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Composition and distribution patterns of eukaryotic microbial plankton in the ultra-oligotrophic Eastern Mediterranean Sea

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ABSTRACT: Marine microbial eukaryotes play crucial roles in water-column ecosystems; however, there are regional gaps in the investigation of natural microbial eukaryote communities, and uncertainties concerning their distribution persevere. This study combined 18S rRNA metabarcoding, biomass measurements and statistical analyses of multiple environmental variables to examine the distribution of planktonic microbial eukaryotes at different sites and water layers in the ultra-oligotrophic Eastern Mediterranean Sea (Western Levantine Basin). Our results showed that microbial eukaryotic communities were structured by depth. In surface waters, different sites shared high percentages of molecular operational taxonomic units (MOTUs), but this was not the case for deep-sea communities (≥1000 m). Plankton biomass was significantly different among sites, implying that communities of a similar composition may not support the same activity or population size. The deep-sea communities showed high percentages of unassigned MOTUs, highlighting the sparsity of the existing information on deep-sea plankton eukaryotes. Water temperature and dissolved organic matter significantly affected community distribution. Microeukaryotic distribution was additionally affected by the nitrogen to phosphorus ratio and viral abundance, while nano- and pico-communities were affected by zooplankton. The present study explores microbial plankton eukaryotes in their natural oligotrophic environment and highlights that, even within restricted oceanic areas, marine plankton may follow distribution patterns that are largely controlled by environmental variables.

KEY WORDS: Marine plankton · Eukaryotic microorganisms · Microbial communities · Eastern Mediterranean Sea · Plankton biomass · Metabarcoding

1. INTRODUCTION

Marine eukaryotic plankton has been in the research spotlight in previous decades due to its importance in biogeochemical cycles and the unlimited biotechnological potentials related to it. Even so, the study of unicellular eukaryotic communities (protists) has been particularly delayed in comparison to prokaryotes. A reason for this is that protists have consistently been studied based on cell morphology and their means of acquiring energy (Caron et al. 2012, Caron & Hu 2019). It was once common prac-

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tice to study protists by grouping them into amoeboid, ciliated and flagellated forms, or to separate cells according to skeletal structures or pigments (Caron et al. 2012, Leray & Knowlton 2016). However, like the prokaryotic division (Solden et al. 2016 and references therein), the largest part of marine protist diversity has not been cultured (Shi et al. 2009, Massana et al. 2014) and therefore characterization based solely on morphological features is not possible.

Following the 'metabarcoding revolution' (Leray & Knowlton 2016), the field of protistan diversity has rapidly progressed, and much information has been gathered regarding lakes (Filker et al. 2016), marine sediments (Zhang et al. 2018), coastal waters (Massana et al. 2015) and the ocean (Flaviani et al. 2018); even more remarkable are the studies that have explored eukaryotic plankton diversity on a global scale (de Vargas et al. 2015, Pernice et al. 2016, Villarino et al. 2018). Recently, important information has come to light by examining the pico- and nanoplankton fraction, a decade after its particularly high diversity was highlighted (Not et al. 2009). The diversity of plankton pico- and nano-eukaryotes has been studied in the Baltic Sea, and an important connection between salinity and community composition has been revealed (Y. O. O. Hu et al. 2016). The pico- and nano-eukaryotic community has been found to differentiate according to depth in the water column of the Mariana Trench (Xu et al. 2018), while the study of the global ocean has indicated vertical patterns in pico-community composition and important differences in the pico-eukaryotic diversity of the meso- (200–1000 m) and the bathypelagic (≥1000 m) zones (Giner et al. 2020). Even by focusing only on photosynthetic pico-eukaryotes, differences between lake communities of similar physicochemical and climate characteristics have been found, particularly, that different groups of eukaryotes dominate different lakes (Metz et al. 2019).

Despite the vast amount of knowledge acquired to date, the study of microbial eukaryotic plankton communities in their natural environment remains intriguing, and numerous questions remain to be addressed. In the interest of increasing the knowledge and the certainties concerning microbial eukaryotes, more studies at global and regional scales are necessary, with an extra focus on extreme environments such as the deep sea (≥1000 m) (Leray & Knowlton 2016, Xu et al. 2017). Information concerning the relationship of microbial eukaryotes to environmental factors and parameters such as geography, depth, physics and chemistry is also needed. Additionally, the combination of absolute biomass measurements of plankton populations with community diversity and richness results is expected to expand the insights of the modern field of microbial eukaryotes (Caron & Hu 2019) by connecting the previous to current understanding and by comparing and contrasting biomass and diversity patterns.

In this study, we explored the diversity and distribution of unicellular planktonic eukaryotes in the Western Levantine Basin of the Eastern Mediterranean Sea. Micro-eukaryotes (20–200 µm) and pico- and nano-eukaryotes (0.8–20 µm) were studied using DNA metabarcoding and microscopy in order to explore both community composition and biomass. Taking into account the ultra-oligotrophy (Krom et al. 2014) and the phosphorus-limited character (Krom et al. 2010) of the Eastern Mediterranean, we examined (1) the diversity and composition of unicellular eukaryote communities in this area, from surface water layers (0–75 m) to the deep sea (≥1000 m). An additional focus was given to (2) the spatial distribution of plankton eukaryotes; specifically, we hypothesized that the composition and biomass of eukaryote communities follow horizontal and vertical patterns in space. Finally, apart from depth and geographic distance, this study tested (3) whether community distribution is associated with environmental variables and, more specifically, if the ultra-oligotrophic nature of the studied area plays a role in the differentiation of the community composition and biomass.

2. MATERIALS AND METHODS

2.1. Study area and sampling

Sampling of marine plankton took place aboard the RV 'AEGAEO' in April 2016. Plankton samples were collected from 4 oceanic stations in the Western Levantine Basin of the Eastern Mediterranean Sea (Stns 03, 10, 13 and 18; Fig. 1). Seawater was collected in Niskin bottles during morning hours and was subsampled for the different analyses. For the biomass estimation of the different plankton populations, subsamples were collected from multiple depth layers (5, 20, 50, 75, 100, 120, 200, 500, 1000, 2000 m) and from the deepest point at each station (Table 1). Surface seawater for community composition analysis came from 5, 50 and 75 m, while deep-seawater samples came from 1000 m and from the deepest point at each station (Table 1).

Subsamples for plankton biomass estimation were fixed and stored immediately after sampling. For pico-

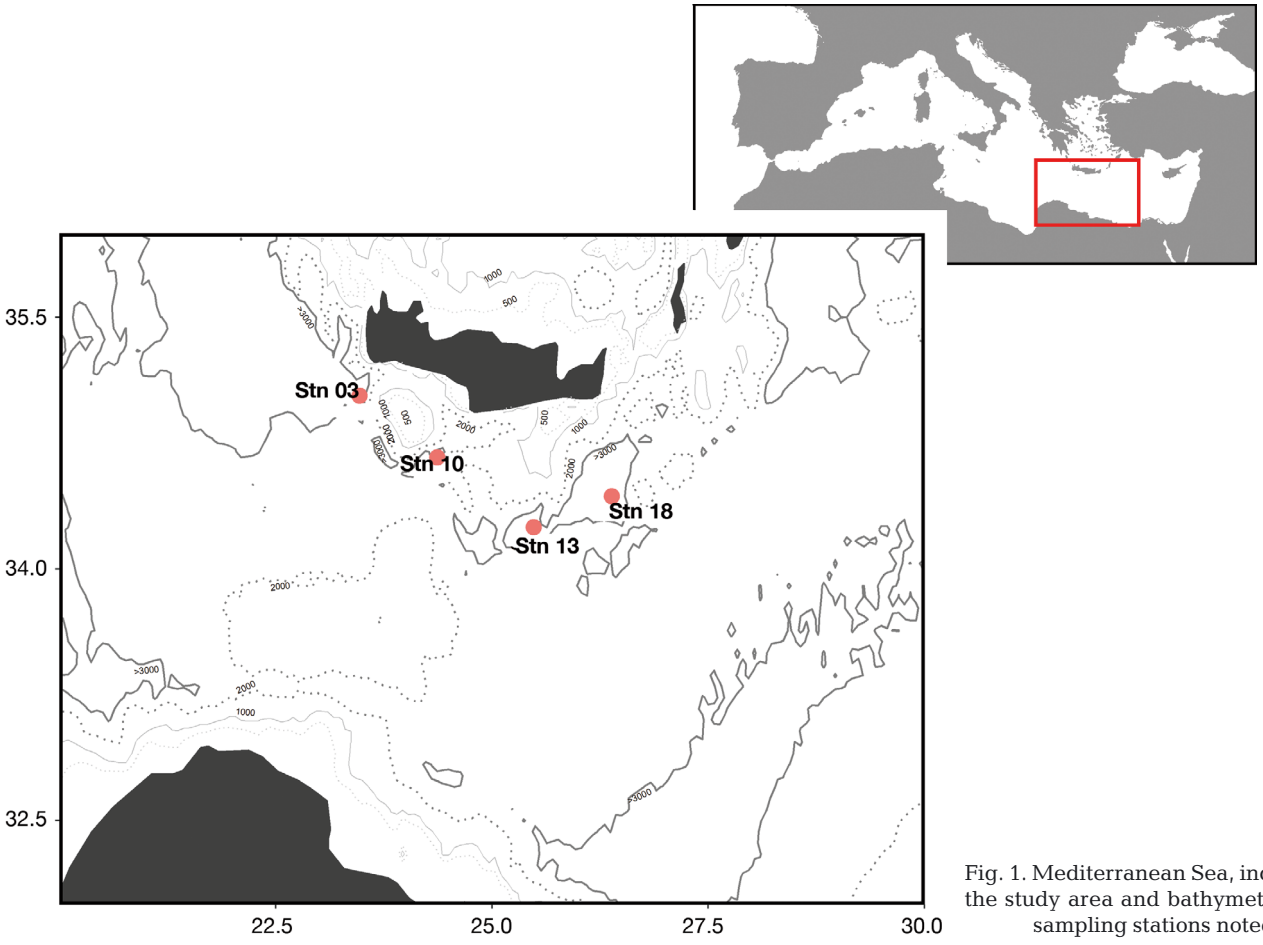

Fig. 1. Mediterranean Sea, indicating the study area and bathymetry, with sampling stations noted

and nanoplankton enumeration (piconanoplankton: 0.8–20 µm), subsamples of 50 ml (surface water layers) and 200 ml (deep-water layers) were fixed with 1.8% buffered formaldehyde (0.45 µm pre-filtered). After fixation for 2 h at 4°C, samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) and filtered through 0.8 µm black polycarbonate membranes according to Porter & Feig (1980). Filters were mounted on glass slides and stored at −20°C until enumeration. Seawater volumes of 500 and 2000 ml (for surface and deep-water layers, respectively) were collected for microplankton enumeration and were fixed with acidic Lugol's solution (2% final concentration); microplankton samples were stored at 4°C until analysis.

