Capítulo 4

COMPOSITION AND DISTRIBUTION OF EUKARYOTIC PICOPLANKTON ACROSS HYDROGRAPHIC FRONTS IN THE SOUTHERN OCEAN

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En preparación.

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Abstract

Two different molecular fingerprinting techniques were used to analyze the distribution and composition of eukaryotic picoplankton along two latitudinal transects in the Southern Ocean. First, primers specific for eukaryotic 18S rDNA were used in a polymerase chain reaction (PCR). The amplification products were then subjected to denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphism (T-RFLP) in order to compare the different picoeukaryotic assemblages. Transect DOVETAIL (44°W) went from the ice-edge (at 60°S) across Weddell–Scotia confluence and north to 58°S and was sampled in January 1998 (summer). Transect DHARMA (between 53ºW and 58ºW) went from the ice edge in the Weddell Sea (63°S) across the Drake Passage to the South American continental plataform (55ºS). It was sampled in December 1998 (late spring). DGGE band patterns and T-RFLP chromatograms were used to build dendrograms combining samples from each cruise. Samples grouped in several distinct clusters that were generally consistent with the hydrography of the area. The upper mixed layer showed the same composition of the eukaryotic picoplankton at all depths. The most dominant DGGE bands were excised and sequenced, and were identified as belonging to *Mantoniella antarctica* and *Phaeocystis antarctica*. Assignment of T-RFLP fragments was consistent with these identifications. The relative abundance of these OTUs could then be reconstructed for both transects. Other

well known components of the plankton such as dinoflagellates an diatoms were also present. A significant number of sequences were related to novel phylogenetic groups, including novel stramenopiles and alveolates or to poorly known microorganisms such as cercomonads. Obviously, these latter groups could not have been detected or followed with any other technique. Thus, these two fingerprinting techniques were proven to be useful for a rapid evaluation of the spatial distribution of picoeukaryotic assemblages in the oceans.

Introduction

Marine picoeukaryotes (between 0.2 and 2-3 μm in diameter) are probably 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 global mineral cycles (Li 1994, Fogg 1995). Marine picoeukaryotes seem to belong to widely different phylogenetic groups, but the extent of their diversity and the distribution and abundance of the different taxons in situ remain unknown (Partensky et al. 1997). In the open oceans most picoeukaryotes are coccoid or flagellated forms, with or without chloroplasts (phototrophic or heterotrophic, respectively), and with few morphologically distinct features (Thomsen 1986, Simon et al. 1994, Caron et al. 1999). In this way, 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 SSU rRNA genes (Amann et al. 1995, Partensky et al. 1997). Three very recent papers describe 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. 2001a). These studies have revealed a high phylogenetic diversity of these assemblages and the presence of novel lineages. Yet, these studies were carried out with extremely few samples. The cloning approach was used in just 10 samples, a ridiculous number compared to the whole ocean. Are these samples representative of whole ecosystems or are they peculiar to the spots that were casually sampled? And, how are the picoeukaryotic assemblages distributed in the ocean? Do they conform to water

masses or do they make a very homogeneous assemblage across hydrographical structures? These questions of representativenes and of distribution in the sea require analysis of many samples. This cannot be achieved by cloning and sequencing, but it can be addressed through the use of molecular fingerprinting techniques.

We have shown that DGGE and T-RFLP provide a very reasonable estimate of the abundance of the dominant members of natural microbial assemblages including bacteria (Casamayor et al. 2000) and eukarya (Díez et al. 2001b). Here we illustrate how this fingerprinting approach can be used to describe the distribution of eukaryotic picoplankton in relation to large-scale hydrographic features. We chose two transects in the Southern Ocean as examples, since the frontal areas crossed provided well known discontinuities in the structure of the ocean. The frontal areas have been identified as transition zones among different hydrographic regions characterized by particular temperature and salinity properties. One of the most well-marked fronts is the Antarctic Polar Front (PF), which separates Antarctic from Sub-Antarctic waters, and that is characterized by a dramatic latitudinal change in temperature (from 1 to 5 °C). Sub-Antarctic Front (SAF) is the name of the northern boundary zone of the Polar Frontal Zone (PFZ). The Weddell-Scotia Confluence (WSC), is the boundary zone between the Weddell and Scotia Seas. The Drake Passage is particulary interesting to study because in this region the distinctive Antarctic water masses and fronts are compressed into a narrow zone and large differences in the physical and chemical environment can be observed over relatively small distances. This environment with such marked physical gradients was the optimal case study to investigate the composition and variability of picoeukaryotic assemblages.

Materials and Methods

Sample collection. Samples were collected during cruises DOVETAIL and DHARMA on board BIO Hespérides. Several stations were sampled across the Scotia-Weddel Confluence during the DOVETAIL cruise (23-26 January 1998) and 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 continuously with a General Oceanics MkIII WOCE or a MkV conductivity-temperature-depth profilers (CTD). Chlorophyll *a* (Chl *a*) concentration was determined by measuring the fluorescence in acetone extracts with a Turner Designs fluorometer (Yentsch et al. 1963). Phytoplankton samples were fixed with formalin (4% final concentration) during DOVETAIL or with Lugol's solution during DHARMA. Phytoplankton counts (nano- and microplankton size ranges) were carried out by inverted microscopy (Utermöhl 1958). One hundred millilitres 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 400x to enumerate the more frequent taxa. Additional transects and the whole chamber bottom were scanned at 100x 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”.

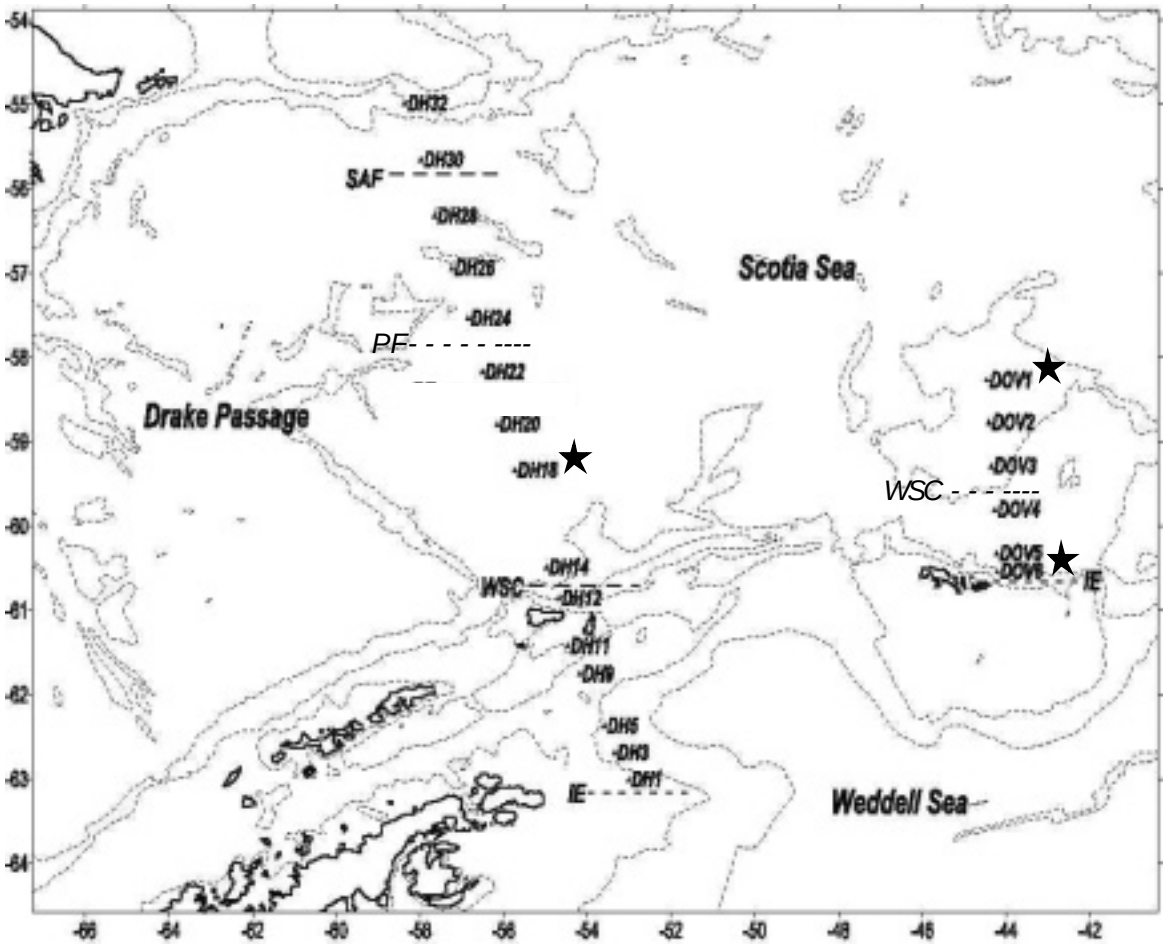


FIG. 1. Map of the area where cruises DOVETAIL (stations labeled DOV) and DHARMA (stations labeled DH) took place. The approximate position of the ice-edge is indicated by a discontinuous line (IE). The main fronts at the time of sampling are indicated by discontinuous lines: WSC, Weddell-Scotia Confluence; PF, Polar front; SAF, Sub Antarctic Front. The three stars indicate stations where clone libraries are available.

