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Eukaryotic picoplankton communities of the Mediterranean Sea in summer assessed by molecular approaches (DGGE, TTGE, QPCR)

Dominique Marie¹, Fei Zhu^{1,3}, Vanessa Balagué², Joséphine Ras³, & Daniel Vaultot¹

¹Station Biologique, UMR7144 CNRS et Université Pierre et Marie Curie, Roscoff, France; ²Department de Biologia Marina i Oceanografia, Institut de Ciències del Mar, CMIMA, CSIC, Passeig Marítim de la Barceloneta, Barcelona, Catalonia, Spain; and ³Laboratoire d'Océanographie de Villefranche-sur-mer, UMR 7093 CNRS et Université Pierre et Marie Curie, Villefranche-sur-Mer Cedex, France

Correspondence: Daniel Vaultot, Station Biologique, UMR7144 CNRS et Université Pierre et Marie Curie, Roscoff, France. Tel.: +33 2 98 29 23 34; fax: +33 2 98 29 23 24; e-mail: vaultot@sb-roscoff.fr

Present address, Fei Zhu: Department of Pharmaceutical Sciences, University of Maryland, Baltimore, USA

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Keywords

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Introduction

Despite its relatively small size, the Mediterranean Sea displays a range of environmental conditions. In winter, strong wind mixing and mesoscale circulation induce in the Western Basin high nutrient conditions and localized blooms (Bosc *et al.*, 2004). In summer, some areas, in particular in the Eastern Basin, can become extremely oligotrophic (Berman *et al.*, 1985; Bosc *et al.*, 2004). Moreover, moderate riverine inputs and absence of denitrification induce N:P ratios higher than the standard Redfield value (16:1) in the Eastern Basin leading to phosphorus limitation (Moutin *et al.*, 2002; Krom *et al.*, 2004). When oligotrophic conditions prevail in the Mediterranean Sea, i.e. throughout all basins in summer or in the Eastern Basin in winter, the smaller size fractions (i.e. picoplankton < 2–3 µm) dominate the plankton biomass and production (Raimbault *et al.*, 1988; Li *et al.*, 1993; Ignatiades *et al.*, 2002). However, Mediterranean Sea picoplankton have been relatively little studied at the basin scale.

This is especially true of its eukaryotic component, for which until recently we were lacking proper study tools.

Abstract

The composition and abundance of eukaryotic picoplankton (defined here as cells smaller than 3 µm) was investigated in the Morocco upwelling and throughout the Mediterranean Sea in late summer using flow cytometry and molecular methods (gradient gel electrophoresis and quantitative PCR). The picoplankton displayed characteristics typical of oligotrophic oceanic areas with concentrations down to 1000 cells mL⁻¹ in the Eastern Basin. The most abundant eukaryotic sequences recovered by gradient gel electrophoresis were related to uncultivated marine groups: alveolates I (16%) and II (26%) and a newly discovered group (env Nansha, 17%) for which sequences have been recently obtained from the South China Sea and that could be related to Acantharians. Prasinophyceae (photosynthetic green algae) accounted for 10% of the sequences, whereas Cercozoa, Stramenopiles, Polycystinea, dinoflagellates and ciliates provided minor contributions. The use of quantitative PCR coupled with taxon-specific primers allowed us to estimate the relative abundance of several taxa belonging to the Prasinophyceae. Of the three genera assessed, *Bathycoccus* appeared as the most abundant, forming localized maxima at depth.

Microscopy is unable to resolve the often simplistic morphological traits of many species, whereas biochemical approaches such as pigment analyses provide only limited taxonomic resolution. In the past 5 years, molecular approaches previously restricted to prokaryotes have been applied to eukaryotes allowing the study of their diversity (Díez *et al.*, 2001b; Moon-van der Staay *et al.*, 2001) and abundance (Not *et al.*, 2002). However, such approaches have not been yet used in the Mediterranean Sea, except in coastal areas (Massana *et al.*, 2004a) or on a few pelagic samples (Díez *et al.*, 2001a).

In the present study, we investigate picoplankton communities at stations sampled during the PROSOPE cruise, which covered the Mediterranean Sea as well as the Morocco upwelling. In addition to traditional approaches (flow cytometry and high-performance liquid chromatography (HPLC) pigment analyses), we used both denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient gel electrophoresis (TTGE) to describe the diversity of eukaryotic assemblages. Both techniques are based on the amplification of a specific gene by PCR using two primers, a regular primer and a modified primer fitted

with a long terminal GC chain (GC-clamp, about 50 bases). Under denaturing conditions, the presence of the GC-clamp prevents the complete separation of double strands. As a consequence, PCR products of identical sizes but with different sequences can be separated on acrylamide gels submitted to chemical (DGGE) or temperature (TTGE) denaturing gradients. DGGE of PCR amplified genes (in general rRNA) is now widely used to analyze the diversity of bacteria, viruses or archaea in different environments (Muyzer & Smalla, 1998) and it has been applied more recently to marine eukaryotes (Díez *et al.*, 2001a; Zeidner & Béjà, 2004). In contrast, TTGE has been very little applied to aquatic communities (Bosshard *et al.*, 2000) and not yet for marine eukaryotes. Another technique for which application is progressing very rapidly in environmental microbiology is quantitative PCR (QPCR) (Suzuki *et al.*, 2000). Based on the real-time monitoring of PCR products by fluorescence during the amplification reaction, it allows the quantification of specific groups of microorganisms and has been applied recently to photosynthetic picoeukaryotes (Zhu *et al.*, 2005). In the present study, QPCR allowed us to assess the abundance of specific taxa belonging to the green algal lineage (Prasinophyceae), an important component of picoplankton (Not *et al.*, 2004).

Materials and methods

Cruise samples

Samples were collected using Niskin bottles during the oceanographic cruise PROSOPE (9 September–3 October

1999) in the Mediterranean Sea on board the NO Thalassa along a transect from the Moroccan upwelling to the Eastern Basin near Crete, and then to the Gulf of Lyons (Fig. 1). Ancillary data were obtained from the PROSOPE web site (<http://www.obs-vlfr.fr/jgofs/html/prosope/home.htm>).

For flow cytometry, 1.5 mL samples were fixed with 0.1% of glutaraldehyde (final concentration), incubated at room temperature for at least 15 min and then deep frozen in liquid nitrogen. For pigment and DNA analyses, samples were pre-filtered through 3 µm pore-size, 47 mm diameter, Nuclepore filters (Whatman International Ltd, Maidstone, UK) using moderate vacuum (200–280 mmHg) in order to separate picoplankton. For pigment analyses, 2.8–5.6 L of pre-filtered samples were filtered onto glass fiber filters (GF/F, 25 mm diameter, 0.7 µm particle retention). After being subjected to an absorbance measurement, the filters were placed into cryovials and immediately stored in liquid nitrogen until HPLC analysis. For DNA, 1.45–5 L of pre-filtered samples were collected at a subset of stations on a 47-mm diameter membrane filter (Pall Supor-450, Pall, Ann Arbor, MI) with a 0.45 µm pore size. The filter was transferred into a cryovial containing 3.5 mL of DNA lysis buffer (0.75 M sucrose, 50 mM Tris-HCl, pH 8) and immediately frozen in liquid nitrogen.

