Molecular and pigment studies of the picophytoplankton in a region of the Southern Ocean (42–54°S, 141–144°E) in March 1998

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Abstract

Seven filtered seawater samples (depths between 30 and 55 m) collected during the SAZ project of the Austral summer of 1997–1998 were used for a simultaneous study of the picophytoplankton pigments based on high-performance liquid chromatography (HPLC) analyses and flow cytometry, and of the molecular diversity of the picophytoplankton based on their rDNA sequences. The sampling sites could be divided into three temperature zones, distinguished by their proximity to the Sub-Antarctic and Polar Fronts.

HPLC analysis of total chlorophylls and carotenoids showed fairly low phytoplankton concentrations (77–262 ng chl $a \, l^{-1}$), with minimal values of the pigments in the two samples of the Polar Front Zone around 54°S (water temperature of 4°C at time of collection). In this zone, a similar decrease of particles, identified as cyanobacteria on the basis of their fluorescence, was observed by flow cytometry.

Sequences very similar to the 16S rDNA sequence of *Synechococcus* WH8103 were present in all samples. This *Synechococcus* genotype is thus found in the Southern Ocean in addition to the Atlantic and Pacific locations where it has been previously observed. The yield of PCR products was lower in the two samples taken in the Polar Front Zone, showing a good agreement between molecular and pigment data.

16S rDNA sequences of plastids of eukaryotic algae also were retrieved, mostly related to those of an environmental clone called OM164, which has not been cultivated but has phylogenetic affinities to the Raphidophyceae. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

The picophytoplankton of size 0.2–2.0 μm includes picocyanobacteria and picoeukaryotes. The picocyanobacteria are of two types distinguished by their different pigment composition, *Synechococcus* (Johnson and Sieburth, 1979; Waterbury et al., 1979) with phycobiliproteins as antenna pigments, and *Prochlorococcus* (Chisholm et al., 1988) with divinyl chlorophyll *b* (Partensky et al., 1999). In addition, a particular phycoerythrin also was observed in one *Prochlorococcus* strain (Hess

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et al., 1999). The picoeukaryotes belong to diverse taxa, including the Pelagophyceae (Andersen et al., 1993), the Bolidophyceae (Guillou et al., 1999a), the Prasinophyceae (a primitive class of green algae), the Chlorophyceae, and the Haptophyceae (Potter et al., 1997). Recent molecular ecology studies have shown that 18S rDNA sequences from new lineages of picoeukaryotes can be retrieved from tropical waters (Moon-van der Staay et al., 2001).

In the Southern Ocean, data on picophytoplankton diversity and distribution are still fragmentary. Available studies are scarce, and many authors do not separate the pico- and nano-phytoplanktonic fractions (2–20 μm). In addition, several of the techniques generally used do not allow differentiation of the *Synechococcus*-, *Prochlorococcus*-types of cells, from the picoeucaryotes (e.g. measurement of chlorophyll *a*, counting of fluorescent cells). Though the global abundance of picoplanktonic cells appears lower in the Southern Ocean than in warmer waters (Marchant et al., 1987), their biodiversity and their variations in horizontal and vertical space, as well as in time, have yet to be elucidated.

During the Ant XII/4 cruise in March/May 1995 along the 90°W meridian, van Leeuwe et al. (1998) used high-performance liquid chromatography (HPLC) to study the pigment variations from 51° to 69°S. They showed that the cyanobacteria were present in very low concentrations, compared to Haptophyceae (Prymnesiophyceae), diatoms, and green algae. However, the pigment characteristic of cyanobacteria (zeaxanthin) was still detected at 68°S, though it was not continuously present along the transect.

Recently, Fouilland et al. (1999) have published a flow cytometric analysis of the picoplanktonic biomass at the surface during a transect La Reunion–Kerguelen (38–46°S) in September/November 1995, especially in relation to the subtropical frontal zone. They found that the biomass remained constant but that the taxonomic composition changed. The prochlorophytes that were abundant in the subtropical zone disappeared in the ACC frontal zone. This might be due to a decrease in temperature or due to changes in other water mass characteristics. In contrast, the picoeu-caryotes and cyanobacteria of the *Synechococcus*-type were constantly present, but their abundance decreased in higher latitudes.

Not only the distribution but also the taxonomy of the picocyanobacteria is still imperfectly defined. This is specially true of the organisms classified as "*Synechococcus*", a genus that may need to be split (Wood et al., 1985; Komárek, 1999). Marchant et al. (1987) have observed two different ultrastructural types of cyanobacteria in the Southern Ocean. Molecular analyses are necessary to sort out the evolutionary lineages gathered under these names. Most of these studies are based on the use of 16S rDNA sequence analysis, but sequences of the genes *psb*B, *pet*B, *pet*D, *rpo*A, *rbc*L also have been used (Palenik and Haselkorn, 1992; Urbach et al., 1992; Shimada et al., 1995). 16S rDNA sequences from 11 *Prochlorococcus* and six *Synechococcus* strains have recently been determined (Urbach et al., 1998) and show that they all belong to the same lineage.

The goal of the present study was to determine the genotypic diversity of the organisms present in the picoplanktonic fraction of the Southern Ocean waters during a N–S transect south of Australia crossing the Polar Front, and to compare this data with cell counts obtained by flow cytometry and with pigment analyses.

2. Material and methods

2.1. Sampling strategy

The samples were taken by Dr. Frank Dehairs (VUB, Belgium) during the SAZ cruise (V6 of Aurora Australis) organised by the Australian Antarctic Division during the austral summer 1997–1998. Dates and position of the stations are given in Table 1. Samples CTD4, CTD29, 1 and 2 were taken north of the Sub-Antarctic front. Samples 5–7 belonged to the Sub-Antarctic front area, and samples 3 and 4 were taken in the Polar Front zone, as shown by water temperature (Fig. 1). Samples for total phytopigment determination, flow cytometry analyses, and molecular study of the picophytoplankton fraction were

