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RESOURCE ARTICLE

Evaluation of sequential filtration and centrifugation to capture environmental DNA and survey microbial eukaryotic communities in aquatic environments

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Abstract

Sequential membrane filtration of water samples is commonly used to monitor the diversity of aquatic microbial eukaryotes. This capture method is efficient to focus on specific taxonomic groups within a size fraction, but it is time-consuming. Centrifugation, often used to collect microorganisms from pure culture, could be seen as an alternative to capture microbial eukaryotic communities from environmental samples. Here, we compared the two capture methods to assess diversity and ecological patterns of eukaryotic communities in the Thau lagoon, France. Water samples were taken twice a month over a full year and sequential filtration targeting the picoplankton (0.2–3 μm) and larger organisms (>3 μm) was used in parallel to centrifugation. The microbial eukaryotic community in the samples was described using an environmental DNA approach targeting the V4 region of the 18S rRNA gene. The most abundant divisions in the filtration fractions and the centrifugation pellet were Dinoflagellata, Metazoa, Ochrophyta, Cryptophyta. Chlorophyta were dominant in the centrifugation pellet and the picoplankton fraction but not in the larger fraction. Diversity indices and structuring patterns of the community in the two size fractions and the centrifugation pellet were comparable. Twenty amplicon sequence variants were significantly differentially abundant between the two size fractions and the centrifugation pellet, and their temporal patterns of abundance in the two fractions combined were similar to those obtained with centrifugation. Overall, centrifugation led to similar ecological conclusions as the two filtrated fractions combined, thus making it an attractive time-efficient alternative to sequential filtration.

KEYWORDS
biomonitoring, eDNA capture, metabarcoding, microbial community ecology, phytoplankton, protists

Angélique Gobet and Ariane Atteia equally contributed to the study.

1 | INTRODUCTION

Our understanding of microbial communities and their functions in aquatic ecosystems relies on the ability to detect and monitor species distributions and abundances. Microbial communities differ according to the origin of the ecosystem (e.g. open ocean waters, coastal waters, freshwaters), the trophic level of the ecosystem, the season and contextual conditions. Biomonitoring of aquatic microorganisms has long relied on light microscopy observations and cultivation methods, being thus limited to the most abundant and the better-known living organisms. The development of environmental DNA (eDNA) metabarcoding has greatly improved aquatic biomonitoring, as it made possible the tracking of abundant and rare species across the whole tree of life (Burki et al., 2021; Drummond, 2015; Taberlet et al., 2012; Zinger et al., 2012). Metabarcoding involves a series of steps, including eDNA capture, eDNA extraction, gene amplification and taxonomic identification, but there is still no consensus in the processing of these steps (Lobanov et al., 2022). eDNA capture is a crucial step as it may affect eDNA recovery and abundance. For aquatic environments, water filtration is the most commonly used capture method to target microbial eukaryotes as it allows processing large volumes (Grossart, 2010). Among the major size fractions classifying microbial eukaryotes, there is the picoplankton (cell sizes between 0.2 and 2 μm), the nanoplankton (2–20 μm) and the microplankton (20–200 μm) (Sieburth et al., 1978). Sequential filtration allows, in theory, to target separately these different planktonic size fractions. For example, picoplankton may be targeted with filters of 3 and 0.2 μm or of 5 and 0.8 μm in pore size, nanoplankton with filters of 20 μm and 3 or 5 μm and microplankton with filters of 180 and 20 μm (de Vargas et al., 2015; Sieburth et al., 1978). Metabarcoding studies have, however, shown that in sequential filtrations designed to target picoplankton, sequences from larger protists (notably dinoflagellates and ciliates) and metazoans were also found (Lovejoy et al., 2006; Massana et al., 2004; Vaulot et al., 2008). The deformation of flexible-walled protist and cell breakage are believed to account for the DNA to pass through the 3 μm filters (Massana et al., 2004; Terrado et al., 2015), while a large part of metazoan sequences in the smaller fractions likely come from metazoan gametes (López-Escardó et al., 2018). Sequential filtrations have several clear advantages as, for example, to enrich the diversity within targeted size fractions and to capture poorly abundant species (de Vargas et al., 2015; López-Escardó et al., 2018; Massana et al., 2015). Still, this method has inherent issues including the choice of filtration material and parameters (e.g. pump & filter holder) and filter membrane type (e.g. porosity, chemical composition) which will have an impact on the captured DNA quality (Goldberg et al., 2016; Majaneva et al., 2018), time required to process the samples (Taberlet et al., 2018) and the cost of multiplying samples to analyse.

An alternative to sequential filtration for the capture of aquatic microorganisms is gentle centrifugation. Low-speed centrifugation, a common laboratory practice typically used for harvesting cultivated bacteria and microbial eukaryotes, can also be used for compacting cells from environmental samples. It is a technically simple eDNA capture method which does not discriminate for size and offers the possibility to process simultaneously multiple samples. Centrifugation has been used to collect eDNA from macroinvertebrate species in water samples but was found less efficient than filtration to capture diversity (Deiner et al., 2015; Spens et al., 2017). A recent study using *Escherichia coli* cells harbouring synthetic target DNA as a case study has shown that centrifugation outperformed filtrations for DNA capture (Bockrath et al., 2022). To our knowledge, the efficiency of the two eDNA capture methods, namely sequential filtration and centrifugation, to survey aquatic microbial communities within environmental samples has not been evaluated.

The Thau lagoon, one of the largest Mediterranean coastal lagoons in France, is economically important due to its shellfish farming production representing around 8–10,000 $t.year^{-1}$ since 2010 (Derolez et al., 2020). This activity largely depends on the quality of phytoplanktonic communities, with diatoms being the main food for oysters (Pernet et al., 2012). During the year 2018, an episode of exceptionally warm temperatures was accompanied by the development of anoxic zones in the lagoon (Lagarde et al., 2021). This extreme climatic event severely impacted the ecosystem, with an important mortality of mussels and oysters as well as of many fish species and benthic invertebrates (Richard et al., 2022). Through the autumn, a massive algal bloom of the chlorophyte *Picochlorum* developed in the waters and lasted over almost all winter (Lagarde et al., 2021). As part of a larger study to assess the recovery of the lagoon ecosystem, monitoring of microbial eukaryotic communities using an eDNA metabarcoding approach was carried out with a bimonthly sampling from February 2019 to January 2020 at the site of Bouzigues, a shellfish farm area. To assess the eukaryote diversity, two eDNA capture methods were tested: On the one hand, sequential filtration to target the picoplankton (0.2–3 μm) and the nanoplankton and larger organisms (>3 μm), and on the other hand, centrifugation which does not discriminate for size. Using metabarcoding, we evaluated the efficiency of the two capture methods (i) to identify potential differences in detecting taxonomic groups in the eukaryotic community and (ii) to evaluate their respective performance in describing diversity patterns.

