Dynamics of phytoplankton groups in three contrasting situations of the open NW Mediterranean Sea revealed by pigment, microscopy, and flow cytometry analyses

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ABSTRACT

A detailed study of phytoplankton composition and dynamics was carried out during three contrasting situations (cruises F1, F2, and F3) in the northwestern (NW) Mediterranean Sea. Haptophytes, diatoms, and green algae dominated in F1, during the spring bloom, with high nutrients and high phytoplankton biomass. In F2, the post-bloom situation with a still weak stratification and lower nutrient concentrations, we found a high spatial variability. Stations were clearly dominated by either *Synechococcus*, haptophytes or cryptophytes; with *Synechococcus* reaching the highest abundance (4×10^5 cells mL⁻¹, 60% of the integrated chlorophyll a) reported to date for the open Mediterranean Sea. Cryptophytes accumulated close to the surface in very shallow mixed layer stations. In late summer, F3 revealed a fully developed stratification with low nutrients and a marked deep chlorophyll maximum (DCM). *Prochlorococcus* was present only during this cruise, mainly in deep layers together with haptophytes and pelagophytes, while haptophytes and *Synechococcus* dominated the upper mixed layer. Flow cytometry (FCM) and pigment-based abundance estimates for *Prochlorococcus*, *Synechococcus* and cryptophytes were well correlated, as happened also between small picoeukaryotes (FCM) and green algae (pigments), and between large picoeukaryotes (FCM) and haptophytes (pigments). Dinoflagellate abundance by microscopy and by pigments did not agree well, probably due to the presence of heterotrophic forms or because they contained pigments other than peridinin, the standard dinoflagellate marker. The decrease in size of the FCM large picoeukaryotes group with depth was presumably related to the increasing contribution of pelagophytes, with smaller cells than haptophytes,

the other main component of this fraction. Cell size increase of *Prochlorococcus* and *Synechococcus* with depth suggests vertical segregation of genotypes or photoadaptation. The groups' ecological preferences are presented with respect to depth and nutrient concentrations. *Synechococcus* and cryptophytes occupied shallow layers; diatoms, green algae and *Prochlorococcus* showed a tendency for deep layers and pelagophytes for even deeper layers, while haptophyte and dinoflagellate allocations were less clear. As for nutrients, the maximum relative contributions of green algae and especially diatoms occurred when dissolved inorganic phosphorus (DIP) concentrations were highest, of *Prochlorococcus*, dinoflagellates and pelagophytes when lowest, and of *Synechococcus* and cryptophytes when DIP concentrations were low but not minimal. The contribution of haptophytes did not show a relationship with DIP concentration. These results from individual groups stand as significant exceptions to the general relationship between phytoplankton cell size and nutrient availability.

1. INTRODUCTION

The composition of phytoplankton has profound implications for the functioning of marine ecosystems. Taxonomic affiliation and size are among the most important traits in determining the role of phytoplankton in aspects such as physiology, ecology, or biogeochemistry. The NW Mediterranean Sea is an excellent system for studying phytoplankton in relation to the environment. The hydrodynamics of the area share the characteristics of both temperate and subtropical oceans and have clear consequences on the composition and succession of phytoplankton. In the NW Mediterranean, this succession has been described in considerable detail

using oceanic (Marty et al., 2002) and coastal time series (Gutiérrez-Rodríguez et al., 2011; Nunes et al., 2018) or relating the dynamics of phytoplankton groups and hydrographic conditions (Bustillos-Guzman et al., 1995). Briefly, these studies have shown a situation of water column mixing in late autumn and winter with moderate concentrations of chlorophyll *a* (chl *a*) and a phytoplankton community dominated by green algae, haptophytes, and diatoms. In late winter to early spring, a bloom of diatoms and haptophytes with a notable presence of green algae coincides with the beginning of stratification. From this moment on, increasing stratification leads to a progressive decrease in phytoplankton biomass together with an increase in the relative contribution of haptophytes and a substitution of diatoms and green algae by cyanobacteria, mainly *Synechococcus* in early summer and then *Prochlorococcus* in autumn. The cycle closes with a stepwise break in the stratification due to autumn storms and a return to the winter situation. In all this cycle, the contribution of other phytoplankton groups is low except for some sporadic increases. The studies cited above revealing these dynamics are based on the High-Performance Liquid Chromatography (HPLC) analysis of phytoplankton group marker pigments, but there are other works using different techniques that reinforce and add detail to this image. For instance, the prevalence of *Phaeocystis*, a key genus with an important role in oceanic biogeochemical fluxes (Schoemann et al., 2005; Verity et al., 2007), could be revealed in haptophyte blooms reported in the area with the higher taxonomic resolution of microscopy (Arin et al., 2013; Estrada, 1991).

The chemotaxonomic pigment approach has recognized advantages and disadvantages. Among the main advantages are that it captures the entire

phytoplankton community and that with appropriate protocols it can be operator independent. Among the disadvantages are its low taxonomic detail (usually only to class level), its lack of an exact correspondence between taxonomy and pigments, and its reference to phytoplankton biomass as chl *a*. Despite this, it is the main technique used to differentiate phytoplankton groups in global studies or time-series (Anderson et al., 2008; Kramer and Siegel, 2019; Schofield et al., 2017; Uitz et al., 2006). Microscopy, flow cytometry (FCM) and molecular approaches are commonly applied for the study of phytoplankton composition, while other new techniques such as LISST (Laser In-Situ Scattering and Transmissometry), FISH (Fluorescence In Situ Hybridization) or imaging flow cytometry, have shown great promise but are not yet widespread for routine use (Cabello et al., 2016; Hrycik et al., 2019; Leroux et al., 2018). Microscopy offers the best quantitative taxonomic resolution but it depends heavily on operator expertise, presents optical limitation for small size organisms (< 5 µm), routinely uses fixatives that can affect morphology and sample preservation (Caron, 1983), and maintains a low throughput. Flow cytometry is very widespread as it is a relatively simple, fast, and cheap technique and has no bias in picoplankton counting. As a disadvantage, it might suffer the effect of preservatives, it is routinely used to count only pico- and small nanoplankton (< 5 µm), and its taxonomic resolution is limited to *Prochlorococcus* and *Synechococcus* cyanobacteria, and small cryptophytes, although it has occasionally been applied to identify also coccolithophores (von Dassow et al., 2012). Molecular techniques are advancing rapidly and their potential advantages are enormous, although uncertainties associated with commonly used DNA amplification (PCR) or gene copy number variability limits the results to a semi-quantitative approach. In conclusion, the shortcomings of each

technique mean that their combined use provides a great improvement for the study of phytoplankton.

In this study, we use pigments, microscopy and flow cytometry to present a detailed view of phytoplankton composition in three contrasting, well-defined situations of the annual cycle in the NW Mediterranean comprising the MEDOC area centered at 42°N, 5°E, where deep winter convection often mixes the whole water column down to the bottom, at more than 2000 m depth (MEDOC Group, 1970). By combining these techniques, we confirm not only the general dynamics of phytoplankton in the area, but also describe exceptional findings, e.g. the absolute dominance of certain groups during the post-bloom period. Weaknesses of standard techniques for phytoplankton studies are also identified, such as discrepancies in estimates of nanophytoplankton or autotrophic dinoflagellate biomass, for the resolution of which some alternatives are offered. Finally, the ecological preferences of the different phytoplankton groups with respect to depth and nutrient concentrations are revealed and discussed in the context of other ecological features.

2. MATERIAL AND METHODS

2.1. Sampling

This study was conducted in 2009 during three different cruises on board the R/V Sarmiento de Gamboa within the framework of the FAMOSO project. FAMOSO 1 (F1, 6 -23 of March) sampled the pre-bloom to bloom transition, FAMOSO 2 (F2,

29 April - 14 May) the post-bloom period, and FAMOSO 3 (F3, 14 - 22 of September) a fully developed stratification period. The three cruises surveyed a small area of about 100 x 100 km (Fig. 1), far from coastal fronts and centered in a distinct oceanic province of the Mediterranean Sea (D'Ortenzio and Ribera D'Alcalà, 2009). Conductivity, temperature, and depth (CTD) data were obtained with a SeaBird 911Plus multiparametric probe, with additional sensors to measure dissolved oxygen concentration, fluorescence, turbidity, light transmission, and Photosynthetically Active Radiation (PAR) attached to a rosette frame. The water sampling was performed with 24 L Niskin bottles. The sampling strategy was designed for the study of vertical fluxes and adopted a Lagrangian approach. Drifting particle interference traps were deployed, and their movements were followed during 72 h. Therefore, the intra-cruise variability reflects both spatial and temporal changes between stations and within a particular water body. Additional details on sampling can be found in Estrada et al. (2014). The correct functioning of the conductivity and dissolved oxygen sensors was monitored by analysis of a selected number of water samples using onboard salinometer Portasal and Winkler titration methodology, respectively. Oceanographic parameters and their derived variables (e.g. Brunt-Väisälä frequency) were visualized through Ocean Data View software (Schlitzer, 2016). The Mixed Layer Depth was defined as the depth where the density was 0.03 kg m^{-3} higher than at the 5 m reference depth. Integrated values of primary or derived variables obtained by the rosette discrete samples, such as nutrient concentrations or phytoplankton biomass (chl a and C), were estimated for the water column down to a reference depth (usually 100 m) following the trapezoidal rule. For the average values in the water column, the integrated values were divided by the reference depth.

2.2. Nutrients and fluorometric chlorophyll *a*

Samples for inorganic nutrient analyses were collected at 4-6 depths in the upper 100 m in pre-cleaned (10% HCl washed and Milli-Q water rinsed) 1L polycarbonate bottles. Samples for dissolved inorganic phosphorus (PO_4^{3-} , DIP) and dissolved inorganic nitrogen ($\text{NO}_3^- + \text{NO}_2^-$, DIN) were immediately filtered through pre-combusted (450°C, 4 h) 47 mm diameter glass fiber filters (Whatman GF/F) in an acid-cleaned glass filtration system, under low N_2 -flow pressure. Dissolved inorganic phosphorus (PO_4^{3-} , DIP) was determined spectrophotometrically on board using a 10 cm cuvette to increase the detection limit to $0.01 \mu\text{mol L}^{-1}$, while water samples for the determination of dissolved inorganic nitrogen ($\text{NO}_3^- + \text{NO}_2^-$, DIN) were stored in acid-cleaned 60 ml polycarbonate bottles at -20°C for later analysis on land with an AA3 autoanalyzer (detection limit of $0.03 \mu\text{mol L}^{-1}$). Silicate (SiO_4^{3-}) determinations failed and are unavailable for the FAMOSO cruises. Methods were as described in Grasshoff et al. (1999).

For fluorometric chl *a* analysis, 100 mL water samples from 6 - 8 depths between surface and 100 m depth were filtered through 25 mm diameter glass fiber filters (Whatman GF/F) under low vacuum. Filters were frozen for several hours and subsequently placed in 10 mL of 90% acetone and kept at -20°C overnight. Fluorometric chl *a* was estimated from fluorescence readings in a Turner Design fluorimeter calibrated with chl *a* standard (Sigma-Aldrich). No phaeopigment correction was applied.

