

## Capítulo 5

### VARIABILITY IN ABUNDANCE AND COMPOSITION OF EUKARYOTIC PICOPLANKTON IN THE ALBORÁN SEA (SW MEDITERRANEAN)

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En preparación.

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### Abstract

Within the marine phytoplankton, the picoplanktonic fraction is regarded as an ubiquitous assemblage quasi invariable in terms of biomass. However, its variability in terms of taxonomical composition is very much unknown, mostly due to the technical difficulties to identify these small organisms. Here we present an study on the variability in abundance and composition of the eukaryotic picoplankton in the Alborán Sea (SW Mediterranean Sea) during two cruises performed in Spring 1998 and Fall 1999. In this marine area there are different macroscale and mesoscale physical structures due to the input of surface Atlantic waters into the Mediterranean basin. While it is known that these structures cause a high spatial variability in the composition of large phytoplankton, their influence on the distribution of picoplankton is unknown. We first used flow cytometry to quantify the picophytoplankton and observed higher abundance in Spring 1998 than in Fall 1999 samples, mostly due to higher numbers of *Synechococcus* and picoeukaryotes during the first cruise. During each cruise, the variability was large and in general prevented differences among stations or transects to be significant. Second, we used two fingerprinting techniques, DGGE and HPLC pigment analysis, to analyze the spatial and temporal variability of the eukaryotic picoplankton. These results also showed that the main differences in community structure occurred between seasons/years. Moreover, DGGE data grouped the 1998 samples (but not the 1999 samples) in a shore – offshore gradient. Finally, we investigated the taxonomic composition of picoeukaryotes by converting the HPLC pigment matrix to the relative contribution of different groups using CHEMTAX and by sequencing selected DGGE bands. Both techniques agreed in presenting chlorophyll b-containing algae, such as prasinophytes, as one of the main components of the picoplankton during our Alborán Sea cruises. Apart from this predominant group, HPLC also detected a similar amount of prymnesiophytes and minor levels of cryptophytes and pelagophytes, whereas DGGE retrieved mostly novel lineages among the stramenopiles and alveolates. This apparent discrepancy could be explained by the presence of heterotrophic organisms in the picoplankton that would be detected by DGGE but not by HPLC. In summary, our results show a large variability of picoplankton in the studied region and a predominance of prasinophytes and prymnesiophytes.

## Introduction

Planktonic organisms of very small size are now widely recognized as dominating biomass, production and metabolic activity in the open oceans (Azam et al. 1983; Li et al. 1983; Murphy and Haugen 1985). Their importance has been well documented in oligotrophic regions of the ocean (Li et al. 1983; Stockner and Antia 1986; Chisholm 1992), where picoplankton generally account for more than 50% of the chlorophyll *a* (Chavez 1989; Peña et al. 1990; Bidigare and Ondrusek 1996). The larger size fractions of phytoplankton tend to be more prevalent in eutrophic areas (Binder et al. 1996; Latasa and Bidigare 1998; Bidigare and Ondrusek, 1996). Contrasting with the ecological importance of the picoplankton, their taxonomy is poorly known because of their small size and lack of distinct morphological features. These problems have been sometimes circumvented by using more sophisticated methods such as electron microscopy (Andersen et al. 1996) or cloning and sequencing of taxonomical informative genes (Díez et al. 2001a; López-García et al. 2001; Moon-van der Staay et al. 2001), but these approaches are too laborious and time-consuming to be applied to the large number of samples routinely collected and processed in an oceanographic study. Denaturing Gradient Gel Electrophoresis (DGGE) is a good candidate among the molecular tools for oceanographic studies, and can provide information complementary to that provided by other techniques such as pigment analysis by HPLC or cell counting by flow cytometry. All these techniques can be used to monitor picoplanktonic organisms, and all of them have the capacity to process many samples relatively fast. DGGE and HPLC have the additional advantages of giving information about phylogenetic composition of the picoplankton and actually quantifying the different groups.

The use of marker pigments in the identification of algal classes has become established due to the constant improvement in HPLC analytical techniques. Analyses of marine ecosystems have attempted to estimate the abundance of different phytoplankton classes from the concentrations of their marker pigments (Gieskes and Kraay 1986; Gieskes et al. 1988; Letelier et al. 1993; Tester et al. 1995; Andersen et al. 1996). By analyzing the marker pigments in size-fractionated samples, this approach allows to identify the main algal classes of picoplankton. A remarkable improvement in the HPLC field has been the introduction of CHEMTAX, a program able to integrate large sets of pigment data and deliver the relative abundance of different algal groups (Mackey et al. 1996). A limitation of HPLC pigment analysis is the impossibility to know what genera or species inside each algal class are present in the samples. Thus, it can be combined with other techniques such as electron microscopy (Andersen et al. 1996) or 18S rDNA phylogenetic analysis (Fawley et al. 2000). And, an additional shortcoming is that only autotrophic microorganisms can be studied.

Variations in abundance of different microorganisms can also be followed by DGGE. This technique has the potential to provide information about the phylogenetic composition of the small plankton including both autotrophs and heterotrophs. The main question about its usefulness in marine studies is whether the extraction of DNA and/or the PCR steps, which are necessary preliminary steps for DGGE, introduce biases that may provide a false image of the composition of natural plankton (Suzuki et al. 1998). In a previous study (Díez et al. 2001b) we optimized this technique for the analysis of marine picoplanktonic eukaryotes and compared it to other molecular techniques such as cloning and sequencing and T-RFLP (Chapters 3 and 4). Here we test the capacity of DGGE to be used in oceanographic cruises as a tool complementary to more conventional techniques such as flow cytometry and HPLC.

For this purpose we chose three cruises in the Alborán Sea, hoping to find a large diversity of hydrographic conditions and of trophic status (Raimbault et al. 1988a; Rodríguez et al. 1994; García 1994; Rodríguez et al. 1998; Morán et al. 2001). The water circulation in this area is characterized by two main anticyclonic gyres of approximately 100 km in diameter, the Western and Eastern Alborán Sea Gyres (Minas et al. 1991; Tintoré et al. 1991), which are easily seen in surface temperature satellite images (see for instance Fig. 12 in Chapter 1). These quasi-permanent gyres (Heburn and La Violette 1990) are formed by the surface inflow of Atlantic water which crosses the Strait of Gibraltar and gradually mixes with the resident Mediterranean waters, originating the so-called Modified Atlantic Water (Parrilla and Kinder 1987). Upwelling events are common in the northern part of the Western Alborán Gyre (Gil and Gomis 1994; Rodríguez et al. 1998; Morán et al. 2001). Due to the episodic nature of the physical forcing in the entire Western basin, the position of the water masses can fluctuate considerably at short-time scales (Perkins et al. 1990). The presence of anticyclonic (oligotrophic) and cyclonic (productive) gyres within relatively short distance, with accompanying fronts and upwelling phenomena (Tintoré et al. 1991, Rodríguez et al. 1998), make the Alborán Sea a natural laboratory that offers an opportunity to study the variability of the picoeukaryotic populations under very different conditions.

Picoplankton in the Alborán Sea has been studied by Rodríguez et al. (1998). During a cruise performed in Summer 1992 these authors found that picoplankton was generally invariable in terms of biovolume independently of the type of hydrographic circulation or dynamics, whereas nano- and microplankton increased their abundance under upwelling conditions. However, no reports exist about the taxonomic composition and variability of picoplankton in this area, especially the eukaryotic picoplanktonic fraction. We analyzed three stations in one transect along the Western anticyclonic gyre four times in a spring cruise in 1998 and three times in an autumn cruise in 1999. This sampling strategy was performed in order to maximize the spatial and temporal variability of picoplanktonic assemblages.

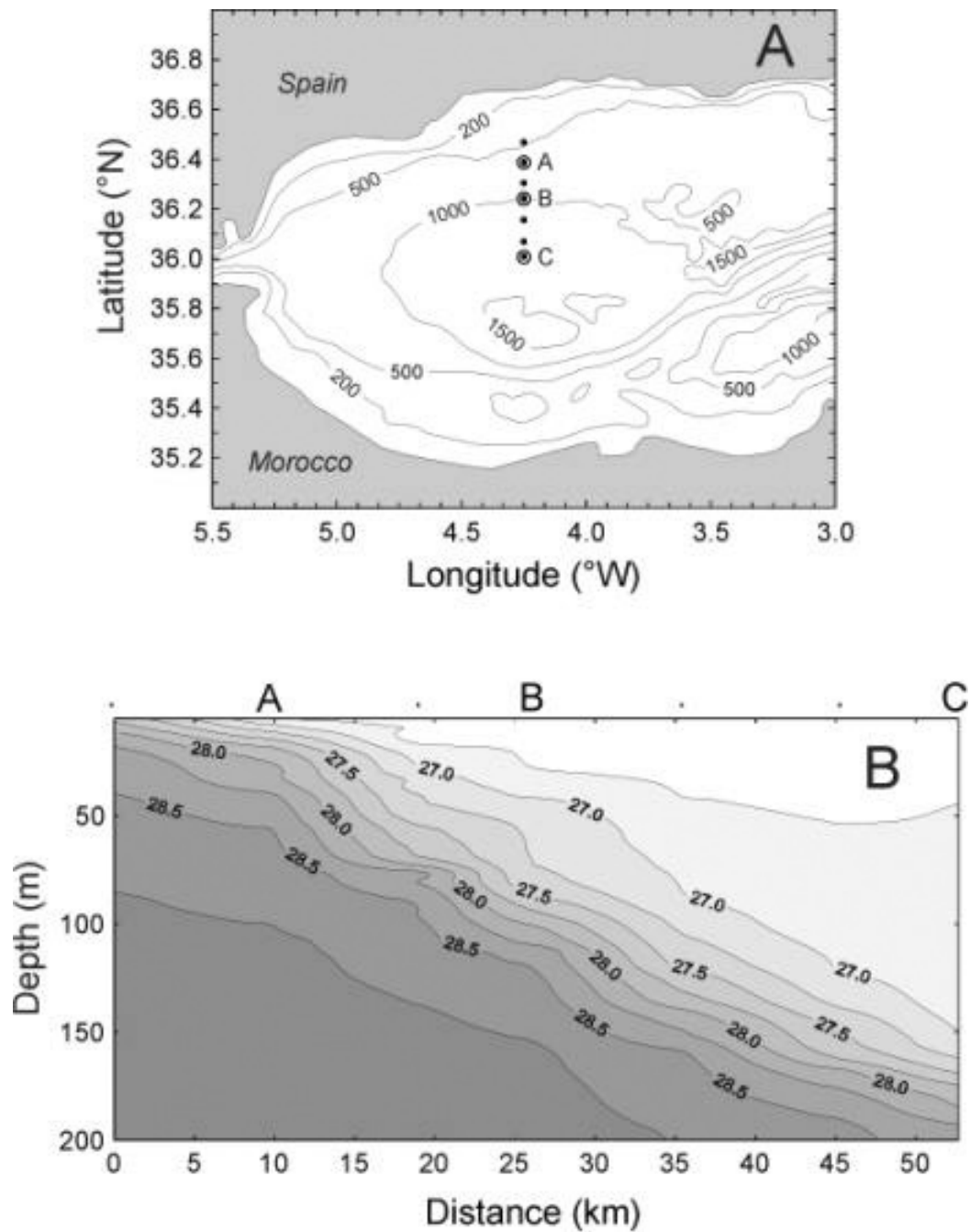


FIG. 1. (A) Map showing the positions of the three repeatedly sampled stations A, B and C and the stations of the transect shown in B. (B) Seawater density ( $\sigma\text{-t}$ ) in the first 200 m across a 50 km transect including stations A, B and C during the fourth transect in the ME98 cruise (between 10/05/98 and 12/05/98). (courtesy of Morán et al. 2001).

## Materials and Methods

**Seawater sampling.** Three stations located in the Western gyre of the Alborán Sea (SW Mediterranean Sea) were visited several times during different oceanographic cruises: station A at 36°23' N, 4°15' W, station B at 36°14' N, 4°15' W, and station C at 36°0' N, 4°15' W (Fig. 1A). Only station B was sampled during the first cruise in November 1997 (MTP-III-MATER/97, on board B/O García del Cid). The three stations were sampled four times the second cruise in May 1998 (MTP-II-MATER/HESP/98, on board BIO Hespérides) and three times in the third cruise in September 1999 (MTP-I-MATER/HESP/99, on board BIO Hespérides). The actual dates when the stations were sampled are indicated in Table 1.

TABLE 1. Sampling dates of the stations visited in the present study.

Cruise	Station	Date (day/mo/yr)
MATER-97	ME97-B0	11/9/97
MATER-98	ME98-A1	2/5/98
	ME98-A2	5/5/98
	ME98-A3	8/5/98
	ME98-A4	11/5/98
	ME98-B1	3/5/98
	ME98-B2	6/5/98
	ME98-B3	9/5/98
	ME98-B4	12/5/98
	ME98-C1	4/5/98
	ME98-C2	7/5/98
	ME98-C3	10/5/98
	ME98-C4	13/5/98
MATER-99	ME99-A1	17/9/99
	ME99-A2	21/9/99
	ME99-A3	24/9/99
	ME99-B1	16/9/99
	ME99-B2	20/9/99
	ME99-B3	23/9/99
	ME99-C1	15/9/99
	ME99-C2	19/9/99
	ME99-C3	22/9/99

Surface seawater (5 m) was collected with 12-liter Niskin bottles attached to a rosette carrying a CTD (conductivity, temperature, and depth probe). Seawater was then transferred to a 25-liter plastic container previously rinsed three times with the same water. Microbial biomass was collected on a 0.2  $\mu\text{m}$  pore-size Sterivex unit (Durapore, Millipore) by filtering 5 to 10 liters of seawater through a 5  $\mu\text{m}$  pore-size prefilter (Durapore in the first cruise and polycarbonate in the other two) and the Sterivex unit in succession with a peristaltic pump, using filtration rates of 50 to 100  $\text{ml min}^{-1}$ . Thus, biomass from the 0.2 - 5  $\mu\text{m}$  and > 5  $\mu\text{m}$  fractions were obtained after filtration. The prefilter and the Sterivex unit were covered with lysis buffer and frozen at  $-70^{\circ}\text{C}$  until nucleic acid extraction was performed in the laboratory.

Between 1.5 and 2 liters of sample were filtered with positive pressure (0.3 bars) through 25 mm 5  $\mu\text{m}$  Poretics and Whatman GF/F filters in-line for HPLC analysis of pigments. Filters were frozen and stored until pigment analyses were carried out in the laboratory. Another aliquot of the sample (between 100 and 200 ml) was filtered through Whatman GF/F filter and chlorophyll *a* was determined by measuring the fluorescence in 90% acetone extract with a Turner Designs fluorometer (Yentsch et al. 1963). Subsamples for determination of picoplankton abundance using flow cytometry were collected by fixing 1.2 ml of seawater with glutaraldehyde-paraformaldehyde (final concentrations of 0.05 and 1%, respectively). After 10 minutes of fixation in the dark, samples were frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$  until analysis. Populations of *Synechococcus*, *Prochlorococcus* and photosynthetic picoeukaryotes were identified by their distinct size and pigment properties with a FACScalibur flow cytometer (Beckton Dickinson) as described in Olson et al. (1993).

**Nucleic acid extraction.** Nucleic acid extraction was performed as described by Massana et al. (1997). Essentially, lysozyme was added (final concentration, 1  $\text{mg ml}^{-1}$ ) and the filters were incubated at  $37^{\circ}\text{C}$  for 45 min. Sodium dodecyl sulfate (final concentration, 1%) and proteinase K (final concentration, 0.2  $\text{mg ml}^{-1}$ ) were added, and the filters were incubated at  $55^{\circ}\text{C}$  for 60 min. The lysates were purified twice by extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and the residual phenol was removed by extraction with an equal volume of chloroform-isoamyl alcohol (24:1). Finally, nucleic acid extracts were further purified, desalted, and concentrated with a Centricon-100 concentrator (Millipore). The integrity of the total DNA was checked by agarose gel electrophoresis. The DNA yield was quantified by a Hoechst dye fluorescence assay (Paul and Myers 1982). Nucleic acid extracts were stored at  $-70^{\circ}\text{C}$  until analysis.