2 | MATERIALS AND METHODS

2.1 | Field collection

Surface water samples (1 m depth) were collected at the shellfish farming site of Bouzigues in the Thau Lagoon, France (GPS WGS84 coordinates: Long 3.66463°E, Lat 43.43429°N), every 2 weeks from February 2019 to January 2020. The water samples were stored in DNA-free 2 L bottles and transported in a cooling box to the laboratory. The same day, the samples were processed for DNA capture

by centrifugation and sequential filtration. For each water sample, three replicates of 150 mL were centrifuged for 30 min at 3100 *g* at 4°C. The supernatants were removed carefully with a pipette without drying out the pellets. The pellets were resuspended in the remaining supernatants (<1 mL), transferred to 2 mL Eppendorf tubes and centrifuged for 10 min at 6000 *g* at 4°C. After carefully removing the supernatants, the pellets were flash-frozen. For sequential filtration, three replicates of 150 mL water samples were each filtered on a 3-μm pore size filter (Whatman Nucleopore Track-Etch membrane filter) using a peristaltic pump. The resulting filtrates were pooled and homogenized. To prevent clogging, only 15 mL of the 3 μm filtrate was used and filtered using a porosity of 0.2 μm (Polycarbonate Track Etch PCTE filter), and this was done three times. Filters and pellets were stored at −80°C until DNA extraction.

2.2 | Molecular analysis

DNA was extracted using a phenol/chloroform method as in Lacroux et al. (2022). In brief, biological matter from pellets and filters was resuspended in a TEN buffer (10 mM Tris pH 8.0, 10 mM EDTA, 150 mM NaCl) and further lysed at 37°C for 30 min in SDS-EB buffer (100 mM Tris pH 8.0, 400 mM NaCl, 40 mM EDTA, 2% SDS) containing RNase A at a final concentration of 40 $\mu g\,\mu L^{-1}$. Extraction of nucleic acids was performed using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1 (v:v:v)) and then a mixture of chloroform:isoamyl alcohol (24:1 (v:v)). Nucleic acids were precipitated using isopropanol, washed in cold 70% ethanol, air-dried and resuspended in Tris-EDTA buffer, pH 8.0. For each DNA triplicate, aliquots of 20 μL were pooled and cleaned with OneStep PCR Inhibitor Removal (Ozyme). DNA was quantified using the dsDNA BR Assay Kit on a Qubit fluorometer (ThermoFisher Scientific).

Libraries and MiSeq sequencing were performed by the LGC Biosearch Technologies platform (Berlin, Germany). Libraries included negative controls (filtered MilliQ water for DNA extraction and molecular grade water for the PCR) and were prepared using one amplification step, with barcodes directly attached to the primers. The PCRs included about 1–10 ng of DNA extract (total volume 1 μL), 15 pmol of each forward primer and reverse primer in 20 μL volume of 1 x MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 μL of BioStabII PCR Enhancer (Sigma). The V4 variable region of the 18S rRNA gene was amplified using the following eukaryotic-specific universal primers: TAReuk454FWD1 (5′-CCAGCASCYGCGGTAATTCC-3′) and TAReukREV3mo (5′-ACTTTCGTTCTTGATYRATGA-3′) (Piredda et al., 2017; Stoeck et al., 2010). For each sample, the forward and reverse primers had the same 10-nt barcode sequence. PCRs were carried out for 30 cycles using the following parameters: 1 min 96°C pre-denaturation; 96°C for 15 s, 50°C for 30 s, 70°C for 90 s. DNA concentration of amplicons of interest was determined by gel electrophoresis. About 20 ng amplicon DNA of each sample was pooled for up to 48 samples carrying different barcodes. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other small mispriming products, followed by an additional purification on MiniElute columns (Qiagen). About 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Illumina libraries were pooled and size selected by preparative gel electrophoresis. MiSeq sequencing was done using a V3 kit, allowing for paired-end sequencing, 300 cycles (Illumina, San Diego, CA, USA).

2.3 | Sequence data analysis

Raw Illumina sequences were preprocessed through the LGC Biosearch Technologies pipeline: Libraries were demultiplexed using the Illumina bcl2fastq 2.17.1.14 software (https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html); Illumina adapters, barcode and primer sequences were removed; and sequences without barcodes/primers or conflicting barcode/primer pairs were discarded. Sequences were then processed in R v4.2.2. (R Core Team, 2021) using the dada2 package (Callahan et al., 2016), where default parameters were used for all functions but for *filterAndTrim* where maxEE = c(2,5) was chosen. This sequence processing allowed removing low-quality sequences, merging overlapping paired-end sequences together, clustering sequences into amplicon sequence variants (ASV), removing PCR chimeras (Table S1) and taxonomically assigning ASV using the PR2 SSU database version 4.14.0 (https://pr2-database.org/ (Guillou et al., 2013)).

Amplicon sequence variants represented by only one sequence in the sum of at least two samples were removed to avoid ASV with small mean and large coefficient of variation (McMurdie & Holmes, 2013). Alpha diversity indices (i.e. observed richness, Shannon index, Simpson index) were calculated from this raw data set. Significant mean differences between alpha diversity indices calculated for each eDNA capture method were searched for by calculating an ANOVA followed by a post hoc Tukey test (Brunet et al., 2021). The data set was further transformed by normalizing the number of sequences in each sample using median sequencing depth (Gérikas Ribeiro et al., 2018), then used for analyses on taxonomic composition and the next analyses as well. Beta diversity patterns were visualized by calculating dissimilarities using the Bray–Curtis dissimilarity index before non-metric multidimensional scaling (nMDS) (Bray & Curtis, 1957). A permutational analysis of variance (PERMANOVA, 999 permutations) was then applied to compare the centroid and dispersion of the samples for each eDNA capture method (Anderson, 2001). Analyses of differential abundance of ASV were done after log-ratio transformation of the data set. A generalized linear model test was then applied with a Benjamini–Hochberg corrected p value > .05 to select for significantly differential ASV between the methods (Benjamini & Hochberg, 1995).

Multivariate and statistical analyses were performed in R v4.2.2. with the packages phyloseq_1.42.0, vegan_2.6-4, ggplot2_3.4.1 and ALDEx2_1.30.0 (Fernandes et al., 2013; McMurdie & Holmes, 2013; Oksanen et al., 2013; R Core Team, 2021; Wickham, 2016).

3 | RESULTS

To evaluate the efficiency of the two eDNA capture methods, we surveyed the eukaryotic community in Thau lagoon's waters over 1 year with bimonthly samplings using sequential filtration, targeting the >3 μm and the 0.2–3 μm size fractions, and low-speed centrifugation, targeting the whole community. The composition of the eukaryotic community was surveyed by targeting the V4 region of the 18S rRNA gene.

