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Abundance and diversity of prymnesiophytes in the picoplankton community from the equatorial Pacific Ocean inferred from 18S rDNA sequences

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Abstract

Picoplankton, i.e., cells smaller than 2–3 μm, dominate in most open oceanic regions, such as in the Pacific Ocean. In these areas, the dominant carotenoid of photosynthetic eukaryotes is 19′-hexanoyloxyfucoxanthin (19HF), considered to be a diagnostic marker for prymnesiophytes. This suggests that this class could be a major component of eukaryotic picoplankton, despite the fact that virtually no prymnesiophyte has been described to date from this size class. To address this question, we assessed prymnesiophyte diversity and abundance in natural picoplankton communities, using a molecular approach. Total genomic DNA was isolated from 3-μm-filtered samples collected in the Pacific Ocean. Small subunit (18S) ribosomal RNA genes (rDNA) were amplified by the polymerase chain reaction (PCR) using universal eukaryotic primers. The relative abundance of 18S rDNA from prymnesiophytes was quantified using group-specific and eukaryotic 18S rDNA probes. The percentage of the prymnesiophyte versus total 18S rDNA was much lower than the percentage of prymnesiophytes calculated on the basis of pigment analyses for the same samples. 18S rDNA libraries from five samples were screened using a prymnesiophyte-specific oligonucleotide probe, and 14 nearly complete 18S rDNA sequences were retrieved. Phylogenetic analysis of these sequences established the presence of several prymnesiophyte lineages with no equivalent among cultivated species.

It is now well established that cells smaller than 2–3 μm (picoplankton), rather than the larger microalgae, dominate the phytoplankton community in the open ocean. In particular, the eukaryotic component of picoplankton, the so-called picoeukaryotes, has been recognized to contribute significantly to both primary biomass and production in open oceanic regions (Campbell et al. 1994; Li 1994). Its taxonomic composition, however, is poorly known. Major classes or divisions known to have picoplanktonic representatives include the pelagophytes (Andersen et al. 1993), the bolidophytes (Guillou et al. 1999), the chlorophytes (Tschermak-Woess 1999), and the prasinophytes (Courties et al. 1998). However, it is paradoxical that the most abundant eukaryotic carotenoid in tropical waters (Letelier et al. 1993; Bidigare and Ondrusek 1996) is often 19′-hexanoyloxyfucoxanthin (19HF), a diagnostic pigment for prymnesiophytes (Hooks et al. 1988). This suggests very strongly that prymnesiophytes could be a major component of eukaryotic picoplankton, despite the fact that very few prymnesiophyte species described or isolated in culture qualify as picoplanktonic. For example, *Imantonia* sp. PLY 18561 (Plymouth Collection), one of the smallest strains available, has an average size of 3.5 μm (Simon et al. 1994). In fact, prymnesiophytes (class Prymnesiophyceae, division Haptophyta; Jordan and Green 1994; Edvardsen et al. in press) are one of the most abundant nanoplanktonic group (i.e., in the 2–20 μm size range) in many oceanic regions, ranging from tropical to polar systems (Thomsen et al. 1994; Andersen et al. 1996). Some genera, such as *Phaeocystis* or *Emiliania*, make significant contributions to the global sulfur and carbon cycles (Westbroek et al. 1994), whereas others, such as *Chrysochromulina*, have the potential to develop noxious blooms (Richardson 1997). The aim of the present paper is to assess

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whether prymnesiophytes could also be important in the picoplanktonic size range.

Conventional approaches to estimate the abundance of any algal group in oceanic waters suffer limitations, especially for the picoplankton size class. Smaller cells are difficult to identify using light microscopy, often appearing as little balls (Potter et al. 1997). More sensitive techniques, such as epifluorescence microscopy or flow cytometry, can be used to estimate the total abundance of eukaryotic picoplankton (Campbell et al. 1994), but these techniques cannot distinguish taxa (Simon et al. 1994), in contrast to the prokaryotes such as *Prochlorococcus* and *Synechococcus*, which can be easily separated based on the orange phycoerythrin fluorescence of the latter (Partensky et al. 1999). Similarly, both scanning and transmission electron microscopy provide exquisite taxonomic details (Andersen et al. 1996), but are very time consuming, with heavy cell losses occurring during sample preparation. Pigment analysis by high-pressure liquid chromatography (HPLC) is the most widely used method to date to estimate abundance and diversity (Bidigare and Ondrusek 1996). In the case of prymnesiophytes, the relative concentration of 19HF can be used to assess their biomass. However, this method has several limitations. First, it cannot resolve diversity at lower taxonomic levels. Second, algorithms require calibration using laboratory cultures. However, obtaining isolates in culture that are truly representative of the open ocean is difficult (Jordan and Chamberlain 1997). Moreover, culture methods are always selective: the diversity of cultivated species probably does not reflect the environmental diversity of the group. Finally, the absolute and relative cellular concentrations of diagnostic pigments, such as 19HF, vary both with environmental conditions (van Leeuwe and Stefels 1998) and species (Jeffrey and Wright 1994). Thus, alternative approaches are necessary to gain insights into the diversity and abundance of prymnesiophyte populations in situ.

Molecular biological techniques have been used to analyze marine communities directly, avoiding the selective steps of laboratory cultivation (Giovannoni et al. 1990). Because of the large number of available sequences and its amenity to phylogenetic analyses, sequence comparisons of rDNA, the gene coding for the small subunit (SSU: 18S for eukaryotes and 16S for prokaryotes) of ribosomal RNA (rRNA) provide the most useful tool for community analysis. However, the application of this tool for marine samples has been almost completely restricted to Bacteria and Archaea (Pace 1997). For eukaryotic plankton, the only application of this technique has been the design of oligonucleotide probes recognizing specific taxa (Simon et al. 1995; Lange et al. 1996). Application of such probes to natural communities and detection by flow cytometry or epifluorescence microscopy is possible for nanoplankton (Miller and Scholin 1998; Lim et al. 1999), but has proved difficult for picoplankton (Simon et al. unpubl.). Here we use 18S rDNA to infer the abundance and diversity of picoplanktonic prymnesiophytes in a transect ranging from high to low nutrient waters of the equatorial Pacific Ocean. Our major conclusions are that molecular techniques yield significantly lower estimates of prymnesiophyte abundance than pigments and that, as in the case of prokaryotes (Giovannoni et al. 1990), a large fraction of the sequences recovered are very different from those available to date from cultured species.

