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Optimized routine flow cytometric enumeration of heterotrophic flagellates using SYBR Green I

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Abstract

Heterotrophic flagellates (HF) are the major consumers of bacteria in aquatic ecosystems and dominate heterotrophic nanoplankton in numbers and in biomass. A DNA-staining based flow cytometry (FC) protocol to enumerate HF was described by Zubkov et al. (2007), but has not yet been widely adopted. We tested extensively the method and its limitations using a wide range of sample types and trying several fixation and conservation alternatives. We evaluated simplification of some steps of the method, seeking the best compromise between precision and the quality of distinction of HF from bacteria and phytoplankton in the cytograms. We found that a flow rate of 120-220 µL min^{-1} without using a syringe-pump enhanced machine modification, and running times of 8-10 min allowed enumeration of HF even at values below 10^2 cells mL^{-1}. SYBR Green I, at final concentrations of 1:10000 and a minimum staining time of 10 min at room temperature in the dark, was adequate for staining and detecting HF. No significant differences were found between cell numbers obtained from freshly analyzed samples and those previously frozen in liquid-N. FC and epifluorescence microscopy (EpiM) were in good agreement and FC yielded lower variability between replicate samples than EpiM. One limitation we encountered was that, in the presence of large bacteria and/or bacterial aggregates, enumeration was difficult. However, in absence of bacterial aggregates samples with Bact/HF ratios > 1000, HF could be well-enumerated.

Tiny planktonic microbes, autotrophs and heterotrophs < 100 µm in size, represent the bulk of biomass production and dominate biogeochemical cycles in the world's oceans. Related with their small size, they have generation times in the order of several hours to a few days. To track them in space and time scales commensurate to their dynamics, development of techniques for the rapid detection and enumeration of microbes has been one of the major objectives of marine biologists. Flow cytometry (FC) is now routinely used for rapid, easy, and accurate analysis of eukaryotic and prokaryotic autotrophs on the basis of their distinct pigments (e.g., Olson et al. 1993; Li 1994; Marie et al. 2000), and of heterotrophic bacteria (Robertson and Button 1989; Monger and Landry 1992; Li et al. 1995, Marie et al. 1996; del Giorgio et al. 1996; Lebaron et al. 1998; Troussellier et al. 1999) and viruses (Marie et al. 1999; Brussaard 2004) using a variety of fluorochromes. One of the major advantages of highly sensitive stains, such as SYBR Green I, is that it can be used with fixed samples analyzed in compact flow cytometers that can be employed on ships. Consequently, its usage has become routine in oceanographic studies.

Heterotrophic flagellates (HF), mainly between 2 to 5 µm in size, occur in marine waters at densities of 10^2-10^3 mL^{-1} and dominate heterotrophic nanoplankton in numbers and in biomass. HF, through their grazing activity, play a crucial role in

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heterotrophic bacterial C transfer toward higher trophic levels (e.g., Sherr and Sherr 1994) and in nutrient remineralization (e.g., Caron and Goldman 1990). HF also graze on autotrophic cells, both prokaryotic and eukaryotic. Relatively little attention has been paid to the role of heterotrophic flagellates as grazers of <5 µm phytoplankton, although there have been a number of papers focusing on this role over the last couple of decades (Sherr and Sherr 1992, 1994, 2002; Sakka et al. 2000; Christaki et al. 2001, 2005). The additional trophic role of HF as herbivores makes them even more important in marine waters. Detection and enumeration of fixed heterotrophic protists with flow cytometry has been possible, mainly in cultures, with nucleic acid stains such as SYBR Green I, SYTO 13, TO-PRO1, and YOYO 1 (e.g., Zubkov et al. 2001; Lindström et al. 2002; Guindulain-Rifà et al. 2002; Zubkov and Sleigh 2005). Additionally, LysoTracker Green, a fluorescent stain that has affinity for acidic organelles (Rose et al. 2004; Sintes and del Giorgio 2010; Heywood et al. 2010), has been proposed for counting live cells. However, the Lysotracker protocol is impractical for large-scale studies involving many samples, as changes in HF concentrations occur within a few hours in live samples (Gifford and Caron 2000). For that reason, for routine use on environmental samples which require sample fixation, the nucleic acid approach is preferable.

In practice, the FC analysis of HF in open ocean samples has proven difficult. There are two major problems: (1) The separation of heterotrophic protists from other heterotrophs or autotrophs, and (2) the relatively low concentrations of HF given the working limits of common cytometers (lower limits ca. 100–1000 mL^{-1}, e.g., Shapiro 2003, Gasol and del Giorgio 2000).

To address the latter problem of relatively low HF concentrations in natural samples, tangential flow filtration or centrifugation, while perhaps adequate for prokaryotes (e.g., Porter et al. 1993; Wallner et al. 1997), can result in significant loss of cells in the case of fragile protists. A significant advance was reported in 2007 when Zubkov et al. published data of spatial and vertical distribution of HF in oligotrophic oceanic waters using FC. Their approach relied on increasing the sample flow with a syringe pump (Zubkov and Burkill 2006), but the fixation, storage, and staining methods used were similar to those routinely applied for bacteria and phytoplankton. However, in contrast with bacteria protocols that have become widespread, this HF protocol, which also uses bench top flow cytometers, has not become common. This is perhaps due to its requirement of an additional pump. The aim of our study was 2-fold: First, to experiment with the fixation, storage, staining conditions, and sample flow used by Zubkov et al. (2007) to justify and determine the most effective and efficient combinations. Second, to determine the limits of the protocol, by testing it in a wide range of samples ranging from very low concentrated ones, such as deep ocean samples, to those with high particle concentrations and large bacterial cells.

Materials and procedures

Origin of samples

The samples used in the different tests came from natural environments or laboratory manipulations. Coastal samples were collected from the stations SOLA (42°29′300N, 03°08′700E, 27 m depth), Blanes Bay Microbial Observatory (BBMO, 41°40′N, 02°48′E; 20 m depth) in the NW Mediterranean, and SOMLIT (50°40′75 N, 1°31′17 E, 25 m depth) in the Eastern English channel. Open ocean samples were collected from stations MOLA (42°27′330N, 03°32′665E, 600 m depth) and Station MD (40°49′74 N 02° 26′80 E, 1706 m depth) in the MicroDeep cruise in the NW Mediterranean.

