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Picoeukaryotic diversity in the Gulf of Gabès: variability patterns and relationships to nutrients and water masses

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ABSTRACT: Marine picoeukaryotes show high phylogenetic diversity worldwide, notably in oligotrophic waters. In the Gulf of Gabès (south-eastern Mediterranean), characterized by oligotrophic conditions and a complex water mass circulation, information on picoeukaryotic diversity is still lacking. In this study, we investigated the diversity and spatial variability of picoeukaryotic assemblages in relation to nutrient availability, physical parameters and water masses in 3 cruises carried out in the Gulf of Gabès in June of 2008, April of 2009 and November of 2009. High-throughput sequencing revealed a dominance of sequences from non photosynthetic picoeukaryotes, mostly represented by the presumably parasitic marine alveolate MALV-II (33.20%) and the bacterivorous Bicosoecida (13.56%). Differences in picoeukaryotic assemblages were higher between coastal and open-sea stations, and depth in the water column also affected community differences, with surface (5 m), intermediate (25–100 m) and mesopelagic (>200 m) samples forming separate groups. A clear temporal variability was also evident, particularly for communities collected from the surface layer and open-sea stations. Co-inertia analysis revealed that picoeukaryotic groups were more affected by salinity in deep waters, whereas at the surface, they were dependent on nutrients and temperature. During the November cruise, samples that shared similar water mass properties generally clustered together. The Levantine water mass, observed for the first time in this area, was characterized by the presence of Acantharia and Polycystinea. Our study highlights the role of physical and chemical features, such as water mass origin, the wide continental shelf and trophic status, in determining the diversity of marine picoeukaryotes.

KEY WORDS: Picoeukaryotes · V4 · 18S rDNA · Diversity · Inorganic nutrients · Water masses · Mediterranean Sea · Gulf of Gabès

INTRODUCTION

Marine picoeukaryotes (protists up to 3 µm in size) are important players in planktonic food webs of coastal and offshore ecosystems (Massana 2011). Picoeukaryotic diversity has been widely studied in different types of aquatic ecosystems, showing high phylogenetic diversity worldwide. This diversity seems to be crucial in maintaining the functional stability and resilience of ecosystems (Caron & Countway 2009). In the past, diversity of natural picoeukaryotic assemblages has been widely analyzed using Sanger sequencing of cloned environmental genes (Díez et al. 2001a, Massana et al. 2004, Countway et al. 2010, Wu et al. 2014), as well as denaturing (DGGE) and temperature gradient gel electrophoresis and terminal restriction fragment length polymorphism fingerprinting tools (Díez et al. 2001b, Zeidner & Beja 2004, Marie et al. 2006, Wu et al. 2009, Lie et al. 2013). High-throughput

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sequencing (HTS) of ribosomal DNA (rDNA) gene markers revolutionized the field of microbial ecology by allowing an exhaustive characterization of the entire microbial eukaryotic community within an environment, confirming their large diversity and high proportion of novel taxa (Stoeck et al. 2010, Logares et al. 2012, de Vargas et al. 2015, Massana et al. 2015, Hu et al. 2016).

Several studies have investigated relationships between picoplankton diversity and environmental factors and have demonstrated that these microorganisms respond to shifts in temperature, carbon chemistry, nutrient and oxygen content, and alterations in ocean stratification and currents (Hamilton et al. 2008, Doney et al. 2012, Grossmann et al. 2016). In a complex region of Atlantic–Arctic confluence, picoeukaryotic assemblages were influenced by both contemporary conditions (salinity, photosynthetically active radiation, transmissivity, total phototrophic biomass and size class) and water mass origin (Hamilton et al. 2008), while in Tibetan lakes they were mostly affected by the chemical composition of the water, which covaried with altitude and latitude (Wu et al. 2009). In the Coorong Lagoon, diversity of picoeukaryotes was mainly controlled by geographic distance rather than salinity despite the existence of a high salinity gradient (Balzano et al. 2015). Furthermore, in Alpine freshwater lakes, changes in protistan communities were mostly dependent on pH and nutrient concentrations (Grossmann et al. 2016).

In the oligotrophic eastern Mediterranean, chlorophyll biomass is generally very low and is attributed to picoeukaryotes (Siokou-Frangou et al. 2010). Particularly in the Gulf of Gabès, picoplankton is the main contributor within the ultraphytoplankton (cells up to 10 µm) (Hamdi et al. 2015), which together with the nanoplankton accounts for up to 90% of the overall chlorophyll biomass (Bel Hassen et al. 2009a). Despite the oligotrophic character of the eastern Mediterranean Sea, considered to be one of the most oligotrophic regions of the world's oceans (Berman et al. 1984, Krom et al. 2005), the Gulf of Gabès exhibits a relatively high nutrient content throughout the year (Drira et al. 2009). In addition, this area presents a complex water circulation pattern with water masses from Mediterranean and Atlantic origins (Bel Hassen et al. 2009b). Previous studies on phytoplankton community composition and distribution in different water masses prevailing in the Gulf of Gabès revealed that Mediterranean and Atlantic water masses did not affect the autotrophic biomass variability (Bel Hassen et al. 2009b), but a distinction between these 2 water masses based on ultraphytoplankton abundance was noted (Hamdi et al. 2015). However, in the Gulf of Gabès, the picophytoplankton fraction, and notably the eukaryotic component (cells ≤3 µm in diameter, Vaulot et al. 2008), has received much less attention than other planktonic groups. The only data available, derived from a chemotaxonomic pigment analysis, revealed a diverse pattern of community composition (Bel Hassen et al. 2009a), which contrasted with the general feature of prymnesiophyte dominance in the Mediterranean basin (Claustre et al. 1994, Vidussi et al. 2000).

Despite the putative importance of picoeukaryotes in the Gulf of Gabès, no studies have targeted their genetic diversity, including the effects of the particular oligotrophic status and the water mass features. In the present study, we compared the communities of picoplanktonic eukaryotes in this area by DGGE of the 18S rRNA gene, in a first step, and their phylogenetic diversity by high-throughput sequencing of the V4 region of the 18S rRNA gene in a second step. Species richness, community structure and spatial distribution of picoeukaryotic assemblages were determined in June of 2008, April of 2009 and November of 2009 in coastal and open-sea regions of the Gulf of Gabès. The influence of physical parameters and nutrient availability on their composition and distribution was investigated, as well as the relationship between picoeukaryotic assemblages and distinct water masses.

MATERIALS AND METHODS

Study site, cruises and sampling

The Gulf of Gabès is located south of the Tunisian coast in the southern Ionian Sea. It stretches from 'Ras Kaboudia' at 35° N latitude to the Tunisian–Libyan border at 33° N (Fig. 1). The Gulf presents one of the widest continental shelfs in the Mediterranean, with the 50 m isobath located 110 km from the coastline. In the Gulf, different water masses related to Mediterranean and Atlantic origins circulate. Modified Atlantic Water (MAW) is characterized by strong advection during winter, whereas in summer, advection is weakened and the MAW is observed in the deeper layers. Mediterranean Mixed Water (MMW) is located in the coastal mixed layer (Bel Hassen et al. 2008). Ionian Water (IW), which was firstly observed in the Gulf by Hamdi et al. (2015), is located beneath the MAW.

