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Distribution of eukaryotic picoplankton assemblages across hydrographic fronts in the Southern Ocean, studied by denaturing gradient gel electrophoresis

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Abstract

We used a molecular fingerprinting technique to analyze the distribution and composition of eukaryotic picoplankton along latitudinal transects in the Southern Ocean. First, primers specific for eukaryotic 18S rDNA were used in a polymerase chain reaction (PCR) with environmental DNA. The amplification products were subjected to denaturing gradient gel electrophoresis (DGGE). Transect DOVETAIL (44°W) went from the ice edge (at 60°S) across the Weddell–Scotia confluence and north to 58°S; it was sampled in January 1998 (summer). Transect DHARMA (between 53°W and 58°W) went from the ice edge in the Weddell Sea (63°S) across the Drake Passage to the South American continental platform (55°S); it was sampled in December 1998 (late spring). DGGE band patterns were used to build dendrograms combining samples from each cruise. Samples were grouped in several distinct clusters that were generally consistent with the hydrography of the area. In DOVETAIL, the upper water column was stratified and the DGGE band patterns varied with depth. In DHARMA the upper mixed layer showed the same composition of the eukaryotic picoplankton at all depths. The most dominant DGGE bands were excised and sequenced. Some were closely related to well-known components of the plankton such as prasinophytes, prymnesiophytes, dinoflagellates, and diatoms. A significant number of sequences were related to previously unknown phylogenetic groups, including novel stramenopiles and alveolates or to poorly known groups such as cercomonads. This fingerprinting technique is useful for a rapid evaluation of the spatial distribution of picoeukaryotic assemblages in the oceans.

Marine picoeukaryotes (between 0.2 and 2–3 μm in diameter) are the most abundant eukaryotes on Earth. They are found throughout the world's oceans in concentrations between 10^2 and 10^4 cells ml^{-1} in the photic zone, and they constitute an essential component of microbial food webs, playing significant roles in the major biogeochemical cycles (Li 1994). Marine picoeukaryotes seem to belong to widely different phylogenetic groups, but the extent of their diversity and the distribution and abundance of the different taxa in situ remain poorly known (Partensky et al. 1997). In some cases, careful microscopy combined with culture techniques has allowed the identification and quantification of some marine picoeukaryotes. For example, Throndsen and Kristiansen (1991) determined that *Micromonas pusilla* reached numbers up to 10^5 ml^{-1} in some marine environments. In the open oceans, however, many picoeukaryotes are coccoid or flagellated forms, with or without chloroplasts (phototrophic or heterotrophic, respectively), and with relatively few morphologically distinct features (Thomsen 1986; Simon et al. 1994; Caron et al. 1999). Thus, many of the conventional characterization techniques have a limited capacity to identify these small cells.

An alternative approach to characterize the phylogenetic diversity of marine picoeukaryotes is provided by analyses of small subunit rRNA genes (Partensky et al. 1997; Rappé et al. 1998). Three recent papers described the diversity of picoeukaryotes by gene cloning and sequencing of rDNA in one sample from the equatorial Pacific Ocean (Moon-van der Staay et al. 2001), several deep-sea samples from the Southern Ocean (López-García et al. 2001), and five surface samples from the Southern Ocean, the North Atlantic, and the Mediterranean (Díez et al. 2001*b*). These studies have revealed a large phylogenetic diversity in these assemblages and the presence of novel lineages. Yet these studies were carried out with just 10 samples, an insignificant number to characterize the whole ocean. This situation leads one naturally to ask how the picoeukaryotic assemblages are distributed in the ocean. Can different assemblages be associated with particular water masses or environmental characteristics at a mesoscale level? Or is there a certain set of species that tends to be widely distributed across hydrographic boundaries throughout the world oceans, as appears to happen with marine archaea (Massana et al. 2000) and cyanobacteria (Partensky et al. 1997)? A second question, related to the patterns of variability of picoeukaryotic assemblages, is to what extent a particular sample is representative of the sampling locality. This depends on the relationship between variability at different scales. How does small-scale variability, among samples from neighboring localities within the same mesoscale hydrographical region, compare with variability among samples from different regions? These questions of variability and whether samples are represen-

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tative of a certain area require analysis of many more samples than would be practical with the cloning and sequencing approach. Both, however, can be addressed through the use of molecular fingerprinting techniques such as denaturant gradient gel electrophoresis (DGGE, Muyzer et al. 1993; Murray et al. 1996; Van Hannen et al. 1998).

We have shown that DGGE band patterns are a robust characteristic of natural microbial assemblages of both bacteria (Casamayor et al. 2000; Schauer et al. 2000) and eukaryotes (Díez et al. 2001*a*). Briefly, the total DNA of the microbial assemblage is extracted, a PCR amplification is carried out with general primers for the SSU rDNA gene of eukaryotes, and the PCR products are loaded in a gel with a gradient of a denaturant. Upon electrophoresis, each rDNA fragment denatures at a given point in the gradient, depending on the particular sequence. The result is a series of bands that ideally correspond to the most abundant members of the initial assemblage. Each microbial assemblage results in a distinct and characteristic band pattern or fingerprint. Here we illustrate how this fingerprinting approach can be used to describe the distribution of eukaryotic picoplankton assemblages in relation to mesoscale hydrographic features.

We chose two transects in the Southern Ocean as examples, since the frontal areas crossed provided representative discontinuities in the structure of the ocean. Starting at the Antarctic continent and moving toward the north, several fronts and zones are found in succession (Orsi et al. 1995). The area close to the Drake Passage is particularly interesting to study because in this region the distinctive Antarctic water masses and fronts are compressed into a relatively narrow region, and large differences in the physical and chemical environment can be observed over relatively small distances. An environment with such marked physical gradients provided an excellent case study to investigate the composition and variability of picoeukaryotic assemblages.

Materials and methods

Sample collection—Samples were collected during cruises DHARMA (Diversidad, Heterotrofía, Autotrofía, y relaciones entre Microorganismos Antarticos) and DOVETAIL (Deep Ocean Ventilation Through Antatctica Intermediate Layers) on board *RV Hespérides.* Several stations were sampled across the Scotia–Weddell confluence during the DOVETAIL cruise (23–26 January 1998) and in a longer transect across the polar front during the DHARMA cruise (6–14 December 1998) as shown in Fig. 1. Seawater from different depths was collected with Niskin bottles attached to a rosette. Temperature, salinity, conductivity, and fluorescence were determined with General Oceanics MkIII or MkV conductivity–temperature–depth (CTD) profilers. Samples were screened with a 200-μm net prior to collection to exclude large organisms. Chlorophyll *a* (Chl *a*) concentration was determined by measuring the fluorescence in acetone extracts with a Turner Designs fluorometer (modified from Yentsch and Menzel 1963). This method does not measure accurately either chlorophylls *b* or *c*, but it is used here only as a general indicator of phytoplankton biomass. A parallel high-performance liquid chromatography (HPLC) study

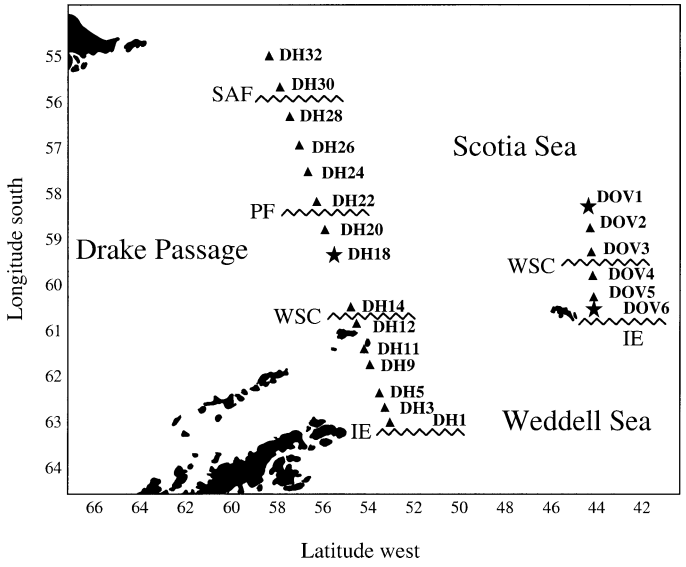

Fig. 1. Map of the area where cruises DOVETAIL (stations labeled DOV1–6) and DHARMA (stations labeled DH1–32) took place. The approximate position of the ice edge is indicated by a broken line (IE). The main fronts at the time of sampling are indicated by broken lines: WSC, Weddell–Scotia confluence; PF, polar front; SAF, sub-Antarctic front. The three stars indicate stations where clone libraries were constructed and published in Díez et al. (2001*b*) for DOVETAIL and López-García et al. (2001) for DHARMA.

of the DHARMA transect indicated that, on average, chlorophylls *b* and *c* accounted for 10% and 16% of Chl *a*, respectively (M. Latasa pers. comm.). Phytoplankton samples were fixed with formalin (4% final concentration) during DOVETAIL or with Lugol's solution during DHARMA. Phytoplankton counts (nanoplankton and microplankton size ranges) were carried out by the inverted microscope method (Utermöhl 1958). One hundred milliliters of water were allowed to settle in chambers. One or more transects of the chamber (equivalent to 1–2 ml of sample) were examined at 400× to enumerate the more frequent taxa. Additional transects and the whole chamber bottom were scanned at 100× to count the less frequent, relatively large organisms. Cells were identified to species when possible, but many could not be classified and were lumped into categories such as ''flagellates'' or ''small flagellates.'' Subsamples for flow cytometry were fixed with glutaraldehyde-paraformaldehyde (0.05% and 1% final concentrations) and stored frozen until processed. Autofluorescing picoeukaryote counts were carried out with a FACSCalibur flow cytometer. When possible, distinct picoeukaryotic populations were distinguished in the cytometry graph and analyzed separately.