To test the upper and lower limits for FC detection relative to EpiM, to evaluate the interference by large bacteria, bacterial aggregates and inorganic particles, and to compare replication of counts by FC relative to EpiM, the following manipulations were performed:

Unamended incubations to promote the development of HF, prepared by gravity filtration of surface seawater from BBMO through a 3-µm poresize polycarbonate filter and incubation of the filtrate at in situ temperature in the dark, as in Massana et al. (2006)

Enrichment incubations to promote the development of bacterial aggregates along with HF, prepared by gravity filtration of 5 L of 24 m depth water from the SOLA station through 5 µm Nuclepore filters followed by incubation in the dark of the filtrate enriched with 75 mg L^{-1} marine broth.

Fractionated replicates to test the lower detection limit for FC and the coherence with epifluorescence counts. These were prepared with 3 m depth SOLA whole seawater and the < 10, < 5, < 2 and < 1 µm fractions after gravity filtration through Nuclepore filters.

Inorganic particle addition to observe the degree of interference of inorganic particles with flow cytometry analyses and compare FC and EpiM counts, established by the dark incubation of 3 m SOLA water filtered through 10 µm Nuclepore filter in 200 mL triplicate bottles. Zero (control), 1, 10, and 50 mg L^{-1} of commercially available kaolinite ($Al_2O_7Si_2.2H_2O$) particles (Sigma-Aldrich, product reference: 03584) were used as a model aluminosilicate clay particle. All particles were smaller than 45 µm with a mean particle size of 2.1 µm.

Testing fixation and storage conditions

Individual subsamples (5 mL for cytometry and up to 100 mL for epifluorescence microscopy) were fixed at a final concentration of 1% of fixative for 2-4 h at 4°C in the dark before analysis or freezing. Particle-free neutralized formaldehyde (FA) was prepared from borax-buffered 37% formaldehyde (FA), then passed through 0.2 µm cellulose filters. FA was kept in the dark at room temperature. Electron microscopy grade distillation purified 25% glutaraldehyde (GA) was kept at ~4°C in the dark. GA was obtained in individual 10-mL sealed glass tubes, which guaranteed the best storage conditions.

Paraformaldehyde (PFA) used here corresponded to the solution routinely used for bacterial fixation (e.g., Pernthaler and Amman 2004). PFA preparation yields 250 mL of a 20% PFA solution in 10 mM sodium phosphate buffer. PFA was heated to ~60°C on a stirring hot-plate until it formed a solution (~40 min). The cooled solution was then passed through 0.2 µm polypropylene filters, dispensed into 10 mL tubes and stored in the freezer. Before its use, PFA was thawed at room temperature. FC and EpiM counts were also compared relative to fixation and storage conditions.

Staining conditions

Concentration kinetics were tested with 1:10000 (Lebaron et al. 1998), 1:5000 (Zubkov et al. 2007) 1:2500, and 1:1250 final SYBR Green I by SYBR Green I from an initial stock diluted to 1:10 with DMSO. Time of staining was tested on GA-fixed samples. Samples stained for over 1 h were kept at ~4°C in the dark and returned to room temperature 15 min before analysis. For optimization of the staining solution the addition of potassium citrate 30 mM final concentration (Lebaron et al. 1998, Zubkov et al. 2007) was tested. Samples from 3 to 220 m depth (MOLA station) fixed with FA or GA, fresh or stored in liquid-N, were analyzed in presence or absence of potassium citrate (30 mM final concentration). To minimize the dilution of our sample with the potassium citrate addition, the best compromise that we found was to add 30 µL per mL of sample of a 1M stock solution.

Flow cytometry (FC) adjustments

Samples were analyzed with a FACSCalibur flow Cytometer (BD-Biosciences) equipped with an air-cooled argon laser (488 nm, 15 mW). One of the fixed flow rates of the FACSCalibur was increased from ~10-40 to ~220 µL mn^{-1}, which allowed analysis of 1-2 mL sample within 5-10 min (we chose to modify the "Medium" rate). The flow rate modification was adjusted using the corresponding potentiometer to modify the differential pressure applied to the sample surface, thus modifying flow rate. Bead solutions (1.002 µm, Polysciences) were used to adjust and calibrate the flow rate increase, and bead CV's (coefficient of variation) measured on fluorescence histogram were used as indicator of *correct analysis*: i.e., increased flow rates imply that more particles cross in front of the laser with misalignment due to the degradation of laminar fluxes between the sheath and the sample liquid into the FC flow cell. A way to measure the degree of that problem is by analyzing the CV of the fluorescence detection of a set of particles that all have the same fluorescence (i.e., fluorescence reference beads). Up to 220 µL mn^{-1}, the bead-fluorescence CV were below 9%, a value which was considered as the highest limit acceptable.

The HF protocol for FC analysis was modified from the standard one used to count bacteria in marine environments (e.g., Marie et al. 1997, 2000; Bouvier et al. 2007). The main modification was that a reduction of the fluorescence detector voltage was applied on side light scatter (SSC) and green fluorescence (FL1 530 + 15 nm) photomultipliers (PMT), so that fluorescence particles of the bacteria type would be mostly below the detection threshold (as in Guindulain-Rifà et al. 2002). We set up the acquisition so that in environmental samples only a part of the HNA (high nucleic acid) bacteria were visible within the first two decades of a SSC versus FL1 dot-plot, while HF and nanophytoplankton occupied the 2 other decades. Low voltages applied on the PMT present also the advantage of avoiding physical and electronic coincidences during FC analysis, which are due to the high natural bacteria concentrations and the high speed sample flow rate used (220 µL mn^{-1}). Triggering was applied on FL1, with a threshold value at 125 and 300 volts on the FL1 PMT (530/30 nm). A secondary threshold at 54 was placed on SSC, with 280 volts applied on the PMT. Finally, to discriminate autotrophic large cells, red fluorescence (FL3, > 650 nm) was collected with 400 volts on the FL3 PMT (LP 670 nm). These settings need to be understood as an orientation only, as they will vary with machine types; and even within the same type, will vary between individual machines.

FL1 and SSC cell properties were acquired with log amplification on a four- decade scale and data were analyzed using Cell-Quest software (BD-Biosciences). Fluorescent 1.002 µm beads were analyzed simultaneously with the samples to normalize cell fluorescence and light scatter emissions, thus allowing comparison of results. To estimate cell concentration, the exact volume of sample analyzed was determined by measuring sample volumes before and after analysis.

