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Metabarcoding vs Microscopy: Comparison of Methods To Monitor Phytoplankton Communities

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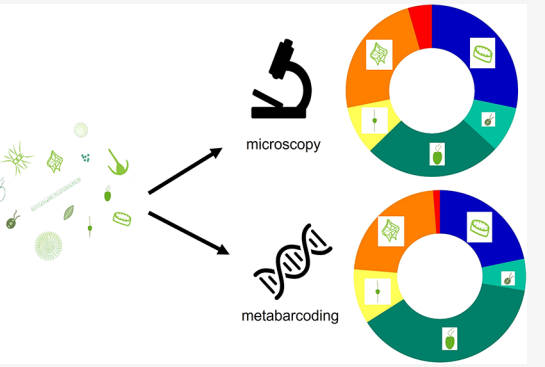
ABSTRACT: Phytoplankton are used worldwide to monitor the environmental status of aquatic systems. Long-time series of microscopy-analyzed phytoplankton are available from many monitoring stations. The microscopy method is, however, time-consuming and has shortcomings. DNA metabarcoding has been suggested as an alternative method, but the consistency between different methods needs further investigation. We performed a comparative study of microscopy and metabarcoding analyzing micro- and nanophytoplankton. For metabarcoding, 25−1000 mL of seawater was filtered, DNA extracted, and the 18S and 16S rRNA gene amplicons sequenced. For microscopy, based on the Utermöhl method, we evaluated the use of three metrics: abundance, biovolume, and carbon biomass. At the genus, species, and unidentified taxa levels, metabarcoding generally showed higher taxonomic diversity than microscopy, and diversity was already captured at the lowest filtration volume tested, 25 mL. Metabarcoding and microscopy displayed relatively similar distribution patterns at the group level. The results showed that the relative abundances of the 18S rRNA amplicon at the group level best fitted the microscopy carbon biomass metric. The results are promising for implementing DNA metabarcoding as a complement to microscopy in phytoplankton monitoring, especially if databases were improved and group-level indices could be applied to classify the environmental state of water bodies.



KEYWORDS: *sampling volume, diversity, carbon biomass, gene abundance, group/class-level consistency*

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INTRODUCTION

Phytoplankton are of fundamental importance in aquatic ecosystems as they constitute the base of the food web.[1,2] They respond quickly to environmental change, for example, anthropogenic nutrient load and climate change.[3] They show wide size variations, spanning from pico- to microplankton, ∼0.7 to >100 μm.[4] There are approximately 4000 known phytoplankton species in marine systems;[5] of these, around 2000 are reported to be found in the Baltic Sea.[6] The phytoplankton species composition and the community structure are useful for classifying the ecological status of an aquatic system. In healthy aquatic ecosystems, phytoplankton are edible and efficiently grazed by zooplankton, which in turn are consumed by planktivorous fish. Due to the key function and fast responses of the phytoplankton communities, they are suitable as indicators for monitoring environmental change and marine management. Phytoplankton are therefore used worldwide to assess ecological status in aquatic systems, as in the European Union's two directives: the Water Framework Directive and the Marine Strategy Framework Directive,[7] as well as many other marine management plans. Quantitative parameters such as biomass, taxonomic composition, or frequency and intensity of algal blooms can be included in the assessment.

In the monitoring programs, the composition of the phytoplankton community and abundances of different taxa are traditionally analyzed using inverted microscopy.[8] Then, the biovolume and carbon biomass concentration can be estimated using conversion factors.[8−10] These are all valuable metrics, which can be used to analyze time trends and to classify the ecological status of the aquatic environment. However, the analysis of the samples is time-consuming and requires expert taxonomic skills. Furthermore, small phytoplankton (nano- and picoplankton) are difficult or impossible to detect with the Utermöhl method, which excludes important primary producers from the analysis.[11] Another drawback is that not all phytoplankton can be identified at the species level from preserved samples due to a lack of clear morphological characters. Therefore, molecular methods (e.g., metabarcoding) are receiving increased attention as an alternative approach to assess biodiversity due to the combination of

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high sensitivity and potential for the highest taxonomic resolution in a cost- and time-effective methodology.[12]

In monitoring, it is of crucial importance to produce long time series and keep the used methods unchanged. Therefore, there is a need to intercalibrate new and current method to avoid artificial disruptions in the time series data. However, there is a challenge to intercalibrate microscopy and DNA metabarcoding, since microscopy is a quantitative method, while metabarcoding is more a qualitative or semiquantitative method.[13] DNA metabarcoding has been proposed to complement and even replace phytoplankton microscopy.[14,15] However, there are still many open questions on how comparable the two methods are. For example, it is not known how a large volume of water needs to be filtered to capture the best diversity and community composition with metabarcoding, and how the abundance and the diversity of taxa differ between the two methods. Furthermore, it is not known at which taxonomic level the best match can be found between the methods. To our knowledge, only a few previous studies have been performed comparing microscopy with metabarcoding, and often these studies do not comprise the same size range of phytoplankton.[16,17]

The overall aim of this study was to compare the analysis of a coastal phytoplankton community, using a metabarcoding method and an established microscopy technique. We performed a nondiscriminating assessment, by focusing on nano- and microphytoplankton, which are covered by both the Utermöhl and metabarcoding methods. We wanted to elucidate how comparable is the identification of the phytoplankton taxa, species, genera, and diversity from the two methods. Furthermore, we attempted to evaluate the optimal filtration volume for DNA metabarcoding and to find out what microscopy metric (abundance, biovolume, or carbon biomass) best matches metabarcoding.

