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A Simple Method to Preserve Oceanic Phytoplankton for Flow Cytometric Analyses

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A simple method was developed to preserve marine phytoplankton populations so that delayed flow cytometric analyses could be performed. The method consisted of immediate fixation with 1% glutaraldehyde (final concentration) followed by storage in liquid nitrogen. The method was tested on individual algal species and on natural samples from both coastal and pelagic waters. In most cases, it caused little cell loss and preserved well both forward angle light scatter and chlorophyll fluorescence, but phycoerythrin fluorescence sometimes was significantly increased. The technique performed best for the small-sized picoplankton (below 2 μm) such as *Synechococcus* cyanobacteria or the newly discovered oceanic prochlorophytes. For larger-sized cells it had to be applied on a case by case basis as some fragile species, particularly dinoflagellates and cryptophytes, were poorly preserved.

Key terms: Glutaraldehyde, chlorophyll, phycoerythrin, light scatter

The past 5 years have witnessed a rapid development in the applications of flow cytometry to the analysis of photosynthetic plankton, both in the laboratory (20,13,27,23,24) and in the field (14,10,5,4,15,6,12). On natural samples, flow cytometry is used to discriminate and enumerate several cell types (cyanobacteria, prochlorophytes, eukaryotes), and to assess their cellular characteristics (light scattering properties, pigment composition). This technique is especially suited for picoplankton, which is difficult to measure by other techniques because of its very small size (below 2 μm). These analyses, which until now had to be performed on fresh samples, have required the use of flow cytometric equipment embarked on board oceanographic ships, resulting in high operating costs (14,15,6,12). Furthermore, operation of flow cytometers at sea is only possible in relatively quiet weather (4). Therefore it is highly desirable to be able to preserve marine phytoplankton samples in order to perform flow cytometric analyses in land-based laboratories. This will allow flow cytometry to be used in a more routine way in biological oceanography, although embarkment of flow cytometers on board oceanographic ships will still be necessary to study fragile forms that cannot be well preserved, and to sort live cells in order to establish new algal strains or to measure the incorporation of radioactive precursors (17).

A method to preserve phytoplankton samples for flow cytometric analyses must meet several criteria. It must be simple enough to be easily applicable on board ships, it must cause minimal cell loss, and it must preserve the individual scattering and pigment fluorescence properties of the different phytoplankton populations. Classical phytoplankton preservation methods such as formalin or Lugol's fixation do not generally meet these criteria, as the former modifies cell shape (3) and the latter affects drastically fluorescence (22), although there have been very few quantitative studies of these effects (2,3). Preservation methods routinely used for flow cytometric analyses of animal cells, such as ethanol fixation, are not suitable either since they induce photosynthetic pigment extraction and therefore loss of cell autofluorescence (13). This paper presents a simple preservation method that has been tested quantitatively on both laboratory cultures and field samples.

Table 1
Time Elapsed Between Fixation and Re-analysis

Samples	Days
Cultures	5
English Channel	20–120
Sargasso Sea	260

MATERIALS AND METHODS

Cultures

Several algal strains were isolated by flow cytometric cell sorting: *Synechococcus* (strain ROS04), *Micromonas pusilla* (strain ROS09), and a small (5 μm) unidentified flagellate (strain ROS07) from English Channel coastal waters; *Synechococcus* (strain MAX41) from the Sargasso Sea; and *Gymnodinium* cf. *nagasakiense* (strain Tinduff) from aquaculture tanks located at a shellfish farm (Tinduff, France). Other strains were obtained from algal collections: *Hymenomonas carterae* (clone Cocco II) from R. Guillard (Bigelow Laboratory, Boothbay Harbor, ME, USA); *Dunaliella primolecta*, and *Cryptomonas maculata* from the Cambridge Culture Collection (UK); and *Chaetoceros curvisetus* from the Station Zoologique in Villefranche-sur-Mer (France).

Duplicate batch cultures were maintained in exponential growth at 20°C in K medium (9) at a light intensity of 100 μEinst m^{-2} s^{1} (except for strain MAX41, maintained at 10 μEinst m^{-2} s^{-1}) and a 12:12 L:D cycle.