For the community composition analyses, sequential filtration through filter membranes of different pore sizes was performed in order to collect plankton communities of different sizes. Seawater volumes of 21 and 30 l, for the surface and the deep-water layers, respectively, were filtered at room temperature. A 200 µm mesh was used at the beginning of the filtration sequence to remove organisms larger than 200 µm. Subsequently, nylon mesh membranes of 20 µm pore size were used to collect microplankton cells (20–200 µm). The 20 µm filtrate then passed through 0.8 µm polycarbonate membranes that collected the 0.8–20 µm cells and comprised the pico- and nano-community, i.e. piconanocommunity, including the nanoplankton (2–20 µm)

Table 1. Sampling stations, coordinates and deepest depth layers where seawater was collected. For all stations, biomass samples were collected from 5, 20, 50, 75, 100, 120, 200, 500, 1000, 2000 m and the deepest water layer, while for metabarcoding, samples were collected from 5, 50, 75, 1000 m and the deepest water layer

Stn	Longitude (°N)	Latitude (°E)	Deepest sampled layer (m)
03	35.0333	23.4667	3500
10	34.6667	24.3667	3300
13	34.2500	25.4833	3900
18	34.4333	26.3833	3500

and part of the picoplankton (0.8–2 µm). Sequential filtration for each sample was completed within 75 min. After filtration, all membrane filters were flash frozen in liquid nitrogen and, at the end of the sampling cruise, stored at –80°C until further analysis.

2.2. Enumeration and biomass determination of plankton populations

Filters for piconanoplankton enumeration were examined under the UV light of an epifluorescence microscope. At least 100 and 500 fields were scanned for surface and deep-water layers, respectively; blue light excitation was used to distinguish autotrophs and heterotrophs based on the chloroplast auto-fluorescence. Cells were categorized into size classes (0.8–2, 2–3, 3–5, 5–10 and >10 µm) using a micrometer ocular, and the biovolume of each size class was calculated assuming an ellipsoid shape. Subsequently, the biomass was calculated by accounting for 183 fg C μm^{-3} (Caron et al. 1995).

For surface seawater microplankton enumeration, subsamples of 100 ml were left to settle in Utermöhl chambers for 24 h. Deep-water microplankton samples were first concentrated to a volume of 200 ml by gently removing the supernatant after 24 h of settling and subsequently left to settle in Utermöhl chambers. Microplankton cells were enumerated and identified using an inverted microscope, and the dimensions of each individual cell were measured using image analysis software (Image-Pro Plus 6.1). The biovolume of each individual cell was calculated using the measured dimensions and the geometry assigned by Hillebrand et al. (1999) and Olenina et al. (2006). Finally, the carbon content of each cell was calculated using coefficients provided by Putt & Stoecker (1989) and Davidson et al. (2002).

2.3. DNA metabarcoding analysis

DNA was extracted from the biomass accumulated on the filters using the PowerWater DNA isolation kit (Qiagen) following the manufacturer's instructions. DNA was double eluted, initially with the kit-suggested solution and subsequently with PCR-grade water to maximize yields. The V9 region of the 18S rRNA gene was the target DNA barcode, and the specific primers of Amaral-Zettler et al. (2009) for this region were used. PCR amplification of the targeted region was performed in triplicate, PCR products were purified using magnetic beads (Agencourt AMPure XP; Beckman Coulter), and subsequently PCR triplicates were pooled. The samples were multiplexed for sequencing using the 2-step parallel multiplexing approach (Shokralla et al. 2015). In this approach, the second-step reaction adjusts short index sequences for post-sequencing sample identification and flow cells adaptors for the sequencing platform. Products of the second-step reaction were again purified using magnetic beads (Agencourt AMPure XP, Beckman Coulter) and normalized by gel quantification so that equimolar amounts of each sample were pooled for sequencing. Paired-end sequencing was performed using the MiSeq Reagent Kit v2 on the Illumina MiSeq platform at the Institute of Marine Biology Biotechnology and Aquaculture of the Hellenic Centre for Marine Research, Greece. Negative control samples were created by filtering Nanopure water on board and treated as normal samples during the whole analysis. Raw sequence data were submitted to ENA-GenBank under accession number PRJEB26382.

Raw sequence reads were trimmed to a median Phred quality score >40, paired reads were assembled with at least 50 nucleotides overlapping, and pairs of higher-than-40 alignment quality score were selected using the OBITools metabarcoding software suite (Boyer et al. 2016). Only sequences of 80–200 bp were selected by applying a length filter (Boyer et al. 2016). Sequences were dereplicated, with the subsequent removal of sequences occurring only once in the dataset. PCR and sequencing errors, as well as chimeras, were removed using the 'obiclean' algorithm of OBITools that preserved sequences without related sequences counting for more than 5%. Molecular operational taxonomic unit (MOTU) clustering was performed using SWARM (v2), a stepwise deterministic aggregation algorithm (Mahé et al. 2015). Taxonomy was assigned using the 'ecotag' algorithm (Boyer et al. 2016) and the National Center for Biotechnology Information (NCBI) as a taxonomic reference. Reference sequences were acquired from the EMBL-EBI databank (www.ebi.ac.uk/), and subsequently, the 18S amplicon was targeted with *in silico* ecoPCR (Ficetola et al. 2010) using the primers used for the actual PCR (Amaral-Zettler et al. 2009).

The taxonomically assigned dataset was refined by correcting for the negative control sequences, removing false positive results because of random index swapping (Wangensteen & Turon 2016), minimal abundance filtering (removal of MOTUs with <5 reads) and contaminant removal (Wangensteen & Turon 2016). Fungal and metazoan MOTUs were

removed from the dataset since this study focused only on eukaryotic protists. Finally, samples with fewer than 10 000 final sequence reads were removed from the dataset. Processing metrics and the rarefaction curves of samples are presented in Table S1 and Figs. S1 & S2 in the Supplement at www.int-res.com/articles/suppl/a084p155_supp.pdf.

2.4. Data analyses and statistics

The MOTU dataset was normalized by the total number of reads per sample for all comparisons and statistical analyses. The Shannon-Wiener index (H') was calculated using the metabarcoding MOTU dataset and was used as an estimation for alpha diversity. The number of shared MOTUs among sample pairs, as well as the number of common MOTUs for each sample, were calculated and visualized as a network. For each MOTU, the abundance (number of reads) versus the number of samples in which it appeared was plotted (Fig. S3) in order to determine which MOTUs appear with high abundance in more than 75% of samples and which MOTUs appear with high abundance in less than 10% of the samples, as in Barberán et al. (2012). The sample differentiation according to station, depth and size-fraction was checked with permutational analysis of variance (PERMANOVA) at 999 permutations using a Bray-Curtis dissimilarity matrix. Accordingly, PERMANOVA was used to test if each of the surface microplankton, surface piconanoplankton, deep-sea total eukaryotic community, plankton biomass of different populations and seawater chemistry differed according to station or depth.

The Bray-Curtis dissimilarity of the MOTU composition of each micro- and piconano-community and the Bray-Curtis dissimilarity of the plankton population biomass were the response (y) variables in generalized linear models (GLMs) based on beta distribution. The explanatory (x) variables used in the models were the Euclidean distance among stations based on geographic coordinates, the temperature difference among stations and depths, the total dissolved inorganic nitrogen (nitrate and nitrite) to dissolved inorganic phosphorus (phosphate) ratio (N:P), the dissolved organic carbon to nitrogen ratio (DOC:DON), the abundance of viruses and the abundance of zooplankton. Backward stepwise selection based on Akaike's information criterion (AIC) values was performed to obtain the final models. Collinearity among covariates was checked *a priori*, and the final models were validated by checking the residuals versus the fitted values according to Cribari-Neto & Zeileis (2010). The methodology for the measurement of explanatory variables can be found in Text S1 in the Supplement. All statistical analyses were performed using R v3.5.0, the community ecology package 'vegan' v2.5-2 (Oksanen et al. 2017) and the beta regression package 'betareg' v3.1-1 (Cribari-Neto & Zeileis 2010).

3. RESULTS

3.1. Eukaryotic plankton diversity and community composition

The alpha diversity (estimated as H') of the micro-eukaryotes was generally lower than that of the piconano-eukaryotic community (Fig. 2). The difference between the 2 communities was larger in surface waters; indeed, the lowest H' value for piconano-eukaryotes (H' = 4.05) was higher than the highest value for micro-eukaryotes (H' = 3.95). Piconano-alpha diversity was lower in deeper water than in surface water (Fig. 2). Exceptions were Stn 10 at 1000 m and Stn 03 at >3000 m that accounted for the outlier and high upper hinge and whisker values in Fig. 2; these 2 deep layers sustained high piconano-alpha diversity, similar to that of the surface.