MATERIALS AND METHODS

Field Sampling. To approach the addressed questions, we performed a field study in the Öre estuary, northern Baltic Sea (Figure S1), where the productive season lasts approximately from April to October.[4]

We sampled seawater at five different stations in the estuary on October 20, 2016 (Figure S1, Table S1). The samples were collected at 1.5 m depth using a Ruttner sampler. The phytoplankton communities were analyzed in parallel using microscopy and DNA sequencing. For microscopic analysis, the method followed the Helcom guidelines that are used in the whole Baltic area for monitoring of phytoplankton.[18] 100 mL of seawater was preserved with 0.3% acidic Lugol's solution (final concentration) and stored in dark at 4 °C until analysis. For DNA analysis, 25, 500, and 1000 mL of seawater were filtered onto 0.2 μm sterile filters (47 mm Supor filter, PALL Life Science). The volumes were chosen to match, respectively, the volume sampled by microscopy, an intermediate volume and a volume commonly used to assess biodiversity with DNA metabarcoding. The filtration was performed within 1 h after returning to the laboratory. The filters were stored in TE buffer at −80 °C until DNA extraction was performed.

Morphological Identification of Phytoplankton. Micro- and nanophytoplankton were analyzed microscopically using the Utermöhl method according to the Helcom Combine manual and the PEG biovolume file (2015).[8,10,18] Picophytoplankton were not included in this study. Lugol fixed samples (25 mL) were settled in sedimentation chambers for at least 24 h and counted in an inverted microscope (Nikon Eclipse Ti, Japan) using phase contrast. Microphytoplankton (>20 μm) were counted in half chamber at 100× magnification and nanophytoplankton (2−20 μm) in one diagonal at 400× magnification. The analyzed seawater volume was thus 12.5 and 0.3 mL for the microphytoplankton and nanoplankton, respectively. Autotrophic and mixotrophic phytoplankton were identified to the highest possible taxonomic level. For all taxa, three different parameters were estimated: cell abundance, biovolume concentration, and carbon biomass. Biovolume and carbon biomass were calculated using the size classes according to the PEG biovolume file of 2015. The biovolume file is annually updated, and the latest version can be found on the HELCOM webpage with a changelog therein (https://helcom.fi/helcom-at-work/projects/peg/). The carbon biomass calculations followed Menden-Deuer and Lessard (2000).[9]

Molecular Identification of Phytoplankton. DNA analysis was performed using amplicon sequencing of 16S rDNA and 18S rDNA genes, to target both prokaryotic and eukaryotic phytoplankton. The DNA collected on the filters was extracted using a Power Water isolation kit (MO BIO Laboratories, Inc.), following the kit instructions.

16S rRNA V_3-V_4 region was amplified using the primers 341F (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTACGGGNGGCWGCAG-3′) and 805R (5′-AGACGTGTGCTCTTCCGATCTGACTACHVGGGTATCTAATCC-3′).[19] 18S rRNA V_4-V_5 region was amplified using the primers 574*F (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGGTAATTCCAGCTCYV-3′) and 1132*R (5′-AGACGTGTGCTCTTCCGATCTCCGTCAATTHCTTYAART-3′).[20] A two-step PCR was performed with 38 cycles (25 + 13 cycles) using KAPA Hifi HotStart ReadyMix (2X) (Kapa Biosystems, code 25-KK2602). The reaction mixture of the first PCR step for each primer pair consisted of 20 μL, with 12.5 μL of Kapa HiFi HotStart ReadyMix, 1.25 μL of each primer (10 μM) and 8 ng of DNA of each sample. Cycling conditions were 98 °C for 2′, 25 cycles of 98 °C for 20″, annealing temperature of 51 °C (18S) or 54 °C (16S) for 20″ and 72 °C for 15″, followed by a final elongation step of 72 °C for 2′. Illumina sequencing barcodes were added in the second PCR step, with a reaction mixture of 25 μL with 14 μL of Kapa HiFi HotStart ReadyMix, 1 μL of each primer (10 μM) (5′-AATGATACGGCGACCACCGAGATCTACAC-X_8-ACACTCTTTCCCTACACGACG-3′ and 5′-CAAGCAGAAGACGGCATACGAGAT-X_8-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′, where in each case X_8 represents an 8-bp DNA barcode) and 4 ng of template DNA. Cycling conditions were 98 °C for 2′, 13 cycles of 98 °C for 20″, the annealing temperature of 62 °C for 30″ and 72 °C for 30″, followed by a final elongation step of 72 °C for 2′. Each sample was amplified in duplicate, and the duplicates of each sample were then pooled. After each PCR step, the products were cleaned using magnetic beads (Beckman Coulter, Agentcourt AMpure XP A63880). The amplicon concentrations were measured with a Qubit 2.0 Fluorometer (Invitrogen, Qubit dsDNA HS Assay kit). The tagged samples were mixed in an equimolar solution and paired-end sequenced with Illumina MiSeq (Illumina Inc., USA) at SciLifelab (Stockholm, Sweden).