Microbial biomass was collected on 0.2-μm Sterivex units (Durapore, Millipore) by filtering between 10 and 25 liters of seawater through a 1.6-μm Whatman GF/A prefilter (DOVETAIL) or a 5-μm polycarbonate prefilter (DHARMA) and the Sterivex unit in succession, using a peristaltic pump with filtration rates between 50 and 100 ml min^{-1}. Before passing the prefilter, water was screened through a 50-μm net. We have found that this prescreening significantly reduces contamination with DNA from large organ-

isms such as copepods or apendicularians. Sterivex units were filled with lysis buffer (40 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA], 50 mmol L^{-1} Tris-HCl, and 0.75 mol L^{-1} sucrose), capped, and frozen at −70°C until nucleic acid extractions could be carried out. DOVETAIL samples were extracted in the laboratory, and DHARMA samples were extracted on board.

Nucleic acid extraction—Nucleic acid extraction was carried out as described in Massana et al. (2000). Lysozyme (1 mg ml^{-1} final concentration) was added and filters were incubated at 37°C for 45 min. SDS (sodium dodecyl sulfate, 1% final concentration) and proteinase K (0.2 mg ml^{-1} final concentration) were added, and the filters were incubated at 55°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 extracting with an equal volume of chloroform-isoamyl alcohol (24 : 1). Finally, nucleic acid extracts were further purified, desalted, and concentrated in a Centricon-100 concentrator (Millipore). Integrity of the total DNA was checked by agarose gel electrophoresis. DNA yield was quantified by a Hoechst dye fluorescence assay. Nucleic acid extracts were stored at −70°C until analysis.

PCR—Approximately 10 ng of extracted DNA was used as a template in a polymerase chain reaction (PCR) using the eukaryotic-specific 18S rDNA primers Euk1A and Euk516r-GC (Díez et al. 2001*a*). PCR mixtures (50 μl) contained 200 μmol L^{-1} of each dNTP, 1.5 mmol L^{-1} of $MgCl_2$, 0.3 μmol L^{-1} of each primer, 2.5 units of Taq DNA polymerase (Gibco BRL), and the PCR buffer supplied with the enzyme. The PCR program included an initial denaturing step at 94°C for 130 s and 35 cycles of denaturing 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 program, the extension step was held for an extra 6 min. A total of 600 to 1000 ng of product were regularly obtained under these conditions. An aliquot of the PCR product was run in a 0.8% agarose gel, stained with ethidium bromide, and quantified using a standard (low DNA mass ladder, Gibco BRL).

DGGE—Denaturing gradient gel electrophoresis was carried out with a DGGE-2000 system (CBS Scientific) as described previously (Díez et al. 2001*a*). Electrophoresis was run in 0.75-mm thick 6% polyacrylamide gels (37.5 : 1 acrylamide : bisacrylamide) with a linear gradient of denaturing agents from 45% to 65% (Díez et al. 2001*a*), where 100% denaturing agent is defined as 7 mol L^{-1} urea and 40% deionized formamide. Around 800 ng of PCR product were loaded in each lane. Electrophoresis conditions were 100 volts for 16 h submerged in 1× TAE (Tris-acetate-EDTA) buffer (40 mmol L^{-1} Tris, 40 mmol L^{-1} acetic acid, and 1 mmol L^{-1} EDTA, pH 7.4) at 60°C. Gels were stained for 30 min in 1× TAE buffer with SybrGold nucleic acid stain (1 : 10000 dilution; Molecular Probes) and visualized under ultraviolet radiation in a Fluor-S MultiImager (BioRad). Usually, two images with integration times of 1 and 3 min were taken from each gel. The first was intended to determine the intensity of the main bands in an unsaturated image. The second was intended to reveal even the faintest bands.

The presence and intensity of DGGE bands was estimated by image analysis using the Diversity Database software (BioRad) as previously described (Schauer et al. 2000; Díez et al. 2001*a*). 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. Bands occupying the same position in the different lanes of the gel were identified. The number of DGGE bands was considered to be the number of operational taxonomic units (OTUs) in each sample. An intensity matrix was constructed with the relative intensity for individual DGGE bands in all samples from DOVETAIL and DHARMA transects separately. These matrices were used to calculate distance matrices using normalized Euclidean distances (root-mean-squared differences, SYSTAT). A dendrogram showing the relationships among samples was obtained by unweighed pair-group method with arithmetic averages (UPGMA) in cluster analysis.

In order to obtain the sequence of DGGE bands, polyacrylamide fragments were excised from the gel using a sterilized razor blade, resuspended in 20 μl of MilliQ water, and stored at 4°C overnight. An aliquot of supernatant was used for PCR reamplification with the same specific primers as before. Between 30 and 50 ng of the reamplified PCR product was used for a sequencing reaction (with the corresponding forward primer) with the Thermo Sequenase v.2 kit (Amersham, US Biochemical), in an ABI PRISM model 377 (v.3.3, Applied Biosystems) automated sequencer. Sequences obtained (300–400 bp) were submitted for checking similarity by BLAST (Altschul et al. 1997).

Results

Description of DOVETAIL and DHARMA transects—Several stations were occupied along two latitudinal transects in the Southern Ocean (Fig. 1). These transects comprised different hydrographic regions crossing well-defined oceanic fronts: the Weddell–Scotia confluence (WSC) in cruise DOVETAIL, and the Weddell–Scotia confluence (WSC), the polar front (PF), and the sub-Antarctic front (SAF) in cruise DHARMA. The distribution of water density (as sigma-t) down to 500 m depth along both transects is shown in Fig. 2.

The DOVETAIL transect showed sharply stratified waters. Surface temperature (data not shown) ranged between −1.8°C close to the ice edge and +1.8°C in the northernmost waters sampled. The DHARMA transect showed a relatively well-mixed water column down to 100 m depth along the whole transect, both north and south of the PF. The hydrographic features of this transect have been described in Doval et al. (2001). Briefly, five regions were crossed along the transect (schematically represented in Fig. 2). Station 1 was at the ice edge (IE), the Weddell Sea waters extended from Stas. 3 to 12. This region, however, was somewhat interrupted by the South Shetland's ridge between Stas. 9 and 11. Station 14 was in the Weddell–Scotia confluence (WSC) and north of it extended the Antarctic zone (AZ) to the polar front, located around Sta. 22. Next, there was the polar fron-

