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# Fluorescent In Situ Hybridization with rRNA-Targeted Oligonucleotide Probes To Identify Small Phytoplankton by Flow Cytometry

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**Because of their tiny size (0.2 to 2 μm), oceanic picophytoplanktonic cells (either cultured strains or natural communities) are difficult to identify, and some basic questions concerning their taxonomy, physiology, and ecology are still largely unanswered. The present study was designed to test the suitability of in situ hybridization with rRNA fluorescent probes detected by flow cytometry for the identification of small photosynthetic eukaryotes. Oligonucleotide probes targeted against regions of the 18S rRNAs of Chlorophyta lineage (CHLO probe) and of non-Chlorophyta (NCHLO probe) algal species were designed. The CHLO and NCHLO probes, which differed by a single nucleotide, allowed discrimination of chlorophyte from nonchlorophyte cultured strains. The sensitivity of each probe was dependent upon the size of the cells and upon their growth stage. The mean fluorescence was 8 to 80 times higher for specifically labeled than for nonspecifically labeled cells in exponential growth phase, but it decreased sharply in stationary phase. Such taxon-specific probes should increase the applicability of flow cytometry for the rapid identification of cultured pico- and nanoplanktonic strains, especially those that lack taxonomically useful morphological features.**

Photosynthetic oceanic pico- and nanoeukaryotes (0.2 to 2 and 2 to 20 μm in diameter, respectively) contribute significantly to standing stock and primary production in the oligotrophic areas of the world oceans (7, 9, 23) and can be important, at times, in coastal waters (14). These organisms can be distinguished from nonphotosynthetic organisms and photosynthetic picoplanktonic prokaryotes and can be enumerated by either flow cytometry or epifluorescence microscopy (21, 24, 32). Nevertheless, these techniques cannot provide details for species identification or even for the broad taxonomic affinities of most of these organisms. The reliable identification of such species, especially those which lack taxonomically useful morphological features, often requires the combined use of electron microscopy, pigment analysis with high-performance liquid chromatography, and 18S rRNA sequencing (4, 11). Difficulties associated with identifying such cells in culture and, especially, in the marine environment have long been limiting ecological and physiological studies of these small organisms. Some basic questions concerning the population structure and species composition of eukaryotic picoplankton assemblages are still largely unanswered (8, 33).

New approaches, such as immunolabeling (8) or the design of taxon-specific rRNA probes (3, 13, 15), are thus needed to help identify small phytoplanktonic cells and further to estimate the contributions of the different taxonomic groups to a given picoeukaryotic community. Moreover, rRNA probes may provide new information on those species which are not amenable to culture in the laboratory. The use of rRNA probes for the detection and identification of bacterial and archaeal species by epifluorescence microscopy is now common (3, 6, 28). Most recently, this technique was combined with flow cytometry for the detection of bacteria (2, 37) and nanoheterotrophs (25).

In this study, we tested the suitability of a phylogenetic approach based on oligonucleotide probes detected by flow cytometry to identify small photosynthetic eukaryotes. Since the level of phylogenetic diversity of the photosynthetic pico- and nanoeukaryotic community is probably high and since some taxa are probably still undescribed (as exemplified by the recent discovery of the class Pelagophyceae from an oceanic picoeukaryotic isolate [4]), probes which would recognize large taxonomic groups are obviously needed for a first-step discrimination of algal isolates or of algal species within a complex

TABLE 1. Designations and average diameters of the phytoplanktonic strains used in this study

| Class | Species | Strain designation[a] | Cell diam (μm)[b] |
|---|---|---|---|
| Prymnesiophyceae | *P. carterae* | CCMP 645 | 11 |
| | Unidentified | CCMP 625 | 3 |
| | *Imantonia* sp. | PCC 18561 | 3.5 |
| Pelagophyceae | *P. calceolata* | CCMP 1214 | 2 |
| Chlorophyceae | *C. coccoides* | PCC 494 | 7 |
| | *Chorella* sp. | CCMP 253 | 1.5 |
| | *Nannochloris* sp. | CCMP 515 | 1.7 |
| Prasinophyceae | *M. pusilla* | CCMP 490 | 1.5 |
| | *B. prasinos* | Type strain | 1.6 |
| | *P. provasolii* | CCMP 1203 | 2.6 |
| | Unidentified | EUM 16A | 1.5 |

[a] CCMP, Provasoli-Guillard Culture Center for Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine; PCC, Plymouth Culture Collection, Plymouth Marine Laboratory, Plymouth, United Kingdom. Strain EUM 16A was isolated during the EUMELI 3 French cruise in the tropical Atlantic ocean (21°02′N, 31°08′W). The *B. prasinos* type strain was kindly provided by Wenche Eikrem (Blindern, Norway).

[b] Equivalent diameters were measured either with light microscopy for *P. carterae* and *C. coccoides* or with a Coulter Counter for smaller cells (33).

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TABLE 2. Alignment of the CHLO and NCHLO probes with the complementary 18S rRNA regions from algae belonging to 12 classes[a]