The Illumina Miseq sequencing resulted in 16274766 reads for 16S rRNA and 15829588 reads for 18S rRNA. Primers were screened and trimmed by FASTX-Toolkit. The trimmed

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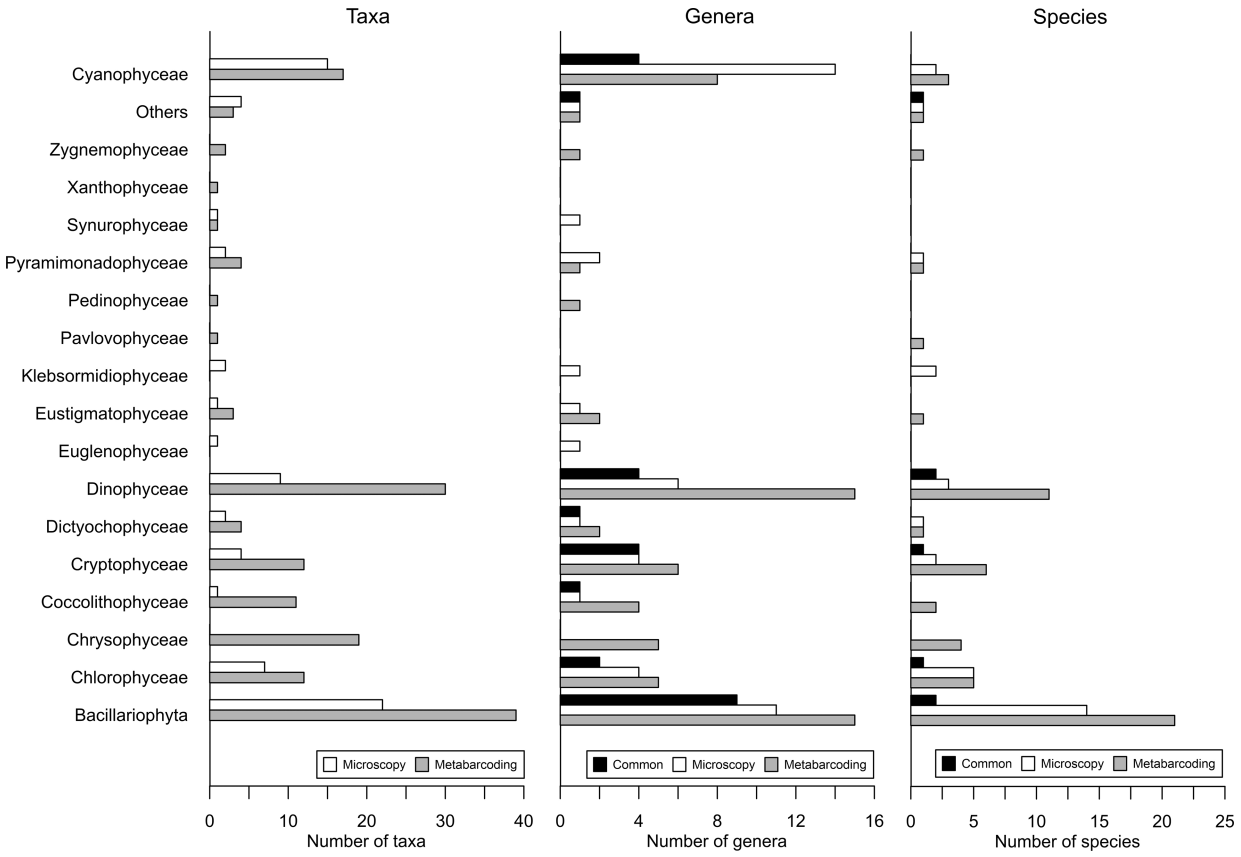

Figure 1. Number of nano- and microphytoplankton taxa, genera, and species identified by metabarcoding (16S and 18S) and microscopy, and number of genera and species common to both methods.

sequencing data were analyzed by DADA2 pipeline version 1.22.0.[21] Sequencing reads were filtered by default parameters (maxN = 0, maxEE = c(2,5), truncQ = 2). 16S sequencing reads were truncated to 260 bp for forward reads and 240 bp for reverse reads, paired-end sequences were merged with the parameters minOverlap 12, and chimeras were removed. There are 4849 amplicon sequence variants (ASVs) generated and annotated by the SILVA 138.1 version database.[22] In order to recover more fragments, 18S sequencing data were merged with the parameters—minOverlap 6. There were 2728 ASVs generated and annotated by the PR2 4.14.0 version database.[23] Rarefaction analysis was applied, subsampled at random to normalize the number of reads per sample to equal the minimum sample size before any further analysis.

All DNA sequences have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number PRJNA882391.

Dataset Preprocessing. The datasets resulting from 16S and 18S were treated separately. The complete 18S barcoding dataset contained 2581 unique ASVs and was preprocessed in order to constrain the dataset to organisms comparable to the microscopic analysis. All ASVs were not only nano- and microphytoplankton but also metazoans, fungi, and other protists not autotrophic or belonging to picophytoplankton; therefore, ASVs belonging to certain groups (at phylum, class, or order level) were excluded (Table S2). The ASVs annotated to the species *Mesodinium rubrum* were included in the analysis, as this species is a major contributor to primary production which is counted together with phytoplankton in the monitoring of the Baltic Sea. In the remaining nano-microphytoplankton datasets (Table S3) of 454 ASVs, ASVs annotated to the same taxon name were merged in a single taxon (i.e., the abundance of those ASVs was summed up). Taxon was defined as the highest taxonomic resolution achieved. ASVs with the same taxonomic annotation, e.g., full species name, genus sp. name, or class name, were summed up. For example, all unidentified diatoms ASVs were merged into one single taxon "Bacillariophyta".