Table 1
Geographical position, date and depth of the samples, and experiments performed on the samples (listed according to their latitude)

| Sample	Latitude	Longitude	Date	Sampling depth (m)	HPLC	Flow cytometry	Molecular study
CTD4	42°05′S	141°53′E	01/03/98	45	N.d.	X	N.d.
CTD29	46°45′S	142°00E	10/03/98	45	N.d.	X	N.d.
1	47°S	141°30′E	09/03/98	55	X	N.d.	X
2	47°S	141°30′E	11/03/98	50	X	X	X
7	51°S	144°07′E	28/03/98	40	X	X	X
5	51°01′S	143°16′E	26/03/98	30	X	X	X
6	51°05′S	143°36′E	27/03/98	30	X	X	X
4	53°46′S	141°44′E	22/03/98	30	X	X	X
3	54°S	141°30′E	17/03/98	30	X	X	X

X: data available; N.d.: not done.
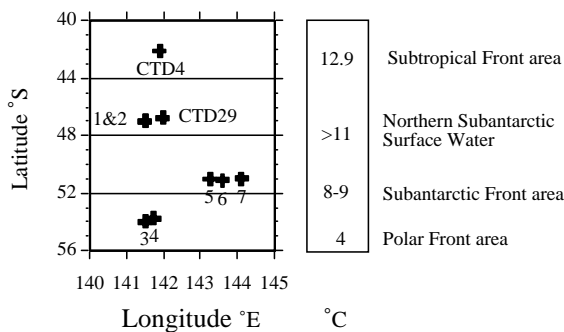
Fig. 1. Positions of the samples according to their geographic coordinates. Water temperature (°C) at the sampling depths is indicated, as are the main hydrological structures.

taken in the mixed layer, above the subsurface chlorophyll maximum (Table 1). The subsurface chlorophyll maximum is persistent in the Sub-Antarctic zone south of Australia and ranges from about 60 m in spring and early summer to 100 m or greater by March (Parslow et al., 2001).

2.2. Pigment extractions and HPLC analyses

The photosynthetic pigments were measured by HPLC and used as biomarkers to characterise the total autotrophic populations (Table 2). Samples for pigment determination (2–2.9 l) were filtered through Whatman GF/F filters and stored at $-20°C$ until analyses. Frozen filters were extracted in 100% methanol using sonication and refiltration to remove cellular debris. Phytoplankton

pigments were separated and quantified by HPLC, following the procedure of Williams and Claustre (1991). With this method, zeaxanthin and lutein coelute. Divinyl chl *a* is not resolved from chlorophyll *a*, and the sum of both chlorophylls is referred as Tchl *a*.

2.3. Flow cytometry analyses

Fresh samples were analysed at sea within 1 h of sampling using a Becton Dickinson FACScan flow cytometer, with a 15 mW argon ion laser exciting at 488 nm. The flow cytometer flow rate and size calibration were determined using calibration-grade "Fluoresbrite" beads (Polysciences), and laboratory cultured cells (e.g. *Synechococcus*, *Pyramimonas*). Lysis II software was used to acquire data from the FACScan. Phytoplankton was characterised and enumerated using red (chlorophyll, 660–700 nm) and orange (phycoery-thrin, 530–630 nm) fluorescence. (Olson et al., 1993; Hofstraat et al., 1994).

2.4. Filtrations, DNA extraction, amplification of the rDNA, cloning, clone screenings, sequence analyses

Water samples were prefiltered by Dr. Frank Dehairs (VUB, Belgium) with a vacuum Millipore pump. A prefiltration on sterile Isopore filters of 2 μm pore diameter (Millipore, Bedford, USA) of 2–2.5 l of seawater was followed by a filtration on sterile Supor filters of 0.2 μm pore diameter

Table 2
Diagnostic pigments used in this study and their taxonomic significance

Pigment	Taxonomic significance	References
Chlorophyll *a*	All photosynthetic microalgae (except prochlorophytes)	[a]
Divinyl chl *a*	Prochlorophytes	[b]
19′-HF	Haptophyceae (Prymnesiophyceae)	[a]
19′-BF	Haptophyceae (Prymnesiophyceae), Chrysophyceae, Pelagophyceae	[a,c]
Fucoxanthin	Diatoms, Bolidophyceae	[a,d,e]
Zeaxanthin	Cyanobacteria, Prochlorophytes	[a,f]
Lutein	Green algae	[a]

[a] Jeffrey et al. (1997).
[b] Goericke and Repeta (1992).
[c] Latasa and Bidigare (1998).
[d] Goffart et al. (2000).
[e] Guillou et al. (1999b).
[f] Vidussi et al. (2000).

(Gelman, Ann Arbor, USA). The filters were placed in sterile plastic tubes with 2 ml of lysis buffer (Giovannoni et al., 1990). All samples were kept at $-20°C$, and transported cooled from Tasmania to Belgium, where they were stored at $-20°C$ until extraction.

The DNA was extracted by a modification of the hot phenol method (Giovannoni et al., 1990). 50 µl of lysozyme (50 mg ml^{-1}) were added to the tube with lysis buffer and filter, and the tube was incubated for 20 min at 37°C. After addition of 100 µl of SDS 10% and 25 µl of proteinase K (20 mg ml^{-1}), an incubation for 2 h at 37°C followed. An equal volume of warm phenol equilibrated with Tris–HCl (Gibco Life Technologies) was added, mixed carefully a few times, and incubated for 10 min at 56°C. The mixture was transferred to a Corex tube and centrifuged at 5000 rpm for 15 min. The supernatant was extracted with an equal volume of warm chloroform/isoamylalcohol (24:1) for 20 min at 56°C and the mixture was centrifuged at 5000 rpm for 15 min. The nucleic acids were precipitated from the supernatant (divided into microcentrifuge tubes) by addition of 0.6 volume of isopropanol and incubation at $-20°C$ for 3 h. The microcentrifuge tubes were centrifuged for 30 min at 13,000 rpm. The pellet was rinsed with 500 µl of ethanol 70% and air-dried before dissolution of the nucleic acids in 40 µl buffer TE^{-4} (10 mM Tris HCl pH 7.4 and 0.1 mM EDTA pH 8.0).