Flow cytometry analyses

Samples were run after the cruise using a FACSsort flow cytometer (Becton Dickinson, San Jose, CA) using the standard protocol described in Marie *et al.* (1999). Three

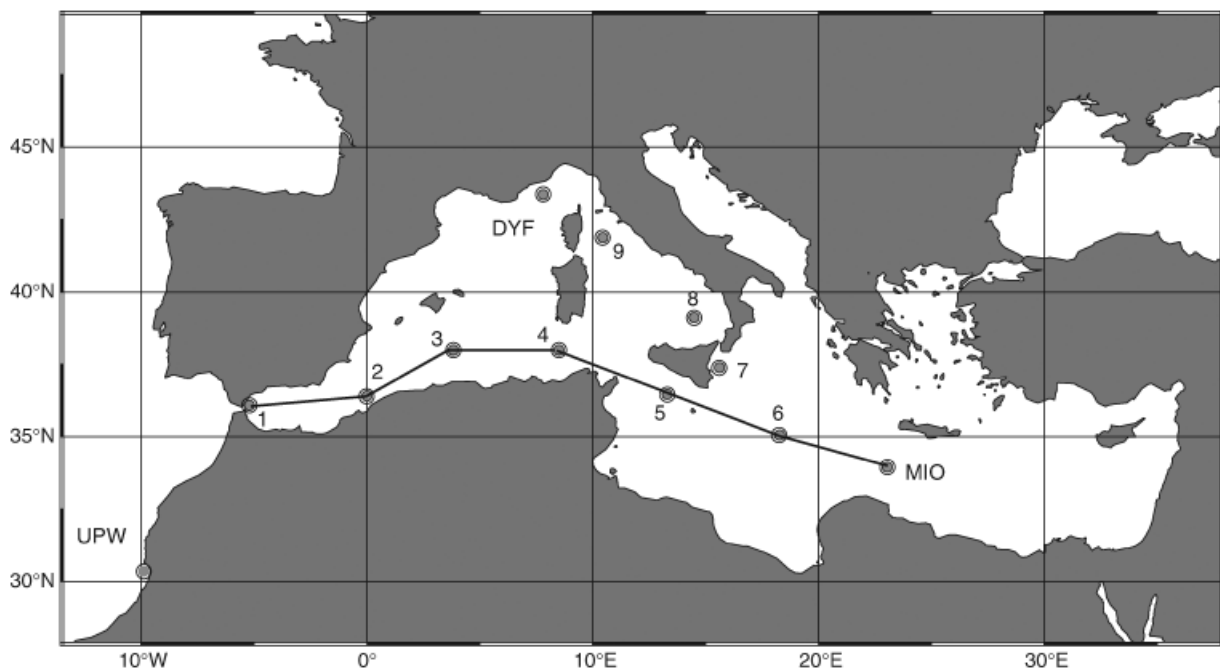


Fig. 1. Map of the PROSOPE cruise. The solid line corresponds to the transect plotted in Fig. 2 and Fig 5.

populations were discriminated on the basis of their fluorescence characteristics: *Prochlorococcus*, *Synechococcus*, and picoeukaryotes.

Pigment analyses

Extraction of the filters was carried out in 3 mL of methanol, according to the method described previously (Vidussi *et al.*, 1996). The HPLC analytical method, based on a gradient between a methanol: ammonium acetate mixture (70:30) and a 100% methanol solution (solvents A and B, respectively), was modified from Vidussi *et al.* (1996). Modifications include the flow rate (0.5 mL min^{-1}), the reversed-phase chromatographic column (RP-C8, Reference: Hypersil MOS, $3 \mu\text{m}$), which has an internal diameter of 3 mm and the gradient program (minutes; % solvent A; % solvent B): (0; 80; 20), (4; 50; 50), (18; 0; 100), (22; 0; 100). The solvents were driven by a binary pump (1100 series, Agilent Technologies, Palo Alto, CA), sample preparation and injection were performed by a refrigerated Thermoquest auto-sampler, and detection was carried out at 440 and 667 nm with a diode array detector (DAD 1100 series, Agilent Technologies). A fluorometer (Thermoquest, West Palm Beach, FL) also allowed the detection of chlorophylls when concentrations were below the DAD detection limits. The pigments were identified spectrally and their quantification was based on peak area, with an internal standard (β -apo-8'-carotenal) correction. The instrument was calibrated with pigment standards supplied by DHI Water & Environment (Danish Hydraulic Institute, Hørsholm, Denmark). The database was finally validated against filter pad absorbance and *in situ* fluorescence data.

DNA extraction

Samples were thawed at 37°C and incubated for 20 min in presence of $70 \mu\text{L}$ of lysozyme (50 mg mL^{-1}). Then $50 \mu\text{L}$ of Proteinase K (10 mg mL^{-1}) and $350 \mu\text{L}$ of sodium dodecyl sulfate (SDS) 10% were added. The mixture was incubated 20 min at 37°C and the reaction was stopped by 10 min at 55°C . Two microliters of phenol were added in each sample, the mixture was quickly vortexed and incubated for 5 min at 65°C to dissolve the filter. Two microliters of chloroform: isoamyl alcohol (24:1) were added and the mixture was gently agitated and incubated for 15 min at 55°C . Then a 10-min centrifugation step at 3000 g allowed the removal of the organic phase. Three microliters of a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) were added and gently agitated for 10 min. The aqueous phase was recovered by a 10-min centrifugation at 3000 g at room temperature, concentrated using Centricon YM-100 (Millipore, Bradford, MA) and washed with 2 mL of milliQ water. The concentration of extracted DNA was estimated by spectrophotometry and DNA was stored at -80°C .

PCR amplification

PCR amplification was performed with about 10 ng of extracted DNA using the primer set Euk1A and 516r-GC (Díez *et al.*, 2001a), which amplifies a fragment of approximately 560 bp. PCR reaction was performed in $50 \mu\text{L}$ containing $5 \mu\text{L}$ of $10 \times$ PCR buffer, $100 \mu\text{M}$ of each deoxynucleotide triphosphate, magnesium chloride (2.5 mM), primers ($0.3 \mu\text{M}$) and 2.5 U of Taq Polymerase (Qiagen, Hilden, Germany). The PCR program consisted in an initial denaturation of 94°C for 4 min and 30 cycles including 1 min of denaturation at 94°C , 45 s of annealing at 56°C and 2 min extension at 72°C . A final extension of 7 min at 72°C followed by cooling at 4°C terminated the PCR program. PCR products were controlled on a 1% agarose gel and their concentration was evaluated in comparison with $5 \mu\text{L}$ of smart ladder (Eurogentec, MW-i700-02, Angers, France).