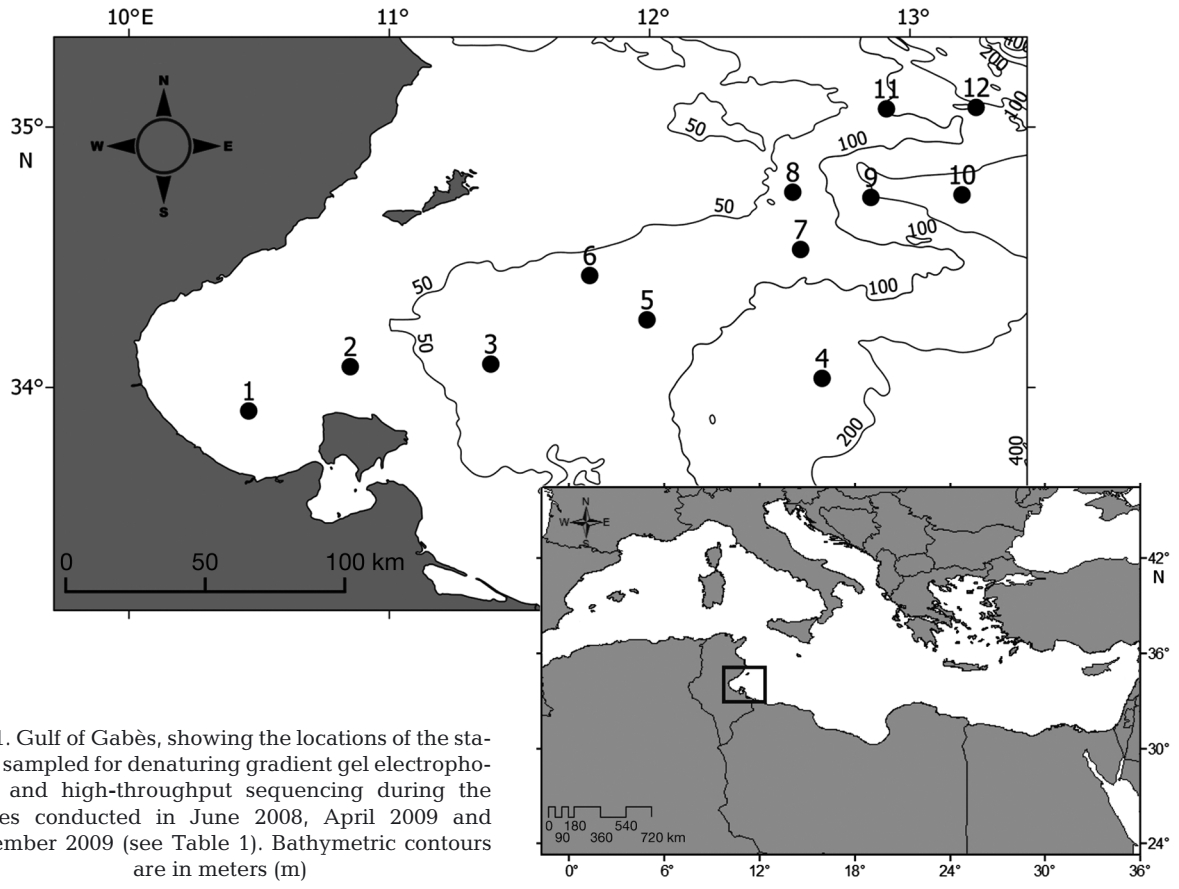

Fig. 1. Gulf of Gabès, showing the locations of the stations sampled for denaturing gradient gel electrophoresis and high-throughput sequencing during the cruises conducted in June 2008, April 2009 and November 2009 (see Table 1). Bathymetric contours are in meters (m)

Three oceanographic cruises on board the RV 'Hannibal' took place, covering the coastal and the oceanic area of the Gulf of Gabès. The first cruise (POEMM3) was conducted at the beginning of summer (12–16 June 2008), the second (POEMM5) in the middle of spring (1–4 April 2009) and the third (POEMM6) at the end of autumn (16–19 November 2009). During June and November cruises, 14 and 15 stations were respectively sampled under calm weather conditions, whereas 23 stations were sampled during the April cruise following a storm in the Gulf (see Fig. 2). Seawater samples were collected with 12 l Niskin bottles attached to a rosette and a conductivity, temperature and depth (CTD) probe (SBE 9, Sea-Bird Electronics). At each station, we sampled at 3 to 5 depths, depending on bottom depth and real-time temperature profile provided by the CTD. For stations whose bottom depth was lower than 65 m, we sampled at the surface, half of the water column and near the bottom. At stations where the bottom depth was between 65 and 90 m, we sampled at the surface, the thermocline, 50 m depth and near the bottom. At stations where the bottom depth was between 90 and 170 m, we added a 75 m sample and in those with a bottom depth deeper than 170 m, we sampled at 100 m instead of 75 m depth. At stations without thermal stratification, we sampled at 20–30 m depth intervals.

Picoplankton biomass was obtained by filter size fractionation. Seawater was pre-filtered through a 100 µm pore size mesh by gravity to remove large organisms, and then filtered by vacuum first onto a GF/D filter (Whatman) and then a 47 mm diameter membrane filter with 0.45 µm pore size (PESU, Sartorius), which was transferred immediately into a cryovial tube containing 3 ml of lysis buffer (0.75 M sucrose, 50 mM Tris-HCl and 40 mM EDTA, pH = 8) (Massana et al. 2004). Cryovials with the 0.45 µm filters containing the picoplankton (0.45–2.7 µm in size) were stored at –20°C until nucleic acid extraction.

For the determination of inorganic nutrients (nitrite: NO_2^-, nitrate: NO_3^-, ammonium: NH_4^+, orthophosphate: PO_4^{3-} and silicate: $Si(OH)_4$), 250 ml of seawater were directly taken from the rosette in a plastic bottle previously washed with acid and rinsed thoroughly with distilled water. Samples were stored at –20°C for subsequent analyses with an auto analyzer. Nutrient analyses were performed with an automatic analyzer type 3 (Bran+Luebbe) using standard methods (Tréguer & LeCorre 1975).

DNA extraction

DNA was extracted according to the phenol/chloroform protocol detailed by Massana et al. (2004). After thawing, 30 µl of lysozyme (100 mg ml^{-1}) were added to the lysis buffer and cryovials were incubated at 37°C for 45 min. Then, 300 µl of sodium dodecyl sulfate (10%) and 15 µl of Proteinase K (40 mg ml^{-1}) were added and the mixture was incubated at 55°C for 1 h. The lysate was recovered and distributed among 4 Eppendorf tubes. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8) was added and the mixture was centrifuged at 18 000 × *g* (5 min). Another extraction with chloroform/IAA (24:1) was performed to remove the residual phenol. After centrifugation at 15 000 × *g* (10 min), supernatant was recovered and DNA was precipitated overnight at −20°C with 2 volumes of ethanol and 1/10 volume of sodium acetate (3 M, pH 4.8). After centrifugation at 18 000 × *g* (10 min), the pelleted DNA was washed with 70% ethanol, air-dried and resuspended in 40 µl of TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8). DNA integrity was checked by agarose gel electrophoresis, and DNA yield was quantified in a Nanodrop spectrophotometer (NanoDrop 2000 Thermo). DNA extracts were stored at −20°C.

DGGE

To determine spatial and temporal variability of picoeukaryotic communities in the Gulf of Gabès, a subset of 54 samples was selected at different depths in the coastal and open-sea areas during the 3 investigated periods and analyzed by DGGE (Table 1). A 560 bp fragment of eukaryotic 18S rRNA gene was amplified by PCR with the oligonucleotide primers Euk1A (5′-CTG GTT GAT CCT GCC AG-3′) and Euk 516r-GC (5′-ACC AGA CTT GCC CTC C-3′ with a 40 bp GC clamp) following PCR conditions described by Díez et al. (2001b). PCR products were quantified using a low DNA mass ladder (Invitrogen). DGGE was performed with a DGGE-2000 system (CBS Scientific) as described previously (Díez et al. 2001b, Not et al. 2008). Approximately 800 ng of each PCR product were loaded into 6% polyacrylamide gels having a linear gradient (35–55%) of denaturant conditions (100% denaturant conditions are 7 M urea and 40% deionized formamide). Electrophoresis was performed in 1× TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) at 100 V and 60°C for 16 h. The gel was stained with SYBR GOLD (Invitrogen), and DNA bands were visualized with UV light and photographed in a ChemiDoc System (Bio-Rad). High-resolution DGGE images were analyzed with Quantity One 4.6.3 (Bio-Rad) to detect bands, calculate their intensity and identify the same band position in the different gel lanes. Binary (presence/absence of bands) and band-intensity matrices were constructed as described before (Díez et al. 2001b, Not et al. 2008). The binary matrix was used to calculate richness (number of bands). The intensity matrix was first normalized such that all lanes had the same band intensity and then log(x+1) transformed. The normalized-transformed DGGE intensity matrix was used to build a dendrogram.

