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Flow Cytometric Detection and Quantification of Heterotrophic Nanoflagellates in Enriched Seawater and Cultures

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Summary

A flow cytometric protocol to detect and enumerate heterotrophic nanoflagellates (HNF) in enriched waters is reported. At present, the cytometric protocols that allow accurate quantification of bacterioplankton cannot be used to quantify protozoa for the following reasons: i) the background produced by the bacterial acquisitions does not allow the discrimination of protozoa at low abundance, ii) since the final protozoan fluorescence is much higher than the bacterioplankton fluorescence (more than 35 fold) the protozoa acquisitions lie outside the range. With an increase in the fluorescence threshold and a reduction of the fluorescence detector voltage, low fluorescence particles (bacteria) are beneath the detection limits and only higher fluorescence particles (most of them heterotrophic nanoflagellates) are detected. The main limitation for the application of the cytometric protocol developed is that a ratio of bacteria/HNF below 1000 is needed. At higher ratios, the background of larger cells of bacterioplankton makes it difficult to discriminate protozoa. The proposed protocol has been validated by epifluorescence microscopy analyzing both a mixed community and two single species of HFN: *Rhynchomonas nasuta* and *Jakoba libera*. Taking into account the required bacteria/HNF ratio cited above, the results provide evidence that the flow cytometric protocol reported here is valid for counting mixed communities of HNF in enriched seawater and in experimental micro or mesocosms. In the case of single species of HNF previous knowledge of the biological characteristics of the protist and how they can affect the effectiveness of the flow cytometric count is necessary.

Key words: flow cytometry – epifluorescence – seawater – bacterioplankton – heterotrophic nanoflagellates – HNF – SYTO 13

Introduction

During last few years, the use of flow cytometry in combination with the increasing number of fluorochromes has provided rapid, easy and accurate detection and quantification of bacteria in aquatic systems (Vives-Rego et al., 2000). Flow cytometry is designed to detect and quantify high-density targets usually at concentrations above 10,000 encounters per ml. Since heterotrophic nanoflagellates (HNF) occur in marine waters at densities ranging between 0.3×10^3 and 4×10^3 cells per ml (Fenchel, 1982; Sherr and Sherr, 1984), no studies on the flow cytometric detection and quantification of aquatic protozoa are available. The nanoflagellate concentration and the proportion of nanoflagellates to bacteria in natural waters are below the working limits of cytometers. Some authors have circumvented these obstacles by concentrating the sample using tangential flow filtration (Porter et al., 1993), centrifugation (Wallner et al., 1997) or simple filtration (Urbach and Chisholm, 1998), prior to standard analysis. The use of molecular probes in low-density targets also suffers from low emission of the targeted cell and high background noise due to the long acquisition times. In addition, these alternatives are time consuming, reduce the precision of the measurements and, in the case of protozoa, may result in loss of cells due to the lysis caused by the concentration method.

The aim of this study was two-fold. First, to develop and optimize a cytometric protocol for the detection and quantification of HNF using a range of seawater enrichments. With an increase in the fluorescence threshold and

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a reduction of the fluorescence detector voltage, low fluorescence particles (bacteria) would be beneath the detection limits and only higher fluorescence particles (most of them HNF) might be detected. Second, to validate the cytometric counting protocol, parallel epifluorescence-cytometric counts were performed. Given the diversity of the seawater protozoan community, the successful detection of a given species may depend on its individual characteristics. Therefore, a mixed nanoflagellate community and two monospecific nanoflagellate cultures were used for the parallel epifluorescence-cytometric counts.

Materials and Methods

Sampling points and seawater incubations

Coastal water was sampled at the surface of the Mediterranean Sea in Barceloneta, the natural beach of Barcelona, 41° 24.2′N;2°13.3′E (Catalonia, northeastern Spain) and in Premià de Mar, 41° 29,2′N;2°21.5′E, a coastal site near Barcelona. Seawater was also sampled from the coastal water of the Bay of Biscay, 43°24.5′N;3°2.7′W (northern Spain). The water was immediately taken to the laboratory in 5 or 10 liter polyethylene containers. During the optimization of the cytometric protocol for HNF, three nanoflagellate enrichment procedures were tested: seawater incubation after the addition of (1) wheat grains, (2) TSB 5 mg · l^{-1} (Triptic Soy Broth, ADSA-Micro, Barcelona, Spain) and (3) by adding an *Escherichia coli* culture. In the seawater incubations with wheat grains, three Erlenmeyer flasks of 5 l were filled with seawater to one-third of their volume and 25 wheat grains were added; once the Erlenmeyers were autoclaved and cooled, 150 ml of fresh seawater were added to each. The seawater for the first flask was previously filtered through a 2 µm pore-filter (Millipore, Bedford, MA), for the second flask through a 10 µm pore-filter, and the seawater for the third flask was not filtered. A fourth flask with only seawater was not treated in any way, and was studied as control. *Escherichia coli* 536 (Berger et al., 1982) to a final concentration of 10^6–10^7 bacteria · ml^{-1}, was added using an overnight culture grown in Luria-Bertani medium (LB) at 30 °C, and washed twice in artificial seawater (ASW, ADSA-Micro, Barcelona, Spain) by centrifugation (5,000 rpm, 5 min). All seawater incubations took place at 20 °C, at 100 rpm and in the dark, for up to 10 days. 2-ml samples were fixed with paraformaldehyde at 2% (final concentration) for 10 min at room temperature, and stored at –20 °C until cytometric analysis. When the aim was the cross-calibration between cytometry and epifluorescence microscopy counting, the seawater was enriched with an infusion of cereal leaves following Lee and Soldo (1992). Seawater (300 ml) in a one-liter Erlenmeyer flask was amended with cereal leaf infusion (Sigma Chemical Co., St.Louis, MO) at 0.03% w/v. When the nanoflagellates reached their maximum abundance, 25 ml were taken and preserved with paraformaldehyde buffered with tetraborate at 2% (final concentration).