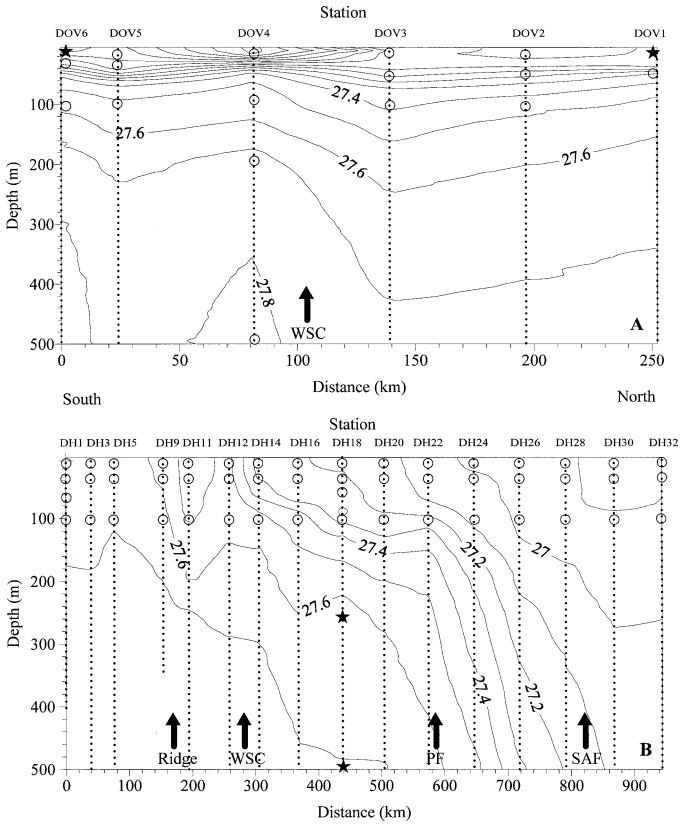

Fig. 2. Distribution of density (sigma-t) with depth and latitude during (A) cruise DOVETAIL and (B) cruise DHARMA. Small dots indicate density data used to build isoclines, and big dots correspond to depths where samples for fingerprinting analysis were taken. Stars indicate depths at which clone libraries were built and published in Díez et al. (2001*b*) for DOVETAIL and in López-García et al. (2001) for DHARMA. In the latter paper libraries from the same station at 2000 and 3000 m were also presented. In both graphs, stations on the left are the southernmost stations. Ridge, South Shetlands ridge; WSC, Weddell–Scotia confluence; PF, polar front; SAF, sub-Antarctic front. Isopycnals units in kg m^{-3}.

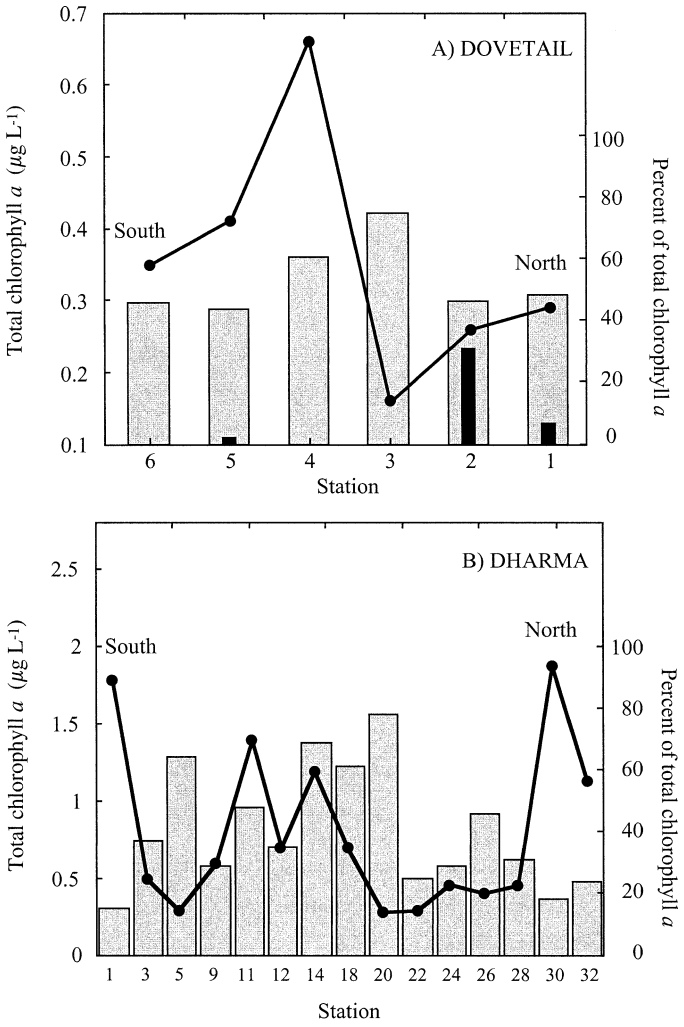

Fig. 3. Concentration of total chlorophyll *a* (dots and lines) in surface samples from (A) DOVETAIL and (B) DHARMA. The bars indicate the percent of total chlorophyll *a* in the fractions smaller than 5 μm (gray) and smaller than 1.6 μm (black).

tal zone (PFZ) from Stas. 22 to 28, the sub-Antarctic front, and the sub-Antarctic zone (SAZ) in Stas. 30 and 32. Temperatures in this transect ranged between −1.5°C in ice-edge waters, around 3°C in the polar frontal zone (PFZ), and +5°C close to the South Antarctic frontal zone (SAF).

Figure 3 shows Chl *a* concentration in the upper mixed layer. In DOVETAIL (Fig. 3A) total Chl *a* was higher in the southern than in the northern stations. Conversely, the percent smaller than 1.6 μm was higher in the northern stations. The percent of Chl *a* passing a 5-μm filter was rather constant along the whole transect (around 50%). In DHARMA (Fig. 3B), Stas. DH1, 11, 14, 30, and 32 showed higher values of total Chl *a*. The maximum in Sta. 1 was due to the effects of the ice melting that stabilizes the water column and allows phytoplankton growth. The maximum in Sta. 11 was probably due to enrichment with coastal waters from the South Shetland Islands. The higher concentrations in Stas. 30 and 32, finally, showed the different conditions in the warmer waters of the SAZ. Stations on both sides of the PF presented the lowest concentrations. Chl *a* in the <5-μm fraction varied considerably between 20% and 80% of the total. The lowest percentage was found in the station closest to the ice edge and in the SAF. The highest percentage was found in Stas. DH14 and DH20, both in the AZ.

Figure 4 shows counts of phototrophic picoeukaryotes obtained by flow cytometry in surface waters along the DHARMA transect. Three populations of differently sized organisms could be identified. The two larger populations, P2 and P3, were present at rather constant numbers along the whole transect (between 400 and 1000 cells ml^{-1} for P2, and between 2 and 200 cells ml^{-1} for P3), whereas the smallest population, P1 (between 300 and 5000 cells ml^{-1}), accounted for the increase in total picoeukaryotic numbers between Stas. DH24 (PFZ) and DH32 (SAZ). In DOVETAIL three different groups of picoeukaryotes were also found in the two stations analyzed by flow cytometry (DOV1 and DOV6, data presented in Díez et al. 2001*b*).

Examination of the samples by inverted microscopy was carried out in order to determine the main nanoplankton and

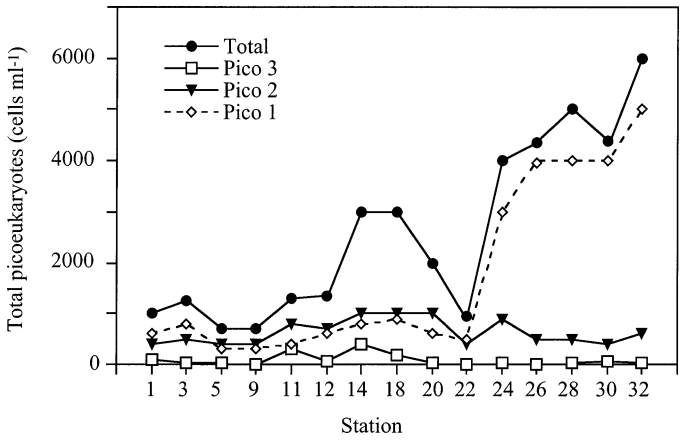

Fig. 4. Abundance of phototrophic picoeukaryotes along the DHARMA transect as determined by flow cytometry. The total numbers and three easily distinguishable populations are shown.