High-throughput sequencing (HTS) and data analysis

In order to determine the phylogenetic diversity of the picoeukaryotic community and their spatial and temporal variability in the Gulf of Gabès, 12 samples were selected to account for the whole area and were processed for HTS (Table 1). The eukaryotic V4 region of the 18S rRNA gene (about 400 bp) was PCR amplified from environmental DNA using the forward primer V4F (5′-CCA GCA SCY GCG GTA ATT CC-3′) and the reverse primer V4R (5′-ACT TTC GTT CTT GAT YRA-3′) (Stoeck et al. 2010). PCR reactions and Illumina MiSeq sequencing were performed at the Research and Testing Laboratories (RTL, Lubbock, TX, USA). Illumina reads released by the sequencing service were analyzed with UPARSE (Edgar 2013, Logares 2017) following an in-house made pipeline (https://github.com/ramalok/amplicon_processing). First, the BayesHammer program was applied to correct sequencing errors introduced by the Illumina MiSeq platform. Paired-end corrected reads were then merged using PEAR (Zhang et al. 2014) and processed through USEARCH (Edgar 2010) for quality check and dereplication. Sequences were grouped into operational taxonomic units (OTUs) using the UPARSE algorithm, with a 99% threshold of similarity (Edgar 2013). Chimera detection was done based on the reference sequences released from SILVA 119 (Quast et al. 2013). A representative sequence for each OTU was then picked for further taxonomic assignment with BLAST against classified reference sequences from 3 different databases: PR^2 (Guillou et al. 2013) and 2 in-house marine microeukaryote databases (available at https://github.com/ramalok) based on a collection of Sanger sequences (Pernice et al. 2013) or 454 reads from the

Table 1. Details of stations sampled for denaturing gradient gel electrophoresis (DGGE) and high-throughput sequencing (HTS) during the cruises conducted in June 2008, April 2009 and November 2009 in the Gulf of Gabès

Stn	Sampling time	Depth (m)	Sampling point label	Latitude (°N)	Longitude (°E)	Type of station	Molecular technique DGGE	HTS
1	Jun 2008	1	1-J-1	33.91	10.46	Coastal	+	
		9	1-J-9				+	
2	Jun 2008	1	2-J-1	34.08	10.85	Coastal	+	+
		8	2-J-8				+	
		16	2-J-16				+	
	Apr 2009	1	2-A-1				+	+
		8	2-A-8				+	
		16	2-A-16				+	
3	Nov 2009	1	3-N-1	34.09	11.38	Coastal	+	+
		27	3-N-27				+	
		54	3-N-54				+	
4	Apr 2009	1	4-A-1	34.03	12.66	Open-sea	+	
		32	4-A-32				+	
5	Jun 2008	1	5-J-1	34.26	11.99	Open-sea	+	+
		25	5-J-25				+	
		78	5-J-78				+	
6	Nov 2009	1	6-N-1	34.40	11.76	Open-sea	+	
		25	6-N-25				+	
7	Jun 2008	1	7-J-1	34.53	12.58	Open-sea	+	
		78	7-J-78				+	
8	Apr 2009	1	8-A-1	34.75	12.55	Open-sea	+	+
		32	8-A-32				+	
		64	8-A-64				+	
	Nov 2009	1	8-N-1				+	+
		32	8-N-32				+	
		64	8-N-64				+	
9	Jun 2008	1	9-J-1	34.73	12.85	Open-sea	+	
		20	9-J-20				+	
	Apr 2009	1	9-A-1				+	
		20	9-A-20				+	
	Nov 2009	1	9-N-1				+	
		20	9-N-20				+	
10	Apr 2009	1	10-A-1	34.74	13.20	Open-sea	+	+
		25	10-A-25				+	
		50	10-A-50				+	
		100	10-A-100				+	
		262	10-A-262				+	+
	Nov 2009	1	10-N-1			Open-sea	+	+
		25	10-N-25				+	
		100	10-N-100				+	
		262	10-N-262				+	+
11	Jun 2008	1	11-J-1	35.07	12.91	Open-sea	+	+
		25	11-J-25				+	
		102	11-J-102				+	+
12	Apr 2009	1	12-A-1	35.07	13.26	Open-sea	+	
		25	12-A-25				+	
		50	12-A-50				+	
		75	12-A-75				+	
		98	12-A-98				+	
	Nov 2009	1	12-N-1				+	
		25	12-N-25				+	
		50	12-N-50				+	
		75	12-N-75				+	
		98	12-N-98				+	

BioMarKs project (Massana et al. 2015). Sequences have been deposited at the European nucleotide archive (ENA) under accession number PRJEB84566.

Based on these results, an OTU table including the 12 selected samples was constructed and then filtered to remove undesired taxa (*Bacteria*, Metazoa, Streptophyta) and nucleomorphs. Singletons and OTUs whose representative sequence was shorter than 340 bp were removed as well. Sample 11-J-1 was discarded due to the low number of reads retrieved. The final OTU table had 11 samples and 12 926 OTUs. The number of reads per sample ranged from 18 945 to 70 485 (34 583 on average). This OTU table was then randomly subsampled to the smallest sample size (18 945) and square root transformed in order to reduce the impact of the very abundant OTUs and increase the impact of the less abundant OTUs in the community. For alpha diversity, the Shannon diversity index (*H*; Shannon & Weaver 1949) and the Simpson-Gini (1–*D*) index (Hurlbert 1971) were calculated based on the rarefacted square rooted OTU table. OTU sequences (https://doi.org/10.6084/m9.figshare.5414578), taxonomy (https://doi.org/10.6084/m9.figshare.5414599) and abundance (https://doi.org/10.6084/m9.figshare.5414527) have been deposited in FigShare.

Statistical analyses

The PRIMER software package (version 6.1.13) was used to calculate Bray-Curtis similarity matrices and to generate dendrograms (Kruskal & Wish 1978). Matrices of pairwise Bray-Curtis similarity values were calculated from the normalized-transformed DGGE intensity matrix and from the rarefacted square rooted OTU table. For dendrograms, we used the function 'Simprof.'

The co-inertia analysis was performed using the multivariate statistical R package version 3.0.2 (Casgrain & Legendre 2001) as detailed by Hamdi et al. (2015). It was carried out to examine the correlation between an array of response variables (samples subjected to HTS analyses) and of independent explanatory variables (phylogenetic group abundance) conditional to a third matrix (physico-chemical parameters). Only groups with relative abundance above 1% (excluding unidentified Eukaryota) were included in this analysis, namely the marine alveolates MALV-I and MALV-II, Dinoflagellata, Bicosoecida, Chrysophyceae, Dictyochophyceae, Mamiellophyceae, Polycystinea, Acantharia and the marine stramenopiles MAST-3 and MAST-4.

RESULTS

Hydrographic characterization

The diagrams of potential temperature (θ) and salinity obtained from samples taken during the 3 cruises revealed the presence of 4 water masses in the Gulf of Gabès (Fig. 2). The MAW was characterized by the lowest salinity in each cruise (between 36.98 and 37.72) and by temperature values ranging from 15.78 to 22.15°C in June, 14.25 to 16.0°C in April and 16.73 to 20.56°C in November. It was detected in the upper water column of the open sea, approximately 110 km off the coast. The widest vertical extent of this water mass was observed in November (Fig. 2F), when it reached a depth of 140 m, whereas in June (Fig. 2D) and April (Fig. 2E) it did not exceed depths of 80 and 60 m, respectively. The highest density ($\sigma > 29$) at depths above 200 m at Stn 10 (Fig. 2H,I), which resulted from low temperatures (<14.8°C) and high salinity values (>38.78 in April and >38.81 in November), corresponded to the Levantine Intermediate Water (LIW) (Theocharis et al. 1999). The LIW was observed and described by Ben Ismail et al. (2012) through the Channel of Sicily, but it was observed for the first time in the Gulf of Gabès during this survey. This dense water mass was observed in November and April but not in June, when the deepest sample was 184 m. The IW, which is a transitional layer between the MAW and LIW, was characterized by relatively warmer temperature (14.95–16.68°C) and lower salinity (37.84–38.79) than the LIW (Fig. 2G,H,I). The IW was located at depths between 100 and 200 m. The coastal waters characterized by the highest temperatures during each cruise and extending to almost 50 m depth corresponded to the MMW (salinities between 37.93 and 38.64 in June, 36.55 and 39.12 in April, 37.75 and 38.89 in November; temperature between 22.0 and 24.81°C in June, 13.94 and 14.95°C in April, 19.8 and 21.81°C in November; Fig. 2).

Table 2 shows the mean, minimal and maximal values of physical variables (temperature, salinity and density) and nutrient concentrations (silicate, nitrate, nitrite, ammonium, orthophosphate, total nitrogen and total phosphorus) during each cruise. Mean water temperature was the same in June (19.12 ± 2.71°C, ±SD) and November (19.14 ± 1.91°C) and was significantly colder in April (14.43 ± 0.17°C). During June, temperature increased towards the coast and from the bottom to the surface layer (Fig. 2A), whereas during November it was almost homogenous in the upper 50 m layer (Fig. 2C). Dur-