2.3. Flow Cytometry Analysis

Samples (1.8 mL) for flow cytometry determination of picophytoplankton abundance and biomass were collected at 5 to 9 depths in the upper 100 m. After fixing with 1% paraformaldehyde and 0.05% glutaraldehyde and being kept 10 min at 4°C in the dark, samples were deep frozen in liquid N₂ and stored at -80°C until further analysis. Most analyses were performed on board using a FACS Aria (BD Biosciences) flow cytometer equipped with a laser emitting at 488 nm. Additional analyses were performed on a similar FACSCalibur (BD Biosciences) flow cytometer at the IEO lab in Gijón/Xixón, for which a flow rate calibration was performed daily to estimate the abundance of the different groups. After thawing at room temperature, the samples were run at high speed (ca. 50 µL min⁻¹) for 300 s. Fluorescent latex beads of 1 µm diameter (Molecular Probes) were used as internal standard. *Prochlorococcus* and *Synechococcus* cyanobacteria, cryptophytes, and large and small picoeukaryotes were distinguished based on the different red fluorescence, orange fluorescence and forward angle light scatter or side scatter (SSC) signal as described in detail in Calvo-Díaz and Morán (2006). Picophytoplankton cell volume was estimated through the empirical calibration between SSC and cell diameter of Calvo-Díaz and Morán (2006), assuming spherical shapes (Table S1). Finally, the conversion between cell volume and carbon content used the values suggested by Worden et al. (2004), which ranged from 230 (*Synechococcus*) to 240 fg C µm⁻³ (picoeukaryotes).

2.4. Microscopy Analysis

Samples for micro- and nanophytoplankton (> 2 µm) microscopy were taken at 2 to 4 depths including the mixed layer and deep chlorophyll maximum (DCM) and fixed

with formaldehyde buffered with hexamine (0.6% final concentration). Cell identification was carried out to the group, genus and, when possible, to the species level with a Nikon Eclipse TE2000-S inverted microscope equipped with bright-field and phase-contrast objectives (total magnifications from 100× to 400) using the Utermöhl method (Utermöhl, 1958) and following the indications of HELCOM (2017). The shape of each taxon was defined according to composite geometric bodies extracted from the literature (Hillebrand et al., 1999; Olenina et al., 2006; Sun and Liu, 2003). Cell biovolume for each taxonomic unit was calculated as a revolution body considering the range of two-dimensional cell size measured in the samples. Cell biovolumes were converted to carbon (C) using the factors of Menden-Deuer and Lessard (2000).

Nano- and microphytoplankton C derived from microscopy were estimated only in samples from the surface and the DCM layer. To improve the vertical resolution and integrated C estimated for the water column down to 100 m we assumed that the C: chl *a* ratios were constant within the mixed and the DCM layers. First, we estimated the C: chl *a* ratios for each group in both the mixed and DCM layers. Next, we used the chl *a* values, sampled more frequently, to re-estimate the C values for each microscopy-defined phytoplankton group. Finally, we integrated phytoplankton C in the water column down to 100 m depth for each group. Autotrophy was determined according to the genera or if plastids could be observed in the cells. However, there was a major proportion of dinoflagellates (mostly athecate) for which neither taxonomic affiliation nor chloroplast presence could be defined. To estimate the autotrophic dinoflagellate C in the >20 µm fraction, we applied a factor for each of the cruises based on the C in identified genera considered as generally autotrophic

(*Margalefidinium* (formerly *Cochlodinium*), *Oxytoxum*, *Tripos* (formerly *Ceratium* or *Neoceratium*), *Gymnodinium*, *Amphidinium*...) and generally heterotrophic (*Gyrodinium*, *Protoperidinium*, *Dinophysis*...). The autotrophs/total ratio from the identified genera averaged for each cruise (0.61 in F1, 0.49 in F2 and 0.40 in F3) was multiplied by the total (autotrophs, heterotrophs plus unknown) dinoflagellates of each sample to obtain the C estimates of autotrophic dinoflagellates in the >20 µm fraction. Despite averaging produced a loss of information, it provided more robustness to the data by reducing uncertainty of the individual samples due to low counts of well-identified dinoflagellate species. For the < 20 µm fraction, cells were mostly counted as flagellates except when clear morphologies could be recognized, e.g. dinoflagellates or coccolithophores. A 50% of autotrophic C was assigned to the < 20 µm fraction of flagellates and dinoflagellates based on the literature (Table 1 in Christaki et al. (1999), Fig. S2 in Edwards (2019), Fig. 7 in Aytan et al. (2018))

2.5. HPLC Pigment Analysis

Pigment concentrations were estimated by HPLC analysis from samples obtained at 4 to 6 selected depths including always the mixed and DCM layers. Two-liter water samples were filtered through glass fiber filters (Whatman GF/F), which were folded, blotted dry and stored in liquid nitrogen on board and at -80 °C later in the laboratory. For pigment extraction, filters were placed in 2.5 mL of 90% acetone, sonicated and stored at -20 °C overnight, and the extract was subsequently filtered through Whatman GF/F filters. Samples were run in an Agilent (Waldbronn, Germany) HPLC 1200 system with a G1311A quaternary pump, a G2258A autosampler, a G1316B column thermostat, and a G1315C diode array detector.

Absorption signals recorded were 472 ± 8 nm for carotenoids and 650 ± 40 nm for chlorophylls. The separation method was based on Zapata et al. (2000) with slight modifications. Solvent A was methanol: acetonitrile: 0.05 mol L⁻¹ aqueous pyridine solution (pH = 5), 50:25:25 (v:v:v); solvent B was acetonitrile: methanol: acetone, 60:20:20 (v:v:v). The mobile phase gradient was (minutes, %A, %B): (0, 100%, 0%), (4, 90%, 10%), (19, 62%, 38%), (26, 20%, 80%), (38, 0%, 100%), (45, 0%, 100%). Flow rate was kept at 1 mL min⁻¹ and temperature at 25 °C. Spectra obtained with the absorption detector showed that during F3 the peak corresponding to the alloxanthin retention time was a 19'hexanoiloxifucoxanthin (19'hex-fuco) derivative, revealing that our method could not separate alloxanthin from this contaminant. Because of the high correlation between 19'hex-fuco and its derivative ($r^2 = 0.94$), we used the 19'hex-fuco measurements to calculate the concentration of this contaminant and subtracted its contribution from the alloxanthin + contaminant peak to obtain the alloxanthin values for F1 and F2 samples.

2.6. Pigment Data Treatment and CHEMTAX Analysis

For exploring and organizing the pigment data, samples were clustered according to the value of the logarithm of pigment to chl *a* ratios of the following pigments: chl *c*₃, chl *c*₂, peridinin, 19'butanoiloxifucoxanthin, fucoxanthin, 19'hexanoiloxifucoxanthin, alloxanthin, zeaxanthin, chl *b*, prasinoxanthin and divinyl chl *a*. When accessory pigment concentrations were below detection, the zero value of the pigment ratio was substituted by 1/3 of the minimum value of that ratio for the whole series to avoid indetermination of the logarithm. The clustering

was based on the Pearson r correlation as distance and using the Ward clustering method (Statistica software). Pigment clustering analysis distinguished 5 major sample associations, which included samples from one or more cruises (Fig. S1). During F1 there were 2 associations of phytoplankton pigments, in F2 there were 4 associations and in F3 there was one association. Cluster 1 samples were located only in the mixed layer during F2. Clusters 2 and 5 comprised adjacent samples, containing all samples of F1, the haptophyte-dominated stations of F2 (see below), and DCM and deeper samples in other stations of F2. Cluster 3 comprised samples only from the upmost surface layer in F2. Cluster 4 comprised exclusively all the samples of F3.

CHEMTAX analysis was applied separately to each cluster of samples following the procedures of Latasa (2007) to estimate the contribution of cryptophytes, diatoms, dinoflagellates, green algae, haptophytes, pelagophytes, *Synechococcus* and *Prochlorococcus* to the total chl a . The final outputs of pigment to chl a ratios used to calculate the contribution of each group to total chl a concentration are shown in Table S2. From now on, the chl a ascribed to each group by this CHEMTAX procedure will be used as an estimator of biomass, rather than FCM and microscopy-derived C . We decided to use pigments as the basis of our comparative study because it is the only method that encompasses the whole phytoplankton community, making the comparison between groups more consistent.

2.7. Phytoplankton Size Fractions

Phytoplankton size fractions were estimated independently using pigments (chl *a* - based) and microscopy plus FCM (C-based). For the pigment approach the different taxonomic groups were associated to nominal size fractions following Vidussi et al. (2001), i.e. *Prochlorococcus*, *Synechococcus* and green algae were considered as picoplankton (< 2 µm); haptophytes, pelagophytes and cryptophytes as nanoplankton (2-20 µm); and diatoms and dinoflagellates as microplankton (>20 µm). Thus, the groups quantified with CHEMTAX were separated into size classes according to this classification. For comparison, we also applied the Uitz et al. (2006) approach (hereafter UITZ), a widely used method based on pigments for estimating the contribution of the three size fractions. Unlike CHEMTAX, this approach gives fixed weights (coefficients) to the groups' marker pigments. In addition, we used Solver in Excel to propose new coefficients for the UITZ approach to fit the groups contributions obtained with CHEMTAX. The target of Solver was to change UITZ coefficients to minimize the sum of the 1-slopes (i.e. the divergence from the 1:1 line) and intercepts between CHEMTAX and UITZ results. Slopes larger than 0.92 and intercepts less than 10 ng L⁻¹ were the only constraints of the model. Because we realized that the output was sensitive to the initial values of the coefficients, we started with a value of 1 for all pigment-marker to chl *a* ratios, since we dealt with major accessory pigments with concentrations comparable to chl *a*.

Phytoplankton C contribution to size fractions was estimated by microscopy and FCM as described above.

2.8. Ecological preferences

The relative contribution of the different groups as a function of nutrient concentration (DIP and DIN) and depth, down to 100 m, was examined to reveal their ecological preferences. The general patterns were described by means of a smooth function (SigmaPlot software). The chosen smoothing method was a Negative Exponential, a Gaussian function to weight the data and a linear fit in our case. The parameters used were: Interval = 25, Bandwidth = nearest neighbor, sampling proportion = 4, polynomial degree = 1 (linear regression). These parameters indicate that there are 25 points (locations or depths in our case) for the x-axis (i.e., every c.a. 4 m for the 100 m water column), and 0.4 (40%) of the data from the closest depths (weighed by the Gaussian function) were used in the smoothing process at each depth. The chosen values represent a compromise between too jagged and too smoothed lines.

3. RESULTS

The hydrographic conditions of the cruises went from almost complete mixing of the water column during the initial stations of F1 to a pronounced stratification in F3, with a somehow intermediate situation in F2 (Fig. 2). Potential density (σ_0) in the upper layer decreased gradually from F1 to F2 to F3, as it became warmer and slightly less saline (Fig. S2). Consequently, the water column stability, expressed by the Brunt-Väisälä frequency, followed the same evolution, with highest values during F3 between 30 and 50 m, at the strong thermocline of the fully developed stratification (Figs. 2 and S2). Superimposed to the seasonal variability, there was substantial mesoscale heterogeneity, as shown by the near-surface high salinity waters between stations 21 and 24 in F1, the low salinity waters in the upper layers

of stations 11-15 in F2, and the slightly lower temperatures and higher salinities at the surface in the last two stations of F3 (Fig. S2).

The concentration of dissolved inorganic nitrogen (DIN) and phosphorus (DIP) in the upper mixed layer decreased from F1 to F2 to F3 (Fig. 2, Table 1). Surface DIN concentration was always above $3 \mu\text{mol L}^{-1}$ during F1 (5.1 ± 2.1 , mean $\pm$ S.D., $n=8$), decreasing to $1-4 \mu\text{mol L}^{-1}$ in F2 (1.9 ± 1.0 , $n=14$), and to $0.7-2.1 \mu\text{mol L}^{-1}$ in F3 (1.7 ± 0.5 , $n=5$). DIP followed the same temporal and spatial pattern as DIN, with concentrations in the upper mixed layer ranging from $0.06-0.36 \mu\text{mol L}^{-1}$ in F1 (0.16 ± 0.11 , $n=10$), $0.009-0.080 \mu\text{mol L}^{-1}$ in F2 (0.030 ± 0.022 , $n=14$) and $0.016-0.049 \mu\text{mol L}^{-1}$ in F3 (0.032 ± 0.012 , $n=6$). Median DIN/DIP ratios in surface waters (excluding ratios with $\text{DIP} < 0.01 \mu\text{mol L}^{-1}$) were 35, 55 and 43 for F1, F2 and F3, respectively.