Field Samples

English Channel coastal waters were sampled at the Estacade station in Roscoff (19) every 2 weeks from the end of January until mid-May (seven samples). Sargasso Sea oceanic waters were sampled at different depths within the euphotic zone (six samples: stations 4 and 624) during the CHLOMAX cruise of the NO Suroit (12).

Fixation Method

Fresh samples (1 ml volume) were fixed in 1.8 ml cryotubes (Nunc, Roskilde, Denmark) with electron microscopy grade glutaraldehyde (Merck, Darmstadt, FRG) at a final concentration of 1% (vol/vol) and immediately stored in liquid nitrogen for a period ranging from a few days to almost a year before analysis (Table 1).

Microscopy

Cells were observed with an Olympus (Chome, Japan) BH-2 microscope under a X40 objective using either interferential contrast (Nomarski) or epifluorescence (B filter set) illumination. Photographs were taken with an OM-2 camera (Olympus) using Kodak T-Max 100 film (100 ASA).

Flow Cytometry

All samples were analyzed with an EPICS 541 (Coulter, Hialeah, FL, USA) using either the 488 nm or the 515 nm line of a 6W argon laser (Coherent, Palo Alto, CA, USA). The laser beam was focused with a confocal lens producing an elliptical beam spot of 40 μm × 16 μm. Table 2 provides a summary of the laser power and filter set-up used for the different analyses. Preserved samples were thawed at room temperature and maintained on ice in the dark. Duplicate volumes, ranging from 0.05 to 1 ml, depending on cell concentration, were analyzed. The use of the standard 76 μm flow cell and the application of a differential pressure of 5 in. of water resulted in a typical sample flow rate of 0.05 ml/mn. Data were stored in list mode. Each of the four measured parameters (forward and right angle light scatter, green and red fluorescence) was recorded on a 3 decade logarithmic scale mapped onto 256 channels. Calibration was achieved with 1 μm (Polysciences, Warrington, PA, USA), 10 μm full-bright, and 10 μm 2% (Coulter) fluorescent beads.

Data collected on the EPICS were transferred to an IBM-AT compatible computer with the EPINET software (Coulter) and analyzed with custom-designed software (CYTOPC). For natural samples, subpopulations were identified according to their relative scatter and green and red fluorescence (see Results). The number of cells and the mean values of cellular parameters for each subpopulation were computed after conversion of the logarithmic scales to linear ones. In order to account for day-to-day variations in instrument settings, mean population parameters were normalized through division by the corresponding parameters measured on standard beads.

RESULTS

Preliminary Tests

We first attempted to preserve algal populations by rapidly freezing unfixed samples and subsequently storing them in liquid nitrogen (25). Although cell characteristics were perfectly recovered when the preserved samples were thawed and immediately analyzed, we observed a marked degradation of the fluorescence of photosynthetic pigments within a few hours. This resulted in artifactual bimodal histograms of pigment fluorescence when analyses were delayed or when partial melting occurred during sample transport (25).

One way to avoid this problem appeared to be fixing cells prior to freezing. Among the fixatives used for phytoplankton, glutaraldehyde seemed to be the most appropriate since it is the fixative of choice for phytoplankton electron microscopy (e.g., 8,21), where preservation of cell shape is important. Moreover, it is also widely used when enumerating phytoplankton by epifluorescence because it has been observed to keep pigment fluorescence (e.g., 11,3). Preliminary tests on picoplanktonic species showed, however, that samples

Table 2
Laser and Filter Set-up

Samples	Laser wavelength (nm)	Laser power (W)	Filter splitting	Filter green fluorescence	Filter red fluorescence
Cultures, English Channel	488	1.3	SP590	LP530	LP690
Sargasso Sea	488	1.5	SP640	SP530	LP630

fixed with 1% glutaraldehyde and simply stored at 4°C in the dark deteriorated in the long term. For example, in the case of *M. pusilla*, 80% of the cells and 50% of the red fluorescence were lost after 2 weeks (data not shown; see also 2,3). Similarly, storage in a standard freezer (−20°C), though somewhat better, still induced cell loss in the long term (> 1 month). Therefore storage at very low temperature appeared necessary.