| Class | Species | GenBank-EMBL accession number or reference | 18S rRNA sequence |
|---|---|---|---|
| | | | 1166 GTGGTGGTCCTCACCTCG 1195 (NCHLO probe) |
| Dinophyceae | *Alexandrium tamarense* | X54946 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Alexandrium fudyense* | U09048 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Prorocentrum micans* | 30 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Crypthecodinium cohnii* | M64245 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Gymnodinium viscum* | L13716 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Symbiodinium corculorum* | L13717 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Symbiodinium meandrina* | L13718 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Amphidinium belauense* | L13719 | ---CACCACCAGG**A**GTGGAGC--- |
| Fucophyceae | *Fucus distichus* | M97959 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Fucus gardneri* | X53987 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Costaria costata* | X53229 | ---CACCACCAGG**A**GTGGAGC--- |
| Bacillariophyceae | *Cylindrotheca closterium* | M87326 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Nitzschia apiculata* | M87334 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Coscinodiscus radiatus* | X77705 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Thalassionema nitzschoides* | X77702 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Rhizosolenia setigera* | M87329 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Bacillaria paxilifer* | M87325 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Skeletonema costatum* | M54988 | ---CACCACCAGG**A**GTGGA**A**C--- |
| | *Stephanopyxis broschii* | M87330 | ---CACCACCAGG**A**GT.GAGC--- |
| Synurophyceae | *Synura spinosa* | M87336 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Mallomonas papillosa* | M55285 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Mallomonas striata* | M87333 | ---CACCACCAGG**A**GTGGAGC--- |
| Chrysophyceae | *Chromulina chromophila* | M87332 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Ochromonas danica* | M32704 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Hibberdia magna* | M87331 | ---CACCACCAGG**A**GTGGAGC--- |
| Eustigmatophyceae | *Nannochloropsis* sp. | M87328 | ---CACCACCAGG**A**GTGGAGC--- |
| Pelagophyceae | *Pelagomonas calceolata* | 4 | ---CACCACCAGG**A**GTGGAGC--- |
| Prymnesiophyceae | *Emiliana huxleyi* | M87327 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Phaeocystis antarctica* (5 strains) | X77475 to 77479 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Phaeocystis globosa* | X77480 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Phaeocystis pouchetii* | X77481 | ---CACCACCAGG**A**GTGGAGC--- |
| Cryptophyceae | *Cryptomonas phi* | X57162 | ---CACCACCAGG**A**GTGGAGC--- |
| Prasinophyceae | *Nephroselmis olivacea* | X74754 | ---CACCACCAGG**A**GTGGAGC--- |
| | *Pseudocourfielda marina* | X75565 | ---CACCACCAGG**C**GTGGAGC--- |
| | *Scherffelia dubia* | X68484 | ---CACCACCAGG**C**GTGGAG.--- |
| | *Tetraselmis striata* | X70802 | ---CACCACCAGG**C**GTGGAGC--- |
| | *Mantoniella squamata* | X73999 | ---CACCACCAGG**C**GTGGAGC--- |
| | *Tetraselmis convolutae* | U05039 | ---CACCACCAGG**C**GTGGAGC--- |
| Chlorophyceae | *Chlamydomonas reinhardtii* | M32703 | ---CACCACCAGG**C**GTGGAGC--- |
| | *Chlorella vulgaris* | X13688 | ---CACCACCAGG**C**GTGGAGC--- |
| | *Dunaliella salina* | M84320 | ---CACCACCAGG**C**GTGGAGC--- |
| | *Chlorella minutissima* | X56102 | ---CACCACCAGG**C**GTGGAGC--- |
| | *Asteromonas gracilis* | M95614 | ---CACCACCAGG**C**GTGGAGC--- |
| | *Nanochlorum eukaryotum* | X06425 | ---CACCACCAGG**C**GTGGAGC--- |
| Pleurastrophyceae | *Friedmannia israelensis* | M62995 | ---CACCACCAGG**C**GTGGAGC--- |
| | | | 1164 GTGGTGGTCCGCACCTCG 1182 (CHLO Probe) |

[a] Spaces separate the different algal lineages (see text). Boldface letters indicate mismatches with the CHLO or NCHLO probe. Sixty-two sequences were examined for group signature sites. Of the 21 chlorophycean sequences that were examined, only a selection of species is presented here. All additional species proved to present perfect target sites for the CHLO probe.

community. Aligned sequences from 50 algal species were examined in order to develop probes for the Chlorophyta lineage (versus non-Chlorophyta taxa). Representative species with various cell sizes (nanoplankton or picoplankton size class) in different growth stages were used (i) to optimize the hybridization conditions and (ii) to evaluate the specificities and sensitivities of the probes. In parallel, we evaluated the impact of the hybridization treatment on the phytoplankton cell properties (light scatter and chlorophyll fluorescence) which are traditionally measured by flow cytometry and used to discriminate these cells from nonphotosynthetic organisms and photosynthetic prokaryotes.

## MATERIALS AND METHODS

**Strains and culture conditions.** Eleven marine photosynthetic nano- and picoeukaryote strains were selected for the hybridization experiments. Mean cell sizes ranged from 1.5 to 11 μm (Table 1). Cells were grown in K medium (19) at