Except for the first biological station (F1-08), which presented strong deep winter mixing and low chl *a* concentrations (Fig. 3), the F1 cruise sampled the winter-spring bloom situation, with a weak stratification, high nutrients and enhanced phytoplankton biomass distributed quite homogeneously in the upper 40 m layer (Fig. 2). The average chl *a* concentration during F1 was $0.84 \mu\text{g L}^{-1}$ in the upper 100 m (Table 2). The most abundant phytoplankton groups according to their contribution to chl *a* were haptophytes (32%), diatoms (23%), and green algae (19%), followed by *Synechococcus* (11%), pelagophytes (9%), dinoflagellates (4%) and cryptophytes (2%) (Tables 2, 3 and S3; Fig. 3). In terms of C, microscopy assessed a greater contribution of diatoms, mainly through large ($>20 \mu\text{m}$) and chain forming diatoms, which made up 37% of the bulk phytoplankton (Table 2),

and a smaller contribution of nanoflagellates (2-20 μm , 9%) compared to pigment-based estimates of diatoms and haptophytes. Microdinoflagellates ($>20\ \mu\text{m}$) and nanodino­flagellates accounted for 5% and 2% of phytoplankton C, respectively. The dominance of diatoms was due to their overwhelming contribution (61-73% in C) at two stations (F1-27 and F1-31, Table 4). The picophytoplankton community was composed by eukaryotes and *Synechococcus*, which contributed 25 and 18% of the total autotrophic C, respectively. FCM analysis revealed very low concentrations of *Prochlorococcus* cells, not detected by divinyl chl *a*, their characteristic pigment marker.

The hydrodynamic and biological conditions during F2 (the post-bloom situation) were very heterogeneous. The stratification was weak and shallow, but stronger than in F1 (Table 1). The shallowest mixed layers of the study were found during this cruise (Table 1). Nutrient concentrations were lower than during F1 in the upper 40 m, and the chl *a* concentration had decreased in relation to the latter stages of F1 to $0.30\ \mu\text{g L}^{-1}$ in the upper 100 m (Fig. 2, Table 2). On average, *Synechococcus* contribution to chl *a* was the highest (36%), followed by haptophytes (25%), pelagophytes (13%), green algae (11%), cryptophytes (8%) and diatoms and dinoflagellates (3% each), which contributed the least (Fig. 3). Microscopy and FCM estimates of C (Table 2) confirmed the high contribution of *Synechococcus* to C (55%) followed further behind by microdinoflagellates (14%), nanoflagellates and picoeukaryotes (9% each), nanodino­flagellates (7%) and microdiatoms (2%). The spatial dynamics of the phytoplankton groups during this cruise in the post-bloom period revealed some uncharted features in this area. The most striking finding was the overwhelming dominance of *Synechococcus*,

reaching concentrations up to 4.2×10^5 cells mL⁻¹, equivalent to 60% of the
 integrated chl *a*, >90% of the picophytoplankton C and > 80% of the total
 phytoplankton C, at the initial stations (Figs. 3, 4 and S3, Tables 5 and S3). A weak
 and broad DCM was present between 30 and 60 m depth. Exceptions to this
 pattern were observed in F2-40 and, especially, F2-44, in the southern part of the
 studied area (Figs. 3 and S3), sampled at the end of the cruise. Here, the
 stratification increased, chl *a* concentrations were higher with a clear DCM around
 50 m, and haptophytes were the most important group representing up to 50% of
 the phytoplankton community (Figs. 3 and S3; Tables 3 and S3). At these stations
 there was also an increase of large picoeukaryotes detected by FCM (Figs. 4 and
 6), and of microscopy-derived abundances of large dinoflagellates, diatoms and
Mesodinium rubrum (Table 4), a ciliate with cryptophyte-type pigments.
 Nanophytoplanktonic flagellates also increased, but to a lesser extent. Another
 interesting feature was found at stations F2-20 to F2-32. We found here the
 shallowest mixed layers (Table 1) and abundant cryptophytes (~20% of integrated
 chl *a*, Figs. 3, 4, and S3) particularly in the upper layers where they tended to
 accumulate (~30% of chl *a* in shallow samples). This increase agreed with high
 counts of nanoplanktonic cryptophytes by FCM (up to 900 cells mL⁻¹, Fig. 4). It is
 worth noting the very low concentrations of diatoms during F2, especially on the
 stations where *Synechococcus* was very abundant. Diatom concentrations were
 similar or even lower than during the highly stratified conditions of F3 in September
 (Tables 2, 3 and 4). There was a discrepancy in the contribution of autotrophic
 dinoflagellates, which represented 3% or 21% of the phytoplankton according to
 pigment (chl *a*) or microscopy (C) data, respectively (Table 2).

The F3 cruise showed a typical fully developed stratification situation of mid-latitudes characterized by a thick mixed layer with low nutrient and phytoplankton concentrations (chl *a* ~0.11 µg L⁻¹), clearly isolated from subsurface nutrient-rich waters by a strong thermocline between 40 and 60 m depth. The depth of a distinct DCM (> 0.6 µg L⁻¹) varied between 50 and 80 m, and the average chl *a* concentration in the upper 100 m was 0.32 µg L⁻¹ (Fig. 3, Table 2). Phytoplankton was dominated by haptophytes and *Synechococcus* in the mixed layer (>40% and >30%, respectively) and by haptophytes and pelagophytes at the DCM and deeper. *Prochlorococcus* contribution was highest (~30-40%) just in the upper part of the DCM (Figs. 3 and 4). Cryptophytes were under our detection limit for pigment, FCM and microscopy analyses (Tables 3, 5). There was a reasonable agreement in the total contribution to the phytoplankton community as pigment or C (Table 2) by *Prochlorococcus* (23% vs 26%) and *Synechococcus* (10% vs 17%). As for F2, there was a discrepancy in the contribution of dinoflagellates, 5 and 35% depending on pigment or microscopy information, respectively (Table 2). Picoeukaryotes (9%) and nanoflagellates (7%) were the other main groups in terms of C.

The distribution of phytoplankton in the three canonical size fractions (pico-, nano- and micro-) was different if estimated as C from FCM and microscopy, or as chl *a* according to CHEMTAX estimates (Table 2, Fig. 5). The major discrepancy was for nano- and microphytoplankton. On one hand, microscopy estimates of microphytoplankton contribution (which includes chain-forming diatoms smaller than 20 µm) to phytoplankton C were 45, 20 and 28% for F1, F2 and F3, respectively. Pigment estimations rendered a substantially lower contribution of

microphytoplankton to total chl *a* across all cruises (F1=27%, F2=6% and F3=12%
Table 2). On the other hand, the estimates for the nanophytoplankton fraction were
11, 16 and 20% in terms of C, markedly lower than the 43, 46 and 50% obtained in
terms of chl *a* for F1, F2 and F3, respectively. Finally, the estimates for the
picophytoplankton fraction using FCM were 44, 64 and 52% in terms of C, and 30,
48 and 38% in terms of chl *a* for F1, F2 and F3 (Table 2).

4. DISCUSSION

4.1. Biomass estimates and methodological considerations

The combination of pigments, FCM and microscopy revealed the strengths and the
weaknesses of the different techniques. On the other hand, the integration of
complementary techniques provides robustness to the interpretation of our results.
HPLC (in the laboratory) and fluorometric estimates (on board) of chl *a* presented a
high correlation ($\text{Fluorometric chl } a \text{ (ng L}^{-1}\text{)} = 1.10 \text{ HPLC chl } a + 8.8$, $r^2=0.92$). The
estimates of phytoplankton size fractions obtained by CHEMTAX and UITZ
methods also showed a high correlation (Fig. 6). In these two pigment-based
approaches, picoplankton is represented by cyanobacteria and green algae,
microplankton by diatoms and dinoflagellates, and nanoplankton by the other
pigment groups. The assignment of fixed sizes to taxonomic groups as well as the
uncertainty caused by some phytoplankton classes sharing some marker pigments
(Chase et al., 2020) makes size classification by pigments a rough approximation.
It is intended to be applied with caution and preferably to large scale and global
data sets, and not as an exact measure for a particular region or ecosystem (Uitz

et al., 2006). To reduce part of this uncertainty for the NW Mediterranean, we tuned the coefficients used in the original UITZ approach to fit our CHEMTAX results (see M&M section) resulting in the following new coefficients (new/original): cryptophytes (alloxanthin) 1.06/0.60, diatoms (fucoxanthin) 0.62/1.41, dinoflagellates (peridinin) 1.21/1.41, green algae + *Prochlorococcus* (chl *b*) 1.02/1.01, haptophytes (19'hex-fucoxanthin) 0.79/1.27, pelagophytes (19'but-fucoxanthin) 0.94/0.35 and cyanobacteria (zeaxanthin) 1.33/0.86. Figure S4 shows the results using these new coefficients. We went further to provide a slightly modified algorithm that discriminates the contribution of green algae, *Prochlorococcus* and *Synechococcus*. The resulting coefficients of the new algorithm are shown in Fig. S4. This adjustment exercise with a different methodology has been applied for other databases (Brewin et al., 2017; Di Cicco et al., 2017). Here, we provide what we consider to be better coefficients of the UITZ approach for the NW Mediterranean Sea.

There was a high correlation between abundance estimates of various phytoplankton groups (*Synechococcus*, *Prochlorococcus* and small cryptophytes) based on FCM-derived C and pigment data (Fig. 7). This comparison revealed a close relationship between FCM-derived C from small picoeukaryotes (overall cell diameter $1.45 \pm 0.12 \mu\text{m}$, Table S1) and the chl *a* biomass of green algae (suggesting that they represented a substantial part of the picoeukaryotes). Also, the strong correlation between FCM-derived C for large picoeukaryotes (cell diameter $1.97 \pm 0.17 \mu\text{m}$) and chl *a* from haptophytes suggests that a significant part of this nanoplanktonic group contributes to the FCM-picoplankton fraction (Fig. 7). Cabello et al. (2016), using epifluorescence observations of FISH-marked algae

in the Cantabrian Sea, estimated that 88% of haptophyte and 93% of pelagophytes cells are in the 2-5 μm fraction, largely recorded with our FCM settings (up to 4 μm). Yet, there was no good correspondence between large (or small) picoeukaryotes (FCM) and pelagophytes (pigments) in our data (Fig. 4). We hypothesize that the lower contribution of pelagophytes (12% vs. 29% of haptophytes over all cruises) was the reason for their minor influence in the variability of the bulk of large picoeukaryotes. An interesting result was the strong correlation between pigment-based and FCM-based cryptophyte abundances (mean cell diameter of 2.4 μm , Table S1), indicating a considerable presence of very small cells in this group (Figs. 4 and 7). It is worth to note that the population dynamics of haptophytes and cryptophytes, usually considered as nanoplanktonic, are well reflected by their representatives in the FCM <3 μm fraction (Figs. 4 and 7). If this result is confirmed by other studies, it opens a window of opportunity for FCM studies since haptophytes and cyanobacteria, with a significant participation of pelagophytes, are usually the main phytoplankton components in oligotrophic areas.

