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Structure and seasonal dynamics of the eukaryotic picophytoplankton community in a wind-driven coastal upwelling ecosystem

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Abstract

We studied the structure and seasonal dynamics of the photosynthetic picoeukaryote community at a coastal site off central Chile that is strongly affected by seasonal wind-driven upwelling. We determined the picoeukaryote phylogenetic diversity by cloning and sequencing the 18S ribosomal ribonucleic acid (rRNA) gene amplified with Chloroplastida-biased primers. Chloroplastida made up 100% (autumn), 61% (spring), and 20% (summer) of the clone libraries. Most sequences belonged to Mamiellophyceae and clustered with *Micromonas* (clades A.BC.1, B.E.3, C.D.5), *Ostreococcus* (clade A), and *Crustomastix* (clade A). Fluorescent in situ hybridization coupled with tyramide signal amplification (FISH–TSA) was used to determine the time variability in abundance of the total picoeukaryotic community, the Chloroplastida, and the genera *Micromonas*, *Ostreococcus*, and *Bathycoccus* over a period of 2 yr with nearly monthly sampling. The abundance of photosynthetic picoeukaryotes was low during the upwelling season (spring and summer) and high during the nonupwelling season (late fall and winter). Chloroplastida made up, on average, 74% of the total picoeukaryotic community, and the sum of the three genera probed accounted for 100% of the Chloroplastida abundances. *Ostreococcus* dominated the picophytoplanktonic community numerically throughout the year and, thus, appears to be a key component of the upwelling picoplanktonic community in the eastern South Pacific.

Size is often used to classify the organisms that inhabit the water column. Picoplankton ($\leq$ 2–3 μm in diameter) comprises the three major domains of life: Archaea, Bacteria, and Eukarya. Photosynthetic marine picoplankton includes both cyanobacteria, represented mainly by two genera (*Synechococcus* and *Prochlorococcus*), and photosynthetic picoeukaryotes (PPEs), that can belong to a wide range of algal classes (Vaulot et al. 2008). Elucidating the diversity of PPEs started just over a decade ago with the application of molecular techniques such as 18S ribosomal ribonucleic acid (rRNA) gene cloning and sequencing (Moon-van der Staay et al. 2001).

Despite the evidence that PPEs can contribute significantly to carbon biomass in the picoplanktonic fraction (up to 75%, Worden et al. 2004), PPEs have been much less extensively studied than their cyanobacterial counterparts, and their ecological and biogeochemical roles are still poorly understood. This is particularly true in productive coastal waters, where large phytoplankton dominates in terms of biomass and productivity, but where PPEs can represent an important carbon source for the heterotrophic protist community that graze on the small size fractions (Bec et al. 2005). The large carbon biomass of PPE is due to their large cell size in comparison with the numerically abundant cyanobacteria (Worden et al. 2004). Thus, PPEs may contribute significantly to the carbon flux through the microbial web in coastal systems (Worden et al. 2004; Bec et al. 2005), especially when larger phytoplankton (nanoplankton and microplankton) is not blooming (Vargas et al. 2007). Furthermore, Richardson and Jackson (2007) have proposed that photosynthetic picoplankton can also play a major role in the carbon export to the deeper ocean, even in eutrophic areas.

Pigment and molecular analyses are important tools to study the presence and abundance of certain groups in the eukaryotic fraction of the picoplankton. So far, studies have found that prasinophytes (Chloroplastida) dominate in coastal waters (Not et al. 2005; Worden 2006), mainly because of the presence of three genera from the class Mamiellophyceae: *Micromonas*, *Bathycoccus*, and *Ostreococcus* (Not et al. 2004; Zhu et al. 2005; Worden 2006). However, little is known about the ecology of these organisms (e.g., abundance and distribution), but it has been observed that a single genus can, in some cases, dominate the picoeukaryotic community (Not et al. 2004). *Micromonas*, despite being widely distributed (Slapeta et al. 2006), seems to be better adapted to nutrient-rich conditions (Not et al. 2004, 2005; Viprey et al. 2008), unlike *Ostreococcus*, which seems to be adapted to mesotrophic conditions (Viprey et al. 2008). Although *Ostreococcus* has been detected generally at low abundances in marine waters (Not et al. 2004; Zhu et al. 2005), high abundances have been reported at some coastal sites during short bloom periods (O'Kelly et al. 2003; Countway and Caron 2006). *Ostreococcus* dominates the picophytoplankton community throughout the year in the Thau Lagoon, where it was originally discovered (Courties et al. 1994). On the other hand, while several intrageneric clades have been identified for *Micromonas* (Guillou et al. 2004; Slapeta et al. 2006; Worden 2006), *Ostreococcus* (Guillou et al. 2004),

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and *Crustomastix* (Viprey et al. 2008), *Bathycoccus* seems to be quite homogeneous genetically (Guillou et al. 2004; Viprey et al. 2008). However, coastal environments can be quite distinct with respect to factors such as coastal circulation, land and river influence, or availability of trace metals, and PPEs have been surveyed in only a few coastal systems, mostly in the Northern Hemisphere. Moreover, microbial time-series studies are still scarce, and, with a few exceptions (Not et al. 2004; Countway and Caron 2006), they mostly report on the PPE community as a whole, without taxonomic resolution (Worden et al. 2004; Sherr et al. 2005).

The role of eukaryotic picoplankton in coastal upwelling ecosystems of the eastern South Pacific, one of the most productive marine systems of the world, has not been extensively studied. In the coastal area off central Chile (30°–40°S), wind-driven upwelling of nutrient-rich subsurface waters is highly seasonal. The system shifts from very productive in spring–summer, because of the presence of large chain-forming diatoms (González et al. 2007), to less productive in autumn–winter, when smaller phytoplankton is responsible for the productivity (Vargas et al. 2007). Similar results have been obtained off the Oregon coast, another eastern boundary upwelling ecosystem (Sherr et al. 2005). There, observations over a period of about 2 yr showed that the abundance of PPEs was lower in summer, coinciding with blooms of large diatoms.

The aim of the present work was to determine the diversity and seasonal abundance of the dominant PPE taxa in the coastal upwelling zone off central Chile. To evaluate the genetic diversity of the community, Chloroplastida-biased primers were used to amplify environmental deoxyribonucleic acid (DNA) in order to clone and sequence the 18S rRNA gene, used to identify picophytoeukaryotic taxa. Fluorescent in situ hybridization coupled with tyramide signal amplification (FISH–TSA, Not et al. 2002) was used to quantify three Chloroplastida genera (*Micromonas*, *Bathycoccus*, *Ostreococcus*) using oligonucleotide probes designed in previous work (Not et al. 2004). More general probes were also used in order to target total eukaryotes and total Chloroplastida.