HF cell sorting

A FACSAria cell sorter (BD-Biosciences) was used to group live and fixed HF stained by SYBR Green I. The excitation-light was 488 nm from the argon laser to differentiate both auto- and heterotrophic cells. The FACSDiva software (BD-Biosciences) was used for cell sorting and data acquirement. Acquisition settings performed on a four-decade scale, were set to discriminate SSC and fluorescence emissions showing a pattern similar to when acquired with the FACSCalibur analyzer. The green fluorescence (FL1) of the SYBR Green I stained HF was collected at 530/30 nm, while a red PMT (>655 nm) was used to collect signals from autotrophic cells, with the trigger on FL1 and a threshold at 200. PMT were set at 380, 380, and 560 volts for SSC, FL1, and FL3, respectively. The sheath fluid was prepared from fresh sterilized particle-free seawater, passed through 0.2 µm (Stericap, Whatman). Analysis and cell sorting were made using a 70 µm nozzle, with a sheath pressure of 70 psi and a sample flow fixed at 11. The real sample flow rate was measured using BD Trucount Tubes (lot 43385, BD-Biosciences), and evaluated at 84.4 ± 1.7 µL mn^{-1} (n = 22). Sorting precision mode was 0/32/0 and total event rates per second during sorts were generally below 1000. According to HF concentration, one or two sorting-ways were simultaneously used, allowing isolation of one or two different populations with 1000 to 3000 sorted cells in sterile vials. HF

fractions were carefully collected on Nuclepore filters (25 mm, 0.2 µm) stained with DAPI, and examined with a BX 61 Olympus microscope equipped with an Olympus DP72 digital camera.

Epifluorescence microscopy

To enumerate heterotrophic flagellates (HF), samples (5-100 mL) were preserved using FA, GA, or PFA at final concentrations of 1%. Samples were filtered onto black Nuclepore filters (poresize, 0.8 µm) and stained with DAPI (Porter and Feig 1980) within 5 h sampling and stored at –20°C until counting. HF were enumerated using a AX-70 Olympus or a BX-61 Olympus microscope at 1000x. To distinguish between autotrophic and heterotrophic flagellates, autofluorescence (chlorophyll) was determined under blue light excitation.

The bacterial abundance counts SOLA and SOMLIT stations were provided by the National Coastal Observation Network (France). Bacterial concentrations in incubation experiments and open Mediterranean Sea stations were obtained from counts of material retained on 0.2 µm black filters after DAPI staining as is described for HF.

The statistical tests presented in this article were performed using Excel-STAT.

Assessment

Cytometric groups detected by FC

Group detection with the FC protocol used here was conducted as follows: A first dot-plot of SSC versus FL1 (Fig. 1a) shows all organisms stained by SyberGreen-I. The settings are chosen so that most bacteria are left out in the lower and left

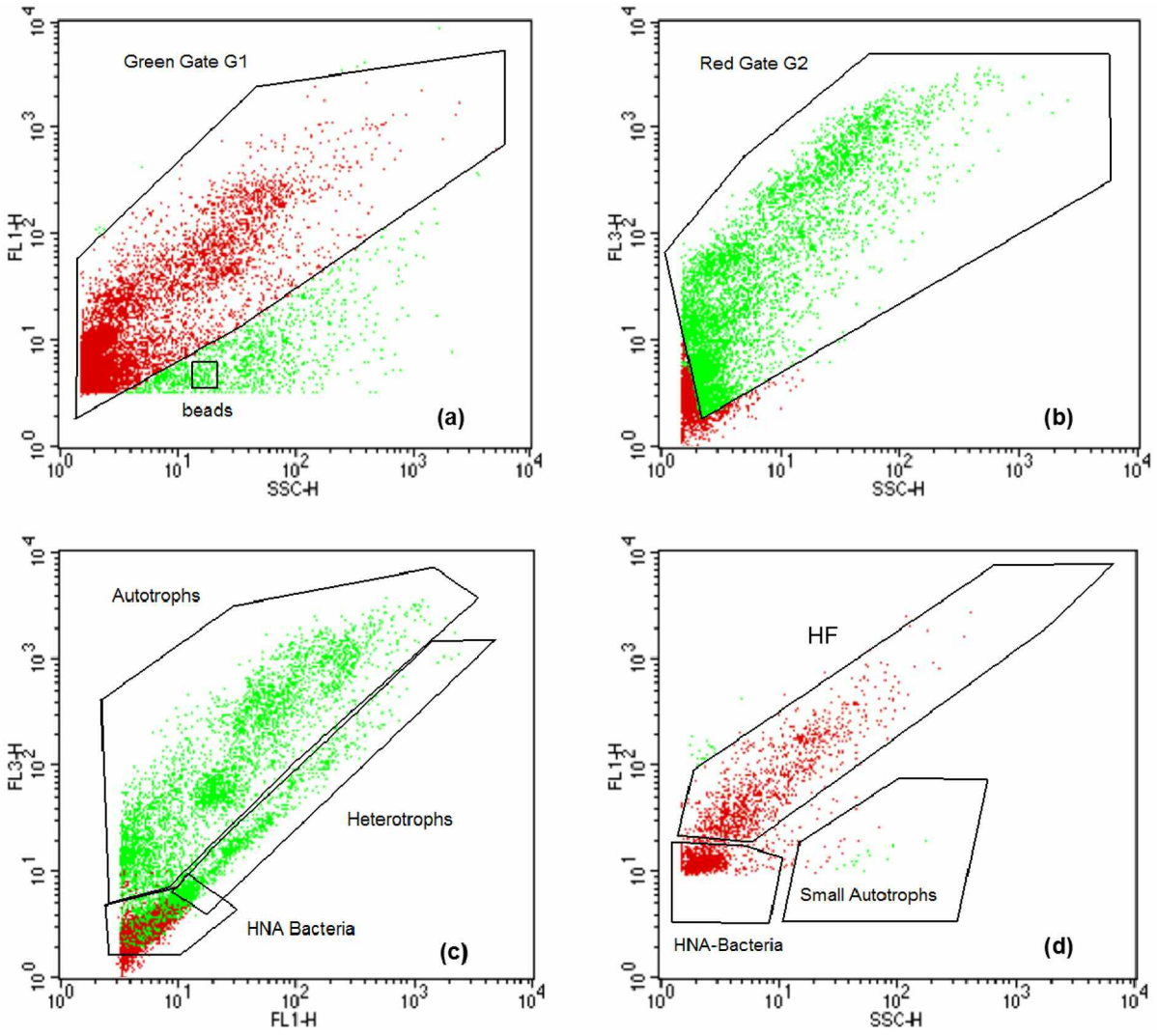

Fig. 1. Detection of heterotrophic nanoflagellates with the flow cytometric protocol: (a) Side scatter (SSC) versus green fluorescence (FL1) showing all organisms stained by SyberGreen I and the establishment of G1, a "Green gate." (b) The data falling inside gate G1 are represented in an intermediate plot (G2) of SSC versus red fluorescence (FL3). (c) The G2 data are then plotted in a graph of FL1 versus FL3 that allows a clear discrimination of the autotrophic cells (gate "Autotrophs") from the heterotrophic population (gate "Heterotrophs," including a small part of HNA Bacteria and the HF). (d) Finally, the data from gate "Heterotrophs" are represented again in a SSC versus FL1 which permits to better discriminate HF (gate "HF") from large bacteria (HNA Bacteria).

