Flow cytometric enumeration of DNA-stained oceanic planktonic protists

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Received June 8, 2006; accepted in principle August 4, 2006; accepted for publication October 12, 2006; published online October 17, 2006

Communicating editor: K.J. Flynn

The aim of this study was to test the practicality of enumerating fixed, DNA-stained heterotrophic protists (H) and phototrophic protists (P) in contrasting regions of the Atlantic Ocean. Oceanic protists were enumerated using a standard flow cytometer (FACSort, BD) at an enhanced flow rate of up to 1.0 mL min$^{-1}$ to increase numbers of counted cells. The enumeration error of protists decreased hyperbolically from 30–40 to $<5\%$ corresponding to the number ($<100$ to $>2000$) of enumerated cells. H and P were discriminated using the extra red chlorophyll-derived plastidic fluorescence of the latter. The relationship between counts of stained and unstained fixed and unfixed P was statistically close to 1:1, confirming the accuracy of stained protist counting by flow cytometry and adequate discrimination of P from H cells. The estimated average abundance of H in the surface mixed layer of the southern and northern oligotrophic gyres was remarkably similar, with $400 \pm 140$ and $450 \pm 60$ cells mL$^{-1}$, respectively, adding further evidence to the suggestion that these regions are in steady state. In agreement with earlier studies in more productive aquatic environments, a significant correlation (correlation coefficient 0.84, $P < 0.0001$) was found between the H and the total bacterioplankton numbers, with an average ratio of $\sim 1300$ prokaryotes to 1 H cell, suggesting a relatively constant trophic interaction between these two groups. This study demonstrates that flow cytometric enumeration of protists is $\sim 100$ times faster compared with microscopy and, thus, represents a major improvement for quantifying protists in ocean waters, including oligotrophic gyres.

INTRODUCTION

Accurate enumeration of planktonic protists is critically important for quantifying microbial trophic dynamics and for understanding the roles of microbes in the main biogeochemical cycles, because protists are key primary producers and consumers of biomass in the world ocean. The fluorescence of chlorophyll and other photosynthetic pigments makes phytoplankton cells well suited for studies by flow cytometry (FC). The majority of oceanic phytoplankton comprises small picocellular algae as well as abundant cyanobacterial cells, which can all be enumerated using established flow cytometric procedures (Olson et al., 1993). Larger, less abundant, eukaryotic nanophytoplankton cells have also been enumerated by FC (Tarran et al., 2001). FC following DNA staining is successfully employed for the enumeration of bacterioplankton (B) including cyanobacteria (Robertson and Button, 1989; Marie et al., 1997). Phagotrophic or heterotrophic protists (H) are usually enumerated using microscopic techniques (Sherr et al., 1993) and discriminated from phototrophic including mixotrophic protists (P), by the presence or absence of autofluorescence of plastidic photosynthetic pigments. Microscopic counting of protists is time consuming and hampered by human error, such as misidentification. Protists could also be enumerated by FC using either DNA stains (Zubkov et al., 2001; Lindstrom et al., 2002; Rifa et al., 2002) or food vacuole stains (Rose et al., 2004). Although the latter method was suggested as a promising technique for enumerating heterotrophic protists in natural aquatic communities, the approach requires FC of live stained cells. These could suffer
mortality during sample incubation and storage before analysis, and so the approach may lack accuracy. In that respect, a technique that involves cell fixation would be preferred. A potential disadvantage of protist fixation is that this approach may introduce errors, such as loss of cells, severe distortion of cell size and shape upon fixation (Zubkov and Sleigh, 1998). However, flow cytometric enumeration of protists should theoretically remain possible, provided their nuclei remain intact.

The aim of the present study was to achieve this and validate DNA-stained protist enumeration by FC in oceanic samples.