For the amplification of the 16S rDNA plus internally transcribed spacer (ITS) by PCR, 0.2–2 µl of the extracted DNA solutions were added to $1 × $ PCR buffer of Super Taq Plus, 0.2 mM dNTPs, 0.4 µM primer 16S5′F, 0.4 µM primer 23S5′R, 1 mg ml^{-1} bovine serum albumin (BSA), and 0.8 U Super Taq Plus polymerase with a proof-reading activity (HT Biotechnology, UK) in a total volume of 50 µl. The primer sequences, derived from Wilmotte et al. (1993) were "16S27F" (AGAGTTTGATCCTGGCTCAG) and "23S26R" (TCTGTGTGCCTAGGTATCC), respectively (5′–3′ direction). The thin-wall tubes were submitted to thermal cycling in the Gene Cycler^{TM} (BioRad, USA): 180 s at 94°C; 10 cycles of 45 s at 94°C, 45 s at 57°C, 120 s at 68°C; 15 cycles of 45 s at 92°C, 45 s at 53°C, 150 s at 68°C, and a final elongation step of 7 min at 68°C. The PCR products were visualised after 1% (w/v) agarose gel electrophoresis and stored at –20°C.

Construction of clone libraries was performed for samples 2, 4 and 5, using the PCR-products purified with the kit PCR Kleen Spin Columns (BioRad, USA). The clones derived from these samples were designated 2X, 4X, and 5X, respectively. Moreover, for sample 5, a separation based on the length of the three bands observed after electrophoresis of the PCR product was carried out before additional cloning reactions. Five PCR reactions were pooled and the bands of different lengths were separated by electrophoresis on 1%

agarose in TAE buffer and excised under sterile conditions from the gel. The DNA was extracted by centrifugation at 7000 rpm for 10 min on a mini-column made with glass-wool (Heery et al., 1990). It was precipitated with ethanol, rinsed and air-dried. The DNA was resuspended in 20 µl of buffer TE^{-4} (10 mM Tris–HCl, 0.1 mM EDTA, pH. 8) for cloning. The clones from the lower, middle, or upper band of sample 5 were designated 5I, 5M, and 5S, respectively. For all the samples, cloning was performed with the kit TOPO™ TA Cloning (Invitrogen, USA) following the manufacturer's instructions. White and light-blue transformants were purified twice by streaking and then screened by PCR on the cells using 16S27F and 23S26R primers. Plasmids from positive transformants were extracted with the Quantum Prep Plasmid Miniprep Kit (Bio-Rad, USA). They were screened by digestion of the PCR products with the restriction enzyme *Rsa*I (Gibco Life Technologies), to obtain a preliminary grouping and select representative clones to sequence.

For sequencing, 25 µl of plasmid DNA was sent to GenomeExpress (Paris, France) for sequencing reactions with the primer 16S27F on an ABI PRISM system 377 (PE Applied Biosystems, USA). For the cyanobacterial clone 5X15, a full insert was sequenced on both DNA strands with primers 16S513R (TTA CCG CGG CTG CTG GCA CGG), 16S533F (CCG TGC CAG CAG CCG CGG TAA), 16S1094R (GGG TTG CGC TCG TTG CGG GAC), 16S1114F (GTC CCG CAA CGA GCG CAA CCC), 16S1494R (GTA CGG CTA CCT TGT TAC GAC) and the consensus sequences obtained with the Genetics Computer Group, Inc. (GCG) package, available remotely at Belgian EMBnet Node (BEN). The complete 16S rDNA sequence of 5X15 was 1479 bases long (including the primer sequence) and was deposited in the EMBL database under the accession number AJ289785. Partial sequences of 5X11 and 5I4, that are representative of different clusters in the phylogenetic analysis, also were deposited and obtained the accession numbers AJ315778 and AJ315779, respectively.

The 26 partial sequences obtained were analysed by a similarity search using the software BLAST widely available on Internet. After detection of chimera using "Chimera Check" of the RDP-II (http://rdp.cme.msu.edu/), an alignment of the cyanobacterial sequences was obtained using the software CLUSTALW and corrected manually with the software BioEdit (http://www.mbio.nc-su.edu/BioEdit/bioedit.html). The sequences of clones 2X4, 2X13, and 2X17 are identical, as are those of clones 5X9 and 5X18, and only 2X4 and 5X9 were included in the analysis. 685 positions (corresponding to *E. coli* positions 101–757) were used for tree construction. The distance tree was constructed with the TREECON for Windows v1.2 software package (Van de Peer and De Wachter, 1994). A distance matrix was computed using the Jukes and Cantor (1969) coefficient option (not taking into account the insertions/ deletions), followed by the construction of a tree according to the Neighbor-joining method (Saitou and Nei, 1987). A bootstrap analysis involving 500 resamplings was performed.

3. Results and discussion

3.1. Pigment analyses by HPLC

HPLC analysis of Tchl *a* and carotenoids are shown in Fig. 2. Mixed-layer Tchl *a* concentration are $< 300 \, \text{ng} \, \text{l}^{-1}$. They range from 77 to 262 ng Tchl $a \, \text{l}^{-1}$, with minimal concentrations in two samples of the Polar Front area (samples 3 and 4). The latitudinal variation of the main taxonomic pigments show that: (1) flagellate carotenoids (19′-hexanoyloxyfucoxanthin (19′-HF) + 19′-butanoyloxyfucoxanthin (19′-BF)) are the dominant accessory pigments throughout the transect; (2) fucoxanthin is ubiquitous, but is present in low concentration $(15–36 \, \text{ng} \, \text{l}^{-1})$; (3) zeaxanthin/lutein does not contribute significantly to the total carotenoid concentration, except in the warmest waters $(>11°C)$. At the southern end of the transect (sample 3), zeaxanthin/lutein concentration is below the detection limit of the instrument $(<1 \, \text{ng} \, \text{l}^{-1})$.