Based on the rarefaction curves, the largest proportion (>76%) of the samples was considerably near richness saturation (Figs. S1 & S2). The relative abundance of sequence reads was calculated as the number of reads per phylum divided by the total number of reads per sample; total number of reads and MOTUs per sample are presented in Table S1. Cercozoa (Rhizaria) was a relatively abundant representative of the micro-eukaryotic community (Fig. 3A). For surface layers at all stations, the relative abundance of Cercozoa (Rhizaria) reads in the micro-eukaryotic community was higher at 50 m (mean ± SD, 37 ± 12% of reads) and 75 m (30 ± 11.5% of reads) in comparison to 5 m (16 ± 6.9% of reads). However, concerning deeper micro-eukaryotic communities, Cercozoa (Rhizaria) were found only at Stn 10 at 1000 m depth. Comparatively, Cercozoa (Rhizaria) were almost absent from the piconano-eukaryotic fraction (<1.5% of reads; Fig. 3B). Dinoflagellata (Alveolata) were important members of both micro- and piconano-communities (Fig. 3). In surface water, the lowest relative Dinoflagellata (Alveolata) read abundance was 7% for micro- while piconano-Dinoflagellata (Alveolata) always

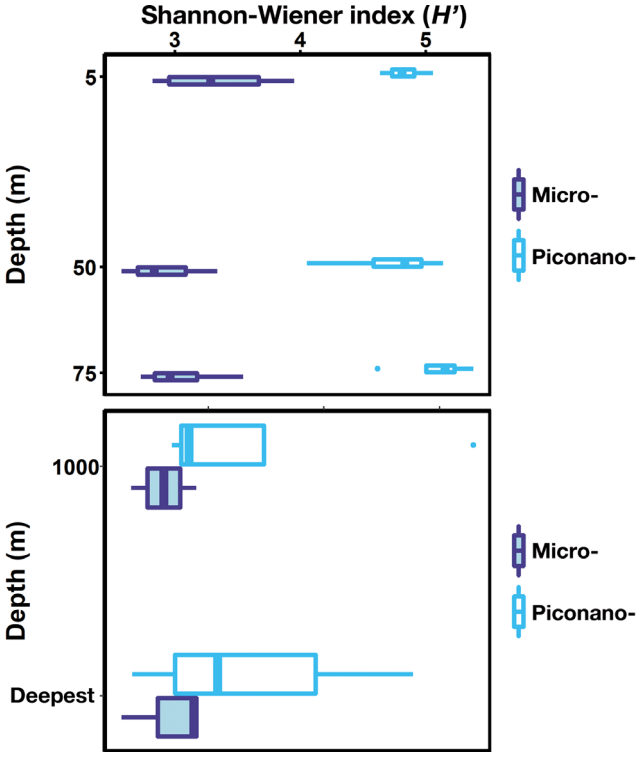

Fig. 2. Shannon-Wiener diversity index (H') representing alpha-diversity of the 2 eukaryotic communities (micro- and piconano-) in the different water layers. The vertical line in the box plots represents the median. The left and right hinges correspond to the first and third quantiles and the whiskers show the minimum and maximum non-outlier values

showed more than 50% relative read abundance. Additionally, Dinoflagellata (Alveolata) dominated by far the deep-water piconano-eukaryotes (Fig. 3B). The contribution of Ciliophora to the micro-eukaryotic community at surface waters slightly varied between depths (Fig. 3A). In contrast, the relative read abundance percentages of Ciliophora (Alveolata) were fairly consistent in the deep-water communities (micro: 4.3 ± 2.8% of reads). Like Cercozoa (Rhizaria), Ciliophora (Alveolata) were almost absent from the piconano-eukaryotic fraction (<1% of reads; Fig. 3B).

The primarily autotrophic groups (Bacillariophyta [Stramenopiles] and Chlorophyta [Chloroplastida]) showed very low relative read abundance percentages within micro-eukaryotes; however, their presence was relatively stronger in the piconano-community (Fig. 3). The same held true for Haptophytes (Haptista), which also had a stronger presence in the surface piconano-eukaryotes (6.6 ± 2.2% of reads) than in the surface micro-eukaryotes (0.7 ± 0.5% of reads) or any deep-water community (3.7 ± 3.4% of reads; Fig. 3). A pattern in relative read abundance with depth was noted for Haptophytes (Haptista) and Cryptophytes (Cryptista), with the former being more abundant at 5 m and the latter at 75 m (Fig. 3).

The phylum Tubulinea (Amoebozoa) was only present in the deep-water communities, with overall low relative read abundance percentages that were relatively higher in the piconano-community (Fig. 3). Although the relative read abundance of Apicomplexa (Alveolata) was low (<5% of reads), there was a clear variation of this phylum with depth and its presence was much higher at 5 m depth (Fig. 3). Oomycota (Stramenopiles) seemed to be an important member of the piconano-community only at a single station and 1 depth layer (Stn 18, 75 m; Fig. 3B). The relative read abundance of unassigned groups was 39.3 ± 10.1% for the micro-eukaryotes and 16.9 ± 3.2% for piconano-eukaryotes at the surface, while percentages were even higher for the deep-sea communities (micro: 57.5 ± 26%, piconano: 33.4 ± 16.9%; Fig. 3).

3.2. Vertical and horizontal patterns of eukaryotic plankton

PERMANOVA revealed that the total eukaryotic community significantly differentiated according to water depth and size fraction, whereas it did not differentiate from station to station (Table 2). Based upon this distinction, eukaryotic communities of different sizes (micro- and piconano-) were tested separately, and a significant distinction according to depth was found for each size fraction in the surface water (Table 2). This depth separation of surface communities was more intense for micro-eukaryotes, as indicated by the higher values of mean of squares, F_{perm} and R^2 (Table 2). Concerning the deep-sea communities, because of the low number of samples per station, permutational analyses were not possible for the different size fractions separately; however, the total deep-sea eukaryotic community was again not separated according to station (Table 2). For all eukaryotic community permutational analyses, a large percentage of variation was not explained by either depth or size fraction (Table 2; residuals R^2 range: 49.4–68.9%). The differences in communities among stations were also visualized using non-metric multidimensional scaling ordination (Fig. S4).

Shared MOTUs between the different samples are shown in Fig. 4 for micro-eukaryotes and in Fig. 5 for piconano-eukaryotes. Micro-eukaryotic MOTUs were widely distributed among the 3 surface layers (5, 50 and 75 m), with the exception of Stn 18 at depths of

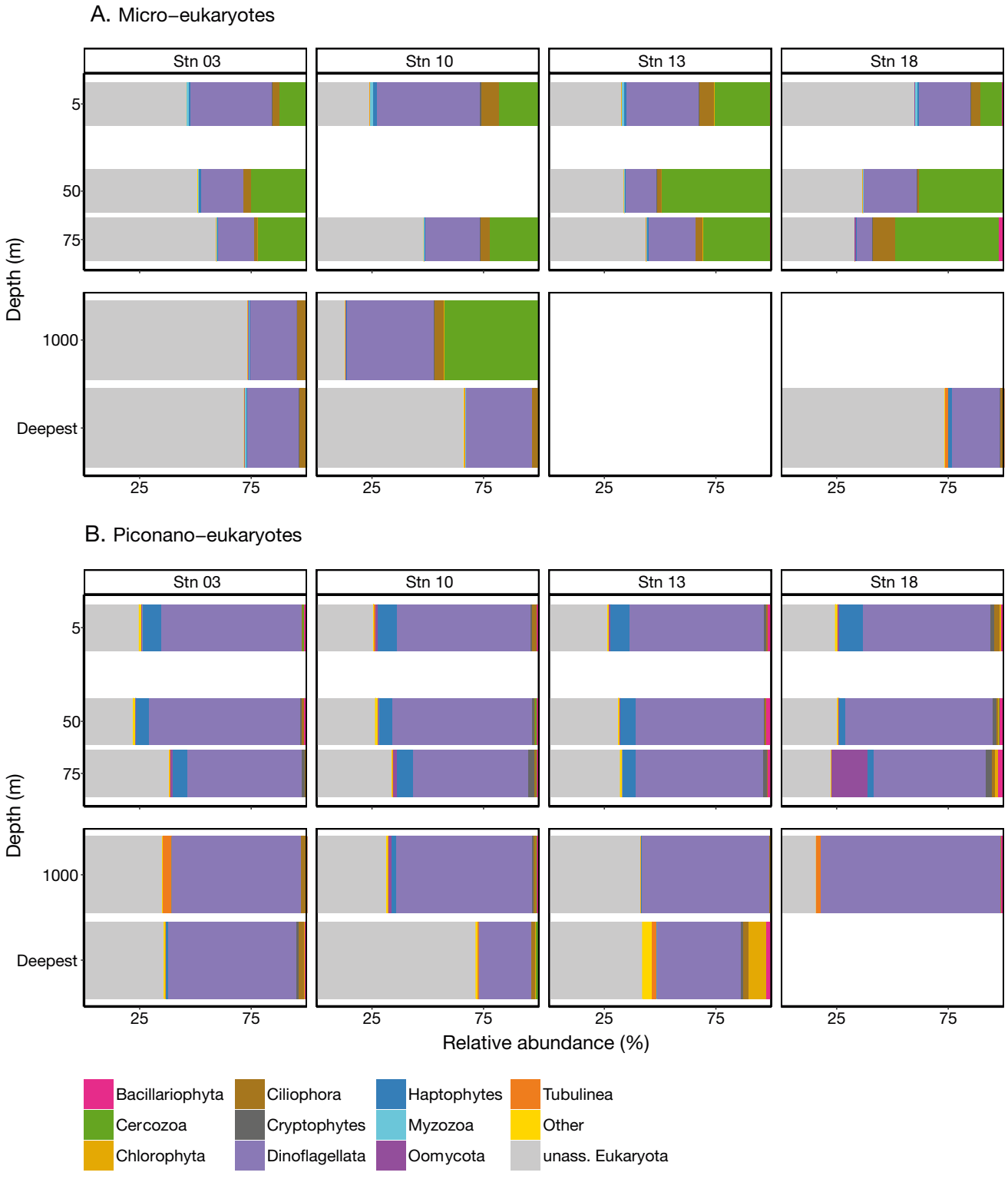

Fig. 3. Composition of (A) micro-eukaryotic and (B) piconano-eukaryotic communities at the different sampling stations and water layers. Relative abundance percentages of reads were calculated from the number of reads of each phylum in a sample versus total number of reads of the sample. Total number of reads per sample is presented in Table S1 in the Supplement. 'Other' includes the phyla with relative read abundance <5% in all cases, i.e. Bigyra, Hyphochytriomycota, Ochrophyta, Rhodophyta, unassigned Viridiplantae, unassigned Alveolata and unassigned Stramenopiles