**Molecular analyses.** PCR and DGGE were performed essentially as described by Díez et al. (2001b). A fragment of the eukaryotic 18S rRNA gene was amplified with eukaryal specific primers Euk 1A and Euk 516r with a GC-rich clamp, which amplify a 560 bp DNA fragment of

eukaryotic 18S rDNA (Díez et al. 2001b). The PCR mixtures (50 µl) contained 200 µM of each dNTP, 1.5 mM of MgCl<sub>2</sub>, 0.3 µM of each primer, 2.5 U of *Taq* DNA polymerase (Gibco BRL) and the PCR buffer supplied with the enzyme. About 10 ng of extracted DNA was used as the template in each PCR reaction. Reactions were performed in an automated thermocycler (Genius, Techne) with the following cycle: an initial denaturation step at 94°C for 130 s, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 130 s. During the last cycle the extension step was increased to 7 min. An aliquot of PCR products were visualized and quantified using Low DNA MASS Ladder (Gibco BRL) in a 0.8% agarose gel stained with ethidium bromide.

DGGE was performed with a DGGE-2000 system (CBS Scientific Company) as described (Díez et al. 2001b). Electrophoresis was performed in 0.75-mm-thick 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37.5:1) for 16 h at 100 V in a linear 45 to 65% denaturant agent gradient (100% denaturant agent was defined as 7 M urea and 40% deionized formamide), submerged in 1x TAE buffer (40 mM Tris, 40 mM acetic acid, and 1 mM EDTA, pH 7.4) at 60°C. Approximately 800 ng of PCR product were loaded in individual lanes in the gel. After electrophoresis nucleic acids were stained for 45 min in 1x TAE buffer with SybrGold nucleic acid stain (Molecular Probes) and visualized with a FluorS Multimager and the MultiAnalyst Imaging software (Biorad).

In order to know the identity of DGGE bands, several DGGE bands were selected, cut, and reamplified with the same set of primers as previously described (Díez et al. 2001b). Between 30 and 50 ng of the reamplified PCR product were used for a sequencing reaction (with the corresponding forward primer) with the Thermo Sequenase v.2 Kit (Amersham, U.S. Biochemical) and an ABI PRISM Model 377 (v.3.3) automated sequencer. The sequences obtained were compared with public DNA database sequences by using BLAST (Altschul et al. 1997).

Digitized DGGE images were analyzed with the Diversity Database software (Bio-Rad) as previously described (Schauer et al. 2000). Bands occupying the same position in the different lanes of the gel were identified. A binary matrix was constructed taking into account the presence or absence of individual bands in all lanes. This binary matrix was used to calculate a similarity matrix with the Jaccard coefficient of similarity, which was used to construct a dendrogram with the software SYSTAT 5.2.1. Finally, the software records a density profile through each lane, detects the bands, and calculates the relative contribution of each band to the total band intensity in the lane after applying a rolling disk background subtraction.



**HPLC pigment analysis.** For the HPLC analysis of pigments the filters were placed in 3 ml of 90% acetone overnight and destroyed with a cell mill cooled with ice. One ml of pigment extract was mixed with 0.2 ml water prior to injection to improve peak separation and avoid pigment precipitation (Latasa et al. 2001). The HPLC system consisted of a ThermoSeparation binary pump (P2000), autosampler (AS3000), UV detector with fast scan capabilities (UV3000) and fluorescence detector (FL2000). All the hardware was controlled via a SN4000 interface and Cromquest software. The HPLC protocol basically followed the indications of Zapata et al. (2000) with very slight modifications (Latasa et al. 2001). Pigments were identified by their retention time and absorption characteristics.

In this paper we report a small portion of the whole pigment dataset which will be discussed elsewhere (Latasa et al. in preparation). This large dataset, containing nine to ten datapoints per station, allowed us to apply CHEMTAX (CHEMical TAXonomy), a public available software tool that allows to use pigment data to estimate relative abundance of different algal classes (Mackey et al. 1996). A previous screening of the data set showed that six major groups made up the bulk of the pigment biomass in surface waters: chlorophyll b-containing phytoplankton, prymnesiophytes, pelagophytes, diatoms, cryptophytes and cyanobacteria. The contribution of this six groups to the (monovinyl) chlorophyll a was estimated with CHEMTAX. In addition, *Prochlorococcus* could be quantified independently because they specifically contain divinyl chlorophyll a.

HPLC pigments were also used as fingerprints to find relationships among stations. The following pigments per station were considered in the analysis: Chlorophyllide *b*, Divinyl Chlorophyll c3, Monovinyl Chlorophyll c3, Chlorophyllide *a*, MgDVP, Chlorophyll c2, Chlorophyll c1, Peridinin, 19'-butanoyloxyfucoxanthin, Fucoxanthin, Neoxanthin, Prasinolaxanthin, Unknown1, Violaxanthin, 19'-hexanoyloxyfucoxanthin, Diadinoxanthin, Unknown mix, Alloxanthin, Diatoxanthin, Unknown2, Zeaxanthin, Lutein, Unknown3, Chlorophyll *b*, Chlorophyll *a* allomer 1, Chlorophyll *c* apolar, Chlorophyll *a* allomer 2 and Divinyl Chlorophyll *a*. A matrix was constructed taking into account the percentage of these pigments based on the total percentage of chlorophyll. The matrix was used to calculate a distance matrix with Euclidian distances, which was then used to construct a dendrogram with Ward minimum variance method with the software SYSTAT 5.2.1.

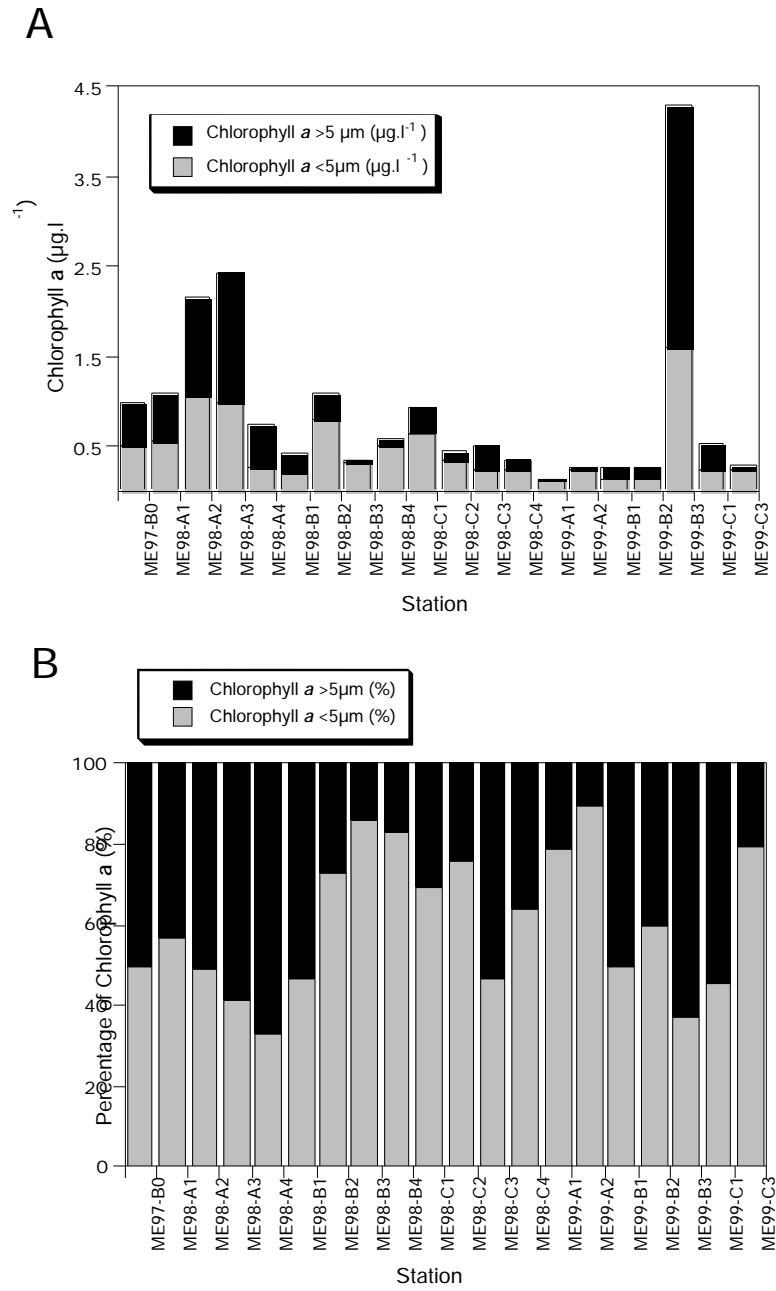


FIG. 2. (A) Concentration of total chlorophyll *a* in surface Alborán Sea samples from ME97, ME98 and ME99 in the fraction larger than 5  $\mu\text{m}$  (black) and smaller than 5  $\mu\text{m}$  (striped). (B) Percent of total chlorophyll *a* in the fraction larger than 5  $\mu\text{m}$  (black) and smaller than 5  $\mu\text{m}$  (striped).

## Results

The three stations sampled followed a coastal offshore gradient across the Western Alborán Sea Gyre, with station A being located at the northern edge of the gyre and station C at the center. These regions were visited several times during a cruise in Spring 1998 (ME98 samples) and in Fall 1999 (ME99 samples), whereas an additional sample from station B was collected in Fall 1997. Surface temperature was markedly different during the spring 1998 cruise (around 15-17°C) and the autumn 1999 cruise (around 20-22°C), while salinity was relatively constant at around 36.5 ‰. Temperature and salinity data from several depths in each station were used to obtain sigma-t values, and the data obtained for one of the transects in spring 1998 is shown in Fig. 1B. This Fig. clearly reveals an upwelling event occurring at station A. Chlorophyll *a* concentrations were between 0.5 and 1 µg l<sup>-1</sup> in most samples (Fig. 2A), and a very significant fraction of these values (between 30 and 85% of the total; 58% on average) was due to the organisms smaller than 5 µm (Fig. 2B).

TABLE 2. Concentrations (10<sup>3</sup> cells ml<sup>-1</sup>) of phototrophic picoeukaryotes, *Prochlorococcus* and *Synechococcus* in whole water samples counted by flow cytometry.

Sample	Picoeukaryotes		<i>Prochlorococcus</i>		<i>Synechococcus</i>		Total picophytoplankton
	Cells ml <sup>-1</sup>	%	Cells ml <sup>-1</sup>	%	Cells ml <sup>-1</sup>	%	Cells ml <sup>-1</sup>
ME97							
B0	18.0	33.3	21.0	38.9	15.0	27.8	54.0
ME98							
A1	1.8	27.3	0.5	7.9	4.3	64.5	6.7
A2	8.0	55.3	0.7	5.2	5.7	39.4	14.5
A3	17.5	30.9	6.0	10.7	33.2	58.4	56.8
A4	2.5	15.5	0.5	3.3	13.4	81.2	16.5
B1	1.9	13.7	0.3	2.1	11.6	84.2	13.8
B2	8.3	19.2	4.4	10.2	30.6	70.6	43.3
B3	12.7	8.4	0.0	0.0	138.0	91.6	151.0
B4	3.7	7.1	1.6	3.1	46.6	89.7	52.0
C1	4.5	20.0	0.5	2.0	17.5	78.0	22.5
C2	4.9	8.4	0.0	0.0	53.7	91.5	58.6
C3	5.1	2.0	0.0	0.0	248.0	98.0	253.0
C4	2.5	6.2	0.0	0.0	38.1	93.8	40.6
Average	6.1		1.1		53.4		60.7
ME99							
A1	0.1	1.0	11.5	69.3	4.9	29.6	16.6
B1	0.7	11.2	3.7	59.8	1.8	28.9	6.2
B2	0.3	16.6	5.6	78.0	0.3	5.4	6.2
C1	0.3	5.6	1.6	89.7	0.1	4.6	2.0
C2	0.1	1.1	6.3	94.8	0.3	4.0	6.7
Average	0.3		5.7		1.5		7.5

Abundance of picophytoplankton measured by flow cytometry is shown in Table 2. Total picoplankton numbers were extremely variable, ranging between  $10^3$  and  $10^5$  cell  $\text{ml}^{-1}$  among samples from different stations and cruises. In general, values from Spring 1998 were much higher than from Fall 1999. This was caused by higher values of picoeukaryotes and *Synechococcus* in 1998, while the opposite was true for *Prochlorococcus*.

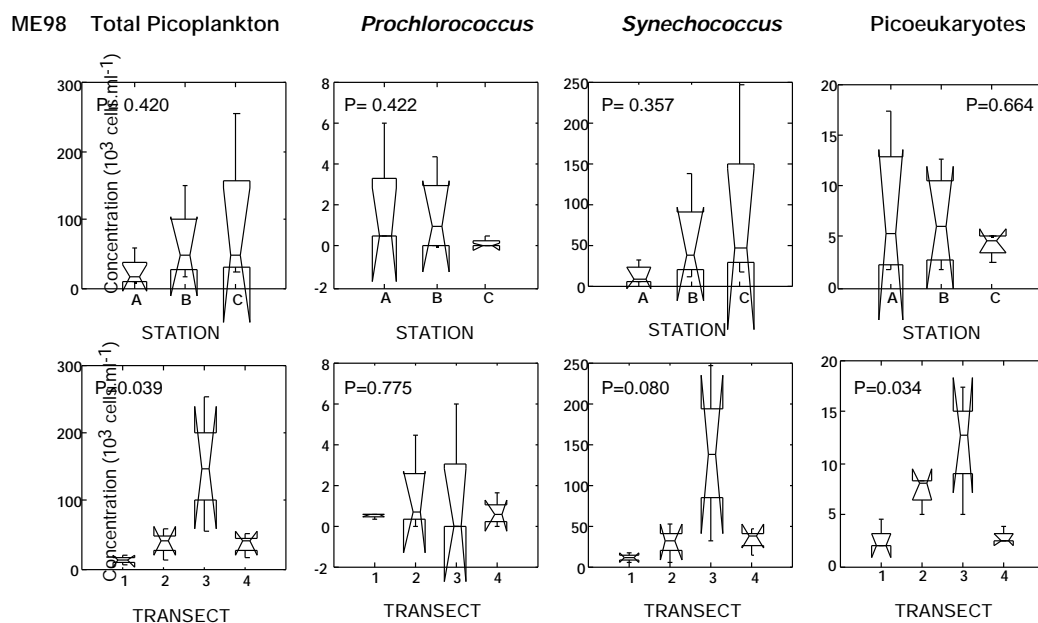


FIG. 3. Graphic significance of differences in picophytoplankton counts (total numbers and partitioned among the three groups easily distinguishable by flow cytometry) among ME98 stations and transects, according to ANOVA analyses. The p value is shown in each case (differences significant when p is smaller than 0.05). The central horizontal line in each box represents the median of the distribution, whereas the other two horizontal lines contain 50% of the values between them. The endpoints of the inclined straight lines indicate the 95% confidence intervals for the median, whereas the whiskers indicate the total range of values without outliers.