Even if the correspondence between marker pigments and algal classes is complex, the major phytoplankton signatures indicate that the nano- and pico-size flagellates containing 19′-HF + 19′-BF,
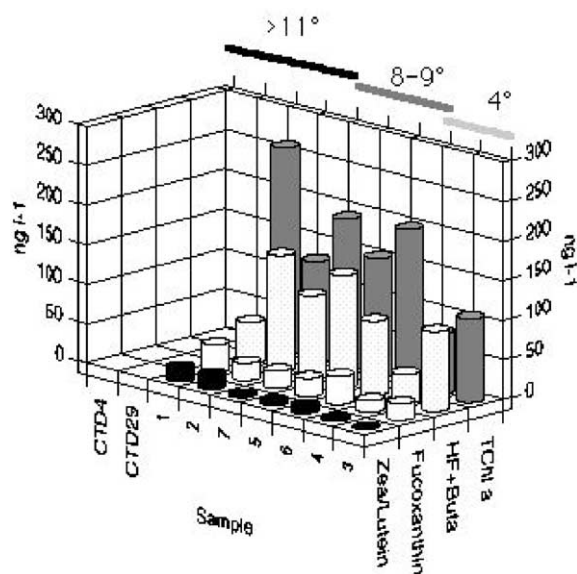
Fig. 2. Distribution of Tchl *a*, 19′-hexanoyloxyfucoxanthin (19′-HF) + 19′-butanoyloxyfucoxanthin (19′-BF), fucoxanthin and zeaxanthin/lutein (ng l^{-1}) along the N–S transect (North at the left side), as a function of the water temperature. Water temperature (°C) at the sampling depth is indicated in the upper part of the figure.

mainly Haptophyceae (Prymnesiophyceae), Chrysophyceae and Pelagophyceae, dominate the phytoplankton community along the transect. Diatoms, characterised by fucoxanthin, are the second group in importance. The presence of Bolidophyceae, a newly described picoplanktonic algal class which contains fucoxanthin as the major carotenoid (Guillou et al., 1999a), is possible but they cannot be distinguished from diatoms based on their pigment signatures. However, as far as reported in the recent literature, this new class is a minor component of the natural picoeukaryotic populations (Guillou et al., 1999b). The contribution of cyanobacteria and prochlorophytes to the total biomass, derived from zeaxanthin concentration, is of reduced importance and decreases towards the south.

3.2. Fluorescent pigment analyses by flow cytometry

Bivariate scatter plots and histograms of particle distribution are here used to analyse population structure. Regions 1–7 have been defined in the scatter plot to separate particles with different optical characteristics, for example of size or auto-fluorescent emission. The regional analysis uses the output from the red fluorescence (FL3) detector plotted against the output from the orange fluorescent (FL2) detector. In this type of plot, particles with similar auto-fluorescent characteristics but dissimilar size or complexity are not differentiated. However, much phytoplankton population information can be gleaned from the simple two-dimensional scatter plots, especially, when the data are considered alongside light microscope cell counts, and HPLC pigment data. The R2 particles are mainly living biliprotein-rich cyanobacterial cells. These are numerous in the upper 45 m of the watercolumn from 42° to 47°S, but scarce south of the Sub-Antarctic front. R3 particles show a low level of red fluorescence, and a few of these particles are intact prokaryotic or eukaryotic cells. However, microscopy of the samples suggests that many particles in the R3, and some in the R4, regions are fragments of cells and cell debris. Much of this debris is fluorescent material, such as isolated chloroplasts, derived from larger cells, and the particle count is increased by filtration procedures. R4 particles have low levels of red fluorescence, and are mostly picoplanktonic eukaryotes, such as small diatoms and silicoflagellates, but some cell fragments are also counted here. R5–R8 are regions of nanoplankton with relatively strong red (chlorophyll) fluorescence (e.g., prasinophytes and diatoms) (Durand and Olson, 1996; Wright et al., 1998). The division into regions of the scatter plot is shown in Fig. 3 for sample 7.

The unfiltered water samples were used to count auto-fluorescent particles (Fig. 4). Increased particle counts in scatter regions R3 and R4 were given by the flow cytometer following 2 μm filtration, and probably include numerous cell fragments as well as intact cells. Murphy and Haugen (1985) pointed out that size fractionation by filtration can lead to cell disintegration, and to changes in cell shape and cell wall flexibility.

The samples CTD4, CTD29 and 2 yielded the highest counts of particles for all scatter regions. Intermediate counts were obtained in samples 5–7.
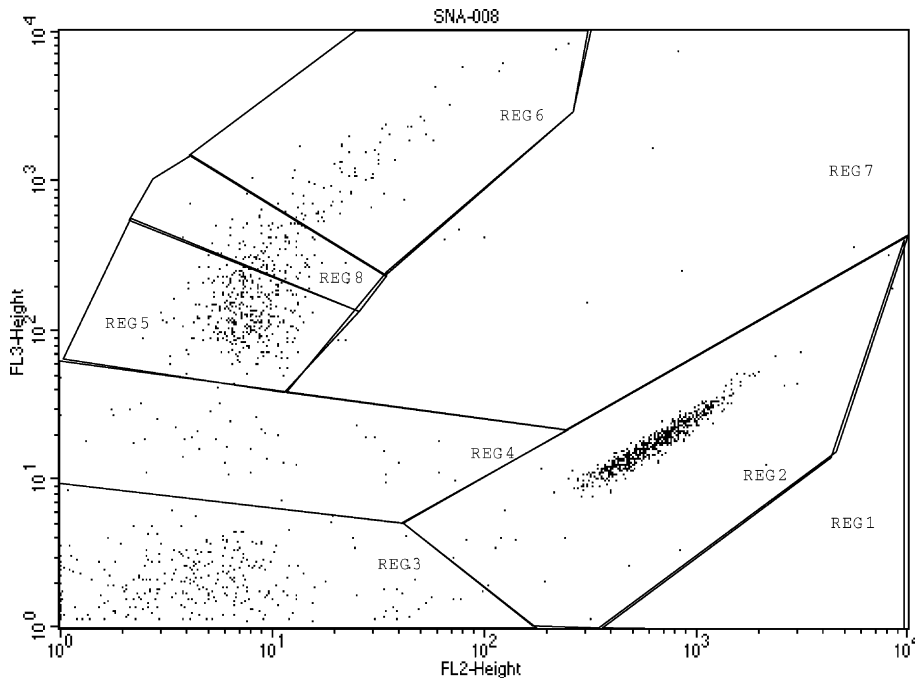
Fig. 3. Scatter plot, obtained by flow cytometry, of sample 7 (SNA-08), showing the regions 1–8 distinguished in this analysis. FL2—orange fluorescence, FL3—red fluorescence.
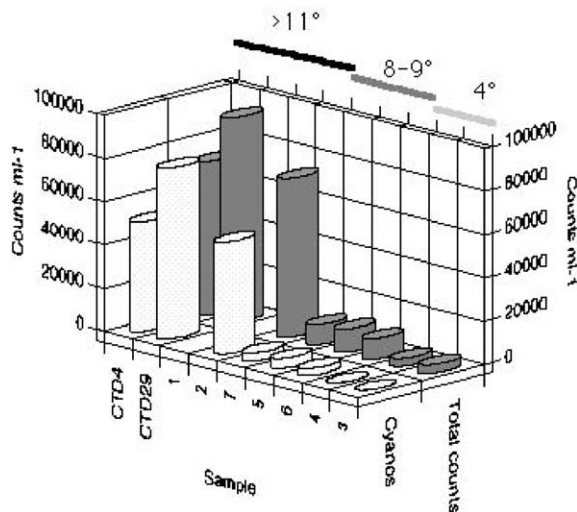
Fig. 4. Abundance of total and cyanobacterial cells (cells ml^{-1}) along the N–S transect (North at the left side). Water temperature (°C) at the sampling depth is indicated in the upper part of the figure.