Methods

Study site and hydrographic sampling—Sampling was performed at a fixed time-series station located on the continental shelf ~ 10 km off Dichato, central Chile (Station 18, 36°30.8′S, 73°07.7′W), with a water-column depth of ~ 94 m (Fig. 1). This station is maintained by the Center for Oceanographic Research in the Eastern South Pacific (COPAS), Universidad de Concepcion (UdeC). Water samples were collected (10-liter Niskin bottles) at a nearly monthly frequency from June 2006 to October 2008 (23 sampling dates) on board the R/Vs *Kay-Kay* and *Kay-Kay* II (UdeC). Samples from the surface (0–5 m) were used for the analyses of picoplankton, since most of the time (~ 82%) the chlorophyll *a* maximum was in the upper 10 m. Hydrographic data (temperature and salinity) were obtained using a SeaBird 25 conductivity–temperature–depth–oxygen sensor. Macronutrients and chlorophyll *a*

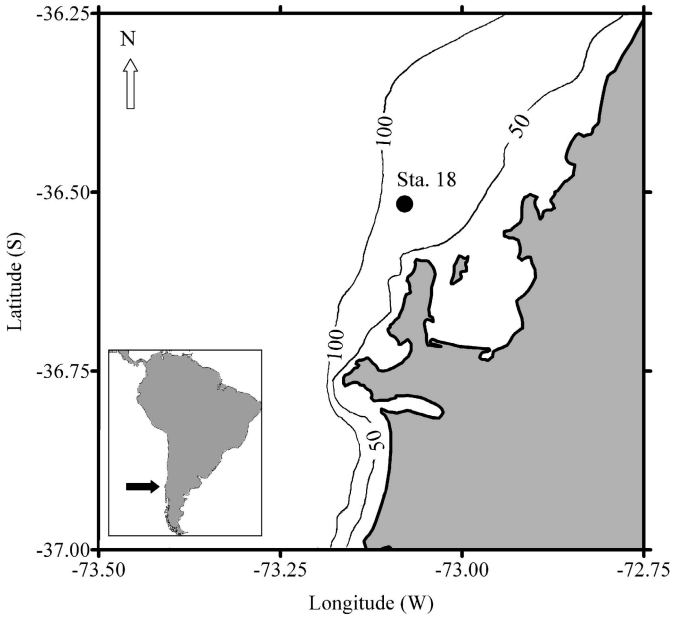

Fig. 1. Location of Station 18 over the shelf in the coastal upwelling area off Concepción (central Chile). Isobaths of 50 m and 100 m are also included.

(total and fractionated through 3-μm filters) were measured using standard methods.

DNA was collected from seawater prefiltered through a 20-μm pore size mesh. With a peristaltic pump, 3 liters of this seawater was filtered through a 3-μm pore size membrane, and microbial biomass was collected on a 47-mm-diameter membrane with a 0.2-μm pore size (Supor 450). The latter was transferred to cryovials and covered with DNA lysis buffer (40 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA], 0.73 mol L^{-1} sucrose, and 50 mmol L^{-1} Tris-HCl pH 8.3), immediately frozen in liquid nitrogen, and stored at −80°C until DNA extraction.

Each sample for FISH–TSA analysis was collected from 45 mL of seawater prefiltered through 3-μm pore size filters and fixed on board with 5 mL of fresh 10% paraformaldehyde for at least 1 h at 4°C. Fixed samples were then filtered onto 25-mm diameter 0.2-μm pore size polycarbonate filters (Millipore) and dehydrated three times with increasing concentrations of ethanol (50%, 80%, and 100%; 2 mL for 3 min each). Filters were stored at −80°C until hybridization.

Samples for flow cytometric analysis were collected from 2 mL of seawater fixed on board with 1% glutaraldehyde and frozen in liquid nitrogen after 10 min at room temperature. Fixed samples were stored at −80°C until analysis.

DNA extraction, polymerase chain reaction (PCR), and cloning—Genetic diversity was assessed on one surface sample per date, on three dates (May, October, and December 2007). May was representative of the autumn–winter condition, when relatively high PPE abundances were found. October and December represented the austral spring and summer conditions, respectively, with relatively low PPE abundances. To obtain the genomic DNA of the

picoplanktonic community, cell lysis was performed by adding lysozyme (1 mg mL^{-1}), proteinase K (0.5 mg mL^{-1}), and sodium dodecyl sulfate (SDS) (1%) to the filters. DNA from the lysate was purified with phenol–chloroform–isoamyl alcohol (25 : 24 : 1) and chloroform–isoamyl alcohol (24 : 1) extractions. It was then precipitated by adding sodium acetate (7.5 M) and isopropanol (0.4 and 1 volume, respectively) and then resuspended in 50 μL of ultrapure DNA- and ribonuclease-free water. Integrity was checked on an agarose gel. DNA was stored at $-20^{\circ}C$ until amplification.

The partial 18S rRNA gene was amplified using the primers EukA (Medlin et al. 1988) and CHLO02r (Zhu et al. 2005), targeting Chloroplastida. This target group was chosen based on preliminary FISH–TSA results demonstrating its importance at the study site (*see* below). The PCR mixture (25 μL final volume) contained $\sim$ 20 ng of environmental DNA template, 200 μmol L^{-1} of each deoxynucleoside triphosphate, 1.5 mmol L^{-1} $MgCl_2$, 0.3 mmol L^{-1} of each primer, 2.5 U of *Taq* DNA polymerase (GoTaq, Promega), and the PCR buffer (1×) supplied with the enzyme. PCR conditions were as follows: initial denaturation at 94°C for 130 s, 30 cycles of denaturation (at 94°C for 30 s), annealing (at 56°C for 45 s), extension (at 72°C for 130 s), and a final extension step (at 72°C for 7 min). PCR products of about 950 base pairs (bp) were purified and visualized after electrophoresis on an agarose gel (1%). Amplified products of three reactions were pooled and concentrated in a volume of 30-μL sterile water. An aliquot of each pooled PCR product was cloned using the kit pGem easy vector (Promega), following the manufacturer-recommended protocol. Positive colonies were selected and grown in multiwell plates with Luria–Bertani medium and glycerol (7%). PCR reamplification was performed with the primers M13F and M13R, using 1 μL of culture as template, to check for the presence of the 18S rRNA gene in the plasmids. Amplified products were visualized using agarose gel electrophoresis.

Sequence analysis—Clones with inserts were sequenced by the Macrogen Inc. sequencing service (Korea). Removal of vector and primer sequences, as well as quality and orientation analyses, were performed using Seqman software (Lasergene 7). Sequences were aligned using the Bosque software (Ramírez-Flandes and Ulloa 2008). The percentage of similarity between sequences was calculated, and sequences with similarities higher than 99.5% were considered to belong to the same operational taxonomic unit (OTU), which corresponds to a species or infraspecies cutoff level (Viprey et al. 2008). One sequence per OTU was sent to the online web tools Bellerephon (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) and KeyDNAtools (http://keydnatools.com/) in order to identify potential chimeric sequences. The KeyDNAtools website was also used to help assign sequences to a specific clade. With this tool, sequences are screened using 15-bp length oligonucleotide probes (''keys'') generated in silico. The taxonomic specificity, at different hierarchical ranges (supergroups, division, class, order, family, genus, species, and clade), of each key is provided based on a carefully annotated reference 18S rRNA gene eukaryotic database. For each environmental sequence, KeyDNAtools provides the number of ''keys'' found and their taxonomic assignation (Guillou et al. 2008). Sequences were also submitted (May 2009) to Basic Local Alignment Search Tool (BLAST) analysis against the GenBank database (http://www.ncbi.nlm.nih.gov/). An alignment was constructed including the sequences obtained in this study, those retrieved from the BLAST analysis, and sequences from cultured Chloroplastida species. Maximum-likelihood (ML) and neighbor-joining (NJ) algorithms were used to construct phylogenetic trees. Bootstrap values were calculated from 100 iterations. Alignments and phylogenetic trees were performed with Bosque (Ramírez-Flandes and Ulloa 2008). Sequences have been deposited in the Genbank database under accession numbers HM997190–HM997343.