DGGE

DGGE analyses were conducted following the protocol of Díez *et al.* (2001a). Electrophoresis was performed on 0.75-mm thick 6% polyacrylamide gels (ratio of acrylamide to bis-acrylamide 37.5:1). A linear gradient of 45–55% denaturant (100% denaturant corresponds to 7 M urea and 40% of de-ionized formamide) was cast after addition of $5 \mu\text{L mL}^{-1}$ of ammonium persulfate 10% (APS) and $1 \mu\text{L mL}^{-1}$ of N,N,N',N'-Tetramethylethylenediamine (TEMED), and left for about 2 h to polymerize at room temperature. Gels were immersed in $1 \times$ TAE buffer (Trizma base 40 mM, EDTA 1 mM, sodium acetate 20 mM, pH 7.5) at 60°C in a DGGE-2000 system (CBS Scientific Company, Del Mar, CA) or a D-Code system (Biorad, Hercules, CA). About 800 ng of PCR products were applied to individual lanes in the gel. One sample was used as a reference to allow comparison between gels. This sample was repeated on both side of the gel (see below Fig. 3). Electrophoresis was conducted for 16 h at 100 V. Gels were stained using $3 \mu\text{L}$ of SYBR Gold (Molecular Probes, Invitrogen, Cergy Pontoise, France) in 15 mL of TNE buffer (10 mM Tris base, 0.1 M NaCl, 1 mM EDTA, pH 7.5) for 45 min in the dark. Gels were rinsed with 500 mL of TAE buffer and bands were visualized with a Fluor-S MultiImager using the MultiAnalyst imaging software (Biorad) or with a STORM FluorImager (Amersham Biosciences, Orsay, France).

TTGE

Electrophoresis was performed with 0.75 mm thick 6% polyacrylamide gels (37.5:1) containing 7 M urea. APS 10% ($7 \mu\text{L mL}^{-1}$) and TEMED ($1 \mu\text{L mL}^{-1}$) were added before casting and the gel was left to polymerize at room temperature for 2–4 h. About 800 ng of PCR product were deposited for each lane and a temperature gradient ranging

from 64 to 67 °C, modified from Ogier *et al.* (2002), with an increment of 0.2 °C h⁻¹ was applied. Electrophoresis was performed for 18 h at 80 V in 1 × TAE buffer. Gels were stained with 3 µL of SYBR Gold and visualized using a STORM FluorImager (Amersham Biosciences).

Comparison of DGGE patterns

For both DGGE and TTGE, digital images of the acrylamide gels were analyzed using the Cross-Checker software (available from <http://www.dpw.wau.nl/pv/pub/CrossCheck/>). A binary matrix of the intersections between the lanes and the most intense bands (determined by eye) was used to calculate distance matrices using normalized Euclidian distances. Dendrograms presenting the relationship within samples were computed (UPGMA) using the DGGESat program designed by Erik Van Hannen (available from http://www.sb-roscoff.fr/marine_microbes/index.php?option=com_simpleboard&func=view&id=46).

Band extraction

Extraction of bands from the gel was performed with razor blades, on a UV-trans-illuminator, equipped with a Visi-Blue Plate and orange glasses (UPV, Upland, CA). Bands were eluted overnight at 4 °C in 30 µL of nuclease-free water.

For DGGE gels, 4 µL of liquid were used for a 50 µL PCR reaction realized with the same conditions and primer set as previously described. The PCR product was then purified through a PCR Cleanup Kit (Millipore). Its quality and quantity were estimated on an agarose gel and then a 20 µL aliquot of each product was used to check for the purity on a new DGGE gel. Then 3 µL samples were used for the sequencing reaction.

For TTGE gels, 2 µL of the liquid containing the extracted band was re-amplified using the same conditions as described above. The PCR reaction was purified on an agarose gel containing both ethidium bromide (0.4 µg for 100 mL) and SYBR Gold (0.5 µL for 100 mL). Bands were excised from the gel on a blue-colored illuminator and purified using mini columns (Wizard PCR Preps, Promega, Madison, WI). The quality and the amounts of purified DNA were checked on an agarose gel. A 20 µL aliquot of each PCR reaction was also used to verify that a single band was obtained for each PCR product by TTGE and 0.5–1 µL was used for the sequencing reaction.

Band sequencing

A PCR reaction was carried out using fluorescent nucleotides (Big Dye Terminator V3.1) from a DNA sequencing kit (Applied Biosystems, Foster City, CA, USA). A PCR reaction of 5 µL was performed using 0.5 µL of Big Dye terminator, 0.75 µL of Buffer (400 mM Tris-HCl, 10 mM MgCl₂, pH 9), 0.5 µL of forward primer Euk1A (10 µM) and 0.5–1 µL of

the PCR product obtained from the excised bands. Pure water was used to complement the reaction volume. The PCR reaction consisted of an initial denaturation of 96 °C for 5 min and 30 cycles including 30 s of denaturation at 94 °C, 30 s of annealing at 56 °C and 2 min extension at 60 °C. A final extension of 7 min at 60 °C following by cooling at 4 °C terminated the PCR program. The sequencing PCR product was then purified into 20 µL of injecting solution to eliminate the remaining fluorescent nucleotides and the sequencing was then performed using an ABI Prism 3100 (Applied Biosystems).

Sequence analysis

The general taxonomic affiliation of each sequence was determined by BLAST analysis on the National Center for Biotechnology Information web server (<http://www.ncbi.nlm.nih.gov/BLAST/>) in February 2005. Sequences were then aligned using the ARB software (Ludwig *et al.*, 2004) obtained from <http://www.arb-home.de> against a database containing more than 28 000 complete or partial SSU rDNA sequences from both eukaryotes and prokaryotes. This database relied on the aligned SSU rDNA database released by the ARB team in June 2002 (<http://www2.mikro.biologie.tu-muenchen.de/download/ARB/data/ssujun02.arb>), to which more than 1800 new sequences, either publicly available or unpublished, had been added. The resulting alignment was checked and corrected manually, and sequence identity was calculated by constructing a identity matrix with the ARB tools. All sequences used in this paper have been deposited to GenBank (DQ001412 to DQ001537). The aligned sequences are available as an ARB database file from the following web site (http://www.sb-roscoff.fr/Phyto/index.php?option=com_doc-man&task=doc_download&gid=181).

