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Oligonucleotide Probes for the Identification of Three Algal Groups by Dot Blot and Fluorescent Whole-Cell Hybridization

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ABSTRACT. Photosynthetic pico- and nanoplankton dominate phytoplankton biomass and primary production in the oligotrophic open ocean. Species composition, community structure, and dynamics of the eukaryotic components of these size classes are poorly known primarily because of the difficulties associated with their preservation and identification. Molecular techniques utilizing 18S rRNA sequences offer a number of new and rapid means of identifying the picoplankton. From the available 18S rRNA sequence data for the algae, we designed new group-specific oligonucleotide probes for the division Chlorophyta, the division Haptophyta, and the class Pelagophyceae (division Heterokonta). Dot blot hybridization with polymerase chain reaction amplified target rDNA and whole-cell hybridization assays with fluorescence microscopy and flow cytometry were used to demonstrate probe specificity. Hybridization results with representatives from seven algal classes supported the phylogenetic affinities of the cells. Such group- or taxon-specific probes will be useful in examining community structure, for identifying new algal isolates, and for in situ detection of these three groups, which are thought to be the dominant algal taxa in the oligotrophic regions of the ocean.

Key Words. Chlorophyta, flow cytometry, fluorescence, Haptophyta, Heterokonta, in situ hybridization, Pelagophyceae, phytoplankton, Prymnesiophyceae, oligotrophic ocean.

SMALL unicellular eukaryotic algae are important in all marine environments (Hall and Vincent 1990; Johnson and Sieburth 1982; Li and Wood 1988; Murphy and Haugen 1985). These pico- and nano-eukaryotic phytoplankton, together with the prokaryotic picoplankton *Prochlorococcus* and *Synechococcus*, are the major contributors to phytoplankton biomass and primary production in oligotrophic open oceans (Campbell, Nolla, and Vaulot 1994; Li 1994) and can also be important in coastal waters (Campbell et al. 1998; Courties et al. 1994; Sieburth, Johnson, and Hargraves 1988). Historically, identification of small eukaryotic algae relied upon morphology, ultrastructure, and characterization of their photosynthetic pigments, but this was possible only after isolates were established in laboratory culture. Electron microscopy has revealed an apparent broad diversity of species and taxonomic groups that show few, if any, discriminating features by light microscopy, the level at which most routine observations are made (Andersen et al. 1996; Johnson and Sieburth 1982). Very often these small phytoplankton cells are simply referred to as unidentified coccoids or flagellates. Yet, pigment analyses performed in several oceanic regions have revealed that the newly discovered class Pelagophyceae (Andersen et al. 1993) together with the class Prymnesiophyceae, and to a lesser extent the division Chlorophyta (both Chlorophyceae and Prasinophyceae), are the most important constituents of the eukaryotic phytoplankton in oligotrophic regions of the oceans (Andersen at al. 1996; Letelier et al. 1993; Ondrusek et al. 1991). Novel groups in this size class are still being discovered (Guillou et al. 1999). Thus, not only is the biodiversity of these small cells underestimated, their distribution and role in marine ecosystems is also poorly known. Species composition and distribution patterns are important factors in understanding ecological interactions, such as competition, predator-prey interactions, and regulation of growth and mortality. Information concerning the diversity of such communities may be essential for assessing and understanding ecosystem stability (Naeem and Li 1997).

In recent years, the application of rRNA-based phylogeny has reshaped our view of evolutionary relationships among organisms (Woese 1987). DNA sequencing and probing techniques have opened up new avenues of research in microbial ecology and taxonomy (DeLong 1991; Fuhrman et al. 1994; Saylor and Layton 1990). Currently, algal phylogeny inferred from 18S rRNA sequence comparisons consists of nine separate lineages including the divisions Chlorophyta, Heterokonta, Haptophyta, Cryptophyta, Dinophyta, Euglenophyta, Chlorarachniophyta, Glaucocystophyta, and Rhodophyta (Ariztia, Andersen, and Sogin 1991; Bhattacharya and Medlin 1995; Bhattacharya et al. 1992; Saunders et al. 1995). The mosaic organization of variable and conserved positions in the rRNA molecule makes it possible to design probes that distinguish organisms at a variety of taxonomic levels. The high copy number of ribosomes in an actively growing cell represents a potentially large number of hybridization target sites for whole-cell identification. Detection of individual cells using kingdom-level fluorescently-labeled rRNA-targeted oligonucleotide probes has been successfully demonstrated (DeLong, Wickhan, and Pace 1989). Subsequently, whole-cell hybridization has been shown to be a suitable tool for determinative phylogenetic and environmental studies in microbiology (Amann et al. 1990).

As more sequences from algal ribosomal RNA genes are deposited in the databases (Maidak et al. 1997; Potter et al. 1997; van de Peer et al. 1997), it is possible to design probes for a finer scale taxonomic resolution. A limited number of ''phylogenetic stains'' for phytoplankton divisions and classes are presently available for the following groups: chlorophytes (CHLO01), non-chlorophyte algae (NCHLO1), and prymnesiophytes (PRYM01) (Lange et al. 1996; Simon et al. 1995). Since the CHLO01 probe was designed (Simon et al. 1995), a larger number of sequences have become available, so we now know that CHLO01 targets only a subset of the chlorophytes. We sought, therefore, to design an improved suite of probes for the algae, in particular the picoeukaryotes (< 3 μm cells), at a class-level taxonomic resolution. This experimental strategy is appropriate for the pico- and nanophytoplankton because many important components are undescribed species (or species for which sequences are not available). New class-specific probes will afford rapid identification of small-sized coccoid and flagellate algal isolates. We report here the design of new group-specific oligonucleotide probes for the division Chlorophyta, the division Haptophyta, and the class Pelagophyceae (division Heterokonta) and the optimized conditions for their use in dot blot hybridization with polymerase chain reaction (PCR, Saiki et al. 1988) amplified target rDNA and in situ whole-cell hybridization assays.