FISH associated with TSA—Filters were covered with 9 μL of hybridization buffer (40% deionized formamide, 0.9 mol L^{-1} NaCl, 20 mmol L^{-1} Tris-HCl pH 7.5, 0.01% SDS, 2% blocking agent [Roche Diagnostic Boehringer]), and 1 μL of horseradish peroxidase–labeled probes (stock at 50 ng μL^{-1}). Specific probes were used to identify and count Chloroplastida and the target genera (*Micromonas*, *Ostreococcus*, and *Bathycoccus*) within this group. For quantifying the whole picoeukaryotic community (photosynthetic and nonphotosynthetic) we used a combination of a general probe that targets total eukaryotes and two probes that target different photosynthetic groups: Chloroplastida (probe CHLO01) and some Chromalveolata algal lineages (probe NCHLO01) (Simon et al. 1995). The details of the probes used are given in Table 1. Hybridization was achieved by incubating the filters at 35°C for 2 h 30 min. Before the signal amplification step, the filters were washed twice with washing buffer (56 mmol L^{-1} NaCl, 5 mmol L^{-1} EDTA, 0.01% SDS, 20 mmol L^{-1} Tris-HCl pH 7.5) at 37°C for 20 min and then put in an equilibration buffer (100 mmol L^{-1} Tris-HCl, pH 7.5, 150 mmol L^{-1} NaCl, 0.074% Tween 20) at room temperature for 15 min. The TSA reaction using the kit New England Nuclear Life Science Products was performed by adding 10 μL of the TSA mix (1 : 1 40% dextran sulfate and amplification diluent, 1 : 50 fluorescein isothiocyanate tyramide with the mixture of dextran sulfate and amplification diluent) and incubating the filters at room temperature in the dark for 30 min. The enzymatic reaction was stopped by washing the filters twice with the equilibration buffer at 55°C. The cells were counterstained with 4′,6-diamidino-2-phenylindole (final concentration 0.5 μg mL^{-1}) mixed with the antifading reagent (Citifluor AF1). A coverslip was fixed over the slides, which were then stored at 4°C until microscopic observation.

Flow cytometry—The abundances of PPEs and *Synechococcus* (no *Prochlorococcus* was detected in the study area) were determined using a FACSCalibur flow cytometer equipped with an ion–argon laser delivering 15 mW at 488 nm (Becton Dickinson). PPEs were discriminated from

Table 1. Probes used in this work. A combination of three probes (EUK1209, CHLO01, NCHLO01) was used to identify and count all (photosynthetic and nonphotosynthetic) picoeukaryotes (Not et al. 2004).

Probe	Sequence (5′–3′)	Target group
EUK1209	GGG CAT CAC AGA CCT G	Eukarya
CHLO01	GCT CCA CGC CTG GTG GTC	Chloroplastida
NCHLO01	GCT CCA CTC CTG GTG GTC	Non-Chloroplastida
CHLO02	CTT CGA GCC CCC AAC TTT	Chloroplastida
MICRO01	AAT GGA ACA CCG CCG GCG	*Micromonas*
OSTREO01	CCT CCT CAC CAG GAA GCT	*Ostreococcus*
BATHY01	ACT CCA TGT CTC AGC GTT	*Bathyococcus*

cyanobacteria by their optical properties: picoeukaryotes emit only red autofluorescence, while *Synechococcus* cells also emit orange fluorescence, as a result of their phycoerythrin content. Light scatter and fluorescence were normalized by adding 1-μm fluorescent beads to the samples. The data generated were processed using the CytoWin software.

Carbon conversion—To estimate the importance of PPEs in terms of carbon biomass, as compared with their counterpart *Synechococcus* in the picophytoplanktonic fraction, we estimated the mean intracellular carbon contents of these two groups for each sampling date from the flow cytometry data. For this, we used the log–log relationship established by Grob et al. (2007) between the mean forward scatter cytometric signal (FSC, normalized to reference beads) and the intracellular carbon content (C) ($C = 2.65 \times (FSC)^2 + 3.27 \times FSC + 2.58$). The intracellular carbon content of each picophytoplanktonic group was multiplied by the number of cells counted by flow cytometry. To estimate the relative contribution of each genus detected by FISH–TSA (*Ostreococcus*, *Bathycoccus*, *Micromonas*) to the picophytoeukaryotic carbon biomass, a conversion factor of 237 fg C μm^{-3} was used. It was proposed by Worden and colleagues (2004) based on carbon–hydrogen–nitrogen measurements of phytoplankton cells from cultures, including *Micromonas pusilla* and *Ostreococcus lucimarinus*. This conversion factor was multiplied by the abundance obtained by FISH–TSA and the cell volume of cultured species (or strains) of each genus obtained from cell lengths in the literature: 0.95 μm for *O. lucimarinus* (strain CCE 9901, Worden et al. 2004), 2 μm for *Bathycoccus prasinos*, and 2 μm for *M. pusilla* (Vaulot et al. 2004).

Nonparametric statistics were used to determine the correlation of picophytoplankton abundances and the PPE's carbon content with biological (chlorophyll $a >$ 3 μm and < 3 μm), chemical (salinity, macronutrients, and nitrogen-to-phosphorus N : P ratio), and physical (temperature) variables. The dependence between the variables was assessed using the Spearman correlation coefficient.

Results

Hydrographic conditions—Surface temperature during the study period ranged from 10.5°C (July 2007) to 16.5°C (December 2006), following the typical cycle of a temperate zone. Despite the influence of colder subsurface water during the austral spring–summer upwelling period (September to March), solar radiation produced higher temperatures in austral spring–summer than in autumn–winter (Fig. 2A). Salinity ranged from 25.9 (July 2006) to 34.4 (February 2008) (Fig. 2A); lower values were related to freshwater inputs by rainfall and/or river discharges. Nitrate and phosphate were well correlated ($p < 0.001$), and values were, on average, slightly lower in spring–summer, mostly because of the high nutrient uptake during phytoplankton blooms (Fig. 2B). Total chlorophyll *a* averaged 5.0 ± 6.8 mg m^{-3}, with a maximum in summer (> 20 mg m^{-3}). The fraction below 3 μm represented, on average, 10% ± 15% of the total chlorophyll *a*, peaking in autumn–winter, with a maximum of ~ 66% in June 2008 (Fig. 3).

Phylogenetic diversity of picoeukaryotes—In total 154 clones were analyzed: 76 from May, 23 from October, and 55 from December. Clones retrieved from May all corresponded to Chloroplastida sequences, whereas 61% and 20% of the clones from October and December, respectively, were affiliated with Chloroplastida. Most of the non-Chloroplastida sequences clustered within Cercozoa: 33% and 76% of the October and December clone libraries, respectively (Table 2). Within Cercozoa, chlorarachniophytes were important in summer, accounting for 66% of the clone library.

Considering only the Chloroplastida sequences, all but one corresponded to prasinophytes, the latter clustering with chlorophytes (Trebouxiophyceae). Prasinophyte sequences clustered into three of the seven clades proposed by Guillou et al. (2004): Pyramimonadales (clade I), Mamiellophyceae (clade II, now established as a class, Mamiellophyceae; Marin and Melkonian 2010), and Prasinococcales (clade VI). Mamiellophyceae were the most abundant in terms of sequences (~ 97%) and were represented by four genera (Table 2): *Micromonas* (32 clones), *Bathycoccus* (10 clones), *Ostreococcus* (52 clones), and *Crustomastix* (8 clones). In the present study, one clade was present for *Ostreococcus* (clade A) and *Crustomastix* (clade A), and three of the five recognized clades for *Micromonas* (A.BC.1, B.E.3, C.D.5), the first one being more frequent (Fig. 4; Table 3). Interestingly, four OTUs clustered outside of the previously established clades for *Micromonas* with good bootstrap support (Fig. 4). *Micromonas* and *Ostreococcus* were detected during the three periods surveyed, whereas

