

Application of fluorescent in situ hybridization coupled with tyramide signal amplification (FISH-TSA) to assess eukaryotic picoplankton composition

Fabrice Not, Nathalie Simon, Isabelle C. Biegala, Daniel Vaulot

▶ **To cite this version:**

Fabrice Not, Nathalie Simon, Isabelle C. Biegala, Daniel Vaulot. Application of fluorescent in situ hybridization coupled with tyramide signal amplification (FISH-TSA) to assess eukaryotic picoplankton composition. Aquatic Microbial Ecology, 2002, 28, pp.157-166. 10.3354/ame028157 . ird-03759406

HAL Id: ird-03759406
https://ird.hal.science/ird-03759406

Submitted on 24 Aug 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Vol. 28: 157–166, 2002 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published June 26

Application of fluorescent *in situ* hybridization coupled with tyramide signal amplification (FISH-TSA) to assess eukaryotic picoplankton composition

Fabrice Not*, Nathalie Simon, Isabelle C. Biegala, Daniel Vaulot

Station Biologique, UMR 7127, CNRS, INSU et Université Pierre et Marie Curie, Place George Teissier, 29680 Roscoff, France

ABSTRACT: Photosynthetic picoeukaryotes (phytoplankton cells with a diameter smaller than 2 to 3 µm) contribute significantly to both biomass and primary production in the oligotrophic open ocean and coastal waters, at certain times of the year. The identification of these organisms is difficult because of their small size and simple morphology, therefore hindering detailed ecological studies of their distribution and role. In this paper, we demonstrate the use of oligonucleotide probes specific to algal classes or to lower order taxa in combination with fluorescent *in situ* hybridization and tyramide signal amplification (FISH-TSA) to determine eukaryotic picophytoplankton diversity. Target cells were detected and enumerated using epifluorescence microscopy. The sensitivity of the technique and the specificity of the probes were tested on pure and mixed picoplanktonic strains, as well as on natural samples from the English Channel. In these samples, the community was dominated by cells belonging to the division Chlorophyta. Haptophyta, Bolidophyceae and Pelagophyceae were also detected at low abundance. The FISH-TSA method is readily applicable to the study of picoplankton diversity in natural communities.

KEY WORDS: Fluorescent *in situ* hybridization · Tyramide signal amplification · Picoplankton · Eukaryotes · Coastal waters · Diversity

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

Cells smaller than 2 to 3 µm (picoplankton) are important in the marine environment (Li & Platt 1987). The eukaryotic component of the picoplankton has been recognized to contribute significantly to both biomass and primary production in oligotrophic areas of the world's oceans (Campbell et al. 1994, Li 1994) and can also be important in coastal waters (Courties et al. 1994, Campbell et al. 1998). Their small size and simple morphology (numerous picoeukaryotes have converged toward a coccoid form, particularly the photosynthetic ones, e.g. Potter et al. 1997) make their identification difficult and hinder diversity and ecological studies. Today, fewer than 40 picoplanktonic species belonging to 10 algal classes have been described from environmental isolates. The classes Pelagophyceae (Andersen et al. 1993) and Bolidophyceae (Guillou et al. 1999a) have been erected based solely on picoplanktonic species, suggesting that this size class is a reservoir of new taxa. More than 50 yr after the first description of photosynthetic picoeukaryotes in coastal waters (Butcher 1952) and 20 yr after their importance in oceanic waters was established (Johnson & Sieburth 1979), we are still unable to determine the dominant taxonomic groups in the various oceanic environments.

The use of molecular tools is greatly improving our ability to explore natural picoplanktonic communities. Phylogenetic analyses based upon 18S rRNA gene sequences obtained from DNA extracted from natural

*E-mail: not@sb-roscoff.fr

© Inter-Research 2002 · www.int-res.com

samples permit culture-independent assessments of diversity and indicate that there is a considerable number of yet uncultured species in eukaryotic picoplankton communities (Lopez-Garcia et al. 2001, Moon-van der Staay et al. 2001). The enumeration of specific taxonomic groups and the estimation of their contribution to eukaryotic picoplankton, however, are more difficult. Photosynthetic pigments have been widely used to estimate the contribution of algal classes to total chlorophyll *a* (chl *a*) biomass (Latasa & Bidigare 1998). However, this method is limited for several reasons. First, it cannot resolve diversity below the class level. Second, it is based on the assumption that the relative cellular content of diagnostic pigments is constant for a given algal group. This assumption is invalid because pigment content varies between species, as well as within species. For a given strain, it is also affected by environmental conditions (e.g. Stolte et al. 2000).

Whole cell fluorescent *in situ* hybridization (FISH) with rRNA-targeted nucleic acid probes has been used more and more extensively to detect bacteria (Amann 1995). This method, which combines identification with quantitative determination of cell number, has been successfully applied to complex bacteria communities such as biofilms (Brümmer et al. 2000), marine sediments (Llobet-Brossa et al. 1998) and soils (Ravenschlag et al. 2000). Fluorescent rRNA probes have not been used as widely for eukaryotic phytoplankton (Simon et al. 1995, Scholin et al. 1996). Attempts to use mono-labeled oligonucleotide probes for the study of photosynthetic picoplankton in marine waters have been unsuccessful (Simon et al. 1995) because signal intensity was too low to distinguish labeled cells from the auto-fluorescence of non-target cells and from background fluorescence. A number of signal amplification methods have been tested in recent years. Lim et al. (1993) combined a biotin-avidin system with rRNA probes and FISH to detect and quantify marine protists. Polyribonucleotide probes with multiple labels have been used to visualize and enumerate marine bacteria (Ludwig et al. 1994, DeLong et al. 1999). Schönhuber et al. (1997) described a hybridization method involving tyramide signal amplification (FISH-TSA). This technique appears very attractive since it increases fluorescence intensity 10 to 20 times over that of mono-labeled probes. Using horseradish peroxidase (HRP)-labeled probes and fluorescent tyramide as substrate for the enzyme, Schönhuber et al. (1999) and West et al. (2001) were able to use FISH for the detection and quantification of cyanobacteria both in cultures and in natural samples. In the present study, we adapted this method to eukaryotic picoplankton and we demonstrate its applicability to both cultured strains and natural communities.