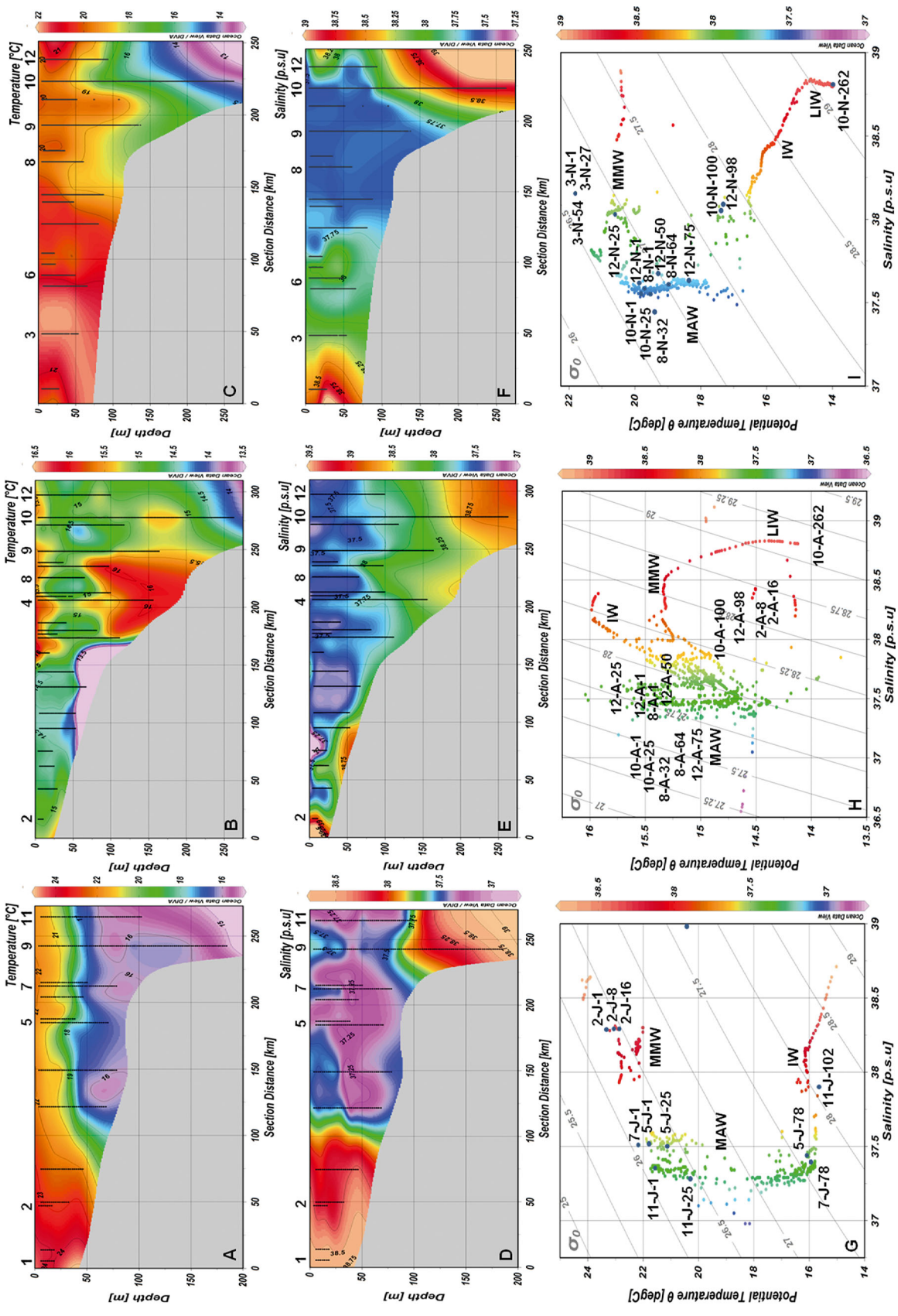

Fig. 2. Vertical profiles of temperature and salinity, and potential temperature–salinity diagrams for cruises conducted in (A,D,G) June 2008, (B,E,H) April 2009 and (C,F,I) November 2009. Vertical profiles were obtained in a cross section through the Gulf of Gabès. Stations sampled for physico-chemical data are indicated by CTD measures (dots). Stations sampled for DGGE and high-throughput sequencing (HTS) are identified. Diagrams show the different water masses identified in the Gulf of Gabès (MMW: Mixed Mediterranean Water; MAW: Modified Atlantic Water; IW: Ionian Water; LIW: Levantin Intermediate Water). Diagonal lines indicate density levels. Samples analyzed by DGGE and HTS are indicated. Vertical profiles and diagrams were plotted with the Ocean Data View software (Schlitzer 2014)

Table 2. Means ± SD, together with minimal and maximal values, of physical parameters and nutrient concentrations during the cruises conducted in June 2008, April 2009 and November 2009 in the Gulf of Gabès. Depths (m) corresponding to minimal and maximal values are also shown

	June 2008				April 2009				November 2009			
	Mean	Min	$Depth_{min}$	Max	$Depth_{max}$	Mean	Min	$Depth_{min}$	Max	$Depth_{max}$	Mean	Min	$Depth_{min}$	Max	$Depth_{max}$
Physical parameters															
Temperature (°C)	19.12 ± 2.71	15.05	184	24.21	6	14.43 ± 0.17	13.74	66	16.04	4	19.14 ± 1.91	14.00	261	21.81	25
Salinity (psu)	37.62 ± 0.41	36.98	36	38.71	184	37.71 ± 0.37	36.55	4	39.12	10	37.87 ± 0.38	37.45	32	38.89	27
Density (kg m^{-3})	27.18 ± 0.85	26.10	4	29.64	184	28.07 ± 0.28	27.48	12	29.18	10	27.44 ± 0.94	26.58	7	30.29	262
Nutrient concentrations (µM)															
Silicate	2.94 ± 0.76	1.55	32	5.29	184	1.17 ± 0.70	0.32	2	3.90	6	1.72 ± 0.79	0.35	50	3.72	20
Nitrite	0.09 ± 0.07	0.03	26	0.31	76	0.11 ± 0.07	0.02	10	0.54	4	0.08 ± 0.04	0.02	100	0.21	80
Nitrate	1.72 ± 0.57	0.23	38	3.75	4	0.67 ± 0.19	0.31	2	1.33	18	0.56 ± 0.19	0.20	5	0.91	5
Ammonium	0.62 ± 0.29	0.16	38	1.88	24	0.41 ± 0.21	0.08	6	0.94	4	0.34 ± 0.15	0.08	100	0.67	5
Orthophosphate	0.06 ± 0.03	0.01	32	0.12	50	0.29 ± 0.06	0.16	12	0.47	16	0.12 ± 0.03	0.09	20	0.20	20
Total nitrogen	10.34 ± 2.46	6.36	50	17.27	78	11.20 ± 1.44	7.96	16	14.26	8	10.04 ± 1.12	8.01	80	12.12	20
Total phosphorus	0.89 ± 0.18	0.54	24	1.44	8	0.81 ± 0.11	0.52	4	1.06	10	1.08 ± 0.28	0.69	5	2.13	5

ing April, highest temperatures were recorded in the open-sea area at about 150 km from the coast in 2 separate layers: 0–25 m and 60–160 m depths (Fig. 2B). The lowest temperature was always observed in the deepest sample except in April, when it was recorded at 66 m (Table 2). A marked thermal stratification was observed in June with a thermocline established at about 35 m (Fig. 2A), whereas in November, a thermocline was observed in offshore stations between 30 and 40 m (Fig. 2C). Mean salinity ranged from 37.62 ± 0.41 in June to 37.87 ± 0.38 in November, with the widest variation (from 36.55–39.10) detected in April.

Phosphate concentrations were generally low, with a maximum of 0.47 µM. On average, the lowest values were reported during the June cruise (0.06 ± 0.03 µM), intermediate values in November (0.12 ± 0.03 µM) and highest values in April (0.29 ± 0.06 µM). The mean concentrations of silicate, nitrate and ammonium were similar in April and November cruises, whereas they were higher in June. Nitrite concentrations were generally low during the survey period, with mean values ranging between 0.08 ± 0.04 µM in November and 0.11 ± 0.07 µM in June. The highest mean concentration of total nitrogen (11.20 ± 1.44) and total phosphorus (1.08 ± 0.28) were recorded in April and November, respectively (Table 2).