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MATERIALS AND METHODS

Design of oligonucleotide probes. An algal database consisting of over 350 published and unpublished chlorophyll *a* + *c* algal 18S rRNA sequences and a large number of representative sequences from five green algal classes (Nakayama et al. 1998) plus higher plants was compiled. The newer sequences of particular relevance to the design of these probes included pelagophyte, prymnesiophyte, and prasinophyte sequences from marine species. We screened this database for signature positions characteristic for the divisions Chlorophyta and Haptophyta and the class Pelagophyceae using the ''probe design'' function of the ARB (from arbor; Latin: tree) program package (W. Ludwig, Technical University of Munich, Munich, Germany). Potential target sites consisted of regions of 15–20 nucleotides that perfectly matched corresponding sites for all the target organisms and presented at least two mismatches with the corresponding regions for non-target organisms. Selection of the potentially ''best'' probes was made according to the following criteria: (1) target sites located in the more conserved regions, (2) target sites with a maximum number of mismatches with non-target organisms, and (3) preference for centrally or near-centrally located mismatches. The specificity of all potential probe sequences was tested by Ribosomal Database Project (RDP) ProbeCheck (http://www.cme.msu.edu/RDP/) and a BLAST (http://www.ncbi.nlm.nih.gov/index.html) search of the GenBank database (Version 110.0) to detect potential matching target sequences in species from non-target and non-algal groups not in our database.

Selected oligonucleotide probes were synthesized first unlabeled by commercial laboratories (MWG-Biotech, Ebersberg, Germany). Subsequently, probes were labeled with fluorescein isothiocyanate (FITC) at the 5′ terminus and purified by HPLC (Genset, Paris, France or Interactiva, Ulm, Germany). Purified probes for flow cytometry were vacuum desiccated (Speed Vac) and subsequently stored at − 80 °C in aliquots. Working stocks were prepared by resuspending aliquots in distilled water at a concentration of 25 or 50 $ng \cdot \mu l^{-1}$. The general eukaryote-specific probe EUK 1209R (Lim et al. 1993) was used as our positive control for all dot blot and most whole-cell hybridization experiments.

Cultures. Representative species of seven marine algal classes were selected for hybridization experiments in order to validate group-specific probes (Table 1). Cells were grown in f/2 (Guillard 1975), IMR/2 (Eppley, Holmes, and Strickland 1967) or K medium (Keller et al. 1987) at 20 °C and 50 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ and harvested in mid-exponential phase.

Dot blot hybridization with nucleic acids. DNA was extracted from 21 species of algae (seven algal classes representing six different divisions) and one bacterium (Table 1) using a 3% CTAB (hexadecyltrimethylammonium bromide) extraction procedure (Doyle and Doyle 1989) as described (Simon et al. 1997). Macroalgae were ground with a mortar and pestle in liquid nitrogen prior to extraction. Total nucleic acid preparations were used as templates for the amplification of the nuclear gene coding for the 18S rRNA molecule using PCR. Polymerase Chain Reaction conditions used for the amplification of the eukaryotic gene followed Simon et al. (1997), whereas PCR primers and conditions for the amplification of the prokaryotic gene followed Kopp et al. (1997). PCR-amplified 18S rRNA genes were denatured by heating at 95 °C for 10 min before blotting on positively charged nylon membranes (Boehringer Mannheim, Germany). Membranes were prehybridized for at least 2 h without probe and then incubated overnight with digoxygenin (DIG)-labeled probes (DIG oligonucleotide 3′-end Labeling Kit, Boehringer Mannheim) at optimum temperatures that were determined empirically: 50 °C for EUK1209, 51 °C for PELA02, 54 °C for PELA01, 55 °C for CHLO02, and 60 °C for PRYM02. Prehybridization and hybridization solutions contained 0.1 $mg \bullet ml^{-1}$ of Poly-A to prevent non-specific binding of the tailed probes. Detection was performed by chemiluminescence with Chemiluminescent Substrate For Alkaline Phosphatase (CSPD) as a substrate (DIG detection kit, Boehringer Mannheim). All steps were performed according to the manufacturer's instructions. Membranes were exposed to X-ray film for 20, 30 or 60 min.

Whole-cell hybridization protocol for epifluorescence microscopy. For examination by epifluorescence microscopy, whole cells from seven algal classes were hybridized with FITC-labeled probes. Cells were harvested by centrifugation and then fixed and stored in 80% ethanol at − 20 °C for several days to remove the chlorophyll before hybridization. Cultures of *Chrysochromulina kappa, Guillardia theta, Heterocapsa triquetra, Nitzschia distans, Pavlova lutheri,* and *Pyramimonas obovata* or *Pelagomonas calceolata* (Table 1) were mixed in approximately equal proportions and hybridized with each of the four probes and, in most cases, EUK 1209R as a positive control following the protocol of F. Brümmer (University of Stuttgart, pers. commun.). Testing of the PELA01 and PELA02 probes against their target rRNA was performed separately from the non-target cell mixture, because the target cells were often lost in the preparation due to their small size.