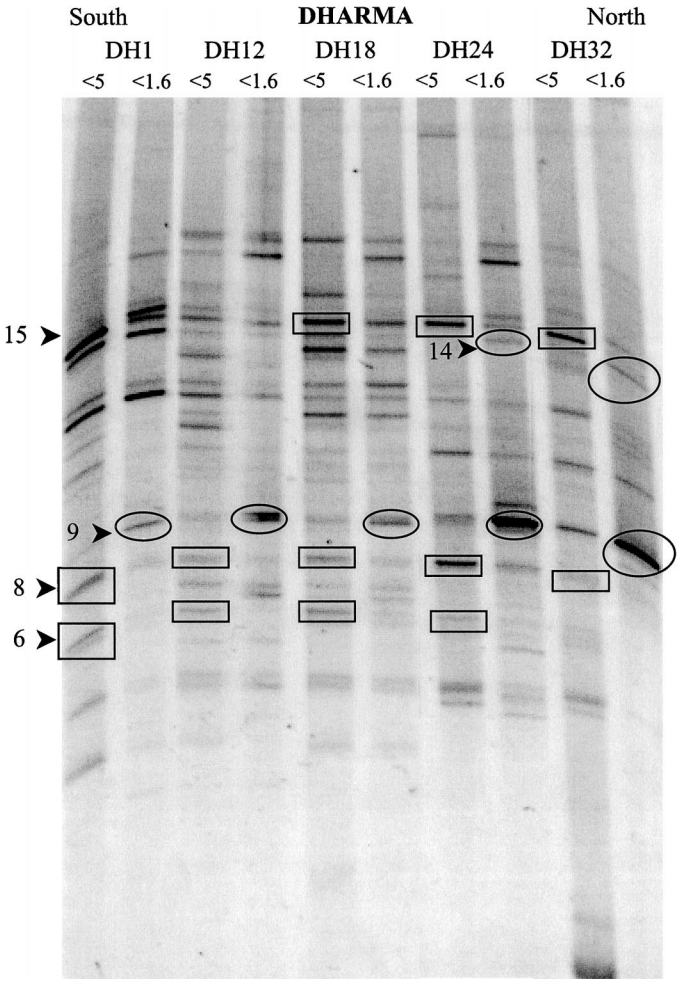

Fig. 5. Negative image of a DGGE gel showing fingerprints for two different size fractions (smaller than 5 μm and smaller than 1.6 μm) at the surface in selected stations along the DHARMA transect. Bands enclosed in a box or an oval correspond to bands identified in other gels and shown in Table 1. Notice that bands 14 (novel stramenopile) and 9 (*Micromonas* RCC434) are more intense in the smaller size fraction (ovals), while the opposite is true for bands 15 (diatom), 6, and 8 (dinoflagellates) (boxes).

microplankton populations. Unidentified small flagellates were the numerically dominant group in both transects. Their concentration in DOVETAIL was approximately 1000 cells ml^{-1} close to the ice edge and 500 cells ml^{-1} toward the WSC. In the DHARMA transect, these small flagellates were found in abundances between 200 cells ml^{-1} in WSC (DH12) and 60–90 cells ml^{-1} in the rest of the stations analyzed (F. G. Figueiras unpubl. data). These numbers are in fact underestimates of the values measured by flow cytometry because the smallest flagellates are very difficult to detect with the Utermöhl technique. The dominant diatoms in both transects were *Corethron criophilum*, *Chaetoceros* sp., *Fragilariopsis* sp., *Pseudo-nitzschia* sp., and *Thalassiosira* sp. *Corethron criophilum* was more abundant close to the ice edge (DOV6 and DH1 to 14), whereas *Fragilariopsis* and *Pseudo-nitzschia* were more frequent away from the ice edge (from DH22 to DH32). We found *Thalassiosira* sp. close to the ice edge in DOVETAIL, but it was homogeneously distributed along the DHARMA transect. Different species of dinoflagellates, essentially gymnodiniales, were distributed more or less homogeneously along both transects. A group of unidentified dinoflagellates, found in concentrations between 60 and 120 cells ml^{-1}, was fairly abundant in DOVETAIL. Cryptophytes were abundant in both transects. Other flagellates, such as *Phaeocystis* sp. and *Pyramimonas*, were only found in DOVETAIL. Some ciliates belonging to the genus *Strombidium* were also found in both transects.

Influence of filter size on DGGE band patterns—In DOVETAIL we had used 1.6-μm prefilters. In DHARMA, we had decided to use larger prefilters in order to get all possible picoeukaryotes. Thus, comparison between the two cruises was complicated. In order to define the influence of these different prefilters on the DGGE band patterns, we compared the fingerprints obtained from DHARMA surface samples prefiltered through 1.6- and 5-μm filters (Fig. 5). Although most bands appeared in both size fractions, the intensities of many bands were quite different. Presumably, the more intense bands in the lower size fraction represented the smallest organisms and vice versa. For example, as will be considered in detail in the discussion, bands 9 and 14 were more intense in the <1.6-μm fraction, while bands 6, 8, and 15 were more intense in the larger size fraction. In effect, when sequenced, bands 9 and 14 corresponded to the prasinophyte *Micromonas* and to a novel stramenopile, respectively. Both organisms are of picoplankton size. On the other hand, the other three bands corresponded to two dinoflagellates and a diatom, which are usually larger organisms. At any rate, this influence of prefilter on band patterns must be taken into account when comparing results from both cruises.

Vertical stratification of DGGE band patterns—DGGE patterns in DOVETAIL were different for each depth (Fig. 6). Differences were very clear between surface and 100-m samples, but they were also apparent between the two upper depths sampled. This was consistent with the sharp stratifi-

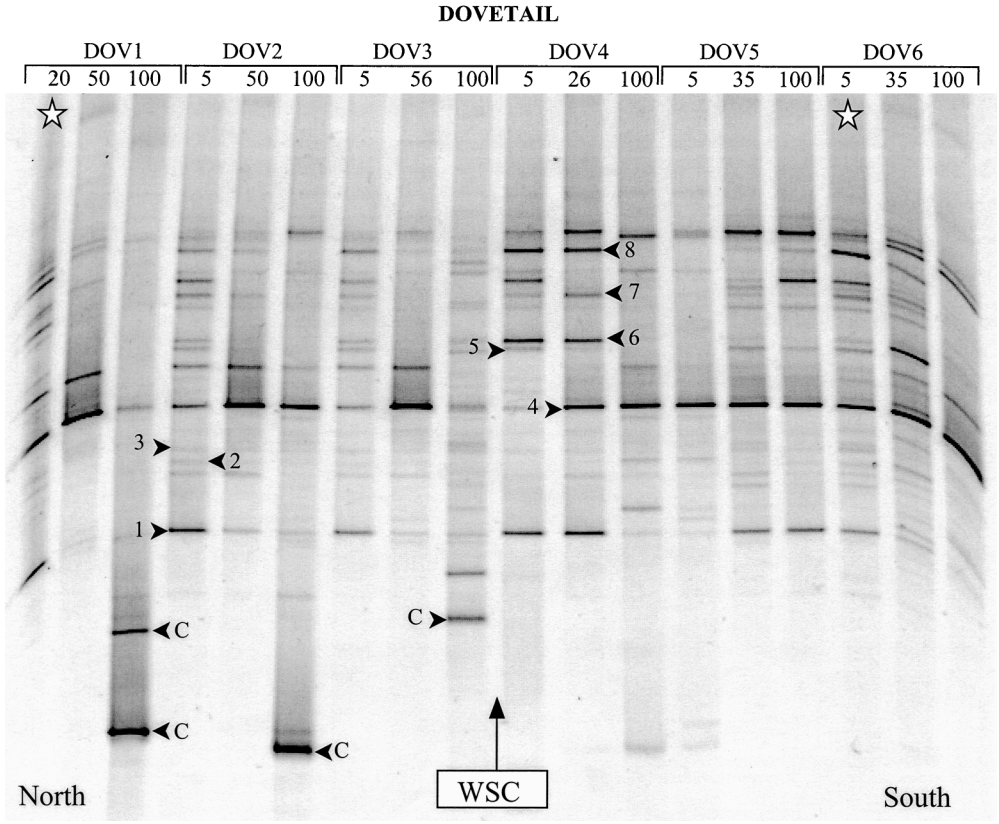

Fig. 6. Negative image of a DGGE gel showing fingerprints of the six DOVETAIL stations at three depths: surface, bottom of the mixed layer, and 100 m. Bands that were sequenced are indicated by a number that corresponds to numbers in Table 1. Clone libraries for the surface samples in DOV1 and DOV6 (white stars) have been published separately (Díez et al. 2001*b*). Bands marked with a C corresponded to copepods.

cation of the water column found during this cruise (Fig. 2A). In DHARMA, on the other hand, the DGGE band patterns were very similar from the surface down to almost 100 m during the whole transect (DGGE gel not shown). A more detailed vertical profile at a single station (Fig. 7) shows that significant differences appeared mostly below 250 m. The relatively large similarity among the upper depths was consistent with the structure of the water column during this cruise, with the upper layer mixed at least down to 100 m (Fig. 2B).

Latitudinal changes of DGGE band patterns—Given the vertical distribution of picoeukaryotic assemblages in the upper layers of the water column, we decided to include two depths of the DOVETAIL transect (surface and bottom of mixed layer, between 26 and 56 m deep, Fig. 6) and only the surface samples of the DHARMA transect (Fig. 8) for the analysis of latitudinal changes. For both cruises, the total number of bands in these samples ranged between 11 and 14 (or between 22 and 25, if bands accounting for $<1\%$ of intensity are considered), indicating the existence of complex and diverse assemblages.