Spatial and temporal variability of picoeukaryotic assemblages revealed by DGGE

To investigate changes of picoeukaryotic assemblages in coastal and open-sea areas and across different water masses, separate DGGE gels were run for June 2008, April 2009 and November 2009 samples (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a081p037_supp.pdf). For each DGGE gel, a dendrogram comparing the picoeukaryotic assemblages was constructed. DGGE patterns shown in Fig. S1 revealed that picoeukaryotic communities of the Gulf of Gabès displayed an important diversity, with the presence of a many bands and a wide variation in band type and relative intensity among samples. Hierarchical cluster analysis revealed large variability in picoeukaryotic assemblages among stations and depths (Fig. 3). The factor that better explained community composition was the gradient from the coast to the open sea during the 3 studied periods. In the coastal zone, samples from different depths always clustered together. This was observed in June and November (Fig. 3A,C), while the 'coastal cluster' was less distinct in April (Fig. 3B). On the other hand, picoeukaryotic assemblages in the open sea changed clearly along the vertical profile. Thus, all surface samples grouped together (with a few excep-

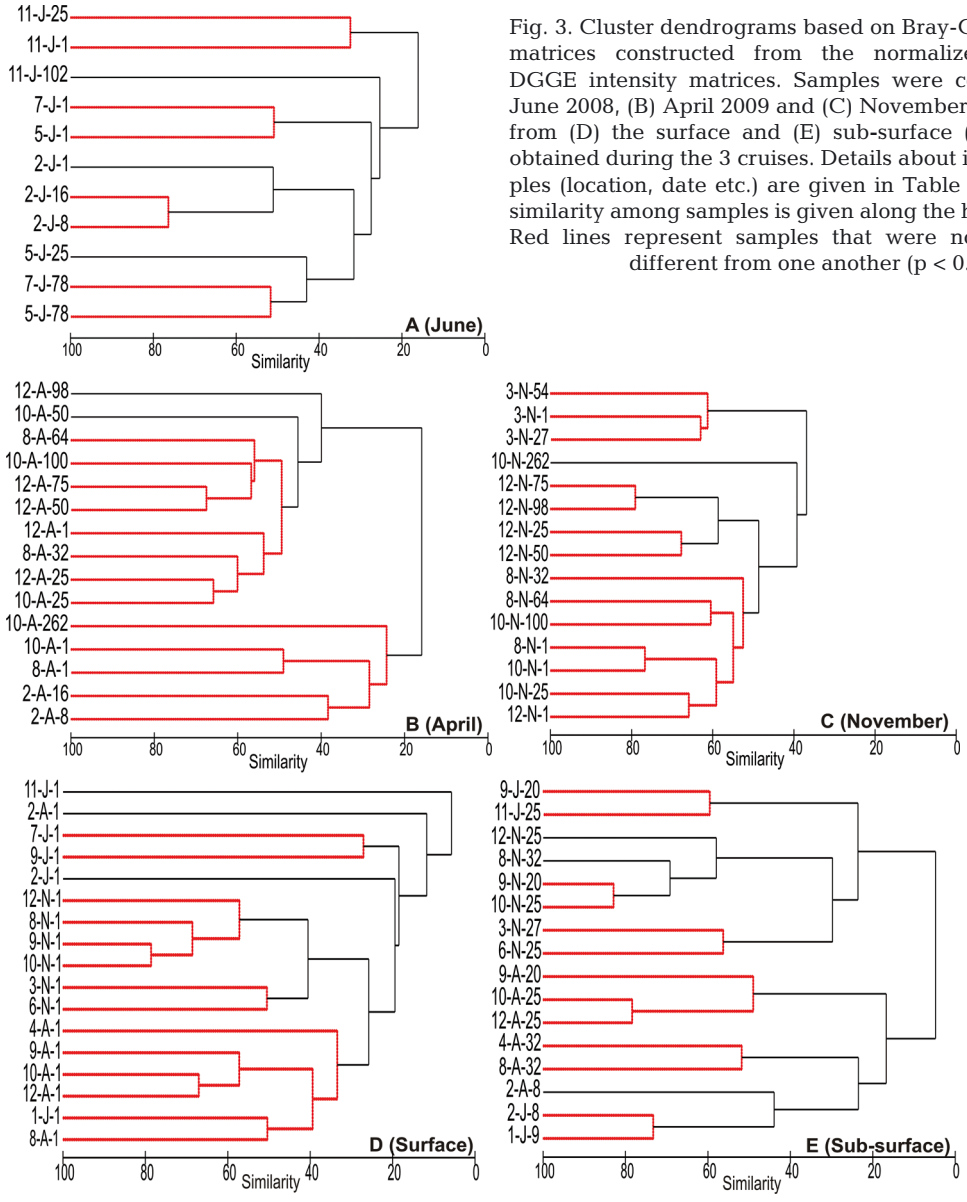

Fig. 3. Cluster dendrograms based on Bray-Curtis similarity matrices constructed from the normalized-transformed DGGE intensity matrices. Samples were collected in (A) June 2008, (B) April 2009 and (C) November 2009. Samples from (D) the surface and (E) sub-surface (8–32 m) were obtained during the 3 cruises. Details about individual samples (location, date etc.) are given in Table 1. The percent similarity among samples is given along the horizontal axes. Red lines represent samples that were not significantly different from one another ($p < 0.05$)

tions: Stn 11 in June and Stn 12 in both April and November), samples from 25–100 m depths formed a distinct cluster in both April and November dendrograms, and the deepest sample from the 3 cruises was always separate.

The variability of picoeukaryotic assemblages in relation to water mass origin was apparent from the clustering pattern (Figs. 2 & 3). Thus, coastal samples corresponding to the MMW clustered together during June (Stn 2) and November (Stn 3), and deepest samples related to the IW (June) or the LIW (April and November) were always separated. In addition, during the November cruise, samples from Stns 8, 10 and 12 formed a single cluster and were located in the MAW, except some of deepest samples (10-N-100 and 12-N-98) that seemed to be in a transitional layer between the MAW and the IW.

In order to better compare samples from different cruises, we ran 2 additional gels with all surface (Fig. S1D) and subsurface (Fig. S1E) samples. This analysis revealed clear differences in the picoeukaryotic community in the 3 studied periods, likely explained by temporal changes as cruises occurred in different seasons. In fact, surface samples generally clustered by study period, forming temporal clusters comprising June, April and November samples (except coastal samples from June and April; Fig. 3D). The cluster analysis of sub-surface DGGE banding patterns was less evident, with April and November samples always

being together, whereas June samples clustered with April (coastal stations) or formed a separate cluster (open-sea samples; Fig. 3E). This clustering revealed the important variability of picoeukaryotic assemblages in the Gulf of Gabès in the 3 sampled periods.

Richness, taxonomic composition and spatial variability of picoeukaryotic assemblages revealed by HTS

The composition and genetic diversity of the picoeukaryotic community in the Gulf of Gabès was investigated by HTS of the hypervariable V4 region of the 18S rRNA gene. The final sequence dataset, rarefied to keep consistency among the different samples, included 208 395 good quality reads clustered into 12 926 OTUs at 99% sequence similarity (Table S1 in the Supplement).

Our data showed that the picoeukaryotes from the Gulf of Gabès were very diverse, including 76 taxonomic groups that covered the 8 major eukaryotic divisions (Table S2). The Alveolata, which consisted mostly of parasitic marine alveolates (MALVs) and phagotrophic ciliates and dinoflagellates, dominated the sequencing dataset, contributing 50.29% of total sequences. Alveolata was mostly represented by MALV-II and MALV-I, detected at all depths and in all 3 sampled months (Table S1, Fig. 4). Other groups like Apicomplexa, Colpodellida, Ellobiopsidae, MALV-III, MALV-IV, MALV-V and Perkinsidae were minor and unevenly distributed components. Ellobiopsidae was mostly detected at 102 m, whereas MALV-V was mostly found during the November cruise. Apicomplexa was detected exclusively at 102 m during June, and Colpodellida was restricted to surface water during April.

Stramenopiles were the second most represented supergroup (26.37%) and the most diverse. In fact,