Materials and methods

Sample collection—In November 1994, during the France Joint Global Ocean Flux Study (JGOFS) OLIPAC cruise (Dandonneau 1999), samples were collected at 10 stations equally spaced on a transect along 150°W from the nutrient-rich equatorial upwelling waters down to the nutrient-depleted waters of the South Pacific gyre off Tahiti (Table 1). For flow cytometry, 1.5-ml samples were collected at 12 depths between 0 and 200 m and analyzed fresh. For DNA sample collection, water from two depths, the surface and deep chlorophyll *a* (Chl *a*) maximum layers (Table 1), was prefiltered using a peristaltic pump through 3-μm pore size, 47-mm-diameter Nuclepore PE filters. Both unfiltered and filtered samples were analyzed by flow cytometry (see below) to detect the presence of cell debris. Only samples from CTD 2 (Table 1) revealed potential cell breakage because of the filtration procedure, probably because of an excessive pump speed, which was reduced for subsequent stations. One liter of the filtrate was then collected onto a glass fiber filter (Whatman GF/F). The filter was put into a cryovial filled with DNA lysis buffer (0.75 M sucrose, 400 mM NaCl, 20 mM ethylenediamine-tetraacetic acid [EDTA], 50 mM Tris-HCl, pH 9.0), immediately frozen in liquid nitrogen, and stored at −80°C. Four liters of water were collected onto a 3-μm pore size Nuclepore PE filter (phytoplankton $>3\mu$m), and 2.8 liters of unfiltered water were collected onto 25-mm GF/F filters (total phytoplankton). Both filters were stored in liquid nitrogen for subsequent pigment analysis.

Pigment analyses, flow cytometry, and culture isolation—Pigment extraction was performed in cold methanol for 30 min, followed by sonication to improve extraction efficiency. After clarification by filtration (Whatman GF/C filter), the extract was mixed with 1 M ammonium acetate (extract: ammonium acetate, 2 : 1 v/v) prior to injection. The HPLC system and the analytical conditions have been detailed elsewhere (Vidussi et al. 1996). Calibration was performed either using commercially available standards (e.g. Chl *a*, β-carotene from Sigma) or standards purified from reference algal cultures. The pigment concentration in the <3-μm fraction was estimated from the difference between the unprefiltered and the >3-μm filtered samples. Pigment algorithms used to estimate the contribution of various eukaryotes to biomass in the <3-μm fraction were based on a value of 0.8 for the ratios Chl *a*/fucoxanthin in diatoms, Chl *a*/19′-butanoyloxyfucoxanthin in pelagophytes and Chl *a*/19HF in prymnesiophytes, a value that falls within the expected range of variation for these ratios (Hooks et al. 1988; Mackey et al. 1996), 1.5 for the Chl *a*/peridinin ratio in dinoflagellates, and 2.1 for the Chl *a*/prasinoxanthin ratio in prasinophytes (Letelier et al. 1993).

Flow cytometric analysis of picoplankton was performed as described elsewhere (Vaulot and Marie 1999). Briefly, samples were analyzed fresh onboard ship on a FACSort instrument. The volume of sample analyzed was computed from the delivery rate and the duration of the analysis. Pi-

Table 1. Conductivity temperature depth probe (CTD) cast number, position, date, depth, and surface nitrate concentration (Raimbault et al. 1999) for all samples. Chl *a* and 19′-hexanoyloxyfucoxanthin (19HF) concentration in the <3-μm phytoplankton fraction as a percentage of total unfiltered phytoplankton. Chl *a* and 19HF concentration (ng L^{-1}) in the <3-μm phytoplankton fraction. Ratio of 19HF to all taxonomically meaningful carotenoids (19HF, 19′-butanoyloxyfucoxanthin, fucoxanthin, prasinoxanthin, peridinin) in the >3-μm and <3-μm phytoplankton. Estimated prymnesiophyte contribution to eukaryotic autotrophic Chl *a* biomass in the <3-μm phytoplankton fraction using the (Bidigare and Ondrusek 1996) model. The last column indicates prymnesiophyte species obtained in cultures originating from <3-μm samples. ND, not determined.

CTD	Latitude (°S)	Date (Nov 94)	Depth (m)	NO_3^- (μM) in surface	Chl *a* <3 μm/total (%)	19HF <3 μm/total (%)	Chl *a* <3 μm (ng L^{-1})	19HF <3 μm (ng L^{-1})	19HF/ carotenoids >3 μm	19HF/ carotenoids <3 μm	Prymnesiophyte (% of Chl *a* <3 μm)	Prymnesiophyte cultures recovered
2*	15.0	5	20	0.00	ND	ND	ND	ND	ND	ND	ND	
			100		ND	ND	ND	ND	ND	ND	ND	
11	11.5	7	15	0.01	75	78	30	18	0.64	0.87	87	
			75		90	92	170	116	0.46	0.66	64	
16	10.0	8	15	0.14	86	83	59	32	0.60	0.66	61	
			75		90	91	137	78	0.45	0.64	61	
21	8.5	9	15	0.69	89	89	71	43	0.59	0.65	55	
			75		85	87	134	69	0.38	0.56	44	
26	7.0	10	15	1.02	84	84	82	47	0.55	0.65	57	*Phaeocystis, Gephyrocapsa*
			60		86	85	136	80	0.52	0.68	64	*Phaeocystis*
31	5.5	11	15	2.43	67	76	58	40	0.43	0.75	67	*Phaeocystis*
			70		79	82	142	75	0.41	0.56	46	
36	4.0	12	15	1.71	64	79	62	43	0.33	0.71	63	
			75		82	91	135	95	0.37	0.70	66	
41	2.5	13	15	2.11	66	82	62	45	0.33	0.71	64	
			75		86	90	94	73	0.44	0.64	62	
45	1.0	14	15	2.49	70	83	89	55	0.30	0.61	64	
			60		81	86	135	72	0.36	0.71	53	
51	0.0	15	15	2.66	78	85	147	89	0.38	0.65	62	
			60		84	92	163	115	0.29	0.65	61	
Mean					80	85			0.43	0.68	61	

* Flow cytometry analysis suggests potential cell breakage due to filtration.

coeukaryotes were discriminated from *Prochlorococcus* and *Synechococcus* based on cell scatter and red fluorescence using the CYTOWIN software (available from http://www.sb-roscoff.fr/Phyto/cyto.html).