Nanoflagellate cultures

The bacterivorous nanoflagellates used were *Rhynchomonas nasuta* and *Jakoba libera*. *R. nasuta* is an ellipsoid bodonid nanoflagellate (4–10 µm long by 2–3 µm wide) common in seawater and mostly associated with suspended particles due to its ability to move over surfaces and graze on attached bacteria, using its proboscis (Burzell Linden, 1973). *J. libera*, a jakobid biflagellate, is very common in seawater. One of its flagella is directed anteriorly and held in a sharp hook, often attaching to the substrate, while the other flagellum lies in a ventral groove (Patterson et al. 1993). It is also ellipsoid-shaped and is 5–12 µm long and 2.5–3 µm wide. Seawater (300 ml) filtered through 0.2 µm and autoclaved, was amended with cereal leaf infusion (Sigma Chemical Co., St. Louis, MO) at 0.03% w/v in an Erlenmeyer flask of 1 l. Appropriate volumes were inoculated to attain a starting abundance of 5×10^2 flagellates · ml^{-1}. When an abundance of 10^4–5×10^4 flagellates·ml^{-1} was reached, 25 ml of the culture was taken and fixed with paraformaldehyde buffered with tetraborate at 2% (final concentration). As a previous requirement, all the samples prepared for the cross-calibration between cytometry and epifluorescence microscopy counting showed a bacteria/HNF ratio between 200 and 800.

Cytometric analysis

Cytometric analysis was performed after staining the thawed samples with SYTO 13 nucleic acid stain (Molecular Probes, Eugene, OR) at 2.5 µM (final concentration) for 20 min in the dark, using frozen stock solutions at 500 µM in dimethyl sulfoxide. Prior to analysis, 10 µm-fluorescence beads (Immunocheck, Epics Division, Coulter Corp. Miami, FL) were added to each sample at a known concentration which was verified with a particle size analyser (Multisizer II, Coulter Electronics, UK). A Coulter Epics XL (Coulter Corp. Miami, FL) flow cytometer equipped with a 15 mW air-cooled 488-nm argon-ion laser was used for sample analysis. The green emission from SYTO 13 was collected with a 525-nm band-pass filter. Beads were detected and counted by their red fluorescence and gated out of the scatter and green fluorescence histograms. Bacterial and nanoflagellate concentrations were deduced from bead counts by ratiometric counting. To reproduce the cytometric settings used in this paper, note that the FSC and SSC numeric values reported are given directly by the instrument after manual adjustment of the Vernier gain and linear amplification. Also, the fluorescence values of the histograms were obtained at the fluorescence voltage and fluorescence threshold specified in Figure 3.

Cytometric sorting

The Coulter Epics Elite cytometer (Coulter Corp. Miami, FL) with Autoclone option consisting of a programmable single-cell deposition system, was used to obtain a confirmation of the protozoan identity of the cytometric population attributed to HNF. Samples stained with SYTO 13 were analysed, and the population attributed to HNF was gated and sorted into a tube containing 200 µl of sodium chloride (0.9%). The sample containing the sorted particles was stained again with SYTO 13 and filtered through a 2 µm pore-diameter polycarbonate filter (Nuclepore, Corning Costar Co. Badhoevedorp, The Netherlands). The filter was placed onto a slide and mounted with a drop of Mowiol non-fading medium prepared with Mowiol 4–88 (Calbiochem, La Jolla, CA, USA) 2.4 g; glycerol 6 g; Tris buffer (0.2 M. pH 8.5) 12 ml and deionised water 6 ml. Epifluorescence observation was performed with a Leica DMRB FLUO microscope (Leica Microsystems Wetzlar, GmbH, Germany) and Metamorph software was used for image acquisition and digitalisation.

Epifluorescence counting

Serial dilutions of enriched seawater or nanoflagellate cultures were performed to obtain several subsamples with abundance ranging from 10 to 10^4 cells · ml^{-1}. Subsamples were counted simultaneously with both epifluorescence microscopy and flow cytometry to cross-calibrate the two counting techniques. For the epifluorescence counting, appropriate volumes of subsamples were DAPI stained (Porter and Feig, 1980) at 3 µg ml^{-1} (final concentration) and filtered onto 0.8-µm pore size black polycarbonate filters (Millipore, Bedford, MA). DAPI solution was previously filtered through 0.2 µm and maintained at

4 °C in the dark. Filters were placed onto a slide and mounted with a drop of low-fluorescence Nikon A oil (Nikon, Tokyo, Japan). All counts were performed in duplicate with Nikon Eclipse E-400 oil (Nikon, Tokyo, Japan) under UV excitation, and at least 100 flagellates were counted for each subsample.

