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Choice of Pore Size Can Introduce Artefacts when Filtering Picoeukaryotes for Molecular Biodiversity Studies

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Abstract Published results of studies based on samples size fractionated by sequential filtration (e.g. 0.2–3 μm) indicate that many ciliate, dinoflagellate and rhizarian phylotypes are found among marine picoeukaryotes. This is somewhat surprising as these protists are typically known as being large organisms (often >10 μm) and no picoplanktonic species have so far been identified. Here, the abundances of ciliate and dinoflagellate phylotypes in published molecular studies of picoeukaryotes are shown to correlate negatively with the pore size chosen for the end filter in the sequential filtrations (i.e. the filter used to collect the microbial biomass). This suggests that extracellular DNA adhering to small particles may be the source of ciliate and dinoflagellate phylotypes in picoplanktonic size fractions. This hypothesis was confirmed using real-time qPCR, which revealed significantly less dinoflagellate 18S rDNA in a 0.8–3-μm size fraction compared to 0.2–3 μm. On average, the abundance of putative extracellular phylotypes decreased by 84–89 % when a 0.8-μm end filter was used rather than a 0.2-μm end filter. A 0.8-μm filter is, however, not sufficient to retain all picoeukaryotic cells. Thus, selection of filter pore size involves a trade-off between avoiding artefacts generated by extracellular DNA and sampling the entire picoeukaryotic community. In contrast to ciliate and dinoflagellate phylotypes, rhizarian phylotypes in the picoplankton size range do not display a pattern consistent with an extracellular origin. This is likely due to the documented existence of picoplanktonic swarmer cells within this group.

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Introduction

The first environmental molecular studies of marine picoeukaryotes (commonly defined as <3 μm) were published in 2001 [9, 18, 29]. Since then, many more have followed and the increasing availability of high-throughput sequencing increases the likelihood that we will see many more such studies in the near future. Before embarking on such studies, it is important that all information relevant to their planning be extracted from the existing literature. One finding from the available literature that deserves some further consideration is the apparent abundance, diversity and ubiquity of ciliates, dinoflagellates and rhizarians found among the picoplankton [26, 39]. Unlike the diverse clades of marine stramenopiles (MAST) [22, 27] and the novel phylum of picobiliphytes [33], microscopic confirmation of these organisms within the picoplanktonic size fraction has not been obtained. The known examples of these groups are usually relatively large (commonly >10 μm). It has earlier been suggested that extracellular (detrital) DNA from larger organisms may explain the apparent occurrence of these phylotypes among the picoplankton [23, 34]. If this is the case, we may be developing an incorrect understanding of biodiversity among the picoplankton.

Here, a systematic meta-analysis is made of picoeukaryotic environmental molecular studies in an attempt to identify potential relationships between the pore size of the end filter used when size fractioning picoeukaryotic samples by filtration and the abundance of ciliate, dinoflagellate and rhizarian DNA sequences. The meta-analysis indicated that less DNA is retained on filters with larger pore sizes compared to those having smaller ones. This suggests that much

of the DNA recorded in such studies may be of extracellular origin. This hypothesis was subsequently tested experimentally using novel dinoflagellate-specific primers in a real-time qPCR set-up. The ability of different end filters to retain *Ostreococcus tauri*, the smallest known eukaryote (cell diameter of 0.8 μm [6]), was also examined in this study.

Methods

Literature Analysis

To test for a correlation between the pore size of the end filter used and the abundance of ciliate, dinoflagellate and rhizarian DNA, relevant data were extracted from all published studies on the molecular diversity of picoeukaryotes found in the literature (Table S1). The abundances of dinoflagellates, ciliates and rhizarians in the collected data were calculated as the relative proportion of the total number of protist clones/tags (i.e. eukaryotes excluding fungi and metazoans). For comparative reasons, studies that presented diversity in a number of operational taxonomic units rather than clones were not included in the statistical analysis. Likewise, studies that used GF/F as the end filter were excluded, as the fibrous nature of glass fibre filters makes their pore size incomparable with those of other types of filters (e.g. Nuclepore).

Real-Time qPCR Experiments

Samples for real-time qPCR were collected in the Fram Strait (78.782°N, 3.116°E) on the 9th of September 2011 at a 15-m depth using a rosette of 10-L Niskin bottles on a cruise with the R/V Lance. Under a gentle vacuum established using a hand pump, a volume of 1 L water was pre-filtered onto a 3-μm filter and the microbial biomass was collected on either a 0.2- or 0.8-μm filter (Nuclepore polycarbonate membranes, Whatman), for three and five samples, respectively. Filters were cut in half and stored at −20 °C until DNA extraction. Only one-half of each filter was used for subsequent analysis.