Enrichment cultures were performed immediately on board. For each sample, five 5-ml polystyrene tubes were incubated at room temperature (roughly 20°C) in a 12h : 12h light : dark cycle at an irradiance level close to that prevailing at the sampling depth. Each tube contained 2 ml of the 3-μm prefiltered sample and 0.2 ml of full-strength K medium (Keller et al. 1987). At the end of the cruise, enrichment cultures were brought back to the laboratory within 24 h and examined for any picoplankton growth by optical microscopy and flow cytometry. Tubes containing viable cells were serially diluted with K medium to obtain unialgal strains. Strain identity was confirmed by scanning and transmission electron microscopy.

Nucleic acid extraction and gene amplification—Frozen filters corresponding to the $<3\mu$m fraction were thawed and homogenized in 3 ml of cold DNA extraction buffer (10 mM Tris Cl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% sodium dodecyl sulfate [SDS]). After homogenization, the SDS concentration was increased to 1%, and the mixture (about 3.5 ml) was incubated with 100 μg/ml proteinase K at 55°C for 3 h. Proteins and polysaccharides were removed by a phenol/chloroform extraction and alcohol precipitation (Sambrook et al. 1989). Total nucleic acid preparations were purified with the Geneclean II kit (BIO 101). DNA was resuspended into 50 μl of 10 mM Tris-HCl pH 8, 1 mM EDTA. From the 20 purified DNA extracts, 18S rDNA was amplified by PCR with the oligonucleotide primers (5′-ACCTGGTTGATCCTGCCAG-3′, 5′-TGATCCTTCYGCAGGTTCAC-3′) complementary to regions of conserved sequences proximal to 5′ and 3′ termini of 18S rRNA genes. The 50-μl of reaction mixture contained about 100 ng of template DNA, 500 nM of each amplification primer, 250 nM total dNTP, 1.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase. Thermal cycle parameters were as follows: denaturation at 94°C for 1 min (initial denaturation 5 min), annealing at 55°C for 2 min, extension at 72°C for 3 min (final extension, 10 min). The reaction was cycled 30 to 35 times. PCR products were purified with the Geneclean II kit or the Qiaquick PCR purification kit (Qiagen).

Dot blot hybridization—Oligonucleotide probes (Table 2) were labeled with Fluorescein-11-dUTP using the enzymatic chemiluminescence (ECL) 3′-oligolabeling kit (Amersham). The probe S-S-E.hux-1702 (*P. globosa*)-a-A-20 (EHUX) was designed in the present study with the aid of the ARB program package (http://www.biol.chemie.tu-muenchen.de). It matches the sequences of *Emiliana huxleyi* and *Gephyrocapsa oceanica* that are identical. It has a minimum of three mismatches with all other sequences available in the GenBank database, except for the prymnesiophyte *Reticulosphaera japonensis* sequence, with which it has only two mismatches.

Amplified target 18S rDNA (80–150 ng) was immobilized on Zeta Probe membranes (Bio-Rad) with a dot-blot device (Bio-Rad) as recommended by the manufacturer. Together with the 18S rDNA from natural community samples, homologous 18S rDNA from pure cultures of the prymnesiophytes *E. huxleyi* and *Phaeocystis* sp. (Naples strain), the pelagophyte *Pelagomonas calceolata*, a yet undescribed picoplanktonic prasinophyte species (Guillou 1999), and PCR 2.1 plasmid vector DNA (Invitrogen) were applied to the membrane as positive and negative control DNA. The undescribed prasinophyte 18S rDNA was used because its sequence (SYM-vdS unpubl. data) has only one mismatch in the target region of the PRYMN01 probe. In addition to these control DNAs, clone OLI26017 (this study) was used as a positive control for the CLADE2 probe, which matched its target sequence. Clone OLI51059 (this study) was used as an additional negative control because its target sequence had one mismatch with the CLADE2 probe.

The membrane was air-dried and baked at 80°C for 2 hours. Prehybridization was performed at 42°C for at least 30 min in a hybridization buffer containing 5× standard saline citrate [SSC], 0.1% (w/v) hybridization buffer component (from the ECL 3′ oligo-labeling and detection kit, Amersham), 20-fold dilution of liquid block (from the same kit), and 0.02% (w/v) SDS. Hybridization with the oligonucleotide probes (5–10 ng/ml) was carried out for 12–17 h at 42°C for all probes except for EHUX, which was done at 55°C, according to the manufacturer's instructions (Amersham). Different posthybridization wash conditions, from low to high stringency, were tested and optimized for the samples. The stringent wash conditions included two subsequent washes (15 min each) with 0.1× SSC/0.1% SDS at 47°C for the PRYMN01 probe, at 52°C for the PRYMN02, the PHAEO01 and the EHUX probes, or at 42°C for the eukaryote probe EUK 1209R. The CLADE2 probe was washed twice with 0.5× SSC/0.1% SDS at 54°C. Detection

Table 2. Probes targeting 18S rRNA used in this study. Probe nomenclature for the *Emiliana huxleyi* probe has been standardized according to the oligonucleotide probe database (Alm et al. 1996). This probe matches positions 1717–1698 on *E. huxleyi* 18S rRNA gene (GenBank accession number L04957).