side. A gate (G1, Fig. 1a) leaves out noise (high SSC and low FL1) as well as the 1 µm beads. At this point, the G1 gate in its lower part can cross through the bacteria points without real separation of bacteria and other particles, and as a result, it includes some of the larger or more fluorescent bacteria (e.g., HNA) and all larger auto- and heterotrophic cells. The data gated in the first step is represented in an intermediate plot of SSC versus FL3 (Fig. 1b), allowing the discrimination of autotrophic and heterotrophic cells. Data inside this second 'red' gate, G2, is presented in a third dot-plot, this time of FL1 versus FL3 (Fig. 1C), which allows a clear discrimination of the autotrophic cells from the heterotrophic population. Note that the HF are located in the prolongation of the largest bacteria. Whereas HF enumeration can be made at this stage (within a third gate, G3), we recommend drawing a last dot-plot, of all the data in gate G3 represented again in a SSC versus FL1 (Fig. 1d). This permits better discrimination of HF from bacteria. To discriminate large bacteria from true HF, it comes out as helpful to change the percentage of events visualized. By changing this number so that the two clouds are distinguishable, it is eventually possible to differentiate HF sub-populations according to their apparent size and green fluorescence (e.g., Fig. 1 in Zubkov et al. 2007).

Effect of fixative and storage

Preliminary experiments

We examined the effects of fixation protocols and sample storage on HF concentration using 15 samples fixed with the 3 fixatives. HF cells were counted using FC and EpiM. The fixation with paraformaldehyde (PFA) gave inconsistent results with our samples. A population appearing in the 'HF region' was always present on cytograms, but microscopic examination of the samples showed that in 9 of 15 samples used here the fixative had damaged the HF cells. The HF numbers obtained in these PFA samples were always much higher than the numbers in formaldehyde (FA) and glutaraldehyde (GA) treatments, most likely due to cell debris 'polluting' the region gated for HF. Of the few samples in which HF were well preserved with PFA, the cytograms provided very good discrimination, in particular between bacteria and HF. However, because of inconsistency of results, we decided not to consider PFA any further in this study. The debris problem with PFA may well be a result of mixing phosphate buffer with seawater. We observed that phosphate often precipitates in the presence of 'stronger salts' and consequently produces colloids which interfere with the FC analysis.

The FC counts in each sample were also compared related to their different storage conditions (Fig. 2). In both GA and FA, fixed samples the HF numbers obtained from 'fresh' samples and from liquid-N frozen samples were similar, whereas samples frozen directly at –80°C showed slightly lower numbers (Fig. 2). Even so, for both GA and FA (Fig. 2), there were no significant differences between EpiM and FC counts (all types of storage considered, ANOVA, $P > 0.523$).

To test the losses of cells over long periods of storage, we analyzed six open Mediterranean water samples. These were fixed with FA 1%, frozen in liquid nitrogen, and stored at –80°C for 2.5 y. Comparing the HF numbers obtained initially by EpiM with those obtained by FC 2.5 y later, the losses of HF were of the order of 50% to more than 70%.

Comparison of FA and GA fixed samples

Fixatives were compared through the analysis of 42 samples taken from 24 m at the SOLA station, from 8 Jun 2009 to 17 May 2010. They were fixed with FA or with GA, and in most cases, analyzed within 6 h of fixation. For the period between Aug 2009 and Feb 2010 they were liquid-N frozen, stored at –80°C, and analyzed 7 months later. We found no significant differences between the 2 fixatives (paired *t* test, $P = 0.66$, a = 0.05) and the regression relationship of FA and GA was significant ($r^2 = 0.73$, GA = 235(± 275) + 0.86(± 0.152) FA, $r^2 = 0.77$ $P < 0.0001$, standard error in parenthesis).

Staining conditions

For the different concentrations of SYBR Green I tested, the HF numbers identified by FC in duplicate varied little (890 ± 80 HF mL^{-1} mean ±SD); the CV was 8.5%, and there was no apparent trend. HF numbers in triplicate samples stained from 5 min to 21 h (at SYBR Green I concentration 1:5000) varied little (950 ± 70 HF mL^{-1}, mean ± SD, $n = 13$) and the CV was 7.5%, with no apparent trend. However, the separation between the bacteria and HF signals was clearer after 10 min staining, which can be considered as the minimum staining time.

Furthermore, the HF numbers estimated by FC in absence and presence of potassium citrate were very similar for the 2 series of samples (mean ± SD, 870 ± 250 and 850 ± 290, respectively, paired *t* test $P = 0.59$, a = 0.05).

Interestingly, the analysis of side scatter (relative SSC) and fluorescence properties (relative green fluorescence, FL1)

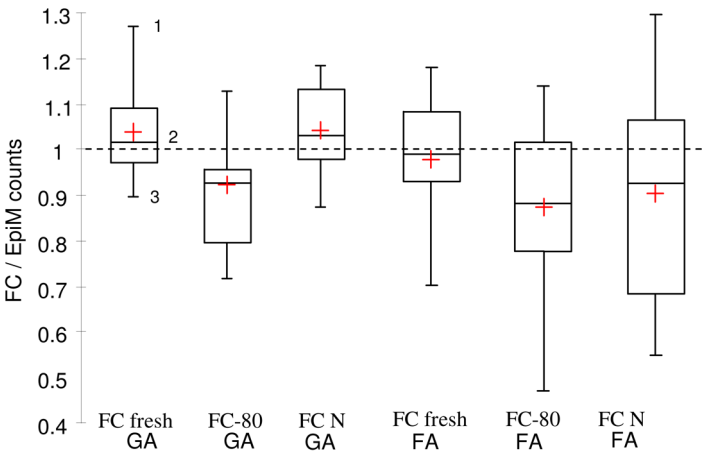

Fig. 2. Box plots of HF mL^{-1} abundance determined with flow cytometry relative to that determined using epifluorescence microscopy (EpiM). The dashed line shows equivalency to EpiM abundances. Cell numbers in these samples ($n = 15$, from 3 m at the SOLA station) varied from ~1000 to ~2200 HF mL^{-1}. Samples, fixed with glutaraldehyde (GA), and formaldehyde (FA) were stained with SYBRGreen I and analyzed by FC within 6 of fixation (FC fresh), frozen directly in –80°C (FC –80) and frozen in liquid Nitrogen (FC N). On box plots: crosses = mean value, 1 = maximum, 2 = median,3 = minimum.