In the most southern samples (3 and 4), there was a sharp decrease in particle numbers. Thus, our samples showed a decline in the concentration of auto-fluorescent particles with higher latitudes, in the Polar Front Zone. A similar effect was observed in flow cytometric counts for R2 (cyanobacteria), for the present stations, and for other samples taken during the same cruise (Hutchins et al., 2001). This decrease was sharp between sample 2 (ca. 5×10^5 particles ml^{-1}) and 7, 6, 5 (ca. 4×10^3 particles ml^{-1}) and reached minimal values for samples 3 and 4 (ca. 10^2 particles ml^{-1}).

3.3. Molecular diversity of the picoplanktonic rDNA sequences

3.3.1. Visualisation of the band lengths of the PCR products

PCR amplification of a part of the ribosomal operon (16S rDNA + ITS) from seven picoplanktonic fractions (0.2–2 μm) of seawater samples has given three different banding patterns, showing that the biological diversity was probably different between the samples belonging to these three patterns. As shown in Fig. 5, the band around 2300 bp, which corresponds to the length of the

Fig. 5. Gel electrophoresis on 1% agarose of 10 μl PCR products obtained from the DNA of the fraction 2–0.2 μm. M: length marker is λ DNA digested with HindIII and the band sizes are indicated at the right of the gel. The numbers of the samples are given above the gel.

3.3.2. Genotypic diversity of the picocyanobacterial and plastidial clones

The clone libraries yielded 31, 108, and 54 recombinant clones for samples 2, 4 and 5, respectively. A restriction digest with *Rsa*I was performed and the sequences with similar banding patterns were grouped (data not shown). Representatives were partially sequenced to assign the groups to broad taxonomic clusters. For sample 2, 80% of the clones belonged to the *Synechococcus* cluster, 10% to a cluster with sequences related to alpha-proteobacteria and the remaining 10% were not assigned to any defined cluster. In the case of sample 4, 2% of the clones belonged to the *Synechococcus* cluster, 28% to the alpha-proteobacteria cluster and 32% to a cluster of sequences similar to the uncultivated clone OM164, of which the closest relative, according to Rappé et al. (1998) was *Heterosigma akashiwo* (Raphidophyceae) (sequence not available in Genbank nor RDP-II). For sample 5, 4% of clones belonged to the *Synechococcus* cluster, 20% to the alpha-proteobacteria cluster and 39% to the cluster with OM164-types. A direct comparison is difficult because the total number of clones in each library was different, but the trend is clearly that the majority of clones in sample 2 belong to the *Synechococcus* cluster and that this proportion is much lower in samples 4 and 5.

The bands with a length of 1800–2000 bp correspond probably to plastid and bacteria sequences, based on observations of ITS sequence lengths available in Genbank. Unfortunately, the cloning efficiencies of the bands separated on an agarose gel on the basis of their lengths were very low and did not yield conclusive results because the separation was not complete. However, the partial sequences of the clones 5I3, 5I4 and 5I18 were used in the analysis.

Among 25 partially sequenced clones, 10 appeared to have an eubacterial origin. An alignment of the 15 partial remaining cyanobacterial and plastid sequences with clone 5X15, is given in Fig. 6.

A distance tree depicting the relationships of 13 clone sequences from samples 2 and 5 to other picocyanobacteria and plastids was built (Fig. 7). Clones 2X18 and 2X20 possess only 1 (position

PCR product from clone 5X15 (that belongs to the *Synechococcus* cluster), is extremely strong in samples 1 and 2, almost invisible for samples 3 and 4, and moderate in samples 5–7. This observation is reproducible. Brüggemann et al. (2000) have shown that quantitative estimates could be obtained after 20 PCR cycles using a quantitative standard in bacterial laboratory mixtures and compost. Because we start from a very low biomass, it is likely that the PCR process is still in its exponential phase after 25 cycles, and thus, we infer that these differences in band yields are probably related to the abundances of target organisms in the samples. The intensity of the 2300 bp-band signal falls with decreasing temperature and increasing latitude. A similar effect was observed in flow cytometric counts for R2 (cyanobacteria) during the same cruise (Hutchins et al., 2001) and in another Sub-Antarctic area (Fouilland et al., 1999). Overall there are correlations between temperature, cyanobacterial count and the 2300 bp-band signal.

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                       10        20        30        40        50        60        70        80        90       100       110
picoclone 5X15     AGTGGCGGACGGGTGAGTAACGCGTGAGAATCTGCCCTCAGGAGGGGGATAACAGCTGGAAACGGCTGCTAATACCCCATATGCC-GCGAGGTGAAA-TGAATT-TC-GC
picoclone 5I3      ........................................................................................................
picoclone 5X14     ......................G.............................G..C.........C.....................A.................
picoclone 2X23     ......................G.................................................................................
picoclone 2X15     ........................................................................................................
picoclone 2X22     ........................................................................................................
picoclone 2X4      ........................................................................................................
picoclone 5X9      ...................A...CT...T...AA.......TA......C.........GT......A...TTTT.......A..G.AA..T..
picoclone 5X18     ...................A...CT...T...AA.......TA......C.........GT......A...TTTT.......A..G.AA..T..
picoclone 5X11     ...................A...CT...T...AA.......TA......C.........GT......A...TTTT.......A..G.AA..TA..
picoclone 5I18     ...................A...MT...T...AA.......TA......C.T......GT......A...TTTT.......A..G.AA..T..
picoclone 5X17     ...................TC...AT...AA.......TA......C.....AGT......A...TTTT.......CA..G.AA..T..
picoclone 5X10     ...................TC...AT...AA.......TA......C.....AGT......A...TTTT.......CA..G.AA..T..
picoclone 5I4      ...................T...T.T.......A.C...T.A....T.A......G...........TATAATA......CG..T.CG..C..