Probe name	Short name	Target	Sequence (5′ to 3′)	Source
EUK 1209R	EUK 1209R	Eucaryotes	GGGCATCACAGACCTG	Giovannoni et al. 1988
PRYMN01	PRYMN01	Prymnesiophyte	ACATCCCTGGCAAATGCT	Lange et al. 1996
PRYMN02	PRYMN02	Prymnesiophyte	GGAATACGAGTGCCCCTGAC	Lange 1997
PHAEO01	PHAEO01	*Phaeocystis*	CGGTCGAGGTGGACTCGT	Lange et al. 1996
CLADE2	CLADE2	*Chrysochromulina*	AGTCGGGTCTTCCTGCATGT	Simon et al. 1997
S-S-E.hux-1702 (*P. globosa*)-a-A-20	EHUX	*Emiliania huxleyi* *Gephyrocapsa oceanica*	CGGCGTCGCGGCTGGAGAAC	This study

and quantification of bound probes were performed with a fluorimager (STORM 840, Molecular Dynamics) using the enzymatic chemifluorescence (ECF) signal amplification system (Amersham). The image was analyzed with the ImageQuant software package (Molecular Dynamics). Serial dilutions of the homologous, positive-control DNAs were performed to find optimal concentrations of blotted DNAs that showed a linear response to the probe. Within the range of optimized DNA concentrations, linear regressions were calculated by the least-square method. The slope of the group-specific probe bound per unit of positive control rDNA was divided by the slope of the eukaryote probe (EUK 1209R) bound per unit of positive control rDNA. The slopes from multiple control DNAs were averaged (Giovannoni et al. 1990). For the estimation of the relative contributions of prymnesiophytes in natural samples, the ratios of the hybridization of the bound group-specific rDNA probes and of the bound eukaryote probes were divided by the ones obtained with positive control DNA (Gordon and Giovannoni 1996). Values were corrected for background, which was computed as previously described (Giovannoni et al. 1990). After detection, the oligonucleotide probe was stripped from the membrane immediately by washing the membrane twice for 20 min in a large volume of 0.1× SSC/0.5% SDS at 80°C for reprobing.

Gene cloning and screening of prymnesiophyte clones—To clone the PCR product, the ends of the amplified DNA fragments were modified for blunt-ended ligation using T4 kinase and polymerase. The blunt-ended 18S rRNA genes were purified with the Geneclean kit and were inserted into the calf intestinal phosphatase–treated *Hin*cII restriction site of the pGEM3Zf(−) plasmid vector (Promega) and transformed into *Escherichia coli* DH5-α cells. Some PCR products were inserted directly into the PCR 2.1 vector (Invitrogen) and transformed into *E. coli* INVαF′ cells (Invitrogen), following the manufacturer instructions. To detect the 18S rDNA of prymnesiophyte species, colony hybridization with the probe PRYMN01 was performed. Selected recombinants were grown on a nylon membrane disc, Magna Lift (Micron Separation) placed on LB (Luria-Bertani) agar. Lysis of cells, DNA denaturation, and removal of bacterial debris were performed as previously described (Sambrook et al. 1989). Hybridization and posthybridization washes were performed under the same conditions as for the dot blot hybridization.

Sequencing and phylogenetic analysis—Plasmid DNA containing amplified 18S rRNA genes from environmental samples was purified with the FlexiPrep kit (Pharmacia Biotech). 18S rRNA genes were sequenced using a VISTRA automated sequencer (Amersham). Sequencing primers including two plasmid primers and those specific for conserved regions of 18S rRNA genes were 5′ end labeled using the Texas Red labeling kit (Amersham). Both strands of the 18S rRNA genes were sequenced. Sequences were checked for chimeric gene artifacts with the Ribosomal RNA Database Project program CHECK_CHIMERA (Maidak et al. 1994). Nucleotide sequences were aligned with CLUSTALW (Thompson et al. 1994) and adjusted manually based on primary and secondary structure consideration. The percentage of similarities between sequences was computed with CLUSTALW, excluding the positions with gaps. Only 1,742 positions considered to be unambiguously aligned were considered for phylogenetic analysis. Distance analysis (neighbor-joining) and bootstrap analysis were performed using the PHYLIP program (Felsenstein 1993). The distance matrix of aligned sequences were generated using the program DNADIST by the use of the Kimura 2-parameter model (Kimura 1980), with a transition/transversion ratio of 2.0.

Nucleotide sequence accession numbers—The 18S rRNA gene sequences determined in the present study have been filed in GenBank under accession numbers from AF107080 to AF107092.

Results

The general conditions prevailing in the study area are described elsewhere (Claustre et al. 1999; Stoens et al. 1999). Briefly, the transect analyzed along 150°W ranged from very oligotrophic conditions at 15°S with no detectable nutrients in surface to mesotrophic conditions at the equator (Table 1). There was a clear divergence zone around 7°S (Claustre et al. 1999). Photosynthetic biomass was dominated by the lower than 3 μm size fraction that made up on average 80% of Chl *a* (Table 1). The abundance of picoeu-

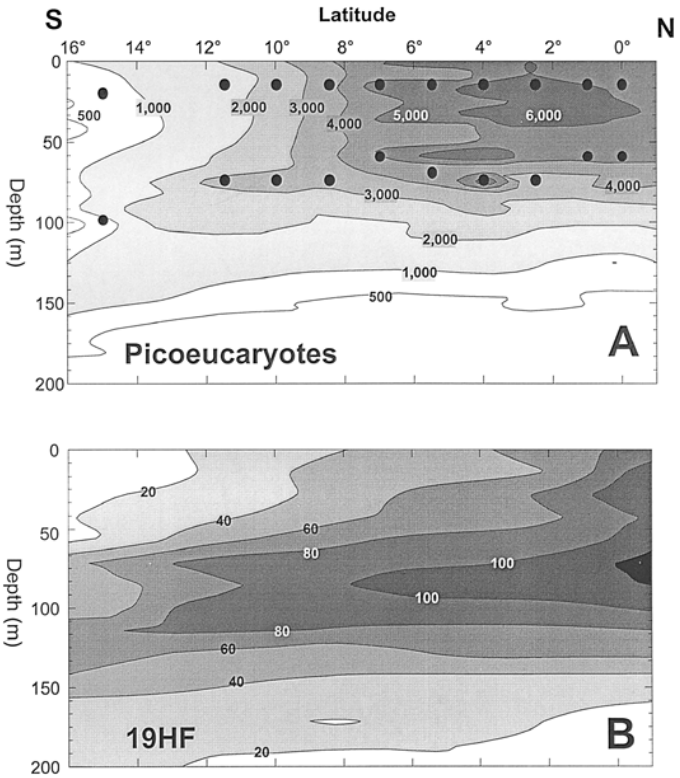

Fig. 1. (A) Concentration of photosynthetic picoeukaryotes in the equatorial Pacific during the OLIPAC cruise determined by flow cytometry. Contours levels are expressed as cells per milliliter. Solid circles correspond to the location where DNA samples have been taken. (B) Concentration of 19′-hexanoyloxyfucoxanthin (19HF) in unfiltered samples expressed in nanograms per liter.