Briefly, cell mixtures were pelleted in a 0.5 ml tube in a microcentrifuge (3 min, 5,000 *g*) and washed once in 200 μl phosphate buffered saline (PBS, 130 mM NaCl, 10 mM phosphate buffer [pH 7.2]) and once in 200 μl hybridization buffer (6× SSC, 0.1% Tween-20). Next, the cells were resuspended in 100 μl hybridization buffer and in situ hybridization performed in a PCR machine with 2.5 ng of probe per μl for 2 h with occasional mixing. Subsequently, cells were washed three times in 200 μl hybridization buffer for 20 min at the hybridization temperature. Hybridization temperatures were 46 °C for a mixture of PELA01 and PELA02, 52 °C for CHLO02 and 54 °C for PRYM02. Finally, the cell mixtures were pelleted and resuspended in 10 μl of Citifluor (Citifluor Ltd., Canterbury, UK) containing DAPI (1 $\mu g \bullet ml^{-1}$) as a counterstain. Two μl of this suspension were spread onto a slide and viewed with an Axioskop 20 epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with Zeiss filter sets 02 (DAPI) and 09 (FITC). Photographs were taken with 1600 ASA Fuji film.

Whole-cell hybridization protocol for flow cytometry. The protocol for whole-cell hybridization was modified from Wallner, Amann, and Beisker (1993). Cells were fixed with paraformaldehyde (10% stock, stored frozen, and thawed immediately prior to use) at a final concentration of 1% for 1 h on ice. Next, cells were pelleted (3 min, 4,000 *g*) and resuspended in a cold mixture (70:30) of ethanol and phosphate buffered saline (PBS, Sigma, St. Louis, MO; 120 mM NaCl, 2.7 mM KCl in 10 mM phosphate buffer [pH 7.4]). The cells were pelleted again and resuspended in hybridization buffer (0.9 M NaCl, 20 mM Tris HCl [pH 7.8], 0.01% SDS, and 0–30% formamide, depending on the probe). To 20 μl of the cell suspension in hybridization buffer, 2 μl of probe stock solution (stock concentration at 25 $ng \bullet \mu l^{-1}$) was added. Cells were then incubated for 3 h at 46 °C, pelleted, and resuspended in a washing buffer containing no formamide, 20 mM Tris HCl [pH 7.8], 0.01% SDS and NaCl. In order to achieve the same stringency conditions in both washing and hybridization buffers, NaCl concentrations in the washing buffer were 0.9, 0.45, 0.225 or 0.112 M for hybridization buffers with 0, 10, 20 or 30% formamide respectively. After a 15 min incubation at 46 °C, hybridization

Table **1**. Summary of algal species tested in the development of 18S rRNA probes CHLO02, PRYM02, PELA01, and PELA02 by dot blot hybridization (DB) and whole-cell in situ hybridization by fluorescence microscopy (FISH) and by flow cytometry (FCM). The number of mismatches to each of the probes is listed, if the rDNA sequence was available.

Species	Source/strain[a]	Test	Number of mismatches for each probe			
			CHLO02	PRYM02	PELA01	PELA02
Division Chlorophyta						
Acrosiphonia cercta	AWI, C. Wiencke	DB	na[b]	na	na	na
Bathycoccus prasinos Type strain	UIO, W. Eikrem	DB	na	na	na	na
Chlamydomonas concordia	PLY 491	FCM	0[c]	2[c]	8[c]	3[c]
Dunaliella salina	CCMP 1303	DB	0	3	6	3
Micromonas pusilla	CCMP 490	DB	0	4	6	3
Pyramimonas obovata	CCMP 723	FISH	na	na	na	na
Pyramimonas parkae	CCMP 724	FISH	0	4	6	3
Tetraselmis suecia	CCMP 904	DB	na	na	na	na
Tetraselmis sp.	RG-07	DB	0	3	6	4
Unidentified prasinophyte	CCMP 1220	DB	0	4	6	4
Division Cryptophyceae						
Guillardia theta	CCMP 327	FISH	2	8	7	3
Division Dinophyta						
Alexandrium lusitanicum	A1–18[b], S. Franca	DB	3[c]	5[c]	6[c]	3[c]
Amphidinium carterae	CCMP 1314	DB	2[c]	6[c]	6[c]	4[c]
Heterocapsa triquetra	CCMP 448	FISH	2	5	6	3
Division Haptophyta						
Prymnesiophyceae						
Chrysochromulina kappa	UIO EN3	FISH	2	0	7	3
Chrysochromulina scutellum	UIO G7	DB	2	0	7	3
Emiliania huxleyi	CCMP 373	DB	2	0	7	3
Imantonia rotunda	UIO 101	DB	2	0	7	3
Pavlova lutheri	CCMP 1325	FISH	2	0	7	3
Phaeocystis globosa	CCMP 1524	DB	2	0	7	3
Prymnesium parvum	PLY 94	DB	2	0	7	3
Prymnesium patelliferum		FCM	2	0	7	3
Division Heterokonta						
Class Bacillariophyceae						
Nitzschia distans	AWI XM118	FISH	2[c]	5[c]	5[c]	3[c]
Skeletonema costatum	CCAP 1077/3	DB	2	5	5	3
Class Pelagophyceae						
Ankylochrysis lutea	ALGO CHR46	FCM	na	na	na	na
Aureoumbra lagunensis	CCMP 1507	DB	2	7	0	1
Unidentified coccoid	CCMP 1145	DB	2	7	0	0
Unidentified coccoid	CCMP 1395	DB	2	7	0	0
Pelagomonas calceolata	CCMP 1214	DB	2	7	0	0
Pulvinaria sp.	CCMP 292	DB	2	7	0	1
Sarcinochrysis marina	CCMP 770	DB	2	7	0	1
Division Rhodophyta						
Helgolandica atropurpurea	AWI, C. Wiencke	DB	na	na	na	na
Bacteria						
Unidentified marine sp.	AWI 6SN9	DB	na	na	na	na

[a] ALGO = Algobank, Culture Collection, Caen, France; AWI = Alfred Wegener Institute, Bremerhaven, Germany; CCMP = Provasoli-Guillard Culture Center for Marine Phytoplankton, Bigelow Laboratory, W. Boothbay Harbor, ME, USA; PLY = Plymouth Culture Collection, Plymouth, UK; UIB = University of Bergen, Dept. of Biology, Norway; UIO = University of Oslo, Marine Botany, Norway.