                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                      120       130       140       150       160       170       180       190       200       210       220
picoclone 5X15     CTGAGGATGAGCTCGCGTCTGATTAGCTAGTTGGTGTAGGTAATGGCTCACCAAGGCGTCGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACAC
picoclone 5I3      ..................................................................A.....................................
picoclone 5X14     ...........C...............A...-...GA.......A.................................................
picoclone 2X23     ..................................................................A.....................................
picoclone 2X15     .......................................G...............A.....................................
picoclone 2X22     ..................................................................A.....................................
picoclone 2X4      ..................................................................A.....................................
picoclone 5X9      ..A...G..................A-G...A...CT.........AT.......T...T......C..C..
picoclone 5X18     ..A...G..................A-G...A...CT.........AT.......T...T......C..C..
picoclone 5X11     ..A...G..................A-G...A...CT.........AT.......T...T......C..C..
picoclone 5I18     ..A...G..................A-G...A...CT.........AT.......T...T......C..C..
picoclone 5X17     ..AT...GA................-G...A...CT.........AT.......T.............
picoclone 5X10     ..AT...GA................-G...A...CT.........AT.......T.............
picoclone 5I4      ..AGA...AA................T...A-.......A...CT.........A.............T.............A........

                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                      230       240       250       260       270       280       290       300       310       320       330
picoclone 5X15     GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGCAAGCCTGACGGAGCAACGCCGCGTGAGGGATGAAGGCCTCTGGGCTGTAAACCTCTTTT
picoclone 5I3      .........................................A.............................................................
picoclone 5X14     .........................................A.............................................................
picoclone 2X23     .........................................A.............................................................
picoclone 2X15     .........................................A.............................................................
picoclone 2X22     .........................................A.............................................................
picoclone 2X4      .........................................A.............................................................
picoclone 5X9      .....................A...C.........A.............TA..............C...T...T...........
picoclone 5X18     .....................A...C.........A.............TA..............C...T...T...........
picoclone 5X11     .....................A...C.........A.............TA..............C...T...T...........
picoclone 5I18     .....................A...Y.........A.............TA.........A...C...T...T...........
picoclone 5X17     .....................A.............A.............TA..............C...T...T...........
picoclone 5X10     .....................A.............A.............TA..............C...T...T...........
picoclone 5I4      ..T...................T.............A...TA.............CG....TC............

                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                      340       350       360       370       380       390       400       410       420       430       440
picoclone 5X15     ATCAAGGAAGAAGATCTGACG-GTACTTGATGAATAAGCCACGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGGAGTGGCAAGCCGTTATCCGGAATTATTGGGCGTA
picoclone 5I3      ........................................................................................................
picoclone 5X14     ........................................................................................................
picoclone 2X23     ........................................................................................................
picoclone 2X15     ........................................................................................................
picoclone 2X22     ........................................................................................................
picoclone 2X4      ........................................................................................................
picoclone 5X9      T......G...T......T.......A.....AT.......C...........G...AG.AT.....T.....................
picoclone 5X18     T......G...T......T.......A.....AT.......C...........G...AG.AT.....T.....................
picoclone 5X11     T......G...T......T.......A.....AT.......C...........G...AG.AT.....T.....................
picoclone 5I18     T......G...T......T.......A.....AT.......C...........G...AG.AT.....T.....................
picoclone 5X17     T......G...T......T.......A.....AT.......C...........G...AG.AT.....T.....................
picoclone 5X10     T......G...T......T.......A.....AT.......C...........G...AG.AT.....T.....................
picoclone 5I4      T......G...T......T.......A.....AT.......C...........G...AG.AT.....T....------------------

                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                      450       460       470       480       490       500       510       520       530       540       550
picoclone 5X15     AAGCGTCCGCAGGCGGCCCTTCAAGTCTGCTGTTAAAAAGTGGAGCTTAACTCCATCATGGCAGTGGAAACTGAGGGGCTTGAGTGTGGTAGGGGCAGAGGGAATTCCCG
picoclone 5I3      ..........................................................................G...T....................
picoclone 5X14     ..........................A...........................................TT....C..................
picoclone 2X23     ........................................................................................................
picoclone 2X15     ........................................................................................................
picoclone 2X22     ........................................................................................................
picoclone 2X4      ........................................................................................................
picoclone 5X9      ......T.T...TT...TAA.........T.......GGT.AA.........TT.AA.CT....CA.......TTA.........ACA..C.A..T.........TY.T
picoclone 5X18     ......T.T...TT...TAA.........T.......GGT.AA.........TT.AA.CT....CA.......TTA.........ACA..C.A..T.........T..T
picoclone 5X11     ......T.T...TT...TAA.........T.......GGT.AA.........TT.AA.CT....CA.......TT.........ACA..C.A..T.........T..T
picoclone 5I18     ......T.T...TT...TAA.........T.......GGT.AA.........TT.AA.CT....CA.......TTA.........ACA..C.A..T.........T..T
picoclone 5X17     ......T.TG..TT...TAG.......AT.......GGT.AA.........TT.AA.CT....A.....CTAA.........ACA..C.A..T.........T..A
picoclone 5X10     ......T.T...TT...TAG.......AT.......GGT.AA.........TT.AA.CT....A.....C.CTAA.........ACA..C.A..T.........T..A
picoclone 5I4      --------------------------------------------------------------------------------------------------------