Table 3. Comparison of percent contribution of 18S rDNA of prymnesiophytes (PRYMN01, PRYMN02), *Chrysochromulina* (CLADE2), *Phaeocystis* (PHAEO01), and *E. huxleyi* (EHUX) to total 18S rDNA of eukaryote picoplankton (<3 μm) communities. ND, not detected (<0.5%). Mean values were not computed for probes CLADE2 and EHUX because they gave undetectable results for a majority of samples.

Latitude (°S)	Depth (m)	PRYMN01 (%)	PRYMN02 (%)	CLADE2 (%)	PHAEO01 (%)	EHUX (%)
15.0	20	0.9	3.6	ND	ND	ND
15.0	100	ND	1.6	ND	ND	ND
11.5	15	ND	3.2	ND	ND	ND
11.5	75	2.5	4.2	ND	ND	ND
10.0	15	ND	3.7	ND	ND	ND
10.0	75	5.9	3.8	ND	1.0	ND
8.5	15	3.5	3.2	ND	0.9	ND
8.5	75	5.3	3.2	ND	1.1	ND
7.0	15	5.5	7.3	0.6	1.8	ND
7.0	60	8.2	3.8	ND	1.4	ND
5.5	15	12.1	2.9	ND	4.4	ND
5.5	70	2.6	2.6	ND	0.6	ND
4.0	15	4.1	3.5	ND	1.3	ND
4.0	75	3.2	3.7	ND	0.7	ND
2.5	15	8.1	5.0	ND	3.0	ND
2.5	75	4.4	2.3	ND	1.8	ND
1.0	15	2.9	4.5	ND	1.3	ND
1.0	60	3.6	2.4	ND	0.7	ND
0.0	15	2.8	7.3	1.1	ND	ND
0.0	60	12.3	5.7	ND	3.5	ND
Mean		4.40	3.88		1.18	

karyotes determined by flow cytometry was maximum in the divergence at about 6,000 cells ml^{-1} (Fig. 1A) and decreased by almost an order of magnitude at 15°S. The concentration maximum deepened from 30 m near the equator to about 100 m at 15°S. 19HF was the dominant carotenoid in the less than 3 μm fraction (Table 1), suggesting that prymnesiophytes were the major contributor to eukaryotic photosynthetic biomass. Surprisingly, pigment analyses indicated that the relative contribution of prymnesiophytes was nearly constant in all picoplankton samples, although pigment biomass clearly increased with depth, as well as from the oligotrophic edge of the Pacific gyre toward the equator (Fig. 1B), following closely the distribution of picoeukaryotes (Fig. 1A). In the larger than 3 μm fraction, however, 19HF was less dominant (Table 1), whereas the relative contribution of fucoxanthin, indicative of diatoms, increased near the equator at the expense of 19HF (data not shown). Prymnesiophyte cultures (Table 1) were isolated only between 5°S and 7°S, i.e., in the divergence zone, and consisted of well-known cultivated genera (*Phaeocystis*, *Gephyrocapsa*, and *Emiliania*, the latter isolated at 5°S from a sample from which no DNA was collected).

Probe based estimates—We used the class-specific probes PRYMN01 and PRYMN02 as well as the clade-, genus-, and species-specific probes CLADE2, PHAEO01, and EHUX to detect prymnesiophytes in natural samples from this area. When the two class-specific probes were used for quantitative hybridization against the 20 PCR-amplified samples, prymnesiophytes were detected in all samples (Table 3). They accounted for 1–12% of the total eukaryotic-amplified rDNA in the less than 3 μm fraction, with no clear trend along the transect or with depth. The two class-specific probes gave roughly equivalent estimates on average, although PRYMN01 gave higher prymnesiophyte contributions at the equator and in the 5–7°S divergence zone (Table 3). Probe-based estimates are more variable than pigment-based estimates: for example, the PRYMN01-based estimates range from 0.9 to 12.3% (i.e., at least a 12-fold range), while pigment-based estimates range from 44 to 87% (i.e., at most a twofold range).

Phaeocystis 18S rDNA was detected (1–4%) in the nutri-

Table 4. Frequency of clones with a positive signal to the PRYMN01 probe among recombinants and clones sequenced from representative sampling sites.

Latitude (°S)	Depth (m)	% positive clones	Clone reference	GenBank accession number
10	75	0.8	OLI16010	AF107081
			OLI16029	AF107080
			OLI16108	AF107082
7	15	0.8	OLI26017	AF107083
			OLI26041	AF107084
			OL126047	AF107085
7	60	1.0	OLI26038*	
0	15	0.6	OLI51004	AF107086
			OLI51050	AF107088
0	60	1.3	OLI51033	AF107087
			OLI51056	AF107087
			OLI51059	AF107089
			OLI51076	AF107090
			OLI51080	AF107091
			OLI51102	AF107092

* Chimeric sequence.

ent-rich equatorial region, but not in the water column at 11.5°S and 15°S or at the surface at 10°S and at the equator (Table 3). As with the PRYMN01 and PRYMN02 probes, no clear latitudinal or depth-related trend was observed with the PHAEO01 probe. The *Chrysochromulina* group was detected only in two surface samples in the mesotrophic region (7°S, 0°S), whereas the *Emiliania*/*Gephyrocapsa* group was never detected.

Clone library—Another estimate of prymnesiophyte abundance was obtained by screening clone libraries using the PRYMN01 probe at three stations representative of low, intermediate, and high nutrient surface levels, respectively (Table 4). Colony hybridization with the eukaryote probe and restriction enzyme digestions of randomly selected recombinant clones from all three sites showed that approximately 80% of recombinants contained an 18S rRNA gene insert. Given this percentage, the contribution of prymnesiophyte 18S rDNA was estimated to be 0.6–1.3% of the total 18S rDNA clones (Table 4). These values were far lower than those estimated from dot blot hybridization for the same locations.

Fifteen PRYMN01-positive clones were completely sequenced. The target sequences of the positive clones matched the PRYMN01 probe perfectly. Sequence similarity search using the EMBL gene data bank showed maximum similarity of the sequences to the prymnesiophyte 18S rDNA. One clone (OLI26038) was identified as a chimeric gene product. This was confirmed by independent phylogenetic analyses using putative chimeric fragments, and this sequence was discarded in the final analysis. Two sequences (OLI51033 and OLI51056) originating from the same sample were identical. The full-length sequences determined in this study were aligned with available 18S rRNA gene sequences of prymnesiophytes. Phylogenetic analyses confirmed that all the sequences were from the prymnesiophytes.