METHODS

Sampling sites

The field study was carried out on three cruises. Two cruises were meridional transatlantic transects on board the Royal Research Ship (RRS) James Clark Ross (cruise no. JR101) in May 2004 and on board the RRS Discovery (cruise no. D299) in September–October 2005. The third cruise was in the Celtic Sea on board the Research Vessel (RV) Terschelling in July 2004. During the transect cruises, seawater samples were collected from 8 to 24 depths in the top 90–300 m with a rosette of 20-L Niskin bottles mounted on a conductivity–temperature–depth (CTD) profiler. Abundances of planktonic H, P and B were determined at 18 stations (Fig. 1a). The samples were collected in acid-washed 50-mL tubes. Subsamples were fixed with 1% paraformaldehyde (PFA) and analysed after DNA staining for H, P and B as well as being analysed unstained for phytoplankton. Live phytoplankton samples were kept in the dark at ~4°C until counted within 1–2 h after sampling. During the Celtic Sea cruise, seawater was continuously centrifugally pumped from 3-m depth, and water samples were taken and fixed with 1% PFA every 12 min by a Miniprep-60 autosampler (Tecan, Reading, UK) during 4 days of sampling to enumerate protists (Fig. 1b).

FC of stained protists

Absolute concentrations of protists were determined by FC (FACSort, Becton Dickinson Biosciences, Oxford, UK) by fixing 3-mL subsamples of natural water with particle-free 20% w/v PFA, dissolved in seawater, 1% final concentration, at 4°C for 2–8 h. Protists were

Fig. 1. A chart with station locations (grey and black triangles) and schematic presentation of the waters studied in the North and South Atlantic Ocean (a) and the Celtic Sea (b). The ocean gyres, marked by dashed lines, were defined by surface waters numerically dominated by Prochlorococcus cyanobacteria at concentrations $>10^5$ cells mL$^{-1}$. The arrows point to a cruise track in the Celtic Sea shown with a solid line and small dots representing sampling points. Symbols indicate stations at which microorganisms including stained protists were enumerated by flow cytometry.
stained with SYBR® Green I DNA stain (Sigma-Aldrich, Poole, UK), 1:5000 final dilution of initial stock, in the presence of potassium citrate, 30 mM final concentration, in the dark at 4°C for 2–4 h. A subset of fixed samples were frozen at –80°C and brought ashore for flow sorting and microscopic examination in the laboratory, for comparison with shipboard analyses.

A yellow-green 0.5-μm bead (Fluoresbrite Microspheres, Polysciences, Warrington, PA, USA) concentration standard was added at known dilution to determine absolute cell concentrations (Zubkov and Burkill, 2006). Protists were enumerated at ~180 μL min⁻¹ flow rate for 2–3 min triggering on 90° light scatter (Fig. 2a, R₁ gate). On the second transect cruise, the stained protists were counted at 1 mL min⁻¹ flow rate for 2 min using a syringe pump (Zubkov and Burkill, 2006) and the same acquisition protocol. The measurements of 90° or side light scatter (SSC), green (FL1, 530 ± 15 nm), orange (FL2, 585 ± 21 nm) and red (FL3, >650 nm) fluorescence were made with log amplification on a four-decade scale. Flow cytometric data were analysed using CellQuest software (Becton Dickinson) and were plotted using WinMDI software 2.8 (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

H and P were discriminated on the bivariate plots of FL3 versus FL1 using the characteristic extra red fluorescence of plastidic chlorophyll containing protists as a discriminator (Fig. 2b). The particles with proportional green and red fluorescence, i.e. a-aplastidic protists without chlorophyll, were gated by a region H, and pico- and nanoplanctonic plastidic protists with extra red fluorescence were gated by regions P₁ and P₂, respectively, thus being discriminated from the largest bacterioplankton cells (Fig. 2b, B₁). Fig. 3 shows characteristic histograms of green, stained DNA fluorescence of H, P₁ and P₂ populations accompanied by micrographs of representative cells flow cytometrically sorted using single-cell sort mode, sorting rate 1–50 particles per second from the respective gated regions. Sorted cells were collected on polycarbonate filters, 0.2 μm pore size, and filters were mounted on microscopic slides to observe under an epifluorescence microscope (Zeiss Axiosvert 200M) equipped with a ×100 objective. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) DNA-specific dye to visualize cell nuclei (Fig. 3, N) using a filter set 1: excitation, 390–420 nm; beamsplitter, 425 nm; emission, 430–480 nm. Red chlorophyll autofluorescence of cell chloroplasts (Fig. 3, Chl) was microscopically observed using a filter set 2: excitation, 450–490 nm; beamsplitter, 510 nm; emission, long pass 515 nm.