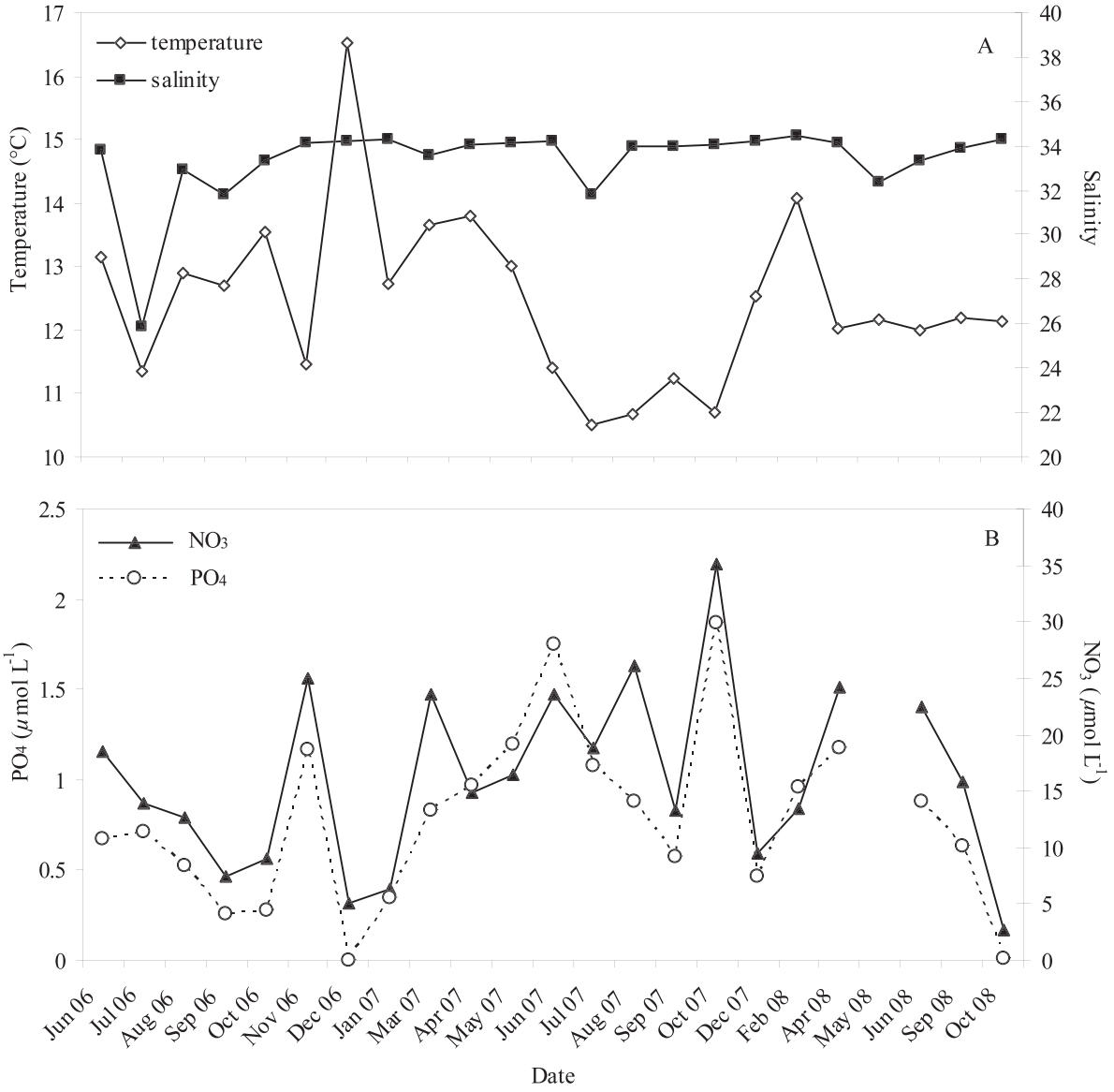

Fig. 2. Variations in (A) physical (temperature, salinity) and (B) chemical (phosphate and nitrate) parameters in the surface waters (0–5 m) of the study area.

Bathycoccus and *Crustomastix* were not observed in summer and autumn, respectively (Table 2). The only spring and summer OTUs (ST100705.F10 and ST120705.G1, respectively) that belonged to *Micromonas* clustered into a single clade (A.BC.1), which also accounted for several autumn clones. *Ostreococcus* was dominant in May (58% of the clones), and its contribution decreased from October (33%) through December (9%). *Micromonas* was only dominant during the spring transition (39%), and *Crustomastix* during summer (64%). Pyraminonadales were detected exclusively in summer, and Prasinococcales and Trebouxiophyceae (Chlorophyta) only appeared in spring (Table 2).

Picoplankton community composition—Photosynthetic picoeukaryote abundance measured by flow cytometry ranged from 10^2 to 10^4 cells mL^{-1}. Seasonal variability was high, with concentrations higher in autumn–winter and lower in spring–summer (Fig. 5A). *Synechococcus* abundance varied between 10^2 and 10^5 cell mL^{-1} (data not shown), and for almost half of the sampling dates, it was one order of magnitude higher than that of their eukaryotic counterpart. No *Prochlorococcus* cells were detected during the entire study period. Abundance of photosynthetic and nonphotosynthetic picoeukaryotes measured by FISH–TSA (Fig. 5A) showed the same seasonal pattern as above, but the range of values was higher (10^3–10^4 cells mL^{-1}). Chloroplastida (probe CHLO02) accounted for up to 100% of the total picoeukaryotic community (74% ± 29% on average). At eight sampling dates, Chloroplastida represented $< 70\%$ of the total picoeukaryotes, six of these dates corresponding to spring–summer months. Moreover, for the two December sampling dates, Chloroplastida represented a minor fraction of the total picoeukaryotes (4% and

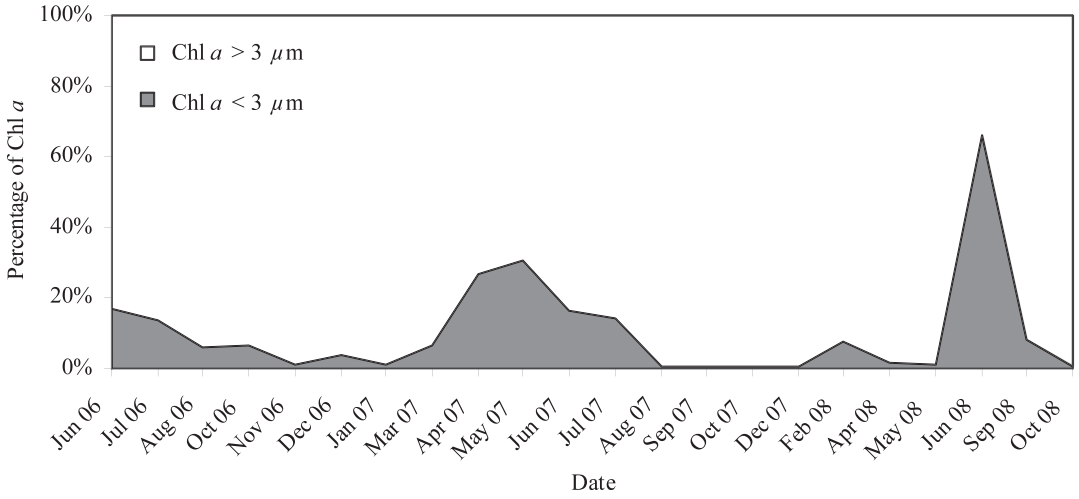

Fig. 3. Relative contribution of two different size fractions (> 3 μm, < 3 μm) to total chlorophyll *a* (Chl *a*), measured in mg m^{-3}, in the surface waters off Concepción.

13%, respectively), consistent with the low number of PPEs detected by flow cytometry.