Only two 18S rDNA sequences were closely affiliated with known prymnesiophyte sequences (Fig. 2). Clone OLI16108 grouped with *C. leadbeateri*, with 96.7% sequence similarity. Clone OLI51004, which perfectly matched the PHAEO01 probe at the target site, grouped with *P. globosa*, with 99.3% sequence similarity.

Clones OLI26017, OLI16108, OLI51102, and OLI16029 were included in the *Chrysochromulina* clade. The lineage comprising clones OLI51033, OLI51056, and OLI51059 showed a weakly supported sister-group relationship with the clade that includes some species of *Chrysochromulina* (*C. hirta, C. ericina, C. kappa, C. polylepis*) and the genus *Prymnesium* (Fig. 2). Two environmental lineages represented by six clones (OLI51050, OLI26041, OLI51080, OLI51076, OLI16010, OLI26047) appeared to be affiliated with the coccolithophorids (*Emiliania, Pleurochrysis, Coccolithus, Cruciplacolithus*), *Isochrysis*, *Reticulosphaera,* and *Phaeocystis* rather than with the Prymnesiales (Fig. 2). However, this relationship was not strongly supported by bootstrap values.

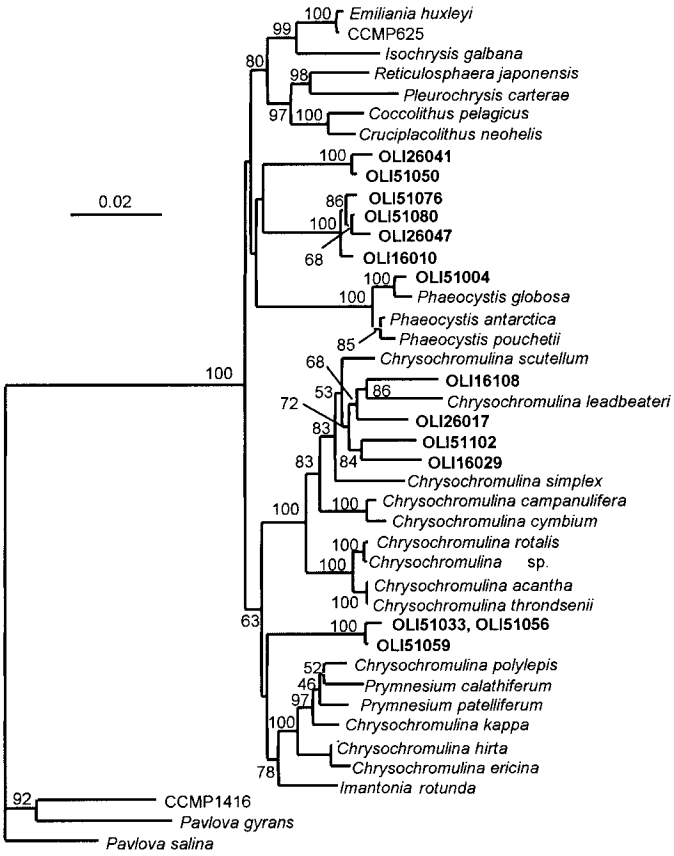

Fig. 2. Phylogenetic relationship (neighbor-joining) among prymnesiophytes. *Pavlova salina* was used as outgroup. Numbers at nodes represent the bootstrap percentage from 100 replicates. Values below 50% are not shown. The scale bar indicates substitutions per nucleotide position.

Discussion

Prymnesiophyte abundance in picoplankton—Picoplankton, and among them small eukaryotes, are major contributors to the photosynthetic biomass in mesotrophic and oligotrophic waters. Estimates of photosynthetic picoeukaryote abundance obtained by flow cytometry in the equatorial Pacific in 1994 agree with previous surveys in the same region (Landry et al. 1996), exhibiting in particular a deepening of the concentration maximum away from the equator. Concentrations at the edge of the south Pacific gyre are very similar to those observed in the north Pacific gyre off Hawaii (Campbell et al. 1994). Flow cytometry, however, does not permit any further characterization of the different eukaryotic components. Until recently, quantification of photosynthetic pigments has been the most common method available for this purpose, and algorithms have been developed to quantify the major groups of algae based on their specific pigmentation (Bidigare and Ondrusek 1996; Mackey et al. 1996). These algorithms clearly point toward the prymnesiophytes as the major eukaryotic class in most mesotrophic and oligotrophic waters. In equatorial Pacific waters, Bidigare and Ondrusek (1996) and Mackey et al. (1998) reported that prymnesiophytes, together with pelagophytes and dia-

toms, were the major eukaryotic phytoplankton components. Off Hawaii, Letelier et al. (1993) estimated their contribution at 22% of the total phytoplankton biomass in the central North Pacific gyre. At the same location, Andersen et al. (1996) estimated by transmission electron microscopy (TEM) prymnesiophyte abundance to vary between 10 and 50% of photosynthetic eukaryotes over a vertical profile. In their study, TEM-derived estimates were always lower than pigment-based estimates by up to 10-fold (at 200 m).

Pigment data from the present study are comparable to those obtained previously in the same region and show the remarkable dominance of 19HF both in whole water samples and in the less than 3 μm fraction (Table 1). However, we demonstrate here that in fact 19HF is relatively (i.e., as a fraction of taxonomic significant carotenoids) more abundant below 3 μm than above. For the former fraction, the contribution of prymnesiophytes ranges from 44 to 87%, suggesting that they are dominant among picoeukaryotes measured by flow cytometry (Fig. 1A).

Although pigment algorithms provide invaluable information on the major algal classes present in the ocean, they have some drawbacks, which have been alluded to in the introduction: restriction to class-level information, reliance on culture data, and variability in pigment content and ratios among species and as a function of environmental conditions.