3.1 | Distribution of eukaryotic divisions with the two capture methods

Sequential filtration and centrifugation allowed to recover 28 divisions, belonging to seven supergroups: Alveolata, Archaeplastida, Hacrobia, Opisthokonta, Rhizaria, Stramenopiles and, finally, Apusozoa which accounted for very few sequences in the data set (Figure 1, Table S2). These supergroups were differently distributed in the two size fractions. Opisthokonta, Alveolata and Stramenopiles dominated the >3 μm size fraction with about one-third of the total sequences each. Among each of these supergroups, few divisions clearly dominated with Metazoa within Opisthokonta, Ochrophyta in Stramenopiles and Dinoflagellata and Ciliophora in Alveolata representing 30%, 25%, 18% and 11% of the total number of sequences in this size fraction respectively. The other sequences identified in the >3 μm fraction, although in minority, belonged to the divisions Chlorophyta, Cercozoa and Cryptophyta, each represented by <6% of the total sequences in the size fraction. For the 0.2–3 μm size fraction, Archaeplastida and Alveolata were the most abundant with more than half of the sequences, corresponding to 30% and 27% respectively. Opisthokonta and Stramenopiles were also abundant, each representing about 14% of the sequences (Figure 1). In the smaller size fraction (0.2–3 μm), Archaeplastida were represented only by Chlorophyta. Sequences identified as Dinoflagellata, Metazoa, Ochrophyta and Cryptophyta in the 0.2–3 μm size fraction represented 20%, 14%, 11% and 9% of the total number of sequences respectively. The overall eukaryotic diversity in the centrifugation pellet was composed primarily of Alveolata, Archaeplastida, Stramenopiles and Hacrobia, with 34%, 22%, 18% and 13% of the total number of sequences respectively (Figure 1). Dinoflagellata and Chlorophyta dominated the pellet with 28% and 22% of the sequences, respectively, followed by Ochrophyta, Cryptophyta and Metazoa with, respectively, 16%, 10% and 9% of the total number of sequences. The taxonomic identification of the sequences recovered in the pellet was comparable to that obtained with both filtration fractions for the most abundant divisions.

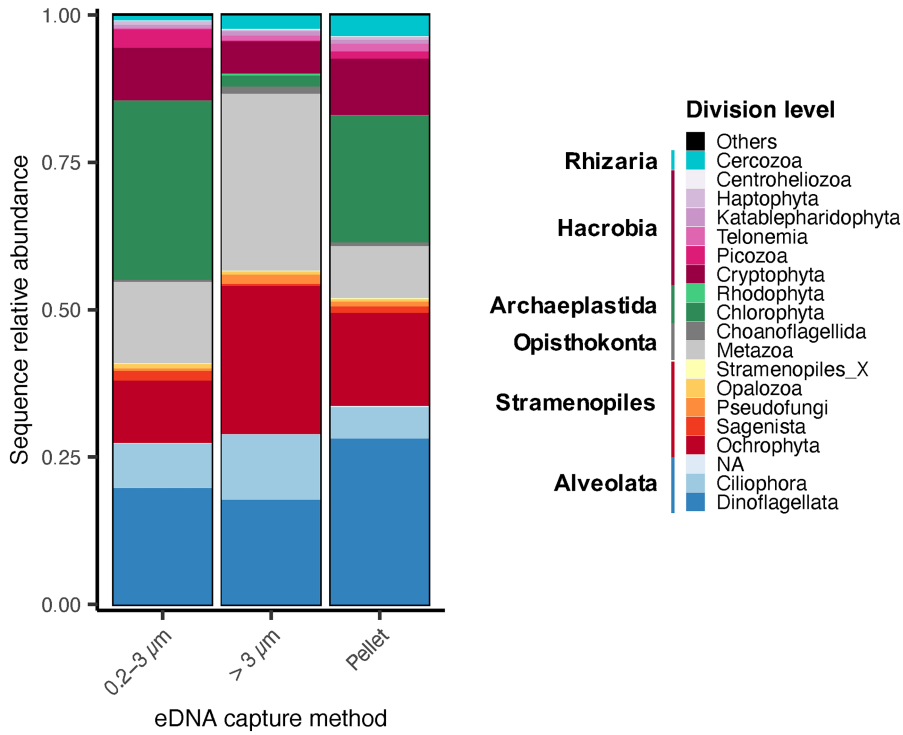

FIGURE 1 Taxonomic composition at the division level of the eukaryotic community using the two methods. The 19 most abundant divisions represent 99.9% of the sequence abundance in the whole data set and the remaining nine divisions representing 0.1% are depicted as 'Others' (Table S2). Divisions were represented by their sequence relative abundance in a given size fraction or in the pellet. Divisions were ordered by sequence abundance within each supergroup level. 0.2–3 μm and >3 μm, size fractions obtained with sequential filtration; Pellet, centrifugation.

3.2 | Most abundant eukaryotic genera identified with each capture method

The taxonomic composition of the five most abundant divisions was analysed at the genus level (Figure 2, Figure S1, Table S3). Dinoflagellata identified in the Thau lagoon waters consisted in 82 distinct genera, belonging to two distinct classes, Dinophyceae and Syndiniales. Dinoflagellata diversity captured by sequential filtration clearly differed between filters: in the >3 μm size fraction Dinophyceae sequences accounted for 85%, and Syndiniales for 15% sequences (Figure 2a). Inversely, most of the sequences in the 0.2–3 μm fraction stemmed from Syndiniales with up to 82% sequences and only 18% sequences were identified as Dinophyceae. The most abundant Dinophyceae genera in the >3 μm fraction included, namely, Dinophyceae_NA (18% sequences), *Heterocapsa* (17%) and *Gymnodinium* (9%), were not as dominant in the 0.2–3 μm fraction, with sequence abundances of about 3% each. Inversely, the most abundant Syndiniales genera in the 0.2–3 μm fraction: Dino-Group-II-Clade-10-and-11_X (12% sequences), Dino-Group-II-Clade-1_X (11%) and Dino-Group-II-Clade-12_X (11%) were also less abundant in the larger fraction with 0.6%, 1% and 1% of the sequences in this fraction. In the centrifugation pellet, about two-thirds of all Dinoflagellata sequences were identified as Dinophyceae and one-third as Syndiniales. The distribution of the most abundant genera in the centrifugation pellet resembled a combination of the two fractions, with the most abundant genera belonging to Dinophyceae found in the >3 μm size fraction: Dinophyceae_NA, *Gymnodinium*, *Heterocapsa* representing 9%–14% sequences, and the most abundant genera belonging to Syndiniales found in the 0.2–3 μm size fraction: Dino-Group-II-Clade-10-and-11_X, Dino-Group-II-Clade-1_X and Dino-Group-II-Clade-12_X, representing 3%–6% sequences.

Although the main focus of our study was on eukaryotic microbes, we also examined the metazoan sequence distribution as their presence in the samples could have an effect on the resulting microbial eukaryotic diversity recovered (López-Escardó et al., 2018). Metazoa were identified by the two capture methods and were amid the most abundant divisions in the whole data set. Sequences identified as Metazoa represented about 14% of the sequences in 0.2–3 μm size fraction, 30% in the larger size fraction and 9% in the centrifugation pellet (Figure 1). A total of 74 metazoan genera were identified throughout the whole year (Figure S1). Metazoan sequences in the picoplankton fraction were mostly represented by the copepod *Oithona* (60% of the sequences) and the tube-forming serpulid worm *Hydroides* (7% of the sequences). The predominant genera in the centrifugation pellet were *Oithona*, *Hydroides* and the mollusca *Abra*, represented by 33%, 11% and 12% of the sequences respectively.