QPCR analysis

Six sets of primers were used to target different taxonomic levels within eukaryotes (Eukaryotes, Chlorophyta, Mamielales, *Micromonas*, *Ostreococcus*, *Bathycoccus*). Primer sequences are provided in Table 2 of Zhu *et al.* (2005). Reactions and calibrations were performed as detailed for natural samples in Zhu *et al.* (2005) on the DNA extracted as described above and diluted 10-fold. Briefly, for each sample reactions were performed in triplicate with optical tubes (Applied Biosystems) in a final volume of 12.5 µL containing 2.5 µL of template DNA, 1.25 µL SYBR Green PCR buffer (Applied Biosystems, Courtaboeuf, France), 200 µM each of dATP, dCTP and dGTP, 400 µM dUTP, 0.25 U of AmpErase uracyl N-Glycosylase (AmpErase UNG), and 0.05 U of Platinum Taq DNA polymerase (Life Technologies, Cergy Pontoise, France). All reactions were performed with an ABI 5700 sequence detection system (Applied

Biosystems) programmed with a soak step of 2 min at 50 °C, allowing AmpErase UNG to hydrolyze PCR amplicons possibly carried over from previous reactions. An enzyme activation step (94 °C, 2 min) followed the initial soak step. Forty cycles of 15 s of denaturation at 94 °C, annealing-extension at 60 °C with the times listed in Zhu *et al.* (2005), and 25 s of data collection at 77 °C were performed. All data were analyzed using Sequence Detection System v 1.3 software (Applied Biosystems). Three linear plasmids from cloned 18S rRNA gene for *Micromonas* RCC 114 (pMIC), *Bathycoccus* RCC 113 (pBAT), and *Ostreococcus* RCC 614 (pOST) were used as standards as detailed in Zhu *et al.* (2005).

Results and discussion

Hydrology and picoplankton distribution

The PROSOPE cruise (September–October 1999, Fig. 1) began first to sample the highly eutrophic Morocco upwelling off Agadir (St. UPW) and then proceeded to a transect through the Mediterranean Sea from the Strait of Gibraltar (St. 1) to the Eastern Basin off Crete (St. MIO). It also sampled the Tyrrhenian and Ligurian Seas (St. 8, 9 and DYF). The temperature and salinity of surface waters increased from the upwelling to the Eastern Basin (16.4 °C and 36.1‰ for St. UPW to 26 °C and 38.9‰ for St. MIO,

respectively). As observed previously in summer (Moutin & Raimbault, 2002), nitrates were very low at the surface at most Mediterranean Sea stations except off the Algerian coast (St. 2). The nitracline deepened steadily eastwards and reached 100 m off Crete. Phosphates were always below the limit of detection of classical methods, ranging from 3 nM near Gibraltar to 0.2 nM in the Ionian Sea (Moutin *et al.*, 2002). Levels of chlorophyll were very high in the Morocco upwelling, where they peaked at the surface (around 2 mg m⁻³) with high concentrations of the carotenoids fucoxanthin and peridinin, associated to diatoms and dinoflagellates, respectively. In the Mediterranean Sea, chlorophyll profiles (Fig. 2) exhibited a subsurface maximum, the concentration of which decreased from Algeria (St. 2, 0.8 mg m⁻³) to Crete (St. MIO, 0.2 mg m⁻³) and whose depth increased down to about 100 m off Crete. Off Sicily (St. 5), a sharp chlorophyll maximum was observed at 50 m (Fig. 2).

The distribution of picoeukaryotes, estimated by flow cytometry (Fig. 2d), was very similar to that of total chlorophyll *a* (Fig. 2a). Concentrations were relatively high (around 20 000 cell mL⁻¹) in the Morocco upwelling (St. UPW, Table 1). In the Mediterranean Sea, picoeukaryotes formed a subsurface peak that deepened eastward. Maximal abundances were observed off Gibraltar, in the Alboran Sea, and off Sicily (St. 1, 2, 5). These maxima of the order of 10 000 cell mL⁻¹ corresponded also to local maxima for

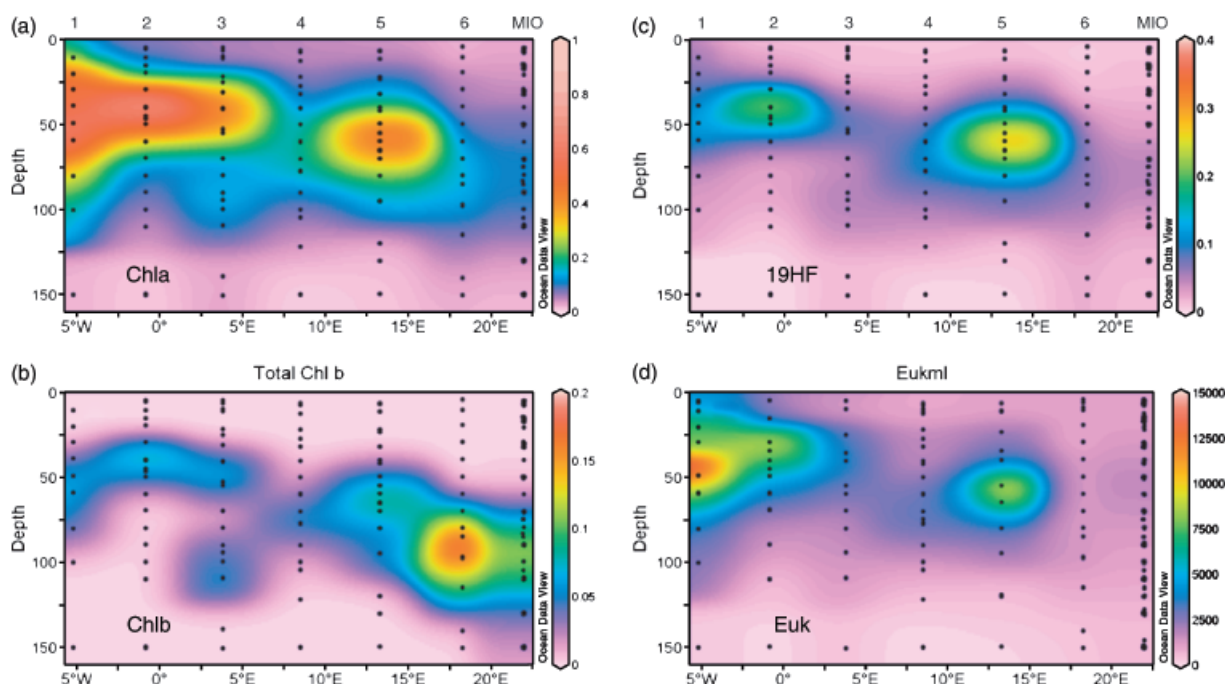


Fig. 2. Mediterranean Sea transect from Stations 1 to MIO. Concentration (mg m⁻³) of chlorophyll *a* (a), total chlorophyll *b* (b), and 19' hexanoyloxyfucoxanthin estimated by high-performance liquid chromatography (c) and abundance (cell mL⁻¹) of photosynthetic picoeukaryotes estimated by flow cytometry (d).