In view of these initial trials, we decided to use 1% glutaraldehyde fixation followed by storage in liquid nitrogen. The final pH of fixed samples ranged between 6.4 and 6.6. We did not attempt to counter the acidic effect of glutaraldehyde since buffering does not seem to improve cell preservation (2). We found that it was important to freeze samples rapidly, as some species develop green fluorescence when left at room temperature (see below). Sufficient time must be allowed however for the glutaraldehyde to fully penetrate the cells (21), optimal lapse between fixation and freezing being about 10 minutes. We also noticed that samples once thawed could be refrozen without damage.

Cultures

The preservation procedure did not seem to affect cell size and shape drastically. For example, in the case of the naked dinoflagellate *G.* cf. *nagasakiense*, a large and fragile cell, the different cell structures such as the nucleus and the chloroplasts were still clearly visible (Fig. 1A,C); moreover, chlorophyll fluorescence appeared to be well preserved (Fig. 1B,D). The most conspicuous changes were the thickening of the cell wall and the disappearance of unstable features such as flagellae and furrows (Fig. 1A,C). The usefulness of freezing was clearly illustrated for this species since fixed cells which were simply stored at 4°C rapidly developed a dull intracytoplasmic green fluorescence and had modified chloroplasts (Fig. 1E,F).

The preservation method induced changes in cell concentration ranging from 46% (decrease) to 126% (increase) of the fresh sample value (Fig. 2A). The least affected species were *Synechococcus* and the small flagellate strain ROS07. Both the dinoflagellate *G.* cf. *nagasakiense* and the cryptophyte *C. maculata* experienced an important reduction in cell concentration, which was expected since species belonging to these groups are usually fragile (3). For *M. pusilla*, *D. primolecta*, and *H. carterae*, cell concentration increased in preserved samples. In these cases we suspected that fresh sample concentrations were underestimated as a consequence of cell clumps, which were probably dissociated by the freezing process.

Forward angle light scatter was affected very little by fixation, except in the case of *M. pusilla* and *G.* cf. *nagasakiense* (Fig. 2B). In contrast, right angle scatter decreased for almost all species (Fig. 2C), the largest decrease being registered for *H. carterae*. In the latter species this was because the fixed cells lost their coccoliths, the calcium carbonate plates covering the cell surface that are responsible for the anomalously large right angle scatter observed for fresh cells (although they have no effect on forward angle scattering) (16). Conversely, the large increase in right angle scatter of *G.* cf. *nagasakiense* might be explained by the increase in the refractive index of the cell wall, clearly visible in photographs (Fig. 1A,C).

Chlorophyll red fluorescence showed very little change (Fig. 2D), ranging from 80% to 120% of the fresh sample value, with the exception of the *Synechococcus* strains, for which it increased to about 150% of its initial value. Phycoerythrin green-orange fluorescence was unchanged in the cryptophyte *C. maculata*, but significantly increased in both strains of *Synechococcus* (Fig. 2E). The same increase was observed for *Synechococcus* cells that were analyzed immediately after fixation prior to freezing, as well as for cells that were frozen but not fixed (data not shown), indicating that it is linked to cell death.

In order to test whether the preservation method modifies the spectral characteristics of photosynthetic pigments, we measured for a subset of species the ratio of pigment fluorescence excited at 488 nm to that excited at 515 nm, on fresh and preserved samples (Fig. 3). This ratio was virtually unchanged in the case of chlorophyll fluorescence and slightly reduced in the case of phycoerythrin fluorescence, indicating that the preservation method causes a shift towards longer wavelengths in the excitation spectrum of the latter pigment. Finally, some of the species containing only chlorophyll (*G.* cf. *nagasakiense*, *H. carterae*, *D. primolecta*) exhibited a little increase in green fluorescence (Fig. 2E), presumably due to intracytoplasmic effects of glutaraldehyde fixation (see Fig. 1F).

