetum* Balech, *Alexandrium margalefii* Balech, *Gonyaulax polygramma* F.Stein, *Gonyaulax whaseongensis* A.S.Lim, H.J.Jeong & Ji Hye Kim) and phytoflagellates species, in particular 11 non-calcified coccolithophyceans (14%, particularly by V9-PR2), ten mamiellophyceans (13%, V4-PR2 and V9-PR2), nine pyramimonadophyceans (11%, particularly by V9-PR2), seven dictyochophyceans (9%, V9-PR2) and seven cryptophyceans (9%, mostly found by V4-SILVA).

For those species not revealed by metabarcoding but by microscopy, primers for V4 and V9 regions of the 18S rRNA gene were checked and found to be suitable for them (i.e. no mismatches were observed or more rarely just single mismatches in the middle of the primers were detected).

Phytoplankton communities

The results of the non-metric multidimensional scaling (NMDS), performed on the richness of genera and species in the different phytoplankton groups, are shown in Fig. 5a and b, respectively.

In both cases, a clear divergence between microscopy and metabarcoding can be observed, while no clear difference was observed among approaches based on metabarcoding (V4-SILVA, V4-PR2, V9-SILVA and V9-PR2).

Considering the presence–absence of genera and species (Fig. S2a, b), a clear difference between microscopy and metabarcoding (V4-SILVA, V4-PR2, V9-SILVA and V9-PR2) is evident at both genus and

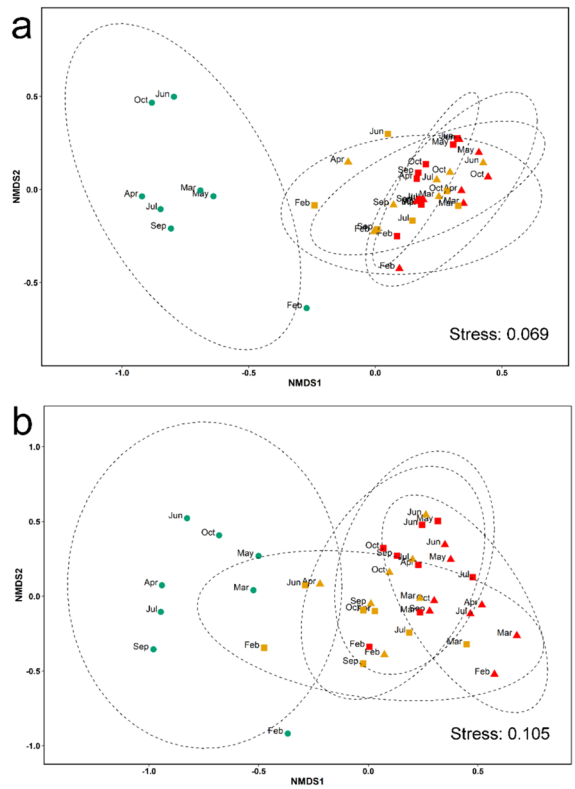

Fig. 5 NMDS performed on the richness of genera (**a**) and species (**b**) in the different phytoplankton groups of the five applied approaches: microscopy (green, ●), V4-SILVA (yellow, ■), V4-PR2 (yellow,▲), V9-SILVA (red,■) and V9-PR2 (red,▲)

species levels. At species level, a distinct difference is noticeable even within the metabarcoding approach between the two different markers, V4 (V4-SILVA and V4-PR2), and V9 (V9-SILVA and V9-PR2).

Comparing the five approaches, the PERMANOVA analysis highlighted significant differences among approaches, both considering the richness in the phytoplankton group and the presence–absence of genera ($p < 0.01$, $p < 0.001$, respectively) and species ($p < 0.001$).