Chlorophyta sequences identified in the Thau lagoon belonged to 33 genera. The predominant genera fell primarily within two classes, Mamiellophyceae and Trebouxiophyceae, representing 82% and 14% of the sequences respectively (Figure 2b). The dominant genera were *Ostreococcus* (51% of the sequences), *Micromonas* (22% sequences), *Picochlorum* (12% sequences) and *Bathycoccus* (11% sequences), all belonging to the picophytoplankton. These chlorophytes were mostly found in the 0.2–3 μm size fraction. Most of the sequences found in the >3 μm size fraction included *Picochlorum* (52% of the sequences), and two Pyramimonadophyceae: *Pyramimonas* (18% sequences) and *Cymbomonas* (4% sequences). As observed in the 0.2–3 μm fraction, the most abundant genera in the centrifugation pellet were *Ostreococcus* (51% of the sequences), *Micromonas* (19% sequences), *Picochlorum* (13% sequences) and *Bathycoccus* (9% sequences).

Ochrophyta in the Thau lagoon were mostly represented by two classes, Bacillariophyta and Chrysophyceae, with 86% and 10% of the sequences in the whole data set (Figure 2c). On the >3 μm filter, the colonial diatom *Chaetoceros* was predominant, followed by *Rhizosolenia*, *Bacteriastrum* and *Nitzschia*, representing 50%, 10%, 10% and 7% of the total number of sequences in this fraction respectively. In the picoplankton fraction, Ochrophyta were dominated by *Chaetoceros* (22% of the sequences) and *Nitzschia* (17% of the sequences). The Chrysophyceae_Clade-H_X represented 14% of the sequences in the 0.2–3 μm fraction, but only 2% of the sequences in the 3 μm fraction. The centrifugation pellet was dominated by Bacillariophyta. As in the >3 μm fraction, the same four genera dominated in the centrifugation pellet; *Chaetoceros*, *Rhizosolenia*, *Nitzschia* and *Bacteriastrum*, representing altogether 72% of the Ochrophyta sequences.

The Cryptophyta *Teleaulax*, *Plagioselmis* and Cryptomonadales_X_NA were the most abundant genera in the >3 μm fraction with 49%, 34% and 13% sequences respectively. The picoplankton fraction was also dominated by *Teleaulax* (36%), *Plagioselmis* (23%) but also by *Falcomonas* (24%) and *Hemiselmis* (12%). All five genera were found in the centrifugation pellet and their sequence abundance resembled that found in the 0.2–3 μm size fraction (Figure S1b).

Overall, the taxonomic analysis indicated that the most abundant genera present in each size fraction were also found with centrifugation for each of the five most abundant divisions. This raises the question whether the two capture methods lead to the same alpha diversity, and whether they share the same ecological patterns or the same temporal fluctuations.

3.3 | Diversity and ecological patterns of the microbial eukaryote community with the two eDNA capture methods

To evaluate the potential of each method to identify microbial eukaryotic diversity in each sample, observed richness, the Shannon and the Simpson indexes were calculated at the ASV level. Observed richness indicated a similar number of ASV for the two size fractions, with the lower size fraction values significantly lower than with centrifugation (ANOVA, $F_{2,69}=4.35$, $p<.05$; $\text{Tukey}_{0.2\text{-centrifugation}}$, $p_{adj}<.05$, Figure S2). Alpha diversity indices such as the Shannon and Simpson index, however, led to similar

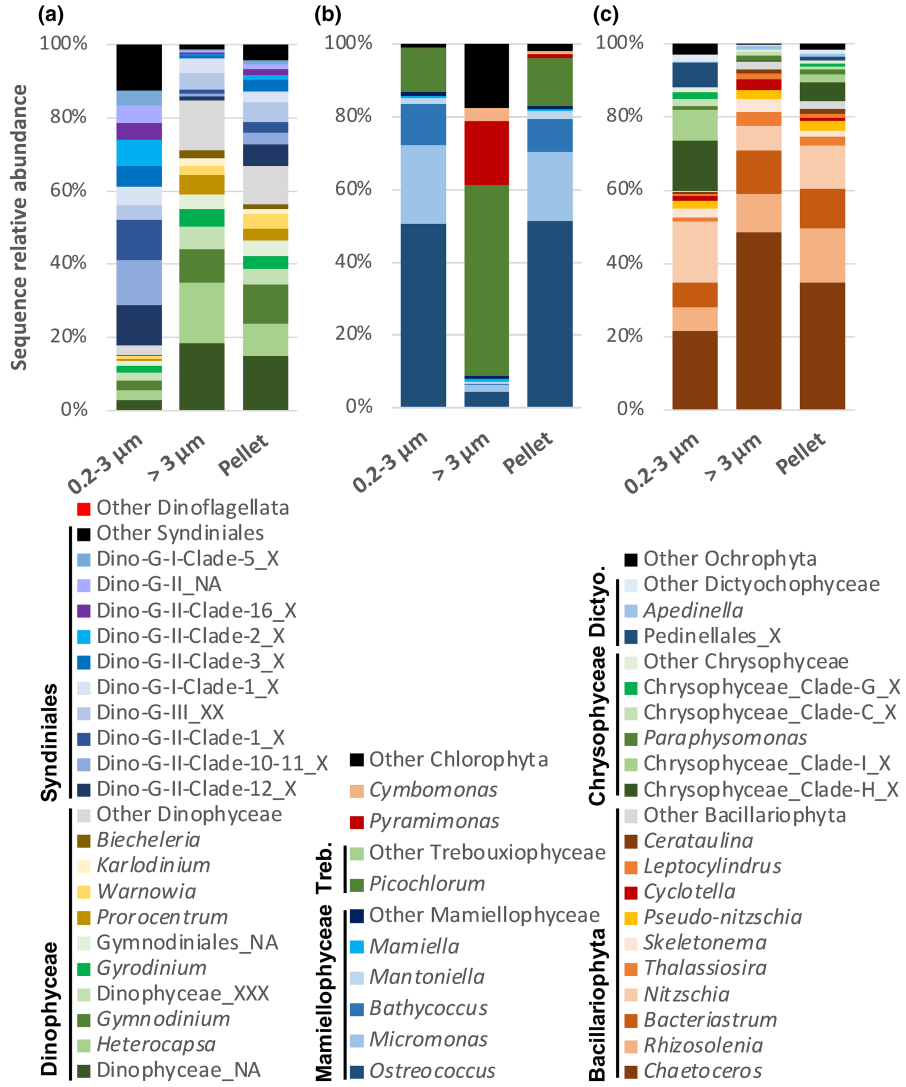

FIGURE 2 Taxonomic composition at the genus level of the eukaryotic community using the two methods. Three of the five most abundant divisions are represented in the figure. Dinoflagellata (a), Chlorophyta (b) and Ochrophyta (c). Taxonomic composition of each sample was surveyed on the data set normalized by the number of sequences in each sample using median sequencing depth. For each division, genera were represented by their sequence relative abundance in a given size fraction or in the pellet. Genera were ordered by sequence abundance within each class level. Genera with a lower number of sequences were grouped as 'Other' for each class and division level. The list of 'Other' genera can be found in the Table S3. Genera identified as NA were not taxonomically identified in the database PR2 and genera identified as _XX were identified in the PR2 database but not yet described at this taxonomic level in the database. Dictyo., Dictyophyceae; Treb., Trebouxiophyceae.