Subsamples for phototrophic picoeukaryote counts 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 (Gasol and del Giorgio 2000). 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 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}. Sterivex units were filled with lysis buffer (40 mM EDTA, 50 mM Tris-HCl and 0.75 M sucrose) and frozen at -70°C until nucleic acid extractions could be carried out. DOVETAIL samples were extracted in the laboratory. DHARMA samples were extracted on board and the extracts frozen at -70°C.

Nucleic acid extraction. Nucleic acid extraction was carried out as described in Massana et al. (1997). 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 (Paul and Myers 1982). Nucleic acid extracts were stored at -70°C until analysis.

PCR. Approximately 10 ng of extracted DNA was used as template in a polymerase chain reaction (PCR) using eukaryal-specific 18S rDNA primers. Primers Euk1A and Euk516r-GC were used for DGGE (Díez et al. 2001b), and HEX-Euk1A and Euk516r for T-RFLP. PCR mixtures (50 µl) contained 200 µM

of each dNTP, 1.5 mM of $MgCl_2$, 0.3 µM of each primer, 2.5 units of Taq DNA polymerase (Gibco BRL) and the PCR buffer supplied with the enzyme. 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. 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 Company) as described previously (Muyzer et al. 1997, Schauer et al. 2000, Díez et al. 2001b). Electrophoresis was run in 0.75 mm-thick 6% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) submerged in 1x TAE buffer (40 mM Tris, 40 mM acetic acid, and 1 mM EDTA, pH 7.4) at 60°C. Around 800 ng of PCR product were applied to individual lanes in the gel. Electrophoresis conditions were 100 volts for 16 hours in a linear gradient of denaturing agents from 45% to 65% (Díez et al. 2001b), where 100% denaturing agent is defined as 7 M urea and 40% deionized formamide. Gels were stained for 30 min in 1x TAE buffer with SybrGold nucleic acid stain (1:10000 dilution; Molecular Probes) and visualized under UV 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. 2001b). 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. Finally, 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 OTUs (Operational Taxonomic Units) in each sample.

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 4º 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) automated sequencer. Sequences obtained (300-400 bp) were submitted for checking similarity by BLAST (Basic Local Alignment Search Tool; Altschul et al. 1997).

T-RFLP. PCR for Terminal-Restriction Fragment Length Polymorphism used the same program and primer set as DGGE, with the exception that the forward primer was 5´-labeled with HEX (hexachlorofluorescein, Operon technologies) and the reverse primer did not have the GC-clamp. Fluorescent labeled PCR products were purified by using WIZARD PCR purification columns (Promega) and were separately digested with the tetrameric restriction enzymes *Msp*I and *Rsa*I (Boehringer Mannheim Biochemicals). Each digest contained 1-10 µl (approximately 200 ng) of a purified PCR product, 10 U of the restriction enzyme with its respective buffer and was filled up to a final volume of 15 µl with sterile distilled water. Incubation was done at 37ºC for 3 h, and 64ºC for 10min. Terminal Restriction Fragments (TRFs) were resolved by electrophoresis at 3000 volts for 14 hours in a 6%-denaturing acrylamide gel (acrylamide-NN-methylenebisacrylamide, 19:1) in an ABI Automated Sequencer (ABI 373).

TRFs were sized with the softwares GeneScan (v. 2.1), and Genotyper up to a one base pair resolution using the size standard TAMRA-2500 (ABI). The intensity of each TRFs was measured using the peak area. Peaks with an area smaller than 150 units of fluorescence intensity were excluded from further

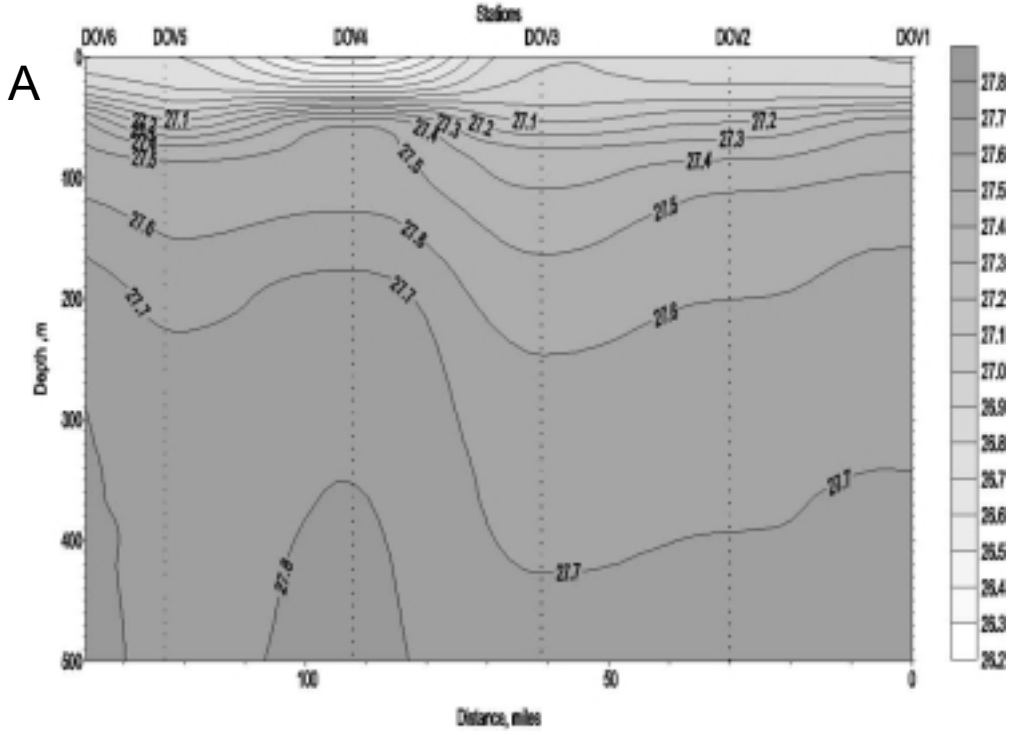
analysis. Moreover, peaks that represented a percentage lower than 1% of the total peak area were excluded when they were present only in one or two samples or when they did not match any TRF in the database. The remaining number of TRFs was taken to be the number of OTUs in each sample. Phylogenetic candidates for each TRF could be searched with the complete sequences extracted from the Ribosomal Database Project (Maidak et al. 2000) using the pattern searching algorithm PatScan (Dsouza et al. 1997). This data was used to identify the putative phylogenetic affiliation of the measured TRFs. In cases where the experimental fragment corresponded to several possible organisms, a "most likely" candidate was chosen based on additional information. This information was gathered from sequencing of DGGE bands from the same samples, from two genetic clone libraries from DOVETAIL (Díez et al. 2001a), or from microscopical examination.

For both DGGE and T-RFLP, an intensity matrix was constructed with the relative abundance data for individual DGGE bands and individual TRFs in all samples. 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 UPGMA (Unweighted Pair-Group Method with Arithmetic averages) in cluster analysis.

Results

Several stations were occupied along two latitudinal transects in the Southern Ocean (Fig. 1). These transects followed different hydrographic regions and crossed well defined oceanic fronts: the Weddell-Scotia Confluence (WSC) during the cruise DOVETAIL, and the Sub-Antarctic Front (SAF), the Polar Front (PF), and the Weddell-Scotia Confluence (WSC) during the cruise DHARMA

along the Drake Passage. The distribution of water density (as sigma-t) up to 100 m depth along both transects is shown in Fig. 2. The DOVETAIL transect showed sharply stratified waters. Surface temperature ranged between -1.8ºC close to the ice-edge and +1.8ºC in the northernmost waters sampled. The DHARMA transect showed a well mixed water column up to 100 m depth along all the transect, both north and south of the Polar Front. The characteristic signature of the Polar Front can be seen between stations DH22 and DH24. Temperatures in this transect ranged between -1.5ºC in ice-edge waters, around 3ºC in the Polar Frontal Zone (PFZ), and +5ºC close to the South Antarctic Frontal Zone (SAF).


A
Stations
DOV6
DOV5
DOV4
DOV3
DOV2
DOV1
Depth, m
Distance, miles

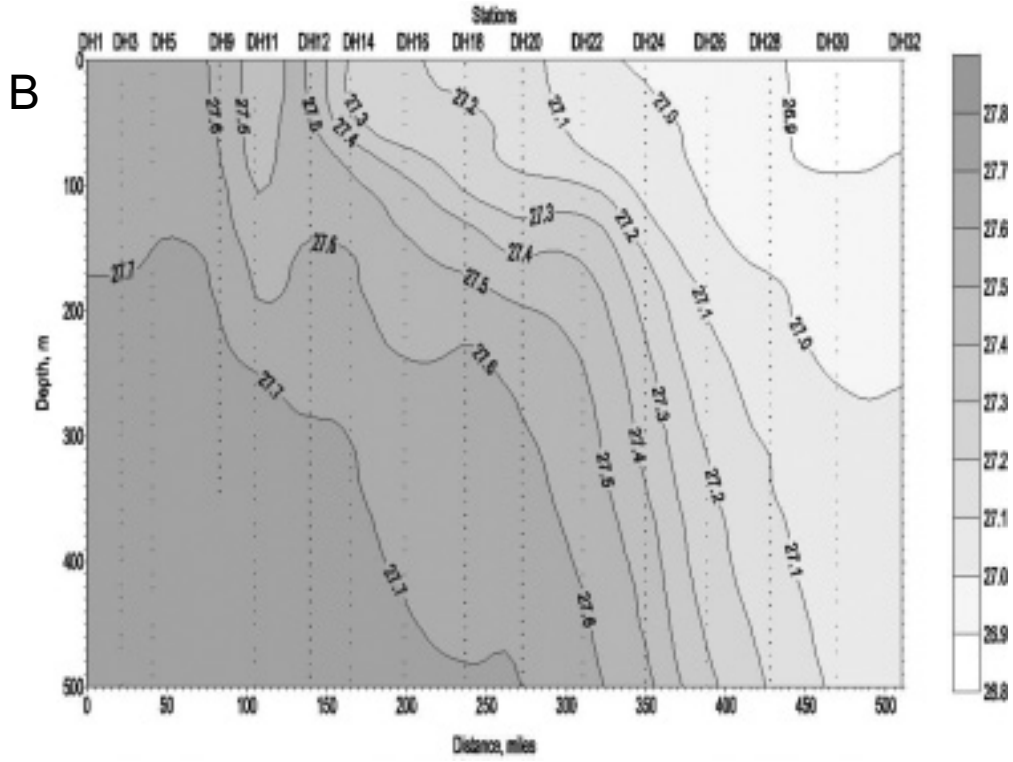

B
Stations
DH1
DH3
DH5
DH9
DH11
DH12
DH14
DH16
DH18
DH20
DH22
DH24
DH26
DH28
DH30
DH32
Depth, m
Distance, miles

FIG. 2. Distribution of density (sigma-t) with depth and horizontal distance during (A) the DOVETAIL cruise, and (B) the DHARMA cruise. Small dots indicate density data used to build isolines. In both graphs, stations on the left are the southermost stations.