In the 16S barcoding dataset, ASVs annotated to the phylum Cyanobacteria, class cyanobacteriia, and orders Cyanobacteriales, Phormidesmiales, Pseudanabaenales, and Synechococcales (Table S4) were selected to compare the detection and annotation of nano- and micro-sized taxa with microscopy (57 ASVs in total). As for the 18S dataset, 16S ASVs annotated to the same taxon were merged into a single taxon.

The taxonomic classification between the metabarcoding and microcopy datasets was harmonized following the classification of AlgaeBase.[24]

Comparison of Methods. The diversity, assessed by the number of taxa, was compared between methods using ANOVA and Tukey's HSD post hoc test. The analysis was performed in PAST 4.06.[25]

To be able to contrast the results obtained with the microscopy and metabarcoding methods, we selected six main comparable phytoplankton groups from the resultant datasets: Bacillariophyta (sensu lato), Chlorophyceae, Cryptophyceae, Dictyochophyceae, Dinophyceae, and Pyramimonadophyceae.

The proportion of the six different phytoplankton groups analyzed by microscopy and metabarcoding of the DNA extracts from 25, 500, and 1000 mL of seawater was compared

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using one-way ANOSIM, with a matrix based on Bray−Curtis similarity index as input. The SIMPER analysis was later used to break apart the contribution to the dissimilarity between the phytoplankton groups. These analyses were performed with the software Primer-e 7.0.13.

The diatom/dinoflagellate index was compared between methods using the Kruskal−Wallis test, followed by the Mann−Whitney pairwise comparison. These tests were performed in PAST 4.06.[25]

RESULTS

Identification of Taxa, Genera, and Species. Considering the total taxa list (including all filtered volumes), the number of taxa annotated to nano- and micro-phytoplankton groups was higher in metabarcoding (454 unique ASVs and 126 taxa) than in microscopy (55 taxa), but the outcome varied between different groups (Figure 1). For example, microscopy identified 15, 3, 9, and 22 taxa of Cyanophyceae, Cryptophyceae, Dinophyceae, and Bacillariophyta (sensu lato) while metabarcoding recognized 18, 11, 27, and 36 taxa, respectively. Generally, groups that are not so prominent in the study area, as, for example, Synurophyceae, showed a similar number of identified taxa using the two methods.

The number of genera partly showed a different pattern (Figure 1). For Cyanophyceae, more genera were identified in microscopy, and some commonly occurring genera in the area were missing in the 16S metabarcoding dataset, for example, *Woronichinia* and *Romeria* (Figure 1, Table S5). For some groups, more genera were identified with metabarcoding, for example, Dinophyceae and Bacillariophyta. The number of genera identified by both microscopy and metabarcoding was in general lower than for each method itself. For example, within Dinophyceae 6 genera were identified by microscopy and 15 by metabarcoding, but only 4 Dinophyceae genera were characterized by both methods (Figure 1).

The number of identified species was in general higher for metabarcoding, for example, for Bacillariophyta, Cryptophyceae, and Dinophyceae (Figure 1). Similar to the genera, the number of species in common was always lower than the number of species found with the two methods separately.

In the Baltic Sea, the ciliate *M. rubrum* is a major contributor to primary production and is considered a mixotroph in the national monitoring programs.[26] In this study, *M. rubrum* was identified by both microscopy and metabarcoding with the 18S primer pair used.

Diversity. DNA metabarcoding showed higher alpha diversity than microscopy, as observed by analyzing the number of taxa of eukaryotic nano- and microphytoplankton (Figure 2). The number of taxa did not differ between seawater filtration volumes ranging from 25 to 1000 mL. The ANOVA showed no difference between filtered volumes ($F = 86.73$, $p > 0.1$). In contrast, the microscopy alpha diversity was much lower, constituting only about 50% of the metabarcoding number of taxa.

Distribution of Different Phytoplankton Groups. The distribution of six commonly occurring eukaryotic phytoplankton groups was analyzed at the five different sampling stations: Bacillariophyta, Chlorophyceae, Cryptophyceae, Dichtyochophyceae, Dinophyceae, and Pyramimonadophyceae (Figures 3 and S2). The different volumes used for metabarcoding and the different microscopy metrics were compared. Independent of what method was used, the phytoplankton community showed rather small variations between the sampling stations (Figure S2).

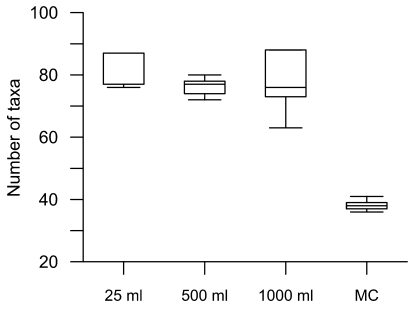

Figure 2. Number of comparable taxa for DNA metabarcoding (18S) 25, 500, and 1000 mL and microscopy (MC, excluding cyanobacteria).

With metabarcoding, the relative contribution was the highest for Cryptophyceae, followed by Dinophyceae, Bacillariophyta, Dichtyochophyceae, Chlorophyceae, and Pyramimonadophyceae (Figures 3 and S2). For microscopy, the relative distribution looked rather different depending on what metric was considered. Carbon biomass showed the highest similarity to metabarcoding, for example, for Bacillariophyta and Dinophyceae (Figure 3). However, for smaller-sized groups like Cryptophyceae and Pyramimonadophyceae, microscopy abundance compared relatively well with the relative number of reads.