The comparison between pigments and microscopy results revealed some important information. Table 2 shows that for the three situations (cruises) the contribution of nanophytoplankton is lower as C (microscopy) than as chl *a*. For microphytoplankton the situation is the reverse: higher contribution as C than as chl *a*. For diatoms, a main microphytoplankton group, the contribution is rather similar as C or chl *a* (Table 2), C and chl *a* co-vary significantly (Fig. 7) and the average C:chl *a* ratio for the 3 cruises is 44 ($\pm$ 65, n = 23, not shown), a reasonable value for all the different environments sampled. On the other hand, the estimates for

dinoflagellates, the other dominating microphytoplankton group, are quite
 intriguing. Dinoflagellate C and chl *a* do not show a correlation overall but co-vary
 within F1 and F2 (Fig. 7). The average C: chl *a* of dinoflagellates was 31, 490 and
 150 for F1, F2 and F3, respectively (not shown). The very high C: chl *a* ratios
 indicate that either C was overestimated or chl *a* underestimated for dinoflagellates
 in F2 and F3. The presence of dinoflagellates with pigments other than peridinin,
 the standard pigment used here for pigment estimates, is not uncommon (Zapata
 et al., 2012) and would have resulted in pigment underestimation of the real
 contribution of dinoflagellates. On the other hand, there are some lines of evidence
 that indicate that dinoflagellate C was overestimated by microscopy, especially in
 the microphytoplankton fraction. First, the contribution of microphytoplankton
 during F2 and F3, when meso- and oligotrophic conditions prevailed, is expected to
 be minimal. Delgado et al. (1992) using epifluorescence microscopy estimated that
 the C contribution of nano- and microphytoplankton in stratified conditions in the
 area was 72% and 6%, respectively. The C contributions of dinoflagellates larger
 than 20 µm in our samples were the second highest after *Synechococcus* in F2
 (12%) and *Prochlorococcus* in F3 (23%). Therefore, autotrophic dinoflagellate
 contribution in these two cruises was likely overestimated. In summary, the best
 explanation to our results of F2 and F3 is a combination of a significant portion of
 heterotrophic dinoflagellates incorrectly assigned as autotrophic and the presence
 of autotrophic forms with pigment markers different from peridinin.

The low C biomass and contribution of nanophytoplankton relative to pigment-
 based values (Table 2) was intriguing. To begin with, the nanophytoplankton
 contribution of pigment groups is overestimated since haptophytes and

pelagophytes (considered nanoflagellates) appear to make a substantial
 contribution to the FCM picoeukaryote size fraction. In addition, the nanoplanktonic
 fraction was likely underestimated by microscopy because of technical limitations
 to observe the smallest cells in this fraction. We made an effort to count cells down
 to 2 μm but there are some inherent difficulties such as the unsatisfactory fixation
 and settlement of small cells in the samples, which could contribute to the
 underestimation of the nanophytoplanktonic fraction by microscopy. Indeed, Leroux
 et al. (2018) using the Laser In-Situ Scattering and Transmissometry technique
 (LISST) in the same area during the spring bloom of 2013 claimed that microscopy
 estimates of nanoplankton could be as low as 15% ($\sim 1/6$) of the real value.
 Because LISST cannot differentiate between autotrophic, heterotrophic and
 detritus particles, it is likely that the real underestimation by microscopy, although
 existing, is smaller than the values presented by these authors. Leroux et al (2018)
 reported that LISST overestimated traditional FCM values by a factor of 3. Since
 we consider that FCM is a reliable counting method, we conclude that C estimates
 of nanophytoplankton by microscopy could be around half of the real value. In
 agreement with this, microscopy estimates are 30 - 60% of the contribution of
 nanophytoplankton estimated by the pigment approach (Table 2). Mena et al.
 (2019) also concluded that light microscopy underestimates the abundance of the
 nanophytoplankton fraction in comparison to epifluorescence microscopy by a
 factor of 2.5. By applying our correction (x2), the nanophytoplankton C contribution
 would increase to 28% and 33% in F2 and F3, respectively, and
 microphytoplankton C contribution would decrease accordingly to 17 and 23%
 (data not shown). Because of the apparently overlooked importance of small
 nanophytoplankton (Leblanc et al., 2018; Nunes et al., 2019) we suggest extending

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574 routine FCM analyses, perhaps by running the sample a second time, to include a
575 representative number of cells in the 2-10 μm range.

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577 As a precautionary methodological note regarding the extrapolation of cell
578 concentrations to C using fixed conversion values, it should be noted that changes
579 of cell size with depth in the picophytoplankton components (Fig. 8) were very
580 similar to those described by Latasa et al. (2017) in the Cantabrian Sea and by
581 Mena et al. (2019) in the western Mediterranean under stratified, oligotrophic
582 conditions. Cell size increase with depth for *Synechococcus* and *Prochlorococcus*
583 is well known (Binder et al., 1996; Olson et al., 1990). Casey et al. (2013)
584 suggested that this pattern in *Prochlorococcus* and *Synechococcus* is due to the
585 fact that a larger pigment system is needed to cope with low irradiances, resulting
586 in an increase in the size of the small cells of these groups. As an alternative,
587 possible changes in *Synechococcus* or *Prochlorococcus* strain assemblages with
588 depth or throughout the seasons cannot be discarded (Hunter-Cevera et al., 2016;
589 Johnson et al., 2006; Scanlan and West, 2002). The comparison of the profiles in
590 the different cruises (Fig. 8) indicates that the change in volume with depth is a
591 temporal process occurring at scales of weeks or longer, which would allow the
592 rearrangement of different clades in the water column (Malmstrom et al., 2010). On
593 the other hand, more than to physiological or ecological responses, we attribute the
594 decrease in size of large picoeukaryotes to the increasing contribution with depth of
595 pelagophytes (Fig. 3), a group with smaller cell sizes than haptophytes (Cabello et
596 al., 2016), the latter making up the bulk of FCM-defined large picoeukaryotes.

597 598 4.2. Biomass distribution

Total phytoplankton biomass followed the general trend described for this area and the surrounding coastal waters, with a late winter - early spring bloom and increasing oligotrophic conditions until early fall (Bosc et al., 2004; D'Ortenzio and Ribera d'Alcalà, 2009; Nunes et al., 2018). Averaging over the 3 cruises, the dominant groups in terms of pigment biomass were haptophytes followed by *Synechococcus*, pelagophytes, green algae, diatoms, *Prochlorococcus*, cryptophytes, and dinoflagellates (Table 2). The temporal patterns of variability of these groups, a bloom dominated by diatoms and haptophytes with a significant contribution of green algae, a post-bloom situation dominated by haptophytes and *Synechococcus* and a stratification period dominated by haptophytes, pelagophytes and *Prochlorococcus* was comparable to the general trends found in previous studies (Latasa et al., 2010; Marty et al., 2002).

4.2.1. F1 Bloom

For the discussion of the spring bloom we will leave apart the deeply mixed situation before any stratification set on (F1-08); this station had low biomass ($0.2 \mu\text{g L}^{-1}$) and a phytoplankton community dominated by green algae, diatoms and haptophytes, typical of winter in the area (Bustillos-Guzman et al., 1995; Gutiérrez-Rodríguez et al., 2011; Nunes et al., 2018).

During the bloom ($\text{chl } a > 0.5 \mu\text{g L}^{-1}$), there was a complex situation with haptophytes, diatoms and green algae dominating the phytoplankton community. Mesoscale processes, baroclinic instabilities and stratification-destratification events associated to the end of deep, violent mixing episodes (Salat et al 2010), as

well as some storms (the cruise had to stop twice, on March 16 and 20 because of strong winds) produced an interleaving of high and low salinity waters in the surface layers (Estrada et al., 2014) and precluded the establishment of clear temporal or spatial patterns in water column stratification and bloom development. It appears that we sampled the early, unstable stages of the bloom onset typical of these dates (Lévy et al., 1999; Severin et al., 2014). This could explain the important contribution of green algae, a picoplanktonic group not often revealed in spring bloom studies but with increasing evidence of its important contribution during this period (e.g. Bolaños et al., 2020). The mixed dominance of diatoms and haptophytes, however, is well documented (Latasa et al., 2010; Marty et al., 2002; Severin et al., 2017). Severin et al. (2017) explained that, for their 2013 study, only stations where previous convection mixing had reached the seafloor presented silicate to nitrogen ratios high enough for diatoms to outcompete other groups. Our results seem to confirm this hypothesis. Our silica analysis failed and the relatively low number of stations (8) with little variance in nitrogen concentrations prevented us from seeing clear patterns, but the contribution of diatoms tended to be higher with higher salinities in the upper 50 m ($p=0.13$, data not shown) indicative of deep mixing of surface waters. Occasional samples obtained with a phytoplankton net (20 μm mesh size) were observed fresh during F1 and allowed us to qualitatively distinguish two plankton communities: a more mature one in which zooplankton, detritus and some *Phaeocystis* colonies were evident, and a younger one with presence of diatoms and healthy-looking cells, and less abundant zooplankton (Fig. S5). The presence of *Phaeocystis* in phytoplankton proliferations in the NW Mediterranean has already been reported in association with recently advected Atlantic waters (Estrada, 1991; Estrada et al., 1999) and shows that this genus can

649 compete well with diatoms when the water column starts to stratify after shallow
650 mixing conditions and lower Si/N ratios.

652 4.2.2. F2 Post-bloom

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654 During the F2 post-bloom cruise in April the stratification was relatively weak and
655 shallow (Fig. 2), nutrients in the surface layer had decreased relative to F1, and
656 phytoplankton biomass was also lower (Table 1, 2). According to previous studies
657 during this period in the area (Latasa et al., 2010; Marty et al., 2002; Vidussi et al.,
658 2000) we expected a diverse community typical of pre-oligotrophic conditions, with
659 haptophytes, pelagophytes, green algae, cryptophytes and *Synechococcus* making
660 up the bulk of the phytoplankton biomass. However, we found a high spatial
661 variability with stations clearly dominated either by *Synechococcus*, haptophytes or
662 cryptophytes (Figs. 3 and S3). This spatial heterogeneity in phytoplankton group
663 distribution during F2 was not clearly related to the environmental variables
664 analyzed in this study. It must be noted that subtle differences in physico-chemical
665 properties of different water bodies may be a consequence of different
666 hydrographical histories and of distinct selection pathways of their associated
667 phytoplankton populations.

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669 The most unexpected result was the very high concentrations of *Synechococcus* in
670 a large area, a new report for the Mediterranean Sea although Marty et al. (2002)
671 in a 8-year time-series in the area present spikes of zeaxanthin concentration
672 above 5 mg m⁻² in two occasions (roughly half the values measured in F2). Except
673 for the exceptional Costa Rica Dome, where *Synechococcus* concentrations can

reach 10^6 cells mL⁻¹ during the summer upwelling season (Saito et al., 2005), dominance of *Synechococcus* in open waters has been seldom described. To our knowledge, it has only been reported in the oligotrophic Eastern Mediterranean (Denis et al., 2010), the Arabian Sea (Bidigare et al., 1996), and the Atlantic Ocean (Partensky et al., 1996), the latter two in mesotrophic conditions. Denis et al. (2010) reported *Synechococcus* contributions >60% of the ultraplankton (<5 µm) biomass in an anticyclonic gyre, but with only 6% of the total chl *a* concentration we found in F2. Partensky et al. (1996) reported abundances up to 5×10^5 cells mL⁻¹, comparable to those found here, in a community with an important contribution of diatoms, suggesting a post-bloom situation. Although the average concentration of chl *a* in the water column during F2 was similar to that in F3 (297 vs. 318 ng L⁻¹, Table 2) the surface concentration during F2 was 3-4 times higher than during F3 (Fig. 3), therefore, hinting that these waters were not as oligotrophic as indicated in terms of integrated phytoplankton biomass. Also, primary production was about 3 times higher in F2 than in F3 (Estrada et al., 2014), and additional hydrographic data (not shown) hinted at a weak deep upwelling reaching the euphotic zone in F2 (Salat et al., 2013). It is intriguing that, in coincidence, the Costa Rica Dome is a recurrent, weak upwelling system. In the coastal Mediterranean Sea, Bustillos-Guzman et al., (1995) found the highest *Synechococcus* concentrations under intermediate mixing conditions, and data from Nunes et al. (2018) in a 15-year time-series study of a coastal site in the NW Mediterranean, show a consistent peak of *Synechococcus* after the spring bloom. High temperature has been considered a beneficial factor since *Synechococcus* blooms are commonly described in coastal tropical and sub-tropical waters (Beardall, 2008), and their optimal growth temperature in the coastal NW Mediterranean has been reported at

~24 °C, with no growth when temperature drops below 11 °C (Agawin et al., 1998).