Fig. 3 shows Chl *a* concentration within the mixed layer. In DOVETAIL (Fig. 3A) total Chl *a* increased towards the ice-edge, and this increase correlated with a decrease of the percent of Chl *a* <1.6 µm. The percent of Chl *a* passing a 5 µm filter was rather constant along the whole transect (40 to 70%). In DHARMA (Fig. 3B), stations DH1, 11, 14, 30 and 32 showed higher values of total Chl *a*, whereas stations corresponding with the PFZ (DH20 to DH26) presented the lowest concentrations. Chl *a* in the <5 µm fraction varied between 20 and 80% of the total. The lowest percentage was found in the stations close to the ice edge and in the SAF. The highest percentage was found in stations DH14 and DH18 in the WSC area.

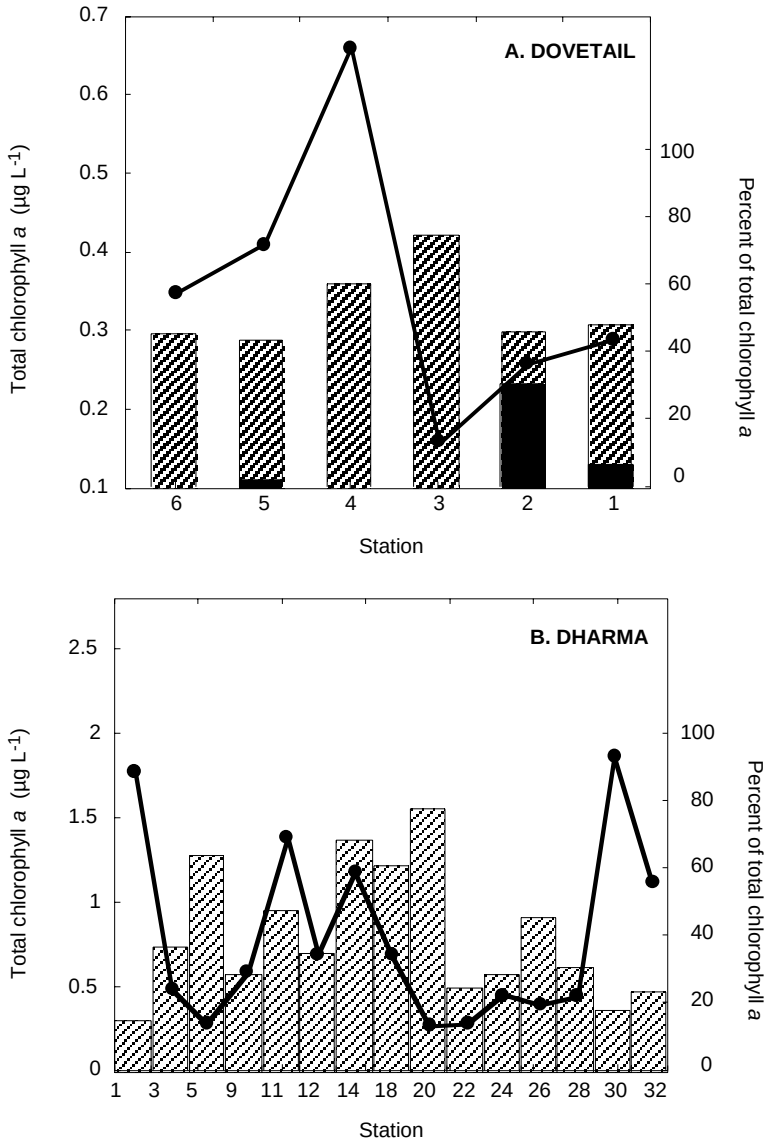


FIG. 3. Total chlorophyll *a* (dots) in surface samples from (A) DOVETAIL and (B) DHARMA. The bars indicate the percent of chlorophyll *a* in the fraction <5 µm (striped) and <1.6 µm (black).

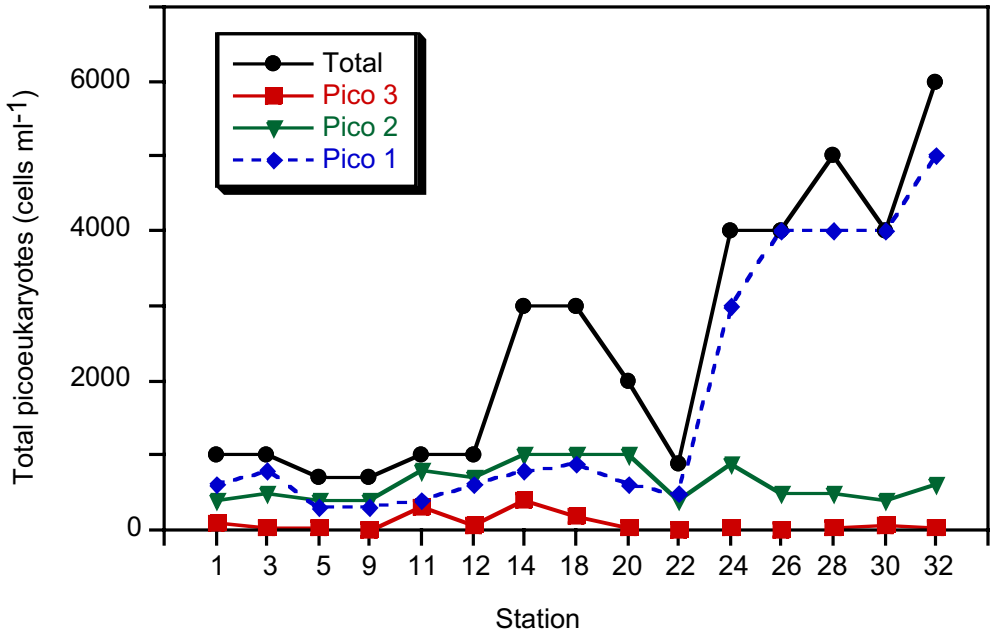
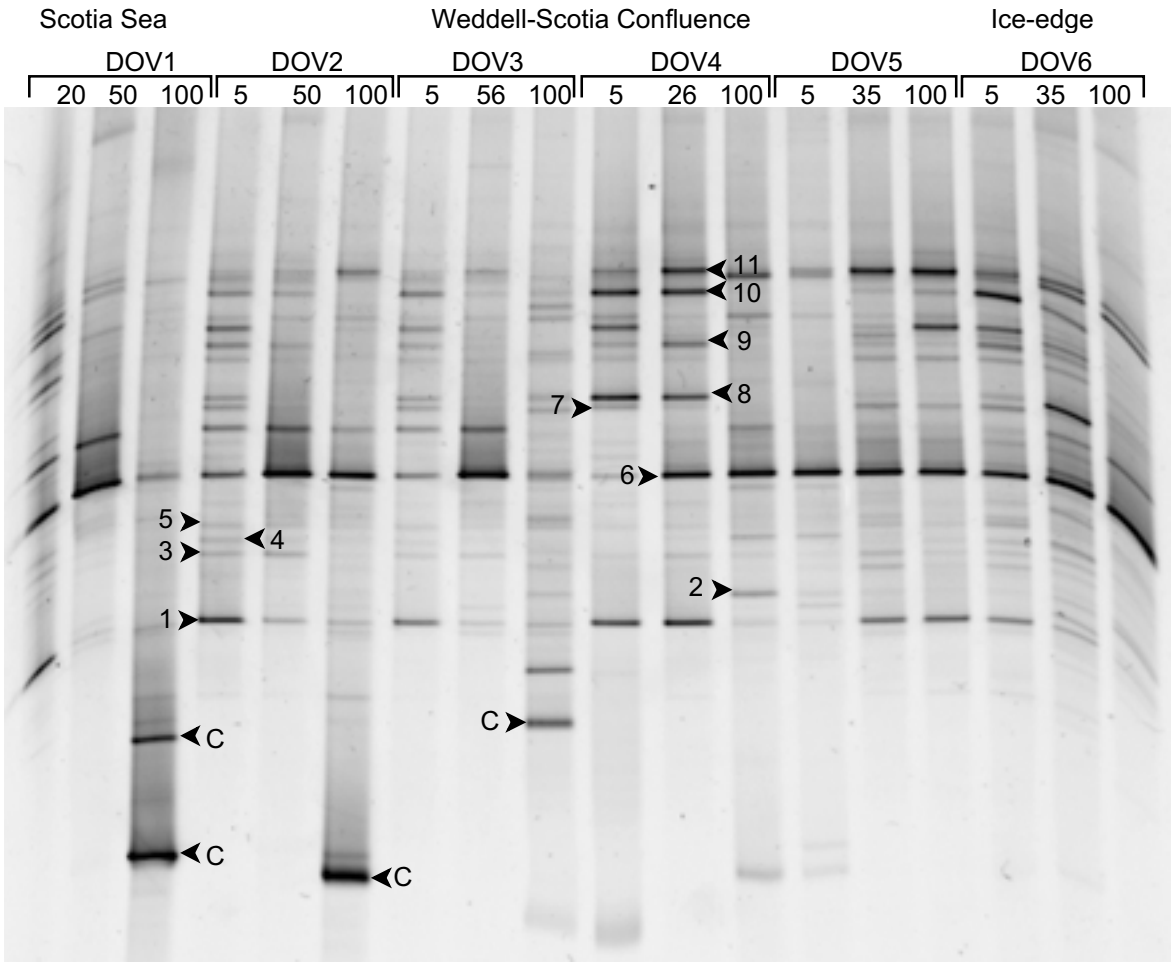