Molecular biology offers clearly a wealth of alternative approaches to quantify taxa in the ocean, as demonstrated for heterotrophic bacteria communities. However, there are several possible strategies to choose from, each with its advantages and drawbacks. Hybridization of probes directly to genomic DNA extracted from natural samples may not be efficient because some oligonucleotide probes could possibly bind randomly to other genes (Fuhrman et al. 1994). Bulk extraction of RNA would be the method of choice (DeLong 1992), if the necessary volumes of seawater to handle were not impractical when target populations are in low abundance. The detection of a specific group using the retrieval of the specific rDNAs from a shotgun-cloned community DNA library is not efficient because rDNA-bearing recombinants are very rare (Olsen et al. 1986; Bond et al. 1995). Selective retrieval of the SSU rDNA sequences by PCR reduces these problems, and this approach has been used to probe marine prokaryotic communities. It has provided invaluable insights into the composition of target groups in a given ecosystem as well as a description of the phylogenetic diversity (Giovannoni et al. 1996).

In the present work, PCR amplification of rDNA followed by detection with class-specific probes confirmed the widespread distribution of prymnesiophytes but did not detect strong trends in their relative abundance with depth or nutrient status throughout equatorial Pacific waters (Table 2). However, the estimates based on class-specific probes are between 5 and 27 times lower than those calculated from photosynthetic pigment data.

First, pigment analyses only detect phototrophs, whereas the amplification primers and the eukaryote probe target both phototrophs and heterotrophs. Therefore, pigment data would yield a higher prymnesiophyte contribution. Andersen et al. (1996) determined phototrophs to comprise 62–74% of total eukaryotic cells off Hawaii. In fact, compilation of data from Chavez et al. (1996) yield estimates of prymnesiophytes making up 12–13% of the sum of autotrophic and heterotrophic carbon of eukaryotes, a figure at the top of the range we found (Table 2).

Second, several factors linked to the PCR and probing processes could explain disparities between pigment and probes. PCR, at least as used in the present work, is not fully quantitative, and all target genes may not be amplified with the same efficiency. Some of the biases inherent to PCR of natural mixtures of rRNA genes are only beginning to be investigated (von Wintzingerode et al. 1997). For example, templates with high G+C content are less well amplified than those with low G+C content, as suggested by the preferential amplification of yeast rDNA from a mixture of thermophilic archaea and yeast DNAs (Reysenbach et al. 1992). In contrast, when degenerate primers are used, templates with G/C in the degenerate position instead of A/T are better amplified (Polz and Cavanaugh 1998). Noncomplementarity of one or both of the PCR primers may also affect the PCR yield. For example, *Phaeocystis* sequences display one mismatch between the 3′ end of the 18S rRNA gene and the corresponding primer used for 18S rRNA amplification (Lange 1997), so that this primer may not bind to all targets at high annealing temperatures. Amplification of some sequences can be inhibited by DNA flanking the template region (Hansen et al. 1998). In addition, reannealing of templates present in excess during the PCR might result in reduced amplification of templates initially in high abundance (Suzuki and Giovannoni 1996). Although there is little information on rRNA gene copy number in algae, this factor also probably plays a critical role during amplification (Farrelly et al. 1995). If there is a positive correlation between genome size and rRNA gene copy numbers (Cavalier-Smith 1985), then algae with large genomes relative to their size will be overestimated by any method based on rRNA gene abundance. Dinoflagellates, in particular, are known to have very large genomes compared with other phytoplankton groups (Rizzo 1987). Boucher et al. (1991) found that dinoflagellates had about 10 times more DNA per unit volume than other microalgae. Since this group is well represented in the equatorial Pacific (see below), it is probably a source of underestimate for the other components.

The probes used may not recognize all prymnesiophyte species, yielding underestimates. Although PRYMN01 matches most of the cultured species tested so far (Lange et al. 1996; Simon et al. 1997), it cannot be excluded that it does not match some species from the natural environment. Furthermore, three environmental clones (OLI16029, OLI51033, OLI51059) did not match the PRYMN02 sequence. This could explain the differences observed between hybridization values for the two class-specific probes (Table 3) and underlines the need to obtain more sequences from natural populations for probe design.

Third, reasons inherent to the structure of the microbial community may explain the discrepancies observed. The relative content of 19HF from natural populations could be significantly higher than in laboratory cultures from which model values have been obtained. Indeed, even within a given genus, such as *Phaeocystis*, relative carotenoid abundance

varies widely among strains (Vaulot et al. 1994). Also pigment ratios vary with environmental conditions. For example 19HF increases dramatically relative to the other fucoxanthin pigments in *Phaeocystis* under iron stress (van Leeuwe and Stefels 1998), conditions that are probably encountered in the equatorial Pacific (Landry et al. 1997). Higher 19HF content in field populations than those assumed in models would induce overestimation of prymnesiophyte abundance using pigment data.

More importantly, the pigment 19HF may not be really specific of, or restricted to, prymnesiophytes. Only 60% of the prymnesiophyte strains surveyed by Jeffrey and Wright (1994) contained 19HF. Conversely, 19HF has been reported in certain dinoflagellates, such as *Gyrodinium aureolum* (Tangen and Björnland 1981), which harbor prymnesiophyte plastids (Schnepf 1993). In fact, dinoflagellates are clearly well represented in the waters we sampled. Data from Chavez et al. (1996) suggest that the biomass of dinoflagellates (both auto- and heterotrophs) account for 43 and 52%, respectively, of the total eukaryotic carbon biomass during two surveys in the equatorial Pacific.

19HF could also well be harbored by other taxonomic groups yet to be discovered. The fact that we have isolated during the OLIPAC cruise several cultures belonging to novel lineages (Guillou 1999), including a new class sister-group to the diatoms (Guillou et al. 1999), attests that the diversity of oceanic photosynthetic picoeukaryotes is largely underestimated, even at the class level. Moreover, many sequences recovered from a 11°30′S clone library from a 3-μm-filtered sample (SYM-vdS unpubl. data) are closely related to, but distinct from, dinoflagellates, pointing to potentially novel taxonomic groups not yet isolated in culture.