The strong dominance of *Synechococcus* in the open NW Mediterranean at 15 °C suggests that coastal clades are likely different from those found in our study area. Hunter-Cevera et al. (2020) showed that *Synechococcus* reached maximum growth rates at c.a. 14 °C for the spring population in the northeast U.S. shelf. The existence of multiple strains of *Synechococcus* with different physiological capabilities (Pittera et al., 2014; Scanlan et al., 2009) confounds the interpretation of the ecological features of this group, often considered as a single population, highlighting the need to consider the diversity of this genus. The lower contribution of *Synechococcus* in the southern stations coincided with an increase in phytoplankton biomass and haptophyte contribution (Figs. 3 and S3). The only clear difference was the stronger stratification in these latter stations, further supporting that semi-mixed conditions appear as a determinant factor for the dominance of *Synechococcus* under nutrient limitation (Bustillos-Guzman et al., 1995). The existence of only few reports of open ocean *Synechococcus* blooms (Bidigare et al. 1996; Partensky et al., 1996; this study) indicates that these episodes are quite unusual and the potential factors driving them are still unclear.

Cryptophytes were especially abundant in the surface layers of some stations of F2. Significant contribution of cryptophytes in the NW Mediterranean had been reported (Estrada, 1985; Latasa et al., 1992), and Marty et al. (2002) results show 3 cruises in their 8 year time series studies with concentrations of alloxanthin around 4 mg m⁻², in the same order as the 2.2 mg m⁻² reached in F2 (data not shown). Flow cytometry showed a parallel increase of small cryptophytes (Figs. 4 and 7). Microscopic observation confirmed that *Mesodinium rubrum*, a ciliate that

feeds on cryptophytes and incorporates their chloroplasts as endosymbionts, was
 abundant (Table 4) but its distribution differed slightly from cryptophyte pigment
 concentrations and FCM abundances, perhaps indicating the detrimental effect of
 this mixotroph on cryptophyte abundances (Johnson et al., 2013). We suspect that
 our previous report of cryptophytes as an eutrophic group with preference for deep
 waters (Latasa et al., 2010) could have been misled by the contamination of the
 alloxanthin peak described in M&M section, although Marty et al. (2002) and
 Vidussi et al. (2000), also using pigments, reported high cryptophyte
 concentrations during nutrient-rich conditions. During F2, cryptophytes dominated
 in some northern stations (Figs. 3 and S3) without any obvious explanation except
 very shallow mixed layers (Table 1). High concentrations of cryptophytes have
 been described in estuaries usually related to low salinities (Johnson et al., 2013)
 and in surface layers associated to stratified, relatively low salinity water masses in
 coastal Antarctica (Nunes et al., 2019; Rodriguez et al., 2002; Schofield et al.,
 2017). Since surface salinity in cryptophyte-dominated stations in F2 was higher
 than in the *Synechococcus*-dominated stations (Fig. S2) it appears that strong
 shallow stratification is more important than low salinity for cryptophytes to thrive in
 the NW Mediterranean.

A noteworthy result was the very low concentrations of diatoms during the cruise,
 confirmed by microscopy (Table 4). Unfortunately, silicate samples were lost and
 the influence of this essential nutrient for diatoms on their dynamics cannot be
 assessed. Based on studies on competitive exclusion, Cermeño et al. (2011)
 indicated that taxa with slow nutrient uptake are favored over opportunistic taxa
 such as diatoms in bloom-post bloom transitions (after reduction of nutrient supply).

Vidussi et al., (2000) in a study in the area during the same transition period suggested, based on their vertical distribution, that diatoms are more limited by phosphorus than nanophytoflagellates. In addition to nutrient dynamics, the biomass of the mesozooplankton community was the highest among the 3 cruises (Isla et al., 2015). We hypothesize that this high zooplankton presence together with low DIP concentrations produced the minimum values of diatoms found in our study. The contribution of diatoms considered as typical of summer assemblages (e.g. *Leptocylindrus* spp, *Hemiaulus hauckii*) increased appreciably from F1 to F2 but not from F2 to F3, discarding the explanation of an increase of diatoms adapted to the severe oligotrophic conditions in F3 (Table S4).

4.2.3. F3 Stratification

The physical and chemical conditions in F3 were typical of a fully developed stratification period in temperate seas. The phytoplankton community was composed mainly by haptophytes, pelagophytes and *Prochlorococcus*, with lower contributions of *Synechococcus*, diatoms, dinoflagellates, and green algae (Table 2). There was a clear vertical differentiation, with haptophytes and *Synechococcus* contributing the most in the surface mixed layer, and haptophytes, *Prochlorococcus*, and pelagophytes peaking around the DCM layer. This partition agrees with Latasa et al. (2010) for the study area, and with systems with low biomass but not extreme oligotrophy (Araujo et al., 2017; Barlow et al., 1993, 1997). There was some spatial variability in the distribution of the groups, especially *Prochlorococcus*. The low number of samples preclude a clear

773 explanation, but a lower contribution of this group (Table S3) seems related with
 774 higher values of DIP (Table 1).
 775 The disappearance of *Prochlorococcus* in spring and mid-summer, their
 776 reappearance in late summer and fall and persistence throughout the winter is a
 777 recurrent phenomenon in the NW Mediterranean and other temperate areas
 778 (Calvo-Díaz et al., 2008; Nunes et al., 2018). Because *Prochlorococcus* is a good
 779 competitor when nutrients are scarce, it is puzzling that this group is absent in the
 780 post-bloom cruise with DIP concentrations at the same level as in F3. Regarding
 781 other environmental variables such as temperature, which displayed a strong
 782 difference among cruises (Fig. S2), models (Flombaum et al 2020) indicate that
 783 *Prochlorococcus* contribution increases from 10° to 30°C. However, their main
 784 contribution during F3 occurred close to the DCM (up to 2×10^5 cells mL⁻¹, Figs. 3
 785 and 4) with temperatures around 16°C (Fig. S2) and only 2°-3° C above those of
 786 F2 at the same depths. This result rules out that temperature was the main
 787 determining factor of *Prochlorococcus* distribution in the studied area. We can think
 788 of alternative, non-excluding hypotheses to explain their low concentrations in the
 789 spring and mid-summer period. It is likely that after being practically wiped out
 790 during the bloom conditions, the seed population in the area was too small to
 791 produce a noticeable recovery of the *Prochlorococcus* concentration between April
 792 and August. A suggested weak deep upwelling during F2 (Salat et al., 2013) could
 793 have produced unfavorable conditions for this group preventing its proliferation at
 794 depth. On the other hand, the UV levels, to which *Prochlorococcus* appears
 795 especially sensitive (Sommaruga et al., 2005), are quite high during this period
 796 probably delaying the recovery of its population at surface. The decline of
 797 picophytoplankton and *Prochlorococcus* populations in the surface mixed-layers

and the DCM has been linked to increased productivity and grazing pressure on heterotrophic bacteria (Kuipers and Witte, 2000; Taylor and Landry, 2018), and could also explain the lack of *Prochlorococcus* in F2. In this period, heterotrophic bacteria production was very high, but their concentrations were kept in check (Gomes et al., 2015) probably by an enhanced grazing that could have helped to maintain *Prochlorococcus* concentrations very low. This scenario would imply that *Synechococcus* was less affected by grazers feeding on smaller bacteria, including *Prochlorococcus*.

4.3. Groups' Ecological Preferences

The most obvious environmental change explaining the distribution of the phytoplankton groups during the three cruises studied here was the development of the water column stratification and the associated decrease in nutrient concentrations from F1 to F3. Stratification fixes temperature, irradiance, and nutrients for phytoplankton at the different layers of the water column. The general pattern of the relative contribution of a phytoplankton group with depth, as shown in Fig. 9, indicates its depth-preference relative to the rest of the groups. As expected, vertical patterns were better defined under stratified conditions. Our results confirm previous knowledge on the relative vertical distribution of phytoplankton groups (Bracher et al., 2020; Miranda-Alvarez et al., 2020; Riegman and Kraay, 2001). *Synechococcus* and cryptophytes showed a strong preference for shallow layers. In contrast, diatoms, green algae, and *Prochlorococcus* showed a tendency for deep layers and pelagophytes for even deeper layers, while haptophyte and dinoflagellate allocation preferences were less clear. The notable presence of

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823 mixotrophic forms in these two latter groups could partly explain their relatively high
824 contribution in the low-nutrient surface waters of F3.

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826 The concentration of nutrients at the surface has been studied as a reasonable
827 indicator of the nutrient input into the euphotic zone (Otero-Ferrer et al., 2018), but
828 there was no clear relationship of DIP at 5 m with either the absolute concentration
829 or the relative contribution of each phytoplankton group (data not shown). Some
830 patterns appear, however, when comparing the groups' contribution with the DIP
831 integrated over 100 m depth (Fig. 10). Thus, *Prochlorococcus*, dinoflagellates and
832 pelagophytes made the highest contribution when DIP was lowest. *Synechococcus*
833 and cryptophytes made their maximum contribution to the phytoplankton
834 community when DIP concentrations were low but not minimal. The contribution of
835 haptophytes did not show a relationship with DIP concentration, while the
836 contributions of green algae and specially diatoms were maxima when nutrient
837 concentrations were highest. Similar patterns could be observed when using DIN
838 as the reference nutrient (Fig. S6) This group distribution can be recognized in the
839 trophic classification established by Latasa et al. (2010), although with some
840 differences. In the present study, diatoms and green algae emerge again as the
841 most eutrophic groups, *Prochlorococcus* as the most oligotrophic one and
842 *Synechococcus* and haptophytes as typically mesotrophic. However, pelagophytes
843 and dinoflagellates contribute more when nutrients are low, while in Latasa et al.
844 (2010) these groups were considered as mesotrophic and eutrophic, respectively.
845 There could be some explanations for this discrepancy. Nunes et al. (2018)
846 observed an increasing trend in the contribution of pelagophytes in a coastal site of
847 the NW Mediterranean when nutrient input decreased, therefore reinforcing the

idea that pelagophytes might be more oligotrophic than suggested by Latasa et al. (2010). On the other hand, dinoflagellates, with heterotrophic, autotrophic and mixotrophic forms, show a great ecological heterogeneity and can be important in both eutrophic and oligotrophic conditions (Jeong et al., 2010; Smayda and Reynolds, 2003). In addition, their pigment composition is quite variable and although peridinin is considered their main pigment marker, other diagnostic pigments are also described (Zapata et al., 2012). In our study, the biomass of large dinoflagellates increased from bloom to stratified non-bloom conditions (Tables 2 and 4), typical for summer or subtropical oligotrophic stratification (Cullen et al., 2002; Margalef, 1978; Smayda and Reynolds, 2003). Since it is difficult to justify that nutrient levels or other conditions in F1 could become physiologically detrimental, our results would confirm that dinoflagellates lose their competitive advantage when turbulence increases and other groups take over (Margalef, 1978; but see Smayda and Reynolds, 2003). Cryptophytes have not been extensively studied in open ocean systems. Their notable contribution in surface waters at some stations with very shallow mixed layers during F2 hints that, as for dinoflagellates, not only chemical, but physical factors might affect their population dynamics. On the other hand, their significant presence in F1 and total absence in F3 also indicate that cryptophytes develop better under meso- through eutrophic conditions, even though their mixotrophic capabilities could provide some advantage under oligotrophic conditions (Yoo et al., 2017). As a precautionary note affecting our interpretation of ecological preferences, biomass accumulation is not always associated with optimal physiological conditions but with the resulting balance between growth and losses, something that is more evident for small size

phytoplankton usually more susceptible to heavy microzooplankton grazing (Hunter-Cevera et al., 2020; Taylor and Landry, 2018).