Table 1. Stations sampled for denaturing gradient gel electrophoresis, temporal temperature gradient gel electrophoresis and quantitative PCR during the PROSOPE cruise with concentrations of chlorophyll *a* estimated by high-performance liquid chromatography and of photosynthetic picoplanktonic eukaryotes estimated by flow cytometry. Additional stations were sampled for pigment and flow cytometry (see Fig. 2)

Station	CTD	Latitude	Longitude	Depth (m)	Chl <i>a</i> (mg m ⁻³)	Eukaryotes (cell mL ⁻¹)
UPW	2	30.97	- 10.05	5	2.82	7347
				30	1.49	8120
1	11	36.09	- 5.20	5	0.13	3882
				30	0.54	7078
				80	0.34	3523
3	17	38.00	3.83	5	0.07	1558
				25	0.32	3174
				55	0.39	3311
				80	0.11	1409
				95	0.18	1547
5	23	36.48	13.32	110	0.08	1131
				25	0.07	2673
				55	0.77	12 467
				65	0.45	8871
				95	0.15	2424
MIO	28	33.99	22.02	5	0.02	790
				30	0.05	1111
				50	0.11	975
				90	0.19	646
				110	0.13	679
9	70	39.12	14.08	5	0.08	962
				30	0.11	1058
				65	0.29	1671
				75	0.16	
				90	0.09	819
DYF	81	43.39	7.81	15	0.10	1093
				25	0.14	2287
				50	0.43	3109
				60	0.23	1968
				75	0.16	1633
				110	0.02	117

chlorophyll *b* (Fig. 2b) and 19' hexanoyloxyfucoxanthin (19HF, Fig. 2c), two pigments associated, respectively, with Prasinophyceae and Prymnesiophyceae, which are key groups of the picoplankton (Moon-van der Staay *et al.*, 2000; Not *et al.*, 2004). At the most oligotrophic station (St. MIO) picoeukaryote concentration was low (1000–2000 cell mL⁻¹), although slightly higher than was found further East (Li *et al.*, 1993).

Eukaryotic picoplankton diversity determined by DGGE and TTGE of the rDNA gene

PCR amplified 18S rRNA genes from 0.2–3- μ m samples were analyzed by denaturing gradient electrophoresis using both DGGE and TTGE (Fig. 3). In general DGGE displayed a better resolution than TTGE, whereas TTGE analyses were easier to perform (no gradient in the gel). Between 8 and 18 (mean 14) major bands as defined by eye were observed per sample using DGGE (Fig. 3) and 10–16 (mean 12) with

TTGE. DGGE and TTGE patterns appear not to be directly comparable. For example, at St. 3, the most intense band from the surface sample co-migrates with the most intense bands from the lower depths with TTGE; in contrast with DGGE, the most intense band from the surface sample is located higher in the gel compared to the most intense bands from the other depths.

The comparison of the banding patterns found for the 21 samples analyzed, allowed 44 major bands for DGGE gels and 40 for TTGE gels to be defined (Fig. 3). The presence or absence of these bands in individual lanes allowed us to perform a cluster analysis for both DGGE and TTGE. Only the latter is discussed here (Fig. 4) as the clustering based on DGGE was difficult to interpret. With TTGE, the two samples from the Morocco upwelling clustered together apart from all other Mediterranean Sea samples. For the latter, two major groups appeared, one corresponding to Ionian, Tyrrhenian, and surface Ligurian Sea samples and the other to Western Basin stations plus the deep samples

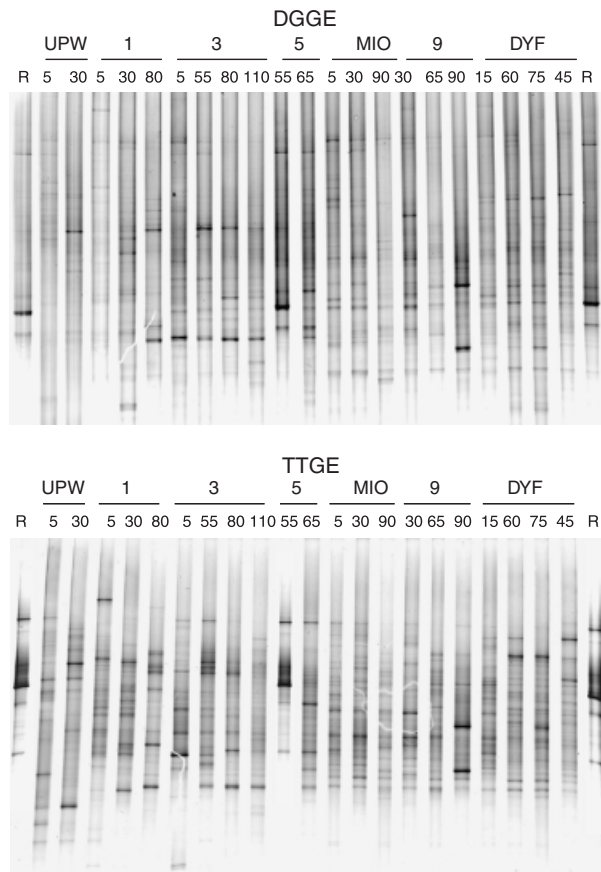


Fig. 3. Denaturing gradient gel electrophoresis (a) and temporal temperature gradient gel electrophoresis (b) gels for selected stations (first line) and depths (second line, meters). One of the sample (St. 5, 55 m) was used as a reference and therefore is also repeated in the first and last lanes.

from the Ligurian Sea. At a finer level, samples from the same station were more closely related to each other than samples from different stations. This suggests that eukaryote communities are quite homogeneous across the euphotic zone but differ from one station to the other, in contrast to what is found for *Prochlorococcus*, for which populations in the surface and at the bottom of the euphotic zone often differ genetically (West & Scanlan, 1999). However, eukaryote populations below the euphotic zone are probably quite different, as demonstrated in the Antarctic by DGGE (Díez *et al.*, 2004).

Among 157 electrophoresis bands excised from both DGGE and TTGE gels, a total of 126 could be sequenced. The 31 remaining were either mixtures of phylotypes or could not be sequenced. BLAST analysis confirmed the specificity of the primers used, as all sequences could be affiliated to eukaryotic groups.

Overall, the vast majority of sequences (96%) had more than 90% of identity to available 18S rRNA gene sequences (Table 2, Table S1) from the following divisions: Metazoa

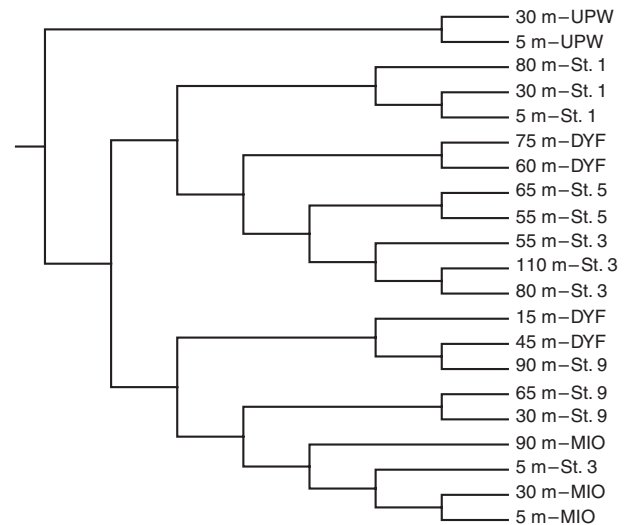


Fig. 4. Clustering of samples based on the analysis of band patterns for temporal temperature gradient gel electrophoresis gels (see Materials and methods).