[b] na = sequence for species, or related species within the same genus, not available.

[c] Number of mismatches correspond to another closely related species within the same genus if the sequence for the strain tested was not available in public databases.

[d] Obidos Lagoon, Portugal. The sequences for *Chlamydomonas reinhardtii, Alexandrium fundyense, Amphidinium belauense,* and *Nitzschia apiculata* were used to calculate the number of mismatches for *Chlamydomonas concordia, Alexandrium lusitanicum, Amphidinium carterae,* and *Nitzschia distans.*

was stopped by the addition of 500 μl ice-cold PBS, pH 9.0. Hybridized cells were kept on ice no longer than 24 h until analysis with flow cytometry.

Detection and fluorescence quantification. Detection and quantification were performed with a FACSort flow cytometer (Becton Dickinson, San Jose, CA) equipped with an air-cooled 488 nm laser delivering 15 mW and the standard filter setup. Cells were identified by their remaining red fluorescence and right angle light scattering characteristics. Mean cell green fluorescence values were normalized to 0.95 μm diameter beads (Polyscience Inc., Washington, PA) using CYTOPC software (Vaulot 1989).

Table **2**. Eighteen S rRNA probe sequence and localization of the target sites on the secondary structure model for the 18S rRNA from *Chlamydomonas reinhardtii* (Neefs et al. 1993).

Probe	Target group	Sequence (5′ to 3′)	Site	Helix
EUK1209[a]	Eukaryotes	GGGCATCACAGACCTG	1422–1437	36
CHLO02	Chlorophyta	CTTCGAGCCCCCAACTTT	973–990	27
PRYM02	Haptophyta	GGAATACGAGTGCCCCTGAC	868–887	24/5
PELA01	Pelagophyceae	ACGTCCTTGTTCGACGCT	937–954	24/6
PELA02	Pelagophyceae[b]	GCAACAATCAATCCCAATC	1531–1549	47

[a] From (Lim et al. 1993).
[b] Clade containing: *Aureococcus, Pelagomonas, Pelagococcus, Pulvinaria,* CCMP 1395.

RESULTS

Probe design. Using the ARB program package and visual inspection of aligned sequences, we selected one target site for a chlorophyte-specific probe (CHLO02), one site for a prymnesiophyte-specific probe (PRYM02, division Haptophyta), and two target sites for pelagophyte-specific probes (PELA01 for all species and PELA02 for a subset of species forming a distinct clade within the Pelagophyceae) (Table 2). The probes are 18 to 20 nucleotides long and present perfect or near perfect target sites for all target organisms and 2 to 3 (CHLO02), 3 to 7 (PRYM02), 4 to 6 (PELA01), and 1 to 3 (PELA02) mismatches with all other available non-target sequences (Table 1). PELA02 presented a single near centrally-located mismatch with four pelagophycean sequences that form a separate clade within the class: *Aureoumbra lagunensis,* CCMP 1410, *Sarcinochrysis marina,* and *Pulvinaria* sp. (Saunders, Potter, and Andersen 1997). Among the 63 sequences of prymnesiophytes that were available, *Chrysochromulina leadbeateri* and three sequences of unknown prymnesiophytes from a clone library (Moon-van der Staay et al., unpubl. data) presented a target site that did not match perfectly with the PRYM02 probe (one mismatch) (Table 1). CHLO02 shows a single mismatch with the Dasycladales, the Characeae, *Chlamydomonas moewusii, Chlorococcum hypnosporum, Chlorella luteoviridis,* and *Spirogyra grevilleana.*

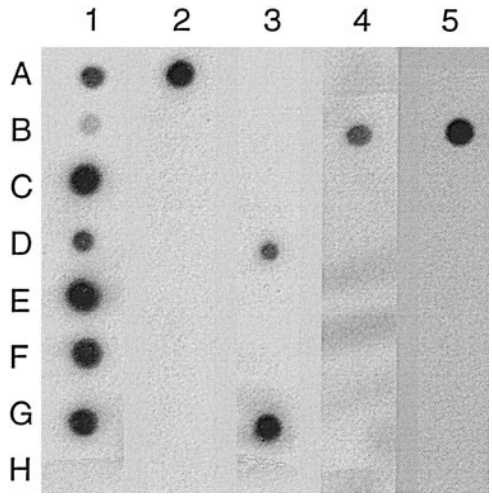

Fig. **1**. Specificity of algal 18S rRNA probes tested by dot blot hybridization with representatives of seven algal classes (see Table **1**). PCR-amplified cytoplasmic SSU rRNA gene bound to the filter was hybridized with five digoxigenin-labeled oligonucleotide probes; 1- EUK1209R, 2- CHLO02, 3- PRYM02, 4- PELA01, and 5- PELA02. For columns 1–3, rows show the results for the following taxa: **A**: *Acrosiphonia cercta,* **B**: *Pelagomonas calceolata,* **C**: *Skeletonema costatum,* **D**: *Prymnesium parvum,* **E**: *Alexandrium lusitanicum,* **F**: *Helgolandica atropurpurea,* **G**: *Chrysochromulina scutellum,* **H**: Unidentified marine bacterium strain 6SN9. For columns 4–5, rows show results for the following taxa: **A**: *Dunaliella salina,* **B**: coccoid pelagophyte, CCMP 1145, **C**: *Skeletonema costatum,* **D**: *Prymnesium parvum,* **E**: *Alexandrium carterae,* **F**: *Helgolandica atropurpurea,* **G**: *Chrysochromulina kappa,* **H**: Unidentified marine bacterium strain 6SN9.