showed that GA fixation generated higher SSC and FL values than FA (Fig. 3). Whatever the fixation used, the frozen samples showed higher scatter and fluorescence values than the samples analyzed within a few hours of fixation (Fig. 3). As a result, we found that side scatter and fluorescence were more affected by sample treatment, in terms of fixative used and freezing treatment, rather than SYBR Green I concentration or staining time.

The range of HF concentrations that is detectable by FC

Effect of inorganic particles:

The eastern English channel is a meso-eutrophic coastal ecosystem influenced by considerable physical forcing (tides, winds). Inorganic particles and detritus in these samples appeared below the region gated for SYBR Green I (G1 in Fig. 1) due to their lower fluorescence. The same was true for the kaolinite particles (inorganic particle addition) that were added to a concentration up to 50 mg L^{-1}. Particles did not impair HF detection.

Effect of large bacteria and bacterial aggregates

In the incubation enriched with marine broth, the presence of large bacteria, and in particular, that of bacterial aggregates, prevented accurate distinction of HF. In fact, because HF are located in the prolongation of the largest bacteria (Fig. 1c), HF and aggregates appeared together in the region gated for HF. This resulted in marked overestimations of HF abundance, 2-fold—or even more—higher than the ones obtained with EpiM. As expected, the first 2 d of incubation were characterized by low HF and high bacterial numbers (on the order of 10^2-10^3 and high 10^7 mL^{-1}, respectively) while days 3 and 4 were characterized by high HF numbers of the order of 10^4 and bacterial aggregates to which HF were often attached. From day 5, the aggregates were less abundant and HF numbers counted by EpiM and FC were 8×10^4 and 9×10^4 mL^{-1}, respectively. Consequently, only the numbers of days 6 and 7 of this experiment are included in the EM versus EpiM comparison shown below.

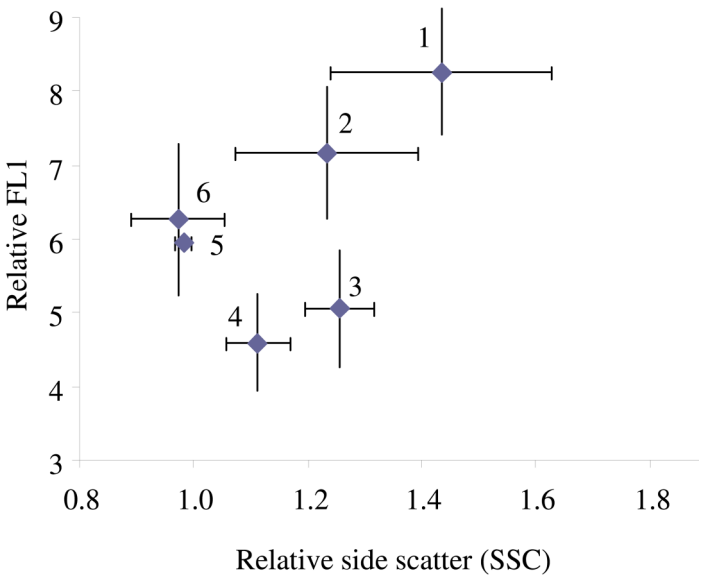

Fig. 3. Normalized light scatter and green fluorescence from HF with different fixative and preservation treatments: 1 = Glutaraldehyde (GA) 1%, stored in liquid nitrogen; 2 = Formaldehyde (FA) 1%, stored in liquid nitrogen ; 3 = GA 1%, analyzed after 3-5 h of sampling; 4 = FA 1%, analyzed after 3-5 h of sampling; 5 = GA 1%, stored in liquid nitrogen + potassium citrate (30 mM final); 6 FA 1%, stored in liquid nitrogen + potassium citrate (30 mM final). Error bars: sd, n = 11.

Lower limit of detection and replication

We had no problem in detecting and enumerating HF at 1500 m in the bathypelagic realm of the NW Mediterranean, with ca. 90 HF mL^{-1}. In another test, we size-fractionated a sample through a series of polycarbonate filters (Fig. 4). After fractionation through 2 µm, we enumerated less than 100 HF mL^{-1}, while the lowest number of HF detected by FC was 10 cells mL^{-1} in the < 1 µm fraction. Incidentally, the serial size-fractionation demonstrates the possibility to estimate the average cell size of the population based on cell size distribution (here estimated as ~2.4 µm). The difference between the 2 replicates counted by FC varied from 3 to 40 cells, whereas by EpiM this difference was 100-200 cells, suggesting a better replication for FC counts (Fig. 4).

Counts of replicate samples were also compared between FC and EpiM for the 'inorganic particle experiment.' In this case, we had 3 replicates of each treatment (3 additions + 1 control) and 3 sampling times (0, 48, 96 h) yielding 12 triplicate observations and HF ranged from ~ 600 to 2500 cells mL^{-1} over the incubation. The CV between triplicates was systematically higher for EpiM counts (amplitude 52%, mean 18%) than for FC (amplitude 24%, mean 8%).

Comparison of counts by EpiM and FC

The comparison of HF abundance in 79 samples (obtained over an annual cycle at the SOLA station) fixed with GA and counted by EpiM and FC (Fig. 5) showed that the counts were similar with a regression of FC = 310(± 190) + 0.79(± 0.110)*EpiM (n = 79, r^2 = 0.82, P < 0.0001). Looking at all the samples analyzed by EpiM and FC in this study (n = 142), the range was from 10^1 to10^4 HF mL^{-1} and the regression relation-

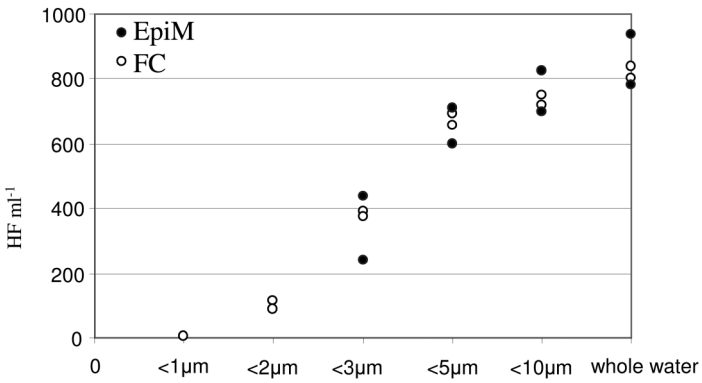

Fig. 4. Duplicate counts of HF numbers in fractionated seawater enumerated by EpiM (Epifluorescence Microscopy) and FC (Flow Cytometry). The <2 µm fraction was below the detection limit for EpiM.