(14), Fungi (4), Chlorophyta (11), Cercozoa (3), Stramenopiles (18), Alveolates (53) and Polycystinea (5). The remaining 18 sequences matched a group of recently published environmental sequences (see below).

Bands extracted from the bottom of the DDGE gels, corresponding to PCR products most resistant to the denaturation, matched sequences of metazoans. These bands appeared less pronounced with TTGE and were not excised. Apart from this case, band position on the gel did not provide any clue to the phylogenetic affiliation of the corresponding sequence. For example, three of the four sequences presenting identities with fungi were recovered from the upper part of the TTGE gels, whereas the last one was located in the lower part. Bands presenting identities with the Prasinophyceae were in general found in the middle of the gels, but a few were located in both the upper and lower parts of the gels.

The overall composition of the picoplankton community was fairly similar when derived from either DGGE or TTGE, despite the fact that we obtained three times more sequences from TTGE than DGGE gels, excluding Metazoa and Fungi (Table 2). Some groups (Cercozoa, Stramenopiles MAST-4 and Ciliophora) were only recovered with TTGE, but this is probably due to the better sampling of bands from these gels, because the same primers (Euk1A and 516r-GC) were used for both techniques.

All Metazoa sequences recovered corresponded to typically marine groups (Table S1), mostly Crustacea (9), Vestimentifera (3) and Mollusca (1). Such sequences are often recovered from marine picoplankton clone libraries (Massana *et al.*, 2004b; Romari & Vaultot, 2004) and could originate from sperm cells that are small enough (Beninger

Table 2. Phylogenetic affiliation of sequences recovered from denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient gel electrophoresis (TTGE) gels (Metazoa and fungi were excluded from the calculation of percentages for both DGGE and TTGE)

Division/class	Total		DGGE		TTGE	
	Nb	%	Nb	%	Nb	%
Metazoa	14		14			
Fungi	4				4	
Chlorophyta Prasinophyceae	11	10	3	12	8	10
Cercozoa	3	3			3	4
Stramenopile Bacillariophyceae	4	4	1	4	3	4
Stramenopile MAST-3	9	8	1	4	8	10
Stramenopile MAST-4	5	5			5	6
Alveolate Group I	17	16	5	19	12	15
Alveolate Group II	28	26	8	31	20	24
Alveolate Ciliophora	4	4			4	5
Alveolate Dinophyceae	4	4	1	4	3	4
Polycystinea	5	5	3	12	2	2
env Nansha	18	17	4	15	14	17
Total (excluding Metazoa and Fungi)	108		26		82	

& Le Pennec, 1997) to pass through the 3 µm filters used to separate picoplankton. In the same way, fungal sequences are often found in picoplankton (Moon-van der Staay *et al.*, 2001; Massana *et al.*, 2004a; Romari & Vault, 2004), but their origin remains less clear. These nonprotist sequences will not be discussed any further here.

A relatively high proportion (50/108 i.e. 46%) of protist sequences had more than 98% identity to existing sequences (Table S1), a level that corresponds roughly to that shared by organisms belonging to the same genus (Romari & Vault, 2004). Among these matching sequences, 15 corresponded to known genera: *Bathycoccus* (8), *Ostreococcus* (2), *Leptocylindrus* (2) and *Cryothecomonas* (3). The remaining 35 matching sequences have been recovered from the environment, many of them quite recently, emphasizing the fact that as the number of available sequences increases, completely novel sequences (i.e. with low identity to anything known) become rarer.

Eleven sequences were related to the order *Mamiellales* (Prasinophyceae, Chlorophyta), a group that contains picoplanktonic species (Guillou *et al.*, 2004). Nine of them matched the species *Bathycoccus prasinos* with 97–100% identity, whereas the genus *Ostreococcus* was represented by two sequences with 100% identity to strain RCC143 isolated in the tropical Atlantic Ocean (Table S1). Prasinophyte sequences were found at all the Mediterranean Sea stations but not in the Morocco upwelling.

Eighteen sequences, originating in particular from the oligotrophic station MIO, were affiliated to the stramenopiles (Table 2). Four of them matched sequences of the diatom genus *Leptocylindrus* with 94–98% identity (Table S1). This is quite surprising as species belonging to this genus are quite large and diatom sequences are quite infrequent in picoplankton samples: for example, none were

found during a seasonal survey in the English Channel, an area that witnesses dense diatom blooms (Romari & Vault, 2004). Fourteen sequences clustered with environmental sequences belonging to the yet uncultivated groups MAST-3 and MAST-4 (Massana *et al.*, 2002, 2004a) with homologies ranging from 92% to 99%. Interestingly, the other MAST groups were not recovered, confirming the findings by Massana *et al.* (2004a) that these two groups are the most frequently found in nonpolar open ocean waters.

With 53 sequences (Table 2), alveolates represented the most important group of the sequences distributed as follows: 4 Ciliophora (95–98% identity), 4 Dinophyceae (89–98% identity), 17 Alveolates group I (97–100% identity), 28 Alveolates group II (89–99% identity). None of the alveolate sequences matched closely (i.e. above the 98% threshold) known species, but in contrast some were nearly identical to other oceanic environmental sequences, in particular from the Pacific Ocean.

Three sequences from the Morocco upwelling corresponded to the genus *Cryothecomonas* (Cercozoa), which contains heterotrophic flagellate species feeding on diatoms (Drebes *et al.*, 1996).

Five sequences from St. 1 (Gibraltar) clustered with Polycystinea (Radiolaria), prominent members of the pelagic plankton (Febvre-Chevalier & Febvre, 1999), with 95–97% of identity to the species *Collozoum inerme* (Table S1). Eighteen sequences presented 93–99% identity to environmental sequences with no cultured relatives recently recovered from samples from the Nansha Islands in the South China Sea (Yuan *et al.*, 2004). This group, named here 'env Nansha', may be related phylogenetically to the Acantharians and Polycystinea (Yuan *et al.*, 2004). It was found at all Mediterranean Sea stations, but not in the Morocco upwelling. Related sequences have also been found

Table 3. Picoplankton community composition at the different stations (Metazoa and fungi were excluded from the calculation of percentages for both denaturing gradient gel electrophoresis and temporal temperature gradient gel electrophoresis)

Station Division/class	UPW		1		3		5		MIO		9		DYF	
	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%
Metazoa Crustacea			1		11				2					
Fungi Ascomycota					1		1		2					
Chlorophyta Prasinophyceae			4	14	1	4	2	67	2	7	1	13	1	11
Cercozoa	3	43												
Stramenopile Bacillariophyceae					1	4			3	11				
Stramenopile MAST-3			3	10					4	14	1	13	1	11
Stramenopile MAST-4									3	11	2	25		
Alveolate Group I	1	14	1	3	4	17			9	32			2	22
Alveolate Group II	1	14	10	34	6	25			3	11	3	38	5	56
Alveolate Ciliophora			2	7					2	7				
Alveolate Dinophyceae	2	29			1	4			1	4				
Polycystinea			5	17										
env Nansha			4	14	11	46	1	33	1	4	1	13		
Total (excluding Metazoa and Fungi)	7		29		24		3		28		8		9	

in the Equatorial Pacific (S. Y. Moon-van der Staay, pers. comm.) and therefore this group may be restricted to oligotrophic blue waters.