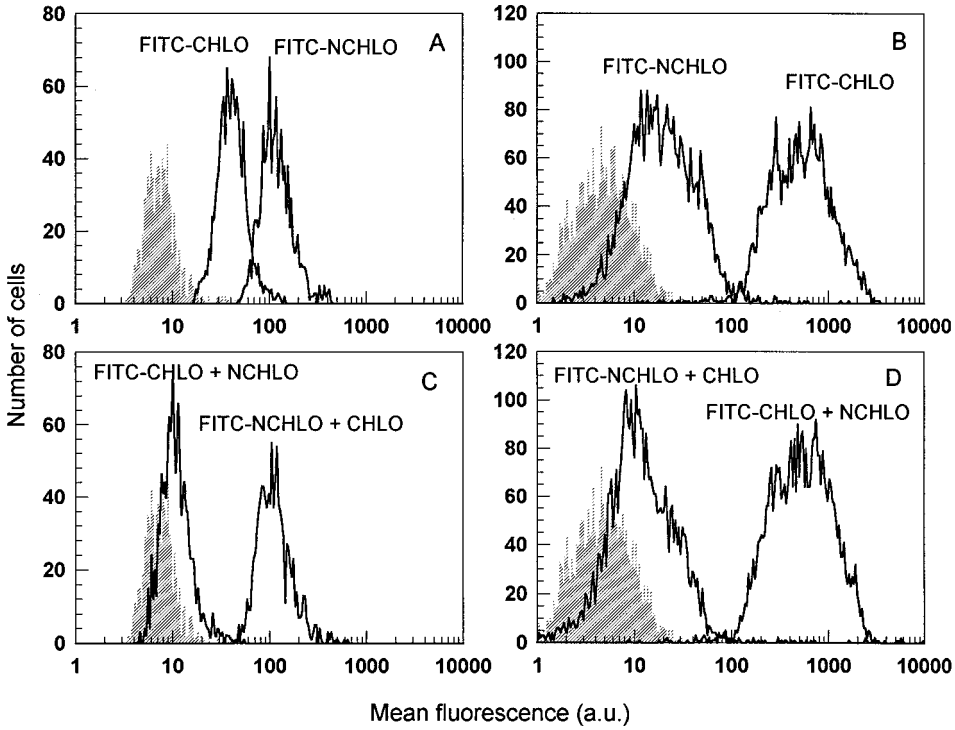


FIG. 1. Effect of the addition of competitor nonfluorescent-oligonucleotide probes on the specificity of hybridization. Histograms show the distribution of green fluorescence (in arbitrary units [a.u.]) of *P. carterae* (A and C) and *C. coccoides* (B and D) cells after hybridization with 2.5 ng of FITC-CHLO or FITC-NCHLO $\mu l^{-1}$ in the absence (A and B) and in the presence (C and D) of 2.5 ng of competitor unlabeled probes $\mu l^{-1}$. Shaded histograms show the autofluorescence of fixed cells incubated in hybridization buffer without probe (control).

17°C and with white light at 50 microeinsteins $m^{-2}$ $s^{-1}$ (provided by Sylvania Daylight fluorescent bulbs).

**Design of oligonucleotide probes.** The complete 18S rRNA coding sequences from 50 eukaryotic algae (26 Chlorophyta, 19 Heteroconta, 4 Dinophyta, and 1 Cryptophyta) were retrieved from the GenBank-EMBL database and aligned by using the Pileup program of the Genetics Computer Group software (16). The alignment was refined by hand, and signatures for the phylum Chlorophyta were searched by visual inspection of the aligned sequences. Two oligonucleotides, one characteristic for the algae belonging to the phylum Chlorophyta (probe CHLO) and the other characteristic for the rest of the algae (probe NCHLO) were designed (Table 2). Both electrophoretically purified unlabeled probes and fluorescein isothyocianate (FITC)-labeled oligonucleotide probes (FITC-CHLO and FITC-NCHLO) were obtained from Eurogentec (Seraing, Belgium). Labeling was performed by linking a fluorescein molecule to the 5′ end of the oligonucleotide via a six-carbon spacer arm.

**Cell fixation and whole-cell hybridization.** The protocol for in situ hybridization was a modification of a method previously described (37). Samples were fixed with fresh paraformaldehyde to a final concentration of 1% and stored at 4°C for 1 h. Cells were then pelleted (3 min, 4,000 × *g*) and resuspended in a cold (−80°C) mixture (70:30, vol:vol) of ethanol and phosphate-buffered saline (PBS) (obtained from Sigma, St. Louis, Mo.; 120 mM NaCl and 2.7 mM KCl in 10 mM phosphate buffer, pH 7.4). The cells were pelleted again and resuspended in 60 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris HCl [pH 7.8], 0.01% sodium dodecyl sulfate [SDS], and 25% deionized formamide). Two 20-μl aliquots of the cell suspension were incubated at 46°C for 3 h with either the FITC-CHLO or the FITC-NCHLO probe (2.5 ng $\mu l^{-1}$) in order to compare specific and nonspecific hybridizations. An aliquot incubated under the same conditions but with no probe added served as a control for autofluorescence. Hybridization was stopped by the addition of 1 ml of cold PBS at pH 9.0. Samples were then stored at 4°C until analysis with flow cytometry.

**Flow cytometry.** Analyses were performed with a FACSsort flow cytometer (Becton Dickinson, San Jose, Calif.). A 488-nm laser was used for excitation, and green fluorescence of fluorescein was collected through a 525 band pass filter. Cell mean fluorescence values were normalized to that of 0.95-μm-diameter beads (Polyscience Inc., Washington, Pa.) by using CYTOPC software (36).

## RESULTS

**Design of group-specific probes.** The alignment screened contained 18S rRNA gene sequences from 50 species belonging to 12 classes (12) segregated into 5 phyla (10): the Dinophyceae (phylum Dinophyta); Prymnesiophyceae (phylum Prymnesiophyta); Cryptophyceae (phylum Cryptophyta); Eustigmatophyceae, Fucophyceae, Bacillariophyceae, Synurophyceae, Chrysophyceae, and Pelagophyceae (phylum Heteroconta); and Chlorophyceae, Pleurastrophyceae, and Prasinophyceae (phylum Chlorophyta). In the relatively conserved region of helix 33 in the secondary structure model for eukaryotic small-subunit rRNA (31), a signature position (38) for the phylum Chlorophyta was observed, corresponding to position 1163 of the 18S rRNA sequence of *Saccharomyces cerevisae* (Table 2). A probe (CHLO) was designed to match this position (18 nucleotides from position 1153 to 1170) and to be complementary to all of the Chlorophyta sequences in this region. A second probe (NCHLO) was designed to hybridize to non-Chlorophyta algae (Table 2).