                   ....|....|....|....|....|....|....|....|....|....|....|....|....|
                      560       570       580       590       600       610
picoclone 5X15     GTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCATC
picoclone 5I3      ................................................................
picoclone 5X14     ........................-----------------------------------------
picoclone 2X23     ................................................................
picoclone 2X15     ................................................................
picoclone 2X22     ................................................................
picoclone 2X4      ................................................................
picoclone 5X9      ...G...................G..A.G.......A......A.A...A..A...TT.T
picoclone 5X18     ...G...................G..A.G.......A......A.A...A..A...TT.T
picoclone 5X11     ...G...................G..A.G.......A......A.A...A..A...TT.T
picoclone 5I18     ...G...................G..A.G.......A......A.A...A..A...TT.T
picoclone 5X17     ...AG..................T..A.G.......A......A.A...A..A...TT.T
picoclone 5X10     ...AG..................T..A.G.......A......A.A...A..A...TT.T
picoclone 5I4      -----------------------T..A.G.......A......A.A...A..A...TT.T----
```
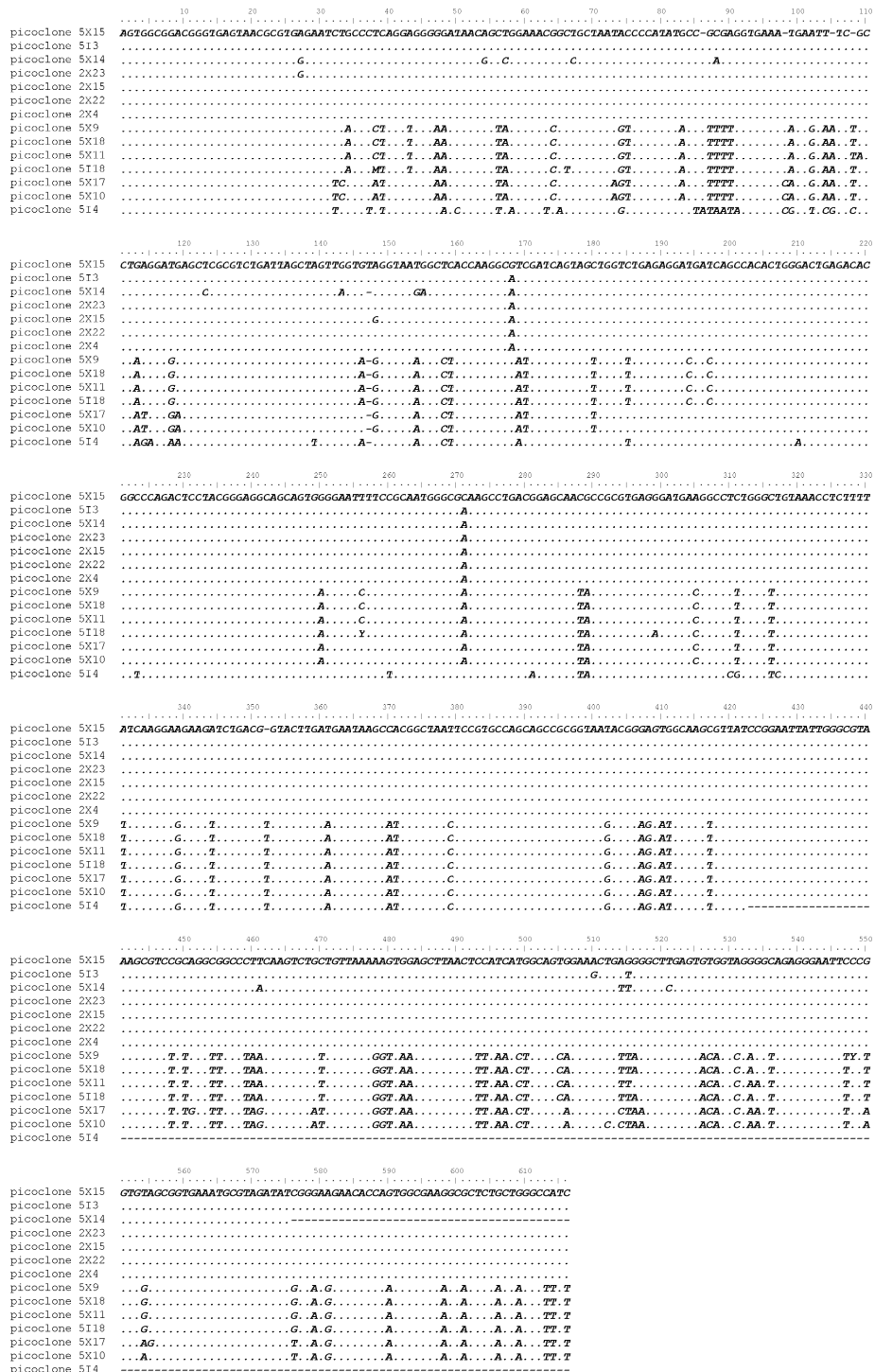
Fig. 6. Alignment of 616 positions of the 16S rDNA of 16 clones obtained in this study, positions identical to those of clone 5X15 are indicated by a dot.
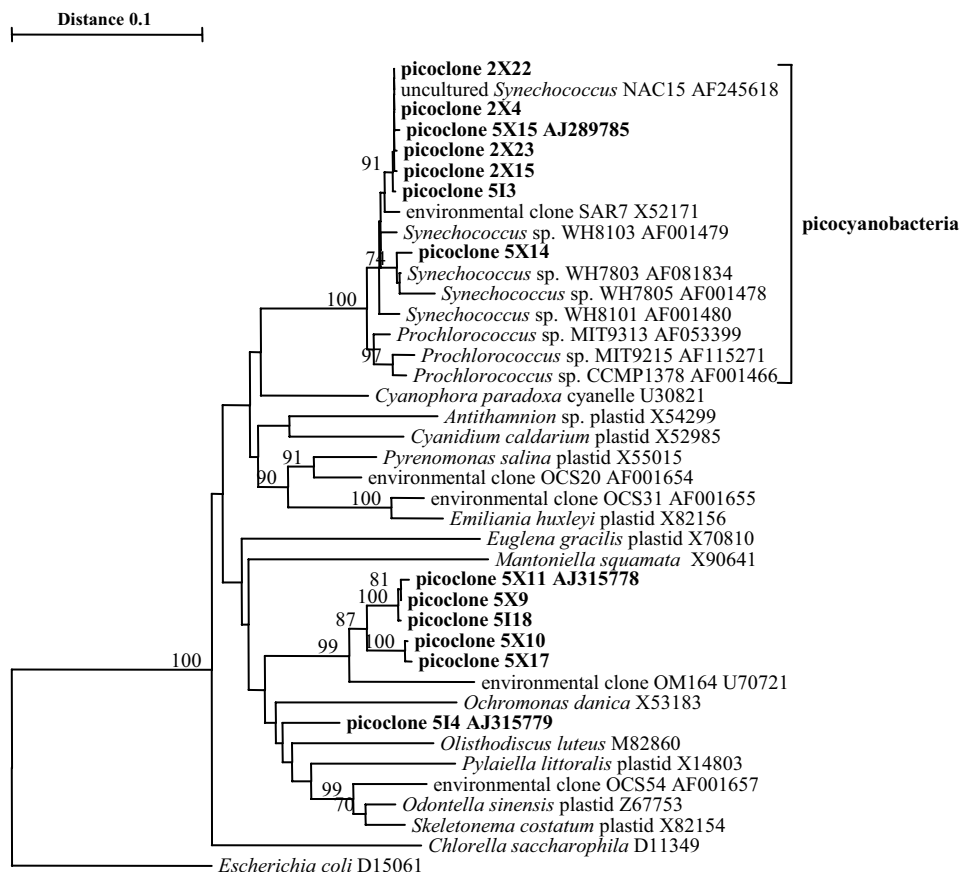
Distance 0.1

Fig. 7. Distance tree based on 685 alignment positions of the 16S rDNA from 25 published cyanobacterial and plastid sequences, the sequence of 14 clones determined in the present study (in bold) and the outgroup *Escherichia coli*. The dissimilarity values corrected with the equation of Jukes and Cantor (1969) were used to calculate the distance matrix and the tree was constructed by the Neighbor-joining method, as implemented in the software TREECON for Windows v1.2 (Van De Peer and De Wachter, 1994). Bootstrap values equal or higher than 70% are shown next to the node concerned. Evolutionary distance between two sequences is obtained by summing the lengths of horizontal branches connecting them, taking into account the scale on top (0.1 mutations/position).

198 in Fig. 6) and 2 base (positions 148, 348 in Fig. 6) differences with clone 2X4 and were not included. Only 685 alignment positions could be used due to limited length of the sequences. The grouping of six Southern Ocean clones from this study (2X4, 2X15, 2X22, 2X23, 5X15, 5I3) with an environmental clone sequence NAC15 (Gonzalez et al., 2000) isolated from the North Atlantic is supported by a high bootstrap value of 91%. The closest related strains are *Synechococcus* sp. WH8103 and WH8101. They show sequence similarities of 97.8% and 97.4% (calculated on 612 positions minus the ambiguities) with clone

5X15, respectively. The sequence of 5X14 is more closely related to those of *Synechococcus* WH7803 (98.7% similarity for 562 positions) and WH7805. Another picoplanktonic cluster, supported by 99% bootstrap, groups five clones of sample 5 (5I18, 5X9, 5X10, 5X11, 5X17) with the plastid sequence of OM164. The similarity of the latter sequence with clones 5X10 and 5X17 is 92% and around 90.5% with the three other clones. Clone OM164 was retrieved from the continental shelf off the North Carolina coast, USA and has affinities to the Raphidophyceae (Rappé et al., 1998). Another Antarctic clone, 5I4, has affinities