On average, the abundances of *Ostreococcus* (probe OSTREO01), *Micromonas* (probe MICRO01), and *Bathycoccus* (probe BATHY01) added up to 102% ± 8% of the total Chloroplastida cells (Fig. 5A). Numerically (Fig. 5B), *Ostreococcus* dominated the Chloroplastida community (52.6% ± 19.3%; range, 290–18,742 cell mL^{-1}), followed by *Bathycoccus* (26.9% ± 15.3%; range, 258–7282 cell mL^{-1}) and *Micromonas* (22.9% ± 12.3%, range: 65–9632 cell mL^{-1}).

Carbon biomass—On most sampling dates, PPEs dominated over *Synechococcus* in terms of carbon (Fig. 6). PPEs represent, on average, 57% of the picophytoplanktonic carbon biomass during the study period, the highest percentages being during spring months. The contribution of each genus to PPE carbon varied depending on the PPE cell size (Fig. 7). *Ostreococcus*, although the most abundant genus, contributed the least to carbon biomass, with an average of 13% of the PPE biomass and a maximum of 40% in April 2007 (Fig. 7). *Bathycoccus* made the largest carbon contribution during the study period, with an average of 47% and a maximum of 78% in November 2006. *Micromonas* contributed on average 40% to the PPE's biomass, with a maximum of 70% in August 2006. In general, the highest values of abundance and biomass were observed in autumn and winter, except for *Bathycoccus*, which showed relatively high values during spring months. During winter, PPE's biomass showed the highest values, and *Micromonas* was the largest contributor to this biomass. Considering only spring and summer, PPE abundance was higher during summer, *Ostreococcus* being responsible for this increase, since *Micromonas* as well as *Bathycoccus* showed lower abundances during this season. However, carbon biomass was higher during spring because *Ostreococcus* has a smaller cell size than the other species. Carbon biomass of PPEs showed a significant negative correlation with temperature and salinity (Table 4), these two variables being lower during winter (Fig. 2). The abundances of some picophytoplankton groups, in particular *Synechococcus* and *Ostreococcus*, were negatively correlated with larger phytoplankton chlorophyll *a*. *Ostreococcus* was positively correlated with

Table 2. Number of clones per taxonomic group identified for each sampling date after a phylogenetic analysis of the clone libraries.

Division	Group	Number of clones		
		May 07	Oct 07	Dec 07
Chloroplastida	*Bathycoccus*	9	1	—
(Mamiellophyceae)	*Micromonas*	24	6	1
	Ostreococcus	43	5	1
	Crustomastix	—	1	7
	Prasinococcales	—	1	—
	Pyramimonadales	—	—	2
	Trebouxiophyceae	—	1	—
Cercozoa	Chlorarachniophyta	—	2	37
	other Cercozoa	—	5	5
Radiolaria	Taxopodia	—	1	—
Alveolata	Dinophyceae	—	—	2

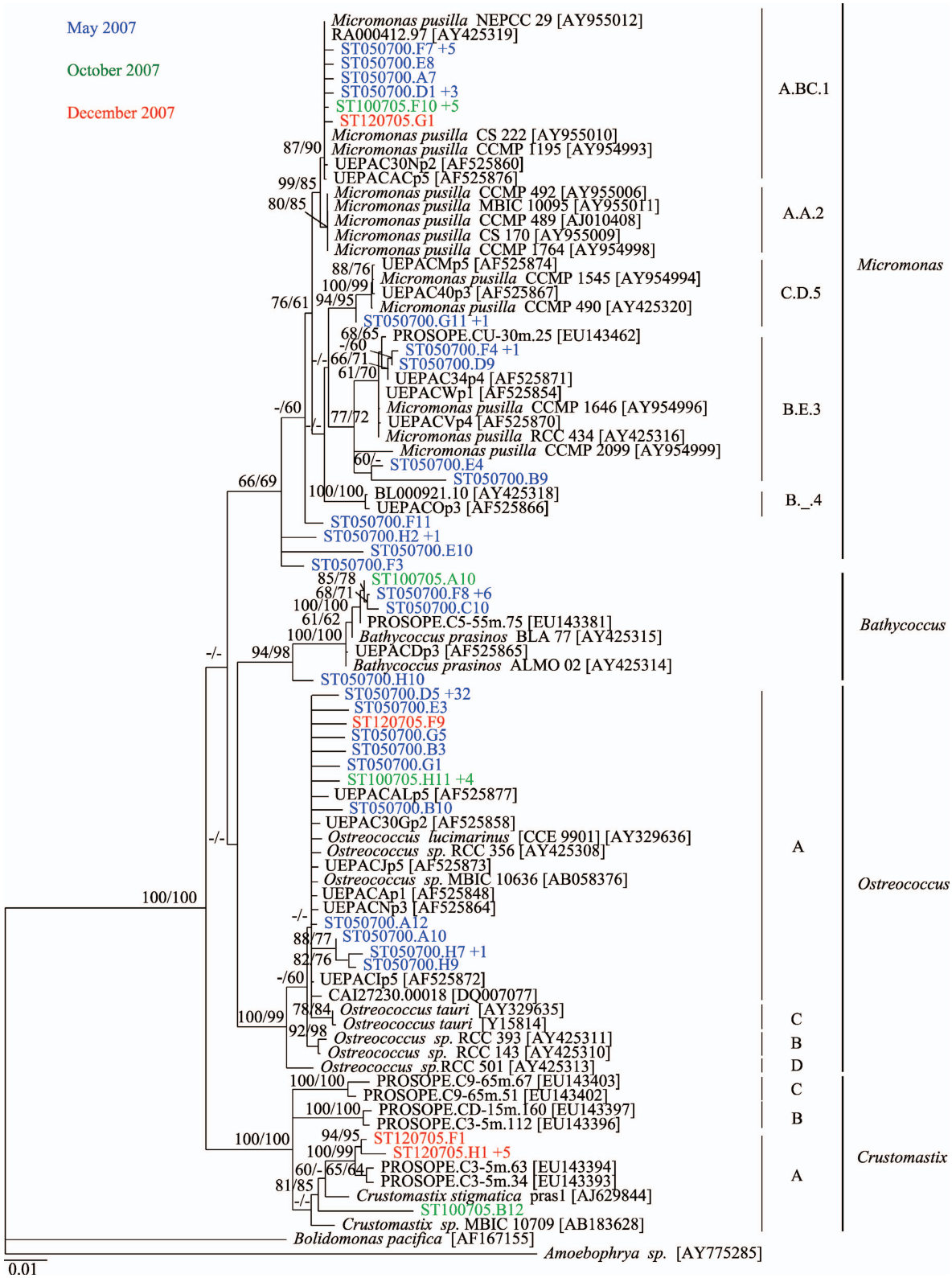

Fig. 4. 18S rRNA gene phylogeny of Mamiellophyceae inhabiting the coastal upwelling ecosystem off Concepción, inferred by ML and considering 996 positions of an alignment of 87 partial and full-length sequences. Significant bootstrap values (≥ 60%) of internal branches, obtained by NJ and ML, are shown in the tree in the order NJ and ML; nonsignificant values are indicated by a dash. Sequences retrieved on the three sampling dates are shown in bold: blue is for May 2007, green for October 2007, and red for December 2007. *Micromonas* clades are according to Worden (2006), *Ostreococcus* are according to Guillou et al. (2004), and *Crustomastix* are according to Viprey et al. (2008).

Table 3. Closest similarity between the clones retrieved in this study and cultured strains of *Micromonas*, *Bathycoccus*, and *Ostreococcus*. Numbers represent percentage of identity (% ID).