Variability in picophytoplankton cell counts in Spring 1998 was partitioned between stations and transects by an ANOVA (Fig. 3). Samples from Fall 1999 were too few to perform the same analysis. The main conclusion was that during the 1998 cruise the variability was as high between stations as between transects, reflecting the very dynamic area studied. There was an increase in cell numbers of *Synechococcus* and picoeukaryotes in the three stations from the first sampling to the third and a decrease in the last sampling. These changes with time can be appreciated better in Fig. 4. The largest increase for *Synechococcus* occurred in

station C and for picoeukaryotes in station A. The numbers of *Prochlorococcus* changed very little with time. The changes in the three populations in station B were intermediate between those of stations A and C. Thus, even within a relatively short period of time, considerable changes in the composition of picoplankton were taking place in the area. This large variability in time within each station prevented changes among stations from being significant (Fig. 3). However, a tendency for higher concentrations of *Synechococcus* offshore and higher concentrations of picoeukaryotes and *Prochlorococcus* inshore was evident. This pattern was not found in 1999 but the number of data points was relatively small and no further conclusion is warranted.

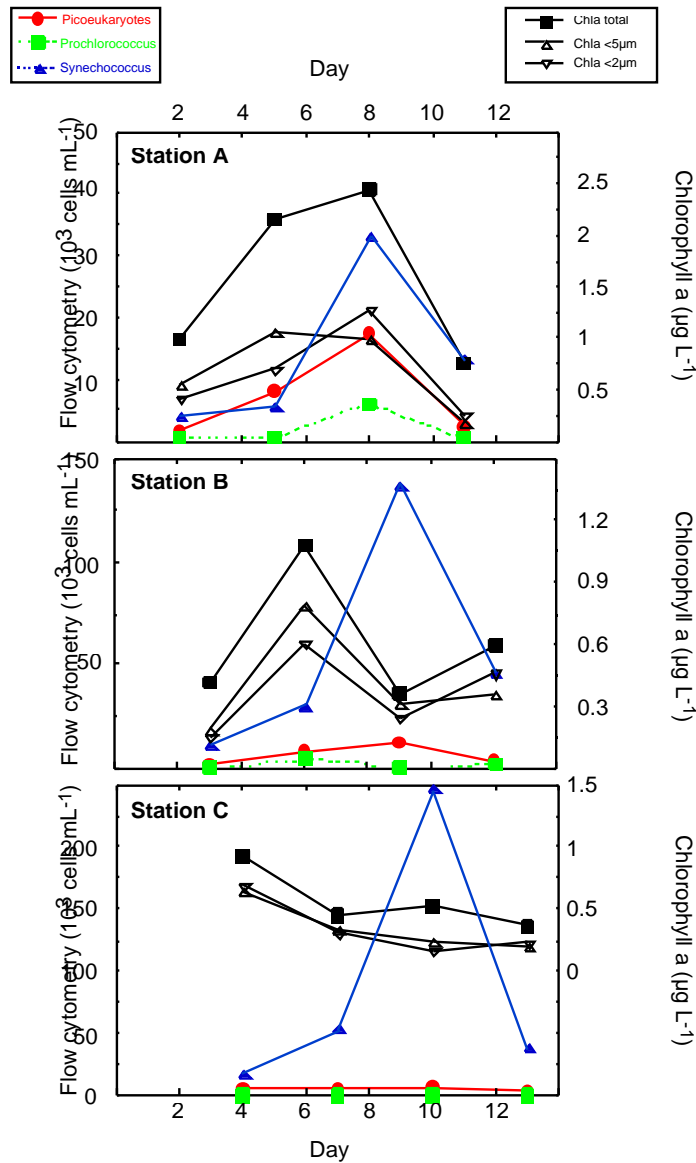


FIG. 4. Changes in the abundance of the three groups of picophytoplankton measured by flow cytometry in the three stations sampled in cruise ME98. Chlorophyll *a* values (total and in two small fractions) are also shown.

DGGE. We first investigated if the size fractionation was really separating the eukaryotic assemblage. In one example from each station (the second transect of ME98), we compared the DGGE fingerprints obtained from the fractions larger and smaller than 5  $\mu\text{m}$  (Fig. 5). As expected, some bands were shared by both fractions, presumably corresponding to cells of a size close to 5  $\mu\text{m}$ . Other bands, however, were exclusively found in one of the two fractions. In general, the large fraction showed more bands than the small one. DGGE fingerprinting for the <5  $\mu\text{m}$  fractions from all surface samples are presented in Fig. 6. The total number of bands ranged between 12 and 24. Three to four bands in each lane were later shown to belong to copepods (see below) and these were excluded from further analysis. Therefore, the number of bands corresponding to picoeukaryotes ranged between 10 and 20.

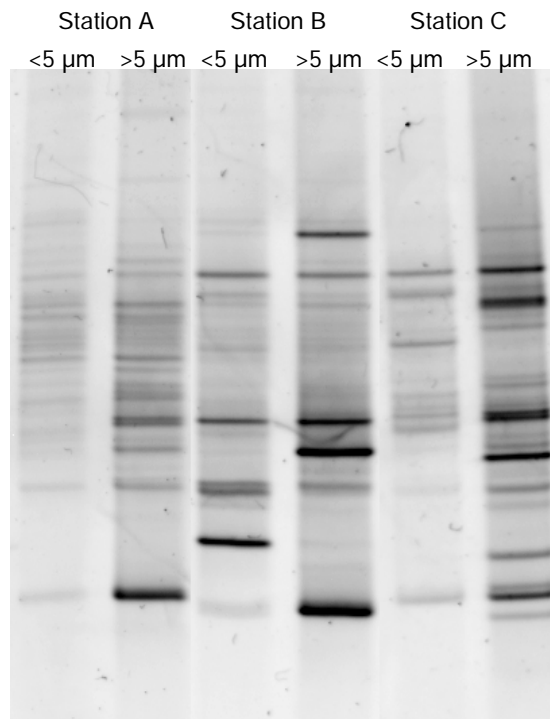


FIG. 5. Negative image of a DGGE gel showing fingerprints of eukaryotic populations smaller and larger than 5  $\mu\text{m}$  found at surface of stations A, B and C sampled during the second transect of ME98.

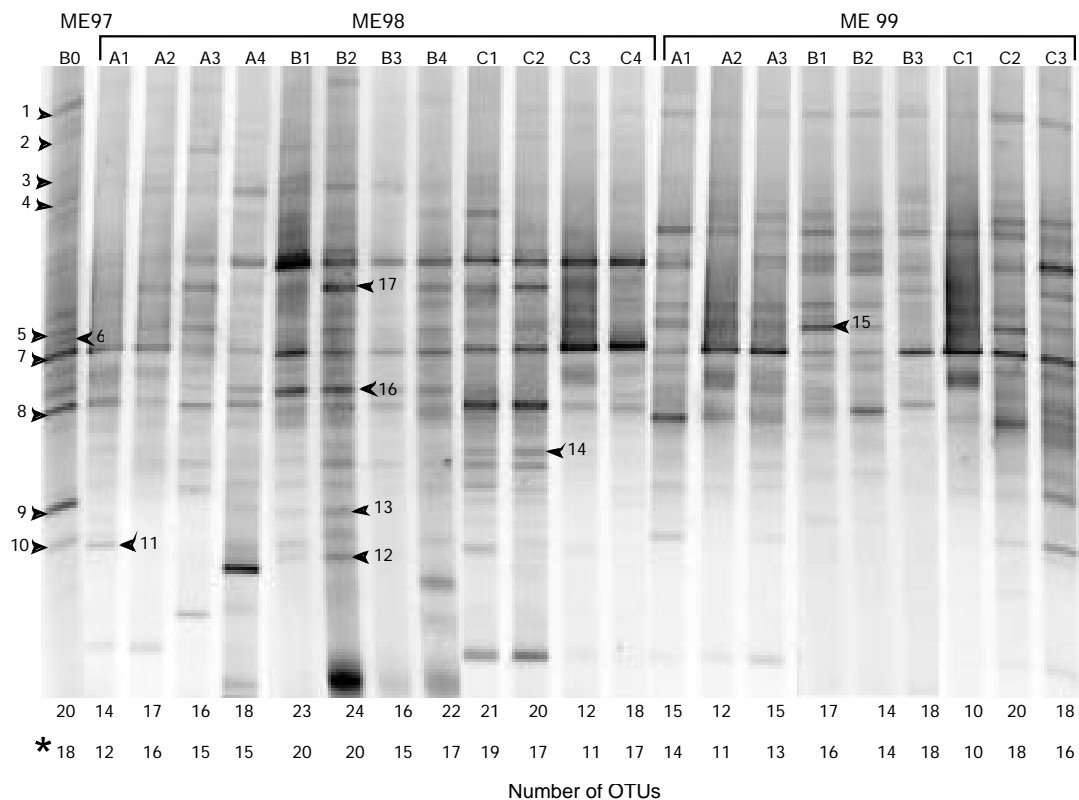


FIG. 6. Negative image of a DGGE gel showing fingerprints of the picoeukaryotic assemblage of all ME97, ME98 and ME99 stations at surface. Bands that were sequenced are indicated by a number that corresponds to numbers in Table 4. The total number of OTUs in each sample is showed at bottom of the DGGE gel. The second row with the asterisk indicates the number of OTUs excluding presumed copepod bands.

A dendrogram was constructed with this DGGE data set (Fig. 7). The single 1997 sample and all the 1999 samples clustered together, while all the 1998 samples formed a separate cluster. Thus, the main difference was found between spring (1998) and autumn (1997 and 1999) samples. The clustering pattern of individual stations was different in both seasons. In the 1998 (spring) cruise, each station formed a separate cluster, indicating that the picoeukaryotic composition was somehow different in the three stations studied and with minor changes during the short time-scale studied. The only exception was station C3 which clustered together with all the samples from station A. Additionally, samples from station A appeared to be slightly closer to those from station B than to those from station C. In the 1999 (fall) samples, however, stations A and C clustered together, while station B formed its own cluster.

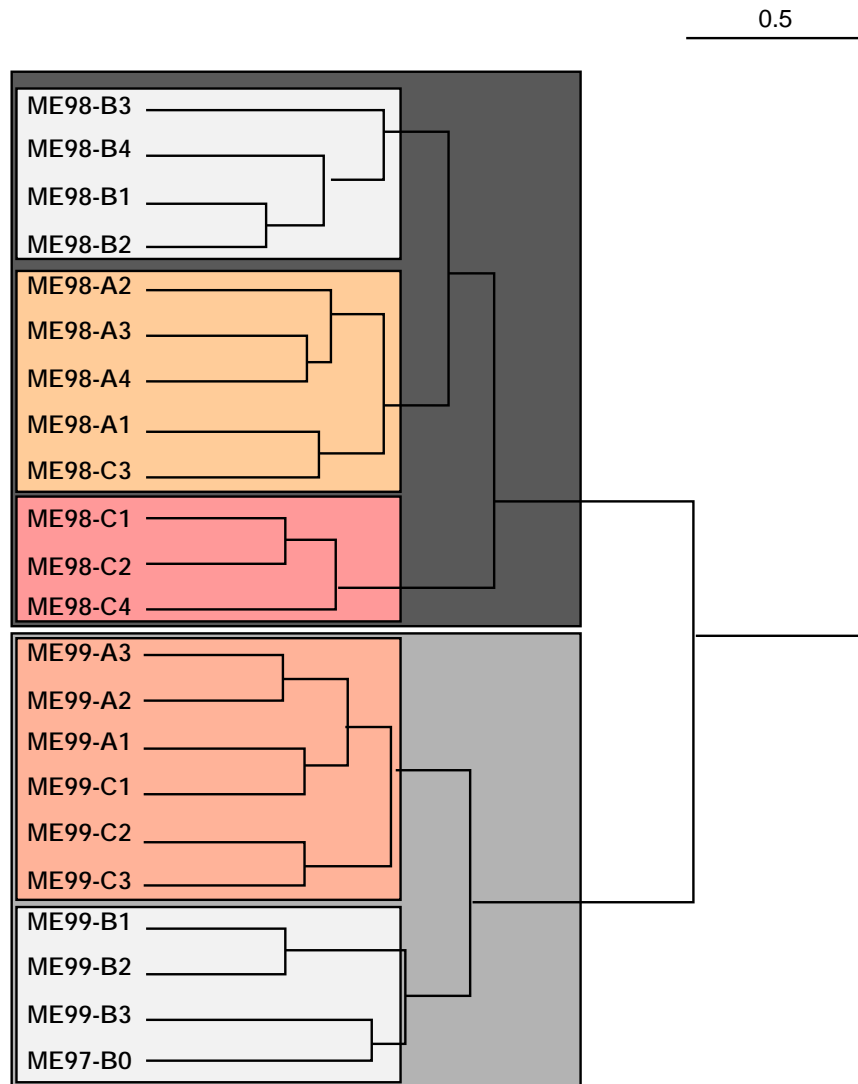


FIG. 7. Dendrogram clustering the samples from all cruises taken into account presence and absence of DGGE bands from each sample profile.

HPLC. Primary pigment data were used to estimate the abundance of different picophytoplankton groups with CHEMTAX. The percent of the chlorophyll *a* <5 $\mu$ m assigned to *Prochlorococcus*, *Synechococcus* and total picoeukaryotes for each sample is shown in Table 3. Data for picoeukaryotes (excluding *Synechococcus* and *Prochlorococcus*) are presented in Fig. 8. The largest component of picophytoplankton according to pigments was *Synechococcus* followed by chlorophyll *b* containing picoeukaryotes. These two groups were abundant in almost all samples. The next group in importance was the prymnesiophytes, which



was present in significant abundance in almost all samples. Cryptophytes were important in 1997 and 1998 but almost completely absent in 1999. Other groups such as diatoms and pelagophytes were present sporadically or low abundance. Finally, dinoflagellates could be detected only in one sample. The dendrogram constructed with HPLC data resembled that obtained by DGGE in that both separated fall and spring samples in two clusters (Fig. 9). However, it was not possible to find clear differences among stations within these two main clusters.

TABLE 3. Participation (in percentages) of the three groups of picophytoplankton on the chlorophyll  $<5 \mu\text{m}$  applying the CHEMTAX approach analysis.