Validation and optimization of probes with extracted DNA by dot-blot hybridization. All probes were specific for the sequences against which they were designed. The probe EUK1209R, chosen as a positive control, labeled all algal strains and showed no signal with the bacterial DNA (Fig. 1). Under the optimized conditions (see below) and for the strains we tested, the four probes, CHLO02, PRYM02, PELA01, and PELA02, labeled only their intended targets, and no signal was detected in tests with PCR products from representatives of other algal divisions, i.e. non-target organisms (Table 1, Fig. 1, and data not shown).

We also tested the ability of each probe to hybridize with target DNA from a variety of species within each target class or division (Table 1 and Fig. 2). All species within each algal group showed a positive signal. The secondary structure of the DNA may explain the faint signal of *Phaeocystis globosa* (Fig. 2, column 4) because the hybridization signal of EUK1209R with the DNA from the five genera of Prymnesiophyceae was equally strong (results not shown).

Within the range of optimum probe concentrations for DIG-labeling suggested by the manufacturer (0.1–2 pmol•ml^{-1}), faint non-specific binding was detected in some cases (see rows F–H, Fig. 2). Attempts to reduce this by increasing the hybridization temperature or stringency of the washes did not improve the blots. By decreasing the probe concentrations to 0.04–0.05 pmol•ml^{-1}, non-specific binding for PELA02 and PRYM02 was eliminated (results not shown). Higher probe concentrations for CHLO02 and PELA01 (0.2 and 0.5 pmol·ml^{-1}, respectively) provided strong results without non-specific binding.

Validation of probes with cultured cells by whole-cell hybridization and fluorescence microscopy. For testing of target-specific binding of the fluorescently labeled probes, seven different genera from six different classes were chosen for in situ hybridization with detection by fluorescence microscopy (Table 1). All probes showed specific signals only with their target organisms and no labeling of non-target species under the conditions chosen (Fig. 3–10). Signal strength of FITC-labeled probes was sufficient to distinguish easily between target and non-target cells with fluorescent microscopy. As expected, the picoeukaryote cells, such as *P. calceolata,* produced a weaker signal than the larger algal species because of their smaller size and lower rRNA content. For example, compare the signal intensity of *P. calceolata* with the PELA01 and PELA02 probes with that of *Pyramimonas parkae* and the CHLO02 probe (Fig. 6). Autofluorescence of residual chlorophyll in cells can sometimes mask the probe signal. By bleach-

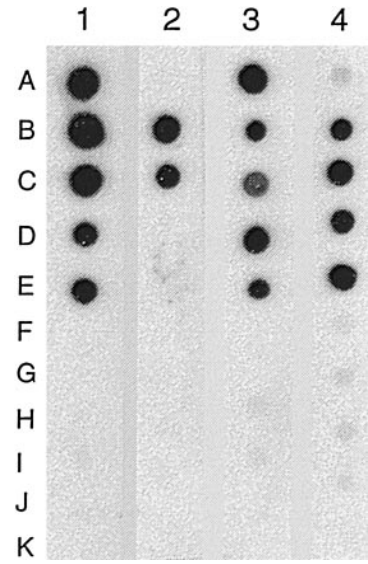

Fig. **2**. Specificity of 18S rRNA probes within each target algal taxon. Dot blot hybridization of filter bound PCR-amplified cytoplasmic SSU rRNA gene with the digoxigenin-labeled oligonucleotide probes; 1- PELA01, 2- PELA02, 3- CHLO02, and 4- PRYM02. For each probe, rows **A** to **E** contain target DNA; except for PELA02 with target DNA in rows B and C only. Rows **F–K** show results for non-target DNA. Strain information is in Table **1**.
Taxa in columns 1 & 2: **PELA01 and 02**: **A**. *Aureoumbra lagunensis,* **B**. CCMP1145, **C**. CCMP1395, **D**. *Pulvinaria* sp., **E**. *Sarcinochrysis marina,* **F**. *Tetraselmis suecia,* **G**. *Emiliania huxleyi*, **H**. *Skeletonema costatum,* **I**. *Amphidinium carterae,* **J**. *Helgolandica atropurpurea,* **K**. Unidentified marine bacterium strain 6SN9.
Taxa in Column 3: **CHLO02**: **A**. *Dunaliella salina,* **B**. *Tetraselmis* sp. (RG 89), **C.** CCMP1220, **D**. *Bathycococcus prasinos,* **E**. *Micromonas pusilla,* **F**. *Emiliania huxleyi,* **G**. *Pelagomonas calceolata,* **H**. *Skeletonema costatum,* **I**. *Amphidinium carterae,* **J**. *Helgolandica atropurpurea,* **K**. Unidentified marine bacterium strain 6SN9.
Taxa in column 4: **PRYM02**: **A**. *Phaeocystis globosa,* **B**. *Imantonia rotunda,* **C**. *Emiliania huxleyi,* **D**. *Pavlova lutheri,* **E**. *Chrysochromulina scutellum,* **F**. *Tetraselmis suecia,* **G**. *Pelagomonas calceolata,* **H**. *Skeletonema costatum,* **I**. *Amphidinium carterae,* **J**. *Helgolandica atropurpurea,* **K**. Unidentified marine bacterium strain 6SN9.

ing the cells in 2 or 3 successive alcohol baths for one day in total we obtained a stronger signal-to-noise ratio for most species, making it easier to distinguish target from non-target cells.