values with the two capture methods (ANOVA, $F_{2,69}=0.43$, $p=.65$, and ANOVA, $F_{2,69}=0.36$, $p=.7$ respectively). Ecological structuring patterns of the microbial community obtained with the two methods were then compared. A pairwise comparison of the sample diversity using the Bray–Curtis dissimilatory index indicated an apparent overlap of the samples of the three capture methods (Figure 3a). Indeed, the structuring of the community was very similar between the three groupings when comparing their sample dispersion (PERMANOVA, $F_{2,69}=2.77$, $p=.001$). The structuring of the community from the 0.2–3 µm and from centrifugation was more similar than that of the >3 µm and the 0.2–3 µm and from centrifugation (PERMANOVA, 0.2–3 µm vs. centrifugation, $F_{1,46}=1.38$, $p=.112$, 0.2–3 µm vs. >3 µm, $F_{1,46}=4.73$, $p=.001$ and >3 µm vs. centrifugation, $F_{1,46}=2.16$, $p=.006$, Table S4). These similarities may be explained when comparing the ASV composition between the 0.2 and 3 µm fraction, the >3 µm fraction and the centrifugation pellet (Figure 3b). Indeed, the two fractions and the pellet shared 62% of the total number of ASV, representing 95% of the total number of sequences. Moreover, when summing up the proportion of ASV shared between the two eDNA capture approaches individually with the proportion of common ASV to all approaches, it represented 96% of all ASV and 99% of all sequences, leaving a low number of ASV and sequences unique to each method. To conclude, the use of sequential filtration or centrifugation led to comparable diversity and community structure patterns.

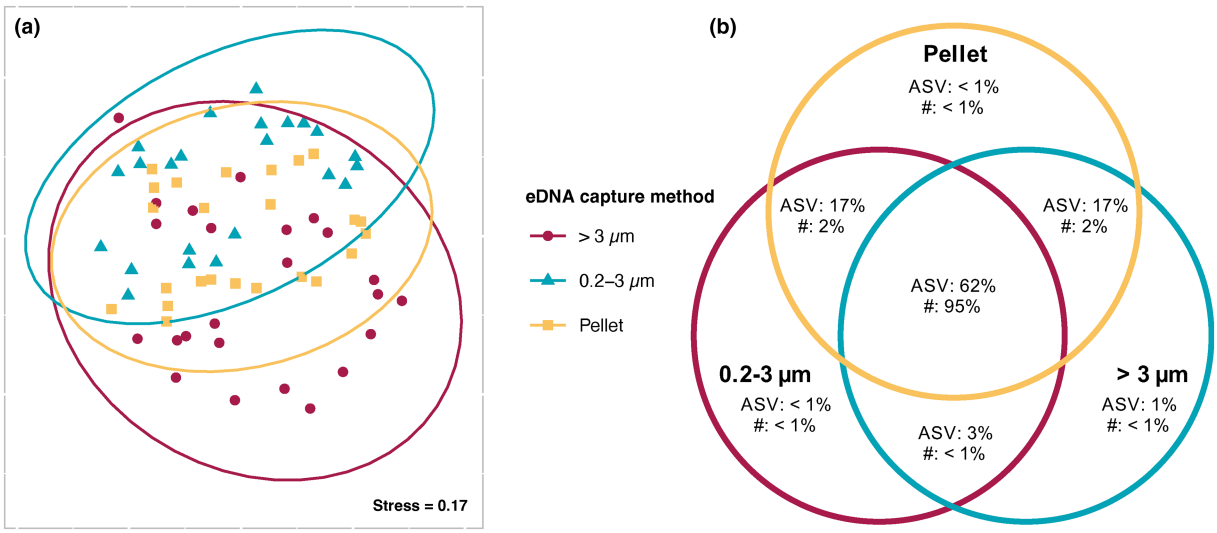

FIGURE 3 Community structure of the microbial eukaryote community using the two capture methods. The Bray–Curtis dissimilarity index was calculated from the metabarcoding data set at the ASV level (a). The low stress value of 17% validates the goodness of fit of the two-dimensional representation compared with the original matrix. Sample dispersion according to the three groupings: sequential filtration for the two size fractions and centrifugation was tested using PERMANOVA (Table S4). Proportion of ASV unique to a filtration fraction or to centrifugation, or shared between the capture methods (b). ASV, relative abundance of ASV; #, relative sequence abundance. 0.2–3 μm, 0.2–3 μm size-fraction; >3 μm, >3 μm size-fraction; Pellet, fraction from centrifugation.

3.4 | Temporal distribution of the 20 differentially abundant ASV between the two size fractions and the centrifugation pellet

To further investigate whether each method discriminated similarly the ASV diversity, we searched for significantly differentially abundant ASV between the two size fractions and the centrifugation pellet from February 2019 to January 2020. The search returned 20 ASV belonging to seven divisions: Dinoflagellata, Chlorophyta, Rhodophyta, Cryptophyta, Picozoa, Ochrophyta and Sagenista (Figure 4). Among Dinoflagellata, four differentially abundant ASV were identified as *Dinophysis* (asv0302_Dinophysis), *Tripos* (asv0308_Tripos), an unknown Dinophyceae (asv0085_Dinophyceae_NA) and *Prorocentrum* (asv0114_Prorocentrum). Cells of these genera are rather large (>10 μm) and diverse in geometry (Table 1). All four ASV were predominantly found in the >3 μm fraction and their temporal patterns were comparable to those in the centrifugation pellet: *Prorocentrum* peaked in summer, and *Dynophysis* and *Tripos* ASV showed no clear seasonal patterns. The succession and temporal fluctuations of seven ASV belonging to five genera of Chlorophyta: *Bathycoccus* (asv0013_Bathycoccus), *Ostreococcus* (asv0020_Ostreococcus), *Mantoniella* (asv0087_Mantoniella), *Micromonas* (asv0108_Micromonas, asv0015_Micromonas, asv0009_Micromonas) and *Picochlorum* (asv0103_Picochlorum) were consistently kept in the 0.2–3 μm fraction and in the pellet: *Bathycoccus* peaked in February, *Ostreococcus* was abundant from May to August with a peak in June, *Mantoniella* was more abundant from March to May and *Picochlorum* was most abundant in May and September. *Micromonas* was represented by three ASV, which showed distinct temporal patterns (Figure 4). There was one Cryptophyta ASV identified as the genus *Falcomonas* (asv0018_Falcomonas) in the small size fraction. The ASV was abundant in the Thau waters throughout the whole year, with similar sequence abundance fluctuations in the small size fraction and in the centrifugation pellet. Two non-identified Picozoa ASV (asv0188_Picozoa_XXXX and asv0032_Picozoa_XXXX) were found in the small fraction, in agreement with their size (Table 1), as well as in the pellet. Patterns of temporal fluctuations of four Ochrophyta and Sagenista ASV (asv0401_Chrysophyceae_Clade-I_X, asv0171_Chrysophyceae_Clade-H_X, asv0149_Thalassiosira, asv0086_Cyclotella and asv0116_MAST-7B_XX respectively) were similar between the smaller size fraction and centrifugation pellet. The genera *Cyclotella* and *Thalassiosira* were more abundant in the >3 μm fraction and this is consistent with their cell size of >5 μm and their cylinder-like morphology (Table 1). There was also one ASV identified as a Rhodophyta seaweed, from the genus *Ceramium* (asv0535_Ceramium) which was only present on the >3 μm fraction. To summarize, patterns of temporal distribution of differentially abundant ASV of the two size fractions combined led to similar conclusion as with centrifugation.