![Fig. 2. Characteristic flow cytometric signatures of SYBR Green I DNA-stained cells larger than bacterioplankton (B), which included heterotrophic (H) and phototrophic (P) protists, were first gated by region 1 (R₁) (a). R₁-gated cells were then plotted separately on a dot plot of their green versus red fluorescence (b) to separate bigger H cells from the largest B and pico- (P₁) and nanoplanctonic (P₂) phototrophic cells. The P cells exhibited greater red chlorophyll fluorescence. Polygon regions were drawn around dominant groups to allow their enumeration. Yellow-green beads, 0.5-μm diameter, were used as an internal standard and these are shown (a). Arrows and polygon regions indicate the beads and segregated dominant groups that were enumerated. As cellular SSC and phytoplankton pigment autofluorescence changed with depth dynamically, rather than statically, group gates were used to analyse sample series. The H and P regions were also used for flow cytometric sorting (Fig. 3). Owing to the large number of organisms, a greyscale density plot rather than a dot plot for clarity is shown (a). The shown sample was collected from 3 m at 50.76° N, 7.32° W in the Celtic Sea.](https://academic.oup.com/plankt/article-abstract/29/1/79/2963066/Flow-cytometric-enumeration-of-DNA-stained-oceanic)

![Fig. 3. Characteristic DNA-SYBR Green I fluorescence histograms of the gated dominant H (a), P₁ (b) and P₂ (c) groups shown in Fig. 2. The H histogram shows a clear double-peak distribution. This indicates the presence of cells with one and two genome copies and is due to cell division rather than to the presence of two distinct groups. The micrographs show examples of cell flow sorted from the corresponding gated groups, stained with 4′,6-diamidino-2-phenylindole (DAPI) DNA dye and photographed using filter sets 1 and 2 (method). The DAPI staining revealed cellular nuclei (N), and filter set 2 illumination revealed chlorophyll fluorescence of cellular plastoplast (Chl) that allowed the identification of P cells. A white bar indicates a 5-μm scale.](https://academic.oup.com/plankt/article-abstract/29/1/79/2963066/Flow-cytometric-enumeration-of-DNA-stained-oceanic)
FC of stained bacterioplankton

For enumeration of total bacterioplankton, i.e. both phototrophic and heterotrophic prokaryotes, 0.8-mL subsamples of natural water fixed with 1% PFA were stained with SYBR Green I (Marie et al., 1997) at ~20°C for >1 h. Cells were counted at 25 or 60 μL min⁻¹ flow rate, depending on cell concentration, for 1 min with triggering on FL1 and using yellow-green 0.5-μm beads as an internal standard. Stained prokaryotic cells were discriminated on the bivariate plots of SSC versus FL1.

FC of unstained phytoplankton

For enumeration of picophytoplankton, 3-mL subsamples of natural water were fixed with 1% PFA, and cells were counted unstained using their characteristic chlorophyll/phycoerythrin autofluorescence (Olson et al., 1993) at a flow rate of ~180 μL min⁻¹ for 2–5 min. To monitor the flow rate and flow cytometer performance, we added 50 or 100 μL of a stock suspension of a known concentration of yellow-green 0.5-μm beads as an internal standard. These were thoroughly mixed with each 3-mL fixed sample immediately before analysis. *Synechococcus*, *Prochlorococcus* cyanobacteria and PP were discriminated and counted using the picophytoplankton protocol. Additionally, P were enumerated live using a nanoplankton protocol (Tarran et al., 2001) and syringe pump sample injection at a flow rate of 1 mL min⁻¹ (Zubkov and Burkill, 2006).

RESULTS

Evaluation of stained protist counting

Our previous studies showed that it is possible to accurately enumerate both cultured heterotrophic protists (Zubkov and Sleigh, 2005) and protists in prey-enriched water samples (Zubkov et al., 2001). Although FC protocols count picophytoplankton and bacteria accurately in the open ocean waters, the low concentration of larger protists required this to be checked qualitatively and quantitatively.