Validation of probes with cultured cells by whole-cell hybridization quantified by flow cytometry. Three reference species (i.e. the chlorophyte *Chlamydomonas concordia*; the prymnesiophyte *Prymnesium patelliferum*; and the pelagophyte *Ankylochrysis lutea*) were selected to test the suitability of each probe for whole-cell hybridization with flow cytometric detection. An optimal balance between probe sensitivity and specificity was determined by adding increasing concentrations of formamide (0, 10, 20, 30%) to the hybridization buffer keeping the ionic strength (0.9 M NaCl) and temperature (46 °C) constant. Specific hybridization required 20% formamide for the probes PELA01 and PELA02 and 30% for the probes CHLO02 and PRYM02. The eukaryotic-specific probe EUK1209R was used with 20% formamide and labeled all three species. Using these conditions, the fluorescence intensity of the specifically hybridized cells was 4- to 16-fold higher than controls for PRYM02 and CHLO02, respectively (Fig. 11). The highest intensity of staining was for *Chlamydomonas concordia* hybridized with the probe CHLO02 (Fig. 11), but this is probably a function of cell size.

DISCUSSION

The specificity of each probe (CHLO02, PRYM02, PELA01, and PELA02) was verified against all known rRNA sequences and was confirmed empirically for representative algal isolates using dot blot and whole-cell hybridization assays (Fig. 1–11). Sequence data added to the database since 1995 permitted the design of these new probes targeting the important eukaryotic members of the picoplankton. Admittedly, the number of 18S rRNA sequences available for the algae is still low compared to the potential number of species present in natural communities, so there is always a risk that probes will have some mismatches with some target organisms (Table 1). For example, the original chlorophyte probe designed in 1993 (CHLO01; Simon et al. 1995) has one mismatch with several 18S rRNA sequences from chlorophyte species (Simon et al. 1995), whereas the present chlorophyte probe has one mismatch with several green macroalgal species and three freshwater microalgal species. Also, both the first PRYM01 (Lange et al. 1996) and the new PRYM02 probes have one mismatch with their target sites on the rRNA molecule for *Chrysochromulina leadbeateri* and three prymnesiophyte sequences from a clone library (Lange et al 1996 and Moon-van der Staay, S.-Y. et al., unpubl. data, respectively). If the goal, however, is to detect all species belonging to a particular phylogenetic group in natural environments, then the use of multiple probes may be more conclusive.

Dot blot hybridization assays with DNA extracted from algal cultures provided the clearest discriminating signals. In this format, our probes may readily be used for the rapid screening of picoplankton isolates, as well as for the study of natural communities. Note, however, the optimized conditions we report are recommended starting points. The actual hybridization conditions used by an individual laboratory should be verified before applying to field testing. Most examples of applications for field populations are found in the microbial ecology literature, and many have not yet been applied to the eukaryotic plankton. For