50 and 75 m. Deep samples shared 20–100 MOTUs among them and with the surface waters layers; the exception to this was again Stn 18, whose deepest sample shared less than 20 MOTUs with the lower depths. Piconano-eukaryotic MOTU-sharing was similar to micro-eukaryotes at the surface, with only Stn 18 (at 50 m depth) being the exception. However, the situation was somewhat different in deeper waters, as the 1000 m sample of Stn 10 shared more than 100 MOTUs with the 3 surface samples, and the deepest sample of Stn 03 shared more than 100 MOTUs with one of the surface stations. In comparison, the number of shared MOTUs for the other deep-sea stations was low.

The biomass of various plankton groups was significantly different among the sampling stations and also according to depth (Table 2). The residual R^2 values of plankton biomass permutational analysis were low, indicating that stations and depth explained well the variation in biomass. Upon detailed examination of the biomass of plankton groups, differences among stations were indeed obvious. The Bacillariophyta (Stramenopiles) biomass pattern with depth was similar for Stns 10 and 18, while the other 2 stations showed a maximum abundance at different depths (Fig. 6). As for ciliated protists, Stn 10 showed a different pattern in comparison to the other 3 stations (Fig. 6). The bathymetric biomass pattern of Dinoflagellata (Alveolata) was similar between Stns 03 and 10 and between Stns 13 and 18 (Fig. 6). The depth of maximum biomass for Prymnesiophyceae (Haptista) was different among stations (Fig. 6). The depth of maximum biomass for piconano-autotrophs was also different among stations (Fig. 7; 50, 20, 50, 20 m for Stns 03, 10, 13, 18, respectively). Piconano-heterotroph maximum biomass was observed at 20 m for all stations except Stn 13 (Fig. 7).

3.3. Generalists and specialists in the communities

The MOTUs that showed more than 75% sample prevalence (i.e. number of samples in which a MOTU appeared) and at the same time had a high mean abundance per sample (abundance higher than 500 reads) are characterized as 'generalists' (Table 3, Fig. S3). In contrast, MOTUs prevalent in less than 10% of the samples that had a high mean abundance per sample (>500 reads) are characterized as 'specialists' (Table 3, Fig. S3). MOTUs of 1 Cercozoa (Rhizaria), 2 Dinoflagellata (Alveolata) and 1 unassigned Eukaryota were prevalent in the surface micro-eukaryote community, while MOTUs of 2 Dinoflagellata (Alveolata) and 1 Haptophyta (Haptista) were prevalent in the surface piconano-community (Table 3, Figs. 8 & 9). The prevalent Dinophyceae (Dinoflagellata, Alveolata) MOTU was the same between the 2 communities. Most of the specialist MOTUs remained unassigned, with the exception of 1 *Karlodinium* (Dinoflagellata, Alveolata) and 1 Dinophyceae (Dinoflagellata, Alveolata) MOTU of the piconano-community. Moreover, one of the unassigned Eu-

Table 2. Permutational analysis of variance (PERMANOVA) testing potential variation among stations, depths and size fractions. Dissimilarity Bray-Curtis matrices were calculated for the response variables and each analysis was based on 999 permutations. MOTUs: molecular operational taxonomic units; DOC: dissolved organic carbon; DON: dissolved organic nitrogen; DOP: dissolved organic phosphorus

Source of variation	df	MS	F_{perm}	R^2	p
Eukaryotic community: all eukaryotic MOTUs (micro- and piconano-)					
Station	3	0.322	1.067	0.076	0.280
Depth	1	1.268	4.204	0.099	0.001
Size fraction	1	1.710	5.670	0.135	0.001
Residuals	29	0.302		0.689	
Eukaryotic community: micro-eukaryote MOTUs of surface water					
Station	3	0.210	1.112	0.275	0.257
Depth	1	0.527	2.796	0.230	0.001
Residuals	6	0.189		0.494	
Eukaryotic community: piconano-eukaryote MOTUs of surface water					
Station	3	0.270	1.158	0.283	0.102
Depth	1	0.421	1.811	0.147	0.002
Residuals	7	0.233		0.570	
Eukaryotic community: eukaryotic MOTUs (micro- and piconano-) of deep water					
Station	3	0.321	0.958	0.239	0.587
Depth	1	0.520	1.553	0.129	0.026
Size fraction	1	0.539	1.611	0.133	0.018
Residuals	6	2.009		0.498	
Plankton biomass: piconano-autotrophs, piconano-heterotrophs, Bacillariophyta, Ciliophora, Dinoflagellata and Prymnesiophyceae (Haptophyta) of surface water					
Station	3	0.006	2.895	0.129	0.024
Depth	1	0.085	39.666	0.589	0.001
Residuals	19	0.002		0.282	
Seawater chemistry: nitrate, nitrite, phosphate, silicate, DOC, DON, DOP					
Station	3	0.001	6.222	0.535	0.001
Depth	1	0.001	9.213	0.264	0.001
Residuals	7	0.000		0.200	

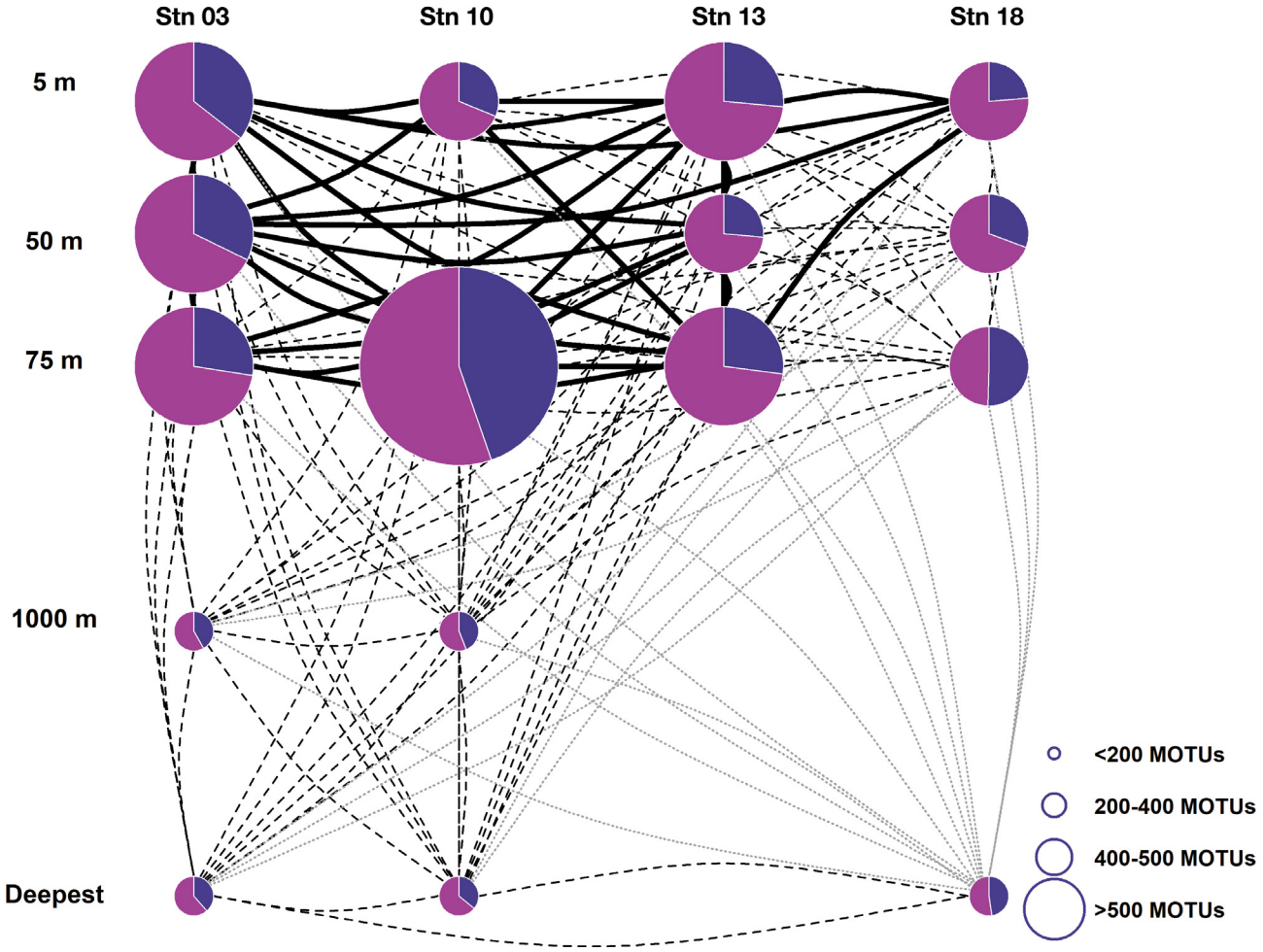

Fig. 4. Shared micro-eukaryote molecular operational taxonomic units (MOTUs) among stations and depths represented as a network. Nodes are samples (depth layer per station) and edges are the number of shared MOTUs between 2 nodes. Nodes are represented as pie charts (light purple: percentage of shared MOTUs of each sample with all other samples; dark purple: percentage of unique MOTUs per sample). Sizes of nodes correspond to the total number of MOTUs per sample. Dotted grey lines: samples share <20 MOTUs; dashed black lines: samples share 20–100 MOTUs; solid black lines: samples share >100 MOTUs

karyota MOTUs was a specialist in surface communities as well as in the deep-sea micro-community (Table 3, Figs. 8 & 9).