Clone	% ID	Closest cultured match
ST050700.F7	99.7	*Micromonas pusilla* CCMP 1195
ST050700.E8	99.7	*Micromonas pusilla* CCMP 1195
ST050700.A7	99.7	*Micromonas pusilla* CCMP 1195
ST050700.D1	99.7	*Micromonas pusilla* CCMP 1195
ST100705.F10	99.9	*Micromonas pusilla* CCMP 1195
ST120705.G1	99.7	*Micromonas pusilla* CCMP 1195
ST050700.G11	99.5	*Micromonas pusilla* CCMP 490
ST050700.F4	99.6	*Micromonas pusilla* RCC 434
ST050700.D9	99.7	*Micromonas pusilla* RCC 434
ST050700.E4	98.4	*Micromonas pusilla* RCC 434
ST050700.B9	97.8	*Micromonas pusilla* RCC 434
ST050700.F11	98.8	*Micromonas pusilla* CCMP 1195 and CCMP 1764
ST050700.H2	97.9	*Micromonas pusilla* CCMP 1195
ST050700.E10	96.7	*Micromonas pusilla* CCMP 1195
ST050700.F3	98.2	*Micromonas pusilla* CCMP 489
ST100705.A10	100	*Bathycoccus prasinos* ALMO 02
ST050700.F8	99.9	*Bathycoccus prasinos* ALMO 02
ST050700.C10	99.7	*Bathycoccus prasinos* ALMO 02
ST050700.H10	97.9	*Bathycoccus prasinos* ALMO 02
ST050700.D5	100	*Ostreococcus* sp. MBIC 10636
ST050700.E3	99.7	*Ostreococcus* sp. MBIC 10636
ST120705.F9	99.7	*Ostreococcus* sp. MBIC 10636
ST050700.G5	99.7	*Ostreococcus* sp. MBIC 10636
ST050700.B3	99.7	*Ostreococcus* sp. MBIC 10636
ST050700.G1	99.7	*Ostreococcus* sp. MBIC 10636
ST100705.H11	99.7	*Ostreococcus* sp. MBIC 10636
ST050700.B10	99.4	*Ostreococcus* sp. MBIC 10636
ST050700.A12	99.9	*Ostreococcus* sp. RCC 356
ST050700.A10	99.3	*Ostreococcus* sp. RCC 356 and MBIC 10636
ST050700.H7	98.6	*Ostreococcus* sp. MBIC 10636
ST050700.H9	98.2	*Ostreococcus* sp. MBIC 10636

nitrogen (nitrate and nitrite) but also with the N : P ratio. The other picoeukaryotic genera did not show any significant correlation with nutrients.

Discussion

The temporal change in the community structure of photosynthetic picoeukaryotes in a temperate coastal upwelling ecosystem of the eastern South Pacific was studied for the first time. We found that Chloroplastida represented the bulk of the picoeukaryotes counted by FISH–TSA. The sum of the three genera *Ostreococcus*, *Bathycoccus*, and *Micromonas* accounted for the Chloroplastida cell counts at all sampling dates. This confirms that these three PPE genera are important genera in upwelling ecosytems (Countway et al. 2010). According to our results, the picoeukaryotic community changed during the annual cycle, both in abundance and diversity. During autumn–winter, PPEs were more abundant, and their abundance decreased as summer approached. Moreover, all clones retrieved from May were Chloroplastida, whereas other lineages appeared in the spring–summer libraries. Thus, along with a decrease in abundance, there was a change in community structure and probably a proliferation of nonphotosynthetic organisms, since total picoeukaryote abundances tended to be higher than PPEs in spring–summer and greatly exceeded them in December 2006. The appearance of nontarget sequences in the clone libraries could be explained by the fact that the primers used are biased toward Chloroplastida but not totally specific. Most of the non-Chloroplastida sequences, which appeared in the warmer months, clustered within Cercozoa, in particular to Chlorarachniophytes. This photosynthetic group, whose sequences contain a single mismatch to the CHLO02 primer, was reported previously in Chloroplastida-biased clone libraries (Viprey et al. 2008). Moreover, the few Dinophyceae and Radiolaria sequences found had two to three mismatches to the Chloroplastida-biased primer. The variability in the number of clones obtained, despite the use of the same protocol and reagents, could be due to differences in clone transformation efficiency.

Chloroplastida, and particularly Mamiellophyceae, were expected to dominate in the study area, since these organisms have been commonly observed in coastal environments (Not et al. 2004; Zhu et al. 2005; Worden 2006) and specifically in upwelling regions (Rodríguez et al. 2006; Countway et al. 2010). They are normally observed in high abundance at the deep-chlorophyll maximum (Not et al. 2005; Countway and Caron 2006). In our case, the maxima in chlorophyll concentration occurred at the

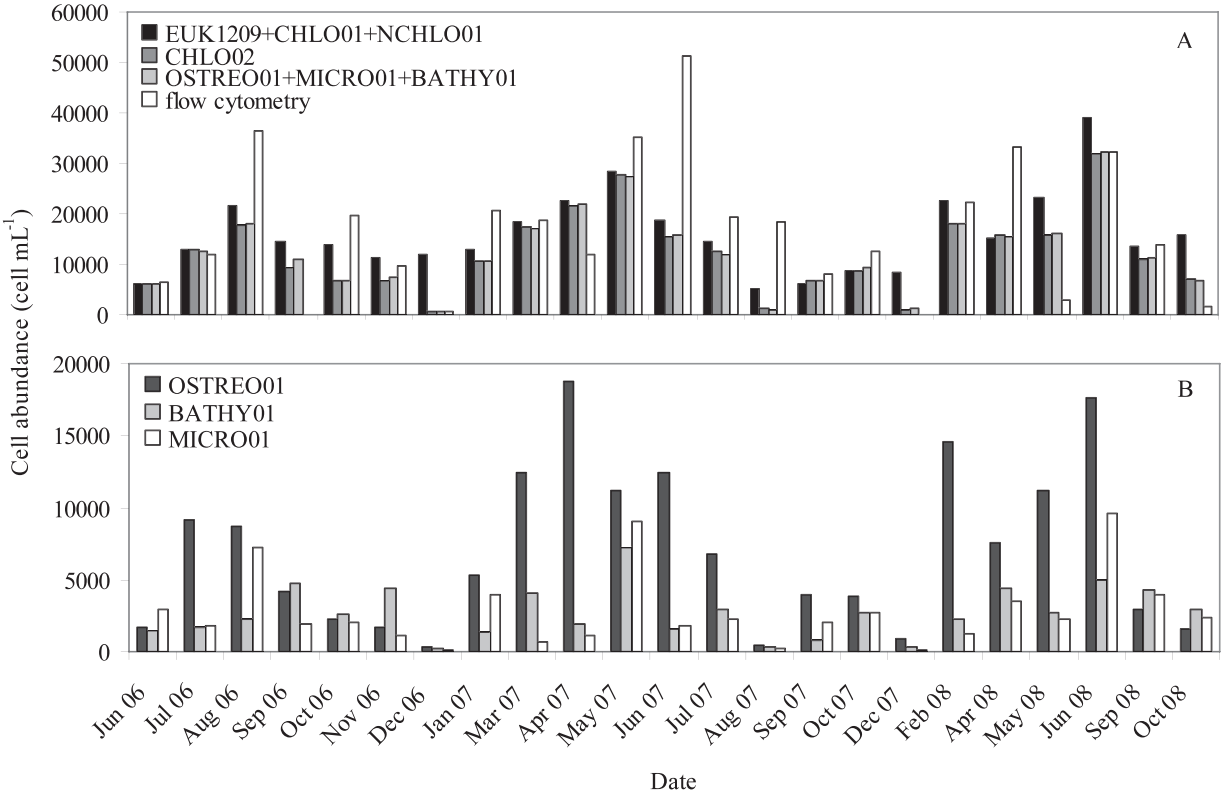

Fig. 5. Abundance of picoeukaryotic cells (expressed as cell mL^{-1}) using FISH–TSA and flow cytometry in the surface waters off Concepción during the study period. (A) Total picoeukaryotes (autotrophs and heterotrophs) were targeted with a mix of three general probes (EUK1209 + CHLO01 + NCHLO01), Chloroplastida with a specific probe (CHLO02), the sum of *Ostreococcus*, *Micromonas*, and *Bathycoccus* with specific probes (OSTREO01 + MICRO01 + BATHY01), and PPE detected by flow cytometry. (B) *Ostreococcus* (OSTREO01), *Bathycoccus* (BATHY01), and *Micromonas* (MICRO01) cells targeted with specific probes. There are no data for flow cytometry on September 2006 and December 2007.