Results

Protozoan detection in enriched seawater and confirmation by cell sorting

Addition of wheat grains to seawater increased the bacterioplankton concentration (Figure 1). In the flask with non-filtered seawater, a strong decrease in bacterial densities was observed after 5 days of incubation, but in seawater filtered through a 2 µm or 10 µm pore-diameter filter, the decrease in bacterioplankton density was less pronounced and only lasted for 24–48 h. (Figure 1). Cytometric analysis of non-filtered seawater at 113 h of incubation revealed a new cytometric population with higher fluorescence and higher scatters than bacterioplankton (Figure 2 A and B respectively). This new population was attributed to HNFs, and it was present in cytometric histograms until the end of the incubation but with decreasing concentration. HNF population detected by flow cytometry also appeared in seawater that was passed through 2 µm or 10 µm filters when bacterioplankton decreased. This detection was less evident because bacterial concentration was still high enough to collapse the cytometric acquisition before it could provide a good definition of the HNF population. Confirmation of the protozoan nature of this population was obtained after cytometric sorting and observation with epifluorescence microscopy. Figure 2 C, shows HNFs previously stained by the green SYTO 13 fluorescence and observed microscopically after sorting. The nucleus is strongly stained but the cytoplasm is also faintly labeled.

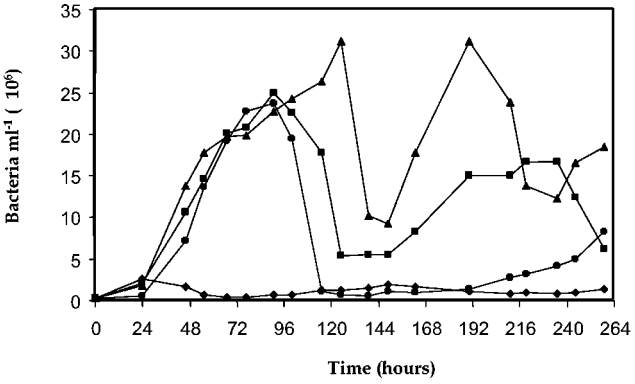


Fig. 1. Evolution of bacterioplankton concentration in seawater enriched with wheat grains, incubated at 20 °C and shaken. ● Non-filtered seawater, ■ Seawater filtered through 10 µm, ▲ Seawater filtered through 2 µm, ◆ Seawater control, non-filtered and non-enriched.

Specific cytometric protocols for HNF quantification

The optimization of the specific cytometric acquisition protocols for the detection and quantification of HNF in seawater was performed in the seawater amended with an *E. coli 536* culture. Two technical variables were changed in the standard protocol used for bacterioplankton: the fluorescence threshold value that discriminates background from the definitive cell counts was increased, and the voltage of the fluorescence detector was lowered. The cytometric settings and the corresponding results of the cytometric acquisition protocols developed for the detection of HNF are shown in Figure 3, where the same seawater sample is analyzed with the three protocols. The main characteristics of these three protocols were:

- **Protocol A:** The standard protocol used for bacterioplankton, the settings were adjusted to detect bacterial cells. Fluorescence was used as the trigger parameter (Figure 3 A).
- **Protocol B:** Using protocol A as a reference, the fluorescence threshold and time of acquisition were increased in order to improve nanoflagellate detection. In these conditions, the bacterial population was partially lost due to the new fluorescence threshold (Figure 3 B).
- **Protocol C:** Developed from protocol B by decreasing the voltage of the fluorescence detector. The bacterial

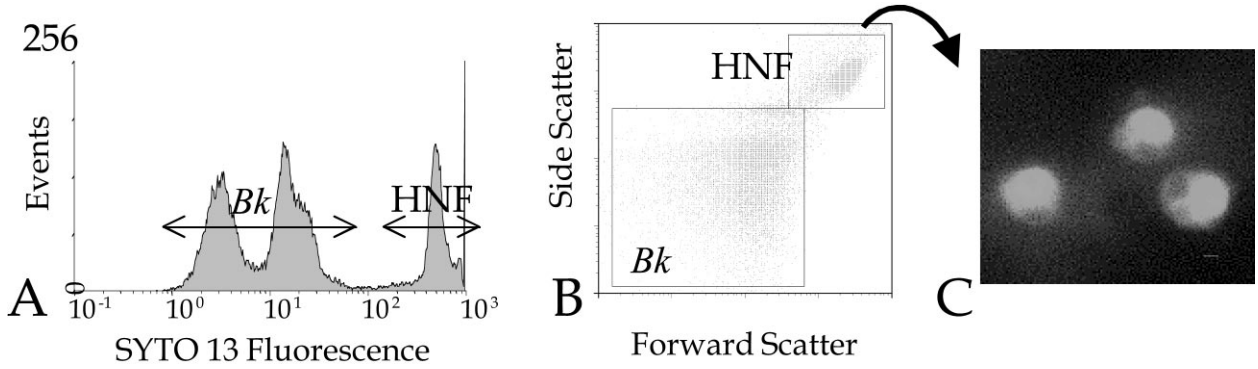


Fig. 2. Flow cytometric histograms and microscopic observations of sorted cells of wheat enriched non-filtered seawater incubated 113 hours. **A** – Fluorescence histogram, **B** – Forward scatter versus side scatter dot plot. *Bk* – bacterioplankton; HNF – heterotrophic nanoflagellates. **C** – Microscopic epifluorescence observation of the sorted HNF population. Scale bar ≙ 1 µm.