4 | DISCUSSION

To survey microbial diversity using eDNA, a number of decisions must be made based on the ecological question raised, the taxonomic groups targeted and the ecosystems explored. Each step of the metabarcoding approach, namely, sampling, nucleic acids extraction, library preparation, sequencing and sequence denoising and cleaning, has inherent biases and an impact on the derived ecological conclusions (Calderón-Sanou et al., 2020; Santoferrara, 2019;

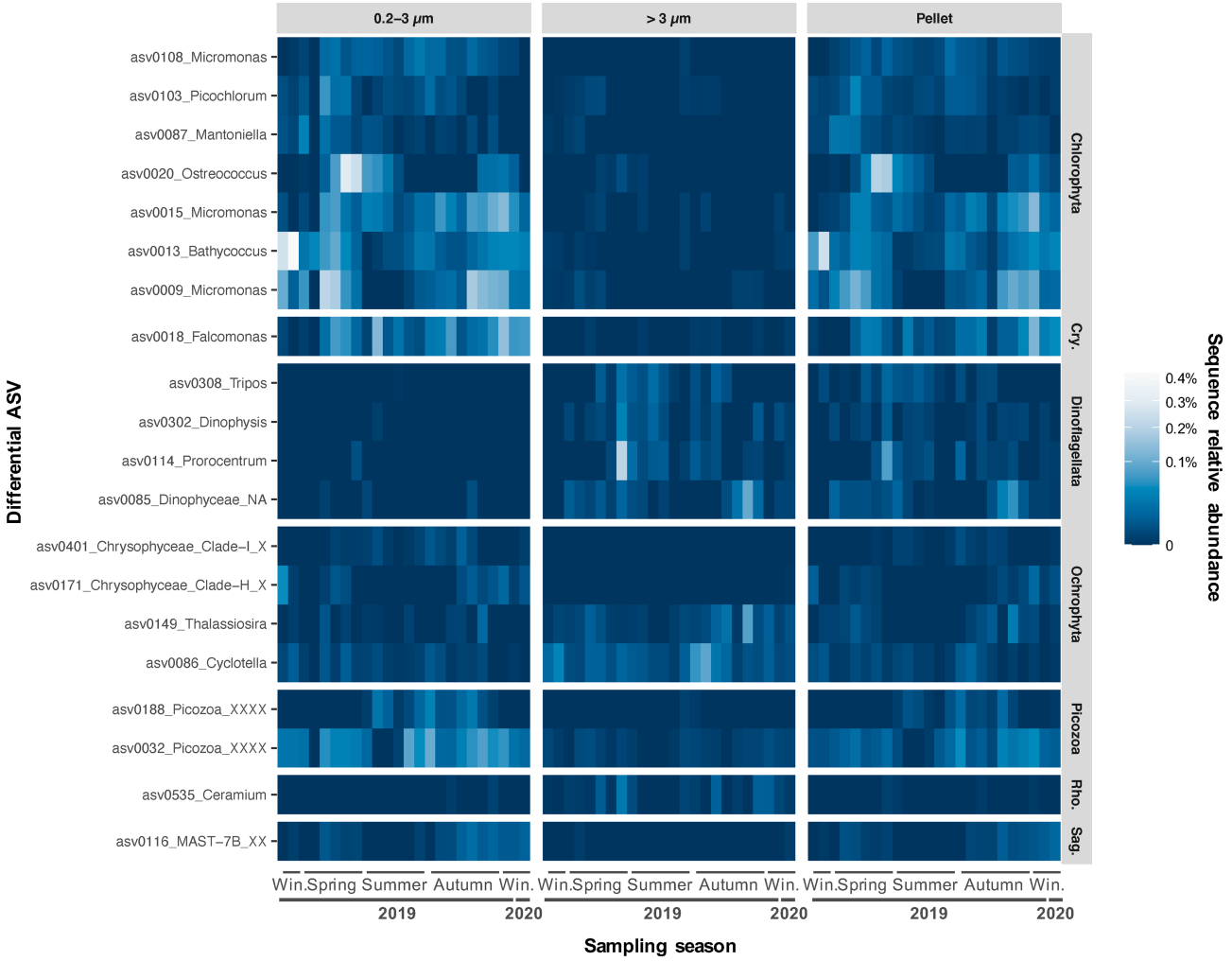

FIGURE 4 Sequence relative abundance of differentially abundant ASV obtained with the two methods. Samples were taken from the Thau lagoon bimonthly over a year from Winter 2019 (11 February 2019) to Winter 2020 (27 January 2020). ASV showing significantly different sequence abundance are shown (Benjamini–Hochberg corrected p value of glm test < .05). ASV are annotated at the genus level and classified at the division level. Rho., Rhodophyta; Cry. Cryptophyta; Sag., Sagenista. 0.2–3 μm, 0.2–3 μm size fraction; >3 μm, >3 μm size fraction. Win, Winter.

Taberlet et al., 2018; Zinger et al., 2019). The accuracy and the replicability of the sampling step are key for the following steps. In metabarcoding studies, the eDNA capture method is crucial for an accurate description of communities of microbial eukaryotes. Our aim was to determine whether microbial community diversity and ecological patterns derived from sequential filtration, a widely used method in aquatic eDNA studies, and centrifugation were equivalent or complementary, and if centrifugation could be considered as a suitable method to survey microbial eukaryotic communities in coastal water samples.