The relative distribution was similar in all three filtered volumes, 25, 500, and 1000 mL (Figures 3 and S2). The ANOSIM showed no difference between different volumes in metabarcoding, large differences between microscopy abundance, biovolume and metabarcoding, while no difference or smaller differences between microscopy carbon biomass and metabarcoding (Table 1). The SIMPER analysis was then used to follow up on which groups were contributing to the differences. Here, Cryptophyceae, Dinophyceae, and Chlorophyceae showed up as phytoplankton groups contributing to 20−40% of the dissimilarity between carbon biomass and metabarcoding (Table 2).

DISCUSSION

Limited Consistency in Annotation by Metabarcoding and Microscopy. Metabarcoding identified about 1.5 times more genera and 3 times more species than microscopy. Moreover, the agreement of genera and species identified in common was limited, especially at the species level. Among genera, 36−55% were identified by both methods, while for species 9−27%, common identifications were found. These results are in agreement with previous reports on freshwater phytoplankton, where only a few common taxa were found by microscopy and metabarcoding operational taxonomic units (OTUs).[16,27,28]

Some species annotated by the metabarcoding in this study are not included in the HELCOM PEG list of species or the list of species in the European standard for Water quality - Guidance on the estimation of phytoplankton biovolume EN 16695. This mismatch can be due to intraspecific dissimilarities, due to evolutionary processes, such as genetic drift, gene duplication, or horizontal gene transfer, and those phenomena could in principle cause a misannotation by metabarcoding.[29,30] For example, the cryptophyte *Urgorri complanatus*[31] is a brackish red-tide species relatively newly

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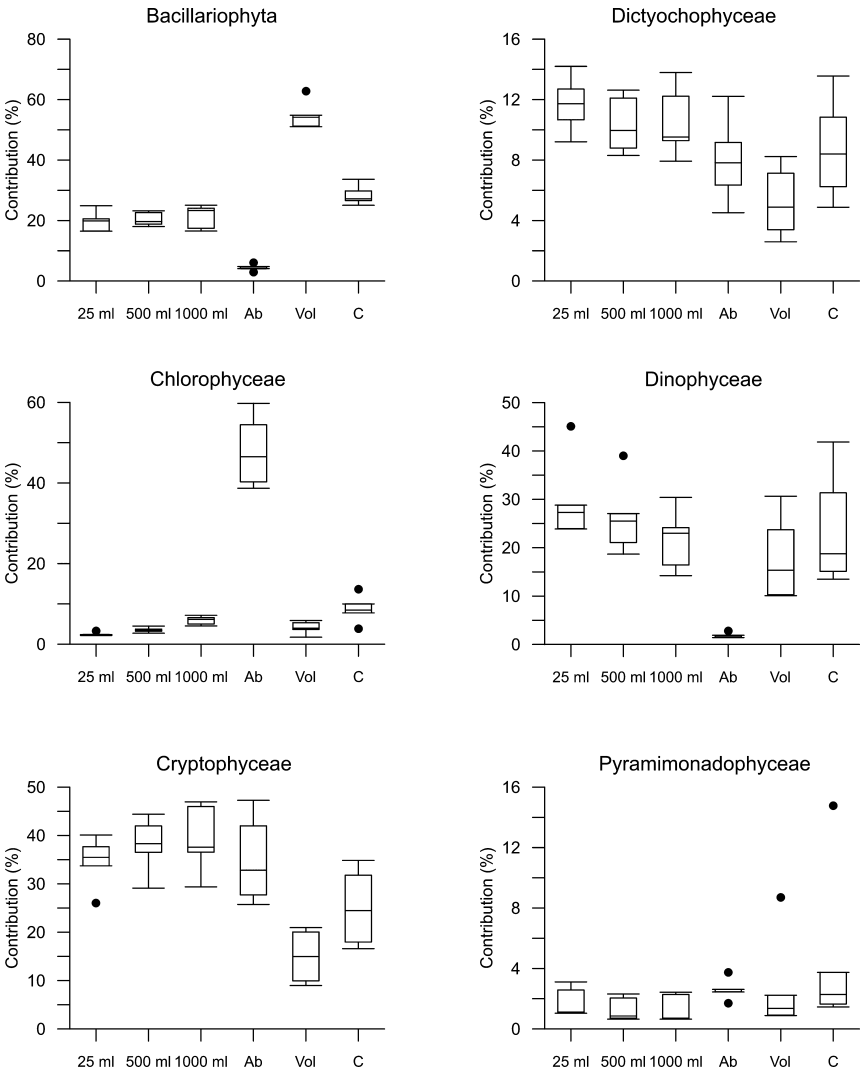

Figure 3. Contribution of each of the six phytoplankton groups to the number of reads (25, 500, and 1000 mL), abundance, biovolume, and carbon biomass of the sum of the six groups (Bacillariophyta, Chlorophyceae, Cryptophyceae, Dictyochophyceae, Dinophyceae, and Pyramimonadophyceae).