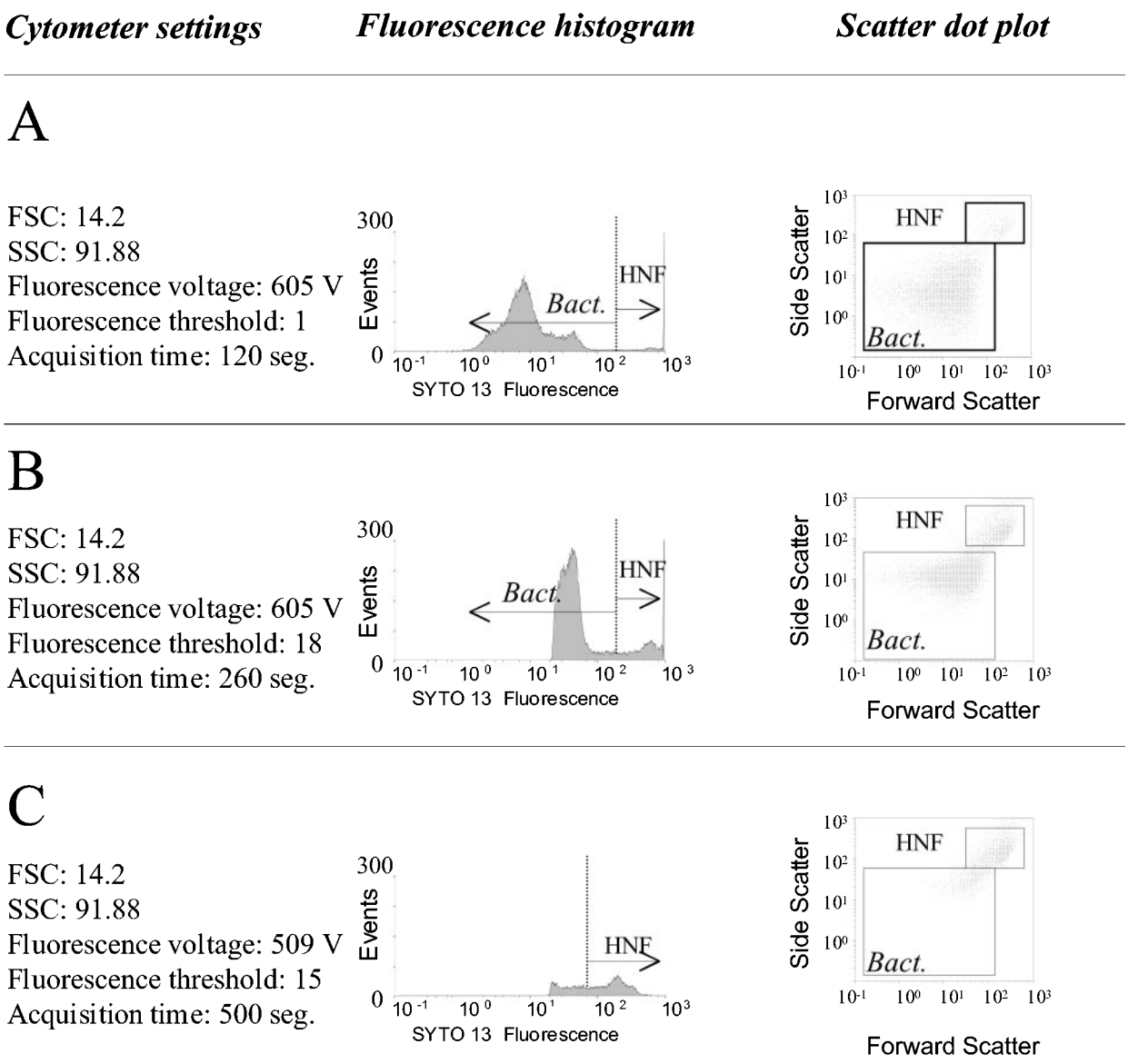


Fig. 3. Cytometric settings and the resulting cytometric detection of bacterioplankton and HNF applied to a seawater sample amended with an *E. coli* culture and incubated 66 hours. The same sample was analysed with the standard cytometric acquisition protocol for bacterioplankton (A), and with the new ones developed for a better detection of HNF in seawater (B and C). Cytometer settings are specified for each protocol. HNF: heterotrophic nanoflagellates, *Bact*: bacteria.

population was not detected, but even large nanoflagellates were not out of range. This protocol allowed longer analysis time, which made it possible for us to analyze larger volumes (Figure 3 C). Fluorescence values were not comparable with those obtained with protocols A and B because the applied voltage was changed. Scatter detection was the same for protocols A, B, and C.