FIG. 4. Abundance of photosynthetic picoeukaryotes along the DHARMA transect as determined by flow cytometry. The total numbers and three, easily distinguishable populations are shown.

Fig. 4 shows picoeukaryotic counts 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 (from 400 to 1000 cells ml^{-1} in P2, and from 2 to 200 cells ml^{-1} in P3) whereas the smallest population, P1 (from 300 to 5000 cells ml^{-1}), accounted for the increase in total picoeukaryotic numbers between stations DH24 (PF) and DH32 (SAF). In DOVETAIL three different groups of picoeukaryotes were also found in the two stations analyzed by flow cytometry (DOV1 and DOV6, Díez et al. 2001a).

Examination of the samples by inverted microscopy were done in order to identify the main nano and 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} towards the Scotia-Weddell Confluence (SWC). In the DHARMA transect, these small flagellates were found in abundances between 200 cells ml^{-1} in SWC (DH12) and 60-90 cells ml^{-1} in the rest of the stations analyzed. These numbers are in fact underestimates of the values measured by flow cytometry. The dominat diatoms in both transects were *Corethron criophilum, Chaetocerus* 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 cell ml^{-1} was fairly abundant in DOVETAIL. Cryptophytes were very 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.



C = copepod

FIG. 5. Negative image of a DGGE gel showing fingerprints of the six DOVETAIL stations at three depth: surface, bottom of the mixed layer and 100 m. Bands that were sequenced are indicated by a number that corresponds to numbers in Table 2. Clone libraries for the surface samples in DOV1 and DOV6 have been published separately (Díez et al. 2001a).

Vertical stratification of picoeukaryotic assemblages. DGGE patterns in DOVETAIL were different for each depth (Fig. 5A). Differences were very clear between 100 m samples and surface samples but they were also apparent between the two upper depths sampled. This was consistent with the sharp stratification 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, and significant differences appeared below 250 m (Fig. 6). The relatively large similarity among the upper depths was consistent with the structure of the water column during this cruise, with the mixed upper layer at least up to 100 m (Fig. 2B). Given this distribution, the two upper depths were included in the analysis of the DOVETAIL transect, but only the surface samples were used for the DHARMA transect (Fig. 7).

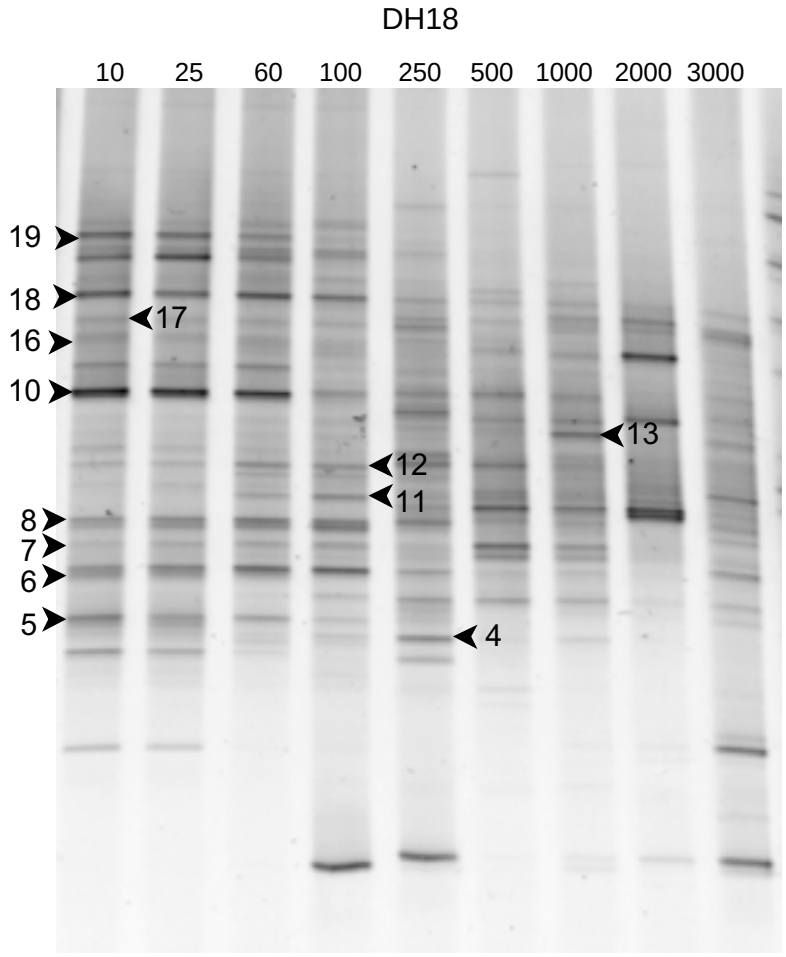


FIG. 6. Negative image of DGGE gels showing fingerprints of a vertical profile at station DH18 from the surface down to 3000 m. Clone libraries for the samples at 250, 500, 2000 and 3000 m have been published separately (López-García et al. 2001). The bands that were sequenced are indicated by a number that corresponds to numbers in Table 2.

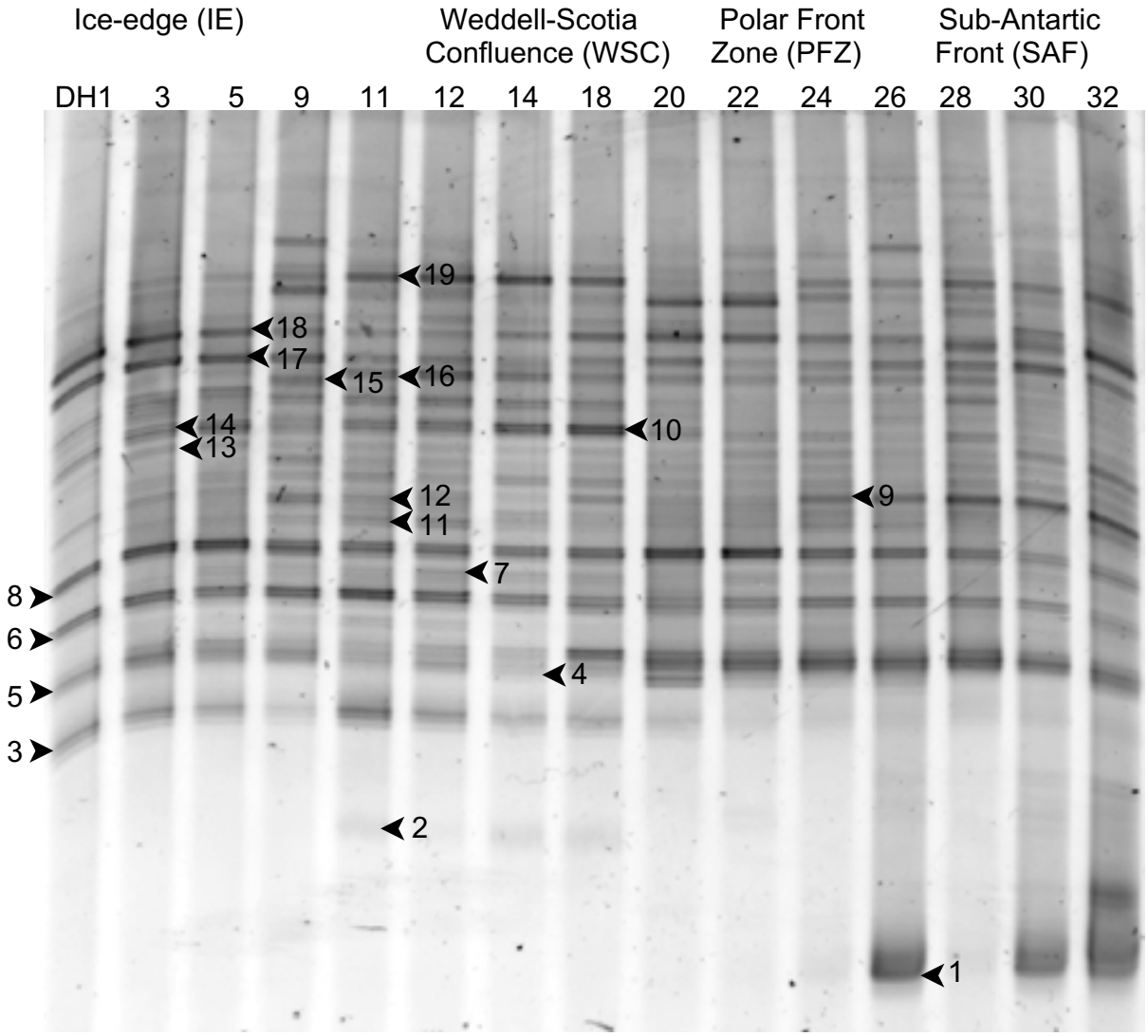


FIG. 7. Negative image of DGGE gels showing fingerprints for the DHARMA surface samples along the transect. The bands that were sequenced are indicated by a number that corresponds to numbers in Table 2.