Qualitative analyses were checked by flow cytometric sorting of cells from different protist populations (Fig 2b). The examination of the DNA-stained cells under a microscope confirmed that the sorted cells have nuclei and, therefore, are true protists (Fig 3) and not bacteria. The bacteria exhibited DNA-SYBR Green I fluorescence comparable with PP cells (Fig 2b) but not PN cells, and this difference was used to differentiate the B_L and H cells. A further confirmation involved the FC discrimination of plastid containing, phototrophic and aplastid, heterotrophic protists based on the red chloroplast fluorescence of the former (Fig. 3).

The discrimination between P_P and H based on the presence or absence of plastids in cells was very clear when P were rich in chlorophyll, and consequently, their autofluorescence was high, e.g. in temperate waters (Fig 2b) or in the deep euphotic zone (Fig. 4a) in

Fig. 4. Depth variation in flow cytometric signatures of DNA-SYBR Green I stained, R₁-gated (Fig 2) large bacterioplankton (B_L), heterotrophic (H), pico- (PP) and nanoplanktonic (PN) phototrophic protists in samples collected from (a) 180 m, (b) 72 m and (c) 2 m and analysed using a syringe pump injection at a flow rate of 1 mL min⁻¹. Arrows and polygon regions indicate the segregated groups that were enumerated. The samples were collected at 18.56°S, 25°W in the Atlantic Ocean.
tropical waters. The decrease of chlorophyll content of \( P_N \) towards the surface (Fig. 4) in the tropics made discrimination in the surface mixed layer less clear and required adoption of a simple separation principle. An \( H \) enumeration region was drawn based on clearly defined \( H \) populations in deep samples (Figs 2b and 4a), and a broad \( P_N \) region was adjoined to account for cell red fluorescence variation with depth. A \( P_P \) region was drawn using a similar principle but adjoined to the tip of a \( B_t \) cluster. The regions remained fixed when analysing samples collected at different depths. There is a possibility, using this counting approach, that some \( P_N \) cells with very low red autofluorescence were counted as \( H \) cells (Fig. 4b and c). However, the percentage of incorrectly identified cells should be small. This was confirmed using counts of fixed stained \( P \) cells and live unstained \( P \) cells in the surface mixed layer using syringe pumping. The differences between these were shown statistically insignificant according to a \( t \)-test comparison made at four stations on the second transect cruise.

To evaluate the efficiency of protist DNA staining, we conducted more extensive FC counting of the same protist samples but using two different protocols. The counts of live unstained \( P \) cells and fixed stained \( P \) cells made using the syringe pump sample injection at the same flow rate of 1 mL min\(^{-1}\) were similar (Fig. 5a and b), confirming insignificant loss of protist cells on fixation and acceptable discrimination of \( P \) and \( H \) cells in stained samples. For more extensive comparison, the group of choice was \( P_P \) because it is often the most numerous group of protists routinely enumerated by FC in unstained samples. The unstained \( P_P \) counts agreed well with the counts of \( P_P \) made using the stained protist protocol both in the transect samples (Fig. 5c and d) and in the Celtic Sea samples (Fig. 5e and f). This confirmed the robustness of the staining protocol.

The quantitative accuracy of counting the \( H \) and \( P_N \) protists was found to be related to their abundance in natural waters. To count more cells, we increased the rate of sample injection (from \(~60 \mu L \text{ min}^{-1}\) compared with \(180 \mu L \text{ min}^{-1}\)). This was done initially, using modification of the conventional air pump of the flow cytometer, and allowed us to analyse 0.54 mL of sample. On the second transect cruise, we increased the analysed volume to 2 mL by further increasing the flow rate up to 1 mL min\(^{-1}\) using a syringe pump. This did not significantly reduce the FC signature quality (Fig. 4). A coefficient of variance (CV) of cell enumeration decreases proportionally to the number of analysed cells, irrespective of the method of sample injection (Fig. 6), from \(~30\% \) when \(<100 \) cells were counted down to \(>10\% \) when \(>1000 \) cells were enumerated.