Natural Samples

From a flow cytometric point of view, natural samples differ from phytoplankton cultures in two ways. Firstly, they contain several phytoplankton populations each occurring at much lower abundances than in

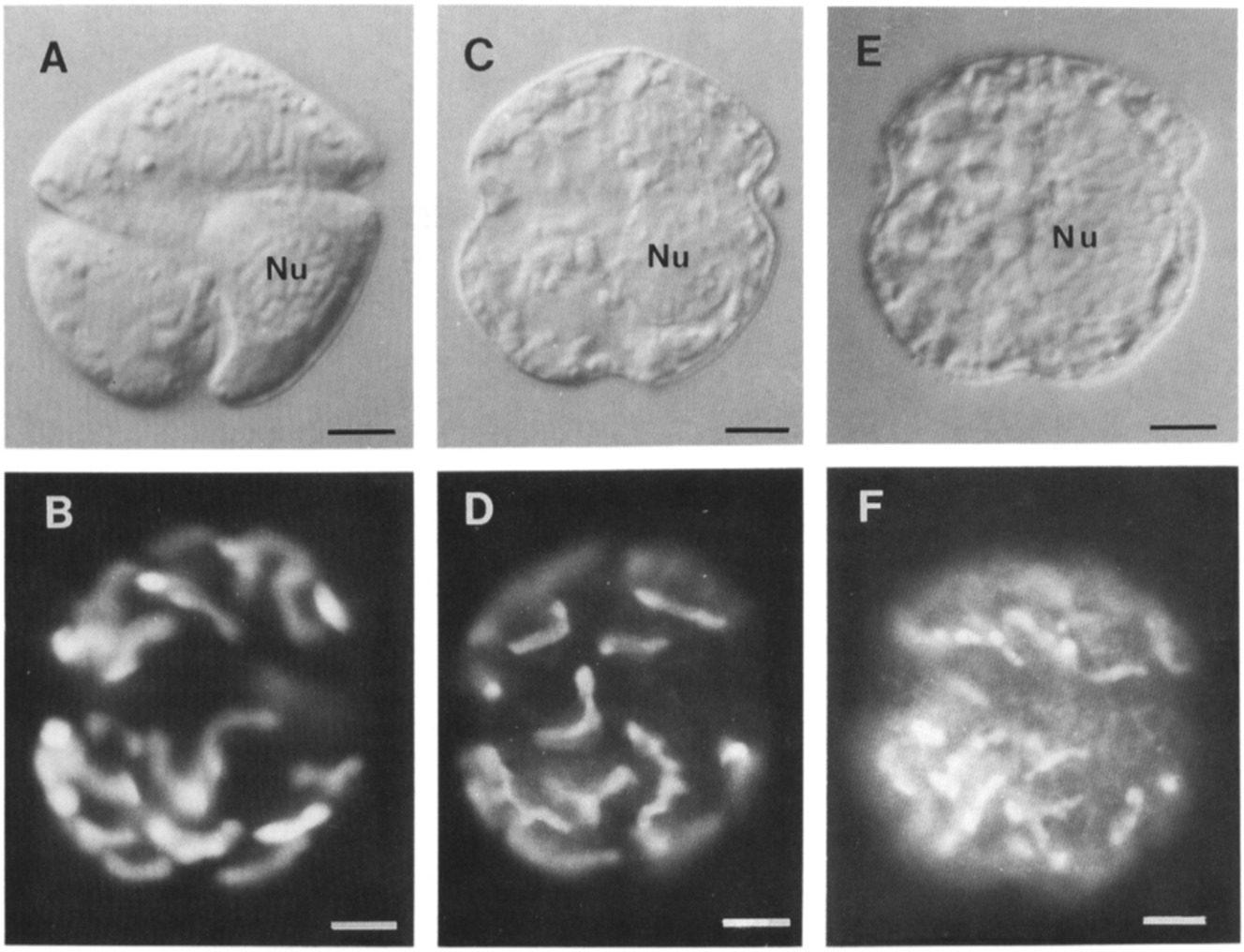

FIG. 1. Effect of the preservation method on the dinoflagellate *Gymnodinium* cf. *nagasakiense*. Scale bar = 5 μm. **A:** Live cell observed with interference contrast (Nomarski). **B:** The same cell observed with blue epifluorescence light. The chlorophyll contained in the chloroplasts fluoresces red. **C, D:** Cell preserved according to the method described in the text and thawed at room temperature. Note the flattening of the cell, the good preservation of the nucleus (Nu) and chloroplasts, and the increased contrast of the cell wall. **E, F:** Cell fixed with 1% glutaraldehyde and kept for 3 hours in the dark at 4°C. Note the shrinkage of cell chloroplasts and the increased green fluorescence of the cytoplasm.

cultures. Secondly, they include a variety of organic and mineral particles besides algal cells. The preservation method did not drastically modify the flow cytometric signature of the natural samples tested (Fig. 4) and the different communities (cyanobacteria, picoeukaryots) were still clearly distinguishable. Moreover, no particles interfering with the signature of these communities were created by the preservation process (Fig. 4), either through glutaraldehyde-induced fluorescence or disruption of large particles following freezing.