MATERIALS AND METHODS

Cultures. Four uni-algal strains of nano- and picoeukaryotes belonging to the Prasinophyceae, Pelagophyceae and Bolidophyceae were selected (Table 1). They were grown in Nalgene flasks at 20°C in K medium (Keller et al. 1987). For hybridization tests, cells were harvested during the mid-exponential phase. In order to estimate the detection limit of 1 target taxa among non-target cells, artificial mixtures were created from pure cultures of *Ostreococcus tauri* (Chrétiennot-Dinet et al. 1995) and *Bolidomonas pacifica* (Guillou et al. 1999a). Eighteen milliliters of each culture was harvested and fixed with 2 ml of 10% paraformaldehyde (PFA) for 1 h (1% PFA final concentration). Cell densities of fixed cultures were estimated using flow cytometry (Marie et al. 1999). Five mixtures of *B. pacifica* and *O. tauri* with 1, 5, 10, 20 and 50% of *B. pacifica* cells, respectively, were prepared. Ten milliliters of these mixtures was filtered onto 0.2 µm Anodisc filters (Whatman). Filters were dehydrated in an ethanol series (50, 80 and 100%, 3 min each) and stored at –80°C until further analysis.

Natural samples. Water samples were collected at 1 m depth, on 3 occasions (July 28, August 9 and August 24, 2000) off Roscoff, France (Stn ASTAN: 48°45′ N; 4°00′ W) in 5 l Niskin bottles. Water temperature at the time of collection was approximately 15°C. Samples were pre-filtered through 3 µm pore size Nuclepore filters (Whatman) and 180 ml of sea water was fixed with 20 ml of 10% PFA for 1 h. Exceeding this duration resulted in cell loss. Samples were then filtered onto Anodisc filters under 200 mm Hg pressure and dehydrated in an ethanol series (50, 80 and 100%, 3 min each), making sure that the filters never dried between filtrations. Filters were stored at –80°C until further analysis. For flow cytometry analyses, 1.5 ml of 3 µm pre-filtered water was fixed with 1% PFA and 0.1% glutaraldehyde (final concentrations), deep-frozen in liquid nitrogen and stored at –80°C.

Flow cytometry. Total photosynthetic picoeukaryote cell counts were obtained by flow cytometry. Analyses were performed on fixed sea water samples with a FACScan flow cytometer (Becton Dickinson) following Marie et al. (1999). Photosynthetic picoeukaryotes were discriminated from cyanobacteria and enumerated using the Cytowin software (see www.sb-roscoff.fr/Phyto/cyto.html, Vaulot 1989).

Oligonucleotide probes. The probes used were chosen to encompass a range of picoplanktonic divisions and classes (Table 2). The theoretical specificity of the probes was checked on an rRNA database containing more than 14 000 sequences, using the probe match function of the ARB software (see www2.mikro.biologie.tu-muenchen.de/arb/). Oligonucleotide

Table 1. Origin and culture conditions of picoplankton strains tested. RCC: Roscoff Culture Collection

RCC number	Class	Species	Strain	Light ($\mu E\ m^{-2}\ s^{-1}$)	Cell diameter (μm)	Origin
116	Prasinophyceae	*Ostreococcus tauri*	OTTH 0595	100	0.8	Thau Lagoon
261	Prasinophyceae	*Pseudocourfieldia marina*	TAK9801	40	4	Takapoto atoll
205	Bolidophyceae	*Bolidomonas pacifica*	OLI 31 SE3	100	1.5	Equatorial Pacific
100	Pelagophyceae	*Pelagomonas calceolata*	CCMP1214	4	2	North Pacific Central gyre

Table 2. Characteristics of oligonucleotide probes used in this study. The bold letters point to the single base change between the NCHLO01 and CHLO01 probes. MW: molecular weight; n: number of bases

Probe	Sequence (5′→3′)	n	%GC	MW (Da)	Target group	Source
EUK1209R	GGG CAT CAC AGA CCT G	16	62.5	5146	Eukaryote	Giovannoni et al. (1988)
NCHLO01	GCT CCA C**T**C CTG GTG GTC	18	66.7	5773	Non-Chlorophyta	Simon et al. (1995)
CHLO01	GCT CCA C**G**C CTG GTG GTC	18	62.5	5798	Chlorophyta	Simon et al. (1995)
CHLO02	CTT CGA GCC CCC AAC TTT	18	55	5661	Chlorophyta	Simon et al. (2000)
PRYM02	GGA ATA CGA GTG CCC CTG AC	20	60	6451	Haptophyta	Simon et al. (2000)
PELA01	ACG TCC TTG TTC GAC GCT	18	55	5732	Pelagophyceae	Simon et al. (2000)
BOLI02	TAC CTA GGT ACG CAA ACC	18	50	5743	Bolidophyceae	Guillou et al. (1999b)

probes were purchased with a 5′ aminolink (C6) from Interactiva. The probes were then labeled with HRP (Roche Diagnostic Boehringer) according to Urdea et al. (1988) and Amann et al. (1992). CHLO02 was also purchased directly labeled with fluorescein isothiocyanate (FITC) at the 5′ terminus and purified by HPLC (Genset) in order to compare the fluorescence intensity conferred by mono-labeled probes to that of HRP-labeled probes.

Fish. The protocol used for *in situ* hybridization with FITC mono-labeled probes was adapted from Amann et al. (1995). Our protocol for *in situ* hybridization with HRP-labeled probes, signal amplification and target cell detection is described by Biegala et al. (2002) and was adapted from Schönhuber et al. (1999). In both cases, dehydrated filter samples were thawed and cut into 12 equal pieces. The filter face that supports the cells was marked with a black pen.