Latitudinal changes. DGGE gels of eukaryotic picoplankton from DOVETAIL and DHARMA transects are shown in Fig. 5 and Fig. 7. The total number of bands in surface samples ranged between 11 and 14 (or between 22 and 25, if bands accounting for <1% of relative intensity are considered, Table 1).

TABLE 1. Number of picoeukaryotic OTUs found by DGGE and T-RFLP in DOVETAIL and DHARMA samples.

Station	Sampling Date (day/month/year)	Depth (m)	Nº. of DGGE OTUs	Nº. of DGGE OTUs(>1% abundance)	Nº. of T-RFLP OTUs	
					*Msp*I	*Rsa*I
DOVETAIL						
DOV1	23/1/98	20	16	15	16	4
		50	17	16	13	2
		100	14	7	ND	ND
DOV2	24/1/98	5	18	16	19	5
		50	18	16	22	4
		100	17	11	ND	ND
DOV3	24/1/98	5	18	15	20	5
		56	17	16	17	3
		200	14	11	ND	ND
DOV4	25/1/98	5	17	14	21	4
		26	21	16	22	5
		100	17	16	ND	ND
DOV5	25/1/99	5	22	16	25	4
		35	24	21	21	5
		100	16	13	ND	ND
DOV6	26/1/98	5	17	14	20	3
		35	26	17	18	4
		100	10	10	ND	ND
DHARMA						
DH1	6/12/98	5	16	13	12	5
		25	16	ND	14	5
DH3	6/12/98	5	20	18	10	5
		25	ND	ND	10	ND

DH5	7/12/98	5	21	19	11	ND
		25	ND	ND	14	ND
DH9	7/12/98	5	22	19	13	6
		25	20	ND	14	7
DH11	7/12/98	5	22	19	ND	1
		25	ND	ND	15	5
DH12	8/12/98	5	23	22	6	6
		25	21	ND	17	8
DH14	8/12/98	5	20	17	13	1
		25	ND	ND	11	1
DH18	9/12/98	5	21	19	16	6
		25	21	ND	14	6
DH20	10/12/8	5	22	16	12	3
		25	ND	ND	11	3
DH22	10/12/8	5	21	16	ND	ND
		25	19	ND	13	ND
DH24	11/12/8	5	21	20	15	2
		25	19	ND	11	ND
DH26	11/12/8	5	22	18	11	ND
		25	22	ND	12	ND
DH28	13/12/8	5	25	20	16	ND
		25	22	ND	17	ND
DH30	13/12/8	5	24	17	20	3
		25	ND	ND	14	ND
DH32	14/12/8	5	22	15	15	ND
		25	ND	ND	13	ND

ND: Not determined

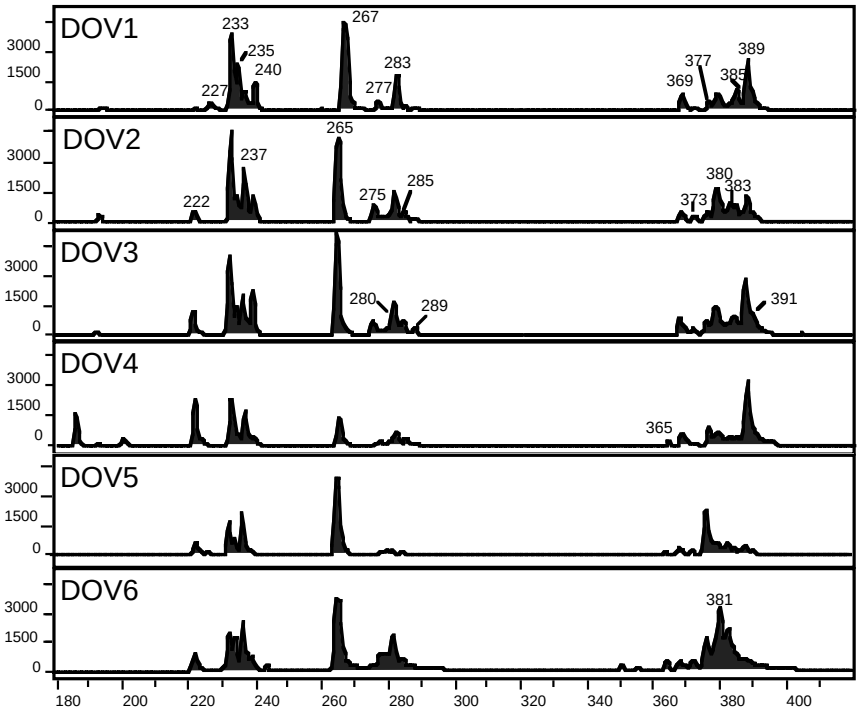
The same samples processed by DGGE were processed by T-RFLP and examples of *Msp*I T-RFLP chromatograms for DOVETAIL and DHARMA are shown in Fig. 8. The number of TRFs detected ranged between 6 and 25 with *Msp*I and between 1 and 8 with *Rsa*I (Table 1). This last restriction enzyme produced very few fragments and, thus, it was judged to provide too little information for direct comparison of picoeukaryotic assemblages. However, the information from this enzyme could be combined with that of *Msp*I when calculating dendrograms. If we consider each TRF obtained by *Msp*I and each DGGE band as one OTU, the number of OTUs detected with both methods were rather similar.

Quantitative DGGE band patterns and T-RFLP chromatograms were used to build dendrograms to compare the grouping of picoeukaryotic assemblages

(Fig. 9). In the case of DOVETAIL, both techniques grouped samples in the same three main clusters. One cluster included the surface samples from stations 1, 2 and 3 and both depths from station 4. A second cluster included the "deep" samples from stations 1, 2 and 3 exclusively. And the last cluster grouped all samples from the stations closest to the ice-edge (station 5 and 6). The main difference between the two techniques was that this third cluster was closer to the "deep" cluster by DGGE, but closer to the "surface" cluster by T-RFLP (Fig. 9A). This clustering of samples is consistent with the hydrography of the area (Fig. 2A), and indicate a clear change in the composition of the assemblages following a spatial gradient (offshore - ice-edge) and a vertical gradient in the water column. We have to take into account that the “deep” samples were collected to a depth of 50 m in DOV1 to DOV3 and a depth of 25 –35 m in DOV4 to DOV6, and this could influence the particular grouping of these samples.

In the case of DHARMA, dendrograms from DGGE (Fig. 9B) showed clustering of samples consistent with the typical hydrography across the Polar Front. Thus, stations formed two main clusters, one with stations south of the PFZ and the other with stations in and north the PFZ. Within each cluster, smaller clusters were also consistent with the water masses crossed along the transect (compare Figs. 9B and 2B). The T-RFLP dendrogram had to be constructed with fragments from one enzyme only (*Msp*I), because *Rsa*I fragments were not available for all samples (Table 1). Thus, although the resultant dendrogram clustered the same stations in a similar way as the DGGE dendrogram, the overall pattern was not totaally consistent with the position of the stations along the transect.

A


DOV1
233
235
240
227
267
283
277
369
377
385
389
DOV2
222
237
265
275
285
373
380
383
DOV3
280
289
391
DOV4
365
DOV5
DOV6
381
3000
1500
0
180
200
220
240
260
280
300
320
340
360
380
400