The NCHLO probe showed no mismatch with positions 1153 to 1170 of the 18S rRNA sequences available for the members of the phyla Cryptophyta, Dinophyta, Prymnesiophyta, and Heteroconta, with the exception of the diatoms *Skeletonema costatum* and *Stephanopyxis broschii* (one mismatch in the 5′ end region of the probe). Overall, the CHLO and NCHLO probes appeared to be the most reliable probes we could design to discriminate Chlorophyta from non-Chlorophyta species. The only exception was *Nephroselmis olivacea* (class Prasinophyceae), whose 18S rRNA sequence was published after we designed our probes (34). This species displayed a perfect target site for the NCHLO probe, although it belongs to the phylum Chlorophyta.

**Optimization of in situ hybridization conditions.** Tests to optimize the sensitivity of this method were performed with the representative nanoplanktonic chlorophycean and prymne-

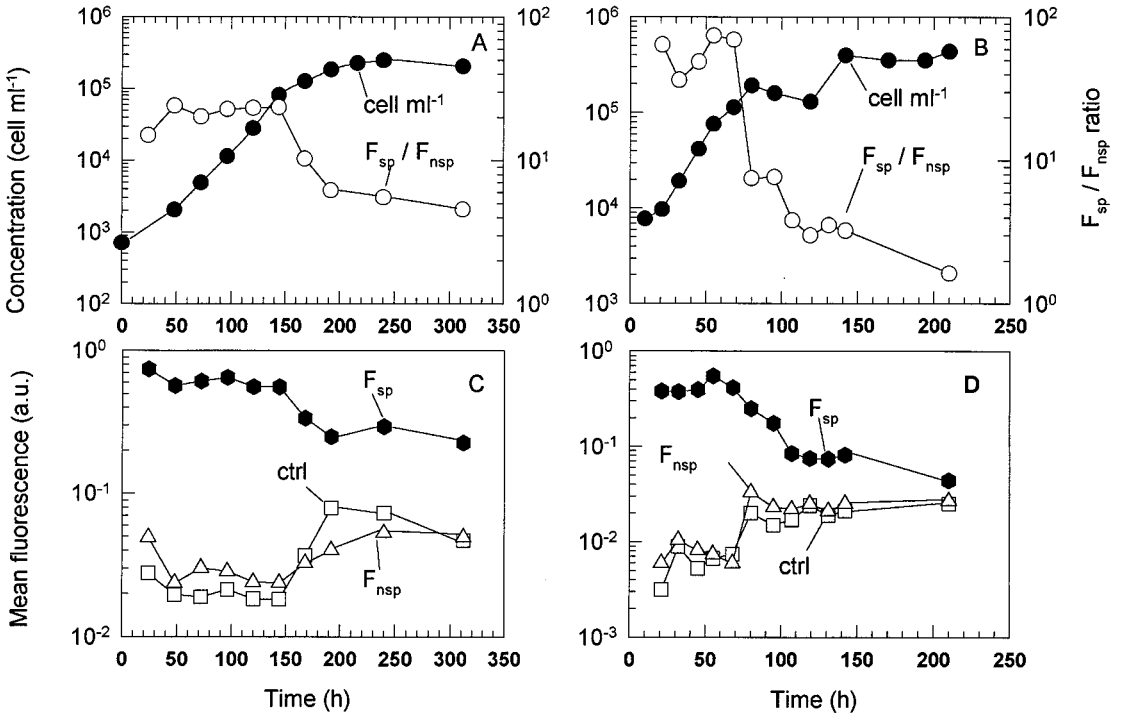


FIG. 2. Variation of cell fluorescence normalized to the fluorescence of 0.95-μm-diameter beads during the growth of the nanoplanktonic species *P. carterae* (A and C) and *C. coccoides* (B and D). (A and B) $F_{sp}/F_{nsp}$. (C and D) Corresponding variations of cell autofluorescence (ctrl), $F_{sp}$ (NCHLO for *P. carterae* and CHLO for *C. coccoides*), and $F_{nsp}$ (CHLO for *P. carterae* and NCHLO for *C. coccoides*). a.u., arbitrary units.

siophycean species *Chlamydomonas coccoides* (7 μm) and *Pleurochrysis carterae* (11 μm), respectively. Since the CHLO and NCHLO probes differed only by one centrally located nucleotide (Table 2), a high stringency was required to discriminate between specific and nonspecific binding of these probes. Despite the addition of 25% formamide, which increases the specificity of hybridization (28), binding of both the FITC-CHLO and FITC-NCHLO probes to nontarget organisms was observed (Fig. 1A and B). Under these conditions, the ratio of the fluorescence of specifically hybridized cells to that of nonspecifically hybridized cells ($F_{sp}/F_{nsp}$) was low for *P. carterae* ($F_{sp}/F_{nsp}$ = 2) and slightly higher for *C. coccoides* ($F_{sp}/F_{nsp}$ = 10). The specificity of each probe was clearly enhanced by the addition of 2.5 ng of competitor oligonucleotide per μl (28) (i.e., of the CHLO probe together with the FITC-NCHLO probe or vice versa) (Fig. 1C and D). Specific binding of the FITC-labeled probes was minimally affected by competition. In contrast, nontarget sequences were blocked by the competitor, and nonspecific binding therefore sharply declined. Consequently, the specific-to-nonspecific signal level was raised by approximately one order of magnitude. In all subsequent experiments, unlabeled competitor oligonucleotides were used to enhance the probe specificity.