### Protist abundance in the surface mixed layer of the southern and northern Atlantic gyres

\( P_N \) were least abundant in the surface mixed layer of the southern Atlantic gyre (Fig. 7a). Their concentration was \( 360 \pm 95 \) cells mL\(^{-1}\) compared with \( 740 \pm 35 \) cells mL\(^{-1}\) in the northern gyre and more variable concentration of \( 1900 \pm 650 \) cells mL\(^{-1}\) in the northern temperate waters. \( H \) concentrations in the surface mixed layer of both gyres were remarkably similar in the southern and northern gyre, \( 400 \pm 140 \) and \( 450 \pm 60 \) cells mL\(^{-1}\), respectively (Fig. 7b) compared with a higher and more variable concentration of \( 820 \pm 340 \) cells mL\(^{-1}\) in the northern temperate waters.

### DISCUSSION

This study shows that discrimination between DNA-stained plastid-containing, phototrophic and aplastidic, heterotrophic oceanic planktonic protists can be achieved by FC. Microscopic analyses of sorted cells proved correct identification of the \( H \) and \( P \) populations as discriminated by FC (Fig. 3). In many cases, the histogram of green DNA fluorescence of \( H \) population showed a clear double peak indicating the presence of cells undergoing DNA replication. The clearly defined FC cluster of \( H \) suggests a dominant population in the waters of the Celtic Sea at that time. The FC clusters of \( H \) in the oceanic waters were broad, indicating the absence of a dominant population (Fig. 4). The results demonstrate that delicate \( P \) cells were adequately fixed by PFA and enumerated accurately using the stained protist protocol (Fig. 5). This should be applicable to the \( H \) cells that differ from \( P \) cells only in the absence of the chlorophyll-derived red autofluorescence.

In many oceanic samples analysed, the concentrations of both \( P_N \) and \( H \) were estimated as \(\leq600 \) cells mL\(^{-1}\) (Fig. 7), when \(<300 \) cells were enumerated on the earlier cruises and \(~1000 \) cells on the later cruise (Fig. 6). Therefore, the CV of these estimates was \(~20\% \) and \(<10\% \) on the first and second transect cruise, respectively. CV of \(<10\% \) could only be achieved by running samples for longer at lower flow rates: for example, 10-min analysis at \(180 \mu L \text{ min}^{-1}\) would allow counting of about five samples per hour. The precision could also be achieved by increasing the flow rate: for example, 2-min analysis at \(1 \mu L \text{ min}^{-1}\) would allow counting of \(~25 \) samples per hour, even if the time for changing samples is taken into account. We believe therefore that this protist staining FC protocol will
facilitate research on protist abundance in open marine waters. Compared with the normal microscopic approach for quantifying protists with typical CV of >30%, the cytometric protocol is ~100 times faster and represents a major improvement for quantifying protists in ocean waters.

The remarkable similarity of average H concentrations in surface mixed layer in the northern and southern gyres (Fig. 7b) adds further evidence to the suggestion that trophic relations in these waters are balanced with bottom-up and top-down impacts on H populations in balance. Considering that both phototrophic and heterotrophic bacterioplanktons are a significant prey source for H, we wondered whether there were first-order relationships between these protist predators and their prokaryotic prey. A comparison of H with total bacterioplankton numbers (Fig. 8) determined in the same samples from the tropical–subtropical,

![Graphs showing data](image.png)

Fig. 5. Comparison of the phototrophic protist (P) or picoplanktonic P (Pp) counts of fixed stained cells and the counts of unstained live [a and b] or fixed [c and d] cells in samples collected in the Atlantic Ocean [a and d] and the Celtic Sea [e and f]. Log scales of plots in the right column [b, d and f] show variation of low counts. Solid lines show linear regressions, lines of short dashes are the unity lines and dotted lines indicate 99% prediction intervals. The corresponding regression slopes, their standard errors, regression coefficients ($r^2$), number of points (n) and probability (P) values are inserted into the graphs.
temperate and shelf waters along the first transect on one scatter plot revealed a statistically significant, but moderate, positive correlation (correlation coefficient 0.84, \( P < 0.0001 \)) between the \( H \) and the total prokaryote abundance. The computed average numerical ratio of 1 \( H \) to \( /C_{24} \) prokaryotes was similar to the 1:1000 ratio (Fig. 8, dashed line) generalized for marine plankton (Fenchel, 1986) and freshwater plankton (Berninger et al., 1991) and the 1:1133 ratio reported for combined freshwater and marine plankton (Sanders et al., 1992).