It is difficult to make inter-station comparison (Table 3) because of the small number of sequences obtained per station. However, one could note that the number of groups recovered was highest at the most oligotrophic station (St. MIO), whereas at the station that exhibited a sharp maximum of photosynthetic eukaryotes (St. 5, Fig. 2), only two groups were recovered: Prasinophyceae and env Nansha.

Only two prior studies of picoeukaryote diversity using molecular approaches have been performed in the Mediterranean Sea. One relied on cloning-sequencing at a coastal site off Spain, Blanes Bay (Massana *et al.*, 2004a), and the other both on cloning-sequencing and DGGE in the Alboran Sea in November (Díez *et al.*, 2001a,b). In these two studies, Prasinophyceae were one of the photosynthetic group most frequently recovered, confirming our results. However, in these studies, other photosynthetic sequences, apart from those of diatoms and dinoflagellates, were also obtained (e.g. Pelagophyceae, Eustigmatophyceae, Prymnesiophyceae, Cryptophyceae). Their absence in the present data set could be due either to insufficient sampling of the eukaryote diversity or to the absence of these groups at the more oligotrophic sites sampled. However, the relative abundance of the carotenoid 19'HF at stations where eukaryotic picophytoplankton is abundant (Fig. 2) suggests that some groups were missed. Among heterotrophic eukaryotes, uncultivated stramenopiles were major groups in Blanes and in the Alboran Sea, in contrast to the present work, in which uncultivated alveolates dominated. This could be linked to the oligotrophic nature of the stations investigated, as alveolates are also important in other open ocean areas such as the Equatorial Pacific (Moon-van der

Staay *et al.*, 2001). However, alveolates, in particular group II, are also abundant in temperate nutrient-rich coastal waters (Romari & Vaulot, 2004). The affiliation of *Amoebophrya*, a parasite of dinoflagellates, with group II (Moreira & López-García, 2002) suggests that this group could in fact co-occur with dinoflagellates and therefore be abundant under environmental conditions favoring the latter, e.g. under calm stratified conditions in coastal waters for photosynthetic dinoflagellates or in the surface layer of oligotrophic areas for heterotrophic dinoflagellates (Vørs *et al.*, 1995).

Chlorophyta distribution from QPCR

As evident from the sequences obtained by DGGE and TTGE, Chlorophyta, and more specifically Mamiellales, are an important component of the Mediterranean Sea picophytoplankton. Therefore, their contribution was investigated in a more quantitative fashion by QPCR (Table 4, Fig. 5). The relative contribution of Chlorophyta to the eukaryotes estimated by QPCR was on average 21% (Table 4), twice as low as in Blanes Bay, a Mediterranean coastal site where the same protocol was used (Zhu *et al.*, 2005). Maximal contributions (around 50%) were in general observed at depths around 50 m or more (e.g. St. 1, 3 or 5, Fig. 5). It should be noted that QPCR estimates of Chlorophyta probably include contribution from other groups such as Polycystinea, which possess a single mismatch to the CHL primer set used (Zhu *et al.*, 2005) and that are amplified with it (M. Viprey, pers. comm.). The contribution of Mamiellales was on average much lower (Mean = 2.8%). Their distribution across the Mediterranean Sea mirrored fairly well the distribution of picoeukaryotes (compare Figs 5b and 2a) with a maximum at depth

Table 4. Abundance of taxonomic groups at the different stations estimated by quantitative PCR. Results are expressed either as number of rDNA gene copies per mL (eukaryotes) or as percentage of rDNA gene copies relative to all eukaryotes

Station	CTD	Depth (m)	QPCR					
			Eukaryotes rDNA copies mL ⁻¹	Chlorophyta % of eukaryotes	Mamiellales	<i>Micromonas</i>	<i>Bathycoccus</i>	<i>Ostreococcus</i>
UPW	2	5	16 610	9.7	0.3	0.0	0.0	0.0
		30	18 101	17.1	1.2	0.1	0.1	0.6
1	11	5	28 942	15.3	2.6	0.2	0.8	0.1
		30	44 724	20.4	9.1	0.6	2.7	0.4
		80	57 474	52.7	2.5	0.1	0.8	0.1
3	17	5	26 767	9.8	0.7	0.0	0.1	0.0
		25	85 515	41.2	1.2	0.0	0.4	0.0
		55	38 854	17.9	2.4	0.0	0.8	0.1
		80	62 260	52.2	1.1	0.0	0.4	0.0
		95	31 538	39.3	1.5	0.1	0.5	0.0
5	23	110	39 465	41.2	1.5	0.1	0.6	0.1
		25	64 834	9.9	1.6	0.1	0.4	0.6
		55	115 519	19.4	12.0	0.3	2.7	5.3
		65	101 098	33.6	18.3	0.2	5.5	5.3
MIO	28	95	52 772	45.6	4.1	0.0	1.5	0.4
		5	12 152	10.5	0.7	0.0	0.0	0.0
		30	53 012	4.8	0.2	0.0	0.0	0.0
		50	30 067	7.5	0.5	0.0	0.1	0.0
9	70	90	54 440	16.7	1.1	0.0	0.0	0.0
		110	51 765	17.7	0.9	0.1	0.1	0.0
		5	20 415	8.9	0.7	0.0	0.0	0.0
		30	45 152	12.2	0.3	0.1	0.1	0.0
DYF	81	65	41 602	25.6	6.1	0.1	2.6	0.1
		75	53 230	23.4	4.1	0.1	1.7	0.1
		90	33 314	15.6	1.7	0.0	0.7	0.1
		15	20 182	12.8	0.6	0.1	0.0	0.0
		25	86 090	18.6	0.4	0.0	0.1	0.0
		50	101 273	13.1	2.0	0.1	0.8	0.0
DYF	95	60	39 944	22.1	4.3	0.2	2.0	0.1
		75	23 698	18.6	2.7	0.1	1.3	0.1
		110	58 814	18.3	1.2	0.0	0.2	0.0
		45	228 240	12.6	3.6	0.0	1.1	0.0
		50	157 693	16.2	3.7	0.1	1.4	0.0
		55	125 614	20.3	3.5	0.1	1.3	0.0
DYF	95	60	67 828	17.3	1.8	0.1	0.8	0.0
		65	60 065	17.0	1.1	0.1	0.6	0.0
		Mean	59 696	21.0	2.8	0.1	0.9	0.4
		Max	228 240	52.7	18.3	0.6	5.5	5.3
		Min	12 152	4.8	0.2	0.0	0.0	0.0