Accordingly, for the deep-sea communities, all generalist MOTUs remained unassigned, with the exception of 1 Dinophyceae (Dinoflagellata, Alveolata), which was also marked as a generalist in the surface communities (Table 3). Several of the specialist MOTUs found in deep-sea samples remained unassigned; however, 1 Tubulinea (*Vermamoeba*, Tubulinea, Amoebozoa), 1 Cercozoa (*Euglypha*, Cercozoa, Rhizaria), 1 Haptophyta (*Isochrysis*, Haptophyta, Haptista) and several Dinoflagellata (*Amoebophrya*, *Lepidodinium*, *Phalacroma* and Dinophyceae, Dinoflagellata, Alveolata) MOTUs were amongst the specialist micro-eukaryotes (Table 3). As for the deep-sea piconano-eukaryotes, 1 *Karlodinium* (Dinoflagellata, Alveolata), 1 *Lepidodinium* (Dinoflagellata, Alveolata), several Dinophyceae (Dinoflagellata, Alveolata) and unassigned Eukaryota were characterized as specialists (Table 3). One representative specialist MOTU of the deep micro-community was also found amongst the surface specialists (Fig. 8). Interestingly, 3 MOTUs found as specialists in the deep-sea communities were among the surface generalists (Figs. 8 & 9). Specialist MOTUs were located at different stations and depths; the deepest water layer of the easternmost station (Stn 18) hosted the most deep-sea specialists (Figs. 8 & 9).

3.4. Physical, chemical and biological variables that designate community variation

Seawater chemical variables (Table S2) varied among stations and depths according to the PERM-

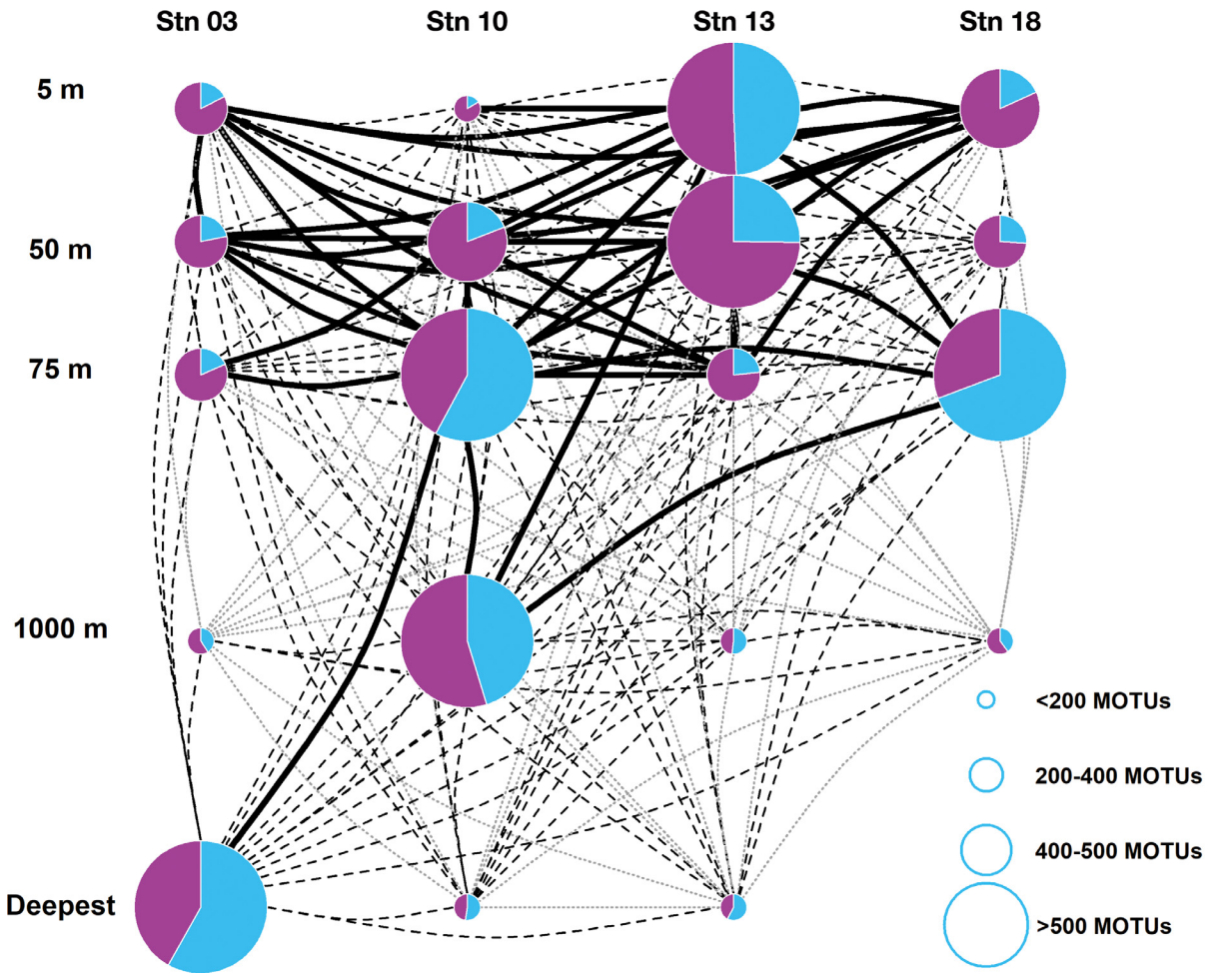

Fig. 5. Shared piconano-eukaryote molecular operational taxonomic units (MOTUs) among stations and depths represented as a network. Details as in Fig. 4, with light blue representing the percentage of unique MOTUs per sample.

ANOVA (Table 2). Additionally, the residual R^2 values of permutational analysis were low, indicating that station and depth explained well the variation in the chemical variables.

In order to assess which explanatory variables affected the eukaryotic beta diversity in the Levantine Basin, a GLM based on beta distribution was applied separately for the 2 size fractions (Table 4, micro- and piconano-eukaryotes). The explanatory variables used in the model are shown in Fig. S5. The micro-eukaryote community was significantly associated with temperature, dissolved N:P ratio, DOC:DON ratio and viral abundance. The significant explanatory variables positively affected the dissimilarity among communities (i.e. the community dissimilarity increased as the difference in temperature, N:P and DOC:DON ratios, and/or viruses increased). For example, at the highest N:P ratio (Fig. S5; Stn 18 at 75 m depth), micro-eukaryotes appeared distinctively dissimilar to the rest of the surface water community (Fig. S4). The estimated slope parameter for viruses was almost double the slopes of the N:P and DOC:DON ratios. Other variables (geographic position and zooplankton abundance) were removed by stepwise model selection based on AIC values.

In contrast, the piconano-eukaryotic community was significantly associated with temperature, DOC:DON ratio and zooplankton abundance (Table 4). The temperature and DOC:DON variables positively affected the dissimilarity among communities, while the association with zooplankton was negative; that is, the community dissimilarity increased as the difference in zooplankton abundance decreased. The estimation of the slope parameter for temperature was much larger than the slope of the DOC:DON ratio and the absolute slope value for zooplankton. Other variables (geographic position, N:P ratio, viral abundance) were removed by stepwise model selection based on AIC values. Differences in the plankton biomass of