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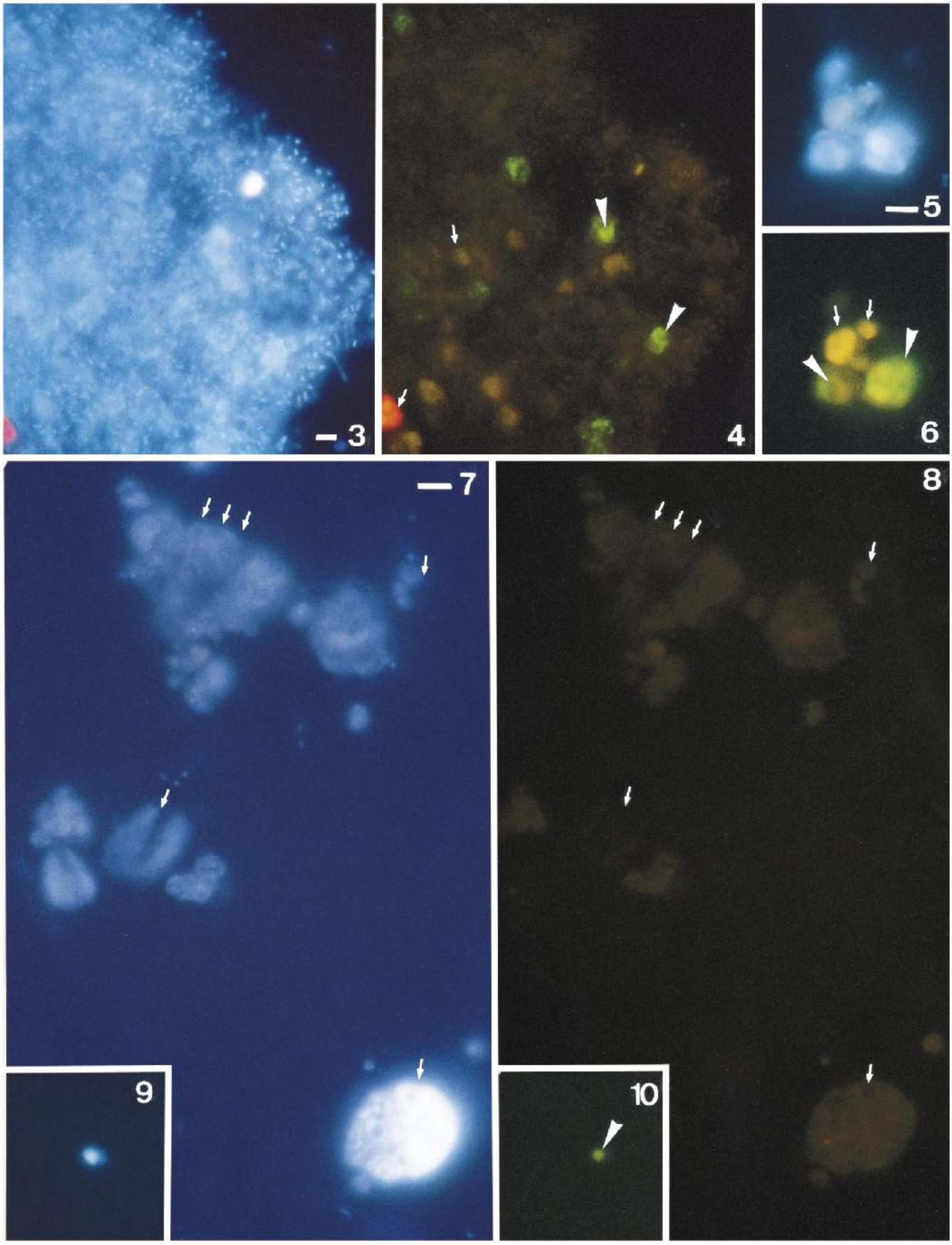
Fig. **3–10**. Whole-cell hybridization for a mixture of cells from different algal classes with the fluorescently labeled 18S rRNA-based probes PRYM02, CHLO02, PELA01, and PELA02 counterstained with DAPI. Cultures in the mixture include: *Chrysochromulina kappa, Guillardia theta, Heterocapsa triquetra, Nitzschia distans, Pavlova lutheri, Pyramimonas obovata,* and *Pelagomonas calceolata.* Scale bar represents 10 μm. Positively-labeled target cells appear green (FITC-label) with 488 nm excitation and are marked with arrow heads only, whereas unlabeled cells appear yellow or red due to autofluorescence of residual chlorophyll and are marked with arrows. Fig. **3**, **4**. Probe PRYM02. Fig. **3**. UV excitation to visualize DAPI staining of all cells; note cloud of bacteria. Fig **4**. 488 nm excitation: Target cells stained green are *Chrysochromulina kappa*; non-target cells in the field of view are *Pyramimonas obovata* and *Guillardia theta.* Fig. **5**, **6**. Probe CHLO02. Fig. **5**. UV excitation: DAPI staining. Fig. **6**. 488nm excitation: Target cells are *Pyramimonas parkae*; non-target cells are *Pavlova lutheri* and *Chrysochromulina kappa.* Fig. **7–10**. Probes PELA01 and PELA02. Fig. **7**. UV excitation: DAPI staining of non-target cells *Heterocapsa triquetra, Nitzschia distans, Chrysochromulina kappa,* and *Pyramimonas parkae,* marked with arrow heads. Fig. **8**. 488 nm excitation: non-target cells in Fig. 7, positions marked with arrowheads for comparison with Fig. 7. Fig. **9**. UV excitation: DAPI staining of target cells, *Pelagomonas calceolata.* Fig. **10**. 488 nm excitation: target cells in Fig. 9.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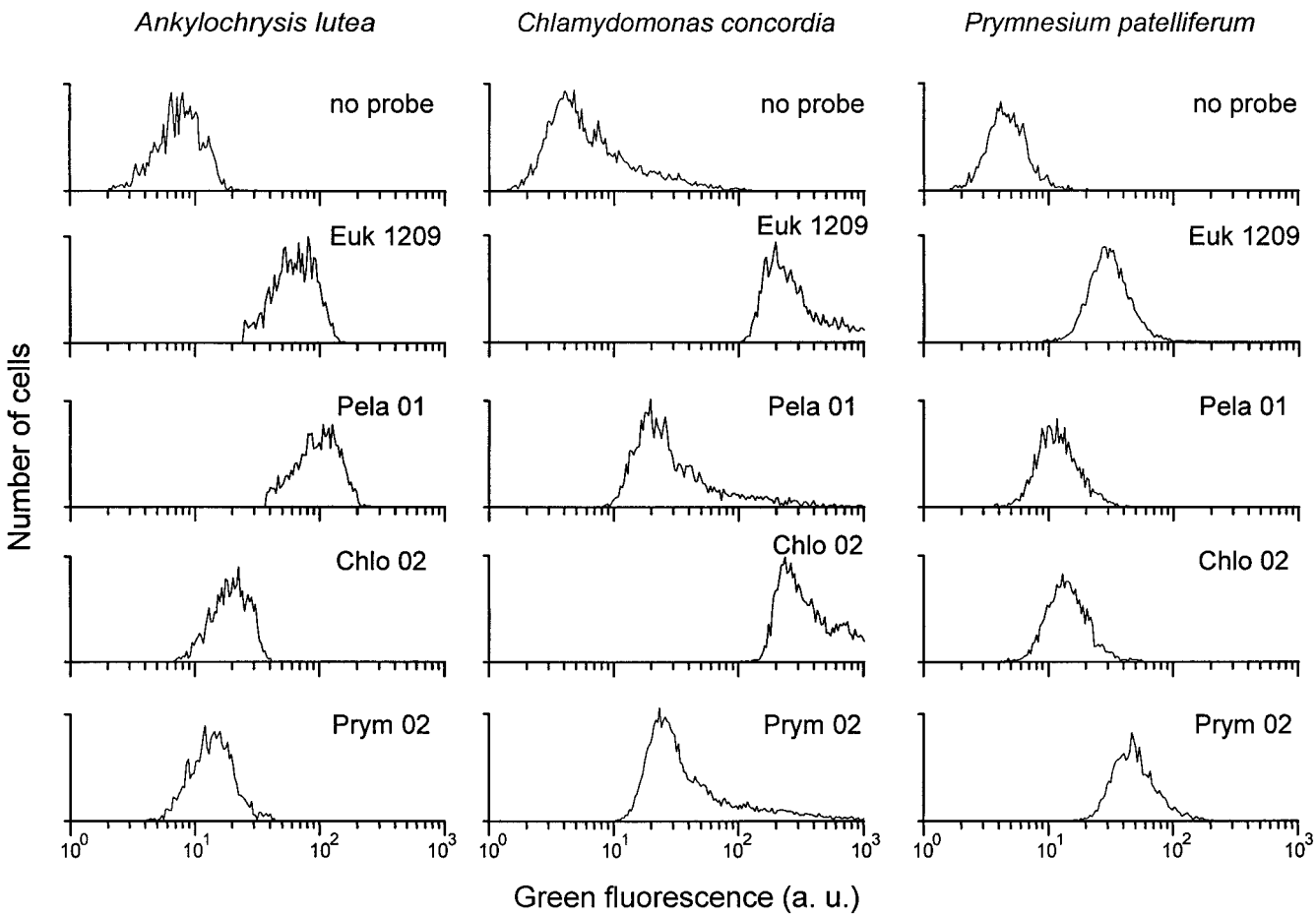