Using 1.5-mL Eppendorf tubes, filters were incubated in 594 μL CTAB and 6 μL β-mercaptoethanol at 65 °C and shaken at 1,000 rpm for 45 min on a PHMT Thermoshaker (Grant Instruments). Samples were then frozen for 30 min at −80 °C, reheated at 65 °C for 30 min at 1,000 rpm and then heated for an additional 15 min without shaking. After adding 500 μL of chloroform:isoamyl alcohol (volume 24:1), the samples were shaken continuously and vortexed twice for 10 min and then centrifuged for 5 min. The water phases were transferred to new Eppendorf tubes, and the procedure with chloroform:isoamyl alcohol was repeated. Two-thirds of the sample volume of ice-cold isopropanol was then added, and the samples were kept at −20 °C for 30 min. The samples were centrifuged for 10 min and washed in 500 μL of 70 % ethanol and 50 μL of 3 M Na–acetate buffer, centrifuged for 2 min and washed in 1,000 μL 96 % ethanol. Residual ethanol was evaporated by putting the opened sample tube on a heating block at 65 °C for 2–5 min. DNA was eluded in 30 μL Milli-Q water and kept at room temperature for approximately 1 h. Extracted DNA was then stored at −20 °C until further processing. All centrifugations were carried out at 16,000×*g* and 4 °C using a Model 157.MP RF centrifuge (Ole Dich).

The following real-time qPCR reaction mixture was used: 0.5 μL of 10 μM CDF670f (forward primer, 5′-GCAT CYTCTTGGWGAACG-3′), 0.5 μL 10 μM CDF1058r (reverse primer, 5′-GTGCTGAAGGAGTCGT-3′), 1.5 μL H_2O, 5 μL Brilliant II SYBR Green QRT-PCR Master Mix (Agilent Technologies) and 2.5 μL sample. Samples (both environmental DNA and plasmids) were diluted 10- or 100-fold. The following qPCR program was used: initial soak at 95 °C for 10 min (following the manufacturer's protocol), 60 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 15 s and extension at 72 °C for 30 s and, finally, melt curve analysis from 56 to 95 °C at 0.5 °C increments. Samples were run in triplicate and only those with a variation of <1 Cq were used. The plasmid from clone 010609_08, a dinoflagellate phylotype from a previous study [37], was purified using the Cycle-Pure Kit (Omega Bio-Tek) and linearised [11] using the restriction enzyme NotI following the manufacturers' instructions and used as a standard in a 10-fold serial dilution. Real-time qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). For the design of the dinoflagellate-specific primer pair CDF670f-CDF1058r, see Supplementary material.

Retention of Picoeukaryotes

To assess how well different filters retain picoeukaryotic organisms, their retention efficiency with respect to the smallest known eukaryote, *O. tauri* [6], was tested. *O. tauri* (RC1114) was obtained from the Roscoff Culture Collection and grown in L1 media [10] at 20 °C and 25 μmol photons $m^{-2} s^{-1}$. A volume of 1 mL culture was filtered onto each of the following filters: GF/F (Whatman); 0.45-μm standard MF mixed cellulose ester membrane (Millipore); 0.6-μm polycarbonate filter (Poretics); and 0.8-, 0.4- and 0.2-μm Nuclepore polycarbonate filter (Whatman). All filtrations were done at the same time from the same culture, which was thoroughly mixed prior to filtration. Except for the 0.2-μm filtration, all filtrates were re-filtered on a 0.2-μm filter, thus creating six size fractions: 0.2–0.8, 0.2–0.6, 0.2–0.45, 0.2–0.4, 0.2–GF/F and >0.2 μm. A drop of immersion oil on

both sides of the filter was used to attach the filter to the microscope slide and cover glass. An inverted microscope (Olympus IX81) equipped with a spinning disc system, epifluorescence and a digital camera (F-View III, Soft Imaging System) was used to take five images at random positions of each 0.2-μm filter, and the number of cells was counted in each image. The 0.2-μm filter was assumed to retain all *O. tauri* cells, and the relative retention of the other filters was calculated by comparing the >0.2-μm size fraction with the pre-filtered size fractions.