In brief, for hybridization with FITC-labeled probes, filters were covered by 10 µl of 40% formamide hybridization buffer (40% deionized formamide, 0.9 M NaCl, 20 mM Tris-HCl, pH 7.5, 0.01% sodium dodecyl sulfate [SDS]) and 1 µl of oligonucleotide probes (stock at 50 ng μl^{-1}) and incubated at 46°C for 3 h. After 2 successive washing steps of 20 min at 46°C in a wash buffer (56 mM NaCl, 5 mM EDTA, 0.01% SDS, 20 mM Tris-HCl, pH 7.5), the filters were dried before being mounted in antifading reagent (see below). For HRP-labeled probes, the hybridization step was slightly different and an additional signal amplification step was necessary. Ten percent (w:v) blocking reagent (Roche Diagnostic Boehringer) was added to the hybridization buffer described above, and hybridization and washing steps were conducted at 35 and 37°C, respectively. Samples were then equilibrated in TNT buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, Tween 20) for 15 min at room temperature. TSA (Kit NEN Life Science Products) was performed for 30 min at room temperature in the dark in 10 µl of TSA mix (1:1 dextran sulfate and amplification diluent, 1:50 FITC tyramide with the mixture of dextran sulfate and amplification diluent). Filters were then transferred in 2 successive 5 ml TNT buffer baths at 55°C for 20 min each, in order to stop the enzymatic reaction and to remove dextran sulfate. Cells were briefly rinsed in 5 ml of distilled water and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) at 5 µg ml^{-1} during 7 min. Filters were finally rinsed in distilled water for 10 min and dried on a slide. Both the filters hybridized with mono-labeled probes and HRP-labeled probes were mounted in antifading reagent AF3 (Citifluor), and the coverslip was fixed to the slide with nail varnish to prevent evaporation of Citifluor. Slides could be stored in the dark at 4°C for 2 wk without significant loss of fluorescence.

Epifluorescence microscopy and image acquisition. The hybridized and DAPI-stained filters were observed with an Olympus BH-2 epifluorescence microscope (Olympus Optical) equipped with a mercury light source and a 40× UV fluorescence objective. Excitation/emission filters were 360/420 for DAPI and 490/515 for FITC. For each natural sample, cells in 10

randomly chosen microscopic fields were visually counted. For probes with a broad taxonomic specificity (e.g. EUK1209R), more than 2000 cells were counted. Images were acquired with an RT-Slider Spot cooled charge coupled device (CCD) digital camera (Diagnostic Instruments). For images of cells in culture, the exposure time and gain were constant for a given species. These values were determined by automatic exposure with the probe specific for the species (e.g. PELA01 for *Pelagomonas* sp.) and then used for the other probes. Images of natural samples were acquired using automatic exposure.

RESULTS

The EUK1209R probe was used in this study as a general eukaryotic probe. However, some species and entire clades such as the Rhodophyta division, as well as some Chlorophyta orders such as the Prasiolales and Dasychladales, which are all probably underrepresented in the picoplankton as suggested by environmental 18S rDNA clone libraries (Lopez-Garcia et al. 2001, Moon-van der Staay et al. 2001), are not targeted by this probe. The CHLO01 and NCHLO01 probes, which differ from each other by a single nucleotide, were initially designed to target the division Chlorophyta and all non-Chlorophyta algae, respectively (Simon et al. 1995). However, our current database search revealed that the CHLO01 probe does not recognize the Pseudoscourfieldiales and Pyramimonadales clades, nor the Prasiolales and Zygnematophyceae, all of which are members of the division Chlorophyta, and this probe cross-hybridizes to several taxa within the Cryptophyceae, Pavlovales and Apicomplexa. Conversely, the NCHLO01 probe recognizes some Chlorophyta taxa such as the Pseudoscourfieldiales or the Pyramimonadales, but not the Rhodophyta, some Cryptophyceae, numerous Apicomplexa and even 1 *Chrysochromulina* clade within Haptophyta. When used in combination, and since they are complementary to each other, the CHLO01 and NCHLO01 probes target most eukaryotic sequences of the 18S rRNA gene database. The probe CHLO02, which was designed more recently (Simon et al. 2000), targets more Chlorophyta species than CHLO01 and has the advantage of not targeting any non-Chlorophyta taxa. The probes PRYM02, PELA01 and BOLI02 are specific for the division or the class they target (Haptophyta, Pelagophyceae, Bolidophyceae).

In order to test the specificity and the sensitivity of FISH-TSA for the detection and identification of picoplanktonic algae, we hybridized cultures of *Pseudoscourfieldia marina*, *Pelagomonas calceolata* and *Bolidomonas pacifica* with the 4 HRP-labeled probes EUK1209R, CHLO01, PELA01 and BOLI02 (Fig. 1). Target species displayed a very bright fluorescence signal, while non-target species showed only a faint fluorescence. For a given probe, the fluorescence intensity of target species was 25 times higher than that of non-target species. Using DAPI counterstaining, we could verify that for a given probe, every target cell was labeled (see Fig. 1 for an example with *B. pacifica*). For all hybridizations, filter fluorescence background and cell auto-fluorescence were very weak and roughly equivalent to those of cells labeled with non-target probes. The signal conferred by HRP-labeled probes is much stronger than that by fluorescein mono-labeled oligonucleotides, as demonstrated for *P. marina* (Fig. 2), *B. pacifica* and *P. calceolata* (data not shown).

The aim of this study was not only to detect a given taxon within a complex community but also to estimate the abundance of the target organisms. As a first test, we analyzed artificial mixtures of 2 picoplanktonic species (*Bolidomonas pacifica* and *Ostreococcus tauri*). These cells were hybridized by FISH-TSA with the BOLI02 and CHLO01 probes (Table 2). Labeled cells were enumerated under epifluorescence microscopy. The fraction of cells hybridized with a given probe (e.g. BOLI02) corresponded very well with the theoretical fraction of cells belonging to the target taxon as determined by flow cytometry before mixing both species (Fig. 3). This was true for all mixtures including those where targeted cell numbers represented as little as 1% of the total population.