Table 1. Analysis of Similarities (ANOSIM) of the Distribution of Different Phytoplankton Groups (Bacillariophyta, Chlorophyceae, Cryptophyceae, Dictyochophyceae, Dinophyceae, and Pyramimonadophyceae) in Different Samples Analyzed by Metabarcoding (25, 500, and 1000 mL of Filtered Seawater) and Microscopy (Three Metrics: Carbon Biomass (C-Biomass), Abundance, and Biovolume)[a]

R	25 mL	500 mL	1000 mL	C-biomass	abundance	biovolume
25 mL		−0.08	0.11	0.32*	1**	1**
500 mL	−0.08		−0.07	0.36*	1**	1**
1000 mL	0.11	−0.07		0.20	1*	1*
C-biomass	0.32*	0.36*	0.20		1**	0.83**
abundance	1**	1**	1**	1**		1**
biovolume	1**	1**	1**	0.83**	1**	

[a]*R* values close to zero indicate short distance between samples. ANOSIM global $R = 0.63$, $p = 0.0001$. Significant differences indicated by *($p < 0.05$) or **($p < 0.01$).

described from Spain, but this species was identified in this study. The species *Chaetoceros pumilum* was also only annotated by the metabarcoding method. This is a species name not accepted by AlgaeBase, but it could be a misspelling of the species *Chaetoceros calcitrans* forma *pumilum*. These examples show the need for harmonization, quality assurance, and curation of metabarcoding databases to comply with the systematics and nomenclature of other well-established taxonomic databases used, e.g., AlgaeBase.

Few cyanobacteria taxa could be annotated at the genus level with metabarcoding, and this highlights not only the need to develop databases with more sequences of identified taxa but also the importance of the choice of primers. In this study, we used a 16S primer pair commonly used to study

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Table 2. SIMPER Analysis Showing the Contribution of Each Phytoplankton Group (%) to the Dissimilarity between Methods (Metabarcoding: 25, 500, and 1000 mL of Filtered Seawater; and Microscopy Three Metrics: Carbon Biomass (C-Biomass), Abundance, and Biovolume)[a]

	25 mL						500 mL						1000 mL					
	bacil	chloro	crypto	dictyo	dino	pyra	bacil	chloro	crypto	dictyo	dino	pyra	bacil	chloro	crypto	dictyo	Dino	pyra
25 mL							14		29	10	38		15	12	27	9	34	
500 mL	14		29	10	38													
1000 mL	15	12	27	9	34		15	10	29	9	34							
abundance	15	45	8		27		16	45	9		25		18	45	10		21	
biovolume	45		25	8	17		44		30	7	15		44		32	7	11	
C-biomass	19	14	23	8	27		18	12	30		25	8	17	9	34		24	9

[a]Only groups contributing to the top 90% of cumulative dissimilarity are shown.

Table 3. Most Common Size Class, Size Class Range, Average Biovolume per Cell, and Average Carbon per Cell of Six Major Phytoplankton Groups

group	most common size class (width × length or diameter, μm)	size class range (width × length or diameter, μm)	mean biovolume (μm^3 $cell^{-1}$)	mean carbon content (pgC $cell^{-1}$)
Bacillariophyta	6−7 × 9−11	3−60 × 3−120	3026	143
Chlorophyceae	1.5−2 × 20−30	2−6 × 5−50	21	4
Cryptophyceae	20−26 × 10−13	3−13 × 4−30	97	15
Dictyochophtyceae	8 × 8	4−10	159	25
Dinophyceae	38−42	5−52 to <10−52	2216	270
Pyramimonadophyceae	5−7 × 5	3−12 × 5−16	247	37

phytoplankton, which targets a wide prokaryotic community.[13,19,32] However, for monitoring applications, where there is a need to identify cyanobacteria with a higher resolution, primers specific to cyanobacteria should be used and developed. The evaluation of primers is often focusing on the recovery of the highest number of taxa and the best coverage over different phylogenetic groups.[20,33] Unfortunately, this approach might in some cases not be the most appropriate for monitoring applications where key species, or harmful species, need to be identified. For example, the ciliate *M. rubrum* is a key species in the Baltic Sea and belongs to a phylogenetic group predominantly considered heterotrophic. The 18S primer pair used in this study could detect this species, while not all commonly used primer pair do detect it in Baltic waters.[34] With the current development of databases, the primers choice might also require to be environment-specific depending on the key species present in the water body to be monitored.

Metabarcoding Indicates Higher Diversity. The biodiversity of eukaryotic nano- and microphytoplankton showed higher values for metabarcoding than for microscopy (Figure 2). This is in agreement with some earlier studies,[16,27] but a recent meta-analysis of marine and freshwater environments showed rather similar alpha diversity of plankton and microphytobenthos for microscopy compared to DNA metabarcoding.[28] Furthermore, a previous study reported that traditional microscopy identified more taxa than metabarcoding for certain phyla, for example, Cryptophyta and Dinophyta.[13]

In this study, we found two-fold more taxa with metabarcoding than with microscopy (Figure 2, left panel), which can be compared to earlier studies observing 9- to 22-fold more taxa in metabarcoding.[14,27] The higher similarity between the methods in our study may partly be due to the focus on the same size range, i.e., nano and micro-sizes, but also by combining ASVs annotated to the same taxa. ASVs are often considered to host inter-specific and intra-specific diversity since a difference in only one nucleotide will lead to a unique ASV.[35] Combining ASVs annotated to the same taxa level should have lowered the importance of intra-specific variation on diversity in a similar way as microscopy. Nevertheless, microscopy likely underestimates the number of taxa due to missing morphological characters of the phytoplankton, while metabarcoding may overestimate the number of taxa, as OTUs or ASVs are based on a sequence similarity cutoff (generally 97−99%) or single-nucleotide variants.