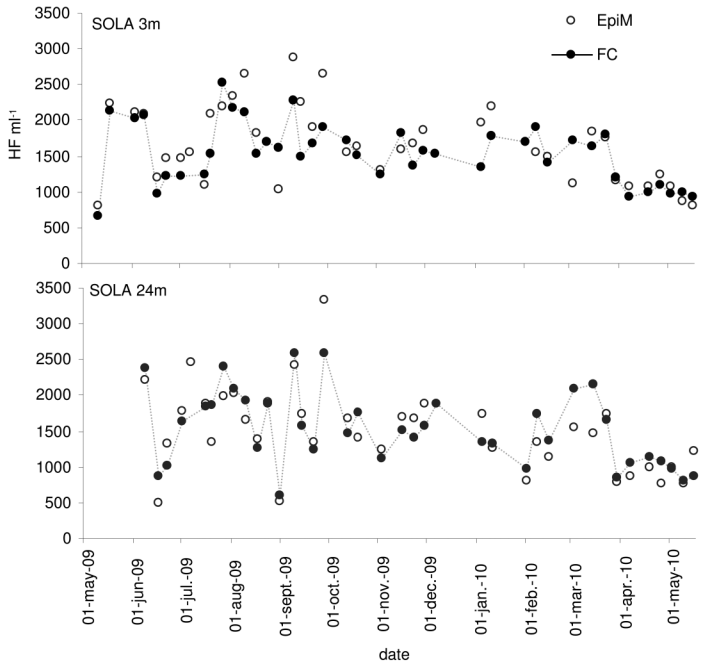

Fig. 5. Temporal variability in 2009-2010 of heterotrophic nanoflagellate (HF) abundance at 3 and 24 m depth at the SOLA station (NW Mediterranean) determined using EpiM (epifluorescence microscopy) and FC (flow cytometry).

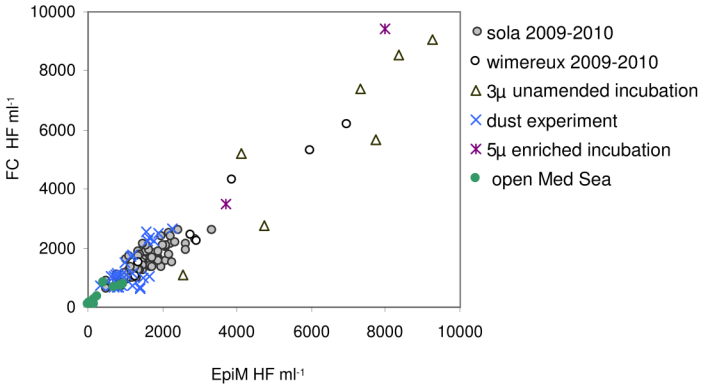

Fig. 6. Comparison of EpiM (epifluorescence microscopy) and FC (flow cytometry) counts. SOLA: coastal Mediterranean annual cycle; Wimereux: coastal E English channel; 3 µm-filtered unamended incubation to promote HF growth; dust experiment: Inorganic particle addition experiment, 10 µm-filtered seawater incubation with kaolinite dust added; 5 µm-filtered enriched incubation: 5 µm-filtered seawater enriched with marine broth; Open Med Sea: Vertical profile down to 1500 m depth at the open oligotrophic Mediterranean Sea. For details, see the *Materials and procedures* section.

ship was FC = 100(± 110) + 0.94(± 0.05)*EpiM, (r^2 = 0.92, P < 0.0001, Fig. 6). Neither the slope nor the intercept were significantly different from 1 and 0, respectively.

Inter-operator variability

Three of the co-authors of this paper independently gated HF on a selection of 50 different samples used in this study, with a range from 10^1 to 10^4 HF mL^{-1}. For cell concentrations up to 2-3 10^3 HF mL^{-1}, the CV was <16%. The differences between users increased for some samples containing higher HF numbers, reaching a maximum CV of 33%. The samples where the higher differences between the 3 users occurred were in those containing large bacteria, and in those where there was some degradation of the autotrophic chlorophyll signal.

Cell sorting

Two populations of HF-1 (smaller) and HF-2 (bigger) were sorted (Fig. 7). The visualization under an epifluorescence microscope, after DAPI staining, revealed that the sorting did not cause damage of cells: we could observe flagella and the typical microvilli collar of choanoflagellates. The HF-2 sorting resulted in almost exclusively HF, while in the HF-1 population, some bacteria were also present on the filter.

Discussion and recommendations

We started from the protocol described by Zubkov et al. (2007). Our objective was to adapt it for the easy handling of a large number of samples, particularly while working onboard. We also intended to determine the best compromises for fixation and storage conditions in terms of their impact on the abundance of HF, as well as the distinction capacity of HF from bacteria and phytoplankton on the cytograms. The procedure is summarized in Table 1.

In the original protocol a syringe pump was used to attain higher sample flow rates (up to 1 mL min^{-1}) to count specific groups of nanophytoplankton (Zubkov and Burkill 2006) and HF (Zubkov et al. 2007). However, as cytometers are rarely used solely for HF counting, this usually involves the installation and removal of the syringe each time HF samples are to be analyzed. In this case, the use of a syringe pump slowed down the process of analysis. Furthermore, at high flow rates (above 250 µL min^{-1}), the detection of beads, commonly used as internal standards, becomes difficult, as apparent bead variability increases (see "Flow cytometry adjustments," Zubkov and Burkill 2006). One should be aware that at high flow rates, the signal of the beads deteriorates along with that of the cells themselves, making more difficult the separation of the different populations. We found that increasing one of the fixed flow rates on the FACSCalibur to ~220 µL min^{-1} gave satisfactory results. We set the MEDIUM position of the FACSCalibur to this flow rate as the MEDIUM position is often not used and, thus, once modified can be more or less permanently dedicated to HF analysis.