**Detection of phytoplankton cells along their growth curve.** The amount of rRNA-specific probe binding to the cell varies with ribosome content and therefore reflects the cell's metabolic activities and growth rate (15). This amount also varies among species, depending in particular on the size of the cells (20). Since these probes are ultimately intended either for the identification of pico- and nanoplanktonic algal isolates or for the detection and identification of the different components of natural communities, with no assumption concerning the physiological status of the cells, we analyzed the hybridization signal for organisms of different sizes at various growth stages. First, we considered two of nanoplankton-sized species, *P. carterae* and *C. coccoides* (Table 1). Cells were harvested every 12 or 24 h until the culture reached the stationary phase of growth. For both species, the $F_{sp}/F_{nsp}$ ratio was high during exponential growth (80 for *C. coccoides* and 23 for *P. carterae*) and dropped sharply within 48 h of entry of the cells into stationary phase (3.5 and 4.5 respectively) (Fig. 2A and B). This phenomenon was due to variations of both specific and nonspecific fluorescence signals. The intensity of the specific fluorescence signal, which presumably reflected the rRNA content of the cells, showed a significant decrease when cells ceased dividing (Fig. 2C and D). Concomitantly, the green fluorescence of cells that were hybridized with the nonspecific probes increased. A similar increase of the nonspecific signal was also observed for nonhybridized control cells. This suggests that the nonspecific fluorescence was due to an increase in the autofluorescence of starving cells and not to nonspecific binding of labeled probes. Nevertheless, for *P. carterae*, labeled cells could be easily discriminated from nonlabeled cells even in stationary phase, as shown in Fig. 3.

Next, we examined picoplankton-sized species, i.e., *Pelagomonas calceolata* and a *Chlorella* sp. (Table 1), which have cell volumes 100 times smaller than that of *P. carterae* and *C. coccoides*. The $F_{sp}/F_{nsp}$ ratio measured during mid-exponential phase was high enough to guarantee an unambiguous identification of both species, although it was lower than those for larger species (Fig. 4, 5, and 6). A general decline of the $F_{sp}/F_{nsp}$ ratio was observed when cells entered the stationary phase (Fig. 4A and B), but the phenomenon was not as clear-cut as it was for larger cells (Fig. 2). At this stage, the fluorescence of specifically hybridized cells was within the level of background noise (Fig. 6). As a consequence, discrimination between specifically labeled species and unlabeled or unspe-

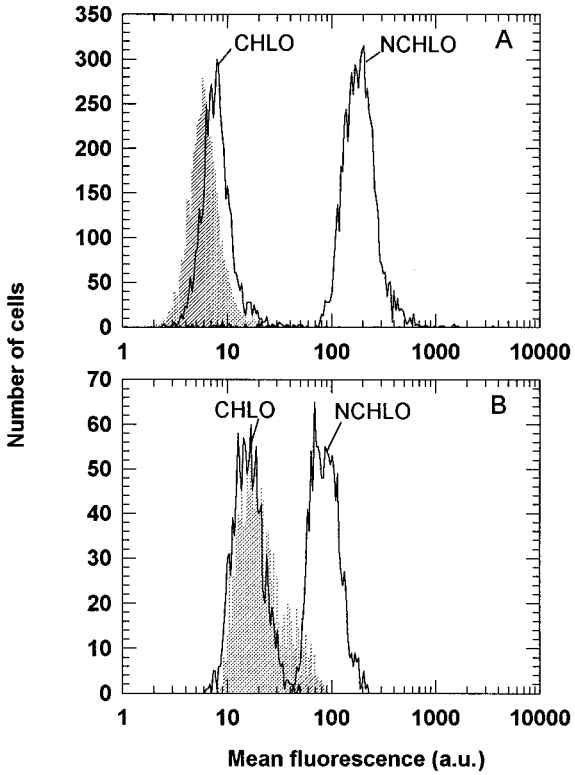


FIG. 3. Fluorescence signals measured after hybridization of *P. carterae* cells in exponential (A) or stationary (B) phase. Histograms show the distribution of green fluorescence for cells hybridized with the CHLO and NCHLO probes and the autofluorescence of control cells. Note that specifically hybridized cells were completely separated from nonspecifically hybridized cells during exponential phase. In stationary phase, the overlap between the CHLO and NCHLO histograms involved only 1% of the total cell number. a.u., arbitrary units.

cifically labeled cells should be difficult for such small organisms in stationary phase.

**Hybridization of the CHLO and NCHLO probes with a collection of picoeukaryotes belonging to different classes.** To check the specificities of the probes and their abilities to penetrate into cells that possess different types of cell walls and cell coverings, the probes were tested with a collection of picoeukaryotes belonging to different taxonomic groups with unknown 18S rRNA sequences. The specific hybridizations of the CHLO and NCHLO probes to the different strains were in agreement with the strain classifications (Table 3) (e.g., the CHLO probe hybridized specifically with the Chlorophyceae and Prasinophyceae, and the NCHLO probe hybridized with the Prymnesiophyceae). The only exception found was for *Pycnococcus provasolii* (class Prasinophyceae), which hybridized with the NCHLO probe and showed no signal with the Chlorophyta-specific probe. Overall, the fluorescence intensity conferred by the specific probe was cell volume dependent (Fig. 7), suggesting that the probes penetrated equally well in all species examined.

**Modification of the cell scatter and chlorophyll fluorescence induced by the hybridization treatment.** With natural seawater samples, flow cytometry enables the discrimination of picoeukaryotes from other organisms (larger phytoplanktonic cells, photosynthetic prokaryotes, bacteria, and nanoheterotrophs) on the basis of their different degrees of red chlorophyll fluorescence and right-angle light scatter (RALS). General information concerning their sizes and chlorophyll contents can also be derived from these parameters (33). We therefore analyzed the alterations of the RALS and red fluorescence induced by the treatment required for in situ hybridization. Both parameters were modified after the treatment, which includes cell fixation with both paraformaldehyde and ethanol followed by incubation in a buffer containing a detergent (0.01% SDS) and 25% formamide. The RALS either increased (*C. coccoides*, *Bathycoccus prasinos*, *P. provasolii*, *Micromonas pusilla*, and strain CCMP 625) by a factor of up to 2 or decreased (an *Imantonia* sp., a *Nanochloris* sp., and *P. calceolata*) without any apparent pattern. For *P. carterae*, two populations of cells with different RALS values were observed both before and after the treatment. These populations corresponded to cells with (low RALS) and without (high RALS) coccoliths, as confirmed by microscopic observations. The mean RALS values of these two populations were not significantly modified by the treatment, but a higher proportion of cells without coccoliths was observed by flow cytometry after the hybridization treatment.