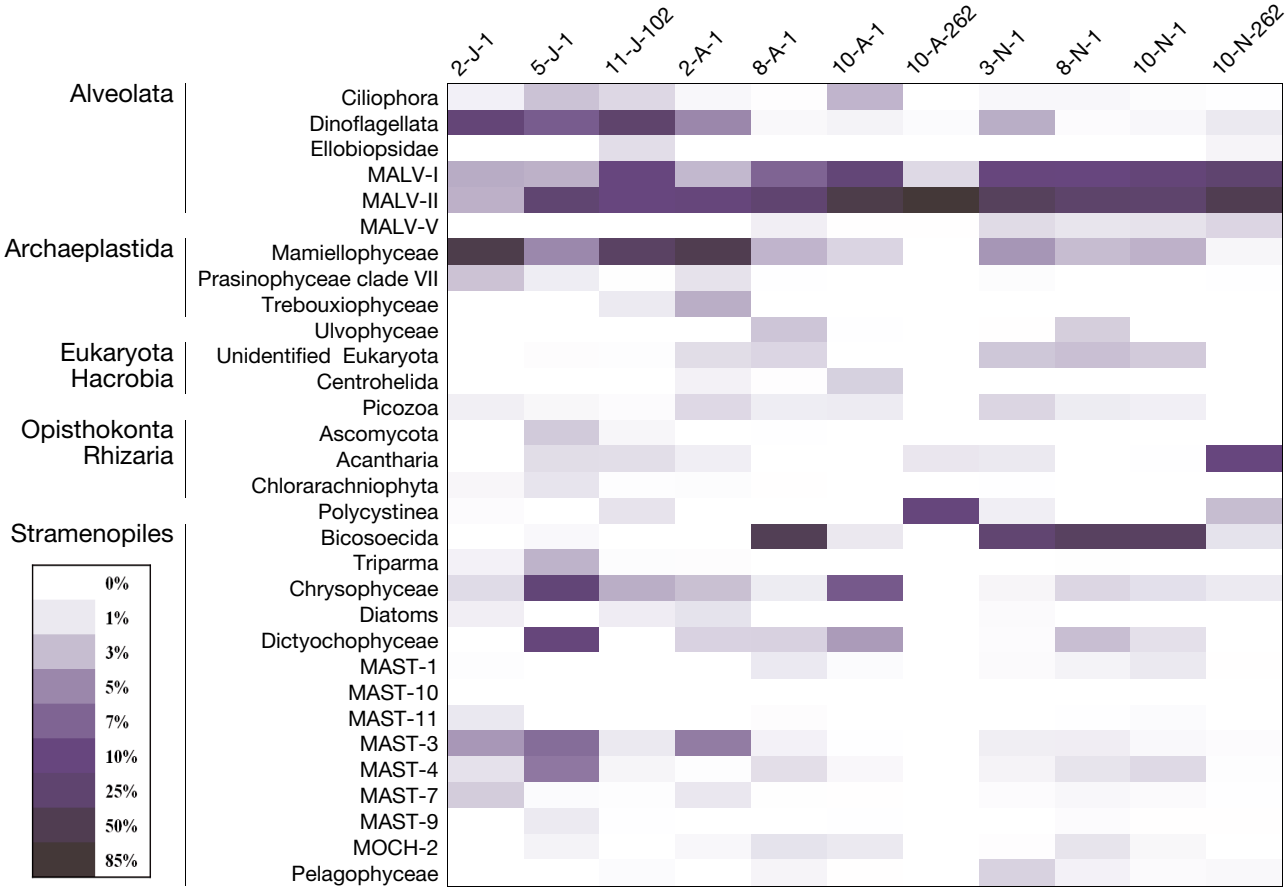

Fig. 4. Heatmap showing the relative abundance of phylogenetic groups in the Gulf of Gabès during June 2008, April 2009 and November 2009 in samples analyzed by high-throughput sequencing. Only groups displaying a relative abundance above 1% in at least 1 sample are represented. Details about individual samples (location, date etc.) are given in Table 1. MALV: marine alveolates, MAST: marine stramenopiles, MOCH: marine Ochrophyta

stramenopile OTUs grouped into 31 taxonomic groups, with Bicosoecida the most represented and the most diverse (2428 OTUs), followed by Chrysophyceae and marine stramenopile (MAST) clades. Among the 18 described subclades of MAST (Massana et al. 2014), 12 were retrieved in the Gulf of Gabès including the newly described MAST-25 that had been detected mainly in offshore surface waters. MAST-3 (1.91%) was the most abundant and diverse group, exhibiting the highest numbers of sequences (3995) and OTUs (220). In addition, 5 new marine Ochrophyta groups (MOCH-1 to MOCH-5) were detected, among which MOCH-2 was the most abundant (1067 reads) and diverse (58 OTUs) relative to the other MOCH groups.

Archaeplastida was dominated by Mamiellophyceae (15.20%), particularly well-represented in the coastal area (mainly *Triparma* and *Micromonas* spp.) and in the deep offshore sample from the June cruise (mainly *Ostreococcus* spp.). Prasinophyceae clade VII and Trebouxiophyceae were detected at higher abundances at the coastal Stn 2 during June and April, respectively, whereas Ulvophyceae were almost exclusively present at Stn 8 in the open-sea region during both April and November. Within Rhizaria (3.45%), Acantharia and Polycystinea groups were the first 2 well represented groups in our survey. They had an important diversity relative to all other Rhizaria groups since they exhibited 170 and 238 OTUs, respectively. Hacrobia, Opisthokonta, Amoebozoa and Eukaryota made up a combined 3.21% of rDNA sequences. Among Hacrobia, Picozoa was the most abundant (1683 sequences) and diverse (90 OTUs) group and was retrieved from almost all samples, without a clear temporal or spatial trend. Fig. 4 illustrates the great seasonal and spatial variability in picoeukaryotic diversity. The dominant groups in the Gulf of Gabès exhibited clear spatial and seasonal trends. During April and November, MALV-II and Bicosoecida were dominant, whereas Mamiellophyceae and dinoflagellates dominated in June. There was a spatial trend of increasing relative abundance of MALV-II during April and November and of MALV-I in November and June. Indeed, an increase in abundance toward the open-sea area was detected for MALV-I and MALV-II during April and for MALV-I and Bicosoecida in November. Among the Stramenopiles, retrieved mainly at surface, Bicosoecida were prevailing in April and November, while Chrysophyceae, Dictyophyceae, MAST-3 and MAST-4 were prevailing in June, particularly at the surface of Stn 5. Within Rhizaria, Acantharia and Polycystinea were detected mainly in the deepest sample, with a dominance of the former in November (9.5% of sequences) and of the latter in April (10.7% of sequences).

Rare taxa, i.e. those found in relative abundances below 1%, were identified in all samples (Fig. 5). On the basis of total abundance, the contribution of the rare biosphere was more or less constant among samples, with values between 5.1 and 8.5%, except a very low number in 10-A-262 (0.6%). On the basis of taxonomic composition, a high variability was found between samples, although there was a tendency of being more similar within the same station. The rare biosphere was composed mainly of Stramenopiles, accounting for almost half of the relative abundance, followed by Alveolata, Rhizaria and Hacrobia.

Shannon-Weaver and Simpson indices of diversity were calculated for all samples (Table 3). Picoeukaryotic diversity was generally higher in November samples compared to those from the other cruises. During April and November, diversity indices were higher in offshore stations relative to coastal stations, with the lowest value detected at 262 m depth at Stn 10 in April. In June, the diversity of picoeukaryotic communities was highest at 102 m depth.

Finally, we used the HTS data to analyze the spatial and temporal variability of picoeukaryotes in the Gulf of Gabès by hierarchical clustering based on Bray-Curtis similarity (Fig. 6). A depth-related trend was clearly apparent among our samples, whereas the temporal trend was less clear. Indeed, pico-

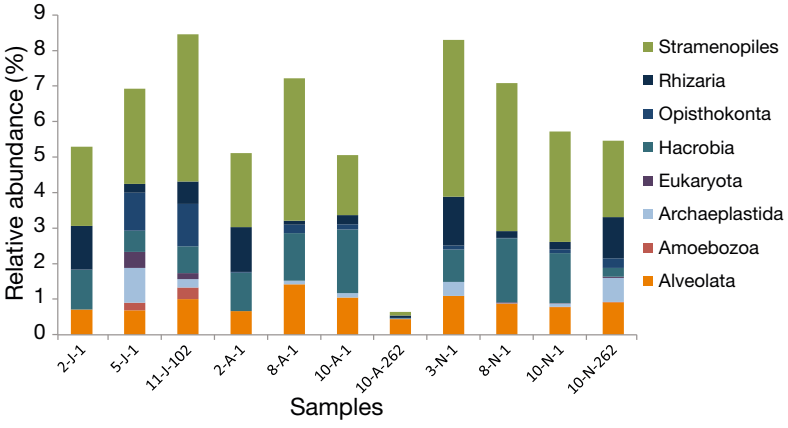

Fig. 5. Relative abundance of phylogenetic supergroups constituting the 'rare biosphere' (taxa with relative abundances below 1%) in the Gulf of Gabès in samples analyzed by high-throughput sequencing. Details about individual samples (location, date etc.) are given in Table 1

Table 3. Shannon diversity index (*H*) and Simpson-Gini index (1–*D*) for each sample derived from high throughput sequencing data. Details about individual samples (location, date etc.) are given in Table 1

Index	2-J-1	5-J-1	11-J-102	2-A-1	8-A-1	10-A-1	10-A-262	3-N-1	8-N-1	10-N-1	10-N-262
H	5.50	5.23	5.78	5.63	6.00	5.81	4.26	6.28	6.30	6.46	6.03
1–*D*	0.987	0.986	0.989	0.989	0.984	0.986	0.911	0.911	0.990	0.990	0.985

eukaryotic assemblages from surface and mesopelagic layers grouped into 3 community clusters regardless of the sampling period (Fig. 6A). Cluster I was composed of the 2 deepest samples (262 m depth at Stn 10) that were distinguished by the presence of Acantharia and Polycystinea. Cluster II included 2 coastal surface samples and a deep sample (102 m depth at Stn 11) characterized by high abundance of Mamiellophyceae (>50% of the total number of DNA sequences). Cluster III grouped surface offshore samples, plus the coastal sample 3-N-1, comprising less than 6% of Mamiellophyceae. It is worth noting that the deep sample from Cluster II (11-J-102) combined phylogenetic groups from the surface Cluster III, such as Mamiellophyceae and Dinoflagellata, and from the deep Cluster I, such as MALV-I, MALV-II, Acantharia and Polycystinea (Fig. 6B).