Considering that a major part of the data reported here was collected in the tropical Atlantic, which is an undersampled oceanic region, the established ratio of \( /C_{24} \) between \( H \) and \( B \) seems to remain remarkably consistent throughout different aquatic environments. The constancy of this relationship at low prey and predator concentrations indicates that capturing bacterioplankton by phagotrophic protists seems to be concentration independent within the studied range. However, such relationship differs from that for \( H \) and \( B \) counts made in the Celtic Sea (Fig. 8, small squares). In these shelf waters, the data points tend to cluster together, but there were orders of magnitude deviations, indicating that these waters subjected to strong tidal processes were in a far from steady state condition.

**Fig. 6.** A scatter plot of an average number of counted stained heterotrophic protists (\( H \)) and nanoplanktonic phototrophic protists (\( P_N \)) versus the corresponding coefficient of variance of their counting, i.e. standard deviation divided on average of 2–7 measurements. Samples were injected into the flow cytometer using either an air (air) or a syringe (syr) pump. The x-axis is in log scale to show variation of low counts.

**Fig. 7.** Latitudinal changes in abundance of (a) nanoplanktonic phototrophic protists (\( P_N \)) and (b) heterotrophic protists (\( H \)) in the surface mixed layer on the two transects. Symbols indicate mean values, and error bars indicate a single standard deviation of 2–7 measurements made in the top 10–50 m. At three stations, single measurements were made at 6.5 m. Dotted drop lines point the latitude of sampled station. Grey lines indicate approximate boundaries of the oceanic provinces; ST, southern temperate waters; SG, southern gyre; EC, equatorial convergence; NG, northern gyre; NT, northern temperate waters.

**Fig. 8.** Comparison of heterotrophic protist (\( H \)) counts, made using the stained protist protocol, versus the total phototrophic plus heterotrophic bacterioplankton (\( B \)) counts made on the transatlantic transect across the tropical–subtropical oceanic (circles), temperate oceanic (triangles) and coastal (black squares) regions. A line of short dashes indicates the 1000:1 ratio. The paired \( H \) versus \( B \) counts (\( n = 415 \)) made in the Celtic Sea (small grey squares) are shown for comparison with the transect counts and for illustration of mesoscale variability.
Thus, this study showed that flow cytometric enumeration of stained protists in oligotrophic oceanic regions is now possible. The technique will be valuable for gaining statistically acceptable assessments of protist populations in environments where the densities of microbial populations have been found to be low and/or remarkably patchy and will facilitate more specific analyses of the roles different classes of protists play in microbial dynamics, trophic relationships and biogeochemical cycles.

ACKNOWLEDGEMENTS

Our thanks are extended to Michael Sleigh for critical comments on the earlier draft of this manuscript. We thank Jane Heywood and Adrian Martin for helping with flow cytometric sample analyses on board the ships. We gratefully acknowledge Patrick Holligan (chief scientist on the transect cruises), the captains, officers, crew and fellow scientists aboard RRS James Clark Ross and RRS Discovery for their support. We are grateful to Captain Nigel Boston and the crew of the RV Terschelling for their invaluable patience and assistance. This work forms a part of the Atlantic Meridional Transect Consortium programme (NER/O/S/2001/00680) and the small grant (NER/B/S/2003/00220) and was funded by the Natural Environment Research Council (NERC), UK, and the National Oceanography Centre Core Programme. The research of M.V.Z. was supported by the NERC advanced research fellowship (NER/1/S/2000/01426). This is contribution number 148 of the AMT programme.

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