The chlorophyll fluorescence of all species dramatically declined after the treatment. For the largest species (*P. carterae*, *C. coccoides*, and *Imantonia* sp., and the unidentified prymnesiophyte CCMP 625), it dropped by 67 to 99%. For the picoeukaryote species (*P. calceolata*, a *Chlorella* sp., a *Nannochloris* sp., *M. pusilla*, *B. prasinos*, *P. provasolii*, and the unidentified prasinophyte EUM 16A), it dropped to the limit of detection by the flow cytometer. Both ethanol fixation and the presence of detergent in the hybridization buffer obviously contributed to the decrease of fluorescence by their deleterious effect on the chlorophyll. Nevertheless, chlorophyll fluorescence dropped (e.g., by 77% for *P. carterae*) even when these compounds were omitted during hybridization (data not shown).

## DISCUSSION

**Specificities of the CHLO and NCHLO probes.** This study shows that highly specific hybridization conditions can be achieved for the flow cytometric discrimination of algal taxa with fluorescent-oligonucleotide probes that differ by only a single nucleotide. One key step is the analysis of the aligned sequences and of the phylogeny of the 18S rRNA gene in order to assess the theoretical abilities of the probes to discriminate between different taxonomic groups of microalgae.

Green algae together with land plants form a monophyletic lineage separated from other algal lineages (see, e.g., reference 29). The signature nucleotide selected with the sequences of the phylum Chlorophyta (Table 2) discriminated these sequences from those of other lineages of algae (Cryptophyta, Prymnesiophyta, Heteroconta, and Dinophyta) for almost all species for which the 18S rRNA gene sequence was available. The only exception was *N. olivacea*, whose 18S rRNA sequence was published after we designed our CHLO and NCHLO probes. This species occupies a basal position among the chlorophytes (34). The selected mutation, which is characteristic of chlorophytes, could have occurred after the divergence of these "early" species from the green alga lineage. Indeed, another prasinophyte species (*P. provasolii*) (Table 3) did not hybridize with the CHLO probe. Nevertheless, all of the other prasinophycean species analyzed here possessed a perfect target site for the CHLO probe.

Although non-Chlorophyta algae do not form a monophyletic assemblage (see, e.g., references 5 and 29), positions 1153 to 1170 (*S. cerevisae* numbering) of the 18S rRNA genes of all species examined presented at least one mismatch with the CHLO probe. Most of these species perfectly matched the NCHLO probe. Considering that the target sequence varies little among eukaryotic 18S rRNA sequences (31), with the

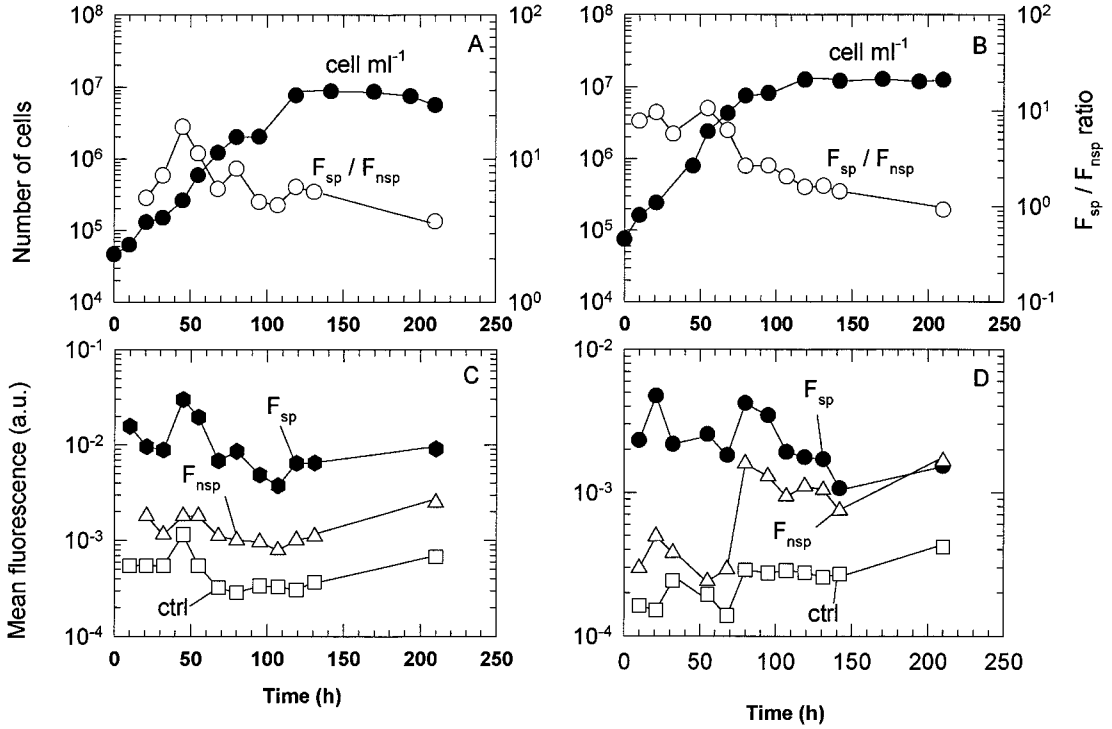


FIG. 4. Variation of cell fluorescence normalized to the fluorescence of 0.95-μm-diameter beads during the growth of the picoplanktonic species *P. calceolata* (A and C) and *Chlorella* sp. (B and D). (A and B) $F_{sp}/F_{nsp}$. (C and D) Corresponding variations of cell autofluorescence (ctrl), $F_{sp}$ (NCHLO for *P. calceolata* and CHLO for the *Chlorella* sp.), and $F_{nsp}$ (CHLO for *P. calceolata* and NCHLO for the *Chlorella* sp.). a.u., arbitrary units.