In this study, the higher number of taxa for metabarcoding might be interpreted as an identification of more rare taxa, which is in agreement with previous findings.[16] However, when comparing the different filtered volumes of seawater, the same number of taxa was identified. This indicated that metabarcoding captured diversity better than microscopy already at a small filtration volume (25 mL).

In marine monitoring, phytoplankton biodiversity indicators have been developed based on microscopic data.[36−38] Since metabarcoding can give 2-fold, or more, the number of taxa compared to microscopy, metabarcoding data cannot directly replace microscopy data in the existing biodiversity indicators, for example, Shannon 95.[39] Instead, new biodiversity metabarcoding baseline data need to be acquired for years in order to have a functional indicator of environmental changes. New biodiversity indicators specific to metabarcoding data should be developed; nevertheless, this would also require years to implement in marine monitoring programs.

Carbon Biomass Shows the Best Fit to Metabarcoding at the Group Level. The relative contribution of the six different eukaryotic phytoplankton groups showed relatively similar patterns between metabarcoding and carbon biomass (Figure 3). Biovolume also showed a relatively similar distribution pattern as metabarcoding, but the differences were larger. Larger phytoplankton cells are expected to contain more gene copies than smaller cells.[35,40−42] Accordingly, the 18S rRNA barcode abundance of relatively large phytoplank-

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ton cells would show better agreement to biovolume or carbon biomass than to microscopy abundance, while for small cells, abundance would show a good relationship.

For the six major eukaryotic phytoplankton groups, the cell size ranged from the smallest cells of 3 × 4 μm to the largest being in the range of 50−70 μm. The largest cells were found within the group of dinoflagellates and diatoms. Cell volume (biovolume) ranged from ca. 20−3030 μm^3 per cell, while the carbon biomass range was only 4−270 pgC $cell^{-1}$ (Table 3). To overcome such a range of size variation, metabarcoding data have been suggested to be corrected by the number of gene copy per cell.[43] However, there is no database with the number of gene copy per cell, and little information is available, i.e., few species in few environments, resulting in very coarse conversion factors per taxonomic groups, and no significant improvement in the comparison between metabarcoding and microscopy.[44]

We found relative carbon biomass to better match the gene relative abundance than biovolume. This can be explained by the carbon conversion used in this study,[9] which takes into account the specificity of diatoms. Diatom (Bacillariophyta) cells enclose vacuoles that do not contain biomass nor DNA. With this conversion, a better relationship between gene copy number and carbon biomass would result in a better match between metabarcoding and microscopy phytoplankton group composition. To our knowledge, this is the first study showing that carbon biomass is the best microscopy metric to compare with metabarcoding in a natural community analysis. The dissimilarity between metabarcoding and carbon biomass was mostly due to Cryptophyceae. This group is commonly overrepresented in metabarcoding datasets from natural waters,[35,45] which could be due to amplification bias from the metabarcoding method but also to the cryptic nature of many Cryptophyceae taxa. For example, *Teleaulax*-like taxa are often connected to the presence of *M. rubrum*, which acquires the cryptophyte organelles from live-preying and can also retain their nucleus.[46,47] In this study, it is likely that under the presence of *M. rubrum*, part of the Cryptophyceae cells would not contribute to carbon biomass, while they would contribute to Cryptophyceae gene copy numbers.

The relationship between cell size and gene copy numbers is not leading to a consistent match between biovolume and metabarcoding. Size is not the only trait affecting the relationship between metabarcoding and microscopy metrics, as discussed for the taxonomy (e.g., diatoms) or cryptic life cycles (e.g., Cryptophyceae taxa), and other traits might play a role. However, in this study, the metabarcoding method used gave comparable phytoplankton group distribution as carbon biomass.

Phytoplankton Group Ratios Indicating Environmental Change. Currently, in monitoring programs, indicators of environmental status are mostly quantitative indices, and only a few semiquantitative indices are developed (e.g., diatom/dinoflagellate or Shannon 95). Using phytoplankton metabarcoding in monitoring would require: (1) evaluating new semiquantitative indicators for monitoring, (2) improving the metabarcoding method to combine prokaryotes and eukaryotes in a single dataset, and (3) developing the metabarcoding method to get quantitative data.

It is promising that we observed comparable results with metabarcoding and carbon biomass, but the usability of the distribution pattern of phytoplankton groups may be discussed. In low-salinity areas, like in the northern Baltic Sea and lakes, nutrient load, eutrophication, and climate change often lead to select cyanobacteria.[48−53] In the Baltic Sea, the cyanobacteria maximum occurs in summer, indicating a positive response to temperature.[54] Therefore, the proportion of cyanobacteria in the total phytoplankton community could be an indicator of eutrophication and climate impact. Unfortunately, it is not possible to co-analyze pro- and eukaryotic phytoplankton using 16S and 18S metabarcoding without standardization. It could be advantageous to use universal primers that target both pro- and eukaryotes. One way would be to use primers for prokaryotes and then analyze chloroplast-specific genes in eukaryotes, which would enable simultaneous detection of prokaryotic and eukaryotic phytoplankton taxa.[13] However, the number of chloroplasts per cell does not relate to size, the semiquantitative outputs were, so far, not convincing, and the taxonomic annotation was weaker compared to 18S rRNA gene barcoding for eukaryotes.[13] The possibility of using 16S primers to identify eukaryotic phytoplankton would depend on the quality and updates of databases.