Minimal cell loss was registered for the small-sized populations (Fig. 2A), including *Synechococcus*, picoplanktonic eukaryots, and the newly discovered oceanic prochlorophytes (6,12). The conservation of the larger-sized populations (nanoplankton from the English Channel) was more variable. This probably reflects the changing nature of this community from sample to sample: in some cases it is dominated by fragile cells such as dinoflagellates and in other cases by more robust cells, which are well preserved. The overall increase that was observed could result from the fractionation of diatom chains, an important component of this community, into individual cells following freezing.

For English Channel populations, the response of the different cell parameters to preservation was very similar to that of cultures. Forward and right angle scatter (signals) were well preserved. For eukaryots chlorophyll red fluorescence was little modified (Fig. 2D), whereas for *Synechococcus* both phycoerythrin and chlorophyll increased to more than 170% of their fresh value (Fig. 2D,E). For Sargasso Sea populations, both

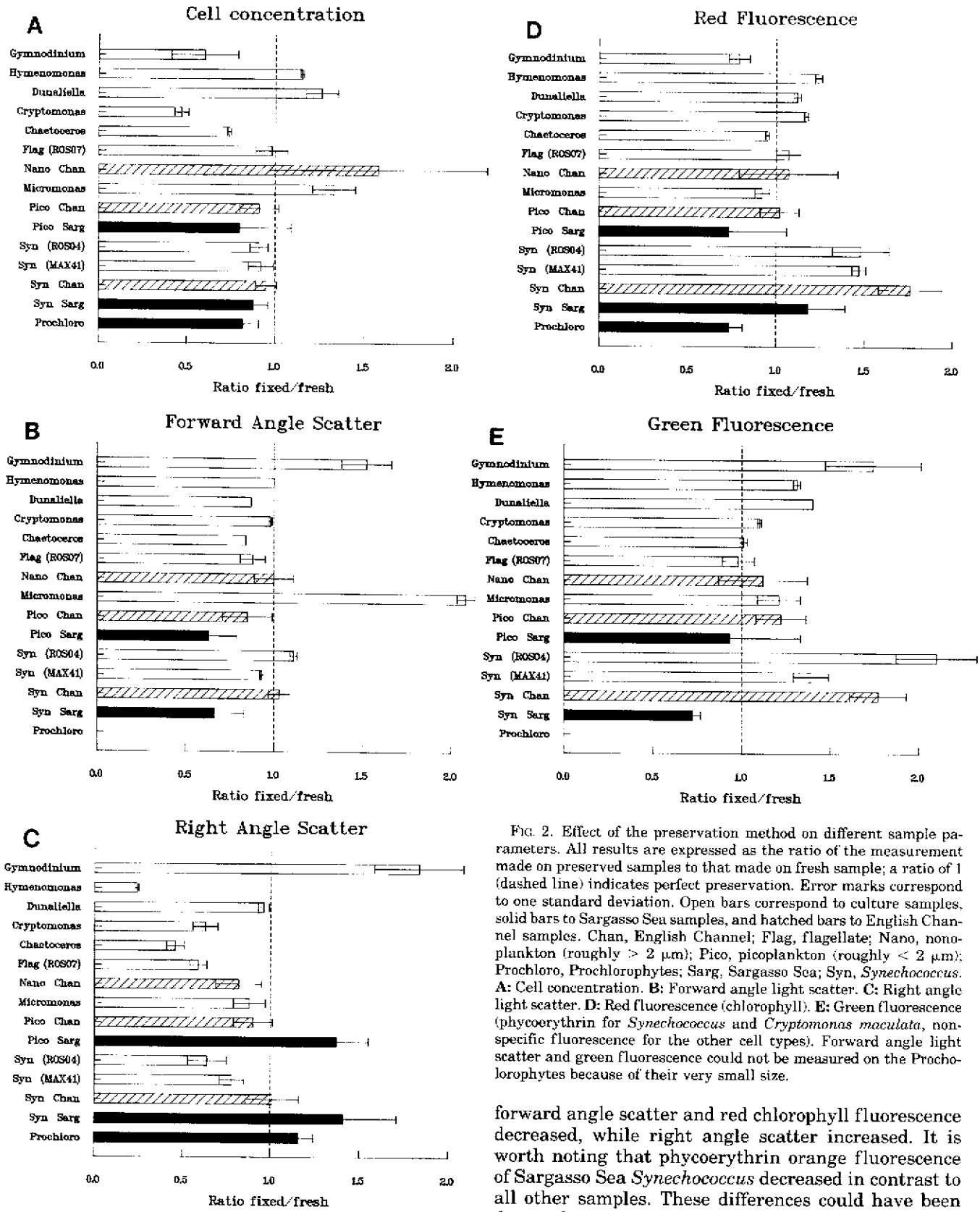

FIG. 2. Effect of the preservation method on different sample parameters. All results are expressed as the ratio of the measurement made on preserved samples to that made on fresh sample; a ratio of 1 (dashed line) indicates perfect preservation. Error marks correspond to one standard deviation. Open bars correspond to culture samples, solid bars to Sargasso Sea samples, and hatched bars to English Channel samples. Chan, English Channel; Flag, flagellate; Nano, nonoplankton (roughly > 2 μm); Pico, picoplankton (roughly < 2 μm); Prochloro, Prochlorophytes; Sarg, Sargasso Sea; Syn, *Synechococcus*. **A:** Cell concentration. **B:** Forward angle light scatter. **C:** Right angle light scatter. **D:** Red fluorescence (chlorophyll). **E:** Green fluorescence (phycoerythrin for *Synechococcus* and *Cryptomonas maculata*, nonspecific fluorescence for the other cell types). Forward angle light scatter and green fluorescence could not be measured on the Prochlorophytes because of their very small size.

forward angle scatter and red chlorophyll fluorescence decreased, while right angle scatter increased. It is worth noting that phycoerythrin orange fluorescence of Sargasso Sea *Synechococcus* decreased in contrast to all other samples. These differences could have been due to the extended preservation period of these samples (Table 1).

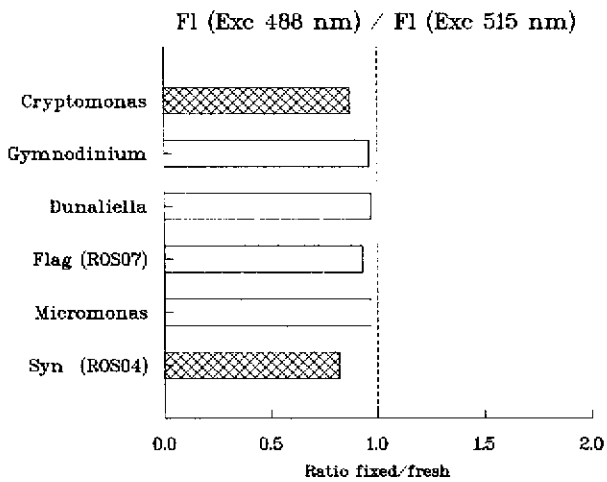

FIG. 3. Effect of the preservation method on the ratio between pigment fluorescence excited at 488 nm [Fl (Exc 488 nm)] and pigment fluorescence excited at 515 nm [Fl (Exc 515 nm)]. Open bars correspond to chlorophyll fluorescence and cross-hatched bars to phycoerythrin fluorescence. For abbreviations, see legend to Figure 2.

DISCUSSION

The method described in this paper worked with a variety of cultured and natural phytoplankton populations. In particular, the good preservation of cell concentrations for picoplanktonic populations (Fig. 2A) made it suitable for quantitative studies of their abundances in the field. When larger and more fragile cells, such as dinoflagellates or cryptophytes are present, results must be interpreted more cautiously since cell loss can be very important.