Fig. **11**. Flow cytometric analysis of fluorescence signals for whole-cell hybridization of exponentially-growing *Ankylochrysis lutea (*Pelagophyceae), *Chlamydomonas concordia* (Chlorophyta), and *Prymnesium patelliferum* (Prymnesiophyceae) with the FITC-labeled probes. For each species, the distribution of green fluorescence intensity•cell^{-1} is plotted on a three-decade log scale. The intensity of green fluorescence per cell was measured for cells incubated without probe (green autofluorescence, no probe) and in the presence of the general eukaryotic probe (Euk1209), the Pelagophyceae-specific probe (Pela 01), the Chlorophyta-specific probe (Chlo 02) and the Prymnesiophyta-specific probe (Prym 02); a. u., arbitrary units.

instance, probes may be used to quantify the relative contribution of rRNA from a target algal group to total rRNA extracted from natural sea water samples. The distribution of the corresponding algal groups throughout the water column may then be investigated (Giovannoni et al. 1996). A limitation of this approach is the reliance on a PCR step, thus results may be biased (Suzuki and Giovannoni 1996). Another approach might be selective recovery of sequences from species belonging to the Chlorophyta, Haptophyta, and Pelagophyceae using colony blots after cloning amplified rRNA genes from natural plankton communities, e.g. (Britschgi and Giovannoni 1991; Rappé, Kemp, and Giovannoni 1995). The PRYM01 and 02 probes have been employed to estimate the contribution of prymnesiophytes to total amplified picoeukaryotic 18S rDNA (dot blot assays) and to recover prymnesiophyte sequences directly from natural samples collected in the Pacific Ocean (Moon-van der Staay, S.-Y. et al. unpubl. data). A further, more promising approach would be to use the probes to construct DNA microchips (Guschin et al. 1997), thus avoiding biases introduced by cloning or PCR.

Flow cytometric detection of probe hybridization presents several advantages over chemiluminescent detection methods to detect and quantify taxa. First of all, it provides measurements of individual cells rather than a bulk measurement. Secondly, additional parameters (such as scattering properties, autofluorescence, and fluorescence of DNA specific dyes) can be measured simultaneously on populations targeted by the probes. Finally, labeled cells can be physically sorted from the rest of the community and further analyzed (Wallner et al. 1997). Unfortunately, because of the repeated centrifugation and washing steps required by the hybridization protocol for flow cytometry, cells may be lost. Consequently, at this time we cannot consider in situ hybridization with flow cytometric detection quantitative. Whole-cell hybridization with epifluorescent microscopic detection suffers from the same limitation but cell loss can be minimized if the assay is performed on filters (Lim et al. 1993). Quantification by microscopy is extremely time-consuming, so the benefits provided by flow cytometry—ease of counting and detection of small cells—are certainly attractive. Nevertheless, improved washing and concentration procedures are needed.

For whole-cell hybridization, we have demonstrated that the intensity of the signal provided by our probes is sufficient for the examination of isolates in culture. In pure cultures, the fluorescence intensity ratio of *specifically hybridized*: *non-specifically hybridized* cells can be calculated from flow cytometric analyses. Although this ratio can be as high as 16 (Fig. 11), it can be lower for other species (e.g. *Prymnesium patelliferum*). The use of a combination of two probes (PRYM01 and

PRYM02) may increase this ratio. Based on these initial results, we now aim to increase the signal intensity obtained from the hybridization of one probe to its target in the cell. Better discrimination of target cells from non-target cells has been obtained using new labeling techniques and signal amplification systems (Lebaron et al. 1997; Lee, Malone, and Kemp 1993; Schönhuber et al. 1997) or competitor oligonucleotide probes to block non-target sites on the rRNA molecule (Simon et al. 1995). The second strategy would require the design of several competitors (especially for probes such as PELA 01) because the number and nature of mismatches with the corresponding sites on non-target species is not conserved through all algal lineages.

Our ultimate goal is to use these probes to determine the composition of natural eukaryotic algal communities dominated by pico- and nanoplanktonic cells using one or more of the detection systems optimized in this study. The use of our probes to detect algal divisions or classes, in addition to more specific probes at the genus and species level (Knauber, Berry, and Fawley 1996; Lange et al. 1996; Simon et al. 1997), will provide a means of obtaining information on the biodiversity of natural picoplanktonic populations, which are notoriously difficult to study.

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