Given the improved sensitivity of this detection and identification system, the next step was to test it on complex communities from coastal sea waters. The picophytoplankton community in the English Channel, off Roscoff, includes *Synechococcus* sp. cyanobacteria and picoeukaryotes. In samples collected during the summer, photosynthetic picoeukaryote cell densities determined by flow cytometry ranged between 4293 and 5627 cell ml^{-1} (Table 3). Using HRP-labeled probes and FISH-TSA combined with epifluorescence microscopy, we were able to detect and enumerate the cells belonging to every group targeted (Fig. 4). The fluorescence of labeled cells was very strong and background fluorescence was very low, as observed for cultures. The density of picoeukaryotic (both autotrophic and heterotrophic) cells hybridized by either the probe CHLO01 or the probe NCHLO01 ranged between 4234 and 5291 cell ml^{-1} (Table 3). Comparatively, the density of cells labeled by the EUK1209R probes was slightly lower. The use of probes targeting lower taxonomic levels of primarily photosynthetic algae in the divisions Chlorophyta (probe CHLO02) and Haptophyta (probe PRYM02), and the classes Pelagophyceae (probe PELA01) and

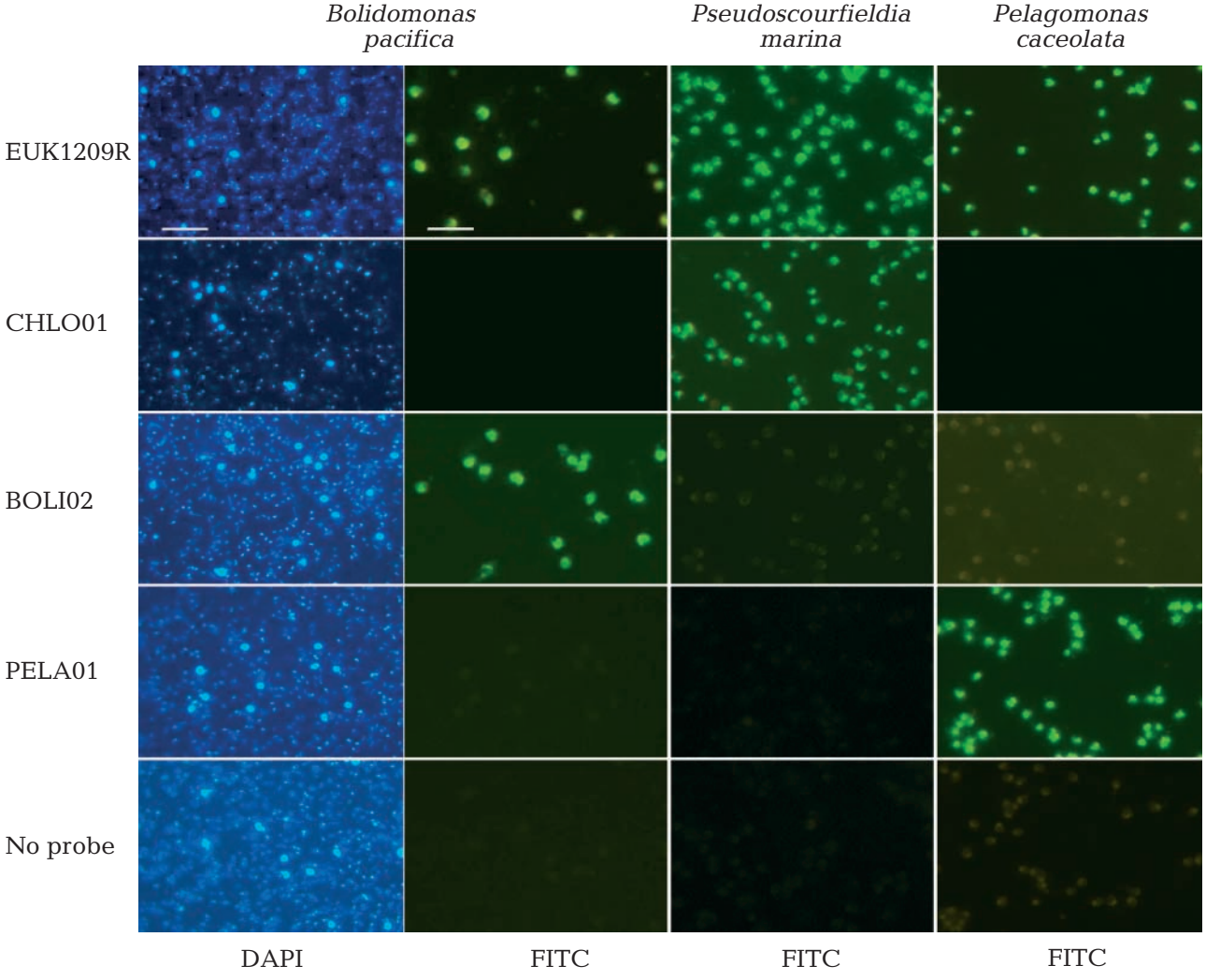


Fig. 1. Epifluorescence microscopy. Picoplanktonic isolates hybridized with taxa-specific probes. All samples were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI), but only results obtained for *Bolidomonas pacifica* are shown. Scale bar = 10 µm. FITC: fluorescein isothiocyanate

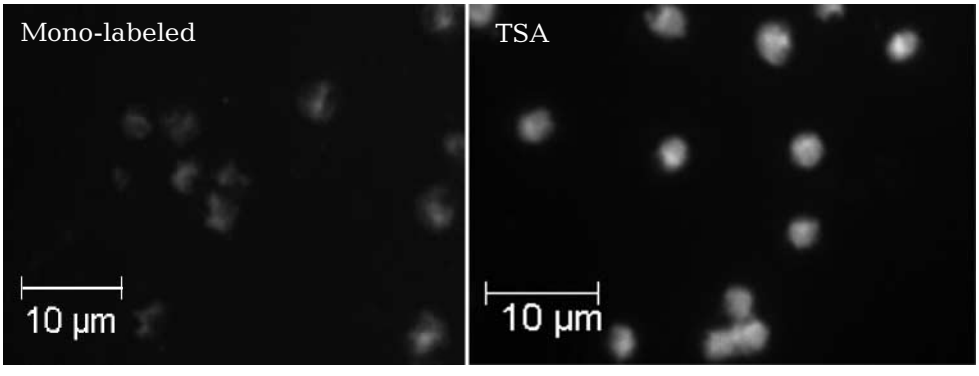


Fig. 2. Epifluorescence microscopy. *Pseudoscourfieldia marina* hybridized with the CHLO02 probe either mono-labeled (left) or horseradish peroxidase (HRP)-labeled (right) revealed by tyramide signal amplification (TSA). Exposure time for mono-labeled probe (left) was 17.5 times longer than for TSA (right)