exception of position 1163 (signature for Chlorophyta), these probes should be reliable for the discrimination of most phytoplanktonic algae. A search revealed that no perfect target sites for the CHLO and NCHLO probes exist in the 16S rRNA gene sequences of prokaryotes that are available in the Ribosomal Database Project database (26). In contrast, the 18S rRNA region from position 1153 to 1170 (*S. cerevisae* numbering) of a few eukaryotic nonphotosynthetic organisms perfectly matched the CHLO or NCHLO probe. An example is a *Cafeteria* sp., a marine heterotrophic nanoflagellate that hybridized with the NCHLO probe. The use of these probes in the natural environment then is under the assumption that photosynthetic organisms can be distinguished from heterotrophs. Both of the currently used flow cytometric parameters for discriminating photosynthetic picoeukaryotes (light scatter and cell natural red [chlorophyll] fluorescence) are affected by the use of ethanol, detergent, and formamide. Thus, alternative, milder cell preparation methods are needed. Although circumventing this problem is not an easy task, it will probably be accomplished in the future.

**Sensitivities of the FITC-CHLO and FITC-NCHLO probes.** Independent from the difficulties associated with the choice of

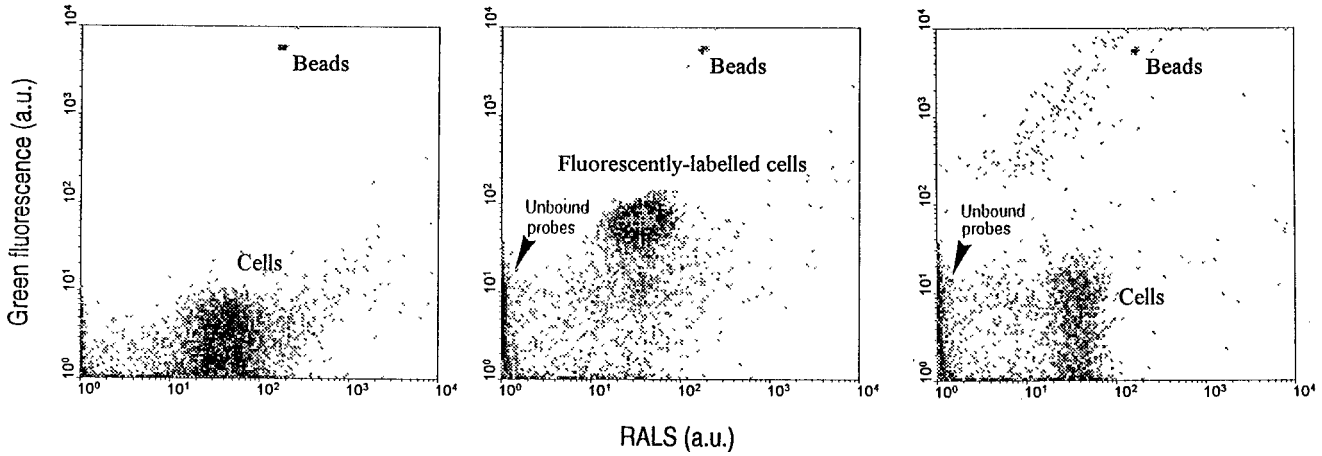


FIG. 5. Cytograms (RALS versus green fluorescence) of a *Chlorella* sp. in exponential phase. (A) Unlabeled cells (control). (B) Cells hybridized with the specific CHLO probe. (C) Cells hybridized with the nonspecific NCHLO probe. Note that in panel C, the intensity of fluorescence of the cells is not higher than the background noise caused by unbound FITC-labeled probes. a.u., arbitrary units.

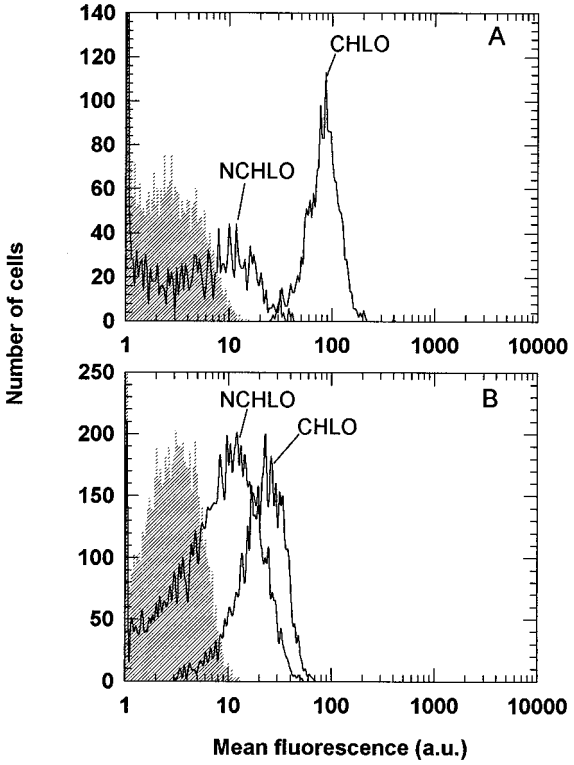


FIG. 6. Fluorescence signals measured after hybridization of *Chlorella* cells in exponential (A) or stationary (B) phase. Histograms show the distribution of green fluorescence for cells hybridized with the CHLO and NCHLO probes and the autofluorescence of control cells. The overlap between the histograms involved only 4% of the cells for exponentially growing organisms (A), compared with 54% of the cells in stationary phase (B). a.u., arbitrary units.