DGGE band patterns were used to build dendrograms that compare the grouping of picoeukaryotic assemblages in both DOVETAIL and DHARMA samples (Fig. 9). In the case of DOVETAIL, one cluster included the surface samples from Stas. 1, 2, and 3 and both depths from Sta. 4 (Fig. 9A). A second cluster included the ''deep'' samples from Stas. 1, 2, and 3 exclusively. And the last cluster grouped all samples from the stations closest to the ice edge (Stas. 5 and 6). This third cluster was closer to the ''deep'' cluster. This clustering of samples is consistent with the hydrography of the area (Fig. 2A) and indicates a clear change in the composition of the assemblages following a spatial gradient (offshore–ice edge) and a vertical gradient in the water column. In the case of DHARMA, dendrograms from DGGE showed clustering of samples consistent with the typical hydrography across the PF (Fig. 9B). Thus, stations formed two main clusters, one with stations south of the PF (1 to 18) and the other with stations close to, and north of, the PF (Stas. 20 to 32). Within each cluster, smaller clusters were also consistent with the water masses crossed along the transect (compare Figs. 9B and 2B). These patterns will be analyzed in more detail in the Discussion section.

Taxonomical identity of the DGGE bands—DGGE gels showing the fingerprints across the two transects were scanned for the most important bands (in terms of intensity and number of samples in which they were present). These were cut and sequenced. We sequenced eight bands in DOVETAIL and fifteen bands in DHARMA (Table 1). In DOVETAIL, three bands could be assigned to novel stra-

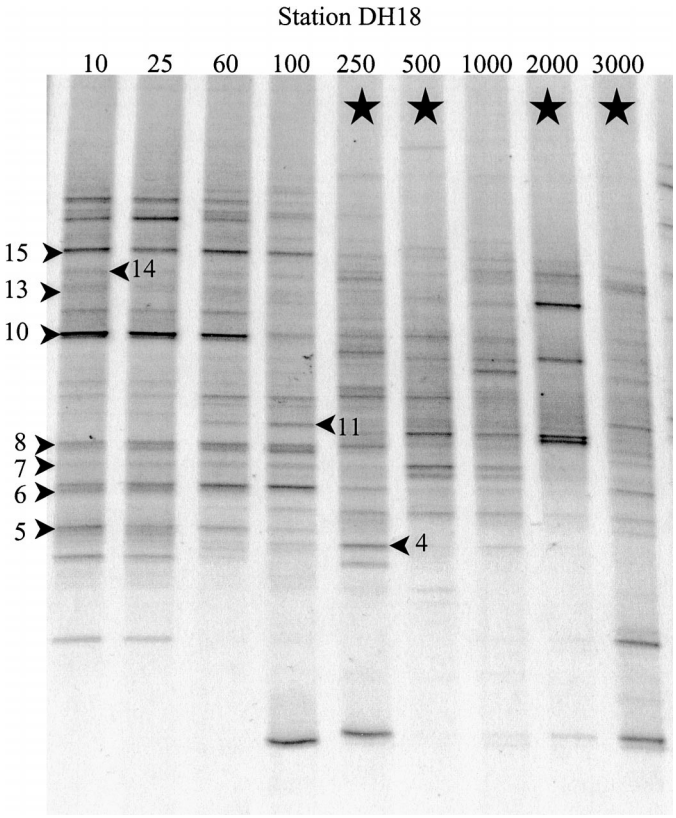

Fig. 7. Negative image of a DGGE gel showing a vertical profile fingerprint at Sta. DH18 from the surface down to 3000 m. Bands that were sequenced are indicated by a number that corresponds to numbers in Table 1. Clone libraries for the samples at 250, 500, 2000, and 3000 m (black stars) have been published in a separate study (López-García et al. 2001).

menopiles, two bands to the prymnesiophytes, and one each to prasinophytes, cercomonads, and novel alveolates. All these groups were also present in DHARMA. Some of these groups, such as prymnesiophytes and prasinophytes, are well-known components of the small Antarctic plankton. Other sequences, however, belong to previously unknown groups that have been discovered only recently, such as the novel alveolates and stramenopiles. Additionally, in the DHARMA transect we also found five bands affiliating to the dinoflagellates, one to diatoms, and one to cryptophytes. These groups are known to generally include larger cells than the previous ones, and their appearance in this transect and not in DOVETAIL was most likely due to the larger size fraction analyzed. This reasoning was partially confirmed by checking the identified bands in the gel shown in Fig. 5. Bands 9 and 14, which were more intense in the $<$1.6-μm fraction, were related to the prasinophyte *Micromonas* RCC434 and to a novel stramenopile. *Micromonas* RCC434 is known to be a very small eukaryote (Guillou et al. in press), and recent data indicate that at least some novel stramenopiles are also 2 to 4 μm in diameter (Massana et al. 2002). Both bands were also well represented in the DOVETAIL transect. Bands 6, 8, and 15, on the other hand, were more intense in the $<$5-μm than in the $<$1.6-μm fractions. These three bands were identified as dinoflagellates and diatoms, which were absent from DOVETAIL but frequent in DHARMA. All described dinoflagellates are larger than 1.6 μm in diameter (Thomsen 1986). Therefore, the presence and relative intensity of these bands is consistent with the size of the known organisms and with the fractionation scheme used in each case.

In addition to these bands, we also found a few bands corresponding to copepods (labeled C in Fig. 6), and in particular DHARMA band 1 could be attributed to *Calanus propinquus.* Obviously, copepods are larger than 5 μm, and the appearance of bands with such sequences must be due to small body or egg fragments that went through the several nets (200 and 50 μm) and the prefilters. Appearance of macroorganisms occurs with a certain frequency in molecular studies of small eukaryotes, and their presence in the smaller size fraction shows that other unknown sequences cannot be automatically assigned to picoeukaryotes. Comparisons such as those in Fig. 5, probe development and analysis of samples by fluorescence in situ hybridization (FISH, fluorescence in situ hybridization; Massana et al. 2002) or, even better, isolation in pure culture, are necessary to clarify the size of the organisms behind a given sequence.

If we accept that the relative intensities of the bands are an indicator of the relative importance of the corresponding organisms in each assemblage (*see Discussion*), the relative changes in composition along the transects can be analyzed as shown in Fig. 10. In DOVETAIL, prasinophytes, prymnesiophytes, and novel stramenopiles were important in essentially all stations (Fig. 10A). Cercomonads and novel alveolates were present in lower proportions. The same groups were again dominant in the gels from DHARMA, with the addition of dinoflagellates and diatoms. In DHARMA there were clear trends with latitude in the proportions explained by some groups (Fig. 10B). Thus, dinoflagellates decreased in importance from the ice edge toward the north, while prymnesiophytes followed the opposite trend. The novel alveolates were present in small abundance along the transect. The two prasinophytes detected were important on opposite sides of the polar front: *Pyramimonas* to the south (*see* intensity of band 10 in Fig. 8) and *Micromonas* to the north (band 9 in Fig. 8). The two together made a significant fraction of total band intensity throughout the transect (Fig. 10B). Finally, diatoms and novel stramenopiles seemed to be present in similar proportions in most of the samples along the transect.

Discussion

Changes in the taxonomic composition of picoeukaryotic assemblages—Despite the fact that different ecotypes have been discovered (Moore et al. 1998), phototrophic prokaryotes in the ocean are closely related phylotypes of only two genera of cyanobacteria: *Synechococcus* and *Prochlorococcus.* This predominance of a few phylotypes in widely distant areas of the oceans seems to be even more marked in the case of marine archaea, where a single phylotype was found to be dominant in 15 clone libraries from the Southern, the Pacific, and the North Atlantic Oceans and the Mediterranean Sea (Massana et al. 2000). For heterotrophic bac-