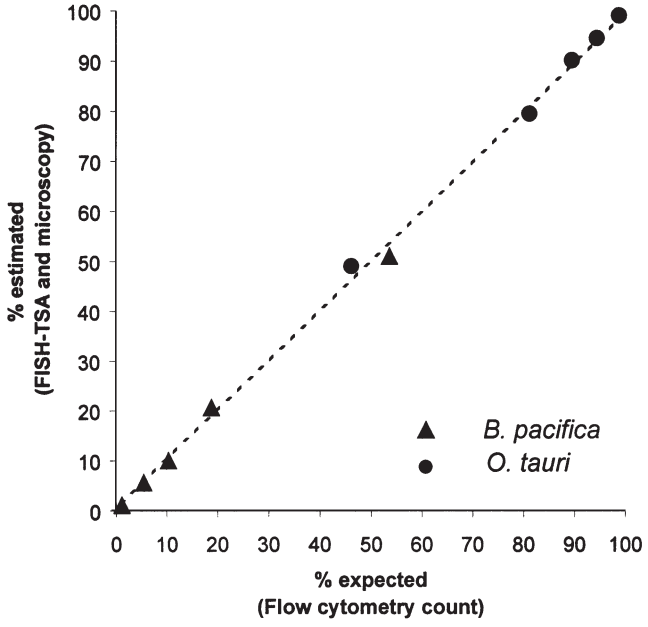


Fig. 3. Percentages of *Bolidomonas pacifica* and *Ostreococcus tauri* in an artificial mixture. Flow cytometry counts were obtained before mixing both species while microscopic counts were obtained from the mixed culture, after hybridization with the probes BOLI01 (specific for the genus *Bolidomonas*) and CHLO01 (specific for the Chlorophyta and targeting *Ostreococcus tauri*). The straight line corresponds to a 1:1 relationship. FISH: fluorescent *in situ* hybridization

Bolidophyceae (probe BOLI02) allowed the assessment of the contribution of specific groups. The CHLO02 probe targeted over 57% of the eukaryotes detected by the sum of CHLO01 + NCHLO01 (Table 3). In contrast, only a small fraction of cells were labeled by the class specific probes PRYM02, PELA01 and BOLI02 (Table 3).

DISCUSSION

The combination of HRP-labeled oligonucleotide probes and of the substrate fluorescein-tyramide has been used for the detection of cyanobacteria that possess strong background auto-fluorescence (Schönhuber et al. 1999, West et al. 2001). Intense and homogeneous fluorescence labeling of the target cells was achieved for these prokaryotic organisms provided that cells were permeabilized with lysozyme before hybridization. In the present study, this detection system (FISH-TSA) intensely labeled photosynthetic picoeukaryotic isolates (Fig. 1). TSA overcomes the problems linked to the low fluorescence levels from mono-labeled probes (Fig. 2). Target cells were easily detected and enumerated under epifluorescence microscopy provided that the following precautions were carefully observed. First, the fixation step should not exceed 1 h, beyond which there is significant cell loss (flow cytometry analyses of fresh vs PFA-fixed samples indicate 11, 30 and 45% of cells lost after 1, 5 and 24 h of fixation, respectively). The inadequacy of PFA to fix picoplankton cells has been recognized for quite a long time, and current protocols used for flow cytometry recommend the combined use of glutaraldehyde at 0.1% final concentration (Marie et al. 1999). However, the latter fixative is not compatible with FISH (Marie et al. 2000). Second, in order to achieve an intense labeling of all target cells, the filter must always remain wet during the filtration and dehydration steps.

Enzymatic permeabilization was not necessary to allow the penetration of the relatively large HRP molecule (molecular weight [MW] 40 000) into the picoeukaryote species tested. Indeed, the cell wall of these species either is lacking (*Bolidomonas pacifica*)

Table 3. Picoplankton abundance in natural samples determined either by flow cytometry or by fluorescent probes and epifluorescence microscopy. FISH-TSA: fluorescent *in situ* hybridization and tyramide signal amplification; dates given as dd/mm/yy

	24/07/00		09/08/00		24/08/00	
	Cell ml^{-1}	% of CHLO01 + NCHLO01[a]	Cell ml^{-1}	% of CHLO01 + NCHLO01[a]	Cell ml^{-1}	% of CHLO01 + NCHLO01[a]
Flow cytometry	5627		5472		4293	
FISH-TSA						
CHLO01 + NCHLO01[a]	5291	100	5213	100	4234	100
EUK1209R	3673	69	3355	64	4187	99
CHLO01	2926	55	3203	61	2562	61
CHLO02	3899	74	4205	81	2400	57
NCHLO01	2365	45	2010	39	1672	39
PELA01	0	0	8	0.2	94	2
BOLI02	14	0.3	7	0.1	19	0.4
PRYM02	416	8	228	4	456	11