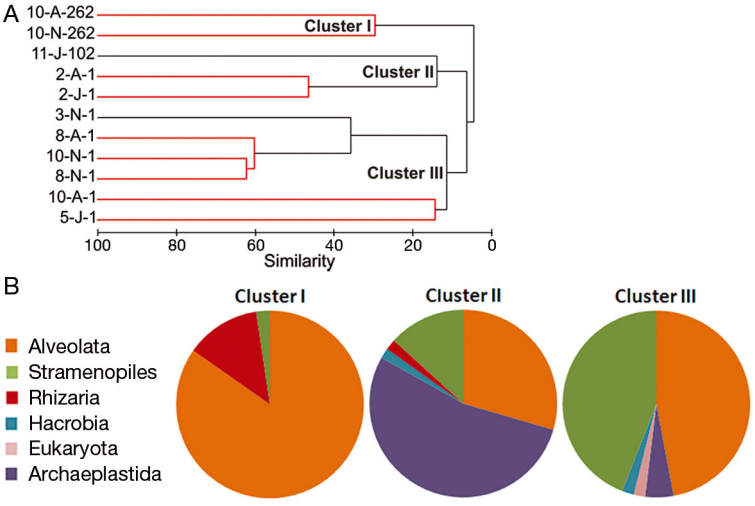

Fig. 6. (A) Cluster dendrogram based on Bray-Curtis similarity matrix from the operational taxonomic unit (OTU) table constructed from high-throughput sequencing (HTS) data. Red lines represent samples that were not significantly different from one another ($p < 0.05$). Details about individual samples (location, date etc.) are given in Table 1. (B) Taxonomic composition (supergroups with relative abundances above 1%) of the 3 clustered communities

Relationship between picoeukaryotic diversity groups and environmental variables

The variability of picoeukaryotic community composition (by HTS data) in relation to environmental factors, including physical parameters and nutrient availability, was examined by co-inertia analysis (Fig. 7). This analysis yielded sample clustering similar to that shown in Fig. 6A and indicated close links between picoeukaryotic composition and abiotic parameters (RV coefficient = 0.60). The relative abundance of Acantharia and Polycystinea was related to high density and salinity, whereas that of MAST-3, Mamiellophyceae and Dinoflagellata was related to inorganic nitrogen. Fig. 7 shows close links between Chrysophyceae, MAST-4 and Dictyophyceae and temperature and total nitrogen on the one hand, and between both MALV-II and Acantharia and orthophosphate and total phosphorus on the other hand. Moreover, the relative abundance of Bicosoecida appeared to be inversely associated with silicate. A co-inertia plot revealed no relationship between MALV-I and the studied environmental variables.

DISCUSSION

Spatial and temporal variability of the picoeukaryotic community

Although the Gulf of Gabès is a relatively narrow area, picoeukaryotic assemblages displayed high variability at horizontal and vertical scales with very marked temporal changes. Indeed, the composition of picoeukaryotic assemblages can be remarkably constant over broad oceanographic regions but can also change abruptly by crossing oceanographic fronts or changing water masses (Díez et al. 2004). Clustering analysis of surface and sub-surface samples investigated by DGGE revealed a clear temporal

variation in picoeukaryotic composition, notably at the surface layer and in the open-sea area. In fact, coastal samples from June and April did not show any temporal clustering pattern (Fig. 3D,E). Similarly, a season-related trend in shallow water assemblages (surface and deep chlorophyll maximum, DCM) was described at the SPOT station (Countway et al. 2010, Lie et al. 2013). The lack of an obvious temporal trend in HTS data was likely due to the low number of samples used.

In our samples, differences in picoeukaryotic assemblage composition and abundance were observed between the coastal and the open-sea areas as well as between surface and mesopelagic layers, with more pronounced changes at the horizontal scale. A similar trend was found in the South China Sea (Wu et al. 2014). This could be explained by the fact that in both studies the water column sampled was relatively shallow (up to 262 m and 60 m, respectively). When deeper sampling into the mesopelagic and bathypelagic layers is performed, such as in the Sargasso Sea (Not et al. 2007) or the Indian Ocean (Not et al. 2008), the vertical gradient was the most important in determining picoeukaryotic diversity. The clustering of coastal samples from different depths suggests homogenous picoeukaryote assemblages throughout the upper water column. In the open-sea region, the vertical distribution of picoeukaryotes exhibited a clear variability between surface, the 25–100 m layer and deeper waters, with a large divergence between surface and deep waters. Some taxonomic groups drove these changes, such as Acantharia, Polycystinea, Mamiellophyceae and Bicosoecida. This vertical trend has already been described in the Mediterranean Sea (Díez et al. 2001b, Marie et al. 2006) with the identification of 3 vertical compartments: surface, DCM and mesopelagic waters. A similar view was obtained at the SPOT station (Countway et al. 2010, Schnetzer et al. 2011, Kim et al. 2014, Hu et al. 2016), where the shallowest samples (5 m and DCM) were clearly different from the deepest samples (150 to 500 m).

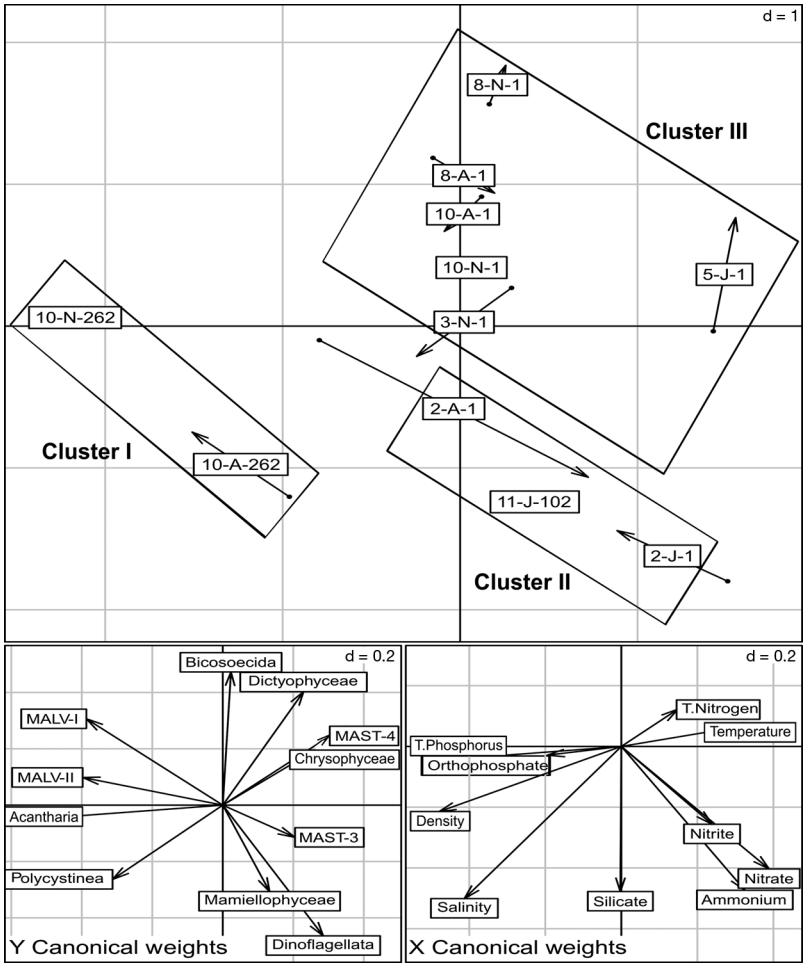

Fig. 7. Co-inertia plot showing the distribution of phylogenetic groups with relative abundances above 1% (excluding unidentified Eukaryota) in the Gulf of Gabès in relation to the environmental variables during the 3 investigated periods (in samples analyzed by high-throughput sequencing). The 3 grouped community clusters from Fig. 6 are shown. Details about individual samples (location, date etc.) are given in Table 1. T.Nitrogen: total nitrogen, T.Phosphorus: total phosphorus, MALV: marine alveolates, MAST: marine stramenopiles

Summarizing our data, picoeukaryotic diversity changes are most important at the horizontal scale, with a clear distinction between coastal and open-sea communities (Fig. 3), whereas at the vertical scale shifts were detected in the open-sea area, particularly between surface and mesopelagic communities (Figs. 3 & 6). The temporal trend seems to be more relevant in the open-sea area (Fig. 3D,E). These diversity patterns could be influenced by the wide continental shelf area of the Gulf of Gabès, one of the widest in the Mediterranean, since the physico-chemical settings showed significant variability between coastal and open-sea areas (Bel Hassen et al. 2009b) likely due to the transition over the continental shelf area.