Results

Literature Analysis

A total of 21 molecular picoeukaryotic libraries using universal eukaryotic primers were retrieved from the literature. Of these, only 15 met the criteria we had set for the analysis [1, 9, 17, 21, 25, 31, 32, 34, 36–38, 40] (Table S1), as three studies presented abundance in terms of operational taxonomical units rather than clones/tags [16, 24, 28] and another three used GF/F as end filters [3, 4, 29] (Table S2). A broad definition of picoeukaryotes is used in this analysis as the pre-filters used ranged from 1 to 5 μm in pore size.

A Spearman's rank correlation found a significant negative relationship between the pore size of the end filter and the summed relative abundance of dinoflagellates and ciliates (r_s=0.63, n=15, p<0.02) and an exponential regression explained r^2=0.51 of the variation (Fig. 1). According to this regression, the abundance of these phylotypes decreases by 89 % when using a 0.8-μm end filter as opposed to a 0.2-μm end filter. Although the correlation is significant, considerable variation was noted for the results using 0.1–0.22-μm end filters. No correlation was found between the abundance of rhizarians and the end filter used (r_s=0.13, n=15, p>0.50), and the summed abundance of dinoflagellates and ciliates in studies using GF/F (Table S2) did not differ significantly from studies using 0.2–0.22-μm end filters (Table S1) in variance (F test, F=1.30, df_1=2, df_2=6, p=0.68) or mean (t test, t=0.26, df=8, p=0.80).

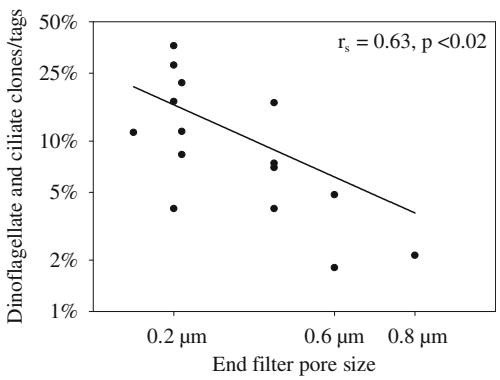

Fig. 1 Summed proportion of dinoflagellates and ciliates observed, plotted against the pore size of the end filter used in published studies. There is a significant negative correlation and an exponential regression with an explained variation of r^2=0.51

Real-Time qPCR

One sample from the 0.8–3-μm size fraction was >20-fold higher than the average of the remaining four which only showed limited variation. This data point was identified as an outlier (Grubb's test, G=1.79, p<0.01) and excluded. The remaining data are shown in Fig. 2, where it can be seen that the amount of dinoflagellate 18S rDNA is significantly higher in the 0.2–3-μm than in the 0.8–3-μm size fraction (t test, t=2.64, df=5, p=0.046) when data were log-transformed for variance homogeneity (F test, F=5.32, df_1=2, df_2=3, p=0.21). On average, the amount of dinoflagellate DNA decreased by 84 % when 0.8-μm filters were used instead of the 0.2-μm end filters, although the 0.2–3-μm size fraction showed a considerable variation.

Retention of Picoeukaryotes

On average, the 0.8- and 0.6-μm polycarbonate filters retained 21 and 75 % of *O. tauri* cells, respectively. The remaining filters (0.4 μm polycarbonate, 0.45 μm mixed cellulose esters and GF/F) all retained 100 %.

Discussion

The meta-analysis of molecular studies reporting the diversity of marine picoeukaryotes shows that the abundance of phylotypes belonging to ciliates and dinoflagellates decreases with increasing pore size of the end filter (Fig. 1). This suggest that either of these phylotypes

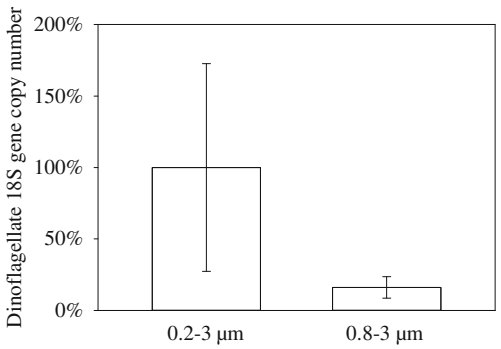

Fig. 2 Relative dinoflagellate 18S rDNA copy number in two different size fractions as determined by real-time qPCR with standard deviations. The 0.2–3-μm size fraction is set to 100 %. There is a significant difference between the two (t test, t=2.64, df=5, p=0.046) when log transforming data for variance homogeneity (F test, F=5.32, df_1=2, df_2=3, p=0.21)