The weak modification of forward angle scatter in most cases (Fig. 2B) reflects the fact that in the size range considered (1–20 μm), this parameter depends mostly on cell size (1,18), which is little changed by glutaraldehyde fixation (3). In contrast, preservation may sometimes strongly affect right angle scatter, which depends for a large part on the cell refractive index. The latter quantity is probably modified by the

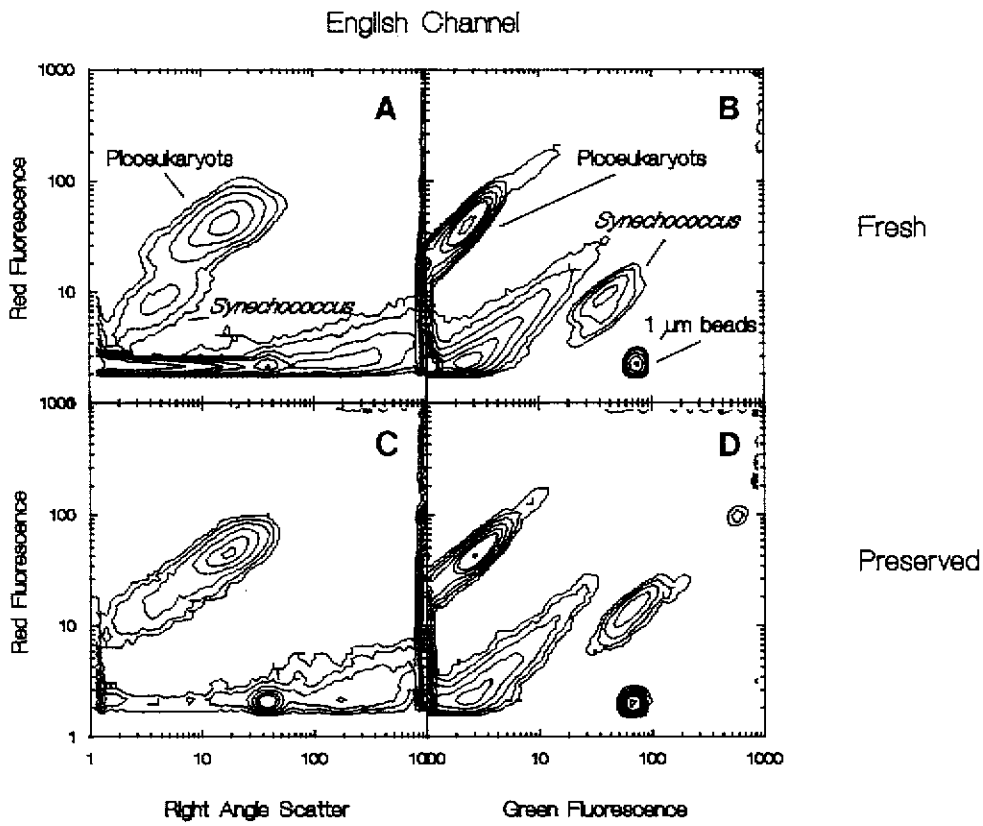

FIG. 4. Effect of the preservation method on the cytograms of natural phytoplankton samples from the English Channel. **A, B:** Fresh sample, right angle scatter vs. red fluorescence (A) and green fluorescence vs. red fluorescence (B). The different populations are well separated on the cytograms located to the right: *Synechococcus* cyanobacteria are characterized by the orange-green fluorescence of phycoerythrin, whereas picoplanktonic eukaryots have mostly red fluorescence. **C, D:** Preserved samples. The position of the picoplanktonic eukaryots is unchanged, while the cyanobacteria have more intense green and red fluorescences.

cross-linking of cell proteins resulting from glutaraldehyde fixation (Fig. 1).

The excitation spectrum and intensity of chlorophyll fluorescence were in general little modified for eukaryots, making this method suitable for quantitative estimates of phytoplankton biomass. For cyanobacteria *(Synechococcus)*, both chlorophyll and phycoerythrin fluorescence were generally increased. This is, however, not linked to the specifics of our method, but rather to cell death, and could be a consequence of uncoupling between the phycobilins and chlorophyll *a* (26). The fluorescence of fixed cells might actually be a better measure of cell phycoerythrin than that of live cells.

In conclusion, this method, although not universal for all phytoplanktonic species, could prove useful in environmental studies. It could well be extended to the preservation of other cell populations such as bacteria (C. Courties, unpublished data) or heterotrophic flagellates. Improvements could be sought through the addition of cryoprotectants such as dimethylsulphoxide (7) or through the use of alternative fixatives such as paraformaldehyde, which might better preserve fragile cells (L. Campbell, personnal communication).

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