surface most of the time and, therefore, were covered by our sampling scheme. Within the Mamiellophyceae, *Micromonas* has been found to be dominant in nutrient-rich coastal waters (Not et al. 2004, 2005, 2007). Our observation on the importance of *Micromonas* clade A in terms of number of OTUs and clones (Fig. 4) concurred with previous studies (Foulon et al. 2008). Observed *Micromonas* abundances were in the range of those reported before for coastal regions such as the English Channel (Not et al. 2004) but were higher than in the northwest (NW) Iberian upwelling system (Not et al. 2007). Despite its high abundance, *Micromonas* did not numeri-

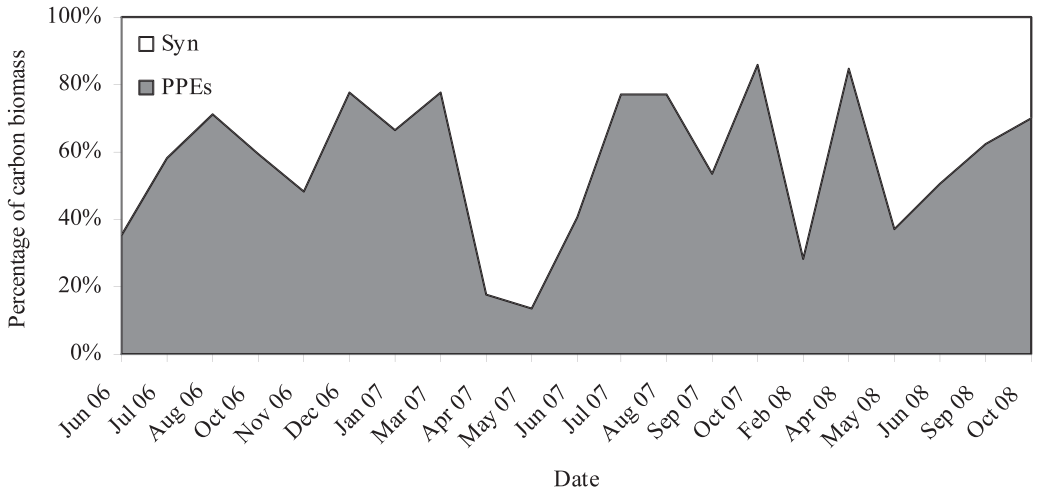

Fig. 6. Relative contribution of *Synechococcus* (Syn) and PPEs to the carbon biomass (μg C L^{-1}) of the picophytoplanktonic fraction during the study period off Concepción. *See* Methods section for conversion factors used.

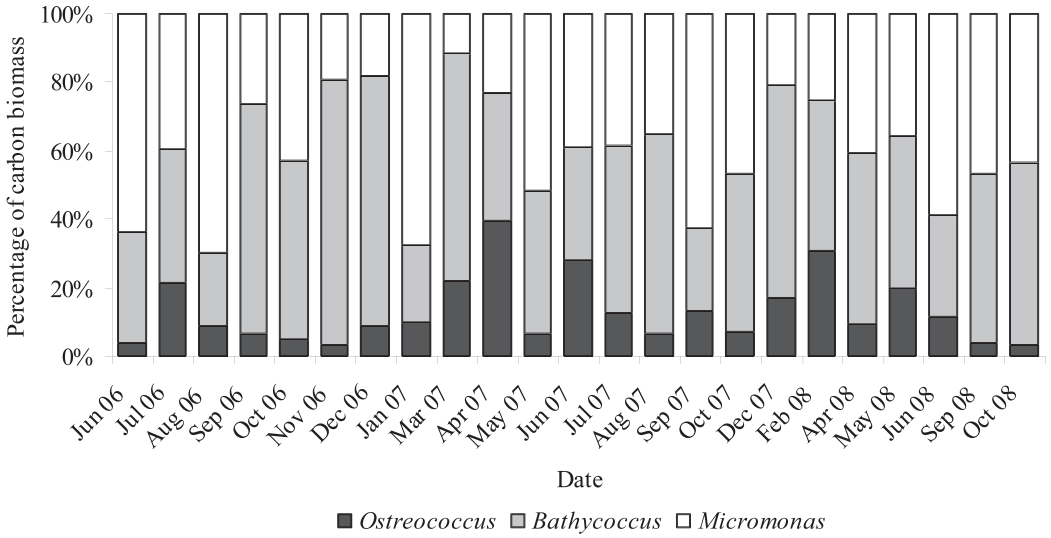

Fig. 7. Relative contribution of *Ostreococcus*, *Bathycoccus*, and *Micromonas* to the picophytoeukaryotic carbon biomass. *See* Methods section for conversion factors used.

cally dominate the PPE community, which contrasts with what has been reported for nearly all other studies in coastal waters of the Northern Hemisphere (Not et al. 2004, 2005, 2008), including those in upwelling regimes (Not et al. 2007). *B. prasinos,* on the other hand, also has been commonly reported in coastal environments, with maximum abundances reaching ~ 2000 cells mL^{-1} in surface waters (Not et al. 2004, 2005). Our observations are comparable, although somewhat higher, with more than 4000 cells mL^{-1}. However, the relative abundance of *B. prasinos* has previously been found to be at most 15% of the Mamiellophyceae community (Not et al. 2004, 2007; Zhu et al. 2005) and always lower than that of *Micromonas*, whereas off Concepcion it was on average ~ 26% of the Mamiellophyceae and surpassed that of *Micromonas* on several sampling dates. Very little is known about the ecology of this species, although comparison of data from two studies that included stations with and without coastal influence (Not et al. 2005, 2008) suggested that *Bathycoccus* reaches maximum abundances in coastal waters. The present study did not uncover any significant relationship between its abundance and environmental variables (Table 4). Finally, *Ostreococcus* inhabiting our study area are members of clade A, a high-light ecotype (Rodríguez et al. 2005) that includes *O. lucimarinus* (strain CCE 9901), which has been isolated from coastal Pacific waters (Worden et al. 2004). *Ostreococcus* occurs generally at very low abundances in coastal environments, with concentrations of no more than 10^2 cells mL^{-1} (Not et al. 2004; Zhu et al. 2005) and at times is absent, as was the case in the NW Iberian upwelling system (Not et al. 2007). However, high concentrations of *Ostreococcus* (e.g., 10^4–10^5 cells mL^{-1}) have been detected in coastal waters during short blooms around Long Island (New York, western North Atlantic; O'Kelly et al. 2003), in the coastal Mediterranean Thau lagoon (Courties et al. 1994), and in the San Pedro Channel (California, eastern North Pacific). The latter is a coastal upwelling area where *Ostreococcus* is present almost year-round but generally in low abundances (Countway and Caron 2006; Countway et al. 2010). In the case of the Chile upwelling ecosystem, we found that *Ostreococcus* was present throughout the study period, with relatively high concentrations (average 6.9×10^3 cells mL^{-1}), although not substantially different from those observed in previous studies during short blooms. A possible explanation for the permanent high abundances we found might be related to the permanent availability of nutrients. Our results suggest that *Ostreococcus* may respond better to new nitrogen (e.g.,

Table 4. Correlation matrix for picophytoplankton groups vs. biological, physical, and chemical variables. All picophytoplankton data concern abundance except for PPE carbon. T, temperature; S, salinity; O_2, oxygen concentration; Chl, chlorophyll *a*.