Evaluation of the optimal bacteria/HNF ratios for the newly developed cytometric protocols.

The addition of an *E. coli* culture to seawater resulted in a rapid increase in HNFs. The time course of *E. coli*, bacterioplankton and nanoflagellate concentration is shown in Figure 4. Cytometric analysis of the seawater also allowed us to differentiate bacterioplankton from *E. coli*, and to quantify them separately. Cells from the *E. coli* culture were larger and as indicated by its fluorescence, metabolically more active than bacterioplankton cells, which differentiated their cytometric signatures. Fluorescence versus forward scatter dot plots of the seawater with the *E. coli* culture are represented in Figure 5. Five hours after the addition of the culture, *E. coli* cells (*Ec* in Figure 5*)* were predominant among the bacterial population and showed higher fluorescence and scatters than the bacterioplankton (*Bk* in Figure 5). At 29 h of in-

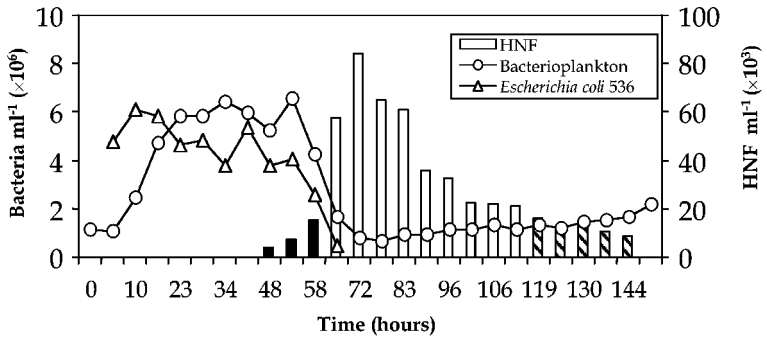


Fig. 4. Evolution of bacterioplankton, *E. coli* and HNF concentration in seawater incubated with an *E. coli* culture. The cytometric acquisition protocol used to detect HNF were: protocol A – white columns; protocol B – stripped columns; protocol C – black columns.

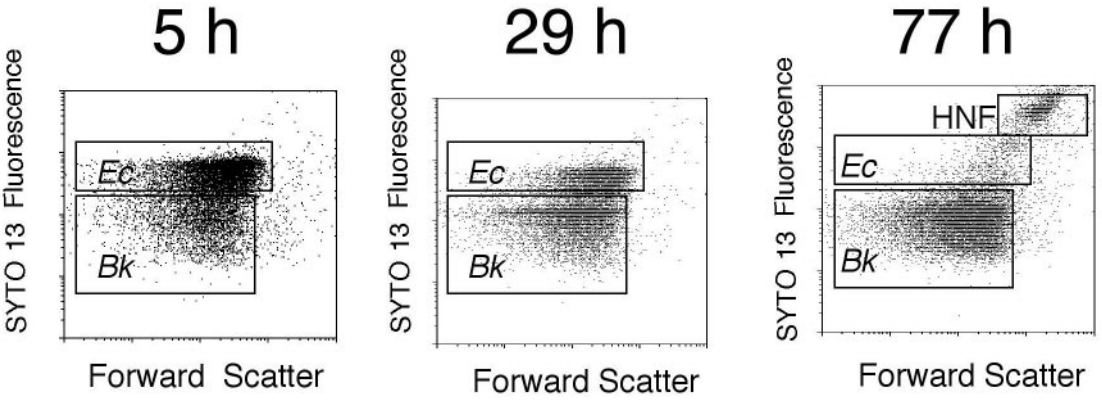


Fig. 5. Evolution of the cytometric signature of seawater with an *E. coli* culture, at 5, 29 and 77 h of incubation. *Ec* – *E. coli; Bk* – bacterioplankton; HNF – heterotrophic nanoflagellates.

cubation, the bacterioplankton concentration had increased and both *E. coli* and bacterioplankton populations were well defined. At 77 h of incubation, the *E. coli* population had disappeared after predation by HNFs, and most bacterial cells belonged to bacterioplankton, with low fluorescence emission and scatters. HNF fluorescence was 8 times higher than *E. coli* fluorescence, which in turn was 5 times higher than mean bacterioplankton fluorescence. The forward and side scatter of *E. coli* was double that of bacterioplankton. The fluorescence and scatter values of the HNF population decreased throughout the incubation.

The strong reduction of bacterioplankton and *E. coli* concentrations took place 66 hours after the beginning of the incubation, and it was coupled with a large increase in HNF concentration. The decision to use protocol A, B or C to quantify HNF was based on the ratio between bacteria and HNF concentrations in the sample. Ratios above 200 required the use of protocol C, (Figure 4, black columns), quotients between 200 and 100 could be analyzed with protocol B (Figure 4, stripped columns) and protocol A was only effective when the quotient between bacteria and HNF was below 100 (Figure 4, empty columns). One advantage of protocol A, or B when possible, is the simultaneous detection of bacteria and HNF in a single run. With protocol C, only HNF and large bacteria were detected. HNF cytometric counting in seawater enriched with TSB gave similar results with all three cytometric protocols. Again, the detection of HNF at high bacterial concentrations (bacterioplankton/HNF ratio above 200) was only possible with protocol C.