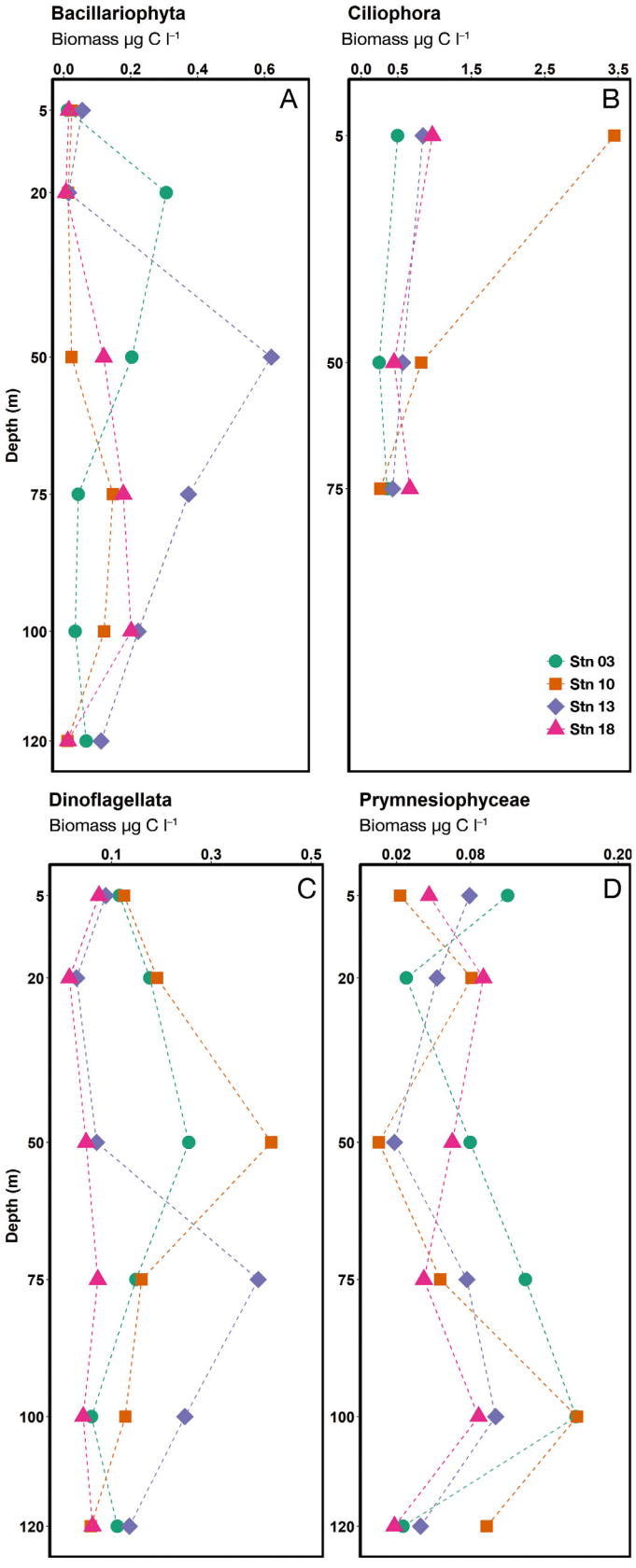

Fig. 6. Microplankton biomass of (A) Bacillariophyta, (B) Ciliophora, (C) Dinoflagellata and (D) Prymnesiophyceae (Haptophyta) at the different stations

the different eukaryotic populations were significantly associated with temperature and N:P ratio (Table 4). The slope parameters for temperature and N:P ratio were estimated to be similar. Other variables (geographic position, DOC:DON ratio, viral abundance, zooplankton abundance) were removed by stepwise model selection based on AIC values.

4. DISCUSSION

4.1. Spatial and bathymetric distribution patterns of plankton eukaryotes in the oligotrophic Eastern Mediterranean Sea

In this study, neither the micro- nor the piconano-eukaryotic communities differed across the sampling stations in the surface seawater (5–75 m) despite the significantly different environmental conditions that characterized the different stations. Rather, eukaryotic communities in the Eastern Mediterranean differentiated according to water depth, suggesting that there are distinct communities at different depth layers. The vertical distribution pattern of plankton indicates that variables such as temperature, salinity, chemical variables and biotic variables that potentially differ among the vertical water layers play an important role in shaping the eukaryotic communities (Schnetzer et al. 2011, Gong et al. 2015). Temperature significantly affected the composition of both communities (micro- and piconano-plankton). Similar studies on prokaryotic plankton have indicated temperature as the main driver of community differentiation (Sunagawa et al. 2015, Lambert et al. 2019). Therefore, it seems that the physiological restrictions and capacities of cells to temperature may influence their distribution and induce bathymetric distribution even within small geographic distances.

Interestingly, community separation with depth was stronger for the micro-eukaryotes, possibly due to the fact that microplankton cells are less physiologically versatile (Litchman & Klausmeier 2008) and more sensitive to changes (Li et al. 2009) in comparison to smaller cells. Therefore, microplankton cells tend to be more prominently separated with depth, occupying subtly different niches formed vertically in the euphotic water column. The N:P ratio, indicating P-limitation in this area, only affected the micro-eukaryotes. Piconano-eukaryotes were not affected by changes in resources (nutrients) and thus appeared to be well-adapted to the oligotrophy of the system; plankton cells smaller than 20 µm have greater abilities to use low nutrient quantities (Moutin

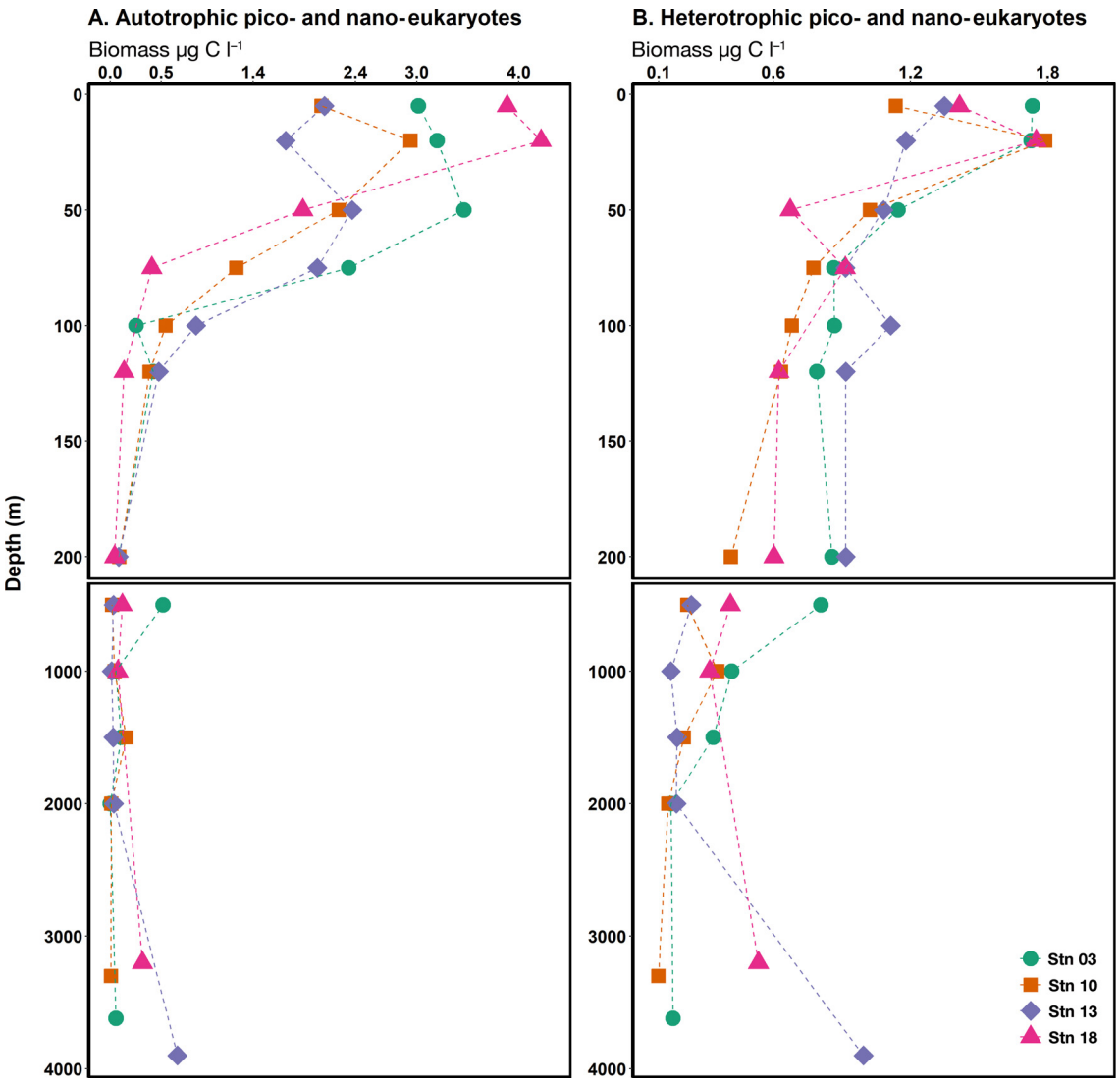

Fig. 7. Biomass of (A) autotrophic and (B) heterotrophic pico- and nano-eukaryotes at the different stations

et al. 2002) and therefore have an advantage in oligotrophic habitats (Unrein et al. 2007, Zubkov & Tarran 2008). Temperature was by far the main factor that influenced piconano-eukaryotes, suggesting that this fraction is adaptable to bottom-up and top-down controls while it is largely affected by seawater abiotic characteristics. In the Eastern Mediterranean Sea, not only were the piconanoplankton not affected by the high N:P ratios (Fig. S5), but they also showed higher alpha diversity (Fig. 2) and biomass (Figs. 6 & 7) in comparison to micro-eukaryotes; all of these facts confirm that piconano-eukaryotes were better fitted to this oligotrophic oceanic area than micro-eukaryotes.

Eukaryotic community composition was also affected by other plankton communities (biological variables): viruses and zooplankton affected micro- and pico-nano-eukaryotes. Zooplankton abundance seemed to influence the piconanoplankton more, and this may relate to zooplankton grazing on piconano-eukaryotes. Small copepod forms, e.g. *Clausocalanus* juveniles, *Oithona* juveniles or *Mormonilla minor* that graze on the nano- and picoplankton size fraction, were predominant in the zooplankton community of the study area (Protopapa et al. 2019). Moreover, juvenile zooplankton is reported to feed selectively according to its metabolic needs (Meunier et al. 2016) and thus may have affected piconano-community composition.

While plankton composition and diversity were similar among stations, the biomass of various plankton groups was different; differences in the biomass of unicellular free-living populations may signify that plankton populations are of different sizes and, additionally, are a strong indication of differences in

activity (S. K. Hu et al. 2016). It seems that while in terms of composition the plankton community in the Eastern Mediterranean Sea is horizontally homogeneous, the activity pattern and the population's size vary horizontally and bathymetrically. To strengthen this, primary productivity has been found different among stations in this area (Livanou et al. 2019). Plankton biomass was influenced only by temperature and N:P ratio. Consequently, it seems that physiology and resource availability (i.e. dissolved inorganic nutrients) were the factors that controlled the biomass differences in the oligotrophic Eastern Mediterranean Sea.