Sequential filtration of samples collected in the Thau lagoon over 1 year allowed identifying taxonomic groups of expected size in each fraction. Metazoa, Ochrophyta, Dinoflagellata and Ciliophora, organisms typically larger than 5 μm (Dupuy et al., 2000; Vadrucci et al., 2013), were the most abundant divisions found in the >3 μm size fraction. This larger size fraction also contained some Chlorophyta, mostly represented by the genus *Picochlorum*. Cells belonging to this algal genus are typically 2–3 μm in diameter and can reach up 5–6 μm during division (Dahlin et al., 2019; Henley et al., 2004). With respect to the smaller size fraction, our study showed taxonomic groups of expected size (<3 μm) but also taxonomic groups representing larger organisms. Chlorophyta dominated the 0.2–3 μm fraction and were mostly represented by the genera *Ostreococcus*, *Micromonas*, *Picochlorum* and *Bathycoccus*, which all belong to the picoplankton (Table 1, (Tragin et al., 2016)). Except for the genus *Picochlorum*, the other three genera are well known in the Thau lagoon (Courties et al., 1994; Domaizon et al., 2012; Trombetta et al., 2022). Cryptophyta was also an abundant division in the smaller fraction. With a cell width ranging from 1.5 to 5 μm (Clay & Kugrens, 1999; Novarino, 2005), most Chlorophyta may pass through the 3 μm filter. The smaller size fraction also contained Dinoflagellata, largely represented by the Syndiniales class with about 82% of the sequences (Figure 2). Syndiniales are parasites of microalgae, other protists and several

TABLE 1 Biological characteristics of the genera representing the 20 differentially abundant ASV.

	Cell geometry	Cell size	Motility	Life form	Sources
Alveolata					
Dinophysis	Flattened ellipsoid	Length 30–60 μm/ width 30–40 μm	Flagella	Solitary	Kudela Lab at the University of California Santa Cruz, 2010, Shin et al. (2016)
Tripos	With horns	Length 50–300 μm	Flagella	Solitary	Shin et al. (2016), Hallegraeff et al. (2022), Ryabov et al. (2021)
Dinophyceae_NA	na	na	na	na	Genus undefined
Prorocentrum	Broad to elongated oval	Length 35–50 μm/ width 20–40 μm	Flagella	Solitary	Verma et al. (2019), Shin et al. (2016), Ryabov et al. (2021)
Archaeplastida					
Bathycoccus	Coccoid, with scales	Diameter 1–2 μm	Non motile	Solitary	Moreau et al. (2012)
Ostreococcus	Coccoid, naked	Diameter 1 μm	Non motile	Solitary	Courties et al. (1994)
Mantoniella	Round, spiderweb-like scales	Diameter 3–5 μm	Two flagella	na	Yau et al. (2020)
Micromonas	Pear-shaped	Length 1–2.5 μm	One flagella	Solitary	Swedish Biodiversity Data Infrastructure, 2010
Picochlorum	Coccoid	Diameter 2–3 μm	Non motile	Solitary	Henley et al. (2004)
Ceramium	Seaweed	Thallus diameter 25–50 μm	na	na	na
Hacrobia					
Falcomonas	Slightly falcate	Length 10 μm/width 5 μm	Two flagella	Solitary	Clay and Kugrens (1999)
Picozoa	Sofa/couch-like	2.5–5 μm	Two flagella	na	Seenivasan et al. (2013)
Stramenopiles					
Cyclotella	Cylinder	5–20 μm	Non motile	na	Spaulding et al. (2021)
Thalassiosira	Cylinder	5–100 μm	Non motile	Solitary (chains)	Round et al. (1990), Olenina et al. (2006)
Chrysophyceae	Spheric	10–100 μm	Flagella	Colonial/ solitary	Nicholls and Wujek (2015), Olefeld et al. (2018)
MAST	na	na	na	na	Genus undefined

Note: The differentially abundant ASV were calculated from their sequence abundance using the two methods.

metazoan, and their free-living stage is found in the picoplankton (Clarke et al., 2019; Guillou et al., 2008). Other Dinoflagellata belonged to the Dinophyceae order, usually large cells of >20 μm, which can be smaller depending on their physiological state or environmental conditions (e.g. cell division, reproduction or nutrient deprived conditions (Chee Yew Leong & Taguchi, 2004; Figueroa et al., 2009)). Still, these smaller cells are larger than 3 μm (>6 μm width) and are not expected to pass through the 3 μm filter (Silva & Faust, 1995). Dinophyceae sequences in the picoplanktonic fraction may thus originate from extracellular DNA, efficiently retained on 0.22 μm filters (Sørensen et al., 2013). Metazoan sequences were also found among the most abundant sequences in the smaller fraction (Figure 1). The presence of metazoan sequences in the smaller size fraction has been reported in various studies targeting marine environments such as the Artic Ocean and adjacent seas (Lovejoy et al., 2006) or European coastal waters (López-Escardó et al., 2018). Metazoa in these water samples represented about 10% of the total number of sequences in the pico-nanoplanktonic fraction (0.8–20 μm), which were dominated by ctenophores and were suggested to be gametes (López-Escardó et al., 2018). This may be the case for the *Hydroides* sequences identified in the Thau lagoon, as this is a benthic genus and its planktonic spermatozoa are about 3 μm in length and could pass through the filter (Hargitt, 1910; Matsuo & Yoshikoshi, 1983) but not for *Oithona* as copepods mate and do not spawn (Titelman et al., 2007). Therefore, the large presence of copepod sequences in the picoplankton fraction is probably due to remnants of broken cells and extracellular DNA. Ochrophyta were abundant in the picoplankton fraction and mostly represented by the genera *Chaetoceros* and *Nitzschia*, part of the microplankton with cell sizes above 10 μm (Dupuy et al., 2000; Leruste et al., 2018). Smaller forms for these genera are known with for instance, *C. tenuissimus* and *C. salsugineus*, with a diameter of about 3 μm (Arin et al., 2022), and this could explain their presence in the picoplankton fraction. Their detection could also result from extracellular DNA released by damaged or dead cells (Vaulot et al., 1989). As observed in earlier aquatic studies (Herbland & Voituriez, 1979; Li et al., 1983; Vaulot et al., 2008), sequential filtration led to a fine resolution of the microbial community composition in the Thau water

samples but with the limit of overestimating the real diversity within the smaller size fraction.

Most abundant divisions and genera recovered in the centrifugation pellet were comparable to those collected by sequential filtration, with differences in relative abundance for some divisions. This is the case for the Chlorophyte picoplanktonic genera, *Ostreococcus*, *Micromonas*, *Picochlorum* and *Bathycoccus* for which the abundance patterns in the pellet were similar to those in the 0.2–3 μm fraction. The most abundant Dinoflagellata genera in the centrifugation pellet were also found in the larger size fraction. They included Dinophyceae whose cell size >3 μm (e.g. *Heterocapsa*, *Gymnodinium*, *Gyrodinium* (Hansen et al., 2003; Iwataki, 2008; Partensky & Vaulot, 1989)) and Syndiniales such as an unidentified genus of the Dino-Group-III order, a group exclusively known from eDNA and whose biology is poorly known despite being regularly found in eDNA samples from marine waters (Guillou et al., 2008; Nagarkar & Palenik, 2023). One Syndiniales genus, Dino-Group-II-Clade-12_X, found among the most abundant of the smaller size fraction, was also abundant in the centrifugation pellet, likely representing the free-living form of the parasite (Guillou et al., 2008). All five Cryptophyta genera identified in the pellet were also detected on the 0.2–3 μm filter, and in similar proportions, suggesting their high biomass in Thau waters in 2019. Surprisingly, the number of sequences for some divisions rather representing larger organisms (i.e. >3 μm), such as Metazoa and Ochrophyta, was much lower in the pellet compared to the larger fraction. This may be explained by (i) the escape of flagellated cells or swimming organisms from the pellet after centrifugation or (ii) a bias in their detection due to the higher eukaryote diversity and the larger biomass of smaller cells in the pellet compared to filters.