[a]Sum of the numbers of cells hybridized with CHLO01 and NCHLO01 probes

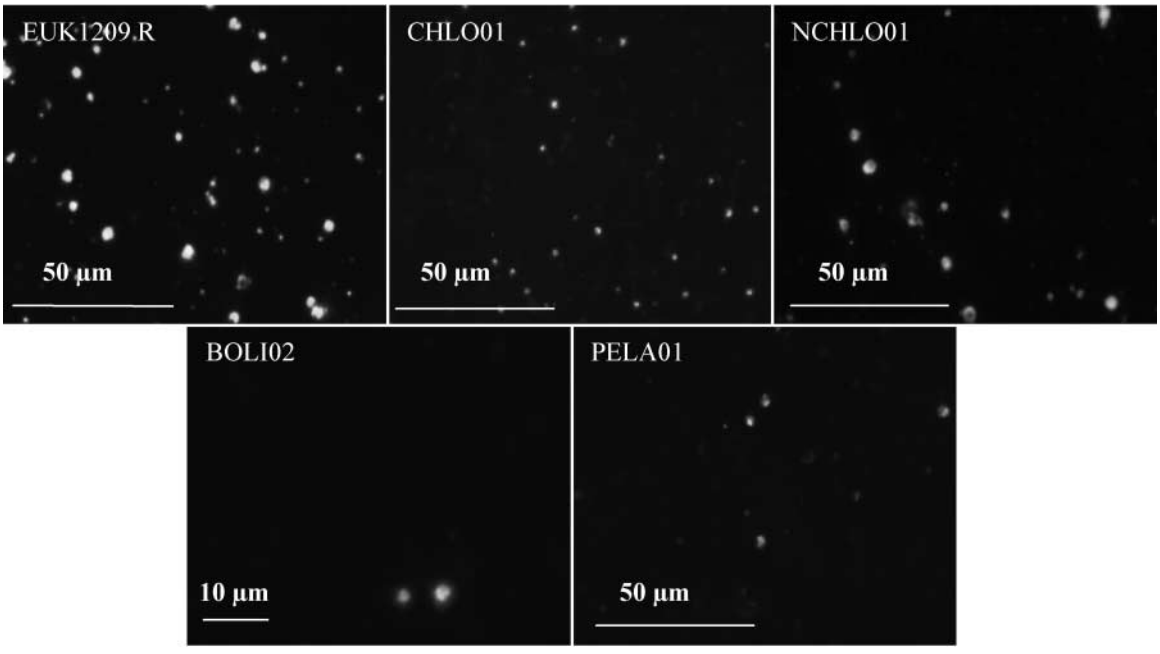


Fig. 4. Epifluorescence microscopy. Picoeukaryote sample from the English Channel in summer 2000. Cells were hybridized with the probes EUK1209R, CHLO01, NCHLO01, BOLI02 and PELA01

or consists of a very thin organic theca (*Pelagomonas calceolata*) or of organic scales (*Pseudoscourfieldia marina*). Yet, for cells with a thicker cell wall such as thecate dinoflagellates, Biegala et al. (2002) had no problem with HRP-labeled probe penetration. However, in the context of another study, we encountered some penetration problems with a few particular species such as *Pycnococcus provasolii* (unpubl.). Extensive tests would be necessary to validate this method on all phytoplankton cell types, and specific protocols will probably be needed for some species. Other parameters may reduce the fluorescence of target cells, in particular low rRNA content linked to low growth rate (Amann et al. 1995, Head et al. 1998). In our experiments, although phytoplankton cells hybridized in the stationary phase are not labeled as uniformly as in the exponential growth phase (data not shown), they are still easily detected. Under the conditions described above, the relative proportion of a target taxon in a mixture can be very accurately estimated (Fig. 3).

In our natural samples from the English Channel, the sum of cells targeted by the probes CHLO01 and NCHLO01, which are complementary to each other, was higher than the number of cells targeted by probe EUK1209R. Surprisingly, database matches revealed that EUK1209R targets more 18S sequences (3269) than the combination of CHLO01 + NCHLO01 (2305). However, the 18S database is not restricted to picoplankton, and conversely many taxa from the picoplankton appear to correspond to novel lineages not yet represented in the databases (Lopez-Garcia et al. 2001, Moon-van der Staay et al. 2001). Therefore the combination of CHLO01 + NCHLO01 may, in reality, target more picoplankton species than EUK1209R. In addition, studies by Fuchs et al. (1998) on *in situ* accessibility of 16S rRNA for fluorescently labeled oligonucleotide probes suggest that the rRNA secondary structure could be more favorable for hybridization with the CHLO01 and NCHLO01 probes than with EUK1209R: CHLO01 + NCHLO01 and EUK1209R probes bind with regions of the rRNA molecule corresponding to Classes III and V, respectively, with Class I giving the brightest fluorescence and Class VI the lowest (Fuchs et al. 1998). Therefore some cells, although targeted by EUK1209R, could fluoresce very weakly and not be detected. One solution to circumvent this problem could be the use of helper probes that have been used to open inaccessible rRNA regions for FISH in bacteria and to increase the fluorescence signal (Fuchs et al. 2000). Our data therefore suggest that the combination of CHLO01 + NCHLO01 may be more adequate than EUK1209R to obtain total eukaryote counts in picoplankton samples.

The number of cells detected using either the probe EUK1209R or the combination of probes CHLO01 + NCHLO01 was, however, lower than the number of

photosynthetic cells detected by flow cytometry (Table 3). This latter estimate (at about 5000 cells ml^{-1}) fell into the range previously observed in the English Channel off Roscoff (1000 to 25 000 cells ml^{-1} over the year, Sournia & Birrien 1995, Vaulot & Marie unpubl.). This discrepancy is surprising since the probe EUK1209R and the combination of probes CHLO01 and NCHLO01 target both photosynthetic and non-photosynthetic eukaryotic taxa and therefore should provide higher cell numbers than flow cytometry. Several hypotheses can explain these results. One source of the discrepancy might be errors in counting. However, the standard deviation for microscopic counts was about 6% for the more general probes and therefore cannot explain this discrepancy. Second, heterotrophic cells may not contribute significantly to the picoplanktonic community. However, in general, heterotrophic taxa make up a sizable fraction of eukaryotic picoplankton (e.g. 18 to 38% in Andersen et al. 1996). Moreover, clone libraries established from Roscoff picoplanktonic samples yielded a large number of 18S rRNA sequences affiliated with heterotrophic taxa (K. R. Romari unpubl.). Third, some cells may be destroyed during fixation or during the treatments associated with FISH (ethanol series and detergents) and some taxa may be impermeable to the HRP-labeled probes (see above). Fourth, some picoeukaryote taxa present in abundance at the station sampled may not be labeled (or only weakly) by the probes used (see above). These last 2 reasons probably explain most of the discrepancy observed.