Discussion

In this study, we combined microscopy and metabarcoding results for the first time in our study area, revealing important differences between the methods. The phytoplankton community of the eLTER SG01 station has been investigated through microscopy since 1988, highlighting a high biodiversity, with 479 species overall identified (Totti et al., 2002, 2005, 2019; Neri et al., 2023). However, metabarcoding, combining the two markers (V4 and V9 of 18S rRNA gene) and reference databases (PR2 and SILVA), revealed a previously unknown diversity, as 99 genera (eight diatoms, 32 dinoflagellates (mainly gymnodinioid forms), one coccolithophore, 58 phytoflagellates), and 151 species (23 diatoms, 50 dinoflagellates, one coccolithophore and 77 phytoflagellates) were recorded for the first time. The higher richness highlighted by the molecular method may be related to different reasons, as previously reported in other studies (Piredda et al., 2017; Santi et al., 2021; Bilbao et al., 2023; Mordret et al., 2023; Pierce et al., 2023). For some taxa, such as for the naked dinoflagellates, raphidophytes that lack a cell wall, and many phytoflagellates, formaldehyde fixation does alter the shape of the cell, preventing the identification (Throndsen, 1978; Menden-Deuer & Lessard, 2001; Fiocca et al., 2014). In such cases, the use of another fixative (e.g. Lugol) would likely provide better taxonomic resolution. Furthermore, some ultrastructural diagnostic characters can be distinguished only under the electron microscopy (scanning or transmission) analysis, which is not suitable in routine monitoring. This explains the new diversity found in the phytoflagellate group, as already observed in other areas (Almandoz et al., 2024), although with some differences among the metabarcoding approaches. It should not be forgotten that the greater diversity revealed by metabarcoding could be also affected by its ability to detect taxa that are not (or not easily) identifiable through microscopy, such as cryptic or pseudocryptic species (e.g. Esenkulova et al., 2020; De Luca et al., 2021) and picoeukaryotic taxa (e.g. species belonging to *Bathycoccus*, *Micromonas*, *Ostreococcus,* see Tables S1–S2) that, together with planktonic cyanobacteria, play a crucial role in the Northern Adriatic Sea by contributing significantly to primary production and supporting the marine food web (Giani et al., 2012).

In addition, it should be considered that the volume of subsamples processed in metabarcoding is much higher than in microscopy, which increases the probability of finding rare taxa (Piredda et al., 2017; Gran-Stadniczeñko et al., 2019; Bilbao et al., 2023). Indeed, in the microscopy analysis, the volume of the subsample settled for the observation (5 to 100 ml), and the portion of settling chamber analysed for counting (1–2 transect, 20–60 random fields) leads to analyse volumes actually much lower (0.05 to 2 ml) compared to metabarcoding that processes a larger volume of seawater (2 L in the present study). Furthermore, as also reported by Mordret et al. (2023), some of the taxa that were recorded for the first time by metabarcoding were parasites and symbionts and thus not targeted in the microscopy analysis (e.g. *Pelagodinium bei*, *Blastodinium* spp., *Zooxanthella* spp.). Nevertheless, it has to be considered that while microscopy could introduce errors related to the operator, the higher diversity inferred from metabarcoding could be inflated by errors arising from PCR and/or sequencing or the subsequent bioinformatic pipeline (e.g. false positives/negatives, artefactual variant diversity estimates) (Behnke et al., 2011; Santoferrara, 2019; Preston et al., 2022; Ershova et al., 2023; Marinchel et al., 2023).

Despite the higher richness obtained by the combination of different markers (V4 and V9) and databases (PR2, SILVA), several genera (three diatoms, three dinoflagellates, four coccolithophores and four phytoflagellates) and species (17 diatoms, 20 dinoflagellates, six coccolithophores and three phytoflagellates) were detected only using microscopy. Some of these taxa (e.g. most of the coccolithophores and all the *Oxytoxum* species) were found to be absent in the databases, underlying the need to implement the reference databases with new sequences, obtained

from monoclonal cultures (Piredda et al., 2017; Santi et al., 2021; Tzafesta et al., 2022; Mordret et al., 2023; Turk Dermastia et al., 2023). Considering the taxa already present in the databases, but not detected by either databases or markers, the reason could rely also on the fact that, in some cases, 18S could have limited resolution at the species level due to high genetic similarity in the 18S rRNA gene (Bittner et al., 2013; Guo et al., 2015; Edvardsen et al., 2016), and the combination with other markers (e.g. either *rbc*L or 23S or 28S) could enhance the captured diversity and improve resolution for closely related species. Moreover, metabarcoding is based on universal primers, which could preferentially amplify other taxa (Stoeck et al., 2010; Pawlowski et al., 2011; Kelly et al., 2019). However, in the present study, the unsuitability of the primers should not be a reason for undetectability of taxa, as no multiple mismatches nor mismatches in critical positions, which could prevent amplification (Stadhouders et al., 2010), were found.