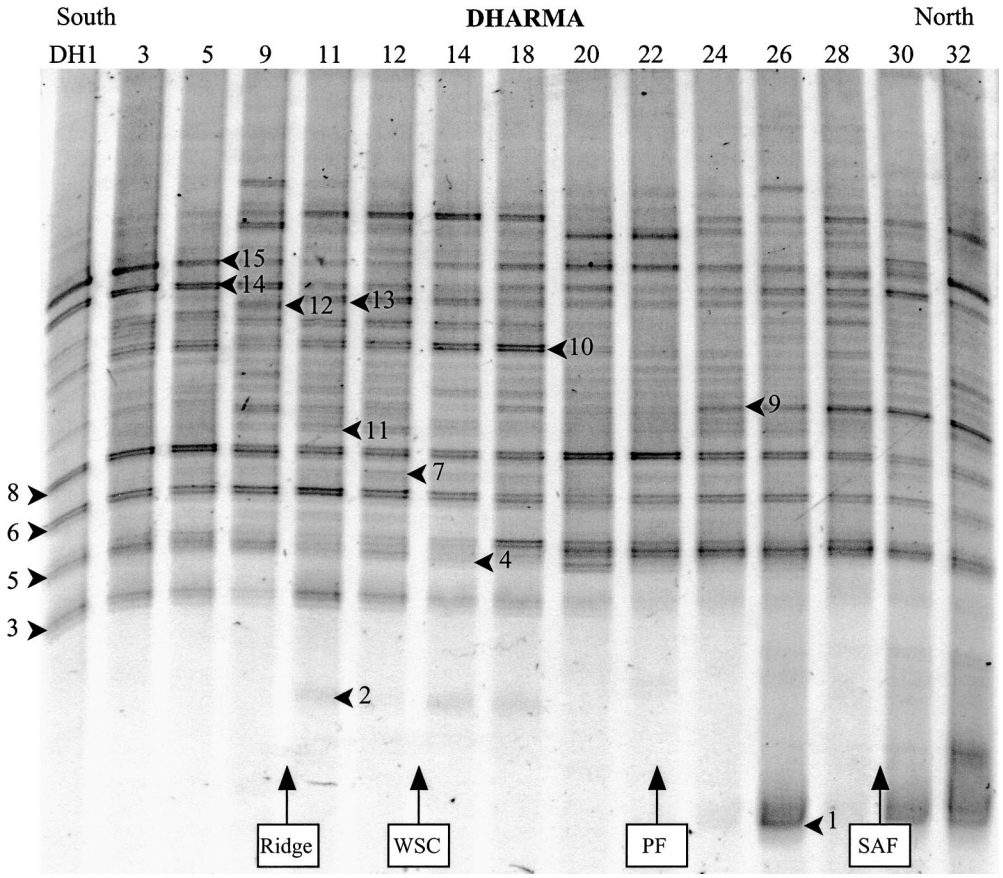

Fig. 8. Negative image of a DGGE gel showing fingerprints for the surface samples along the DHARMA transect. Bands that were sequenced are indicated by a number that corresponds to numbers in Table 1. Ridge, South Shetlands ridge; WSC, Weddell–Scotia confluence; PF, polar front; SAF, sub-Antarctic front.

teria, on the other hand, assemblages are always composed by a large variety of phylotypes (Giovannoni and Rappé 2000). The question of whether the same was true for the eukaryotic picoplankton remained unanswered until cloning and sequencing with eukaryotic primers was applied to size fractionated samples (Díez et al. 2001*b*; López-García et al. 2001; Moon-van der Staay et al. 2001). These three studies clearly showed that the case of marine picoeukaryotes, both autotrophic and heterotrophic, was similar to that of heterotrophic bacteria and different from those of archaea and cyanobacteria, in the number of different taxons present in any given sample. However, these studies were carried out with only 10 samples. The spatial scales at which the composition of the assemblages changed, therefore, remained unknown. Thus, in the present paper we were interested in analyzing how the composition of picoeukaryotic assemblages changed in space.

For this purpose we used the fingerprinting technique DGGE followed by sequencing of the main bands. As with all other PCR-based techniques, DGGE is subject to several biases that have been extensively discussed in the literature. At the very minimum, the presence of a given sequence in the DGGE gel proves the presence of the corresponding organisms in the natural sample. It is well known that the band intensity is not directly proportional to the abundance of the organism. Thus, a band intensity of, say, 20% does not necessarily mean an abundance of 20% of the corresponding cells in nature. However, the relative changes in intensity within a set of samples that have been processed together in the same PCR reaction, and in the same gel, do show changes in the relative importance of the organisms in nature. We have shown that this is the case for cytophagas, cyanobacteria, and sulfur phototrophic bacteria in karstic lakes (Casamayor et al. 2002), and cyanobacteria (Schauer et al. 2003) and the prasinophyte *Micromonas* (Not et al. unpubl. data) in coastal marine environments. Therefore, we feel justified in using the band intensities in Fig. 10 as indicators of changes in the relative importance of different organisms along the studied transects.

We will discuss next the distribution and composition of the different phylogenetic groups identified in the DGGE gels.

Prasinophytes: This was one of the most widely represented groups in our gels. DGGE bands belonging to this algal group accounted for 10–40% of the total band intensity in DOVETAIL. The corresponding percentages in DHARMA were 1–12%. The most frequently retrieved prasinophyte was close to *Micromonas* (DGGE bands 4 in DOVETAIL and 9 in DHARMA). *Micromonas* (approximately 2–3 μm

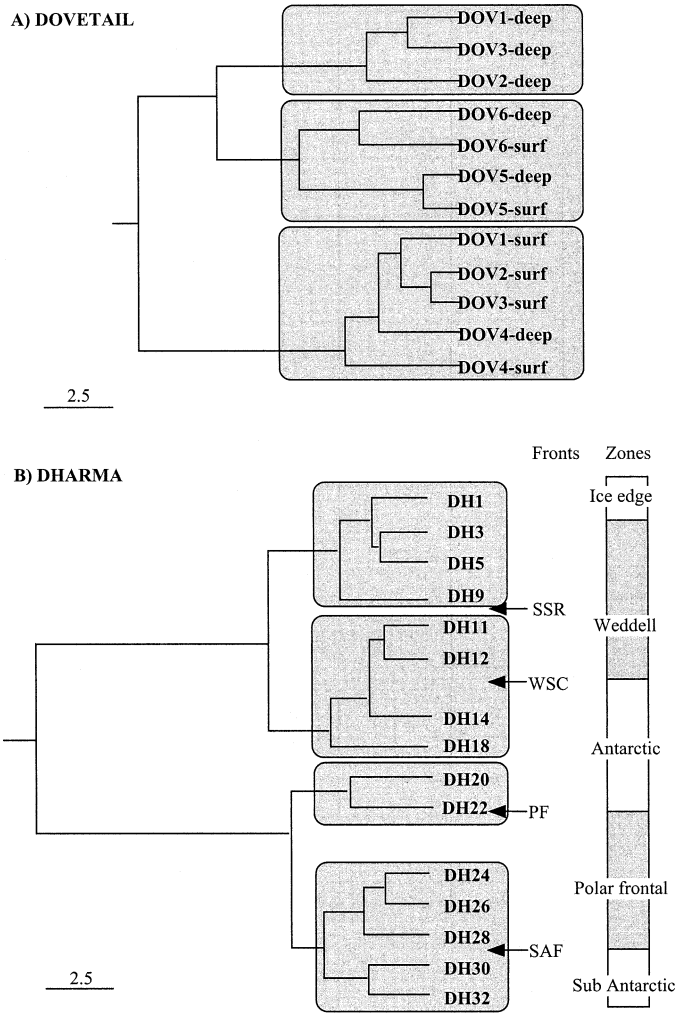

Fig. 9. Dendrograms obtained from DGGE fingerprints clustering the samples from cruises (A) DOVETAIL and (B) DHARMA. Two upper depths were taken into account in DOVETAIL (surf and deep) and only one depth (surface) in DHARMA. The shaded boxes indicate the different clusters found. In panel B, the zones and fronts crossed are indicated.

in diameter) is a cosmopolitan flagellate genus that has been already reported from polar waters by microscopy (Throndsen and Kristiansen 1991). The sequence was very similar to that of clone ME1-2 obtained in a library from the Mediterranean Sea (Díez et al. 2001*b*) and to *Micromonas* RCC434, a prasinophyte we have recently isolated in pure culture from coastal Mediterranean waters (Guillou et al. in press). When we run the ME1-2 clone DNA in a DGGE gel it migrated to the same position as one of the major bands from environmental samples from the Mediterranean (fig. 6 in Díez et al. 2001*a*), the North Atlantic (fig. 1 in Díez et al. 2001*b*), and the Southern Ocean, both in DOVETAIL (band 4, Fig. 6) and in DHARMA (band 9, Fig. 8). Therefore, *Micromonas* RCC434 appears to be a very abundant and widespread prasinophyte.

In a related study (Díez et al. 2001*b*) we constructed clone libraries with surface samples from the stations at both ends of the DOVETAIL transect (DOV1 and DOV6). These two libraries produced numerous clones of prasinophytes. In particular, 16 clones from Sta. DOV6 and 5 from Sta. DOV1 could be assigned to clone ME1-2. Therefore, two different molecular techniques indicated that these flagellates were important members of the picoeukaryotic assemblage. The primers used in cloning were different from those used for DGGE, and, yet, the same sequence was retrieved in significant amounts.