The relationships between nutrient availability and relative group abundance found in this work reveal significant exceptions to the concept that small cells present advantages in low nutrient environments because of their high surface to volume ratio, while large cells outcompete small cells when nutrients are non-limiting. Diatoms and green algae are abundant in coastal waters (Not et al., 2005, 2004; Sarthou et al., 2005) where mixing and high nutrients are more common than in the open ocean. It is striking that these two groups, which comprise the largest and smallest eukaryotic phytoplankton, share the same ecological conditions of nutrients and turbulence. Factors other than size must play a role. Specific diatom advantages are a grazing-protective cell frustule (Mitchell et al., 2013), efficient nutrient assimilation (Olofsson et al., 2019) and the possession of cell vacuoles, which is helpful in fluctuating nutrient conditions (Stolte and Riegman, 1996; Tozzi et al., 2004). On the other hand, the high contribution of green algae under high nutrient concentrations could be due to a diminishing grazing pressure associated to the winter deepening of the mixed layer and subsequent decrease in the prey-predator encounter rate. This decrease has been proposed for the previous and early conditions of the spring bloom (Behrenfeld, 2010). Moreover, green algae should also have some additional physiological advantage under these conditions that would favor them over other similarly sized groups such as pelagophytes. Autotrophic dinoflagellates and *Prochlorococcus* also seem to share the same environmental preferences for low nutrients and strong stratification despite their huge difference in size. The vertical migration capabilities of large dinoflagellates to

obtain nutrients in deeper waters likely contributes to their success under oligotrophic conditions (Hall and Paerl, 2011; Ji and Franks, 2007). As mentioned above, dinoflagellates do not dominate under turbulent conditions while their mixotrophic forms are often abundant during stratification (Stoecker et al., 2017). Finally, cryptophytes and *Synechococcus* appear under similar environmental conditions despite their considerable size differences, although our results showed that a considerable portion of the cryptophyte assemblages were constituted by small cells (Fig. 7).

As a general pattern, a high biomass contribution of small cells occurs under low nutrients, and that of large cells under high nutrient conditions. However, this widespread assumption is far from being fulfilled when life-cycles and ecological strategies are contemplated and the contribution of individual groups to the total biomass are considered. Our study focused on three distinct periods of the annual cycle in the NW Mediterranean illustrates that departures from this general pattern can be captured by combining analytical tools currently available for the study of phytoplankton communities. The wide range of physical and chemical conditions also allowed us to unravel the preferences of the different groups in relation to depth and nutrients. This information, which should be refined with additional data, can be used to identify the direction of changes in phytoplankton communities in relation to possible alterations of physical and nutrient conditions under the climate change scenario.

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Fig. 1

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Figure Legends

Figure 1. Surface chlorophyll *a* ($\mu\text{g L}^{-1}$) and location of the different stations

during the three Famoso cruises, F1: March 6 - 23, F2: April 29 - May 14, F3:

September 14 - 22. Note the different scales between F1 and F2 and F3.

Chlorophyll *a* images are from March 19, May 7, and September 19, and are a

Copernicus merged product obtained from

https://resources.marine.copernicus.eu/?option=com_csw&task=results.

Figure 2. Environmental conditions during the Famoso cruises. From top to

bottom: Potential density, Brunt- Väisälä frequency, Dissolved Inorganic

Nitrogen (NO_3^-) and Dissolved Inorganic Phosphorus (PO_4^{3-}). Station numbers

are displayed above the top panel; the x-axis represents the temporal scale.

Figure 3. Percent contribution of the different phytoplankton groups to

chlorophyll *a*. during the Famoso cruises. Stations are named above the top

panel. Total chlorophyll *a* concentration (TChl *a*, ng L^{-1}) in the bottom panel.

Station numbers are displayed above the top panel; the x-axis represents the

temporal scale.

Figure 4. Biomass distribution of different phytoplankton groups as chlorophyll *a*

(ng L^{-1}) and cell concentrations (cells mL^{-1}) for the three Famoso cruises.

Station numbers are displayed above the top panel; the x-axis represents the

temporal scale.

Figure 5. Carbon to chlorophyll *a* relationship for the bulk phytoplankton and

pico-, nano- and microphytoplankton fractions. Values are averages of the

upper 100 m. F1= green square, F2 = red diamond, F3 = blue circle; black

1 includes all the data. Regression lines are represented when statistically
2 significant ($p < 0.05$).
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5 Figure 6. A comparative of phytoplankton biomass distribution as chlorophyll *a*
6 in different size fractions according to UITZ and CHEMTAX approaches.
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10 Figure 7. Carbon to chlorophyll *a* relationships for different phytoplankton
11 groups. Picoeukaryotes are abbreviated as picoeuk. Large and small
12 picoeukaryotes are populations than can be distinguished by flow cytometry
13 (Table S1). Values are averages of the upper 100 m. Regression lines are
14 represented when statistically significant ($p < 0.05$). F1= green square, F2 = red
15 diamond, F3 = blue circle; black includes all the data.
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27 Figure 8. Cell size changes in the water column for different picoplankton
28 groups. F1= green square, F2 = red diamond, F3 = blue circle.
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33 Figure 9. Percent contribution of the different phytoplankton groups in the upper
34 100 m water column. The line represents a smoothed trend (see M&M) for all
35 cruises (top panel) and for individual cruises. F1= green square, F2 = red
36 diamond, F3 = blue circle.
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44 Figure 10. Percentage contribution of the different phytoplankton groups along
45 the average concentration of dissolved inorganic phosphorus (DIP) in the upper
46 100 m. The line represents a smoothed trend (see M&M). Note the different
47 scale for haptophytes and *Synechococcus*.
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Table 1 Information of stations on date, geolocalization, mixed layer depth (MLD), Brunt- Väisälä (B-V) frequency (cycl h⁻¹), dissolved inorganic nitrogen (NO₃⁻ + NO₂⁻, DIN) and dissolved inorganic phosphate (PO₄³⁻, DIP) concentration (μmol L⁻¹) at surface (5 m), averaged (avg) or integrated (int) over the 100 m water column. N.d., no data. Mean and S.D. of MLD in F1 includes 100 m for station F1-08.

Cruise	Station	Date	Longitude	Latitude	MLD (m)	B-V (int 100m)	DIN (5m)	DIN (avg 100m)	DIP (5m)	DIP (avg 100m)
F1	8	2009-03-10	4.771	41.742	> 100	28.0	n.d.	n.d.	0.357	0.388
F1	13	2009-03-14	5.092	41.587	33	181	5.86	7.26	0.118	0.239
F1	14	2009-03-14	5.221	41.633	8	199	4.33	7.09	0.145	0.267
F1	16	2009-03-15	3.862	41.503	41	178	4.63	6.71	0.144	0.179
F1	19	2009-03-17	3.822	41.274	13	193	n.d.	n.d.	0.056	0.193
F1	21	2009-03-18	4.075	41.501	15	128	5.82	8.08	0.209	0.354
F1	24	2009-03-19	4.257	41.608	24	184	2.97	5.92	0.040	0.141
F1	27	2009-03-21	4.305	41.827	36	120	3.71	5.34	0.068	0.102
F1	31	2009-03-22	4.569	41.794	65	100	3.83	5.15	0.104	0.146
Mean (SD)					37 (±29)	146 (±57)	4.45 (±1.08)	6.51 (±1.08)	0.138 (±0.097)	0.223 (±0.098)
F2	5	2009-04-30	4.874	41.5	26	282	2.78	4.19	0.012	0.143
F2	11	2009-05-03	3.939	41.499	37	339	1.02	4.93	0.019	0.158
F2	12	2009-05-03	3.952	41.512	12	434	1.02	4.93	0.019	0.158
F2	15	2009-05-04	3.958	41.557	26	366	1.30	4.10	0.040	0.111
F2	20	2009-05-07	4.54	42.112	10	261	1.79	4.26	0.026	0.119
F2	26	2009-05-08	4.06	42.026	11	329	1.57	4.44	0.053	0.129
F2	30	2009-05-09	4.098	42.054	11	380	1.34	5.17	0.009	0.106
F2	32	2009-05-10	4.165	42.047	10	382	1.23	4.48	0.021	0.149
F2	36	2009-05-11	4.595	41.459	7	369	1.06	4.26	0.038	0.163
F2	40	2009-05-12	4.532	41.385	15	379	2.16	4.90	0.030	0.121
F2	44	2009-05-13	4.486	41.339	7	428	0.701	3.55	0.057	0.160
Mean (SD)					16 (±10)	359 (±54)	1.45 (±0.60)	4.47 (±0.47)	0.029 (±0.016)	0.138 (±0.021)
F3	5	2009-09-16	4.304	41.483	36	515	n.d.	n.d.	0.032	0.055
F3	8	2009-09-17	4.232	41.473	16	670	1.48	3.34	0.036	0.045
F3	12	2009-09-18	4.208	41.477	26	609	1.87	1.75	0.016	0.050
F3	15	2009-09-19	4.044	41.929	30	524	0.730	3.50	0.024	0.093
F3	20	2009-09-20	4.086	41.937	6	628	1.83	3.64	0.049	0.107
Mean (SD)					23 (±12)	589 (±67)	1.48 (±0.53)	3.06 (±0.88)	0.031 (±0.012)	0.070 (±0.028)

Table 2. Distribution of chlorophyll *a* in the different phytoplankton groups and across size fractions using CHEMTAX, and of phytoplankton carbon estimated from microscopy and flow cytometry cell concentrations and biovolume to carbon conversion. Values (ng L⁻¹ or µg L⁻¹) are averages of the 100 m depth water column integrated following the trapezoidal rule (see M&M). Percentages are estimates per cruise referred to chlorophyll *a* or carbon.