Cross-calibration between flow cytometry and epifluorescence counts

The first step for the cross-calibration was performed using enriched seawater. A good correlation was found between microscopic and cytometric counts ($r^2 = 0.940$) with a slope of 1.19, indicating a slight tendency to obtain higher HNF abundance with flow cytometry than with epifluorescence microscopy (Figure 6). However, different cross-calibration resulted when using cultures of heterotrophic nanoflagellates *R. nasuta* and *J. libera*. With *R. nasuta*, a high correlation was obtained ($r^2 = 0.948$) with a slope of 0.59 (Figure 7), indicating a clear tendency to get almost 2-fold higher counts with epifluorescence microscopy. This was probably due to the great amount of flagellates adhered to aggregates (Figure 8), which could not be discerned by flow cytometry. In the case of *J. libera*, cross-calibration was assayed with two cultures one in the middle of the exponential phase and the other in the late exponential phase. The correla-

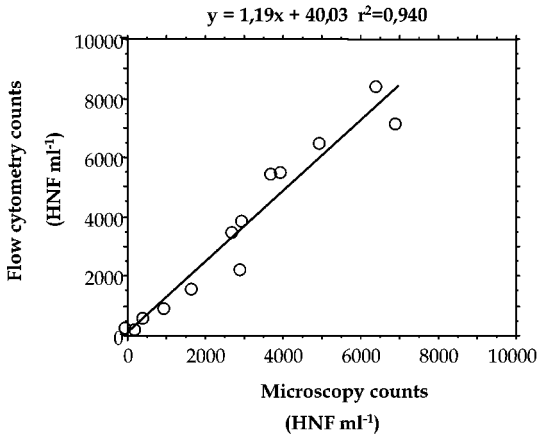


Fig. 6. Cross-calibration between epifluorescence microscopy and flow cytometry HNF counts in seawater enriched with cereal leaves.

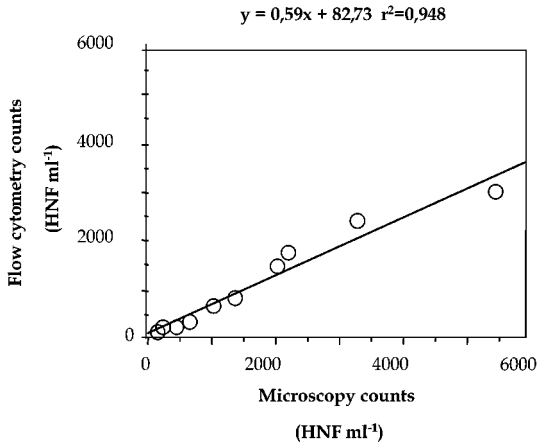


Fig. 7. Cross-calibration between epifluorescence microcospy and flow cytometry counts in a *Rhynchomonas nasuta* culture.

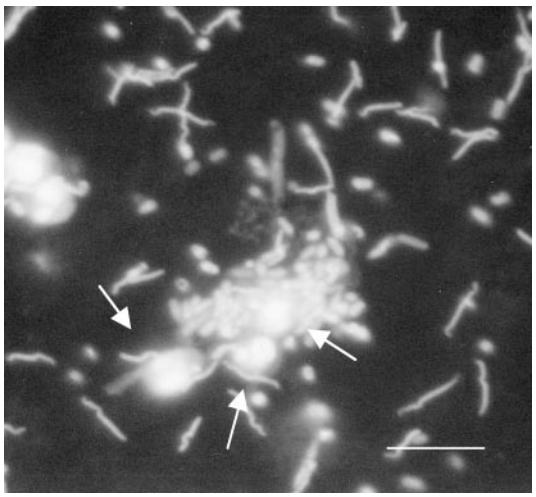
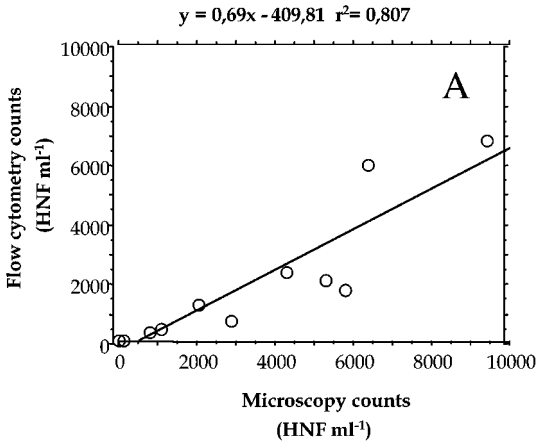


Fig. 8. *Rhynchomonas nasuta* attached to a bacterial aggregate. Scale bar: 10 µm.