Sample	<i>Prochlorococcus</i>	<i>Synechococcus</i>	Picoeukaryotes
ME97-B0	3	9	89
ME98-A1	1	11	88
ME98-A2	1	3	96
ME98-A3	0	6	94
ME98-A4	0	17	83
ME98-B1	0	0	100
ME98-B2	2	12	86
ME98-B3	0	56	44
ME98-B4	3	37	60
ME98-C1	0	16	84
ME98-C2	0	49	51
ME99-A1	3	97	1
ME99-A2	3	78	18
ME99-A3	5	50	45
ME99-B1	1	66	33
ME99-B2	3	50	48
ME99-B3	3	10	87
ME99-C1	0	26	74
ME99-C2	3	61	36
ME99-C3	5	65	30

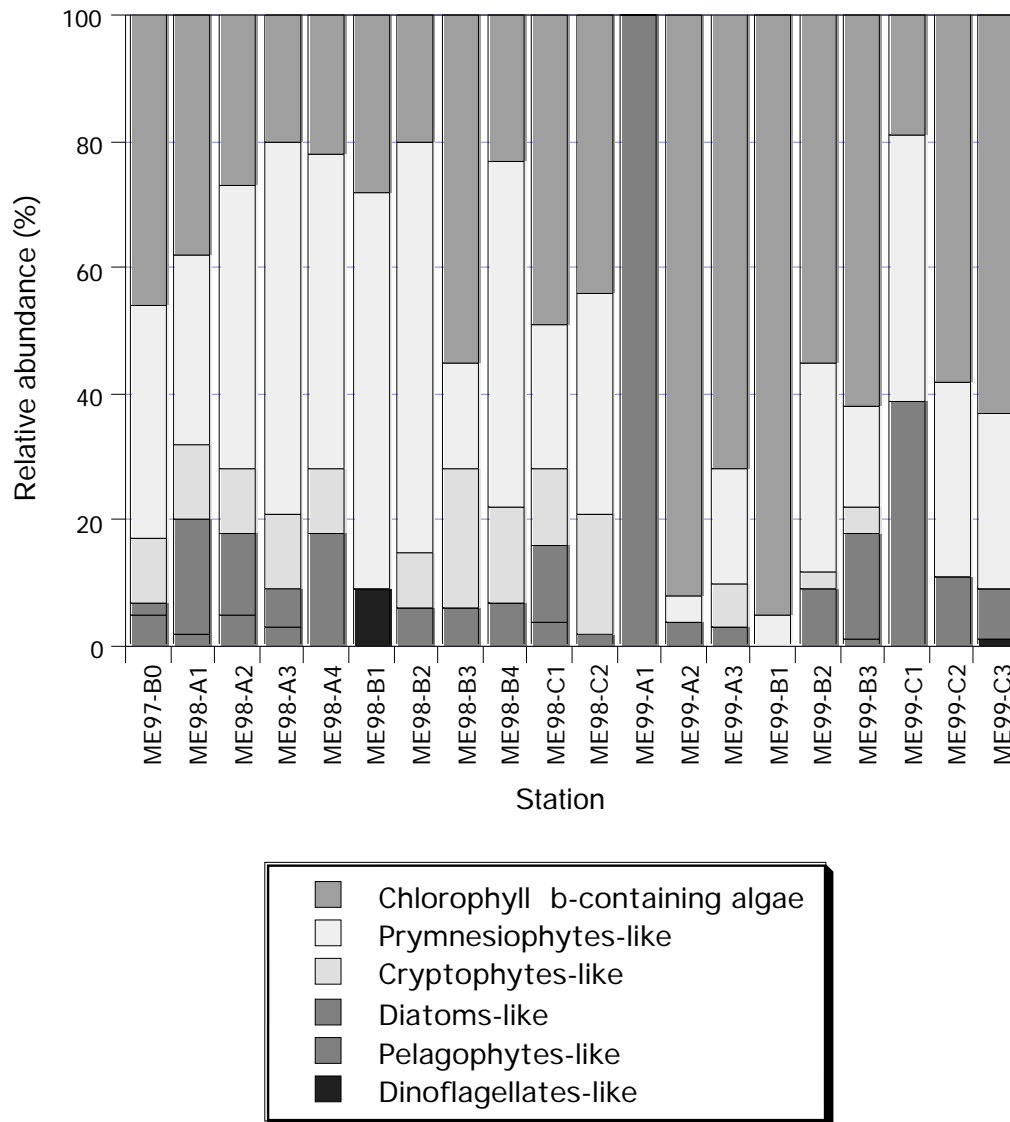


FIG. 8. Contribution of different eukaryotic groups to the picoeukaryotic signal as estimated by CHEMTAX marker pigments analysis.

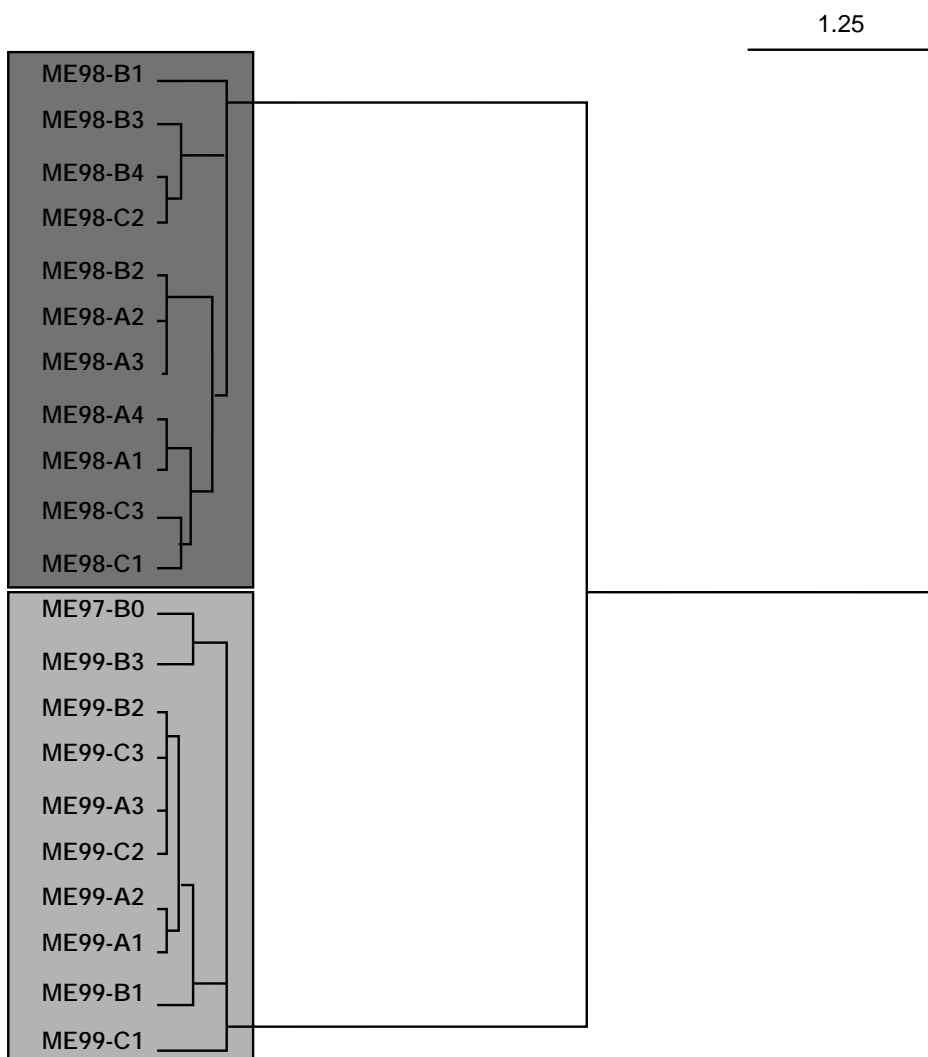


FIG. 9. Dendrogram clustering the samples from all cruises taken into account HPLC pigment analysis matrix generated by the contribution of all marker pigments present at each sample.

**Identity of DGGE bands.** Several bands from the picoeukaryotic DGGE gel (bands 1 to 17 in Fig. 6) were cut and sequenced. Most bands appearing at the bottom of the gel belonged to organisms that are clearly not picoplanktonic, such as the appendicularian *Oikopleura sp.* (band 9) and copepods (bands 10, 12 and 13). Obviously, these bands must correspond to broken cells from these large organisms. Most sequences, however, corresponded to organisms belonging to picoplankton (Table 4).

TABLE 4. Sequence similarities of excised eukaryotic bands that appear in Fig. 6. Bands 1 to 11 correspond to bands shown in Table 2 in Chapter 3 but incorporating more recent BLAST results. The numbers in parentheses are the numbers of bases used to calculate the levels of sequence similarity.

Band	Most closely related organism	% similarity (n° of bases)	Taxonomic group
1	Eukaryote clone OLI11066	98.3 (478)	Novel stramenopile
2	Eukaryote clone OLI11066	94.3 (406)	Novel stramenopile
3	Eukaryote clone OLI11066	92.5 (269)	Novel stramenopile
4	<i>Thraustochytrium multirudimentale</i>	92.2 (414)	Novel stramenopile
5	Unidentified prymnesiophyte	88.6 (88)	Prymnesiophyte
6	Eukaryote clone OLI11011	83.2 (281)	Novel alveolate
7	<i>Mantoniella squamata</i>	96.2 (265)	Prasinophyte
8	<i>Ostreococcus tauri</i>	89.7 (401)	Prasinophyte
9	<i>Oikopleura</i> sp.	97.8 (422)	Appendicularian
10	<i>Clausocalanus</i> sp.	99.3 (489)	Copepoda
11	<i>Geminigera cryophila</i>	90.4 (284)	Cryptophyte
12	<i>Calanus</i> sp.	86.2 (160)	Copepoda
13	<i>Calanus</i> sp.	85.3 (177)	Copepoda
14	Eukaryote clone OLI11511	78.0 (173)	Novel alveolate
15	Eukaryote clone OLI11023	95.0 (364)	Novel alveolate
16	Eukaryote clone OLI11011	96.6 (421)	Novel alveolate
17	Eukaryote clone OLI11006	93.4 (273)	Novel stramenopile

Prasinophytes were present with two bands corresponding to *Mantoniella squamata* and *Ostreococcus tauri*. Band 7 showed a relatively high similarity to *Mantoniella squamata* (96%) and was present in all samples accounting for a relatively large percent of the total band intensity in each lane (between 7 and 35%). The band corresponding to *Ostreococcus* (band 8) was also present in many samples, although in lower relative abundance. Only one band (band 5) was identified as a prymnesiophyte. The short sequence obtained prevented a more accurate identification. Likewise, only one band could be assigned to cryptophytes (band 11). Both bands accounted for a relatively small percent of the total band intensity.

All the remaining bands corresponded to recently discovered groups of picoeukaryotes: the novel stramenopiles (bands 1 to 4 and 17) and the novel alveolates (bands 6 and 14 to 16). Although individually neither of these bands was especially intense, their number and their ubiquitous presence made them important contributors to the eukaryotic picoplankton in the area. Thus, bands belonging to novel stramenopiles made around 20% of the total band

intensity in stations A and B and a lower percentage in station C in 1998. In 1999 they accounted for 5 to 18% in all stations. The novel alveolates, in turn, accounted for 5 to 20% of the total band intensity.

## Discussion

Numerous studies have shown that picoplankton phototrophic organisms are abundant in the surface waters of both freshwater lakes and the sea, with numbers of organisms commonly around  $10^4$  cells  $\text{ml}^{-1}$  for *Synechococcus* (Stockner 1988, Caron et al. 1999),  $10^3$  cells  $\text{ml}^{-1}$  for picoeukaryotes and up to  $10^5$  cell  $\text{ml}^{-1}$  for *Prochlorococcus* (Campbell and Vaultot 1993). In this study phototrophic picoplankton represented a very important fraction of the total chlorophyll in samples taken during three consecutive years. The mean contribution of picoplankton, considered here as the fraction of organisms smaller than  $5 \mu\text{m}$ , to chlorophyll *a* was 60%.

It has been proposed that phototrophic picoplankton forms a more or less constant background of biomass and activity throughout the oceans, while larger phytoplankton appears only under particular conditions where extra nutrients are supplied such as in upwelling areas. Therefore, picoplankton would be the predominant phytoplankton in oligotrophic areas, whereas in eutrophic areas picophytoplankton would still be equally abundant, but their relative contribution would be minimized due to a large biomass of large phytoplankton (Legendre and Le Fèvre 1995). Also, Rodríguez et al. (1998) reported that the biovolume of picoplankton from the deep fluorescence maximum in the Alborán Sea area was fairly constant, while that of larger phytoplankton changed significantly in different stations, increasing in cyclonic gyres and upwelling areas. In other studies, however, phototrophic picoplankton cell numbers have been shown to increase by an order of magnitude between oligotrophic and moderately eutrophic waters (El Hag and Fogg 1986). García (1994) found by flow cytometry, in a frontal station in the same Alborán Sea area, that *Synechococcus* was the dominant group of picoplankton at the surface (around  $10^4$  cell  $\text{ml}^{-1}$ ), *Prochlorococcus* was one order of magnitude less abundant and eukaryotic picoplankton showed an intermediate abundance. Our data showed very similar levels and distributions of picophytoplankton groups in 1998, but a very different pattern in 1999 (Table 2).

In the present study, total cell numbers of phototrophic picoplankton changed by two orders of magnitude. Even within a single cruise, differences reached a factor of 36. And, moreover, total cell number increased by a factor of 10 in just six days at station C during Spring 1998. This variability affected all three components measurable by flow cytometry (Table 2).

Therefore, at least at the surface, the abundance of phototrophic picoplankton was quite variable. In fact, it was as variable as the larger phytoplankton. In effect, Arin et al. (in preparation) determined the biovolume of nano- and microphytoplankton during the 1998 cruise in the same stations. These authors found changes by a factor of two for nanoflagellates and coccolithophorids, a factor of 10 for dinoflagellates and a factor of 18 for diatoms. Therefore, the range of variation in abundance of picophytoplankton was comparable to that of diatoms.

While flow cytometry provides accurate counts of phototrophic picoplankton, it can only discriminate three groups within this assemblage: the two prokaryotic phototrophs and picoeukaryotes. Determination of pigments through HPLC also allows to estimate the relative contribution of these groups to total chlorophyll. To a certain degree, changes in the abundance of pigments can be due to light adaptation of the organisms and not to changes in cell abundance. However, under natural conditions, pigment specific content changes by a factor much lower than the order of magnitude changes of cell abundance and pigment concentration is considered to be a reasonable estimate of biomass. Therefore, the estimates of abundance of *Synechococcus*, *Prochlorococcus* and picoeukaryotes by flow cytometry and HPLC should covary. This is actually what happened with our data set, when cell number from cytometry was compared with chlorophyll *a* concentration accounted for by each one of the three groups. The covariation was good for *Synechococcus* ( $r^2=0.473$ ), reasonable for picoeukaryotes ( $r^2= 0.359$ ) and poor for *Prochlorococcus* ( $r^2= 0.151$ ).

Determination of pigments through HPLC allows separation of the picoeukaryotic group into at least class level taxons. Thus, one can ask the question of whether this group of picoeukaryotes varies in taxonomical composition besides changing in total abundance. As can be seen in Table 3, the chlorophyll *a* corresponding to picoeukaryotes could be assigned to several taxonomic groups. Chlorophyll b-containing algae and prymnesiophytes were the two most important groups followed by cryptophytes, diatoms and pelagophytes. Dinoflagellates were essentially absent from the picoplankton. Pigment data can also be used as a fingerprint of the phytoplankton of the samples and a dendrogram can be build to compare the overall similarity of the samples. When this was done, the phototrophic picoplankton assemblages were clearly different in spring and fall. But within each season, no clear patterns could be discerned.

Pigment determination via HPLC is a powerful approach to characterize small phytoplankton. However, it also has some shortcomings. The main one is that the contribution of each group to total chlorophyll is calculated from the known pigment composition of some algal cultures. As new cultures are analyzed and as new microorganisms are discovered, the pigment ratios considered characteristic of each algal group necessarily change. Therefore, application of

the pigment ratios from known cultures to natural samples of unknown composition needs to be done with caution. And, obviously, the technique is not appropriate to study heterotrophic organisms, since they usually lack any pigments.

Molecular techniques need to be used in order to study all the components of the eukaryotic picoplankton. DGGE has the advantage over other molecular techniques that a large number of samples can be processed. For example, in the present study the numbers of samples processed by DGGE and HPLC was the same. And it also has the advantage, over other fingerprinting techniques, that the bands can be cut and sequenced. If we assume that the nucleic acid extraction and the PCR are not selective, this technique has, thus, the potential to find unknown components of the picoplankton as well as the better known microbes and, furthermore, it can provide a picture of the composition and variability of both autotrophic and the heterotrophic picoplankton. Our main purpose in this paper was to compare the information provided by DGGE to that obtained with the more established techniques such as flow cytometry and HPLC.

Both DGGE and HPLC can be used as fingerprinting techniques to determine the similarity in the composition of picoplankton in different samples. When we built dendrograms, both techniques identified two clusters corresponding to spring and fall respectively. The lower rank clustering however was different. The clusters provided by DGGE were coherent and easily interpreted: the eukaryotic picoplankton assemblage showed more variability among stations than within stations. This is reasonable, since the time span of our sampling was relatively short (around 9 days), while the spatial distance among the stations guaranteed different hydrological conditions, at the very least between the stations A and C. For example, in 1998, station A was experiencing an upwelling event, while station C was more sharply stratified. The number of bands in the DGGE dendrogram was around 20 and, moreover, DGGE included both autotrophic and heterotrophic members of the picoplankton.