The higher richness obtained from metabarcoding is not observed for each 18S region and reference database in the same way, particularly at the species level. Indeed, the use of V4 and V9 led to identify a higher number of species than microscopy when combined with PR2 as database, but this number lowered when SILVA was used. Overall, the combination of V4, as 18S region, and PR2, as reference database, led to assess a higher diversity (both in terms of genera and species), which was spread across the different phytoplankton groups. Only a small component of the total richness was found to be in common to all the different methods and databases (15 genera and 3 species), as previously reported by other studies (e.g. Santi et al., 2021; Akcaalan et al., 2023; Andersson et al., 2023), while an important percentage of genera and species was detected uniquely in V4-PR2, V4-SILVA, V9-PR2, V9-SILVA or microscopy, highlighting that the combination of different methods, markers and databases is necessary to have a more detailed information on the phytoplankton community composition.

The different resolution among approaches (i.e. in general metabarcoding harvest more taxa than microscopy, but some groups are strongly overlooked by the former) strongly affects the phytoplankton group per cent composition in terms of richness: using metabarcoding, the most diversified groups were phytoflagellates (considering genera) and phytoflagellates and dinoflagellates (considering species), while using microscopy, diatoms and dinoflagellates were more diversified. Moreover, metabarcoding failed to identify the majority of genera and species of coccolithophores, likely due to a combination of reasons (see above). The unsuitability of metabarcoding in discriminating coccolithophores could be encompassed (i) trying different primers, combining the V4 and V9 regions with other genes (e.g. the 28S gene, which appears to better distinguish closely related coccolithophore species (Gran-Stadniczeñko et al., 2017)), (ii) utilizing different curated databases, and (iii) increasing efforts to cultivate coccolithophore taxa, which traditionally show challenges in their cultivation (Probert & Houdan, 2004), and obtain more sequences across different taxa enhancing metabarcoding resolution for coccolithophores. Anyway, the absence of the majority of coccolithophore taxa highlights that relying solely on metabarcoding could overlook an important component of the phytoplankton community.

This suggests that the different methods, markers and databases do not always give the same information in terms of seasonal composition of the phytoplankton communities and thus that the two methods (microscopy and metabarcoding) should be combined to obtain a more detailed information on the community.

Conclusions

In conclusion, this study highlights the importance of combining microscopy and metabarcoding for a more comprehensive understanding of phytoplankton communities. Indeed, while microscopy analysis is still essential in the phytoplankton monitoring providing abundance and biomass values, metabarcoding represents a valid approach to implement the evaluation of phytoplankton diversity. In particular, metabarcoding certainly leads to recognize cryptic/pseudocryptic species and/or low-abundant (often potentially toxic) species.

The use of PR2 as reference database led to the highest number of identified phytoplankton genera and species, particularly with V4 as marker, suggesting that this combination is, to date, the most effective for the assessment of phytoplankton diversity. However, different regions and databases should still

be combined with microscopy to counteract the drawbacks of metabarcoding and to have a more detailed information on the community, as many taxa were observed exclusively by each marker and database pairing.

Undoubtedly, the resolution provided by metabarcoding is constantly increasing thanks to the rapidity with which the molecular approach is improving. However, the metabarcoding approach still exhibits significant gaps in the reference databases, especially for underrepresented groups such as coccolithophores. These gaps could be addressed by incorporating sequences of missing species, ideally obtained from the same type locality as in the original species description.

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Data availability Data are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not applicable.

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