Among the cells recognized by the probes used on natural samples, those belonging to the division Chlorophyta dominated the coastal eukaryotic picoplankton in summer (2400 to 4205 cells ml^{-1} targeted by the probe CHLO02). This is in agreement with historical phytoplankton pigment data at this coastal site (Klein & Sournia 1987) and with more recent data showing the dominance of chl *b* in the <3 µm size fraction (M. L. Latasa unpubl.). In addition, this concurs with the large number of sequences attributed to members of the class Prasinophyceae (division Chlorophyta) retrieved from sea water samples in total extracted DNA (K. R. Romari unpubl.). Picoeukaryotic cells belonging to other groups targeted by the NCHLO01 probe are also abundant (1672 to 2365 cells ml^{-1}). Among these cells, the division Haptophyta is the most abundant group detected (4 to 11% of the sum of NCHL0O1 + CHLO01 targeted cells). Only very few cells (<1%) were detected with the probe targeting the Pelagophyceae, assumed to be important in marine waters (Andersen et al. 1996). The presence of Bolidophyceae in coastal waters was unexpected since members of this class have been isolated to date only from oligotrophic marine waters of the Pacific and the Mediterranean Sea (Guillou et al. 1999a,b). The 3 probes, PRYM02, PELA01 and BOLI02, used in this study targeted only 34% of non-Chlorophyta cells, suggesting that a large fraction of cells are composed of non-targeted groups such as the Chrysophyceae and the Cryptophyceae or of unknown organisms such as those from the new Alveolata and Stramenopile lineages identified in clone libraries (Lopez-Garcia et al. 2001, Moon-van der Staay et al. 2001).

If FISH-TSA appears to date as the most appropriate method to directly combine identification and quantification of cells from a complex picophytoplankton community, it is critical to increase considerably the number of available 18S rRNA sequences in databases. This will allow determination, *in silico*, of probe specificity, particularly for those probes that were designed some years ago when there were still a limited number of 18S rRNA sequences available (e.g. EUK1209R, CHLO01 and NCHLO01). Ideally, for general probes such as eukaryotic probes or probes targeting division taxa, the simultaneous use of 2 or 3 probes appears to be the best way to assess the abundance of the corresponding group in a natural sample (Amann & Ludwig 2000).

Phytoplankton ecology has relied heavily on approaches that were developed more than a decade ago, such as flow cytometry (Olson et al. 1985) or HPLC pigment analysis (Gieskes & Kraay 1983). Clearly, it should extensively adopt molecular approaches such as FISH or gene cloning that have proved to be useful for the study of the diversity and the role of oceanic bacteria (Giovannoni et al. 1990, Fuhrman et al. 1992, Béja et al. 2000). An additional advantage of these methods is that they also target heterotrophic eukaryotes for which, until now, it was only possible to estimate total abundance with stains such as DAPI or primulin. Indeed, virtually no information is available on the taxonomic composition of this compartment. The combination of FISH with techniques such micro-autoradiography (Ouverney & Fuhrman 1999) should be very useful to understand more precisely the role of specific heterotrophic taxa in the microbial loop.

Acknowledgements. This work was funded by the following programs: PICODIV supported by the European Union (EVK3-CT-1999-00021), PICOMANCHE supported by the Région Bretagne and PROSOPE supported by PROOF (CNRS). F.N. was supported by a doctoral fellowship from the French Research Ministry. We thank the MYSIS crew for efficient assistance in the sampling and Florence Legall for assistance with phytoplankton cultures. We acknowledge constructive criticisms by anonymous reviewers.

LITERATURE CITED

Amann RI (1995) *In situ* identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. Molecular microbial ecology manual, Vol 3.3.6. Kluwer Academic, Dordrecht, p 1–15

Amann RI, Ludwig W (2000) Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. FEMS Microbiol Rev 24:555–565

Amann RI, Zarda B, Stahl DA, Schleifer KH (1992) Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 58:3007–3011

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Andersen RA, Saunders GW, Paskind MP, Sexton J (1993) Ultrastructure and 18S rRNA gene sequence for *Pelagomonas calceolata* gen. and sp. nov. and the description of a new algal class, the Pelagophyceae *classis nov.* J Phycol 29:701–715

Andersen RA, Bidigare RR, Keller MD, Latasa M (1996) A comparison of HPLC pigment signatures and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Oceans. Deep Sea Res II 43: 517–537

Béja O, Aravind L, Koonin EV, Suzuki MT and 8 others (2000) Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science 289:1902–1906

Biegala IC, Kennaway G, Alverca E, Lennon JF, Vaulot D, Simon N (2002) Identification of bacteria associated with dinoflagellates (*Alexandrium* spp.) using TSA-FISH (tyramide signal amplification-fluorescent *in situ* hybridization) and confocal microscopy. J Phycol 38:404–411

Brümmer IHM, Fehr W, Wagner-Döbler I (2000) Biofilm community structure in polluted rivers: abundance of dominant phylogenetic groups over a complete annual cycle. Appl Environ Microbiol 66:3078–3082

Butcher RW (1952) Contributions to our knowledge of the smaller marine algae. J Mar Biol Assoc UK 31:175–191

Campbell L, Nolla HA, Vaulot D (1994) The importance of *Prochlorococcus* to community structure in the central North Pacific Ocean. Limnol Oceanogr 39:954–961

Campbell L, Landry MR, Constantinou J, Nolla HA, Brown SL, Liu H (1998) Response of microbial community structure to environmental forcing in the Arabian Sea. Deep Sea Res II 45:2301–2325

Chrétiennot-Dinet MJ, Courties C, Vaquer A, Neveux J, Claustre H, Lautier J, Machado MC (1995) A new marine picoeucaryote: *Ostreococcus tauri* gen and sp *nov* (Chlorophyta, Prasinophyceae). Phycologia 34:285–292

Courties C, Vaquer A, Trousselier M, Lautier J, Chrétiennot-Dinet MJ, Neveux J, Machado C, Claustre H (1994) Smallest eukaryotic organism. Nature 370:255

DeLong EF, Taylor LT, Marsh TL, Preston CM (1999) Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent *in situ* hybridization. Appl Environ Microbiol 65: 5554–5563

Fuchs BM, Wallner G, Beisker W, Schwippl I, Ludwig W, Amann R (1998) Flow cytometric analysis of the *in situ* accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. Appl Environ Microbiol 64:4973–4982

Fuchs BM, Glöckner FO, Wulf J, Amann R (2000) Unlabeled helper oligonucleotides increase the *in situ* accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. Appl Environ Microbiol 66:3603–3607