B

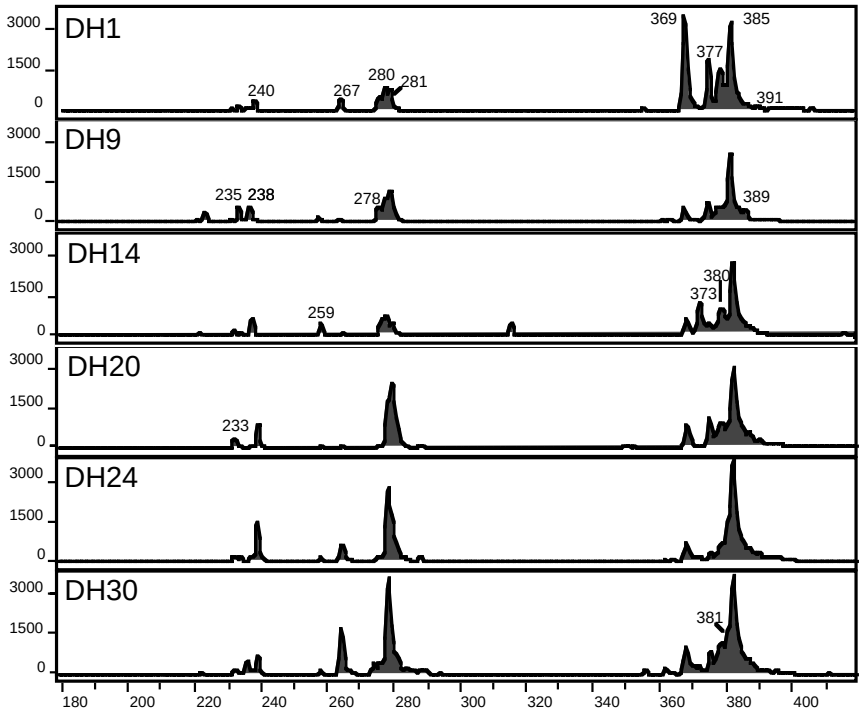
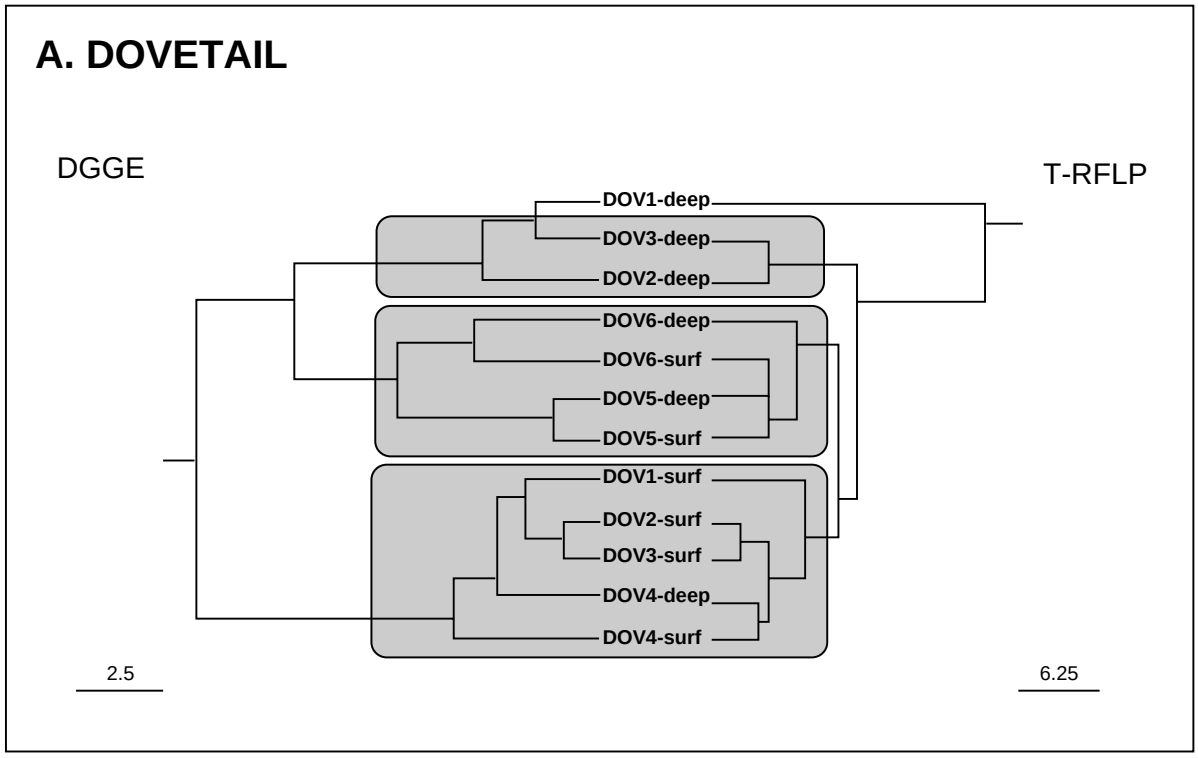

DH1
240
267
280
281
369
377
385
391
DH9
235
238
278
389
DH14
259
373
380
DH20
233
DH24
DH30
381
3000
1500
0
180
200
220
240
260
280
300
320
340
360
380
400

FIG. 8. T-RFLP chromatograms with enzyme *Msp*I for selected surface samples from cruises (A) DOVETAIL and (B) DHARMA. The length of the main fragments in base pairs is indicated.


A. DOVETAIL
DGGE
T-RFLP
DOV1-deep
DOV3-deep
DOV2-deep
DOV6-deep
DOV6-surf
DOV5-deep
DOV5-surf
DOV1-surf
DOV2-surf
DOV3-surf
DOV4-deep
DOV4-surf
2.5
6.25

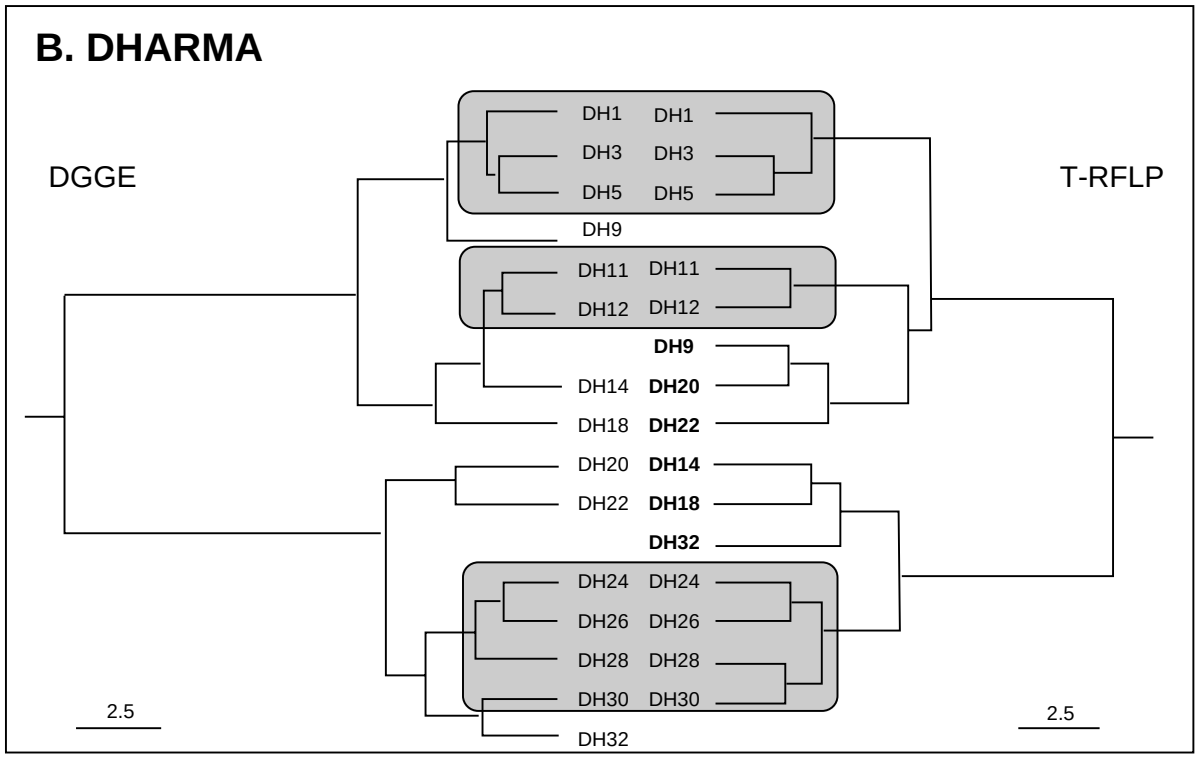

B. DHARMA
DGGE
T-RFLP
DH1 DH1
DH3 DH3
DH5 DH5
DH9
DH11 DH11
DH12 DH12
DH9
DH14 DH20
DH18 DH22
DH20 DH14
DH22 DH18
DH32
DH24 DH24
DH26 DH26
DH28 DH28
DH30 DH30
DH32
2.5
2.5

FIG. 9. Dendrograms clustering the samples from cruises (A) DOVETAIL and (B) DHARMA. The dendrograms for DGGE are shown to the left and those from T-RFLP to the rigth. The T-RFLP dendrogram for DOVETAIL was built with data from two restriction enzymes combined (*Msp*I and *Rsa*I). That for DHARMA was built with data from *Msp*I only. The shaded boxes indicate the main clusters recognized by both fingerprinting techniques.

Taxonomical identity of the OTUs. DGGE gels showing the picoeukaryotic fingerprints across the two transects were scanned for the most important bands. These were cut and sequenced. We sequenced 11 bands in DOVETAIL and 19 bands in DHARMA (Table 2). The short sequences obtained were not adequate to identify precisely the organism present, but they were enough to assign the bands to certain phylogenetic groups with a reasonable degree of similarity. In DOVETAIL, 4 bands could be assigned to the prymnesiophytes, 3 to novel stramenopiles, and one each to prasinophytes, cercomonads, novel alveolates and dinoflagellates. Likewise in DHARMA, 6 bands belonged to dinoflagellates, 2 to novel stramenopiles, prasinophytes, novel alveolates, and diatoms, and one each to cercomonads, prymnesiophytes, cryptophytes, chrysophytes and a copepod. Thus, essentially the same groups made up the bulk of the DGGE bands in both transects. Some of these groups are well known components of the small Antarctic plankton such as prymnesiophytes and prasinophytes. Other sequences, however, belong to previously unknown groups that have been discovered very recently, such as the novel alveolates and stramenopiles.

TABLE 2. Closest relative in the database of the DGGE bands sequenced and their relative abundance in the gel.