FA and GA are the most commonly used fixatives for epifluorescence analysis of marine flagellate samples (e.g., Sherr and Sherr 1993). We did not find significant differences between glutaraldehyde and formaldehyde in terms of HF numbers. GA fixation is reported to be best for fixation of flagellates, at least in grazing experiments (e.g., Pace et al. 1990; Caron et al. 1999), because it minimizes ingested particle loss from cells (e.g., Bloem et al. 1986; Sanders et al. 1989). Fur-

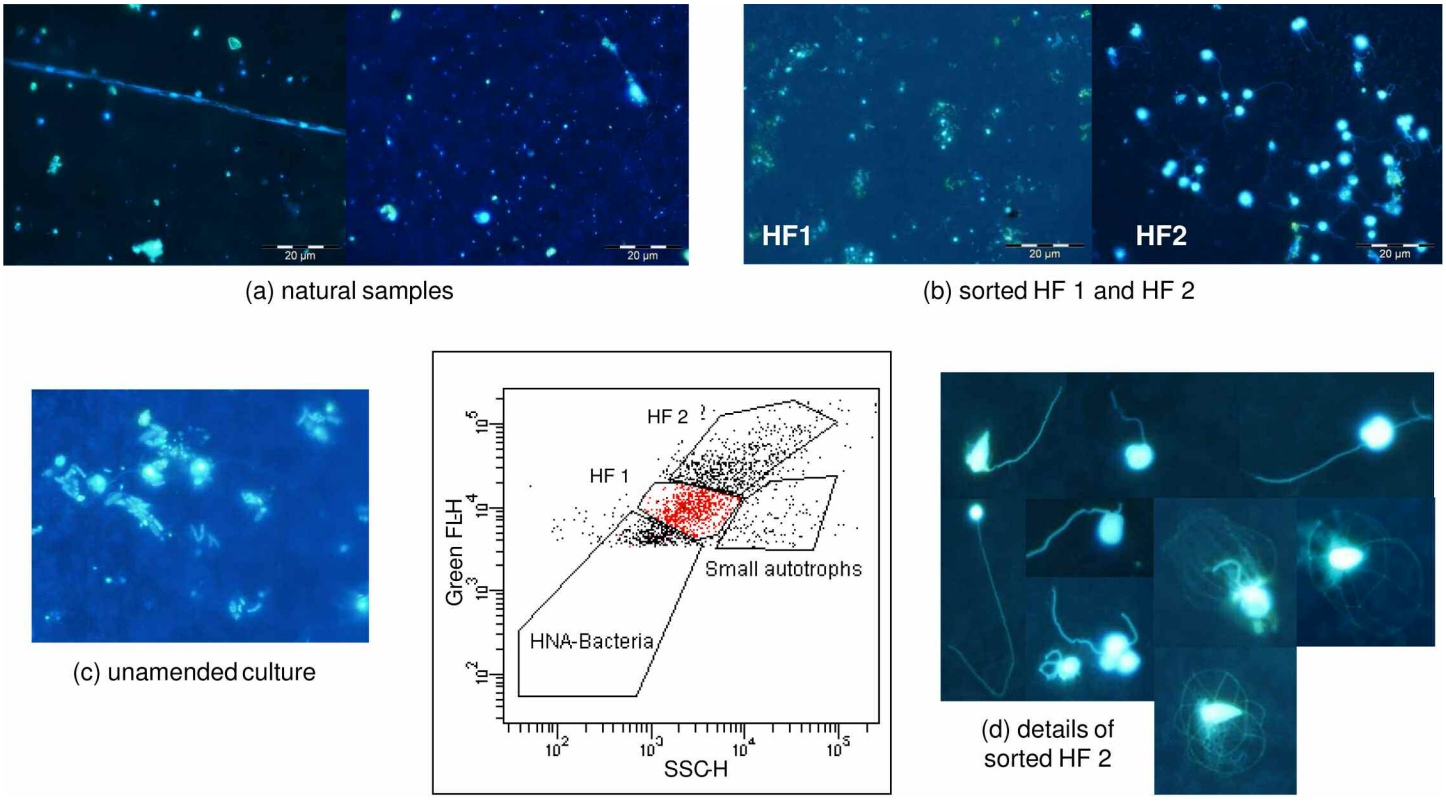

Fig. 7. Characteristic flow cytometric signature of SYBRGreen I–stained cells obtained with the FacsAria BD cell sorter. Two populations of HF-1 (small) and HF-2 (big) were sorted. The pictures show cells observed by epifluorescence microscopy. The original sample was fixed with glutaraldehyde or formaldehyde 1% final concentration. Images on the left show DAPI-stained preparations from the sample before sorting, and on the right, sorted DAPI-stained organisms. (a) Natural sample: MOLA open Mediterranean station whole seawater; (b) sorted cells from sample a; (c) unamended culture: Blanes, oligotrophic coastal Mediterranean seawater, gravity-filtered through a 3 µm pore-size polycarbonate filter and incubated for 4 d in the dark before sorting; (d) sorted cells from sample c. Note the conservation of cell structure and flagella after sorting and the absence and presence of large bacteria in the HF-1 and HF-2 populations, respectively.

Table 1. Summary procedure for HF analysis with FC.

Sampling
Collect 2-5 mL seawater.
Fixation: 1% final v/v, preferentially glutaraldehyde. Formaldehyde, or freshly prepared PFA diluted in seawater, can also be used.
Preservation: preferably analyze fresh samples. Alternatively, freeze in liquid nitrogen and keep samples at –80°C. Analyze within the next few months. Thaw samples at room temperature.
Stain: Minimum concentration of SYBR Green I should be 1:10000 final. Stain for at least 10 min in the dark at room temperature.
Use 1.0 µm beads as internal standard.
Flow cytometer
All the data in gate G3 represented again in a SSC versus FL1. Modify the heterotrophic bacteria protocol by reducing fluorescence detector voltage and the SSC (cf. Fig. 1).
Use a flow rate of 120-220 µL min^{-1}. If necessary, the flow rate of the machine should be modified (cf. supplementary material).
Verify the CV of the fluorescent beads.
Data acquisition from 5-10 min depending on the flow rate and the expected cell concentration.
Recommendations
Avoid freezing directly at –80 and/or storing samples for long periods.
Occasionally check samples by microscopy—in particular, those where large bacteria, aggregates, or degraded phytoplankton cells might be present.