Our sampling area was affected by different oceanic structures: the surface water of the westernmost station by a cyclone (Stn 03; the Cretan Cyclone), the surface water of Stn 13 by an anticyclone and the surface water of the eastern station by a different cyclone (Stn 18; the Rhode Gyre) (Velaoras et al. 2019). These structures constantly influence the conservative properties of the seawater (temperature, salinity and dynamic density) (Velaoras et al. 2019) and the circulation of nutrients (Yilmaz & Tugrul 1998), and differentiate the physical and chemical characteristics of the sampled stations. This significant dissimilarity of the seawater among stations of the same oceanic area was only reflected by plankton biomass and not by its composition. Overall, the Eastern Mediterranean seems to host an invariable community that differs in composition only among depth layers; nevertheless, the biological activity within the community and/or the abundance of the community's populations change among different locations and depth layers.

Table 3. Generalist and specialist eukaryotic molecular operational taxonomic units (MOTUs) in the Western Levantine basin. MOTUs that were found in >75% of the samples with >500 reads per sample are characterized as generalists. MOTUs that were found in <10% of the samples with >500 reads per sample are characterized as specialists. The lowest possible level of taxonomy is given for each MOTU. Numbers in parentheses indicate that more than one MOTU belonging to this taxon were found. Taxa with the same superscripts (a, b, c, d, e) correspond to the same MOTU

	Micro-eukaryotes	Piconano-eukaryotes
Surface water		
Generalists	*Euglypha* (Cercozoa)[a] *Gymnodinium* (Dinoflagellata) Dinophyceae (Dinoflagellata)[b] Unass. Eukaryota[c]	*Lepidodinium* (Dinoflagellata)[d] Dinophyceae (Dinoflagellata)[b] *Prymnesium* (Haptophyta)
Specialists	Unass. Eukaryota (×7)[e]	*Karlodinium* (Dinoflagellata) Dinophyceae (Dinoflagellata) Unass. Eukaryota (×3)[e]
Deep water		
Generalists	Dinophyceae (Dinoflagellata)[b] Unass. Eukaryota (×3)	Unass. Eukaryota
Specialists	*Euglypha* (Cercozoa)[a] *Amoebophrya* (Dinoflagellata) (×2) *Lepidodinium* (Dinoflagellata) *Phalacroma* (Dinoflagellata) Dinophyceae (Dinoflagellata) (×4) *Isochrysis* (Haptophyta) *Vermamoeba* (Tubulinea) Unass. Eukaryota (×10)[c,e]	*Karlodinium* (Dinoflagellata) *Lepidodinium* (Dinoflagellata)[d] Dinophyceae (Dinoflagellata) (×3) Unass. Eukaryota (×2)

4.2. Diversity of eukaryotic plankton in the Eastern Mediterranean Sea

The results of the present study corroborate the findings of de Vargas et al. (2015) that protistan diversity is higher than previously thought. In fact, piconano- (0.8–20 µm) eukaryotic communities were much more diverse in terms of richness (Shannon-Wiener index, H') than microplankton (20–200 µm), in agreement with recent global and regional studies (de Vargas et al. 2015, Brannock et al. 2016, Giner et al. 2020).

The plankton community structure in this marine area challenges the well-known scheme of terrestrial ecological food webs (autotrophs vs. heterotrophs), as in de Vargas et al. (2015) and other Mediterranean studies (Stoecker et al. 2017): taxa that include mixotrophs (Dinoflagellata [Alveolata], Haptophyta [Haptista]) (Mitra et al. 2016, Leles et al. 2017), heterotrophs (Cercozoa, Rhizaria) (Weber et al. 2012) and parasites (Cercozoa, Rhizaria) (Skovgaard 2014) were broadly prevalent and widely present in the area while strictly autotrophic taxa (e.g. Bacillariophyta, Stramenopiles) seemed to play a minimal role in the plankton structure. Groups with an important global presence, such as Diplonemida (Euglenozoa, Excavata) and Collodaria (Retaria, Rhizaria) (de Vargas et al. 2015, Giner et al. 2020), were almost absent from the Eastern Mediterranean.

Despite the small spatial distance, as in the studied area, local or transient environmental conditions might influence the structure and composition of

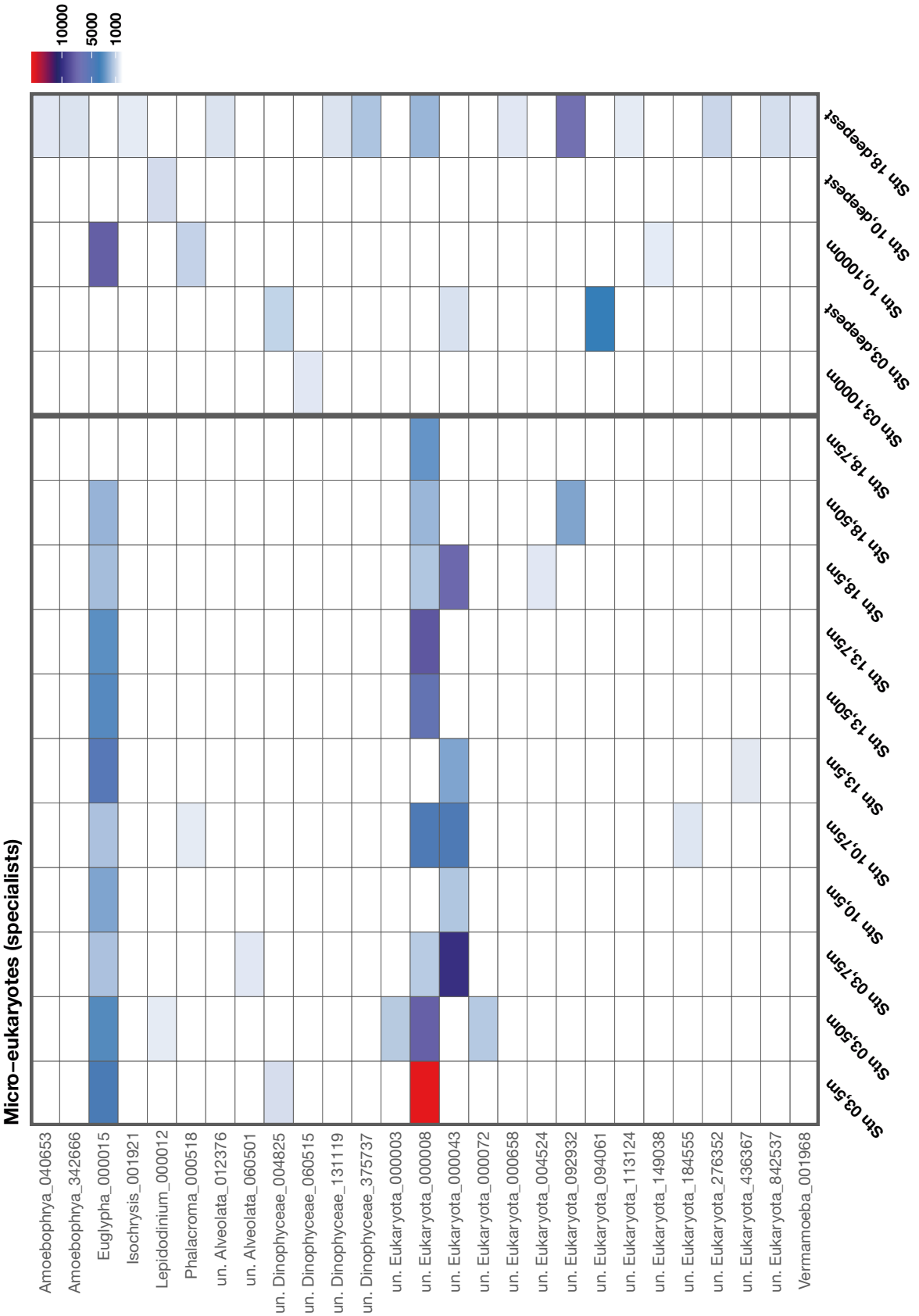

Fig. 8. Distribution of specialist micro-eukaryote molecular operational taxonomic units (MOTUs) visualized as the number of reads on a heatmap. The numbers of reads are represented as a color gradient from blue (low) to red (high). The lowest possible level of taxonomy is given for each MOTU

plankton (Dann et al. 2016). The specialist micro- and piconano-eukaryote MOTUs found in the Eastern Mediterranean are believed to occupy the local niches created by environmental conditions or biotic factors. For example, members of the genus *Karlodinium* (Dinoflagellata, Alveolata) were found to be specialists in a distinct microenvironment characterized by the highest N:P ratio (Stn 18 surface layers; Fig. S5B). *Karlodinium* is a toxic photosynthetic dinoflagellate (Garcés et al. 2006) that is also capable of ingesting prey (Stoecker et al. 2017 and references therein); this mixotrophic behavior of *Karlodinium* (constitutive mixotroph; Mitra et al. 2016) provides an advantage under nutrient-limiting conditions, as the cell may acquire a limiting resource through phagotrophy (Jeong et al. 2010, Lin et al. 2016 and references therein). In fact, *Karlodinium* dinoflagellates have been found to increase their phagotrophy ingestion rate under P-limiting conditions (Lin et al. 2016 and references therein).