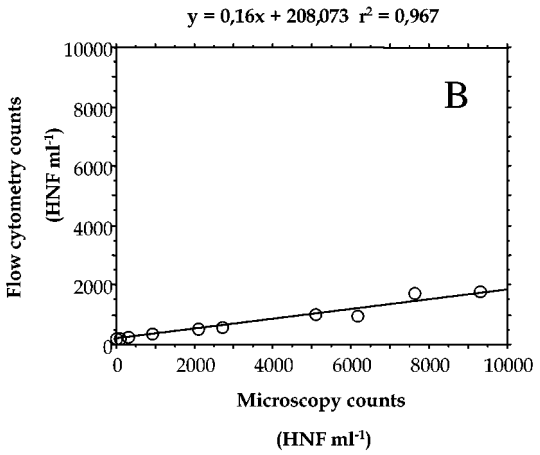


Fig. 9. Cross-calibration between epifluorescence microscopy and flow cytometry counts in two *Jakoba libera* cultures: A) *Jakoba libera* in exponential phase, B) *Jakoba libera* in late-exponential phase.

tion between the two techniques was quite good in both experiments (r^2 = 0.807 and r^2 = 0.967 respectively), but the slopes were different (0.69 and 0.16 respectively) (Figure 9, A and B). The attachment of flagellates to aggregates (Figure 10 A) may explain the low counts obtained with flow cytometry, but other possible explanations may be related to the physiological state of the protists.

Discussion

The standard cytometric protocols that allow accurate quantification of bacterioplankton cannot be used to quantify protozoa mainly for two reasons. First because the background produced by the bacterial acquisitions does not allow the discrimination of protozoa at low densities and secondly because the protozoa population lies outside the range because the protozoan fluorescence is much higher than the bacterial fluorescence (around 35 fold).

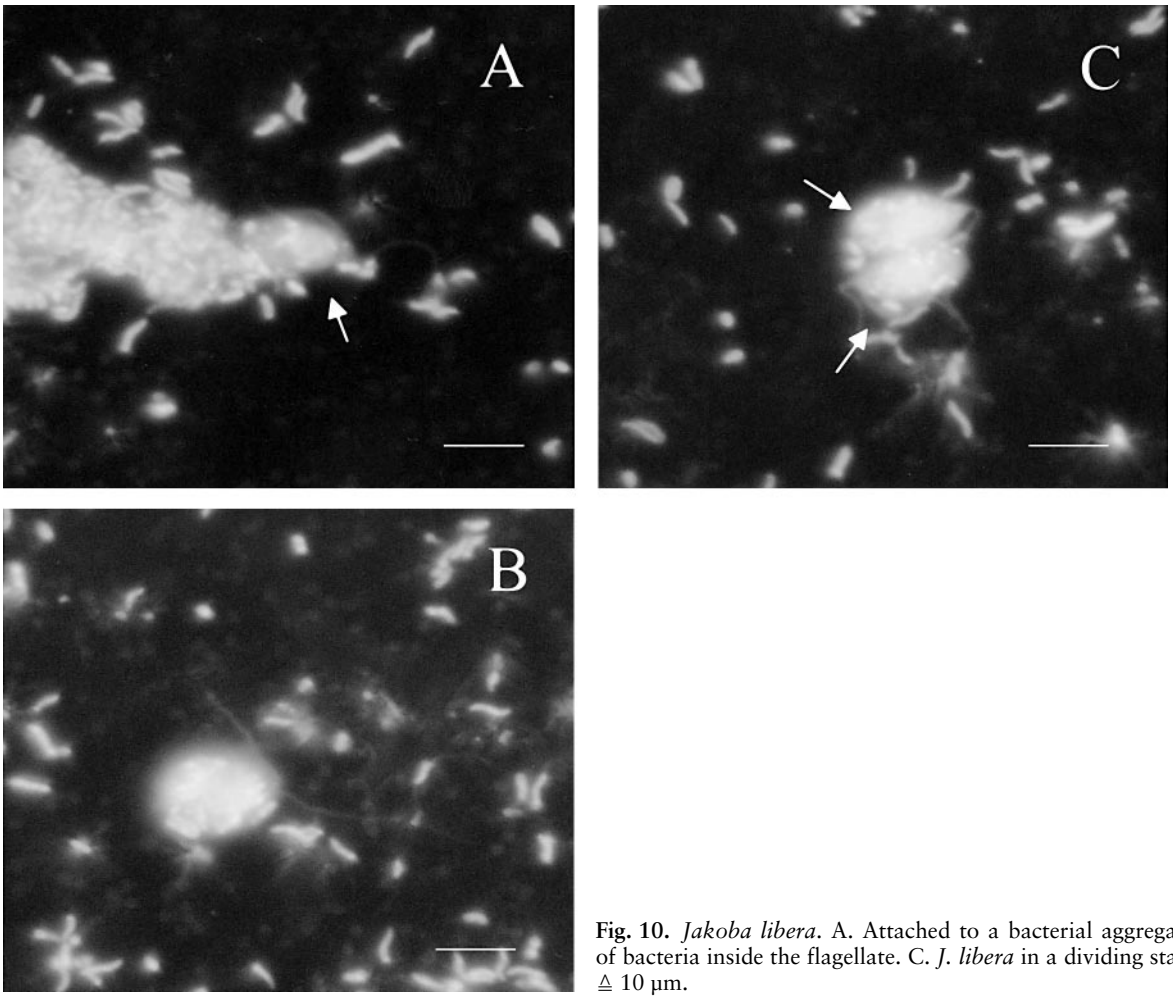


Fig. 10. *Jakoba libera*. A. Attached to a bacterial aggregate. B. Plenty of bacteria inside the flagellate. C. *J. libera* in a dividing state. Scale bar ≙ 10 µm.