A second comparison can be done once the main bands in the DGGE gels are sequenced and identified. We would expect that the autotrophic components of the plankton would show similar relative abundance by HPLC and DGGE. With both approaches the most abundant group was the prasinophytes followed by prymnesiophytes and cryptophytes. The HPLC detected the presence of smaller amounts of diatoms and pelagophytes. Finally, dinoflagellates were only detected in one sample. These latter groups were not detected by DGGE. There are two potential reasons for this discrepancy. First, it must be beared in mind that around 25-50 % of the total band intensity was not identified. Thus, the faint bands that could not be cut and sequenced could belong to the missing groups. And, second, DGGE is known to have a detection limit of about 1% of the total cell count at least for prokaryotes (Casamayor et al.

2000). In the case of eukaryotes, it is likely that the minor algal groups are close to this detection limit, especially since the 1% must be calculated with respect to all the picoeukaryotes (including heterotrophs) and not just the autotrophic ones.

In a previous study (Díez et al. 2001b) we compared results from different molecular techniques for sample ME97-B0. It was shown that cloning and sequencing retrieved the most abundant groups in similar proportions to DGGE. However, cloning retrieved a number of less frequent groups that could not be detected by DGGE. These groups were pelagophytes, diatoms, dinoflagellates and eustigmatophytes. The first three are the same groups identified by HPLC. The presence of the fourth group, the eustigmatophytes cannot be computed by HPLC, because the pigment ratios of this group are not different enough from those of other algae. Therefore, both DNA based and pigment based techniques retrieved the same groups in similar relative amounts. There was no bias against, or in favor of, any of the major groups of autotrophic microorganisms. The conclusion is that DGGE is able to retrieve the most abundant groups but it will miss the less abundant ones. The fact that cloning retrieved these minor groups, where DNA extraction and PCR are also used, suggests that the problem was not due to these steps. We think the problem is that groups found in low abundance produce very faint bands and many times these are difficult to discern from the gel background or, if visible, they contain too little DNA for sequencing.

Finally, DGGE revealed the presence of further groups of microorganisms not detected by HPLC. These were the recently discovered novel stramenopiles and alveolates. In principle, the primers used for the DGGE should not discriminate between autotrophic and heterotrophic eukaryotes. Since no other heterotrophic microorganisms were detected, it seems reasonable to conclude that these two groups of organisms are heterotrophic. This conclusion was reinforced in the case of sample ME97-B0 by the results of the cloning approach mentioned above. In this library 12 clones of novel stramenopiles and 12 of novel alveolates were retrieved. On the other hand only one clone of *Cercomonas* and 3 of crysophytes could correspond to heterotrophic flagellates.

In summary, we have shown that DGGE can be used as an excellent fingerprinting technique for the picoplankton. The number of samples that can be processed and the discrimination power are adequate to address questions of distribution and variability of the smallest eukaryotes in the ocean. The technique is not appropriate to identify all the components of the plankton because it misses those found in lower abundance. However, it allows identification of novel groups, and this is something impossible with most other techniques. The optimal approach to study the composition and distribution of the small plankton would be to analyze a large number of samples by DGGE to get the large picture. Then representative samples could



be selected for a cloning approach in order to retrieve as many different components of the plankton as possible.

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## DISCUSIÓN GENERAL

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Al menos tres razones fueron las que nos llevaron, a estudiar la fracción eucariótica del picoplancton recurriendo para ello a los métodos moleculares:

- son los organismos eucariotas más abundantes del planeta
- son un componente esencial de las cadenas tróficas marina
- su diversidad y distribución son poco conocidas debido por un lado, a que sólo un pequeño porcentaje de ellos puede crecer en cultivo y por otro, a su similar morfología y pequeño tamaño, que hacen difícil su identificación mediante métodos clásicos.

### Aspectos metodológicos

Para llevar a cabo el estudio del picoplancton eucariótico es necesario contar con una técnica optimizada de recuperación de su biomasa. La utilización del fraccionamiento de tamaño por filtración fue la técnica elegida para la recuperación de todos los organismos del picoplancton presentes en una muestra natural. La eficacia de dicha filtración fraccionada es imprescindible para la consecución de las siguientes etapas del análisis. En el **Capítulo 2** de este trabajo se describe detalladamente la metodología llevada a cabo. Aunque estrictamente hablando, el picoplancton está definido como la fracción más pequeña de 2  $\mu\text{m}$  (Sieburth 1978), nosotros decidimos ampliar este rango hasta 5  $\mu\text{m}$  en muchos de los estudios que se presentan en este trabajo, debido a que es a partir de este tamaño cuando se empieza a tener verdaderas dificultades en la identificación de las células mediante la metodología clásica. Así, los diferentes materiales de filtro y tamaños de poro que fueron utilizados (véase por ejemplo **Cap. 2, Tabla 1**) mostraron siempre una diferencia considerable entre las dos fracciones separadas por el filtro: la fracción que atraviesa el filtro y la que es retenida por el mismo (**Cap. 2, Fig. 1**). Se consiguió excluir así la mayoría de las células eucariotas de mayor tamaño, recuperando la mayoría de los piceucariontes. Pero la filtración no es un proceso completamente eficaz. Siempre que se realiza con un sistema de filtración con bombas peristálticas, incluso a bajas presiones como fueron las usadas para la concentración de las muestras en nuestros estudios, constituye una perturbación para los

organismos presentes en la muestra, por lo que es probable que se rompan algunas células en el momento de atravesar el filtro. Este problema hace referencia sobre todo a varias secuencias obtenidas tanto a partir de bibliotecas genéticas como de bandas de DGGE, afiliadas a organismos que no se pueden considerar parte del picoplancton eucariótico. Estos organismos en realidad son de un tamaño superior, tal como la apendicularia *Oikopleura* sp. a la que se hace referencia sobre todo en el **Capítulo 2**, así como copépodos presentes en varias de las muestras que aparecen en los diferentes capítulos de este trabajo.

### **¿Cómo estudiar la diversidad de los picoeucariontes marinos?**

Las recientes técnicas de biología molecular, generalmente desarrolladas y aplicadas para bacterias, pueden ser muy útiles también para el estudio de la diversidad del picoplancton eucariótico. A lo largo de los diferentes estudios recogidos en esta memoria se han descrito detalladamente algunas de ellas. Primeramente se llevó a cabo un análisis de la diversidad genética de los picoeucariontes marinos mediante la secuenciación de clones del gen 18S rRNA obtenidos a partir de cinco bibliotecas genéticas de aguas superficiales del Atlántico Norte, del Océano Glacial Antártico y del Mar Mediterráneo (véase el **Cap. 2**). En general, la diversidad filogenética en cada biblioteca fue bastante elevada, incluyendo muchas unidades taxonómicas diferentes y miembros de grupos filogenéticos distantes (**Cap. 2, Tabla 4 y Fig. 3**). La mayoría de estos grupos taxonómicos se afiliaba a clases de algas, especialmente prasinofíceas, primnesiofíceas, diatomeas y dinoflagelados. Una fracción menor de clones pudo afiliarse a grupos claramente heterotróficos tales como ciliados, algunas crisofíceas, cercozoales y hongos. Finalmente, aparecieron dos nuevos linajes relativamente abundantes: los nuevos estramenópilos y los nuevos alveolados. Estos linajes son muy diferentes de cualquier organismo aislado e indican la existencia de picoeucariontes previamente desconocidos. Este estudio aportó información valiosa acerca de la diversidad y distribución de los picoeucariontes a lo largo de los diferentes sistemas marinos seleccionados y estudiados. Se demostró que numerosas clases de algas contienen representantes de talla picoplanctónica y, por otro lado, se comprobó hasta que punto los océanos constituyen un verdadero reservorio de taxones desconocidos. Algunos de ellos podrían constituir una fracción importante de los microorganismos heterotróficos y jugar así un papel crucial en la red trófica microbiana. Hasta el momento se han descrito muy pocas especies heterotróficas de talla picoplanctónica, lo cual resalta la importancia potencial de las descubiertas mediante la clonación y secuenciación de genes naturales.

A partir de los resultados obtenidos en el **Capítulo 2**, se plantearon otros estudios más amplios acerca de la diversidad y la biogeografía de este grupo de organismos. Estos estudios pudieron ser abordados a partir de la puesta a punto y utilización de técnicas de "fingerprinting" principalmente usadas y optimizadas con otros grupos de seres vivos, sobre todo los procariontes, tales como la DGGE (denaturing gradient gel electrophoresis) y la T-RFLP (terminal-restriction fragment length polymorphism) (véase **Cap. 3**). Estas técnicas permitieron un estudio más rápido y simultáneo del picoplancton eucariótico en un elevado número de muestras obtenidas a partir del medio natural.

### **Optimización de la DGGE para el estudio de las comunidades picoeucarióticas**

Para poder analizar los cambios en las comunidades de picoeucariontes en el mar a lo largo de gradientes, ya sea temporales o espaciales, se hace necesario procesar un número de muestras demasiado elevado como para usar técnicas laboriosas tales como la clonación. En este sentido, nos decidimos por llevar a cabo la optimización y uso de la DGGE. Esta técnica de "fingerprinting" es una de las más utilizadas con buenos resultados en otros grupos de organismos tales como los procariontes

Otra de las ventajas de la DGGE es que posee cierta capacidad filogenética para informar de la composición en especies de una comunidad natural. De este modo es posible hacer un seguimiento, tanto cualitativo como cuantitativo, de estas comunidades como se demuestra en los **Capítulos 4 y 5**. En cualquier caso, a partir del patrón de bandas obtenido en un gel de DGGE, es posible también elegir una o varias de las muestras para su estudio más detallado con otra técnica más resolutive/robusta pero más laboriosa como pueda ser la clonación.

En el **Capítulo 3** de esta tesis se presenta la optimización y aplicación de la DGGE para el estudio del picoplancton eucariótico. Se exploró la utilización de dos parejas de cebadores específicos para eucariontes que amplificaban diferentes regiones en el gen 18S rRNA. Ambas parejas de cebadores amplificaron una sola banda cuando se utilizaron con DNA procedente de cultivos puros de algas (**Cap. 3, Figs. 1 y 2**), y un patrón de bandas complejo cuando amplificaban DNA de comunidades naturales (**Cap. 3, Figs. 1 y 3**). La reproducibilidad de los patrones de bandas fue estimada mediante la cuantificación de la intensidad de las bandas obtenidas a partir de PCRs y de carreras de DGGE independientes

(Cap. 3, Fig. 4), siendo el error estimado menor de un 2%. Se escogió una muestra del Mar Mediterráneo como ejemplo de muestra natural, principalmente porque disponíamos de información complementaria proporcionada por otras técnicas moleculares. Mediante el corte y la secuenciación de bandas del gel de DGGE se obtuvo una estimación de la composición filogenética de picoeucariontes presentes en esta muestra. Los resultados obtenidos mediante el análisis de la biblioteca genética y el "fingerprinting" por T-RFLP a partir de la misma muestra, permitieron comprobar los resultados obtenidos a partir de la DGGE. Los tres métodos basados en PCR, con tres parejas de cebadores diferentes, revelaron una composición de las comunidades muy similar, con la presencia de los mismos grupos filogenéticos (Cap. 3, Tabla 3). Estos resultados mostraron por un lado, la excelente reproducibilidad de esta técnica y, por otro, la gran coincidencia no sólo cualitativa sino cuantitativa entre la misma y el resto de técnicas con las que fue comparada.

En otras ocasiones, como en los Capítulos 4 y 5 donde también se utilizó la DGGE junto con otras técnicas, el objetivo de la utilización simultánea de diferentes técnicas fue el de obtener una información complementaria que ayudara a caracterizar un grupo tan desconocido como es el que se trata en este trabajo.

## **Variabilidad del picoplancton eucariótico**

Una vez cumplido el objetivo inicial de obtener información acerca de la identidad y abundancia relativa de los organismos eucariotas del picoplancton (Cap. 2), y de haber optimizado una técnica de "fingerprinting" como la DGGE que nos ofrecía la posibilidad de procesar muchas muestras a la vez (Cap. 3), el siguiente paso consistió en analizar la distribución de los eucariontes picoplanctónicos en diferentes muestras marinas.

Los estudios de la variabilidad temporal y espacial del picoplancton eucariótico se presentan en los Capítulos 4 y 5 de este trabajo. En el momento de iniciar este trabajo la distribución y variabilidad del picoplancton eucariótico era básicamente desconocida. Solamente se disponía de algunos datos de HPLC en muestras fraccionadas, pero estos datos solamente son relevantes para los componentes fototróficos y, además, solamente proporcionan información sobre la distribución de grandes grupos de organismos. El picoplancton eucariótico podía variar muy poco entre distintos lugares del océano como ocurre con los procariotas fototróficos, o variar tanto o más que el fitoplancton de mayor tamaño.

En el **Capítulo 4** dos técnicas de “fingerprinting” (la DGGE y la T-RFLP) permitieron describir y ratificar la variabilidad de los picoeucariontes a lo largo de dos transectos latitudinales en aguas Antárticas. Las dos técnicas utilizadas mostraron que las muestras generalmente se agrupaban siguiendo el gradiente marcado por las diferentes zonas hidrográficas del área de muestreo. *Phaeocystis* y *Mantoniella* fueron dos de los organismos dominantes identificados por DGGE y por T-RFLP. De nuevo aparecieron organismos relacionados con los nuevos estramenópilos y alveolados abundantemente representados en ambos transectos. En DOVETAIL aparecieron sobre todo estramenópilos, lo que puede apuntar pruebas de su pequeño tamaño, ya que las muestras tomadas en este transecto correspondían a la fracción desde 0.2 a tan solo 1.6  $\mu\text{m}$  de tamaño de poro. En DHARMA ambos grupos fueron muy abundantes. Hay que destacar que en DHARMA se prefiltró por 5  $\mu\text{m}$ , y por lo tanto las diferentes abundancias que aparecen entre los organismos en ambos transectos estudiados podrían deberse al tamaño de estos organismos entre 1.6 y 5  $\mu\text{m}$  y no a factores ecológicos.

En el **Capítulo 5** de nuevo se presenta una combinación de diferentes técnicas. En este caso una técnica dependiente de PCR, la DGGE, otra basada en el análisis pigmentario del picofitoplancton mediante HPLC y la última basada en el recuento de células del picofitoplancton mediante citometría de flujo. Este estudio nos permitió hacer un seguimiento no sólo del picoplancton eucariótico, sino de todas las poblaciones del picoplancton en un área enmarcada dentro del Mar de Alborán. El estudio se llevó a cabo a través no sólo de un gradiente espacial sino también temporal ya que se muestreó en tres años consecutivos. Debido a la episódica naturaleza de las fuerzas físicas que actúan en el área, la posición de las masas de agua puede fluctuar en pequeñas escalas de tiempo, convirtiendo a esta zona en un verdadero laboratorio natural para analizar la variabilidad espacial de las comunidades de picoeucariontes y de éstas respecto a las demás poblaciones del picoplancton.

En este trabajo partíamos del conocimiento de que bajo estas condiciones hidrográficas se producía una alta variabilidad espacial en la composición del fitoplancton de talla mayor, mientras que su influencia en la distribución del picoplancton era desconocida. Los resultados de DGGE, HPLC y citometría de flujo mostraron que aunque la biomasa total del picoplancton permanece relativamente constante a lo largo de las condiciones muestreadas durante tres campañas en el Mar de Alborán, aparecen diferencias significativas entre las diferentes poblaciones picoplanctónicas tales como *Prochlorococcus*, *Synechococcus* y picoeucariontes. La variabilidad espacial entre las estaciones y temporal entre transectos dentro de una misma campaña mostró diferentes patrones en estas poblaciones picoplanctónicas. Así, por ejemplo, el análisis de dendrogramas construidos a partir de los

datos obtenidos con DGGE, reveló que las muestras pertenecientes al muestreo de primavera en 1998 se agrupaban siguiendo un gradiente costa - mar abierto, mientras que las de otoño de 1999 no siguieron este patrón. La influencia de la fracción heterotrófica del picoplancton parece ser una de las causas de las diferencias encontradas entre la técnica de la DGGE, en la que se analiza tanto la fracción fototrófica como la heterotrófica y las técnicas HPLC y citometría de flujo, las cuales sólo analizan la fracción fototrófica del picoplancton.