Dominant DGGE bands	% relative band intensity		Closest relative	% similarity (nº of bases)	Phylogenetic group
	Range	Average			

DOVETAIL					
1	2-29	17.0	*Phaeocystis antarctica*	87.9 (240)	Prymnesiophytes
2	1-5	2.0	*Symbiodinium* sp.	90.3 (62)	Dinophytes
3	1-4	3.2	*Phaeocystis antarctica*	92.3 (39)	Prymnesiophytes
4	1-4	2.7	*Phaeocystis antarctica*	99.0 (212)	Prymnesiophytes
5	1-3	2.5	Clone OLI11009	84.3 (179)	Novel alveolate
6	10-30	15.3	*Mantoniella antarctica*	96.8 (410)	Prasinophytes
7	3-8	4.8	DH148-5-EKD53	87.3 (119)	Novel Stramenopiles
8	3-10	5.8	*Cercomonas sp.*	93.1 (233)	Cercomonads
9	3-10	5.9	Clone OLI11006	91.2 (411)	Novel Stramenopiles
10	3-15	6.7	Clone OLI11066	88.1 (76)	Novel Stramenopiles
11	2-17	5.7	*Phaeocystis antarctica*	98.1 (54)	Prymnesiophytes
DHARMA					
1	ND	ND	*Calanus pacificus*	98.3 (418)	Copepoda
2	<1	<1	*Geminigera cryophila*	95.2 (318)	Cryptophytes
3	5-15	9.1	*Gymnodinium catenatum*	98.6 (451)	Dinophytes
4	3-11	5.7	*Pentapharsodinium tyrrhenicum*	91.3 (301)	Novel alveolate
5	4-25	12.3	*Phaeocystis pouchetii*	87.9 (240)	Prymnesiophytes
6	3-15	9.2	Clone DH48-5-EKD46	85.9 (142)	Dinophytes
7	2-3	1.9	Clone DH144-EKD3	91.5 (320)	Novel alveolate
8	5-15	11.7	Clone DH48-5-EKD46	99.2 (403)	Dinophytes
9	1-8	4.6	*Mantoniella antarctica*	99.0 (401)	Prasinophytes
10	1-12	5.3	*Pyramimonas parkeae*	97.9 (339)	Prasinophytes
11	1-2	1.2	Clone DH48-5-EKD46	95.8 (240)	Dinophytes
12	1-2	1.2	Clone OLI11255	86.9 (46)	Dinophytes
13	1-3	1.5	*Heterocapsa triqueta*	95.3 (43)	Dinophytes
14	1	1.0	Clone DH48-5-EKD54	92.5 (27)	Diatom
15	3	3.0	*Ochromonas* sp.	93.1 (132)	Chrysophytes
16	1-8	3.0	*Cercomonas* sp.	94.0 (50)	Cercomonads
17	2-12	6.0	Clone DH144-EKD10	96.8 (256)	Novel stramenopiles
18	2-15	7.2	*Pseudo-nitzschia* sp.	88.6 (353)	Diatoms
19	1-13	5.6	Clone DH148-5-EKD53	93.5 (31)	Novel stramenopiles

Since T-RFLP with *Rsa*I yielded too few fragments we had to rely on the results from *Msp*I only, which yielded up to 25 fragments of sizes between 222 and 391 bp. However, most fragments identified could belong to a substantial collection of very different organisms (Table 3). For example, the 222 bp fragment could belong to 23 eukaryotes, 22 of which are marine organisms. We have designed a "most likely candidate" in Table 3, based on the information provided by light microscopy, DGGE and the clone libraries available. Considering all this uncertainties, the collection of identified candidates obtained by T-RFLP is similar to that from DGGE.

We used the band intensities in DGGE gels as an indicator of the abundance of each group of organisms. In general, we grouped bands into large groups, since many of the sequences were short. Only in some cases where the sequence was relatively long and the similarity to cultured organisms high, have we used the intensities from individual bands (particularly with *Phaeocystis* and prasinophytes). The changes in the relative band intensities along both transects are illustrated in Fig. 10. Around 20% in DHARMA and 30% in DOVETAIL of the total band intensity could not be assigned to groups, because the corresponding bands were too faint and were not selected for sequencing. The remaining band intensity, however, could be assigned to different groups.

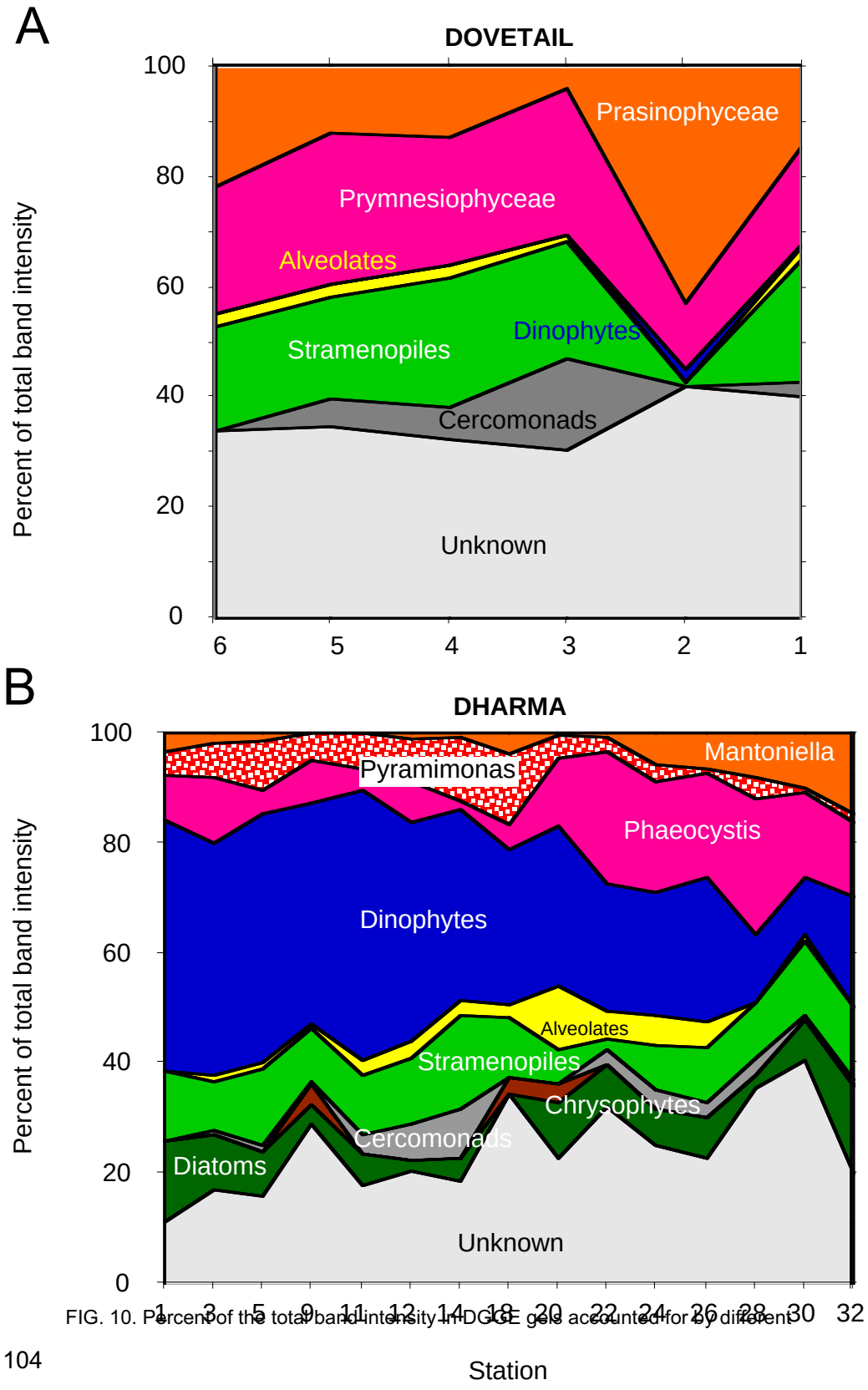


FIG. 10. Percent of the total band intensity in DGGE gels accounted for by different

groups of eukaryotes for cruises (A) DOVETAIL and (B) DHARMA.

In DOVETAIL prasinophytes, prymnesiophytes and novel stramenopiles were important in essentially all stations (FIG. 10A). Cercomonads and novel alveolates were present in lower proportions and dinoflagellates only appeared in one station. The same groups were again dominant in the gels from DHARMA, with the addition of dinoflagellates and diatoms. There were clear trends in the proportions accounted for some groups with latitude (Fig. 10B). Thus, dinoflagellates decreased in importance from the ice edge towards the north, while prymnesiophytes followed the opposite trend. The novel alveolates were more abundant at the polar front and surrounding areas. The two prasinophytes detected were abundant on opposite sides of the polar front: *Pyramimonas* to the south and *Mantoniella* to the north. Finally, diatoms and novel stramenopiles seemed to be present in similar proportions thoughout the transect.

Discussion

Several DGGE bands, numbered from 1 to 11 in DOVETAIL and from 1 to 19 in DHARMA, were excised, sequenced and their closest relative was identified (Table 2). Several T-RFLP fragments obtained with *Msp*I could also be assigned to organisms present in the eukaryotic database (Table 3). Most of the DGGE bands and *Msp*I TRFs obtained were related to well known Antarctic taxons. We will discuss next the distribution and composition of the different phylogenetic groups identified. We have to take into account that in each cruise a different size fraction was analyzed (0.2-1.6 µm in DOVETAIL; 0.2-5 µm in DHARMA), and this may explain many differences in composition of picoeukaryotes between both cruises. Thus, only a relative comparison between samples inside each transect can be done.