	F1	F2	F3	F1 (%)	F2 (%)	F3 (%)
Chl a (ng L⁻¹)						
Total	843 (±313)	297 (±137)	318 (±36)			
Picoplankton	256 (±90)	141 (±43)	122 (±27)	30	48	38
Nanoplankton	360 (±163)	138 (±132)	159 (±27)	43	46	50
Microplankton	227 (±109)	18 (±15)	37 (±17)	27	6	12
Diatoms	197 (±101)	10 (±8.8)	20 (±15)	23	3	6
Dinoflagellates	31 (±15)	8.2 (±7.2)	17 (±10)	4	3	5
Haptophytes	274 (±128)	74 (±94)	88 (±14)	32	25	28
Pelagophytes	73 (±32)	40 (±43)	71 (±15)	9	13	22
Cryptophytes	13 (±11)	24 (±9.2)	0	2	8	0
Green algae	160 (±58)	33 (±10)	17 (±13)	19	11	5
<i>Synechococcus</i>	96 (±34)	108 (±42)	32 (±8.3)	11	36	10
<i>Prochlorococcus</i>	0	0	73 (±26)	0	0	23
Carbon (µg L⁻¹)						
Total	20.02 (±10.26)	18.19 (±6.06)	9.95 (±1.06)			
Picoplankton	8.77 (±3.17)	11.68 (±6.26)	5.21 (±0.61)	44	64	52
Nanoplankton	2.26 (±1.30)	2.92 (±1.08)	1.99 (±0.60)	11	16	20
Microplankton	8.99 (±10.29)	3.59 (±3.32)	2.75 (±1.00)	45	20	28
<i>Prochlorococcus</i>	0.04 (±0.03)	0.00	2.63 (±0.41)	0	0	26
<i>Synechococcus</i>	3.64 (±1.42)	9.92 (±6.38)	1.65 (±0.57)	18	55	17
Picoeukaryotes	4.97 (±1.85)	1.56 (±1.02)	0.92 (±0.33)	25	9	9
Cryptophytes ^a	0.12 (±0.06)	0.18 (±0.12)	0.00	1	1	0
Diatoms <20 µm ^b	0.02 (±0.02)	0.00	0.01 (±0.02)	0	0	0
Dinoflagellates <20 µm	0.46 (±0.29)	1.29 (±0.66)	1.17 (±0.40)	2	7	12
Flagellates <20 µm	1.72 (±1.21)	1.63 (±0.47)	0.73 (±0.25)	9	9	7
Other <20 µm	0.05 (±0.07)	0.00	0.08 (±0.06)	0	0	1
Diatoms >20 µm ^c	7.41 (±9.74)	0.33 (±0.44)	0.29 (±0.19)	37	2	3
Dinoflagellates >20 µm	0.99 (±0.46)	2.47 (±1.89)	2.30 (±0.90)	5	14	23
Other >20 µm	0.60 (±0.38)	0.79 (±1.19)	0.16 (±0.08)	3	4	1

^a Only small cryptophytes measured by flow cytometry

^b Diatoms < 20 µm are non chain-forming diatoms.

^c Diatoms >20 µm include chain-forming species with individual cells <20 µm.

Table 3 Chlorophyll *a* attributed to the different phytoplankton groups by CHEMTAX per station. Average concentration (ng L⁻¹) in the 100 m water column.

Cruise	Station	Cryptophytes	Diatoms	Dinoflagellates	Green algae	Haptophytes	Pelagophytes	<i>Synechococcus</i>	<i>Prochlorococcus</i>	Chl <i>a</i>
F1	8	0.2	46.0	3.7	50.1	29.9	11.6	26.6	0.0	168.2
F1	13	0.4	191.5	43.3	198.0	374.2	110.3	116.9	0.0	1034.6
F1	14	18.7	132.3	24.7	154.9	186.3	62.3	112.0	0.0	691.1
F1	16	15.6	160.6	36.0	249.4	273.2	82.4	139.0	0.0	956.3
F1	19	22.9	231.3	29.3	176.5	476.3	108.5	112.0	0.0	1156.7
F1	21	6.7	163.8	23.9	186.4	275.5	82.1	78.5	0.0	817.0
F1	24	33.1	242.9	58.0	183.4	353.2	89.8	110.0	0.0	1070.4
F1	27	11.9	181.6	25.5	96.7	195.0	40.6	66.7	0.0	618.0
F1	31	8.1	419.3	31.1	145.9	301.9	68.3	102.3	0.0	1076.9
F2	5	18.0	7.3	4.2	39.4	56.1	27.8	162.5	0.0	315.3
F2	11	13.7	3.1	4.1	34.8	49.3	22.6	113.6	0.0	241.2
F2	12	18.2	0.3	7.9	35.0	38.7	17.2	191.0	0.0	308.3
F2	15	18.7	6.5	3.6	34.5	47.5	25.3	122.1	0.0	258.3
F2	20	35.2	3.0	4.6	26.3	13.1	10.5	129.2	0.0	221.9
F2	26	35.0	7.4	3.3	21.9	17.1	13.2	76.5	0.0	174.5
F2	30	31.1	7.9	6.4	29.3	22.0	16.1	109.6	0.0	222.3
F2	32	23.4	7.0	5.1	19.6	43.1	24.9	78.2	0.0	201.3
F2	36	28.8	16.0	10.5	34.4	58.7	49.1	79.4	0.0	276.9
F2	40	33.2	25.7	12.7	35.4	126.4	72.0	68.5	0.0	374.0
F2	44	8.3	26.2	27.8	55.8	341.5	156.7	55.4	0.0	671.8
F3	5	0.0	38.0	22.8	2.2	110.1	83.8	31.5	81.3	369.8
F3	8	0.0	7.6	31.8	17.5	75.8	51.8	44.6	104.8	333.9
F3	12	0.0	1.5	13.2	9.4	84.6	58.8	28.8	87.1	283.5
F3	15	0.0	23.6	7.1	36.1	92.4	86.7	21.5	48.8	316.1
F3	20	0.0	28.5	9.7	21.8	79.4	72.8	31.6	43.0	286.7

Table 4 Microscopy estimations of nano and microphytoplankton carbon (C) biomass per station. Average concentration (ng L⁻¹) in the 100 m water column. Total autotrophic C includes picophytoplankton C estimated from FCM counts.

Cruise	Station	Diatoms >20 µm	Diatoms < 20 µm	Autotrophic dinoflagellates > 20 µm	Autotrophic dinoflagellates < 20 µm	Autotrophic flagellates < 20 µm	<i>Mesodinium rubrum</i> > 20 µm	Coccolithophores > 20 µm	Coccolithophores < 20 µm	Other autotrophs > 20 µm	Total autotrophic C
F1	8	1367	10	221	91	347	9	6	6	154	
F1	13	983	13	530	978	1668	0	33	199	737	10586
F1	14	5617	40	910	374	1427	38	26	25	633	
F1	16	2869	65	588	455	1233	101	46	133	340	19575
F1	19	461	3	521	308	4550	20	7	0	16	16625
F1	21	4015	5	834	544	1364	208	21	4	873	16244
F1	24	1590	44	1399	650	1543	3	45	18	835	16869
F1	27	10893	17	1030	175	651	0	5	2	115	17817
F1	31	31033	15	2002	94	1058	0	12	0	794	42411
F2	5	2	0	808	933	1421	9	0	2	0	29624
F2	11	3	3	1389	644	1260	0	0	0	10	18981
F2	12	4	0	1832	899	1338	0	0	0	0	
F2	15	14	0	2321	1685	2116	5	0	0	51	20650
F2	20	15	0	850	1064	1553	30	0	0	49	15516
F2	26	3	2	753	940	1053	0	0	0	40	8867
F2	30	20	0	1674	867	1046	50	0	0	345	16506
F2	32	613	2	2031	803	1329	0	0	1	100	10919
F2	36	508	0	4282	1769	2211	1089	1	0	80	18140
F2	40	1231	0	3603	1238	2068	3079	0	1	154	17883
F2	44	882	13	6977	2944	2259	2724	0	1	92	24795
F3	5	168	40	2344	1782	913	0	34	56	115	10084
F3	8	55	0	1509	1149	872	0	228	103	31	9179
F3	12	327	5	1416	1125	957	0	38	9	157	8743
F3	15	365	1	2623	667	388	0	40	65	9	10301
F3	20	558	7	3607	1136	537	47	53	161	29	11455

Diatoms >20 µm include chain-forming species with individual cells <20 µm.

Diatoms < 20 µm are non chain-forming cells.

Mesodinium rubrum is a ciliate, autotrophic due to endosymbiotic cryptophytes.

Flagellates < 20 µm refer to non-skeletal, non-colony forming cells.

Other autotrophs > 20 µm include phycoma stages of Prasinophyceae, colonial formas of *Phaeocystis* and non-skeletal, non-colony forming cells and colonies of flagellates.

Total autotrophic C includes picoplankton.

Table 5 Flow cytometry estimations of picophytoplankton carbon biomass in different groups per station. Average concentration (ng L⁻¹) in the 100 m water column.

Cruise	Station	<i>Synechococcus</i>	<i>Prochlorococcus</i>	Small picoeukaryotes	Large picoeukaryotes	Total picoeukaryotes	Cryptophytes	Picophytoplankton	Total autotrophic C
F1	8								
F1	13	2264	17	1360	1711	3071	92	5444	10586
F1	14								
F1	16	6247	93	4153	3125	7278	130	13747	19575
F1	19	3715	28	1891	4876	6767	229	10739	16625
F1	21	2878	37	2521	2845	5366	93	8374	16244
F1	24	4582	41	2187	3769	5956	162	10741	16869
F1	27	2260	14	849	1704	2553	102	4930	17817
F1	31	3509	25	1717	2091	3808	60	7403	42411
F2	5	24816	0	1067	471	1538	95	26448	29624
F2	11	13615	0	998	989	1987	70	15671	18981
F2	12								
F2	15	12877	0	829	655	1484	97	14458	20650
F2	20	10997	0	630	118	636	208	11953	15516
F2	26	5250	0	423	158	581	246	6077	8867
F2	30	11288	0	792	134	926	290	12503	16506
F2	32	5012	0	406	468	874	154	6040	10919
F2	36	6571	0	678	715	1393	237	8200	18140
F2	40	3947	0	779	1357	2137	426	6510	17883
F2	44	4838	0	880	3163	4043	21	8902	24795
F3	5	1282	2719	161	471	631	0	4633	10084
F3	8	1353	3120	200	559	758	0	5231	9179
F3	12	1128	2887	128	564	692	0	4708	8743
F3	15	2444	2304	452	943	1395	0	6142	10301
F3	20	2057	2119	337	807	1144	0	5320	11455

No data for F1-08, F1-14, F2-12.

Average diameter of large and small picoeukaryotes are 1.97 µm and 1.45 µm (Table S1).

Supplementary material

Dynamics of phytoplankton groups in three contrasting situations of the open NW Mediterranean revealed by pigment, microscopy, and flow cytometry analyses

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Table S1. Overall average cell diameter (μm) and carbon content (pg cell^{-1}) of picophytoplankton groups by FCM. Standard deviation and number of samples are indicated in parenthesis.

	Cell diameter	Cell carbon content
<i>Prochlorococcus</i>	0.71 (± 0.164 , 121)	0.053 (± 0.051 , 121)
<i>Synechococcus</i>	1.04 (± 0.071 , 230)	0.152 (± 0.162 , 230)
Small picoeukaryotes	1.45 (± 0.12 , 219)	0.388 (± 0.102 , 219)
Large picoeukaryotes	1.97 (± 0.17 , 217)	0.975 (± 0.277 , 217)
Cryptophytes (small)	2.42 (± 0.31 , 114)	1.805 (± 0.767 , 114)

Table S2. Excel template for initial input random matrices for Chemtax.

	Chlc3	Chlc2	Per	19'but	Fuco	Pras	19'hex	Allo	Zea	Chl b	DVChla	MVCha
1Cryptophytes	-	Random (0.8-0.2)+0.2	-	-	-	-	-	Random (1-0.25)+0.25	-	-	-	1
2												
3												
4Diatoms	Random (0.4-0.1)+0.1	Random (0.8-0.2)+0.2	-	-	Random (1-0.25)+0.25	-	-	-	-	-	-	1
5												
6												
7Dinoflagellates	-	Random (0.8-0.2)+0.2	Random (1-0.25)+0.25	-	-	-	-	-	-	-	-	1
8												
9												
10Prasinophytes	-	-	-	-	-	Random (0.5-0.1)+0.1	-	-	-	0.550	-	1
11												
12												
13Haptophytes	Random (0.6-0.1)+0.1	Random (0.8-0.2)+0.2	-	-	Random (0.4-0.1)+0.1	-	Random (1-0.25)+0.25	-	-	-	-	1
14												
15												
16Pelagophytes	Random (0.6-0.1)+0.1	Random (0.8-0.2)+0.2	-	Random (1-0.25)+0.25	Random (0.5-0.1)+0.1	-	-	-	-	-	-	1
17												
18												
19Synechococcus	-	-	-	-	-	-	-	-	Random (1-0.25)+0.25	-	-	1
20												
21												
22												
23Prochlorococcus	-	-	-	-	-	-	-	-	Random (1-0.25)+0.25	Random (1-0.25)+0.25	1	-
24												
25												

Output Chemtax matrices for the five pigment clusters.