thermore, GA is the most commonly used fixative for FC analysis of bacteria (Marie et al. 1999, 2000), viruses (Brussaard 2004), and phytoplankton (Vaulot et al. 1989). However, GA fixation increases background autofluorescence of cells. Such autofluorescence can interfere with subsequent cell analyses. For example, it can reduce the sensitivity of cell identification using fluorescence in-situ hybridization. On the other hand, PFA fixation does not increase cell autofluorescence making the fixed cells usable for a broader range of downstream analyses. Therefore, Zubkov et al. (2007) fixed HF with freshly prepared PFA dissolved in seawater (1% final concentration), although PFA is not a common fixative for HF. In fact, different protocols for preparing PFA are used in different laboratories worldwide, and sometimes it is difficult to compare them as not enough detail is given in the "*Materials and procedures*" section of most articles. From the experience gained with this work we can make two suggestions: First, it would be good practice if in future studies all authors would specify exactly how they prepared their fixatives, because this is obviously an important variable. Second, if PFA is used for HF fixation, it is compulsory that it is freshly prepared (not frozen) and diluted in filtered seawater (Zubkov et al. 2007). With regard to the effects of storage of fixed samples, the best results were obtained from freshly analyzed samples (Fig. 2), although this is rarely possible during field studies where samples have to be stored for later analysis. For phytoplankton, storage in liquid N minimizes loss of cells (Vaulot et al. 1989). But direct freezing at –80°C was associated with apparent loss of HF cells (Fig. 2). Negative or positive modifications of side scatters and fluorescence due to fixation and freezing have been previously reported and attributed to membrane modifications, better penetration of the fluorochrome in the cell, and/or electrochemical properties of the dyes (Vaulot et al. 1989; Troussellier et al. 1995, 1999; Lebaron et al. 1998). Interestingly, higher scatter and fluorescence values (Fig. 3) were seen in stored samples where lower concentrations of HF were detected (Fig. 2). We suggest that this implies that the small-sized HF (which would have lower scatter and fluorescence), would be the cells lost with storage.

As SYBR Green I is relatively expensive and potentially harmful, it is practical to use the lowest possible concentration for reliable staining. The 1:10000 final concentration used for bacteria (Lebaron et al. 1998) gave identical results in terms of HF numbers and fluorescence intensity at higher concentrations. The minimum staining time was 10 min at room temperature in the dark, suggesting that times of 10-15 min commonly used for heterotrophic bacteria and viruses are also adequate for HF. The addition of potassium citrate to our samples increased the scatter and fluorescence characteristics of HF (Fig. 3), but did not improve analysis in terms of HF numbers. In addition, it added an extra step to the procedure and involved some dilution of the sample. For the above reasons, potassium citrate is not strictly necessary for HF sample analyses.

Guindulain-Rifà et al. (2002) tried FC enumeration of HF in enriched seawater cultures and suggested that the application of their protocol required a Bact/HF ratio well below 1000. Overall bacterial numbers in our samples varied from $5\ 10^5$ to $2\ 10^6$ mL^{-1} and HF from 10^2 to 10^4 mL^{-1}. The ratio of numbers of heterotrophic bacteria versus HF numbers mL^{-1} over an annual cycle at 3 m at the coastal NW Mediterranean (SOLA) varied from 70 to 950. The ratio in the coastal English Channel waters (SOMLIT) was also within this range (360-820), while the maximum Bact/HF ratio in the unmodified <3 µm incubation of coastal Mediterranean water reached values of 1200. High Bact/HF ratios (from 580 to 2100) were observed in samples from a vertical profile of the open Mediterranean sea, where bacteria ranged from $5\ 10^4$ to $5\ 10^5$, yet HF were at 1-3 10^2 mL^{-1}. In the absence of large bacteria and/or aggregates, the Bact/HF ratio was not a limitation in the discrimination between bacteria and HF on the cytograms. By increasing the analysis time to 8-10 min, we were able to detect very low numbers of HF of the order of 20 HF mL^{-1}, which were not easy to enumerate with EpiM. For this reason, we consider that in the case of oligotrophic environments, low HF numbers are not a limitation for FC detection. In the presence of large bacteria and/or bacterial aggregates, the difficulty in discriminating HF from bacteria was due to the overlapping of small HF, large bacteria, and/or bacterial aggregates in the region gated for HF.

Some of the recent cytometer models, like the FACSCanto (Bd-Biosciences), provide a maximum sample flow of 120 µL min^{-1}, which is not easy to modulate. The recent acquisition of a FACSCanto (Bd-Biosciences) by the Banyuls laboratory allowed us to test this protocol on SOLA samples by increasing the time of analysis to 10 min obtaining a good discrimination of HF from bacteria and a good count replication (Bariat pers. comm.)

We found a good agreement between the HF counts obtained using FC and EpiM as previously observed for bacteria (Troussellier et al. 1995; Gasol et al. 1999; and many others). We also found that FC/HF counts varied less between replicate samples than EpiM counts, indicating that the method had higher precision. However, microscopic examination provides information on sizes and shapes of HF and allows detection of large bacteria and bacterial aggregates, which may interfere with HF enumeration by FC. In FC, operator subjectivity in the establishment of gates (i.e., Fig. 1) will always exist. Our examination of inter-operator variability found good agreement between three users for cell concentrations up to 2-3 10^3 HF mL^{-1}. However, there were notable differences between users for specific samples containing large bacteria, and in samples where there was some degradation of the autotrophic chlorophyll signal. This underlines again the utility of EpiM analysis to verify the strength of autotrophic chlorophyll fluorescence and the size distribution of bacterial cells.

With our protocol, cell sorting of nanoheterotrophs by FC was very successful, with microscopic observation of the sorted

cells revealing that their overall structure and flagella were well-preserved subsequent to sorting (Fig. 7), offering an excellent perspective for HF phylogenetic analyses (Yoshida et al. 2009; Heywood et al. 2010). An important remaining limitation of cell sorting is that, because of the relatively low concentration of HF, it is time consuming, in addition to PCR being difficult on aldehyde-fixed samples (e.g., Douglas and Rogers 1998). We also found that concentrating live samples with different devices was not an ideal solution, as it generally resulted in only a very moderate increase in cell concentration, of the order of ×1.5 to ×2 fold (cf. Yoshida et al. 2009). Also, it almost certainly alters sample diversity by damaging the most fragile cells. From our experience, when live cells have to be sorted, it is better to do so directly from natural samples without prior manipulation (e.g., Heywood et al. 2010).

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