Taking as a reference the standard protocol (A) used for our group in previous flow cytometric studies of bacterioplankton (GUINDULAIN et al. 1997), we have developed two new cytometric protocols (B and C) that differ from protocol A in the increase of the fluorescence threshold and the reduction of the voltage of the fluorescence detector. With protocol C we obtained the best HNF quantification; it allowed an HNF count when the bacteria/HNF ratio was around 1000. It might be advisable to use protocols A or B when possible because they allow the detection of bacterioplankton and HNF in a single run, but because low bacteria/HNF ratios are needed, these protocols are restricted to enriched populations. Scatter detection was the same for protocols A, B and C. Fluorescence of the particles detected with protocol C cannot be compared with the fluorescence of particles detected with protocols A or B because the fluorescence voltage in protocol C is lower than in the other two. This is the main disadvantage of this protocol; we cannot compare the fluorescence of the HNF detected with that of a bacterioplankton population detected with the standard protocol.

To validate the flow cytometric counts, we used enriched seawater in HNF as well as cultures of two single species of marine planktonic HNF, *R. nasuta* and *J. libera* (LEE et al., 1985; PATTERSON et al., 1993; ARTOLOZAGA et al., 2000). In all cases the cytometric protocol C was used. In the case of the mixed community of HNF, the cytometric count was not only equivalent but even slightly higher than the microscopic count. An advantage of the cytometric count in natural samples is the quantification of HNF that may go unnoticed using the epifluorescence microscope due to their size, shape or fluorescence distribution. Finally, the counting fatigue of the researcher should be considered as a negative factor when counting with epifluorescence microscopy.

When the single species of the HNF *R. nasuta* and *J. libera* were analyzed, the counts obtained with epifluorescence microscopy were higher than those obtained with flow cytometry. This could be due to the fact that cultures are monoxenic and consequently, the microscopic identification and quantification of only one type of protozoa is easier than in the case of a mixture of protozoan sizes and shapes. In addition, researcher fatigue will be low.

R. nasuta is a planktonic bodonid described as frequently associated with surfaces of suspended particles (LEE et al. 1985; PATTERSON et al.,1993). The bacterivorous protist attaches to the particle previously colonized by bacteria or to bacterial microcolonies using its posterior flagellum, and feeds on these aggregates, detaching and ingesting bacteria using its anterior proboscis (BURZELL LINDEN, 1973). In cultures like those used in this work the proportion of attached protists is variable and depends on the growth phase of the protist as well as on other biotic and abiotic factors closely linked to the aggregation process, such as turbulence, temperature, and composition or concentration of DOM and POM (ALLDREDGE and SILVER, 1988; ALLDREDGE and GOTSCHALK, 1990; IRIBERRI and HERNDL, 1995). In our case, the protists attached to particles and among them (Figure 8) were not numerous and were counted by epifluorescence microscopy. The results we have obtained indicate that our flow cytometric protocols were not able to count attached HNF.

J. libera is a jakobid usually associated loosely with detritus (PATTERSON et al., 1993) although it is an extremely effective grazer on free suspended bacterial prey (CARON, 1987). Two cross-calibrations were carried out with this bacterivore in two different growth states, the exponential and late-exponential phases. In the exponential phase few attached protists were observed (Figure 10 A), and for some protists plenty of DAPI stained bacterial prey (up to 50 bacteria) were also observed (Figure 10 B). The combined fluorescence of the protists plus the bacteria inside them could exceed the cytometric limits of fluorescence established in the protocol. In the late-exponential phase the number of both attached and filled protists increased slightly. However, a higher proportion of dividing protists appeared (Figure 10 C), and due to their higher fluorescence, they probably went out of range when the flow cytometric protocol C was used. This would explain the very low cytometric effectiveness observed in this case. When using the microscopic protocol all the protists were counted. The dividing morphotypes were considered two protists when two individuals could clearly be distinguished.

In spite of low coincidence between cytometric and microscopic counts in HNF cultures, the results obtained show that the use of the proposed flow cytometric protocol C for counting HNF can be useful in experimental work. Since a bacteria/HNF ratio below 1000 is required, this protocol will be applicable to those marine ecosystems that are specially stressed or enriched with an autochthonous or allochthonous input of organic material and the subsequent increase in bacterial prey and HNF grazers. Data-bases elsewhere (SANDERS et al. 1992; GASOL and VAQUÉ, 1993) show a high number of natural aquatic ecosystems with bacteria/HNF ratios below 1000. In addition, the flow cytometric methodology will be useful for monitoring the abundance of the mixed community of HNF through time, in enriched micro or mesocosms like those studied in this work. Finally, cytometry has the advantages of making the count fast, comfortable and repetitive. Epifluorescence microscopy, on the other hand, is essential for providing information about morphotypes, diversity, growth state, free or attached situation and general ecological features of the protistan community.

Since specific protozoa fluorochromes are not foreseen at present, flow cytometry, if it is used to count protozoa in natural non-nriched waters, will need further technical improvement, particularly the incorporation of fluorescence detectors that are adjustable at broader voltages and have a higher capacity to modify the fluorescence threshold.

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