Another intriguing observation was the high percentage of unassigned groups reported here. On the one hand, the Eastern Mediterranean is among the least explored oceanic areas in terms of its eukaryotic plankton community. On the other hand, this area may harbor unique or rare plankton species due to the distinct chemical conditions prevailing (extremely low nutrient content and high N:P ratios, Krom et al. 2010; Fig. S5, Table S2). For these 2 reasons, a large proportion of unassigned eukaryotes may be reasonable and most probably corresponds to unknown microbial species that have not been previously described and hence cannot be taxonomically assigned. The Eastern Mediterranean seems to host a uniquely rare community, and its eukaryotic diversity has not yet been completely captured. The poor taxonomic MOTU assignment of even cosmopolitan eukaryotic plankton has been highlighted in global studies as well (de Vargas et al. 2015) and even in less extreme environments (Zhang et al. 2018).

In this study, we detected a compositionally different plankton community between surface and deep-water layers, as previously reported (Pernice et al. 2016). What was more interesting regarding deep seawater is the large percentage of taxonomically unassigned plankton eukaryotes. Taking into account that the diversity of deep-sea microorganisms is underestimated due to differences in cell survival caused by pressure changes during sampling (Edgcomb et al. 2011), it seems that the real deep-sea plankton community is not only underestimated but also that the part analyzed in studies remains taxonomically unknown. What was also noteworthy in this study was

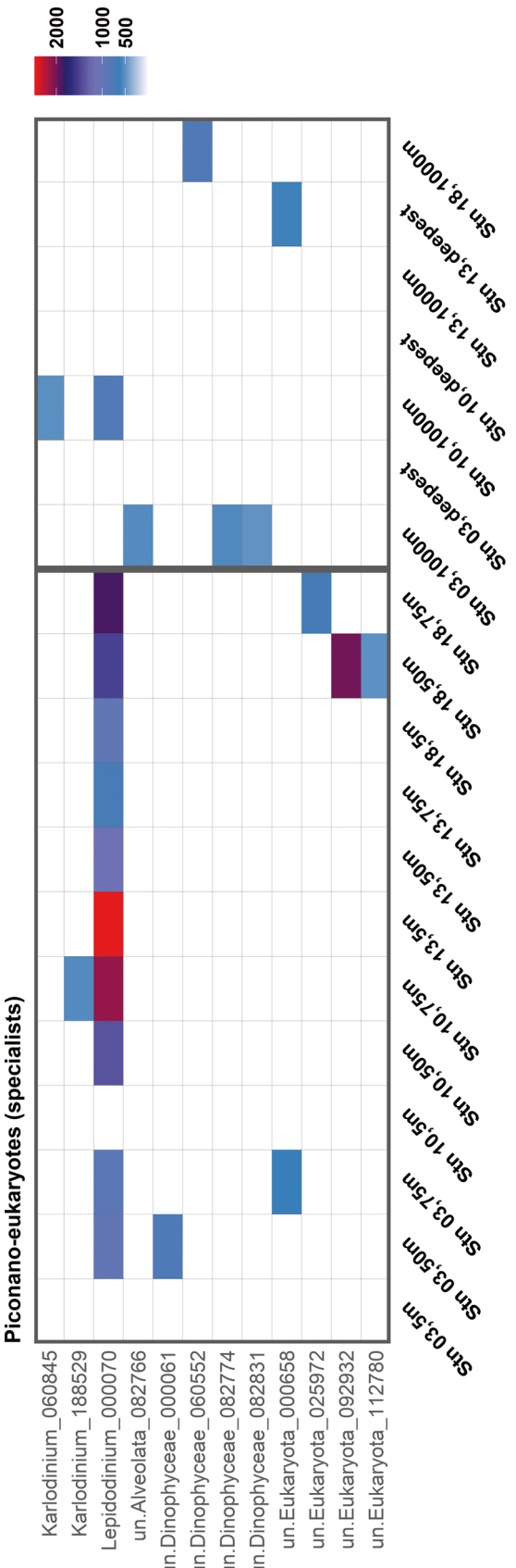

Fig. 9. Distribution of specialist piconano-eukaryote molecular operational taxonomic units (MOTUs) visualized as the number of reads on a heatmap. Details as in Fig. 8

Table 4. Generalized linear models based on beta distributions. MOTUs: molecular operational taxonomic units; DOC (DON): dissolved organic carbon (nitrogen)

	Estimate	SE	*z*	p
Response: micro-eukaryote MOTUs				
(Intercept)	1.197	0.056	21.302	<0.0001
Temperature	0.226	0.091	2.481	0.013
N:P ratio	0.166	0.057	2.907	0.004
DOC:DON ratio	0.170	0.078	2.179	0.029
Viruses	0.336	0.082	4.086	<0.0001
Phi coefficient of beta distribution	15.23	1.94	7.85	<0.0001
				Model R^2: 0.581
Response: piconano-eukaryote MOTUs				
(Intercept)	1.803	0.069	26.188	<0.0001
Temperature	0.850	0.074	11.468	<0.0001
DOC:DON ratio	0.340	0.066	5.152	<0.0001
Zooplankton	−0.129	0.060	−2.135	0.0328
Phi coefficient of beta distribution	10.413	1.179	8.833	<0.0001
				Model R^2: 0.294
Response: plankton biomass (piconano-autotrophs, piconano-heterotrophs, Bacillariophyta, Ciliophora, Dinoflagellata, Prymnesiophyceae [Haptophyta])				
(Intercept)	−0.682	0.045	−15.058	<0.0001
Temperature	0.236	0.046	5.178	<0.0001
N:P ratio	0.292	0.045	6.468	<0.0001
Phi coefficient of beta distribution	17.882	2.256	7.927	<0.0001
				Model R^2: 0.414

that both the rare and the broadly distributed plankton of this deep oligotrophic sea remain unknown.

4.3. Methodological remarks

The interpretation of the MOTU relative abundance should be treated cautiously because of methodological constraints and biological variability concerning metabarcoding analysis (Pawlowski et al. 2016). The copy number of the 18S rRNA gene may vary among the different eukaryotic taxa (Prokopowich et al. 2003, Heywood et al. 2011); for example, ciliates carry between 3 and >300 rRNA gene copies per cell (Gong et al. 2013). Such biases have been attributed to metabarcoding studies and concern even the more established 16S metabarcoding methodologies (Větrovský & Baldrian 2013). Therefore, this study treats metabarcoding results as semi-quantitative (relative abundance of reads or number of MOTUs) and instead uses plankton biomass from microscopical enumeration as quantitative data.

Additional methodological constraints that may affect the results are the sample volume and the duration of filtration. The essential seawater volume needed for adequately capturing the community composition depends on both the sampled environment and the targeted community. To date, no study has extensively considered the required seawater volume for capturing the diversity of microbial plankton eukaryotes, and thus it is difficult to predict the necessary seawater volume prior to sampling. In the coastal Western Mediterranean Sea, the filtration volume for 18S metabarcoding of plankton varies between 1 l (Busch et al. 2016) and 40 l (Grzebyk et al. 2017). To our knowledge, this is the first 18S metabarcoding study that examines the open-sea plankton of the Eastern Mediterranean, and our findings suggest that volumes larger than 20 l are necessary to adequately capture the eukaryotic microbial plankton diversity of oligotrophic waters (Figs. S1 & S2). For the deep-sea water layers (≥1000 m), a seawater volume of 30 l was found to sufficiently represent community diversity (Figs. S1 & S2).

Considering that the doubling times of microbial eukaryotes may range from ~10 h to several days (Christaki et al. 2001, Caron et al. 2017, Grujcic et al. 2018, Šimek et al. 2018), the filtration duration (75 min) in this case hardly affected the community composition. DNA degradation is unlikely to happen at such short intervals, and cell damage was minimized as much as possible by the gentle handling of seawater.

5. SUMMARY AND CONCLUSIONS

This work presents a comprehensive analysis of the biomass, composition and distribution of eukaryotic plankton communities in the ultra-oligotrophic Eastern Mediterranean Sea (Western Levantine Basin). There were many similarities among the eukaryotic plankton composition between the Eastern Mediterranean Sea and other oceanic areas, but several discrepancies were noticed, including the absence of Excavata and Collodaria (Retaria, Rhizaria) (de Vargas et al. 2015). Nevertheless, a large part of the eukaryotic diversity remained unknown and could not be taxonomically affiliated. This unknown frac-

tion was larger in the deep sea, which implies that the distinctive environment of the Eastern Mediterranean may host unique plankton assemblages and emphasizes, once more, the considerable need for studies on eukaryotic plankton in the natural environment. The micro- and piconano-eukaryotes differed in community composition and diversity. In fact, the piconano-eukaryotic fraction was more diverse than the microplankton, as reported for other oceanic areas, and several phyla differentiated the 2 communities: Cercozoa and Ciliophora showed a stronger presence in the micro-fraction, in contrast to Bacillariophyta, Chlorophyta and Haptophyta, which showed a stronger presence in the piconano-fraction.

Eukaryotic plankton communities were similar in composition among the investigated stations in the surface waters; in spite of that, the composition of communities differentiated among the different depth layers, thus indicating a bathymetric distribution of plankton eukaryotic communities in the Eastern Mediterranean. Additionally, it seems that while the sunlit Levantine is geographically homogeneous in terms of eukaryotic plankton composition, the biomass of plankton groups varies among stations and bathymetrically.

Interestingly, there were differences among the environmental factors that affected micro- and piconanoplankton community composition. Temperature and dissolved organic matter were significant for both communities while P-limitation (N:P ratio) and viruses additionally affected microplankton; in contrast, zooplankton was found to influence piconanoplankton. Plankton biomass was only associated with temperature and resource availability (N:P ratio). Our results suggest that biomass and micro- and piconano-eukaryote community composition respond differently to the same environmental conditions.

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