The results of flow cytometry in DHARMA also suggest that *Micromonas* RCC434 was a very important component of the picoplankton. In effect, a significant correlation ($r^2 = 0.740$) was found between the relative abundance of *Micromonas* RCC434 in DGGE gels and the P1 population abundance obtained by flow cytometry (Fig. 4). Thus, we postulate that the P1 population corresponds to RCC434. If this were the case, *Micromonas* RCC434 would be the most abundant picoeukaryotic population detectable by flow cytometry.

In DHARMA another prasinophyte related to *Pyramimonas* (DGGE band 10) was fairly important. The cosmopolitan *Pyramimonas* (approximately 6 × 4 μm) can be identified at the genus level by inverted microscopy, and it has been shown to contribute significantly to phytoplankton biomass in some Antarctic waters (Estrada and Delgado 1990). In effect, we found about 130 cells ml^{-1} of *Pyramimonas* in Sta. DOV6, but we could not detect it at Sta. DOV1 by microscopy. The absence of this sequence from the DOVETAIL gels is likely due to the prefiltration step through 1.6 μm.

Micromonas RCC434 accounted for a very significant fraction of total band intensity in the DOVETAIL transect from the surface down to at least 100 m in depth (Fig. 6). In DHARMA, the band corresponding to *Pyramimonas* showed increasing intensity from Sta. DH1 to DH18 and then disappeared (Fig. 8). *Micromonas* RCC434, on the other hand, was most abundant from Stas. DH24 to DH32. Thus, these two prasinophytes were found on opposite sides of the polar front. Preliminary results obtained by HPLC pigment analysis in DHARMA (M. Latasa unpubl. data) showed that pigments characteristic of prasinophytes, chlorophyll *b*, lutein, and prasinoxanthin, were important in the <5-μm fractions along the transect.

Prymnesiophytes: This was another dominant group of picoeukaryotes. DGGE bands 1 and 2 in DOVETAIL and band 5 in DHARMA were related to *Phaeocystis.* Their relative abundance reached 30% of the total band intensity in both transects, showing that these bands represented one of the most important members of the picoeukaryotic assemblage. In the DHARMA transect *Phaeocystis*-like sequences increased in representation from the ice edge toward more northern samples, while the opposite was true in DOVETAIL (Fig. 10).

In the same study mentioned above (Díez et al. 2001*b*) we found 5 clones from Sta. DOV6 and 17 from Sta. DOV1 that could be assigned to *Phaeocystis.* Again, two different molecular techniques indicated that these flagellates were important members of the picoeukaryotic assemblage. No clone libraries were constructed with surface samples from DHARMA. Results from DGGE, however, can be compared

Table 1. Sequence similarities of the DGGE bands excised from gels in Figs. 5 to 8.

Band no.	Closest match	Squence similarity (%, no. of bases)	Taxonomic group	Band intensity (%)	
				Average	Range
DOVETAIL					
1	*Phaeocystis antarctica*	87.9 (240)	Prymnesiophytes	13	1–22
2	*Phaeocystis antractica*	99.1 (212)	Prymnesiophytes	1	0–2
3	Clone ME1-10	85.2 (216)	Novel Alveolates group II	2	0–3
4	Clone ME1-2 (*Micromonas*)	98.5 (410)	Prasinophytes	18	12–43
5	Clone ME1-22	89.3 (149)	Novel Stramenopiles cluster 1	4	0–5
6	*Cryothecomonas aestivalis*	91.0 (288)	Cercomonads	5	0–16
7	Clone OLI11006	91.2 (411)	Novel Stramenopils cluster 3	4	1–7
8	Clone ME1-24	86.1 (108)	Novel Stramenopiles	10	0–14
DHARMA					
1	*Calanus propinquus*	99.5 (418)	Copepoda	ND	ND
2	*Geminigera cryophila*	95.3 (318)	Cryptophytes	<1	<1
3	*Gymnodinium catenatum*	98.7 (451)	Dinoflagellates	6	0–16
4	*Amphidinium semilunatum*	87.4 (421)	Dinoflagellates	2	0–11
5	*Phaeocystis antarctica*	94.1 (220)	Prymnesiophytes	12	0–25
6	*Gymnodinium* sp.	87.3 (142)	Dinoflagellates	9	3–17
7	Clone DH144-EKD3	91.6 (320)	Novel Alveolates group 1	1	0–3
8	*Gymnodinium* sp.	97.9 (435)	Dinoflagellates	12	5–16
9	*Micromonas* sp.	99.0 (401)	Prasinophytes	4	0–10
10	*Pyramimonas* sp.	97.9 (339)	Prasinophytes	5	1–13
11	*Pentapharsodinium tyrrhenicum*	96.3 (240)	Dinoflagellates	2	0–4
12	Uncultured Chrysophyte	93.2 (132)	Chrysophytes	1	0–4
13	*Cryothecomonas aestivalis*	87.0 (169)	Cercomonads	2	1–9
14	Clone DH144-EKD10	96.9 (256)	Novel Stramenopiles cluster 1	6	3–12
15	*Fragillariopsis sublineata*	89.1 (368)	Diatoms	7	0–15

to a detailed study of phytoplankton pigments by HPLC carried out during the same cruise and using the same size fractions (M. Latasa pers. comm.). In those preliminary results the 19′-hexanoyloxyfucoxanthin, pigment marker to prymnesiophytes, was rather abundant and more or less homogenously distributed along the transect, increasing a little at the northernmost stations.

Dinoflagellates and novel alveolates: Some DGGE sequences were related to dinoflagellates (bands 3, 4, 6, 8, and 11 in DHARMA). These bands showed variable degrees of similarity to the sequences of the dinoflagellates *Gymnodinium* (bands 3, 6, and 8), *Amphidinium* (band 4), and *Pentapharsodinium* (band 11). These bands accounted for a significant percentage of total band intensity (30% on average). Many of the bands were also related to environmental clones of dinoflagellate sequences recently found in the Pacific and the Southern Ocean (López-García et al. 2001; Moon-van der Staay et al. 2001). The sequence derived from band 8 from DHARMA (403 bases), for example, had 99.2% similarity with environmental clone DH148-5-EK46. Bands 6 and 11 might also belong to the same phylogenetic group, but the total length sequenced and the similarity values were lower than those for band 8. Clone DH148-5-EK46 was retrieved from 3000 m in Sta. DH18 of the DHARMA transect. Initially, it was thought to be a deep living organism (López-García et al. 2001). Its presence in surface samples from all stations on the DHARMA transect (bands 6, 8, and 11 in Fig. 8), however, indicates that its most likely environment is the surface layer of the ocean. In fact, the same band can be seen at all depths in Sta. DH18 (Fig. 7). The absence of dinoflagellate sequences in DOVETAIL was consistent with the known dimensions of all described dinoflagellates larger than the 1.6-μm prefilter used.

Finally, two DGGE bands (band 3 from DOVETAIL and band 7 from DHARMA) were associated with a recently described group of novel alveolates. Band 3 from DOVETAIL showed a certain degree of similarity to clone ME1-10 from the southwestern Mediterranean (Díez et al. 2001*b*) and DH148-EKD27 from a Southern Ocean library (López-García et al. 2001). These clones belong to the novel alveolates group II. The DHARMA band, in turn, was closest to clone DH144-EKD3 retrieved from 250 m depth at Sta. DH18 also from the same Southern Ocean library. This clone belongs to the novel alveolates group I. Both novel alveolate bands contributed significant, but relatively low, percentages to the total band intensity (less than 5%).

Bands from dinoflagellates and novel alveolates together accounted for 30–40% of total band intensity in DHARMA and less than 5% in DOVETAIL. Since DOVETAIL samples were prefiltered through 1.6-μm and DHARMA through 5-μm filters, the difference in abundance between the two cruises is likely due to many of these organisms being between 1.6 and 5 μm in diameter and not to other ecological factors. Some support for this explanation can be found in the comparison between size fractions in Fig. 5, where dinoflagellate bands 6 and 8 are always more intense in the larger size fraction.