	Chlc3	Chlc2	Per	19'but	Fuco	Pras	19'hex	Allo	Zea	Chl b	DVChla	MVCha
1Cluster1												
2Cryptophytes	-	0.369	-	-	-	-	-	0.654	-	-	-	1
3												
4Diatoms	0.206	0.462	-	-	0.686	-	-	-	-	-	-	1
5												
6Dinoflagellates	-	0.522	0.648	-	-	-	-	-	-	-	-	1
7												
8Prasinophytes	-	-	-	-	-	0.174	-	-	-	0.689	-	1
9												
10Haptophytes	0.830	0.321	-	-	0.088	-	0.611	-	-	-	-	1
11												
12Pelagophytes	0.333	0.419	-	0.811	0.189	-	-	-	-	-	-	1
13												
14Synechococcus	-	-	-	-	-	-	-	-	0.574	-	-	1
15												
16Prochlorococcus	-	-	-	-	-	-	-	-	0.569	0.542	1	-
17												
18Cluster2												
19Cryptophytes	-	0.402	-	-	-	-	-	0.595	-	-	-	1
20												
21Diatoms	0.271	0.290	-	-	0.574	-	-	-	-	-	-	1
22												
23Dinoflagellates	-	0.432	0.533	-	-	-	-	-	-	-	-	1
24												
25Prasinophytes	-	-	-	-	-	0.131	-	-	-	0.508	-	1
26												
27Haptophytes	0.245	0.206	-	-	0.275	-	0.770	-	-	-	-	1
28												
29Pelagophytes	0.364	0.260	-	0.704	0.200	-	-	-	-	-	-	1
30												
31Synechococcus	-	-	-	-	-	-	-	-	0.525	-	-	1
32												
33Prochlorococcus	-	-	-	-	-	-	-	-	0.535	0.564	1	-
34												
35Cluster3												
36Cryptophytes	-	0.361	-	-	-	-	-	0.590	-	-	-	1
37												
38Diatoms	0.182	0.259	-	-	0.803	-	-	-	-	-	-	1
39												

1	Dinoflagellates	-	0.481	0.596	-	-	-	-	-	-	-	1
2	Prasinophytes	-	-	-	-	-	0.148	-	-	-	0.621	1
3	Haptophytes	0.377	0.275	-	-	0.106	-	0.732	-	-	-	1
4	Pelagophytes	0.191	0.263	-	0.854	0.279	-	-	-	-	-	1
5	<i>Synechococcus</i>	-	-	-	-	-	-	-	-	0.583	-	1
6	<i>Prochlorococcus</i>	-	-	-	-	-	-	-	-	0.620	0.546	1
7	Cluster4											
8	Cryptophytes	-	0.516	-	-	-	-	-	0.504	-	-	1
9	Diatoms	0.260	0.399	-	-	0.639	-	-	-	-	-	1
10	Dinoflagellates	-	0.374	0.620	-	-	-	-	-	-	-	1
11	Prasinophytes	-	-	-	-	-	0.164	-	-	-	0.679	1
12	Haptophytes	0.337	0.261	-	-	0.073	-	0.991	-	-	-	1
13	Pelagophytes	0.470	0.132	-	0.745	0.262	-	-	-	-	-	1
14	<i>Synechococcus</i>	-	-	-	-	-	-	-	-	0.644	-	1
15	<i>Prochlorococcus</i>	-	-	-	-	-	-	-	-	0.400	0.701	1
16	Cluster5											
17	Cryptophytes	-	0.427	-	-	-	-	-	0.546	-	-	1
18	Diatoms	0.152	0.226	-	-	0.645	-	-	-	-	-	1
19	Dinoflagellates	-	0.422	0.516	-	-	-	-	-	-	-	1
20	Prasinophytes	-	-	-	-	-	0.155	-	-	-	0.535	1
21	Haptophytes	0.322	0.194	-	-	0.141	-	0.820	-	-	-	1
22	Pelagophytes	0.400	0.199	-	0.766	0.191	-	-	-	-	-	1
23	<i>Synechococcus</i>	-	-	-	-	-	-	-	-	0.421	-	1
24	<i>Prochlorococcus</i>	-	-	-	-	-	-	-	-	0.606	0.693	1

Table S3 Percent contribution of phytoplankton groups to total chlorophyll a biomass per station.

Cruise	Station	Cryptophytes	Diatoms	Dinoflagellates	Green algae	Haptophytes	Pelagophytes	<i>Synechococcus</i>	<i>Prochlorococcus</i>
F1	8	0.1	27.4	2.2	29.8	17.8	6.9	15.8	0.0
F1	13	0.0	18.5	4.2	19.1	36.2	10.7	11.3	0.0
F1	14	2.7	19.1	3.6	22.4	27.0	9.0	16.2	0.0
F1	16	1.6	16.8	3.8	26.1	28.6	8.6	14.5	0.0
F1	19	2.0	20.0	2.5	15.3	41.2	9.4	9.7	0.0
F1	21	0.8	20.0	2.9	22.8	33.7	10.0	9.6	0.0
F1	24	3.1	22.7	5.4	17.1	33.0	8.4	10.3	0.0
F1	27	1.9	29.4	4.1	15.7	31.6	6.6	10.8	0.0
F1	31	0.8	38.9	2.9	13.6	28.0	6.3	9.5	0.0
F2	5	5.7	2.3	1.3	12.5	17.8	8.8	51.6	0.0
F2	11	5.7	1.3	1.7	14.4	20.5	9.4	47.1	0.0
F2	12	5.9	0.1	2.6	11.4	12.6	5.6	62.0	0.0
F2	15	7.3	2.5	1.4	13.4	18.4	9.8	47.3	0.0
F2	20	15.9	1.4	2.1	11.8	5.9	4.7	58.2	0.0
F2	26	20.1	4.3	1.9	12.5	9.8	7.6	43.8	0.0
F2	30	14.0	3.5	2.9	13.2	9.9	7.3	49.3	0.0
F2	32	11.6	3.5	2.6	9.7	21.4	12.4	38.9	0.0
F2	36	10.4	5.8	3.8	12.4	21.2	17.7	28.7	0.0
F2	40	8.9	6.9	3.4	9.5	33.8	19.3	18.3	0.0
F2	44	1.2	3.9	4.1	8.3	50.8	23.3	8.2	0.0
F3	5	0.0	10.3	6.2	0.6	29.8	22.7	8.5	22.0
F3	8	0.0	2.3	9.5	5.2	22.7	15.5	13.4	31.4
F3	12	0.0	0.5	4.7	3.3	29.8	20.7	10.2	30.7
F3	15	0.0	7.5	2.2	11.4	29.2	27.4	6.8	15.4
F3	20	0.0	9.9	3.4	7.6	27.7	25.4	11.0	15.0

Table S4. Stock forming diatoms ranked by carbon (C) contribution. The C value (ng L⁻¹) is the average of all microscopy samples per cruise. Species marked with asterisks (***) are typical for summer assemblages, as they often form a major component of these communities (Foster and O'Mullan 2008), Gettings et al (2014).

F1	Carbon
<i>Lauderia annulata</i> Cleve 1873	8325
<i>Chaetoceros</i> Ehrenberg 1844 sg <i>Hyalochaete</i>	1692
<i>Ditylum</i> (Bailey ex. L.W. Bailey) spp.	1528
Diatom centric 40-60 µm	191
Diatom disc-like 40-60 µm	138
<i>Cerataulina pelagica</i> (Cleve) Hendey 1937	130
<i>Chaetoceros didymus</i> Ehrenberg 1845	129
<i>Corethron</i> (Castracane 1886) spp.	128
<i>Chaetoceros curvisetus</i> Cleve 1889	98
<i>Dactyliosolen phuketensis</i> (Sundström) Hasle 1996 ***	75
F2	
<i>Leptocylindrus mediterraneus</i> (Peragallo) Hasle 1975 ***	235
<i>Proboscia alata</i> (Brightwell) Sundström 1986	87
<i>Pseudo-nitzschia seriata</i> Complex (Hasle 1965b, 1971) ***	51
<i>Pseudo-nitzschia delicatissima</i> Complex (Hasle 1964, 1965b) ***	16
<i>Guinardia flaccida</i> (Castracane) Peragallo 1892 ***	14
<i>Chaetoceros</i> Ehrenberg 1844 sg <i>Hyalochaete</i>	12
Diatom centric 10-20 µm	2
<i>Ditylum</i> (Bailey ex. L.W. Bailey) spp.	2
Diatom centric disc-like 20-40 µm	2
<i>Corethron</i> (Castracane 1886) spp.	2
F3	
<i>Pseudo-nitzschia delicatissima</i> Complex (Hasle 1964, 1965b) ***	99
<i>Dactyliosolen phuketensis</i> (Sundström) Hasle 1996 ***	82
<i>Cerataulina pelagica</i> (Cleve) Hendey 1937	33
<i>Hemiaulus hauckii</i> Grunow ex. Van Heurck 1882 ***	20
<i>Bacteriastrum</i> spp Shadbolt 1854	15
<i>Proboscia alata</i> (Brightwell) Sundström 1986	13
Diatom pennate oblong 80-100 µm	12
<i>Leptocylindrus mediterraneus</i> (Peragallo) Hasle 1975 ***	10
<i>Leptocylindrus danicus</i> Cleve 1889 ***	9
<i>Chaetoceros</i> Ehrenberg 1844 sg <i>Hyalochaete</i>	9

Figure S1. Clustering of samples according to their pigment composition (top panel). Cluster numbers are shown. Sample ascription to clusters (bottom panel). Station numbers are displayed above the top panel; the x-axis represents the temporal scale.

Figure S2. Temperature and salinity in the first 100 m of the three FAMOSO cruises. Station numbers are displayed above the top panel; the x-axis represents the temporal scale.

Figure S3. Areal contribution of the different phytoplankton groups to chlorophyll a biomass. Note the different scale for *Synechococcus* in F2.

Figure S4. Comparison between CHEMTAX and UITZ methods to estimate the chlorophyll a assigned to the different phytoplankton groups. For the UITZ approach, empty symbols are values estimated according to the original coefficients (Uitz et al 2006), with which *Synechococcus* and *Prochlorococcus* contributions cannot be separated (and are estimated as cyanobacteria) and green algae contribution is estimated by subtracting *Prochlorococcus* contribution to chl b containing groups (*Prochlorococcus* and green algae); red symbols are the values estimated with the new coefficients tuned for our CHEMTAX results in the area. The resulting new coefficients were 1.15 for alloxanthin (cryptophytes), 0.67 for fucoxanthin (diatoms), 1.47 for peridinin (dinoflagellates), 9.91 for violaxanthin (green algae), 1.04 for 19'hexanoiloxifucoxanthin (haptophytes), 1.10 for 19'butanoiloxifucoxanthin (pelagophytes), and 1.31 for zeaxanthin (cyanobacteria). Since the contribution of *Prochlorococcus* can be distinguished as divinyl chlorophyll a (DVChl a), the contribution of *Synechococcus* was estimated as cyanobacteria - DVChl a.

Figure S5. Microscopy pictures of in vivo net samples of a young diatom-dominated community (top panels) and a more mature haptophyte-dominated community (bottom panels) during F1.

Figure S6. Percentage contribution of the different phytoplankton groups along the average concentration of dissolved inorganic (DIN, $\text{NO}_3 + \text{NO}_2$) in the upper 100 m. The line represents a smoothed trend (see M&M). Note the different scale for haptophytes and *Synechococcus*.

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