Tanto por DGGE como por HPLC se comprobó la importancia de los organismos portadores de clorofila b asociada a algas verdes, tales como las prasinofíceas, en el Mar de Alborán. Otros grupos tales como las primnesiofíceas (principalmente por HPLC) y en menor medida criptofíceas y pelagofíceas fueron también detectados por ambas técnicas. De nuevo los nuevos estramenópilos y alveolados aparecieron como grupos relativamente abundantes en la fracción de piceoeucariontes.

A lo largo de los diferentes capítulos de este trabajo se ha conseguido abordar y responder algunas de las preguntas acerca del picoplancton eucariótico marino que estaban sin resolver y se ha llegado a las siguientes conclusiones.



## CONCLUSIONES

## CONCLUSIONES

1. El estudio de la diversidad del picoplancton eucariótico mediante clonación y secuenciación en diferentes ambientes marinos reveló una elevada diversidad filogenética, incluyendo miembros de grupos muy distantes. Los clones de prasinofíceas y de nuevos estramenópilos fueron los más abundantes en todas las bibliotecas genéticas analizadas.
2. El análisis filogenético de picoeucariontes marinos ha revelado la existencia de nuevos linajes de estramenópilos y alveolados que no habrían sido anteriormente descritos. La diversidad genética dentro de estos linajes es elevada y algunos de sus clones tienen una amplia distribución geográfica. Se sugiere que especialmente los nuevos estramenópilos podrían ser heterotróficos de acuerdo a su posición en el árbol filogenético.
3. Se puso a punto la técnica DGGE (Denaturing Gradient Gel Electrophoresis) para picoeucariontes. Esta técnica permitió estudiar la variabilidad y la composición filogenética de las comunidades de picoeucariontes marinos e identificar algunos de sus miembros dominantes.
4. Se puso a punto la técnica T-RFLP (Terminal-Restriction Fragment Length Polymorphism) para picoeucariontes. Con esta técnica se obtenían "fingerprintings" de alta resolución muy útiles para comparar comunidades. Sin embargo, y a diferencia de la DGGE, la identificación de los miembros de la comunidad sólo era posible teniendo en cuenta estudios previos.
5. La aplicación simultánea en la misma muestra natural de tres métodos basados en PCR (clonación, DGGE y T-RFLP) con parejas de cebadores diferentes, revelaron una composición de la comunidad de picoeucariontes muy similar, con los mismos grupos filogenéticos. Las tres técnicas detectaron la presencia de nuevos estramenópilos y alveolados.

6. El estudio de la diversidad del picoplancton eucariótico mediante DGGE y T-RFLP en dos transectos latitudinales en la Antártida demostró cambios en la distribución de poblaciones de picoeucariontes en relación con las condiciones hidrográficas de la zona. Los grupos dominantes fueron de nuevo prasinofíceas, primnesiofíceas, y nuevos estramenópilos y alveolados.

7. La variabilidad temporal y espacial en la comunidad picoplanctónica del Mar de Alborán, estudiado por DGGE, HPLC y citometría de flujo, fue muy alta. La composición y distribución de *Prochlorococcus*, *Synechococcus* y picoeucariontes mostraron una clara dependencia de las condiciones hidrográficas del área estudiada, no respondiendo por igual a las mismas. Las técnicas DGGE y HPLC coincidieron en presentar las prasinofíceas como uno de los principales componentes del picoplancton eucariótico autotrófico. La fracción heterotrófica de picoeucariontes parece ser la causante de las diferencias encontradas entre DGGE y las otras dos técnicas utilizadas en el estudio.

8. Los cambios en la composición del picoplancton eucariótico en un perfil vertical a lo largo de la columna de agua fueron siempre muy marcados, mucho más que los observados siguiendo gradientes horizontales de decenas de kilómetros. En general, la composición dentro de la capa superficial de mezcla fue idéntica, observándose los primeros cambios a partir de la misma y aumentando a medida que nos acercamos a las capas más profundas.

9. En general y a partir de todas las técnicas moleculares utilizadas en este estudio, los grupos dominantes en cuanto a abundancia y representatividad a lo largo de los diferentes sistemas marinos y gradientes estudiados fueron las prasinofíceas (claramente fototróficos), los nuevos estramenópilos (probablemente heterotróficos), y los nuevos alveolados (probablemente con representantes de ambos modos tróficos).

## BIBLIOGRAFÍA

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- Acinas, S. G., J. Antón, and F. Rodríguez-Valera. 1999. Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **65**:514-522.
- Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389-3402.
- Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56**:1919-1925.
- Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**:143-169.
- Andersen, R. A., G. W. Saunders, M. P. Paskind, and J. P. Sexton. 1993. Ultrastructure and 18S rRNA gene sequence for *Pelagomonas calceolata* gen. et sp. nov. and the description of a new algal class, the Pelagophyceae classis nov. *J. Phycol.* **29**:701-715.
- Andersen, R. A., R. R. Bidigare, M. D. Keller, and M. Latasa. 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.
- Andreoli, C., N. Rascio, and G. Casadoro. 1978. *Chlorella nana* sp. nov. (Chlorophyceae): a new marine *Chlorella*. *Bot. Mar.* **21**:235-256.
- Arin, L., and M. Estrada. Influence of mesoscale hydrodynamical processes on the size-distribution of phytoplankton in the Alboran Sea. In prep.
- Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad. 1983. The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.* **10**:257-263.
- Bhattacharya, D., L. Medlin, P. O. Wainright, E. V. Ariztia, C. Bibeau, S. K. Stickel, and M. L. Sogin. 1992. Algae containing chlorophylls a + c are paraphyletic: molecular evolutionary analysis of the chromophyta. *Evolution.* **46**:1801-1817.

**Bidigare, R. R., and M. E. Ondrusek.** 1996. Spatial and temporal variability of phytoplankton pigment distributions in the central equatorial Pacific Ocean. *Deep-Sea Research II*. **43**:809-833.

**Binder, B. J., S. W. Chisholm, R. J. Olson, S. L. Frankel, and A. Z. Worden.** 1996. Dynamics of picophytoplankton, ultraplankton and bacteria in the central equatorial Pacific. *Deep-Sea Research II*. **43**:907-931.

**Booth, B. C., and H. J. Marchant.** 1987. Parmales, a new order of marine Chrysophytes, with descriptions of three genera and seven species. *J. Phycol.* **23**:245-260.

**Britschgi, T. B., and S. J. Giovannoni.** 1991. Population analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. *Appl. Environ. Microbiol.* **57**:1707-1713.

**Butcher, R. W.** 1952. Contribution to our knowledge of the smaller marine algae. *J. Mar. Biol. Assoc. U. K.* **31**:175-191.

**Button, D. K., R. B. Robertson, W. P. Leep, and T. M. Scmiat.** 1998. A small, dilute cytoplasm, high-affinity, novel bacterium isolated by extinction culture and having kinetic constants compatible with growth at ambient concentrations of dissolved nutrients in seawater. *Appl. Environ. Microbiol.* **64**:4467-4476.

**Campbell, L., and D. Vaulot.** 1993. Photosynthetic picoplankton community structure in the subtropical North Pacific Ocean near Hawaii (station ALOHA). *Deep-Sea Research*. **40**:2043-2060.

**Caron, D. A., E. R. Peele, E. L. Lim, and M. R. Dennett.** 1999. Picoplankton and nanoplankton and their trophic coupling in the surface waters of the Sargasso Sea south of Bermuda. *Limnol. Oceanogr.* **44**:259-272.

**Casamayor, E. O., H. Schäfer, L. Bañeras, C. Pedrós-Alió, and G. Muyzer.** 2000. Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: a comparison of microscopy and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **66**:499-508.

**Cavalier-Smith, T.** 1985. Eukaryote gene numbers, non-coding DNA and genome size, p. 69-103. *In* T. Cavalier-Smith (ed.), *The Evolution of Genome Size*. Wiley. Chichester.

- Clement, B. G., L. E. Kehl, K. L. DeBord, and C. L. Kitts. 1998. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *J. Microbiol. Methods*. 31:135-142.
- Courties, C., A. Vaquer, M. Trousselier, J. Lautier, M.-J. Chrétiennot-Dinet, J. Neveux, C. Machado, and H. Claustre. 1994. Smallest eukaryotic organism. *Nature* 370: 255.
- Chavez, F. P. 1989. Size distribution of phytoplankton in the central and eastern tropical Pacific. *Global Biogeochemical Cycles*. 3:27-35.
- Chisholm, S. W. 1992. Phytoplankton size, p. 213-236. *In* P.G. Falkowski, and A. D. Woodhead (ed.), *Primary productivity and biogeochemical cycles in the sea*. Plenum Press, New York.
- Chisholm, S. W., R. J. Olson, E. R. Zettler, R. Goericke, J. B. Waterbury, and N. A. Welschmeyer. 1988. A novel free-living prochlorophyte abundant in the oceanic euphotic zone. *Nature* 334:340-343.
- Chisholm, S. W., S. L. Frankel, R. Goericke, R. J. Olson, B. Palenik, J. B. Waterbury, L. West-Johnsrud, and E. R. Zettler. 1992. *Prochlorococcus marinus* nov. gen. nov. sp.: an oxyphototrophic marine prokaryote containing chlorophyll a and b. *Arch. Microbiol.* 157:297-300.
- Cros, L., A. Kleijne, A. Zeltner, C. Billard, and J. R. Young. 2000. New examples of holococcolith-heterococcolith combination coccospheres and their implications for coccolithophorids biology. *Marine micropaleontology*. 39:1-34.
- Dasikachary, T. V. 1972. *M. squamata* (Manton & Park) Dasikachary, 1972. Dasikachary, T. V. 1972. Notes on Volvocales. I. *Curr. Sci.* 41:445-447.
- De Yoe H. R., D. A. Stockwell, R. R. Bidigare, M. Latasa, P. W. Johnson, P. E. Hargraves, and C. A. Suttle. 1997. Description and characterization of the algal species *Aureoumbra lagunensis* gen. et sp. nov. and referral of *Aureoumbra* and *Aureococcus* to the Pelagophyceae. *J. Phycol.* 33:1042-1048.
- DeLong, E. F. 1992. Archaea in coastal marine environments. *Proc. Natl. Acad. Sci. USA* 89:5685-5689.

DeLong, E. F., T. M. Schmidt, and N. R. Pace. 1989. Analysis of single cells and oligotrophic picoplankton populations using 16S rRNA sequences, p. 697-701. *In* T. Hattori, Y. Ishida, Y. Maruyama, R.Y Morita, and A. Uchida (ed.), Recent Advances in Microbial Ecology. Japan Sci. Soc. Press, Tokyo..

DeLong, E. F., G. S. Wickham, and N. R. Pace. 1989. Phylogenetic stains: Ribosomal RNA-based probes for the identification of single cells. *Nature* 243:1360-1363.

DeLong, E. F., D. G. Franks, and A. L. Alldredge. 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnol. Oceanogr.* 38:924-934.

DeLong, E.F. 1993. Single-cell identification using fluorescently labeled ribosomal RNA-specific probes, p. 285-294. *In* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (ed.), Handbook of Methods in Aquatic Microbial Ecology, Lewis Publishers, Boca Raton. Fla.

DeLong, E. F., K. Y. Wu, B. B. Prézelin, and R. V. M. Jovine. 1994. High abundance of Archaea in Antarctic marine picoplankton. *Nature* 371:695-697.

Díez, B., C. Pedrós-Alió, and R. Massana. 2001a. Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by Small-Subunit rRNA gene cloning and sequencing. *Appl. Environ. Microbiol.* 67:2932-2941.

Díez, B., C. Pedrós-Alió, T. L. Marsh, and R. Massana. 2001b. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. *Appl. Environ. Microbiol.* 67:2942-2951.

Droop, M. R. 1955. Some new supra-littoral protista. *J. Mar. Biol. Ass. U. K.* 34:233-245.

Dsouza, M., N. Larsen, and R. Overbeek. 1997. Searching for patterns in genomic data. *Trends Genet.* 13:497-498.

Eikrem, W., and J. Throndsen. 1990. The ultrastructure of *Bathycoccus* gen. nov. and *B. prasinus* sp. nov., a non-motile picoplanktonic algae (Chlorophyta, Prasinophyceae) from the Mediterranean and Atlantic. *Phycologia* 29:344-350.

El Hag, A. G. D., and G. E. Fogg. 1986. The distribution of coccoid blue-green algae (Cyanobacteria) in the Menai Straits and the Irish Sea. *Br. J. Phycol.* 21:45-54.



- Estrada, M., and M. Delgado. 1990. Summer phytoplankton distribution in the Weddell sea. *Polar Biology* 10:441-449.
- Farrelly, V., F. A. Rainey, and E. Stackebrandt. 1995. Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl. Environ. Microbiol.* 61:2798-2801.
- Fawley, M. W., Y. Yun, and M. Qin. 2000. Phylogenetic analyses of 18S rDNA sequences reveal a new coccoid lineage of the Prasinophyceae (Chlorophyta). *J. Phycol.* 36:387-393.
- Fenchel, T. 1982. Ecology of heterotrophic microflagellates. II. Bioenergetics and growth. *Mar. Ecol. Prog. Ser.* 8:225-231.
- Fenchel, T. 1986. The ecology of heterotrophic microflagellates. *Adv. Microb. Ecol.* 9:57-97.
- Fenchel, T. 1988. Marine plankton food chains. *Ann. Rev. Ecol. Syst.* 19:19-38.
- Ferris, M. J., G. Muyzer, and D. M. Ward. 1996. Denaturing Gradient Gel Electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl. Environ. Microbiol.* 62:340-346.
- Fisher, M. M., and E. W. Triplett. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl. Environ. Microbiol.* 65:4630-4636.
- Fogg, G. E. 1995. Some comments on picoplankton and its importance in the pelagic ecosystem. *Aquat. Microb. Ecol.* 9:33-39.
- Foster, T. D. 1984. The marine environment, p. 345-371. *In* R. M. Laws (ed.), *Antarctic Ecology*. Academic Press, London.
- Fuhrman, J. A., and C. C. Ouverney. 1998. Marine microbial diversity studied via 16S rRNA sequences: cloning results from coastal waters and counting of native archaea with fluorescent single cell probes. *Aquat. Ecol.* 32:3-15.
- Fuhrman, J. A., K. McCallum, and A. A. Davis. 1992. Novel major archaeobacterial group from marine plankton. *Nature* 356:148-149.

Fuhrman, J. A., K. McCallum, and A. A. Davis. 1993. Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. *Appl. Environ. Microbiol.* **59**:1294-1302.

García, C. M. 1994. The size structure and functional composition of ultraplankton and nanoplankton a frontal station in the Alborán Sea, p. 43-52. *In* J. Rodríguez, and W. K. W. Li (ed.), *The size structure and metabolism of the pelagic ecosystem*, vol. 58. Scientia Marina.

Gasol, J. M., and P. A. Del Giorgio. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci. Mar.* **64**:197-224.

Gieskes, W. W. C., and G. W. Kraay. 1986. Floristic and physiological differences between the shallow and deep nanophytoplankton community in the euphotic zone of the open tropical Atlantic revealed by HPLC analysis of pigments. *Mar. Biol.* **91**:567-576.

Gieskes, W. W. C., G. W. Kraay, A. Nontji, D. Setiapermana, and Sutomu. 1988. Monsoonal alternation of a mixed and a layered structure in the phytoplankton of the euphotic zone of the Banda Sea (Indonesia): A mathematical analysis of algal fingerprints. *Netherlands Journal of Sea Research* **22**:123-137.

Gil, J., and D. Gomis. 1994. Circulación geostrofica, dinámica de mesoescala y fertilización de aguas someras en el sector norte del Mar de Alborán. *Bol. Inst. Esp. Oceanogr.* **10**:95-117.

Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace. 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. *J. Bacteriol.* **170**:720-726.

Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field. 1990. Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345**:60-63.

Glover, H. E., B. B. Prézelin, L. Cambell, and M. Wyman. 1988. Pico- and ultraplankton Sargasso Sea communities: variability and algae. *Mar. Ecol. Prog. Ser.* **49**: 127-139.

González, J. M., and M. A. Moran. 1997. Numerical dominance of a group of marine bacteria in the  $\gamma$ -subclass of the class Proteobacteria in coastal seawater. *Appl. Environ. Microbiol.* **63**:4237-4242.

- Good, I. J. 1953. The population frequencies of species and the estimation of the population parameters. *Biometrika* 40:237-264.
- Guillard R. R. L., M. D. Keller, and C. J. O'Kelly. 1991. *Pycnococcus provasolii* gen. et sp. nov., a coccoid prasinococcal-containing phytoplankton from the Western North Atlantic and Gulf of Mexico. *J. Phycol.* 27:39-47.
- Guillard, R. R. L. 1975. Culture of phytoplankton for feeding marine invertebrates, p. 29-60. In W. L. Smith, and M. H. Chanley (ed.), *Culture of marine invertebrate animals*. Plenum Press, New York. N.Y.
- Guillou, L., M.-J. Chrétiennot-Dinet, L. K. Medlin, H. Claustre, S. Loiseaux-de Goër, and D. Vaultot. 1999a. *Bolidomonas*: a new genus with two species belonging to a new algal class, the Bolidophyceae (Heterokonta). *J. Phycol.* 35:368-381.
- Guillou, L., M.-J. Chrétiennot-Dinet, S. Boulben, S. Y. Moon-van der Staay, and D. Vaultot. 1999b. *Symbiomonas scintillans* gen. et sp. nov. and *Picophagus flagellatus* gen. et sp. nov. (Heterokonta): two new heterotrophic flagellates of picoplanktonic size. *Protist* 150:383-398.
- Guillou, L., S. Y. Moon-van der Staay, H. Claustre, F. Partensky, and D. Vaultot. 1999c. Diversity and abundance of Bolidophyceae (Heterokonta) in two oceanic regions. *Appl. Environ. Microbiol.* 65:4528-4536.
- Hall, J. A., and W. F. Vincent. 1990. Vertical and horizontal structure in the picoplankton communities of a coastal upwelling system. *Mar. Biol.* 106: 465-471.
- Hasegawa T., H. Miyashita, M. Kawachi, H. Ikemoto, N. Kurano, S. Miyachi, and M. Chihara. 1996. *Prasinoderma coloniale* gen. et sp. nov., a new pelagic coccoid prasinophyte from the Western Pacific Ocean. *Phycologia* 35:170-176.
- Heburn G. W., and P. E. La Violette 1990. Variations in the structure of the anticyclonic gyres found in the Alboran sea. *J. Geophys. Res.* 95: 1599-1613.
- Herbland A., A. LeBouteiller, and P. Raimbault. 1985. Size structure of phytoplankton biomass in the equatorial Atlantic Ocean. *Deep-Sea. Res.* 32:819-836.

Hibberd, D. J. 1981. Notes on the taxonomy and the nomenclature of the algal Eustigmatophyceae and Tribophyceae (Synonym Xanthophyceae). *Bot. J. Limn. Soc.* **82**:93-119.

Hooks, C. E., R. R. Bidigare, M. D. Keller, and R. R. L. Guillard. 1988. Coccoid eukaryotic marine ultraplankters with four different HPLC pigment signatures. *J. Phycol.* **24**:571-580.

Johnson, P. W., and J. McN. Sieburth. 1979. Chroococcoid cyanobacteria in the sea: an ubiquitous and diverse phototrophic biomass. *Limnol. Oceanogr.* **24**:928-935.

Johnson, P. W., and J. McN. Sieburth. 1982. *In situ* morphology and occurrence of eucaryotic phototrophs of bacterial size in the picoplankton of estuarine and oceanic waters. *J. Phycol.* **18**:318-327.

Jones H. L., B. S. Leadbeater, and J. C. Green. 1994. An ultrastructural study of *Marsupiomonas pelliculata* gen. et sp. nov., a new member of the Pedinophyceae. *Eur. J. Phycol.* **29**:171-181.

Karlson, B., D. Potter, M. Kuylenstierna, and R. A. Andersen. 1996. Ultrastructure, pigment composition, and 18S rRNA gene sequence for *Nannochloropsis granulata* sp. nov. (Monodopsidaceae, Eustigmatophyceae), a marine ultraplankter isolated from the Skagerrak, northeast Atlantic Ocean. *Phycologia* **35**:253-260.

Karpov, S. S., R. Kersanach, and D. M. Williams. 1998. Ultrastructure and 18S rRNA gene sequence of a small heterotrophic flagellate *Siluania monomastiga* gen. et sp. nov. (Bicosoecida). *Europ. J. Protistol.* **34**:415-425.

Klappenbach, J. A., J. M. Dunbar, and T. M. Schmidt. 2000. rRNA operon copy number reflects ecological strategies of bacteria. *Appl. Environ. Microbiol.* **66**:1328-1333.

Komatsoulis, G. A., and M. S. Waltherman. 1997. A new computation method for detection of chimeric 16S rRNA artifacts generated by PCR amplification from mixed bacterial populations. *Appl. Environ. Microbiol.* **63**:2338-2346.

Kopczynski, E. D., M. M. Bateson, and D. M. Ward. 1994. Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. *Appl. Environ. Microbiol.* **60**:746-748.

- Kowalchuk, G. A., S. Gerards, and J. W. Woldendorp. 1997. Detection and characterization of fungal infections of *Ammophila arenaria* (marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18S rDNA. *Appl. Environ. Microbiol.* 63:3858-3865.
- Krienitz, L., D. Hepperle, H. -B. Stich, and W. Weiler. 2000. *Nannochloropsis limnetica* (Eustigmatophyceae), a new species of picoplankton from freshwater. *Phycologia* 39:219-227.
- Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace. 1985. Rapid determination of 16S RNA sequences for phylogenetic analysis. *Proc. Natl. Acad. Sci. USA* 82:6955-6959.
- Lange, M., L. Guillou, D. Vaultot, N. Simon, R.I. Amann, W. Ludwig, and L.K. Medlin. 1996. Identification of the class Prymnesiophyceae and the genus *Phaeocystis* with ribosomal RNA-targeted nucleic acid probes detected by flow cytometry. *J. Phycol.* 32:858-868.
- Latasa, M., and R. R. Bidigare. 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.
- Latasa, M., K. van Lenning, J. L. Garrido, R. Scharek, M. Estrada, F. Rodríguez, and M. Zapata. 2001. Losses of chlorophylls and carotenoids in aqueous acetone and methanol extracts prepared for RPHPLC analysis of pigments. *Chromatografia* 53:385-391.
- Lee, S., and J. A. Fuhrman. 1991. Spatial and temporal variation of natural bacterioplankton assemblages studied by total genomic DNA cross-hybridization. *Limnol. Oceanogr.* 36:1277-1287.
- Legendre, L., and J. Le Fèvre. 1995. Microbial food webs and the export of biogenic carbon in oceans. *Aquat. Microb. Ecol.* 9:69-77.
- Leipe, D. D., S. M. Tong, C. L. Goggin, S. B. Slemenda, N. J. Pieniazek, and M. L. Sogin. 1996. 16S-like rDNA sequences from *Developayella elegans*, *Labyrinthuloides haliotidis*, and *Proteromonas lacertae* confirm that the stramenopiles are a primarily heterotrophic group. *Euro. J. Protistol.* 32:449-458.
- Letelier, R. M., R. R. Bidigare, D. V. Hebel, M. E. Ondrusek, C. D. Winn, and D. M. Karl. 1993. Temporal variability of phytoplankton community structure at the U.S.-JGOFS time-

series Station ALOHA (22°45'N, 158°00'W) based on HPLC pigment analysis. *Limnol. Oceanogr.* **38**:1420-1437.

Lewin J., R. E. Norris, S. W. Jeffrey, and B. E. Pearson. 1977. An aberrant Chrysophyceae alga *Pelagococcus subviridis* gen. et sp. nov. from the North Pacific Ocean. *J. Phycol.* **13**:259-266.

Li, W. K. W. 1994. Primary production of prochlorophytes, cyanobacteria, and eucaryotic ultraphytoplankton: measurements from flow cytometric sorting. *Limnol. Oceanogr.* **39**:169-175.

Li, W. K. W., D. V. Subba Rao, W. G. Harrison, J. C. Smith, J. J. Cullen, B. Irwin, and T. Platt. 1983. Autotrophic pioplankton in the tropical ocean. *Science* **219**:292-295.

Li, W. K. W., and A. M. Wood. 1988. Vertical distribution of North Atlantic ultraphytoplankton: analysis by flow and epifluorescence microscopy. *Deep-Sea Res.* **35**: 1615-1638.

Li, W. K. W., P. M. Dickie, B. P. Irwin, and A. M. Wood. 1992. Biomass of bacteria, cyanobacteria, prochlorophytes and photosynthetic eukaryotic in the sargasso Sea. *Deep-Sea Res.* **39**: 501-519.

Liesack, W., H. Weyland, and E. Stackebrandt. 1991. Potential risks of gene amplification by PCR as determined by 16S rRNA analysis of a mixed-culture of strict barophilic bacteria. *Microb. Ecol.* **21**:191-198.

Lim, E. L., L. A. Amaral, D. A. Caron, and E. F. DeLong. 1993. Application of rRNA-based probes for observing marine nanoplanktonic protists. *Appl. Environ. Microbiol.* **59**:1647-1655.

Lim, E. L., M. R. Dennet, and D. A. Caron. 1999. The ecology of *Paraphysomonas imperforata* based on studies employing oligonucleotide probe identification in coastal water samples and enrichment cultures. *Limnol. Oceanogr.* **44**:37-51.

Liu, W. L., T. L. Marsh, H. Cheng, and L. J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **63**:4516-4522.

Long, E. O., and I. B. Dawid. 1980. Repeated genes in eukaryotes. *Annu. Rev. Biochem.* **49**:727-764.

- López-García, P., F. Rodríguez-Valera, C. Pedrós-Alió, and D. Moreira. 2001. Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. *Nature* 409:603-607.
- Lubian, L. M. 1982. *Nannochloropsis gaditana* sp. nov., una nueva Eustigmatophyceae marina. *Lazaroa* 4:287-293.
- Mackey, M. D., D. J. Mackey, H. W. Hoggins, and S. W. Wright. 1996. CHEMTAX- a program for estimating class abundances from chemical markers: application to HPLC measurements of phytoplankton. *Mar. Ecol. Prog. Ser.* 144:265-283.
- Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, Jr, P. R. Saxman, J. M. Stredwick, G. M. Garrity, B. Li, G. J. Olsen, S. Pramanik, T. M. Schmidt, and J. M. Tiedje. 2000. The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res.* 28:173-174.
- Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, F. K. J. Balndy, and C. R. Woese. 1994. The ribosomal database project. *Nucleic Acids Res.* 22:3485-3487.
- Manton I., and M. Parke. 1960. Further observations on small green flagellates with special reference to possible relatives of *Chloromulina pusilla* Butcher. *J. Mar. Biol. Assoc. U.K.* 39:275-298.
- Manton, I. 1975. Observation of the microanatomy of *Scourfieldia marina* Thronsen and *Scourfieldia caeca* (Korsh.) Belcher and Swale. *Arch. Protistenk.* 117:358-368.
- Marchant, H. J., K. R. Buck, D. L. Garrison, and H. A. Thomsen. 1989. *Mantoniella* in Antarctic waters including the description of *M. antarctica* sp. nov. (Prasinophyceae). *J. Phycol.* 25:167-174.
- Marsh, T. L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol.* 2:323-327.
- Marsh, T. L., W. T. Liu, L. J. Forney, and H. Cheng. 1998. Beginning a molecular analysis of the eukaryal community in activated sludge. *Water. Sci. Techol.* 37:455-460.
- Marsh, T. L., P. Saxman, J. Cole, and J. M. Tiedje. 2000. Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. *Appl. Environ. Microbiol.* 66:3616-3620.

Massana, R., A. E. Murray, C. M. Preston, and E. F. DeLong. 1997. Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. *Appl. Environ. Microbiol.* **63**:50-56.

Massana, R., E. F. DeLong, and C. Pedrós-Alió. 2000. A few cosmopolitan phylotypes dominate planktonic archaeal assemblages in widely different oceanic provinces. *Appl. Environ. Microbiol.* **66**:1777-1787.

Medlin, L., H. J. Elwood, S. Stickel, and M. L. Sogin. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* **71**:491-499.

Minas H. J., B. Coste, P. Le Corre, M. Minas, and P. Raimbault. 1991. Biological and geochemical signatures associated with the water circulation through the Strait of Gibraltar and in the western Alboran Sea. *J. Geophys. Res.* **96**:8755-8771.

Moeseneder, M. M., J. M. Arrieta, G. Muyzer, C. Winter, and G. J. Herndl. 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **65**:3518-3525.

Moestrup, Ø. 1991. Further studies of presumed primitive green algae, including the description of *Pedinophyceae* class. nov. and *Resultor* gen. nov. *J. Phycol.* **27**:119-133.

Moon-van der Staay, S. Y., G. W. M. van der Staay, L. Guillou, and D. Vaultot. 2000. Abundance and diversity of prymnesiophytes in the picoplankton community from the equatorial Pacific Ocean inferred from 18S rDNA sequences. *Limnol. Oceanogr.* **45**:98-109.

Moon-van der Staay, S. Y., R. De Wachter, and D. Vaultot. 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* **409**:607-610.

Morán, X. A. G., and M. Estrada. 2001. Short-term variability of photosynthetic parameters and particulate and dissolved primary production in the Alboran Sea (SW Mediterranean). *Mar. Ecol. Prog. Ser.* **212**:53-67.

Moyer, C. L., J. M. Tiedje, F. C. Dobbs, and D. M. Karl. 1996. A computer-simulated Restriction Fragment Length Polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. *Appl. Environ. Microbiol.* **62**:2501-2507.



- Murphy, L. S., and E. M. Haugen. 1985. The distribution and abundance of phototrophic ultraplankton in the North Atlantic. *Limnol. Oceanogr.* 30:47-58.
- Murray, A. E., J. T. Hollibaugh, and C. Orrego. 1996. Phylogenetic composition of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Appl. Environ. Microbiol.* 62:2676-2680.
- Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural communities. *Curr. Opin. Microbiol.* 2:317-322.
- Muyzer, G., and E.C. de Waal. 1994. Determination of the genetic diversity of microbial communities using DGGE analysis of PCR-amplified 16S rDNA, p. 207-214. *In* *Microbial Mats: Structure, Development and Environmental Significance*.
- Muyzer, G., and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*.
- Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of Polymerase Chain Reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59:695-700.
- Muyzer, G., T. Brinkhoff, U. Nübel, C. Santegoeds, H. Schäfer, and C. Wawer. 1997. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, p. 1-27. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), *Molecular Microbial Ecology Manual*, vol. 3.4.4. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Neefs, J.-M., Y. Van de Peer, P. De Rijk, S. Chapelle, and R. De Wachter. 1993. Compilation of small subunit RNA structures. *Nucleic Acids Res.* 21:3025-3049.
- Neveux, J., D. Vault, C. Courties, and E. Fukai. 1989. Green photosynthetic bacteria associated with the deep chlorophyll maximum of the Sargasso Sea. *C. R. Hebd. Seances-Acad. Sci. III. Paris* 308:9-14.
- Olsen, G. J., D. J. Lane, S. J. Giovannoni, and N. R. Pace. 1986. Microbial ecology and evolution: a ribosomal RNA approach. *Ann. Rev. Microbiol.* 40:337-365.
- Olson, R. J., D. Vault, and S. W. Chisholm. 1985. Marine phytoplankton distributions measured using shipboard flow cytometry. *Deep Sea. Res.* 32:1273-1280.