Another way to make the simultaneous analysis of 16S and 18S metabarcoding data possible would be to get quantitative data by using internal standards. For example, studies have evaluated the possibility to use known DNA sequences as internal standards through the metagenomic analysis for prokaryotes and eukaryotes;[55−57] however, the results were not always conclusive, and the standardization process would need further technical developments.[58]

Ratios between different phytoplankton groups may be useful indicators of environmental change. For example, the ratio between diatoms and dinoflagellates in spring is used as an index of climate change in the Baltic Sea.[59] Even though our sampling was not performed during spring, we tested if this index was calculated from microscopy carbon biomass and metabarcoding reads in the same way. The diatom/dinoflagellate ratio showed the highest value in microscopy followed by metabarcoding 1000, 500, and 25 mL (Figure 4).

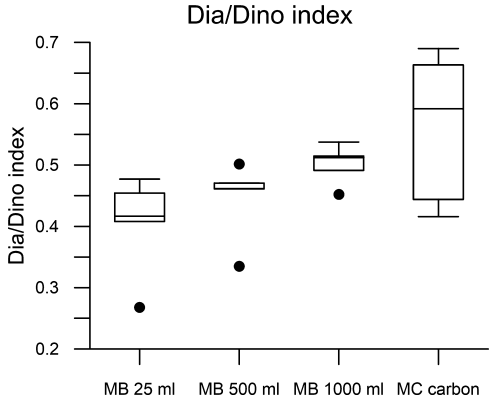

Figure 4. Diatom/dinoflagellate index for metabarcoding data (MB 25 mL, MB 500 mL, and MB 1000 mL) and microscopy carbon biomass.

The average ratio for metabarcoding was lower than that obtained from microscopy carbon biomass; however, there were no statistically significant differences (Kruskal−Wallis $H = 6.42$, $p = 0.0921$). Taken together, analyzing phytoplankton group ratios could potentially be a useful tool to classify environmental change, using microscopy, metabarcoding, or other molecular methods. However, more studies are needed to develop such indices.

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CONCLUSIONS

We show that analysis of relatively small water volumes, 25−500 mL, is sufficient to capture phytoplankton biodiversity by metabarcoding in the study area during autumn. Metabarcoding and microscopy showed some overlap regarding the identification of genera and species. At present, the consistency between metabarcoding and microscopy identification is mostly constrained by the choice of primers and the development and curation of databases. As well, the use of standard could provide a comparable metabarcoding dataset and open the possibility to simultaneously analyze both prokaryotic and eukaryotic phytoplankton in a quantitative way. As one of the first studies of a whole seawater community, we here show that the microscopy metric carbon biomass shows a better fit to the 18S rRNA gene metabarcoding compared to cell abundance when assessing semiquantitative estimates of phytoplankton groups. Our study indicates that the distribution of different eukaryotic phytoplankton, which can be analyzed either by metabarcoding or microscopy, may be useful as an indicator of environmental change. Furthermore, the fitting of carbon biomass with the results from metabarcoding can open a possibility to calibrate preliminary microscopy measurements to the new metabarcoding analysis, at least for some phytoplankton groups, for example, the ones included in our analysis. Nevertheless, more research is needed to confirm this likeness and harmonize the different methods.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.3c00176.

Sampling station coordinates; 18S groups excluded from the comparison; 18S groups included in the comparison; 16S groups included in the comparison; cyanobacterial taxa; sampling location map; and phytoplankton group composition (PDF)

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Author Contributions

A.A. led and coordinated the project. S.B. performed the field sampling. D.F. made the DNA extraction and produced the raw DNA sequences. L.Z. performed the bioinformatics work. S.H. performed the microscopic analysis. S.H. and S.B. carried out statistical analyses. A.A. wrote the paper together with all co-authors. CRediT: **Agneta Andersson** conceptualization (lead), data curation (equal), formal analysis (equal), funding acquisition (lead), investigation (equal), methodology (equal), project administration (lead), resources (lead), software (supporting), supervision (lead), validation (equal), visualization (equal), writing-original draft (lead), writing-review & editing (lead); **Li Zhao** conceptualization (supporting), data curation (lead), formal analysis (lead), investigation (equal), methodology (equal), resources (supporting), software (lead), validation (equal), visualization (equal), writing-original draft (equal), writing-review & editing (equal); **Sonia Brugel** conceptualization (equal), data curation (equal), formal analysis (lead), investigation (equal), methodology (equal), software (equal), supervision (supporting), validation (lead), visualization (lead), writing-original draft (equal), writing-review & editing (equal); **Daniela Figueroa** conceptualization (equal), data curation (equal), formal analysis (supporting), investigation (equal), methodology (equal), software (equal), validation (equal), visualization (equal), writing-original draft (equal), writing-review & editing (equal); **Siv Huseby** conceptualization (equal), data curation (equal), formal analysis (equal), funding acquisition (equal), investigation (equal), methodology (equal), project administration (equal), resources (equal), software (equal), validation (equal), visualization (equal), writing-original draft (equal), writing-review & editing (equal).

Notes

The authors declare no competing financial interest.

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