originate from ciliates and dinoflagellates smaller than 1 μm (and consequently not from organisms measuring 1–3 μm) or from extracellular DNA, which is more efficiently retained on filters with smaller rather than larger pore sizes. In light of the fact that 0.8 μm is the smallest size ever recorded for eukaryotes [39] and the generally large (>10 μm) size of ciliates and dinoflagellates as well as the lack of microscopic observations of picoplanktonic ciliates and dinoflagellates, an extracellular origin of these phylotypes seems the most plausible explanation. Interestingly, the effect of end filter's pore size on the abundance of phylotypes of putative extracellular origin is nearly identical whether assessed by a meta-analysis of molecular studies of picoeukaryotes (Fig. 1) or experimentally using qPCR (Fig. 2). In both study approaches, the 0.2-μm end filter shows a considerable variation and an average decrease of 84–89 % of phylotypes of suspected extracellular origin is observed when compared to the 0.8-μm end filter. For the meta-analysis, the high variation for the 0.2-μm end filters may be caused by a high general variation in ciliate and dinoflagellate abundance, while as a possible explanation for the variability with the qPCR approach, it can be noted that significant differences in the composition of the phytoplankton community have previously been detected at spatial scales much smaller than the resolution offered by the traditional Niskin bottles [30].

On this basis, it seems likely that reported observations of ciliates and dinoflagellates in marine picoeukaryotic molecular studies are filtration artefacts. This conclusion is supported by a study using fluorescence-assisted cell sorting (an approach that does not use sequential filtration, thereby avoiding potential filtration artefacts) to isolate the picoeukaryotic community from the English Channel, in that the study failed to find DNA sequences belonging to either group [20].

Dissolved extracellular DNA is ubiquitous in all marine environments [12] and can range from a few hundred to several thousand base pairs in length [8], fully encompassing the length of partial or complete 18S sequences. Furthermore, it can have a fast turnover time of mere hours [2, 19, 35]. Dissolved DNA is functionally defined as that which passes a 0.2-μm filter [7, 12], but it can bind to particles in the marine sediment, such as sand, thereby inhibiting degradation of the DNA by DNase [14, 15] allowing for a much larger pool of extracellular DNA [5] and, possibly, also for retention on, for example, 0.2-μm filters. Such particle-associated extracellular DNA has been shown to contain highly diverse 18S rDNA sequences [5] and could, possibly, be the source of both ciliate and dinoflagellate sequences in molecular picoeukaryotic studies.

In several molecular studies of picoeukaryotes, GF/F is used as the end filter (Table S2). Despite having a pore size of 0.7 μm according to the manufacturer, GF/F in this study performed similarly to a 0.2-μm filter, with retention of both extracellular DNA and *O. tauri* cells. The fibrous nature of the filter is likely the cause of the apparent discrepancy between nominal pore size and retentive performance.

The largest decrease (84–89 %) in putative extracellular phylotypes compared to a 0.2-μm end filter is seen when using a 0.8-μm filter. Unfortunately, however, this pore size only retained 21 % of *O. tauri* cells in this study. For non-quantitative purposes, the 75 % retention of the 0.6-μm filter might be sufficient but 0.4-μm filters must be used if it is important that the entire picoeukaryotic community is retained. For investigations where only specifically larger picoeukaryotes are of interest (e.g. MAST clades), a pore size of 0.8 μm might be prudent given the decreased retention of extracellular DNA compared to end filters with smaller pore sizes.

This study suggest that it is most likely that the rhizarian phylotypes recorded in the picoplanktonic fraction are not of extracellular origin as their abundance in molecular studies does not decrease with increasing pore size. This might be explained by the presence of rhizarian picoplanktonic swarmer cells which have been described [13].

The high abundance of phylotypes of a putative extracellular origin in molecular studies of picoeukaryotic diversity demonstrates the limitations of a purely molecular approach in describing marine plankton communities, as there is no clear link from phylotype to cellular identity. Visual confirmation (e.g. using flow cytometry or epifluorescence microscopy) of novel molecular diversity is necessary to demonstrate the existence of different phylotypes within the picoeukaryotic size fraction.

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