Fuhrman JA, McCallum K, Davis AA (1992) Novel major archaebacterial group from marine plankton. Nature 356: 148–149

Gieskes WWC, Kraay GW (1983) Unknown chlorophyll *a* derivatives in the North Sea and the tropical Atlantic Ocean revealed by HPLC analysis. Limnol Oceanogr 28: 757–766

Giovannoni SJ, DeLong EF, Olsen GJ, Pace NR (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J Bacteriol 170:2418

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345:60–63

Guillou L, Chrétiennot-Dinet MJ, Medlin LK, Claustre H, Loiseauxde Goër S, Vaulot D (1999a) *Bolidomonas*: a new genus with two species belonging to a new algal class, the Bolidophyceae (Heterokonta). J Phycol 35:368–381

Guillou L, Moon-Van der Staay SY, Claustre H, Partensky F, Vaulot D (1999b) Diversity and abundance of Bolidophyceae (Heterokonta) in two oceanic regions. Appl Environ Microbiol 65:4528–4536

Head IM, Saunders JR, Pickup RW (1998) Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microb Ecol 35: 1–21

Johnson PW, Sieburth JM (1979) Chroococcoid cyanobacteria in the sea: a ubiquitous and diverse phototrophic biomass. Limnol Oceanogr 24:928–935

Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankton. J Phycol 23: 633–638

Klein B, Sournia A (1987) A daily study of the diatom spring bloom at Roscoff (France) in 1985. II. Phytoplankton pigment composition studied by HPLC analysis. Mar Ecol Prog Ser 37:265–275

Latasa M, Bidigare RR (1998) A comparison of phytoplankton populations of the Arabian Sea during the spring intermonsoon and southwest monsoon of 1995 as described by HPLC-analyzed pigments. Deep Sea Res II 45:2133–2170

Li WKW (1994) Phytoplankton biomass and chlorophyll concentration across the North Atlantic. Sci Mar 58:67–79

Li WKW, Platt T (1987) Photosynthetic picoplankton in the ocean. Sci Prog 71:117–132

Lim E, Amaral LA, Caron DA, DeLong EF (1993) Application of rRNA-based probes for observing nanoplankton protist. Appl Environ Microbiol 59:1647–1655

Llobet-Brossa E, Rossello-Mora R, Amann R (1998) Microbial community composition of Wadden sea sediments as revealed by fluorescence *in situ* hybridization. Appl Environ Microbiol 64:2691–2696

Lopez-Garcia P, Rodriguez-Valera F, Pedros-Alio C, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature 409:603–607

Ludwig W, Dorn S, Springer N, Kirchhof G, Schleifer KH (1994) PCR-based preparation of 23S rRNA-targeted group-specific polynucleotide probes. Appl Environ Microbiol 60:3234–3244

Marie D, Brussaard C, Partensky F, Vaulot D (1999) Flow cytometric analysis of phytoplankton, bacteria and viruses. In: Sons JW (ed) Current protocols in cytometry. International Society for Analytical Cytology, New York, p 11.11.1–11.11.15

Marie D, Simon N, Guillou L, Partensky F, Vaulot D (2000) DNA, RNA analysis of phytoplankton by flow cytometry. In: Sons JW (ed) Current protocols in cytometry. International Society for Analytical Cytology, New York,

p 11.12.1–11.12.18
Moon-van der Staay SY, De Wachter R, Vaulot D (2001) Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature 409:607–610
Olson RJ, Vaulot D, Chisholm SW (1985) Marine phytoplankton distributions measured using shipboard flow cytometry. Deep-Sea Res 32:1273–1280
Ouverney CC, Fuhrman JA (1999) Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types *in situ*. Appl Environ Microbiol 65:1746–1752
Potter D, Lajeunesse TC, Saunders GW, Andersen RA (1997) Convergent evolution masks extensive biodiversity among marine coccoid picoplankton. Biodivers Conserv 6:99–107
Ravenschlag K, Sahm K, Knoblauch C, Jorgensen BB, Amann R (2000) Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine artic sediments. Appl Environ Microbiol 66:3592–3602
Scholin CA, Buck KR, Britschgi T, Cangelosi G, Chavez FP (1996) Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridization formats. Phycologia 35: 190–197
Schönhuber W, Fuchs B, Juretschko S, Amann R (1997) Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Appl Environ Microbiol 63:3268–3273
Schönhuber W, Zarda B, Eix S, Rippka R, Herdman M, Ludwig W, Amann R (1999) *In situ* identification of cyanobacteria with horseradish peroxidase-labeled, rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 65: 1259–1267
Simon N, Lebot N, Marie D, Partensky F, Vaulot D (1995) Fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. Appl Environ Microbiol 61:2506–2513
Simon N, Campbell L, Ornolfsdottir E, Groben R, Guillou L, Lange M, Medlin LK (2000) Oligonucleotide probes for the identification of three algal groups by dot blot and fluorescent whole-cell hybridization. J Eukaryot Microbiol 47: 76–84
Sournia A, Birrien JL (1995) La série océanographique côtière de Roscoff (Manche occidentale) de 1985 à 1992. Cah Biol Mar 36:1–8
Stolte W, Kraay GW, Noordeloos AAM, Riegman R (2000) Genetic and physiological variation in pigment composition of *Emiliania huxleyi* (Prymnesiophyceae) and the potential use of its pigment ratios as a quantitative physiological marker. J Phycol 36:529–539
Urdea MS, Warner BD, Running JA, Stempien M, Clyne J, Horn T (1988) A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent, and enzyme labeled oligodeoxyribonucleotide probes. Nucleic Acids Res 16:4937–4956
Vaulot D (1989) CYTOPC: processing software for flow cytometric data. Signal Noise 2:8
West NJ, Schönhuber WA, Fuller N, Amann RI, Rippka R, Post A, Scanlan DJ (2001) Closely related *Prochlorococcus* genotypes show remarkably different depth distributions in two oceanic regions as revealed by *in situ* hybridization using 16S rRNA-targeted oligonucleotides. Microbiology 147:1731–1744

Editorial responsibility: David Caron, Los Angeles, California, USA

Submitted: August 24, 2001; Accepted: March 7, 2002
Proofs received from author(s): May 24, 2002