following the chlorophyll maximum. Of the three genera estimated by QPCR, only *Bathycoccus* had an overall significant contribution and its distribution was very similar to that of the Mamiellales. The contribution of *Ostreococcus* was in general very low with the exception of St. 5 where it was more abundant than *Bathycoccus*. Finally, *Micromonas* was always very minor. The average contribution of these three genera was lower than at the Blanes coastal site. However, in Blanes, *Micromonas* and *Bathycoccus* had similar contributions, and *Ostreococcus* was insignificant. The

absence of *Micromonas* in the open Mediterranean Sea confirms that this species is probably best adapted to temperate nutrient-rich environments (English Channel, coastal Mediterranean Sea, high latitude North Atlantic Ocean, Southern Ocean, Díez *et al.*, 2004; Not *et al.*, 2004, 2005; Zhu *et al.*, 2005). *Bathycoccus* is probably more of a mesotrophic species with good adaptation to low light conditions (it was initially isolated from 100 m depth off Naples, Eikrem & Thronsen, 1990). *Ostreococcus*, despite the fact that it has been isolated from a variety of

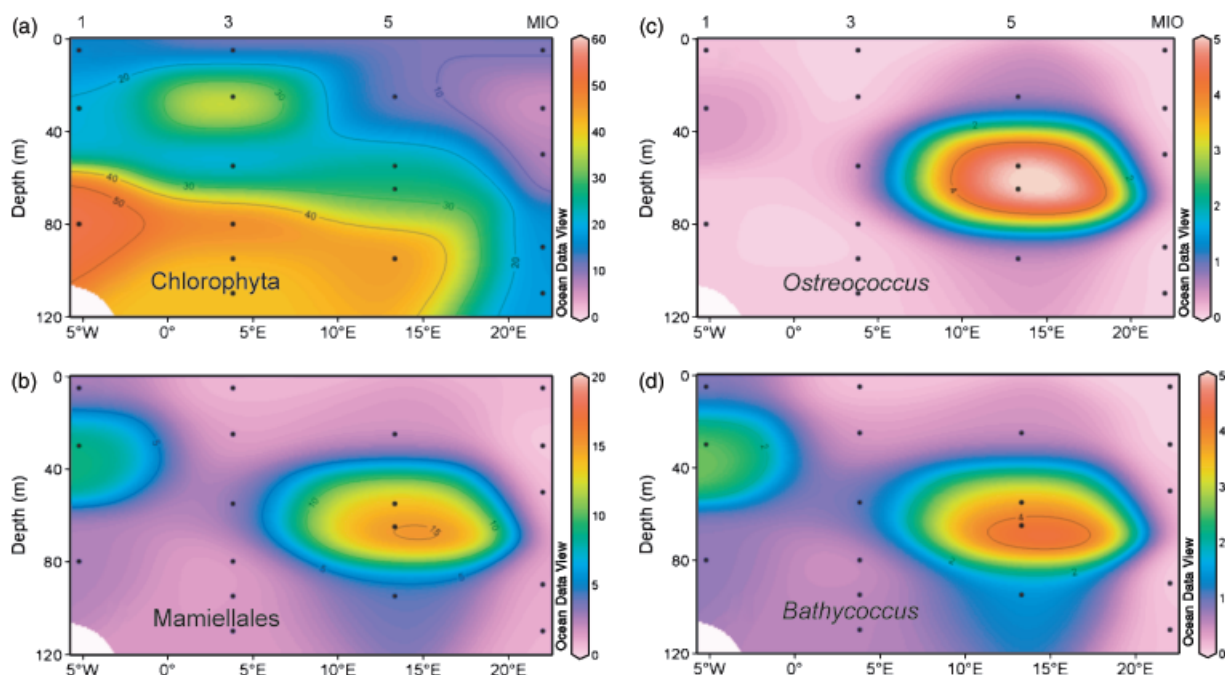


Fig. 5. Mediterranean Sea transect from Stations 1 to MIO of the relative abundance of rDNA gene copies for selected taxa as a fraction of that of total eukaryotes (%) estimated by quantitative PCR. (a) Chlorophyta, (b) Mamiellales, (c) *Bathycoccus*, (d) *Ostreococcus*.

environments (Worden *et al.*, 2004; Rodríguez *et al.*, 2005), could be much more sporadic and occur at high concentrations only under very specific conditions. Moreover, genera such as *Ostreococcus* and *Micromonas* display probably a wide 'ecotypic' diversity as recently shown for the former species for which it is possible to define four clades based on phenotypic and genotypic characters, one of which appear to be adapted to low light conditions (Rodríguez *et al.*, 2005).

Conclusion

The Mediterranean Sea in summer displays overall oligotrophic conditions with a marked gradient between the Western and Eastern Basins, the former being characterized by localized enriched conditions (e.g. off Gibraltar or in the vicinity of the Algerian coast) and the latter strongly limited by phosphorus (Krom *et al.*, 1991; Moutin & Raimbault, 2002). Pelagic eukaryotic picoplankton characteristics reflect oligotrophic conditions as evidenced by the dominance of uncultivated alveolate groups I and II and by the presence of 'blue water' groups such as the acantharians. The oligotrophy of the stations investigated is also confirmed by the observation of sequences related to the newly discovered env Nansha group previously only found in typically oligotrophic environments (South China Sea, Equatorial Pacific) and by the quasi-absence of the genus *Micromonas*, which is abundant in temperate nutrient-rich oceanic and coastal waters (Thronsen & Kristiansen, 1991; Not *et al.*, 2004; Not

et al., 2005) and which is replaced here by *Bathycoccus* and very sporadically by *Ostreococcus*.

Despite these elements, many questions remain concerning the structure of picoplankton communities in the Mediterranean Sea, in particular with respect to its photosynthetic component. Apart from Prasinophyceae, and a few diatom and dinoflagellates sequences, no other photosynthetic groups were recovered, but the importance of pigments such as 19HF (Fig. 2) suggest that Heterokontophyta and/or Haptophyta probably also contribute to this community. Their diversity and abundance will need to be probed using clone libraries built with specific primers amplifying selected phylogenetic groups preferentially (Bass & Cavalier, 2004) and existing probes detected by fluorescence *in situ* hybridization (Simon *et al.*, 2000).

Supplementary Material

The following material is available for this article online: Table S1. Phylogenetic affiliation of sequences recovered from denaturing gradient gel electrophoresis and temporal temperature gradient gel electrophoresis gels.

This material is available as part of the online article from <http://www.blackwell-synergy.com>

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