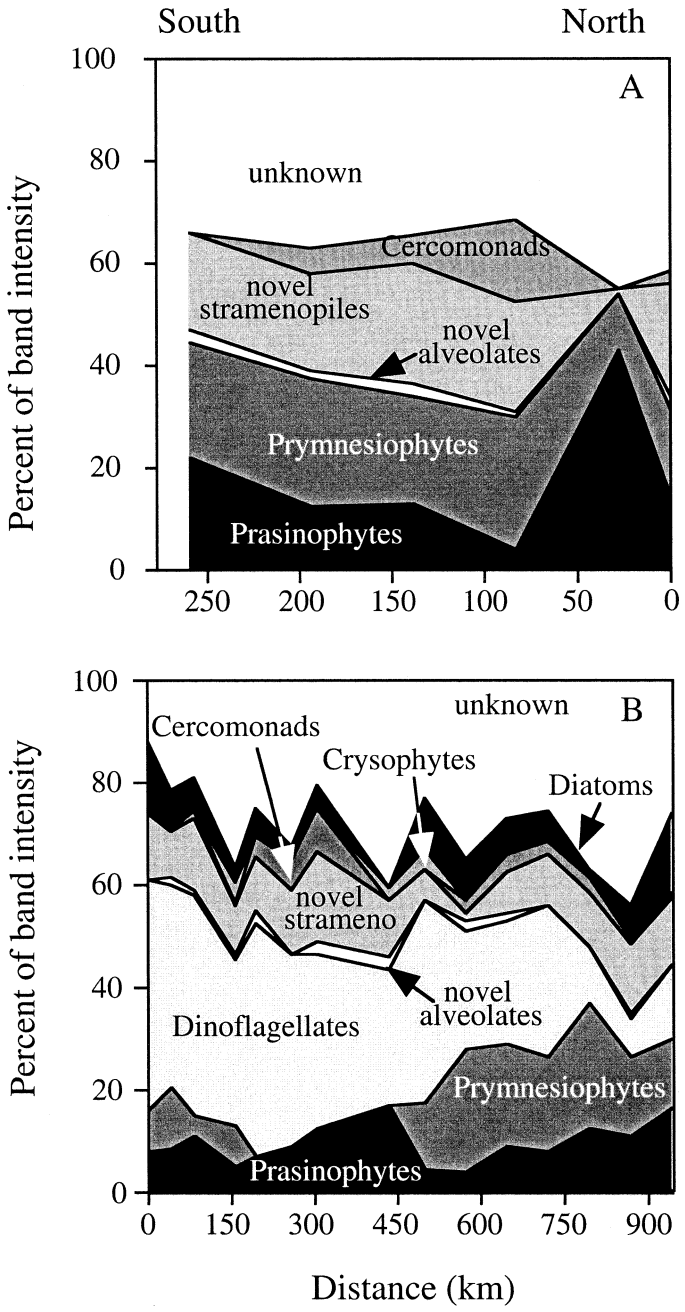

Fig. 10. Percent of the total band intensity in DGGE gels accounted for by different groups of eukaryotes for cruises (A) DOVETAIL and (B) DHARMA.

Novel marine stramenopiles (MAST): Another recently described group of picoeukaryotes is the novel marine stramenopiles (MAST, Díez et al. 2001*b*; Moon van der Staay et al. 2001; López-García et al. 2001; Massana et al. 2002). Bands 5, 7, and 8 from DOVETAIL and band 14 from DHARMA could be assigned to this group. Together, these bands accounted for 17% of the total band intensity in DOVETAIL and 10% in DHARMA. In the two DOVETAIL libraries (Díez et al. 2001*b*) we recovered 11 and 23 clones belonging to these groups (19% and 34% of clonal representation). These sequences are so distant from any cultured organisms that they form completely new lineages (Massana et al. 2002). The fact that they were so abundant in samples from DOVETAIL, both in the clone libraries and as percent of total band intensity in DGGE gels, indicates that the organisms behind these sequences must be truly picoplanktonic. This is further supported by the larger relative intensity of band 14 from DHARMA in the <1.6-μm than in the <5-μm size fractions (Fig. 5). Finally, Massana et al. (2002) have recently shown by FISH with specific probes that at least two of these novel stramenopile clusters are of picoplanktonic size.

Diatoms: Diatoms formed a conspicuous component of the phytoplankton at all stations when examined by inverted microscopy. These cells, however, were in general larger than our prefilters. A parallel study of pigment concentration along the DHARMA transect (M. Latasa pers. comm.) showed that only 10% to 15% of fucoxanthin, the marker pigment of diatoms, went through 5-μm filters. Thus, we only found one DGGE band that could be assigned to diatoms (band 15) in DHARMA (prefiltered through 5 μm) and none in DOVETAIL (prefiltered through 1.6 μm). The sequence obtained from this band showed a low similarity to *Fragillariopsis* (Table 1). Some members of this genus are indeed very small in size, but most of their sequences are not available.

In a separate study we recovered two diatom clones from DOV1 and nine from DOV6 (Díez et al. 2001*b*). These sequences showed between 89% and 95.9% similarity to *Corethron cryophilum* (seven clones), 86.7% to *Chaetoceros* sp. (two clones), or 96.8% to *Skeletonema costatum* (one clone). One last clone was 98.6% similar to *Pseudo-nitzschia multiseries.* All of these diatoms are large celled and they are unlikely to get through the 1.6-μm prefilters used in this study. Our clones could have picked DNA from broken cells or from flagellated life stages. Alternatively, small-celled relatives of these diatoms might exist in Antarctic waters. We do not have enough information to discriminate among these possibilities.

Other groups: The Cercomonads are a little-known group of small heterotrophic flagellates with several strains isolated in pure culture. A few clones of these organisms appeared in the clone libraries we built from surface waters of the Southern Ocean, the Mediterranean, and the North Atlantic (Díez et al. 2001*b*). They showed a relatively low similarity to cultured organisms and may, therefore, be new members of the cercomonads. The two clones from the Antarctic libraries were closest to *Thaumatomonas.* The two DGGE bands (DOVETAIL band 6 and DHARMA band 13), on the other hand, had low similarity to *Cryothecomonas.* In both cases, similarities are so low that the organisms responsible for the new retrieved sequences are probably unrelated to cultured microorganisms. The DGGE fingerprints showed these sequences to be present in essentially all surface samples (Fig. 10), although they always represented a relatively small percentage of the total band intensity (between 1% and 10%, Table 1). Their contribution to band intensity appeared to decrease with depth at least in DOVETAIL (data not shown). Finally, bands belonging to chrysophytes and cryp-

tophytes appeared in a few samples but always accounted for a very small percent of total band intensity.

Spatial scales in the distribution of picoeukaryotic assemblages—The use of a fingerprinting technique allowed examination of enough samples to determine the spatial scales at which given picoeukaryotic assemblages are distributed in the oceans. The DHARMA transect was particularly adequate for this purpose, since several hydrographic features were included. As can be seen in Figs. 8 and 9, band patterns did not change at random, but in an orderly manner: stations close to each other in space tended to cluster together, and different clusters tended to group samples from different zones. Starting from the north, the sub-Antarctic front clearly separated two assemblages: one in the SAZ (Stas. 30 and 32) and a different one in the PFZ (Stas. 24 to 28). This difference in taxonomic composition coincided with a large difference in biomass, since the SAZ waters had about four times more chlorophyll than those of the PFZ. Assemblages from waters close to the polar front (Stas. 20 and 22) formed the next cluster. This cluster was more similar to those of the PFZ and the SAZ than to the Antarctic waters to the south. Apparently, the main biogeographical boundary for picoeukaryotes was south of the PF. The next cluster included stations between the PF and the South Shetlands ridge (Stas. 11 to 18). Within this cluster, two subclusters formed by stations north and south of the Weddell–Scotia confluence could be identified, indicating that this structure was also responsible for some changes in the taxonomic composition of the eukaryotic picoplankton. Stations south of the South Shetlands ridge, finally, formed another cluster. Within this cluster, the stations closest to the ice edge (Sta. 1) and closest to the South Shetlands ridge (Sta. 9) differed from the other two stations, as could be expected from the influence of the ice edge and the islands, respectively. The only discrepancy between DGGE band patterns and hydrography was in the Antarctic zone. While hydrography indicated a relatively homogeneous water mass in this zone, the picoeukaryotic assemblage of the northern station (Sta. 20) clearly differed from the others and was more similar to that of the polar front station (Sta. 22). Altogether, the distribution of particular assemblages followed the hydrography of the area quite closely.

Both the question of whether samples are representative of a given area and that of distribution could be answered by using a fingerprinting technique. At least in the area of the Southern Ocean studied, picoeukaryotic assemblages were characteristic of each water mass. Assemblages were distributed over hundreds of kilometers horizontally and over dozens or hundreds of meters vertically, in accordance with the extension of particular water masses. Clearly, therefore, individual samples can be considered as representative of the particular water mass sampled. Neither of these conclusions relies on the polemical semiquantitative use of DGGE. Rather, they depend exclusively on the use of DGGE as a fingerprinting technique and can, thus, be considered as robust.

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