Olson, R. J., E. R. Zettler, and M. D. DuRand. 1993. Phytoplankton analysis using flow cytometry, p. 175-186. *In* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (ed.), *Handbook of Methods in Aquatic Microbial Ecology*. Lewis Publishers, Boca Raton, Fla.

Olson, R. J., S. W. Chisholm, E. R. Zettler, and E. V. Ambrust. 1988. Analysis of *Synechococcus* pigment types in the sea using single and dual beam flow cytometry. *Deep Sea Res.* **34**:425-440.

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. *Science* **276**:734-740.

Pace, N. R., D. A. Stahl, D. J. Lane, and G. J. Olsen. 1986. The analysis of natural microbial population by ribosomal RNA sequences. *Adv. Microb. Ecol.* **9**: 1-55.

Parrilla G., and T. H. Kinder. 1987. Oceanografía física del Mar de Alborán. *Bol. Inst. Esp. Oceanogr.* **4**:133-165.

Parsons, T. R., Y. Maita, and C. M. Lalli. 1984. *A manual of chemical and biological methods for seawater analysis*, 1st ed. Pergamon Press, NY.

Partensky, F., L. Guillou, N. Simon, and D. Vaultot. 1997. Recent advances in the use of molecular techniques to assess the genetic diversity of marine photosynthetic microorganisms. *Vie Milieu* **47**:367-374.

Patterson, D. J. 1989. Stramenopiles: chromophytes from a protistan perspective, p. 357-379. *In* J. C. Green, B. S. C. Leadbeater, and W. L. Diver (ed.), *Chromophyte algae: problems and perspectives*. Clarendon Press, Oxford. United Kingdom.

Paul, J. H., and B. Myers. 1982. Fluorometric determination of DNA in aquatic microorganisms by use of Hoechst 33258. *Appl. Environ. Microbiol.* **43**:1393-1399.

Pedrós-Alió, C, and T. D. Brock. 1982. Assessing biomass and production of bacteria in eutrophic Lake Mendota, Wisconsin. *Appl. Environ. Microbiol.* **44**:203-218.

Peeken, I. 1997. Photosynthetic pigment fingerprints as indicators of phytoplankton biomass and development in different water masses of the Southern Ocean during austral spring. *Deep-Sea Res. II* **44**:261-282.

- Peña, M. A., M. R. Lewis, and G. Harrison. 1990. Primary productivity and size structure of phytoplankton biomass on a transect of the equator at 135°W in the Pacific Ocean. *Deep-Sea Res.* 37:295-315.
- Perkins, H., T. Kinder, and P. La Violette. 1990. The Atlantic inflow in the western Alboran Sea. *J. Phys. Oceanogr.* 20:242-263.
- Pinhassi, J., U. L. Zweifel, and Å. Hagström. 1997. Dominant marine bacterioplankton species found among colony-forming bacteria. *Appl. Environ. Microbiol.* 63:3359-3366.
- Platt, T. 1989. Flow cytometry in oceanography, vol. 10, p. 500. *In* C. M. Yentsch, and P. K. Horan (ed.), *Cytometry in Aquatic Sciences. Cytometry.*
- Platt, T., and W. K. W. Li. 1986. Photosynthetic picoplankton. *Can. Bull. Fish. Aquat. Sci.* 214:1-583.
- Pomeroy, L. R. 1974. The ocean's food web, a changing paradigm. *Bioscience* 24:499-504.
- Porter, K. G., and Y. S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* 25:943-948.
- Potter, D., T. C. Lajeunesse, G. W. Saunders, and R. A. Andersen. 1997. Convergent evolution masks extensive biodiversity among marine coccoid picoplankton. *Biodivers. Conserv.* 6:99-107.
- Raimbault, P., B. Coste, M. Boulhadid, and B. Boudjellal. 1993. Origin of high phytoplankton concentration in deep chlorophyll maximum (DCM) in a frontal region of the Southwestern Mediterranean Sea (Algerian Current). *Deep-Sea Res.* 40:791-804.
- Raimbault, P., I. Taupier-Letage, and M. Rodier. 1988a. Vertical size distribution of phytoplankton in the Western Mediterranean Sea during early summer. *Mar. Ecol. Prog. Ser.* 45:153-158.
- Raimbault, P., M. Rodier, and I. Taupier-Letage. 1988b. Size fraction of phytoplankton in the Ligurian Sea and the Algerian Basin (Mediterranean Sea): Size distribution versus total concentrations. *Mar. Microb. Food Webs* 3:1-7.

Rappé, M. S., P. K. Kemp, and S. J. Giovannoni. 1997. Phylogenetic diversity of marine coastal picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. *Limnol. Oceanogr.* **42**:811-826.

Rappé, M. S., M. T. Suzuki, K. L. Vergin, and S. J. Giovannoni. 1998. Phylogenetic diversity of ultraplankton plastid small-subunit rRNA genes recovered in environmental nucleic acid samples from the Pacific and Atlantic coasts of the United States. *Appl. Environ. Microbiol.* **64**:294-303.

Reynolds, N. 1974. *Imantonia rotunda* gen. and sp. nov., a new member of the haptophyceae. *Br. Phycol. J.* **9**:429-434.

Riemann, L., G. F. Steward, L. B. Fandino, L. Campbell, M. R. Landry, and F. Azam. 1999. Bacterial community composition during two consecutive NE Monsoon periods in the Arabian Sea studied by denaturing gradient gel electrophoresis (DGGE) of rRNA genes. *Deep-Sea Res.* **46**:1791-1811.

Rizzo, P. J. 1985. Biochemistry of the dinoflagellate nucleus, p. 143-173. *In* F. J. R. Taylor (ed.), *The Biology of Dinoflagellates*. Blackwell Scientific. Oxford. United Kingdom.

Rodríguez, V., B. Bautista, J. M. Blanco, F. L. Figueroa, N. Cano, and J. Ruiz. 1994. Hydrological structure, optical characteristics and size distribution of pigments and particles at a frontal station in the Alboran Sea Working Group 1 Report. *Sci. Mar.* **58**:31-41.

Rodríguez, J., J. M. Blanco, F. Jiménez-Gómez, F. Echevarría, J. Gil, V. Rodríguez, J. Ruiz, B. Bautista, and F. Guerrero. 1998. Patterns in the size structure of the phytoplankton community in the deep fluorescence maximum of the Alboran Sea (southwestern Mediterranean). *Deep-Sea Res.* **45**:1577-1593.

Schauer, M., R. Massana, and C. Pedrós-Alió. 2000. Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. *FEMS Microbiol. Ecol.* **33**:51-59.

Schmidt, T. M., E. F. DeLong, and N. R. Pace. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *J. Bacteriol.* **173**:4371-4378.

Schut, F., E. J. D. Vries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins, and D. K. Button. 1993. Isolation of typical marine bacteria by dilution culture: growth, maintenance,

and characteristics of isolates under laboratory conditions. *Appl. Environ. Microbiol.* **59**:2150-2160.

Sherr, E. B., and B. F. Sherr. 1988. Role of microbes in pelagic food webs. *Limnol. Oceanogr.* **33**:1225-1227.

Sherr, E. B., B. F. Sherr, and L. Fessenden. 1997. Heterotrophic protists in the Central Arctic Ocean. *Deep-Sea Res. II* **44**:1665-1682.

Sieburth, J. McN., V. Smetacek, and J. Lenz. 1978. Pelagic ecosystem structure: Heterotrophic compartments of the plankton and their relationship to plankton size fractions. *Limnol. Oceanogr.* **23**:1256-1263.

Sieburth, J. McN. and P. G. Davis. 1982. The role of heterotrophic nanoplankton in the grazing and nurturing of plankton bacteria in the Sargasso and Caribbean Sea. *Ann. Inst. Oceanogr. Paris.* **58**:285-296.

Sieburth J. McN., P. W. Johnson, and P. E. Hargraves. 1988. Ultrastructure and ecology of *Aureococcus anophagefferens* gen. et sp. nov. (Chrysophyceae): the dominant picoplankter during a bloom in Narragansett Bay, Rhode Island, summer 1985. *J. Phycol.* **24**:416-425.

Silberman, J. D., M. L. Sogin, D. D. Leipe, and C. G. Clark. 1996. Human parasite finds taxonomic home. *Nature* **380**:398.

Simon, N., N. LeBot, D. Marie, F. Partensky, and D. Vaultot. 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., R. G. Barlow, D. Marie, F. Partensky, and D. Vaultot. 1994. Characterization of oceanic photosynthetic picoeukaryotes by flow cytometry. *J. Phycol.* **30**:922-935.

Sogin, M. L., and J. H. Gunderson. 1987. Structural diversity of eukaryotic small subunit ribosomal RNAs. *Ann. N.Y. Acad. Sci.* **503**:125-139.

Stackebrandt, E., and M. Goodfellow. 1991. Nucleic acid techniques in bacteria systematics. John Willey & sons.

Stackebrandt, E., and F. A. Rainey. 1995. Partial and complete 16S rDNA sequences, their use in generation of 16S rDNA phylogenetic trees and their implications in molecular

ecological studies, p. 1-17. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), *Molecular microbial ecology manual*, vol. 3.1.1. Kluwer Academic Publishers, Dordrecht, The Netherlands.

**Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol.* **54**:1079-1084.

**Steele, J. H.** 1974. *The structure of marine ecosystems.* Harvard Univ. Press, Cambridge, Mass., 128 pp.

**Stockner, J. G.** 1988. Phototrophic picoplankton: An overview from marine and freshwater ecosystems. *Limnol. Oceanogr.* **33**:765-775.

**Stockner, J. G., and N. J. Antia.** 1986. Algal picoplankton from marine and freshwater ecosystems: a multidisciplinary perspectives. *Can. J. Fish. Aquat. Sci.* **43**:2472-2503.

**Suzuki, M. T., M. Rappé, Z. W. Haimberger, H. Winfield, N. Adair, J. Ströbel, and S. J. Giovannoni.** 1997. Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. *Appl. Environ. Microbiol.* **63**:983-989.

**Suzuki, M. T., M. S. Rappé, and S. J. Giovannoni.** 1998. Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. *Appl. Environ. Microbiol.* **64**:4522-4529.

**Takahashi, M., and P. K. Bienfang.** 1983. Size structure of phytoplankton biomass and photosynthesis in subtropical and tropical Hawaiian waters. *Mar. Biol.* **76**:201-211.

**Takahashi, M., and T. Hori.** 1984. Abundance of picophytoplankton in the subsurface chlorophyll maximum layer in subtropical and tropical waters. *Mar. Biol.* **79**:177-186.

**Tester, P. A., M. E. Geesey, C. Guo, H. W. Paerland, and D. F. Millie.** 1995. Evaluating phytoplankton dynamics in the Newport River estuary (North Carolina, USA) by HPLC-derived pigment profiles. *Mar Ecol. Prog. Ser.* **124**:237-245.

**Thomsen, H. A.** 1986. A survey of the smallest eukaryotic organisms of the marine phytoplankton. *Can. Bull. Fish. Aquat. Sci.* **214**:121-158.

- Throndsen, J. 1976. Occurrence and productivity of small marine flagellates. *Norw. J. Bot.* 23:269-293.
- Throndsen, J., and S. Kristiansen. 1991. *Micromonas pusilla* (Prasinophyceae) as part of pico- and nanoplankton communities of the Barents Sea, p. 201-207. *In* E. Sakshomy, C. C. E. Hopkins, and N. A. Oeritsland (ed.), *Proceedings of the Polar Marine Symposium on Polar Marine Ecology*, vol. 10. Trondheim, Norway.
- Throndsen J., and A. Zingone. 1997. *Dolichomastix tenuilepis* sp. nov., a first insight into the microanatomy of the genus *Dolichomastix* (Mamiellales, Prasinophyceae, Chlorophyta). *Phycologia* 36:244-254.
- Tintoré, J., D. Gomis, S. Alonso, and G. Parrilla. 1991. Mesoscale dynamics and vertical motion in the Alborán Sea. *J. Phys. Oceanogr.* 21:811-823.
- Tong, S. M. 1995. *Developayella elegans* nov. gen., nov. spec., a new type of heterotrophic flagellate from marine plankton. *Eur. J. Protistol.* 31:24-31.
- Tong, S. M. 1997. Heterotrophic flagellates and other protists from Southampton Water. *U.K. Ophelia* 47:71-131.
- Torsvik, V., J. Goksoyr, and F. L. Daae. 1990. High diversity of DNA of soil bacteria. *Appl. Environ. Microbiol.* 56:782-787.
- Utermöhl, H. 1958. Zur Vervollkommung der quantitativen Phytoplankton-Methodik. *Mitt. Int. Ver. Theor. Angew. Limnol.* 9:1-38.
- Van de Peer Y., J. Jansen, P. De Rijk, and R. De Wachter. 1997. Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res.* 25:111-116.
- Van Hannen, E. J., M. P. Van Agterveld, H. J. Gons, and H. J. Laanbroek. 1998. Revealing genetic diversity of eukaryotic microorganisms in aquatic environments by denaturing gradient gel electrophoresis. *J. Phycol.* 34:206-213.
- Van Hannen, E. J., G. Zwart, M. P. van Agterveld, H. J. Gons, J. Ebert, and H. J. Laanbroek. 1999. Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses. *Appl. Environ. Microbiol.* 65:795-801.

Veldhuis, M. J. W., and G. W. Kraay. 1990. Vertical distribution and pigment composition of a picoplanktonic prochlorophyte in the subtropical North Atlantic: A combined study of HPLC-analysis of pigments and flow cytometry. *Mar. Ecol. Prog. Ser.* **68**:121-127.

Von Wintzingerode, F., U. B. Goebel, and E. Stackebrandt. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* **21**:213-229.

Wang, G. C. -Y., and Y. Wang. 1997. Frequency of formation of chimeric molecules as a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial genomes. *Appl. Environ. Microbiol.* **63**:4645-4650.

Ward, D. M., R. Weller, and M. M. Bateson. 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**:63-65.

Waterbury, J. B., S. W. Watson, R. R. L. Guillard, and L. E. Brand. 1979. Widespread occurrence of a unicellular, marine planktonic cyanobacterium. *Nature* **277**:293-294.

Whittaker, R. H. 1969. New concepts of kingdoms of organisms. *Science* **163**:150-160.

Wilhelm, C., G. Eisenbeis, A. Wild, and R. Zahn. 1982. *Nanochlorum eucaryotum*: a very reduced coccoid species of marine Chlorophyceae. *Z. Naturforsch.* **37**:107-114.

Willians, P. J. leB. 1981. Incorporation of microheterotrophic processes into the classical paradigm of the planktonic food web. *Kiel. Meeresforsch.* **5**:1-28.

Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221-271.

Woese, C. R., and G. E. Fox. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci. USA.* **74**:5088-5090.

Woese, C. R., O. Kandler, and M. L. Wheelis. 1990. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria and Eucarya. *Proc. Natl. Acad. Sci. USA.* **87**:4576-4579.

Wood E. J. F., and P. S. Davis. 1956. Importance of smaller phytoplankton elements. *Nature* **177**:438.

Yentsch, C. S., and D. W. Menzel. 1963. A method for the determination of phytoplankton chlorophyll and pheophytin by fluorescence. *Deep-Sea Res.* **10**:221-231.



Yentsch, C. S., and Horan. 1989. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. *Deep-Sea Res.* **10**:221-231.

Zapata, M., F. Rodríguez, and J. L. Garrido. 2000. Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C-8 column and pyridine-containing mobile phases. *Mar. Ecol. Prog. Ser.* **195**:29-45.

Zingone, A., M. J. Chrétiennot-Dinet, M. Lange, and L. Medlin. 1999. Morphological and genetic characterization of *Phaeocystis cordata* and *P. jahnii* (Prymnesiophyta) two species from the Mediterranean Sea. *J. Phycol.* **35**:1322-1337.