Sequential microbial size fraction is common in aquatic studies and was originally intended to identify a more pronounced diversity than when all the community is considered at once (Grossart, 2010). Indeed, discriminating samples according to organisms' size likely identifies taxa that would be otherwise overlooked. There is thus an expected different equitability of the taxonomic groups in the filtered fractions compared to the centrifugation pellet, leading to a more focused description of the diversity within each size fraction compared to centrifugation. Alpha diversity indices with the two capture methods led to the same alpha diversity for the two size fractions and centrifugation (Figure S2), suggesting that each size fraction contained redundant ASV and/or some specific ASV not found using centrifugation, and also with different equitability between the methods. This was confirmed when comparing the ASV composition from the size fractions and the pellet, which shared 62% of the total number of ASV, representing 95% of the total number of sequences. Still, pairwise comparisons of the diversity in each sample led to similar patterns of the community structure for the two size fractions and centrifugation. This suggests that contextual conditions had a higher effect on the structuring of the community than the capture method. Sequential filtration and centrifugation thus lead to similar ecological conclusions.

As our sampling strategy was designed to survey microbial eukaryotic diversity over a year, we searched for differentially abundant ASV between the two size fractions and the centrifugation pellet and analysed specifically their temporal fluctuations. Some of the 20 differentially abundant ASV followed similar patterns between one size fraction (>3 or <3 μm) and the centrifugation pellet. Similar patterns between the >3 μm fraction and the pellet were obtained for the Dinoflagellata *Dinophysis*, *Tripos*, an unknown Dinophyceae and *Prorocentrum*. Similar distribution and fluctuation patterns were also observed in the smaller fraction and in the pellet for differential ASV belonging to Chlorophyta, Cryptophyta, Picozoa, Ochrophyta and Sagenista. The five differential Chlorophyta ASV were identified as picophytoplanktonic genera (Tragin et al., 2016), and this suggests that centrifugation seems to be an appropriate method to capture DNA from picoplanktonic chlorophytes, likely represented by a larger biomass in the pellet compared to larger cells, as observed in various marine coastal environments (Vaulot et al., 2008; Worden et al., 2004). The differential Cryptophyta ASV belonged to the genus *Falcomonas*, a genus found in Mediterranean coastal waters (Novarino, 2005). One ASV belonged to the MAST-7 group in Sagenista, which are usually found in the picoplanktonic fraction (Massana et al., 2014). Considering the temporal fluctuation patterns of the 20 differentially abundant ASV, results obtained with centrifugation were comparable to the two size fractions combined but not for the three ASV identified as *Cyclotella*, *Thalassiosira* and *Ceramium*. Interestingly, *Cyclotella* and *Thalassiosira* were not or rarely observed in Thau waters during our sampling period using an optic microscopy approach (for *Thalassiosira*, once in 2019 with 100 cells per litre (REPHY—French Observation and Monitoring program for Phytoplankton and Hydrology in coastal Waters 2022)). The filamentous red algal epiphyte *Ceramium* was introduced in the 80s in the Thau lagoon with the shellfish activity (Verlaque, 2001), and the corresponding ASV was mostly found in the >3 μm fraction but hardly detected with centrifugation. This may be due to the predominance of picoplankton cells compared to these three genera in the pellet. As centrifugation does not discriminate the organisms according to their size, the recovered diversity using this capture method is more affected by a higher diversity compared to sequential filtration, a higher cell number and biomass, and inherently the 18S rRNA gene copy number varying according to each species (Gong et al., 2013; Zhu et al., 2005). This may also result from molecular biases such as the differential amplification of some DNA strand which may vary with their molecular composition (e.g. GC% (Reysenbach et al., 1992; Walsh et al., 1992)). To summarize, patterns of temporal distribution of differentially abundant ASV of the two size fractions combined led to similar conclusion as with centrifugation. Altogether our data suggest that the eukaryotic community described using centrifugation is comparable to sequential filtration to some extent and may depend on the biology and the physiology of the organisms at the time they were sampled as well their cell relative abundance in the samples.

Here, we compared two eDNA capture methods on waters from a marine aquatic ecosystem recovering from intense climatic events (heavy rains, heatwaves) (Lagarde et al., 2021) which may have affected the eukaryotic community and physiology. To confirm

that low-speed centrifugation, an approach with no size selection of the aquatic organisms sampled, is effective to assess microbial eukaryotic diversity in general, further analyses may be performed. Additional experiments may be done on other aquatic ecosystems but also in controlled conditions, using mock communities of selected taxonomic groups for instance. Results obtained with centrifugation may also be compared to filtration with no size selection (e.g. the Ocean Sampling Day data set with filtration >0.22 µm (Tragin & Vaulot, 2018)). Metabarcoding studies on both pellets and supernatants resulting from increasing centrifugation speed and time may also be useful to assess the extent of remaining eDNA in the supernatant as it could have consequences on the resulting diversity and ecological conclusions.

To conclude, our study showed that low-speed centrifugation, an approach with no size selection of the aquatic organisms sampled, was effective for assessing the diversity of microbial eukaryotes in the Thau lagoon ecosystem. While sequential filtration likely gives a detailed description of the diversity, centrifugation appears an ideal alternative to filtration in the case of aquatic samples dense in microbial cells and/or rich in organic matter (e.g. coastal eutrophic waters), as it circumvents problems linked to filter clogging and likely limits potential contaminations due to less sample manipulation. As the two eDNA capture methods led to similar ecological conclusions, centrifugation seems a valuable option for laboratories with no filtration equipment but also to process a large number of samples (in this study, there was twice less samples with centrifugation than with filtration) in a timely and cost-effective fashion.

AUTHOR CONTRIBUTIONS

Ariane Atteia and Angélique Gobet concepted and designed the study. Franck Lagarde, Béatrice Bec, Ariane Atteia and Angélique Gobet contributed to funding. Franck Lagarde, Béatrice Bec, Ophélie Serais and Camille Gianaroli performed fieldwork. Ariane Atteia, Camille Gianaroli, Franck Lagarde, Ophélie Serais performed eDNA capture; Ariane Atteia and Camille Gianaroli performed DNA extraction. Angélique Gobet and Isaure Quétel performed bioinformatics analyses. Angélique Gobet and Ariane Atteia interpreted the data and wrote the manuscript. All the authors revised and validated the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Raw 18S rRNA gene sequences are available in the SRA under BioProject ID PRJNA996377.

BENEFIT-SHARING STATEMENT

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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