Abundance of specific prymnesiophyte taxa—Besides providing information at the class level, our probing approach allows also to estimate the contribution of specific genera. For example, *Phaeocystis* is especially known for its significant contribution to the global carbon cycle in coastal and polar regions, where it forms massive colonial blooms (Lancelot et al. 1987; Baumann et al. 1994). However, the present data demonstrate that it is also important in equatorial waters, where it contributes between 17 and 45% to the picoplanktonic prymnesiophyte population. The unicellular form probably dominates because colonies were only infrequently observed in this region at the time of the cruise (Gorsky et al. 1999). The presence of *Phaeocystis* was confirmed by the recovery of cultures (Table 1) and of one sequence (Fig. 2). Although the genus *Chrysochromulina* is present in the equatorial Pacific, as indicated by both probe and sequence data, it may not be as dominant as previously suggested (Thomsen et al. 1994). Neither *Phaeocystis* nor *Chrysochromulina* was detected by probing south of 10°S, i.e., in the region where there is a clear deepening of the picoplankton populations (Fig. 1), suggesting that there is a shift in the prymnesiophytes present in the very oligotrophic region and that these two genera cannot withstand extreme nutrient depletion. The ubiquitous and closely related coccolithophorid genera *Emiliana* and *Gephyrocapsa* have been frequently reported in tropical and equatorial Pacific waters (e.g., Furuya and Maruno 1983). During the period of study, they were observed near the equator at 165°E both in the water column and in the fecal pellets of the appendicularian *Meglocercus huxleyi* (Gorsky et al. 1999). However, these two genera could not be detected with the EHUX probe. These two genera may occur more as blooming species triggered by specific conditions, such as nutrient upwelling (Balch and Kilpatrick 1996), and remain at low concentrations under normal circumstances. It is noteworthy that they were the only coccolithophorids recovered in culture (after filtration through 3 μm) during this cruise. This further suggests that cultures may not always be representative of natural populations. This was recently very convincingly demonstrated for the heterotrophic chrysophyte *Paraphysomonas imperforata,* which accounts for less than 1% of the natural populations off Woods Hole but for 98% of the populations following enrichment (Lim et al. 1999) and for the newly described genus *Bolidomonas* (Guillou et al. 1999) that was repeatedly isolated from the equatorial Pacific, but accounts for less than 1% of picoplanktonic eukaryotes in field samples (Guillou et al. 1999*b*).

Diversity of prymnesiophytes—The design of probes is constrained by available 18S rDNA sequences. Because cultures represent probably only a small fraction of the picoplanktonic taxa present in the ocean, it is critical to obtain sequences directly from environmental samples. In the present work, there were large discrepancies between the values estimated by clonal abundance and those estimated from the quantitative dot blot hybridization. These data suggest that for unknown reasons, the cloning step generates bias in the estimation of taxon composition that results in the underrepresentation of prymnesiophyte genes in the clone library. It is not clear whether this bias is specific to the cloning method we used or more general (von Wintzingerode et al. 1997; Head et al. 1998). Another major problem realized recently with environmental gene cloning is the potential for chimeras. Although one such sequence was identified by the program CHECK_CHIMERA and phylogenetic analyses using putative chimeric fragments (Table 4), we cannot rule out chimeras resulting from very closely related species or from the different rRNA genes within a given genome (Wang and Wang 1997).

Despite these potential biases, we recovered sequences from known prymnesiophyte clades. The *Phaeocystis* sequence recovered is very close to *P. globosa,* which is probably the most common *Phaeocystis* species in temperate and subtropical waters (Vaulot et al. 1994). Seven of the 14 cloned sequences belonged to the *Chrysochromulina* clades, but only one matched (loosely) a *Chrysochromulina* species available in culture. Only one of these clones originated from a site where *Chrysochromulina* could be detected by probing (7°S, 15 m). Although this confirms that sequence abundance in the clone library is not proportional to their initial abundance in the PCR amplified rDNA (see above), this also demonstrates that taxa can be recovered by gene cloning in natural samples even if in low abundance.

More interestingly, we found two new clades in our clone library with no known representative in cultures. These two environmental lineages were represented by six clones and displayed a weak sister relationship to coccolithophorids and

Phaeocystis (Fig. 2). Despite their importance, few coccolithophorid gene sequences have been determined because these algae are difficult to cultivate and fewer than 10 marine species are available in culture (Jordan and Chamberlain 1997). However, those sequences that are available represent two very different coccolithophorid families, which are united by strong bootstrap support into a clade that has been recognized as two distinct orders, the Coccolithophorales and the Isochrysidales (Edvardsen et al. in press). Because the bootstrap values linking these environmental clades either to the coccolithophorids or to *Phaeocystis* are low, we suggest that the environmental clades likely originate from novel taxa. To characterize the evolutionary divergence and the phylogenetic affinities of these environmental lineages, more sequences from the related groups, in particular the coccolithophorids, are necessary. If indeed the novel sequences are not from coccolithophorids, the fact that we did not recover any coccolithophorid sequence despite their known presence and diversity in equatorial and tropical Pacific waters (Kaczmarska and Fryxell 1995; Gorsky et al. 1999) could be because most species have a relatively large size and may be too rigid to pass through the 3-μm filters used in this study. Finally, sequences related to *Pavlova* were not found in the clone library, probably because this genus is mostly restricted to coastal and brackish waters (Chrétiennot-Dinet 1990).

Data from existing cultured species alone may not be sufficient to reconstruct phylogenetic relationships among prymnesiophytes. For example, the hypothesis of a deep divergence of *Phaeocystis*, previously established from a more limited data set (Medlin et al. 1997), was not supported by the present study because of the inclusion of the environmental sequences. In the analyses by Edvardsen et al. (in press), the maximum likelihood analysis recovers an early divergence of the Phaeocystales, but it is not supported by the bootstrap values of the distance and maximum parsimony analyses.

Conclusion

Direct probing and sequencing of genetic material from marine samples has led to the discoveries of novel lineages in Bacteria and Archaea (Pace 1997) and has demonstrated that sole reliance on cultured organisms was providing a somewhat biased view of oceanic diversity. The present study demonstrates for the first time that the same appears to be true for eukaryotic plankton. Such new light shed on marine diversity has important consequences. First, it provides a new assessment of the relative abundance of the various groups of algae to marine biomass, hopefully leading to better models of oceanic production. Second, it reveals new lineages, that may have considerable ecological or taxonomic importance. Third, it reshapes phylogenetic inferences based solely on cultured species (Medlin et al. 1997).

However, it is clear that once molecular analyses have revealed new lineages, it is critical to identify and bring into cultures the corresponding organisms. In this perspective, 18S rRNA probes designed from environmental sequences should be very useful tools to visualize (for example by fluorescent in situ hybridization) target cells in field samples to determine their morphology or in enrichment cultures to optimize culture conditions favoring them (Kane et al. 1993).

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