Relationships between the picoeukaryotic community and environmental factors

The co-inertia analysis showed that the distribution of some picoeukaryotic groups was influenced by nitrogen. Dinoflagellates proliferated during June at the coastal Stn 2 and at 102 m at Stn 11, both characterized by the highest values of inorganic nitrogen. Similarly, a dominance of large dinoflagellates has been recorded in this area during July (Drira et al. 2008). These results suggest that nutrient content might shape the dinoflagellate distribution in the Gulf of Gabès with the co-occurrence of both micro- and pico-sized species. In the South China Sea, relative abundances of dinoflagellates and MALV-II were related to high temperature and irradiance (Wu et al. 2014), while in the Gulf of Gabès the distribution of MALV-II was associated with higher levels of phosphorus. The highest abundances of Chrysophyceae (16.8%), Dictyophyceae (12.0%) and MAST-4 (6.1%) noted at the surface of Stn 5 during June co-occurred with high concentrations of nitrites and total nitrogen. Little has been reported on the relationships between particular marine picoeukaryotic taxa and nutrient content. In Tibetan lakes, Wu et al. (2009) showed that chemical compounds, notably the percentage of chlorine and carbonate, could structure the diversity pattern of eukaryotic plankton assemblages.

The structuring of picoeukaryotic assemblages according to water mass properties has been observed over large areas, such as in the North Water (Hamilton et al. 2008) and in the Southern Ocean (Díez et al. 2004), and this pattern was also detected to some extent in the Gulf of Gabès. Indeed, the clustering based on DGGE fingerprints was consistent with the described water masses in the November cruise (Fig. 2I), when distinct picoeukaryotic assemblages were found in the MMW (Stn 3), the MAW (Stns 8, 10 and 12) and the LIW (Stn 10; 262 m). This was also supported by the co-inertia analysis, where water density affected the picoeukaryotic diversity (Cluster I) in the deeper layer corresponding to the LIW. The fact that the distribution pattern of picoeukaryotes depended on physically identified water masses suggests physiological preferences of given species to these physical properties and/or passive dispersion within the water masses.

Taxonomic composition of the picoeukaryotic community

In the Gulf of Gabès, Alveolata and Stramenopiles clearly dominated the sequencing dataset, with Alveolata accounting for more than 50% of sequences. The same trend has been observed in the northeastern Red Sea (Acosta et al. 2013), in the South China Sea (Wu et al. 2014) and at ocean surfaces (Massana et al. 2011). Moreover, a recent study revealed that alveolates comprised 42% of the piconanoplankton fraction (0.8–5 µm) in the surface layer of the world's oceans (de Vargas et al. 2015). In particular, MALV-II (33.2%) was the most represented in our molecular survey, in agreement with observations reported in many other surveys, such as in the English Channel (Romari & Vaulot 2004), the Sargasso Sea (Not et al. 2007), the northeastern Red Sea (Acosta et al. 2013) and the South China Sea (Wu et al. 2014). In a recent report, the relative abundance of MALV-II was considerably reduced (from about 20 to 2%) when the study used environmental RNA instead of DNA (Massana et al. 2015). Thus, the large contribution of MALV sequences detected here does not directly imply a dominance of active or viable MALV cells in the picoeukaryotic community.

The heterotrophic Bicosoecida represented the major stramenopile group in the Gulf of Gabès (13.6% of sequences), contrasting with previous reports that listed bicosoecids as rarely represented taxa in marine surveys (Massana & Pedrós-Alió 2008, Massana et al. 2014) and instead abundant in freshwater environments (Arndt et al. 2000, Richards et al. 2005). The high diversity of Bicosoecida (2428 OTUs) and Chrysophyceae (283 OTUs) make the Gulf of Gabès a favorable area to shed new light on the diversity and function of these 2 groups in marine microbial food webs.

In most marine environments, including Mediterranean studies at Blanes Bay (Massana et al. 2004) and the Alboran Sea (Díez et al. 2001b), diverse MAST groups collectively dominate within the Stramenopiles, which obviously was not the case at the Gulf of Gabès, even though they were well represented. Although also detected in the deepest samples, the MAST groups were more important at the surface, as also seen at the SPOT station (Countway et al. 2010). The occurrence of MAST-1, -3, -4 and -7 at all stations and at all depths, exhibiting a large variation in hydrologic conditions, suggests that they are cosmopolitan in this area and are important components of the picoeukaryotic community in the Gulf of Gabès. As in other studies (e.g. Logares et al. 2012), MAST-3 was the most abundant and diverse of the groups. FISH (fluorescent *in situ* hybridization) counts revealed that MAST-4 can be one of the most abundant component of marine heterotrophic flagellates, and can be a key bacterivore in both coastal and open

ocean waters (Rodríguez-Martínez et al. 2009). MAST-4 was the second most abundant (in terms of relative abundance) MAST group in the Gulf of Gabès. Novel stramenopile lineages recently described from other areas were also detected here, including the basal heterotrophic MAST-25 group and the 5 new MOCH groups (MOCH-1 to -5). Similarly to results reported by Massana et al. (2014), we observed that MOCH-2 was the most abundant of the 5 groups.

The prevalence of the 2 heterotrophic rhizarian groups Polycystinea (Yuan et al. 2004) and Acantharia in the deeper samples (262 m depth) was consistent with previous observations from other regions such as the Caribbean Sea (Stoeck et al. 2003), the Gulf of California (Edgcomb et al. 2002), Antarctica (López-García et al. 2001) and the Pacific Ocean (Countway et al. 2010, Hu et al. 2016). This finding corroborated the conclusion that these groups appear to be favored in deep waters (Countway et al. 2010). The dominance of Polycystinea in April and Acantharia in November (Fig. 4) indicated a seasonal trend and therefore a putative relationship with environmental factors. Both silicate and total nitrogen were higher in April than in November. The higher amount of silicate could explain the proliferation of Polycystinea, known as an extensively silicified lineage containing silicate transporter genes (Marron et al. 2016). Nitrogen may also play a metabolic role within Polycystinea cells. Rhizarian groups are important yet understudied, and their biological or ecological features require further investigation (de Vargas et al. 2015, Caron 2016)

The dominance of Mamiellophyceae in the coastal region during both June and April was in agreement with previous observations in the English Channel (Romari & Vaulot 2004), Blanes Bay (Zhu et al. 2005) and in the South East Pacific Ocean (Shi et al. 2009, Collado-Fabbri et al. 2011). More recently, Lopes dos Santos et al. (2017) showed that Mamiellophyceae is the dominant Chlorophyta group in coastal areas of oceanic waters.

Our study shows that Prasinophyceae clade VII was present almost exclusively in the coastal area of the Gulf of Gabès; similarly, this group has been detected in coastal waters of the English Channel (Romari & Vaulot 2004) and the South China Sea (Wu et al. 2015). However, Prasinophyceae clade VII is also an important oceanic group (Viprey et al. 2008, Shi et al. 2009, Rii et al. 2016, Lopes dos Santos et al. 2017).

The low abundance of diatoms was in accordance with previous studies in the Gulf of Gabès that showed low diatom contribution to chlorophyll *a* in summer and early spring (Bel Hassen et al. 2009a). Finally, the lack of Prymnesiophyceae in our dataset is consistent with previous data showing a low contribution of Prymnesiophyceae in the Gulf of Gabès (Bel Hassen et al. 2009a), which contrasted with its general dominance in the Mediterranean Sea (Claustre et al. 1994, Vidussi et al. 2000).

The occurrence of rare taxa at similar relative abundances during different sampling periods indicates that they might not be influenced by changes in environmental variables. Rare taxa mostly belonged to the Stramenopiles, which was also the most diverse group in the dataset. The high diversity detected in the rare biosphere further revealed that rare picoeukaryotes could drive the differences in diversity between different samples and be specific to certain environments, and therefore represent the unique characteristics of each environment since the major protistan groups are globally distributed (Fenchel & Finlay 2004, Finlay & Fenchel 2004).

CONCLUSION

This investigation represents the first contribution regarding the distribution and diversity of picoeukaryotic assemblages in the Gulf of Gabès. Although this is a relatively small area, it displays a rather high and variable picoeukaryotic diversity with consistent spatial, vertical and temporal patterns. These patterns were driven by the coast–offshore gradient, the vertical variations in the offshore stations, the sampling period and the co-occurrence of different water masses. Moreover, the picoeukaryotic diversity in the Gulf of Gabès was affected by both physical and chemical factors. In terms of picoeukaryotic composition, the Atlantic, Mediterranean and Ionian water masses did not show specific distinctions, whereas the Levantine water mass was differentiated by the presence of Acantharia and Polycystinea groups.

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