Picophytoplankton group	T	S	O_2	Chl >3 μm	Chl <3 μm	NO_3	NO_2	NH_4	PO_4	N : P
PPE	0	0	−0.3	−0.2	0.7**	0.4	0.3	−0.2	0.2	0.5*
Ostreococcus	0.1	−0.2	−0.2	−0.5*	0.6**	0.5*	0.5*	−0.1	0.2	0.6**
Bathycoccus	−0.1	−0.3	−0.2	−0.4	0.2	0.3	0.1	−0.2	0.3	0.1
Micromonas	−0.1	−0.2	−0.2	−0.1	0.5*	0.1	0.2	−0.4	0	0.1
Chloroplastida	0.1	−0.2	−0.2	−0.5*	0.6**	0.5*	0.4	−0.1	0.2	0.5**
Total picoeukaryotes	0.3	−0.1	−0.1	−0.4	0.5*	0.2	0.1	−0.1	−0.1	0.3
Synechococcus	0.3	0	−0.1	−0.5*	0.8***	0.3	0.2	−0.4	0	0.5**
PPE carbon	−0.5*	−0.6**	−0.1	0.3	−0.2	0	0.5*	0	0.1	−0.2

* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

NO_3) than *Bathycoccus* and *Micromonas*, which is reflected here in the positive correlations found between *Ostreococcus* and nitrate, nitrite, and the N : P ratio (Table 4); statistically significant relationships with nutrients were not observed for the other two eukaryotic genera, but there was a positive correlation between *Synechococcus* and the N : P ratio as well. Our results are consistent with those of Worden et al. (2004), which have shown slightly higher growth rates of *O. lucimarinus* when nitrate is used as the nitrogen source instead of ammonium. In the Chilean upwelling ecosystem, nitrate is found at relatively high concentrations in the euphotic zone during the entire year (Fig. 2), which may explain why *Ostreococcus* is abundant in this region but absent in the NW Iberian coast (characterized by periodic upwelling events), where nitrate concentrations are lower (Not et al. 2007).

The abundance of the photosynthetic picoeukaryote community in the central Chile upwelling area showed a clear seasonal pattern. PPE abundance was higher in autumn–winter and lower in spring–summer. Similar trends have been established for other coastal upwelling ecosystems, such as the Iberian coast (Rodríguez et al. 2006) and the Oregon coast (Sherr et al. 2005). It has also been previously observed that when the larger phytoplankton dominates, picophytoplankton abundances are low (Larsen et al. 2004; Sherr et al. 2005). Here, this also holds as reflected by the negative correlation between the $>$ 3-μm chlorophyll *a* and the abundance of *Ostreococcus* and *Synechococcus*, which are the main contributors to the picophytoplankton abundance in the study area. The reason for this marked seasonal pattern is still poorly understood. One possibility is that picophytoplankters are weak competitors compared with microphytoplankton such as diatoms in high-nutrient environments (Litchman et al. 2007), like those found in upwelling ecosystems. Another possibility is a top-down control by grazing of the heterotrophic microplankton and nanoplankton on the picoplankton fraction (Calbet and Landry 2004; Christaki et al. 2005). While there is no evidence for strong seasonality in the abundance of heterotrophic microplankton and nanoplankton (Böttjer and Morales 2007), grazing rates of heterotrophic nanoplankton on bacteria and cyanobacteria have been shown to be in fact higher during spring–summer in the study area (Böttjer and Morales 2007; Vargas et al. 2007). However, no data are available on specific grazing of PPEs.

The importance of the picophytoplankton community for primary productivity has been assessed and demonstrated in the open ocean (Li 1994; Jardillier et al. 2010), where small cells dominate. But the relevance of this smallest fraction should not be underestimated in coastal environments, especially in highly productive coastal environments, such as upwelling regimes. Cermeño et al. (2006) determined at the Ría de Vigo coastal upwelling system that picophytoplankton accounted for up to 30% of the chlorophyll *a*. We found that the $<$ 3-μm fraction usually contributed less than 20% (on average $\sim$ 11%) of the total chlorophyll *a*, but it did reach up to 66% (June 2008). According to our carbon biomass estimations, more than half of the picophytoplanktonic primary production could be due to eukaryotic picoplankton during most of the sampling dates. This agrees with the observation of Worden et al. (2004), who determined that, on average, PPEs accounted for 76% of the carbon production of the picophytoplankton fraction at a Northern Pacific upwelling coastal system. Both here and in the Northern Pacific (Worden et al. 2004), PPEs contributed a higher carbon biomass than cyanobacteria in this size fraction because of their higher cell size and higher chlorophyll *a* content (Li 1994; Worden et al. 2004). The same is true within the PPE community: *Micromonas* and *Bathycoccus* contributed most to carbon biomass despite being less abundant than *Ostreococcus*. This was recently pointed out by Jardillier et al. (2010), showing that larger photosynthetic picoeukaryotes ($\sim$ 2.8 μm) can account for up to 38% of the total CO_2 fixation in the Atlantic Ocean, despite being almost half as numerically abundant as smaller PPEs ($\sim$ 1.8 μm). One should note, however, that the biovolumes used for the three genera in our study were from cells in culture and are therefore probably overestimated. However, since we are evaluating the relative biomass contribution of each genus, not their absolute contribution to the system, the approach may be valid.

PPE abundances observed off central Chile are similar to those observed previously in coastal upwelling ecosystems (Sherr et al. 2005; Fuller et al. 2006). The PPE contribution to carbon biomass is in the range of what has been reported before for this type of environment, specifically for the NW Iberian upwelling system (Rodríguez et al. 2006). However, the structure of the PPE community from the ecosystem in the eastern South Pacific is somewhat different from those previously observed, as a result of the dominant and persistent abundance of *Ostreococcus*. Although our phylogenetic analysis of the 18S rRNA gene (Fig. 4) revealed that natural populations inhabiting the study area are generally closely related to cultured strains, with similarities above 97% (Table 3), some sequences did not tightly cluster with those previously known for *Micromonas*, *Bathycoccus*, and *Crustomastix*. For example, *Bathycoccus* was not genetically as homogeneous as expected (Guillou et al. 2004, Viprey et al. 2008); one OTU (ST050700.H10) clearly belonged to *Bathycoccus* but clustered out of previous reported sequences with high bootstrap values (Fig. 4).

Further studies are needed to understand why the eukaryotic picophytoplankton decreases in the spring–summer upwelling season but are important during the nonupwelling months. Physiological (e.g., nutrient uptake), ecological (e.g., grazing, virus infection, competition), and even physical (e.g., advection) aspects should be evaluated, as well as the niche partitioning within each genera. The importance of PPEs to the carbon economy of the upwelling ecosystem also needs to be assessed, especially when the larger phytoplankton are less abundant. Although *Ostreococcus*, *Micromonas*, and *Bathycoccus* were the main components of the PPE fraction, attention should be paid to the other photosynthetic groups present in the study area, such as Chlorarachniophytes. Their significant presence in the clone libraries occurred when the abundances of *Ostreococcus*, *Micromonas*, and *Bathycoccus* were

low. Finally, *Ostreococcus* appeared as the dominant PPE in the Chile upwelling ecosystem. Elucidating whether this is based on particular genetic characteristics, e.g., when compared with lineages found in the eastern North Pacific (Countway and Caron 2006; Countway et al. 2010), or on ecological aspects will help in understanding the basis for its global distribution.

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