Prasinophytes. One of the most important groups was the prasinophytes. OTUs belonging to this algal group accounted for 10-30% of the total DGGE band intensity and 7-24% of total TRF peak area in DOVETAIL. The corresponding percentages in DHARMA were 1-12% by DGGE and 1-11% by T-RFLP. The most frequently retrieved prasinophyte was closest to *Mantoniella antarctica* (DGGE bands 6 in DOVETAIL and 9 in DHARMA and 267 bp TRF). *Mantoniella* (approximately 2-3 µm in diameter) is a cosmopolitan flagellate that has been already reported from polar waters by microscopy (Marchant et al. 1989, Throndsen and Kristiansen 1991). In DHARMA another prasinophyte related to *Pyramimonas* (DGGE band 10) was fairly abundant. No TRF could be assigned to this organism, because it was not included in the RDP database and therefore was not processed by the PatScan algorithm. Contrary to *Mantoniella* and other small flagellates, the cosmopolitan *Pyramimonas* (approximately 6 x 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 station DOV6 but we could not detect it at station DOV1 by microscopy. However, since samples for molecular analysis were prefiltered by a 1.6 µm filter in DOVETAIL it is not surprising that we did not retrieve it in this cruise. The DGGE derived sequences from both prasinophytes showed relatively high similarities to sequences in the database (96% and 97% respectively).

M. antarctica 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. 5). In DHARMA, band corresponding to *Pyramimonas* showed increasing intensity from station DH1 to DH18 and then disappeared (Fig. 7). *M. antarctica*, on the other hand, was most abundant from stations DH24 to 32. Thus, these two prasinophytes were found on opposite sides of the Polar Front. In both transects *M. antarctica* was more abundant in stations away from the ice-edge than close to the edge (Fig. 10A, B). Preliminary results obtained by HPLC

pigment analysis in DHARMA (M. Latasa, unpublished) showed that in stations DH1, 11, 14, 30 and DH32 Chlorophyll *b* appear as a very dominant pigment in the total phytoplankton. This pigment was also very important in the < 5µm fraction in station DH1 to DH18 and DH26 to DH32. Lutein, a typical pigment marker from chlorophyceae, was found in DH1 and DH14, and prasinoxanthin, a typical marker pigment to prasinophyceae from DH26 to DH32, which are consistent with the DGGE results of substitution of two different prasinophytes.

A significant correlation (r^2 = 0.740) was found between the relative abundance of *M. antarctica* in DGGE gels and the P1 population abundance obtained by flow cytometry (Fig. 4). The relative abundance of *Phaeocystis* in DGGE gels also showed a significant correlation with P1 (r^2 = 0.348). As can be seen in Fig. 4, however, the distribution of this flagellate did not match that of the P1 population as well as that of *M. antarctica*. Thus, we think the P1 population corresponds to *M. antarctica*. This indicates that the band intensity in DGGE gels, at least in this case, does bear a relationship to the abundance of the corresponding organism in nature.

Prymnesiophytes. This was another dominant group of picoeukaryotes. DGGE bands 1, 3, 4 and 11 in DOVETAIL, band 5 in DHARMA and the 233 bp TRF (found in both cruises) could all be attributed to *Phaeocystis antarctica*. Its relative abundance reached 30% in both transects, showing this to be one of the most important members of the picoeukaryotic assemblage. In both transects *Phaeocystis* increased in representation from the ice-edge towards more northern samples (Fig. 10B).

In a separate study (Díez et al. 2001a) we constructed clone libraries with surface samples from the stations at both ends of the DOVETAIL transect (DOV1 and DOV6 in Table 1). These two libraries produced numerous clones of prasinophytes and prymnesiophytes: 18 clones from station DOV6 and 5 from station DOV1 could be assigned to *Mantoniella* and 5 and 17, respectively, to

Phaeocystis antarctica. 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 T-RFLP and, yet, the same organisms were retrieved in significant amounts. No clone libraries were constructed with samples from DHARMA. Results from DGGE and T-RFLP, however, can be compared to a detailed study of phytoplankton pigments by HPLC carried out during the same cruise and using the same size fractions (M. Latasa, in preparation). In those preliminary results the 19'-hexanoyloxyfucoxanthin pigment marker to *Phaeocystis* was more or less homogeneous distributed along the horizontal transect and increased a little at the northermost stations.

Dinoflagellates and novel alveolates. Some DGGE sequences were closely related to dinoflagellates (band 2 in DOVETAIL and bands 3, 6, 8, 11, 12 and 13 in DHARMA). TRF of 280, 383, and 389 bp could also belong to dinoflagellates. Band 3 from DHARMA was closely affiliated to *Gymnodinium catenatum*. This band accounted for a significant percentage of total band intensity, ranging between 5 and 15%. Many of the other bands were highly related to environmental clones of dinoflagellates recently found in the Pacific and the Southern Ocean (Moon van der Staay et al. 2001, López-García et al. 2001).

The sequence derived from band 8 from DHARMA (400 bases), for example, had 99% similarity with environmental clone DH148-5-EK46, suggesting the presence of this dinoflagellate in DHARMA samples. Bands 6 and 11 might also belong to the same clone, but the total length sequenced and the similarity values were very low (Table 2). Clone DH148-5-EK46 was retrieved from 3000 m in station 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 (Fig. 5), however, indicates that its most likely environment is the surface layer of the ocean. Band 13 could also be assigned to another environmental clone retrieved from station DH18 at 2000 m,

clone DH147-EKD21, although the length of the sequence is very small. Finally, bands 12 in DHARMA and 2 in DOVETAIL were most closely related to an environmental clone from the surface Pacific Ocean (clone OLI11255). Again, the sequence obtained from these bands was very short. Bands from all these environmental clones together accounted for 1-5% of total intensity in DOVETAIL and around 40% in DHARMA. Since DOVETAIL samples were prefiltered through 1.6 µm and DHARMA through 5 µm filters, the difference in abundance between the two cruises might be due to the size of these organisms being between 2 and 5 µm, and not to other ecological factors.

Two DGGE bands (band 5 from DOVETAIL and band 7 from DHARMA) were associated with a recently described group of novel alveolates. The former showed a certain degree of similarity (84%) to clone OLI11009 from the Pacific Ocean library. The DHARMA band, in turn, showed a rather low similarity to both clone OLI11511 from the Pacific (90%) and to clone DH144-EKD3 retrieved from 250 m depth at station DH18. Both bands contributed significant, but relatively low, percentages to the total band intensity.

Novel stramenopiles. Another recently described group of picoeukaryotes is the novel stramenopiles (Díez et al. 2001a, Moon van der Staay et al. 2001). Bands 7, 9 and 10 from DOVETAIL and bands 17 and 19 from DHARMA could be assigned to this group. Together, these bands accounted for 17% of the total band intensity in DOVETAIL and 12% in DHARMA. TRF of 227, 237, 276, 285 and 381/382 bp could potentially belong to this group. In the two DOVETAIL libraries we recovered 11 and 23 clones belonging to these groups (19 and 34% of clonal representation). One of the closest relative in culture is the oomycete *Hyphochytrium catenoides*, but these sequences are so distant from *H. catenoides* that they form completely new lineages. Their relative abundance in DHARMA was very similar to that in DOVETAIL, suggesting that most of these novel organisms has similar filtration efficiency through 1.6 µm and 5 µm filters. 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.

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, in preparation) showed that only 10 to 15% of fucoxanthin, the marker pigment of diatoms, went through 5 µm filters. Thus, we only found two DGGE bands that could be assigned to diatoms (bands 14 and 18) in DHARMA (prefiltered through 5 µm) and none in DOVETAIL (prefiltered through 1.6 µm). Despite the very short sequence obtained, both bands showed relatively high similarities to *Pseudo-nitzschia pungens* and to environmental clone DH148-5-EKD54 (Table 2). This clone had been obtained from a clone library built with DNA from 3000 m depth at a station very close to station DH18, sampled a few days later in the same cruise (López-García et al. 2001). *Pseudo-nitzschia* species are pennate diatoms. Despite being rather long, they are very narrow in diameter (many species are only 3 to 6 µm in diameter) and thus, they may go trough the 5 µm filter pointed-end first. Several TRF might belong to diatoms (see Table 3). However, between 3 and 9 different marine microorganisms belonging to different phylums could also be responsible for these fragments. Thus, for example, the 369 bp TRF could belong to the diatom *Fragilaria striata* or to the chrysophyte *Ochromonas*. sp. Again, T-RFLP cannot be used to identify the unknown components of the assemblage. Use of more restriction enzymes would have probably helped.

In a separate study we recovered two diatom clones from DOV1 and 9 from DOV6 (Díez et al. 2001a). These sequences showed between 89 and 95.9% similarity to *Corethron cryophilum* (7 clones), 86.7% to *Chaetoceros* sp. (2 clones) or 96.8% to *Skeletonema costatum* (1 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 flagellate forms. 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 organisms in pure cultures. 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. 2001a). They showed a relatively low similarity to cultured organisms (83 to 89%) and may, therefore, be new members of the cercomonads. The two clones from the Antarctic libraries were closest to *Thaumatomonas*. The DGGE fingerprints showed these sequences to be present in essentially all surface samples (Fig. 10), although they always represented a relatively small percent of the total band intensity (between 1 and 10%, Table 2). Their contribution to band intensity appeared to decrease with depth at least in DOVETAIL (data not shown). Finally, bands belonging to chrysophytes appeared in a few samples and always accounted for a very small percent of total band intensity.

Acknowledgments

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