to the plastid sequences of *Olisthodiscus luteus* (Raphidophyceae) and *Ochromonas danica* (Chrysophyceae) but does not clearly belong to any cluster.

As reported by other authors working in molecular ecology (Fuhrman & Campbell, 1998), our clone libraries contained sequences that were closely related but not identical to each other. In some cases, this might be the result of an error of the enzyme, though we have used a proof-reading DNA polymerase. In the case of *Prochlorococcus* species, these sequence variations have been shown to coincide with differences in light adaptiveness (Moore et al., 1998). As our samples were not used for strain isolations, it is not possible to know if adaptation to environmental conditions could explain this microdiversity.

4. Conclusion

The picophytoplankton 16S rDNA sequences were obtained from natural samples characterised by moderate phytoplankton concentrations where haptophytes (prymnesiophytes), diatoms and chrysophytes are the major contributors of biomass. The presence of bolidophytes cannot be ruled out on the basis of the pigment composition but until now they have only been reported as minor components of the picoplankton (Guillou et al., 1999b). In the absence of plastid sequences in Genbank, we cannot draw conclusions at the moment on their presence in the Southern Ocean.

The 16S rDNA sequences obtained from the clone libraries of samples 2 and 5 contained a sequence type closely related to Sargasso Sea picoplanktonic *Synechococcus* (e.g., WH8103). Thus, we have confirmed by a molecular approach the presence of picocyanobacteria of the *Synechococcus* type in the waters of the Southern Ocean at 47° and 51°S between Tasmania and Antarctica.

The fact that all the picoplanktonic *Synechococcus* sequences from different oceans are closely related on the basis of the 16S rDNA sequences, which is the standard marker for molecular taxonomy, seems to indicate that a similar genotype has colonised different types of waters and developed physiological adaptations to these different environmental conditions, in particular from the point of view of the light exploitation. Thus, the most ecologically significant variations might be at the level of pigment compositions, but it is uncertain whether they are reflected at the level of the 16S rDNA. However, such a correspondence has been shown for another molecular marker, the *rpo*C sequences of strains of *Synechococcus* from the California Current (Toledo & Palenik, 1997). The use of cultivated strains is necessary to demonstrate whether there is a vertical distribution of picoplanktonic *Synechococcus* with different genotypes in the open waters of the Southern Ocean.

With our PCR primers, we have obtained a characteristic 2300 bp PCR product for clone 5X15 and related sequences. In the seven studied samples, we have observed that with increasing latitude, there is a decrease in the number of autofluorescent particles measured by flow cytometry and in the pigment content of the seawater. The lowest counts were observed in samples taken around 54°S in the Polar Front Zone. In these samples, the PCR yield of the band of 2300 bp (characteristic of the picoplanktic *Synechococcus*) was also lowest. This corresponds to the observation that *Synechococcus* abundance decreases with temperature during transects between Tasmania and Antarctica (Marchant et al., 1987) and between La Reunion and Kerguelen (Fouilland et al., 1999).

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