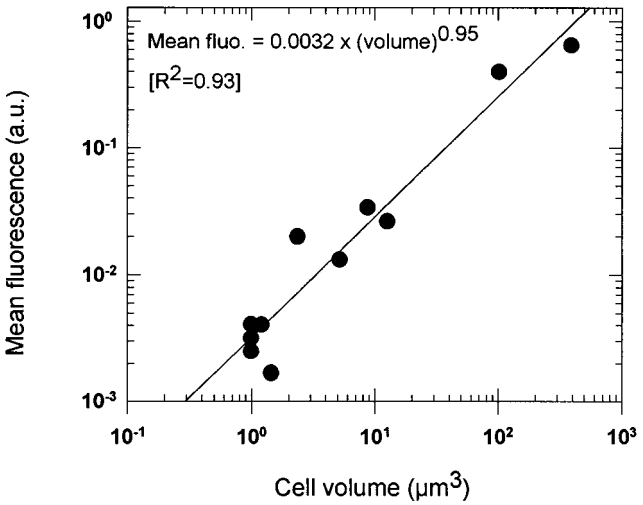


FIG. 7. Whole-cell hybridization for a variety of pico- and nanoeukaryotes, including *P. carterae*, an *Imantonia* sp., and an unidentified species (strain CCMP 625) (class Prymnesiophyceae); *P. calceolata* (class Pelagophyceae); *C. coccoides*, a *Chlorella* sp., and a *Nannochloris* sp. (class Chlorophyceae); and *B. prasinos*, *M. pusilla*, *P. provasolii*, and an unidentified strain (EUM 16A) (class Prasinophyceae). The intensity of the fluorescence (fluo.) of specifically hybridized cells is related to the volume of the cells. a.u., arbitrary units.

taxon-specific probes, problems associated with probe penetration into cells and with inaccessibility of target sites in the rRNA molecule have been reported for other organisms (1, 6). We encountered none of these problems when performing hybridization of the CHLO and NCHLO probes to the nano- and picoeukaryotes. In fact, for exponentially growing organisms, the $F_{sp}/F_{nsp}$ ratio was high for all species (Fig. 7) and especially for organisms with no cell wall, like *M. pusilla* (27), and organisms with a resistant cell wall, like *P. provasolii* (17).

After hybridization, the fluorescent signal emitted by individual cells could be precisely quantified by flow cytometry. We were therefore able to monitor the variations of the signal intensity for different species in different growth stages. This could be exploited to gain insights into the physiological activities of individual phytoplanktonic cells belonging to a given group.

For the smallest eukaryotes, the intensity of the probe-conferred fluorescence was not high enough to allow perfect detection of the hybridized cells in stationary phase (Fig. 5). This problem of signal intensity has been reported repeatedly for the detection of different organisms (22, 25). In our study, tiny eukaryotes in stationary phase probably did not contain enough rRNA to be detected. To enhance the fluorescence of specifically hybridized cells, the use of multiple labeling of longer probes (35), multiple monolabeled specific probes (22), or indirect labeling (25) has been suggested. In particular, the combination of a modified (fluorescence labeled) universal eukaryotic probe with a second taxon-specific probe could enhance the fluorescence of the cells above the level of the background noise and may then allow specific detection of starving small cells.

**Could similar probes be designed for lower taxonomic levels and in particular for classes?** The question of whether similar probes could be designed for lower taxonomic levels has to be discussed separately for each algal division. For example, it may not be possible to design probes that are specific for prasinophytes, at least with the 18S rRNA gene, since this class is polyphyletic within the Chlorophytea lineage (18, 34). There is no unique character, either phenotypic or phylogenetic, that unites the prasinophytes to the exclusion of other green algae. Specific probes for monophyletic lineages among the Prasinophyceae, such as the *Nephroselmis*/*Pseudocourfielda* group (34), should be more reliable. Similarly, it is likely that probes could be designed to discriminate between the different lineages among the nonchlorophyte algae, such as the Dinophyta group and the Heteroconta group. Oligonucleotide probes will soon be available for the discrimination and identification of the classes Prymnesiophyceae (30a) and Pelagophyceae (3a).

TABLE 3. Whole-cell hybridization of picoeukaryote species with the FITC-CHLO and FITC-NCHLO probes

| Class | Species | Strain designation | Hybridization[a] with: | |
|---|---|---|---|---|
| | | | CHLO probe | NCHLO probe |
| Chlorophyceae | *C. coccoides* | PCC 494 | + | − |
| | *Nanochloris* sp. | CCMP 515 | + | − |
| Prasinophyceae | *B. prasinos* | Type strain | + | − |
| | *M. pusilla* | CCMP 490 | + | − |
| | Unidentified | EUM 16A | + | − |
| | *P. provasolii* | CCMP 1203 | − | + |
| Prymnesiophyceae | *P. carterae* | CCMP 645 | − | + |
| | Unidentified | CCMP 625 | − | + |
| | *Imantonia* sp. | PCC 18561 | − | + |
| Pelagophyceae | *P. calceolata* | CCMP 1214 | − | + |

[a] +, $F_{sp}/F_{nsp} > 3$; −, no hybridization.

However, more sequences need to be gathered in order to be able to design reliable probes for each class within the different algal phyla. Probes for lower taxonomic levels (genus and species, etc.) then could be designed, as long as the group of organisms targeted is phylogenetically meaningful. Such probes should be useful especially for the rapid screening of collections of strains whose taxonomy is difficult to resolve by traditional techniques. These probes may also be readily applicable for in situ hybridization of nanoeukaryotes and picoeukaryotes that are metabolically active in natural seawater samples.

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