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Marker pigments and carbon biomass of phytoplankton on the northeastern Mediterranean Sea coast

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ABSTRACT

Marker pigments are used to determine taxonomic composition and biomass of microalgae in different oceanic regions. However, sometimes discrepancies are encountered between microscopy and marker pigment based approaches principally because of altering environmental factors influencing diversity of phytoplankton. In the present investigation, marker pigments from HPLC-CHEMTAX analysis concurrent with carbon biomass estimated by microscopy were investigated during 2015–2016 at weekly intervals in the eastern Mediterranean Sea coast. Counting nanoplankton (in particular non-calcifying haptophytes and prasinophytes) in live samples provided a better correlation between microscopy and pigment-based results than in fixed samples. Nanoplankton and picoplankton constituted ~56% of chlorophyll *a* based on HPLC-CHEMTAX analysis in the sampling location. Diatoms were the most prominent taxa based on both pigments and microscopy results in the study area. A significant positive correlation between PAR values and CHEMTAX derived chlorophyll *a* values of cyanobacteria and cryptophytes was observed. While there was no correlation between carbon biomass and Chl *a* concentrations ($p > 0.05$) for the whole dataset, a significant correlation appeared between these parameters when the data was split as high and low C:Chl *a* samples.

1. Introduction

Determination of carbon biomass of phytoplankton is important in estimation of primary production and carbon flux-based/modelling studies. Carbon (C) biomass of phytoplankton can be estimated using two major approaches: a) from the total biomass of phytoplankton cells in a sample following microscopic determination of cell abundance and individual cell volumes, and (b) from carbon (C): chlorophyll *a* (Chl *a*) ratios estimated from phytoplankton culture experiments using chemical methods (Behrenfeld et al., 2005; Jackson et al., 2017; Sathyendranath et al., 2009). The first approach mentioned here utilises conversion equations of biomass to carbon for each cell shape (Menden-Deuer et al., 2000; Strathmann, 1967). Microscopy however, is a very exhausting and time-consuming technique, not feasible for processing a large number of samples. Furthermore, an experienced taxonomist should be in the research team to obtain accurate analyses in microscopy (Wright and Jeffrey, 2006). For the second approach, factors influencing C:Chl *a* ratios should be considered for a better estimation of carbon content of phytoplankton. This ratio varies substantially (between <10 and > 200) due to changes in nutrient concentration, temperature, irradiance, growth phases and species composition (Laws and Bannister, 1980; Llewellyn et al., 2005; Stelmakh and Gorbunova, 2018).

Chl *a* content of each phytoplankton group could be estimated from marker pigments determined by HPLC coupled with the CHEMTAX program (Mackey et al., 1996) which uses predefined marker pigment: Chl *a* ratios. Carbon biomass of different phytoplankton groups could then be roughly assessed from these group-specific Chl *a* values using C: Chl *a* ratios of each phytoplankton class. However, chemotaxonomic analyses should still be supported by microscopy for sound evaluation of results (Coupel et al., 2015). Because (a) some marker pigments are shared by different phytoplankton groups/species (Jeffrey and Vesk, 1997; Irigoien et al., 2004; Jeffrey et al., 2011), (b) marker pigment:Chl *a* ratios change depending on species composition, growth phase, trophic level (mixo-or heterotrophy) and environmental conditions (Schlüter et al., 2006; Jeong et al., 2010).

In the marine environment, phytoplankton species composition,

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physiological states and environmental conditions change spatially and temporally. Therefore, the estimation of wider regional or annual phytoplankton carbon biomass:Chl *a* ratios utilizing the HPLC-CHEMTAX method requires validation with data obtained through microscopy for different locations comprising frequent sampling through the seasonal cycles. There are a few time series studies in the literature related to marker pigments and phytoplankton abundance in the Mediterranean Sea (Yılmaz, 2006; Garrido et al., 2014; Krivokapić et al., 2018; Nunes et al., 2018). However, based on our literature survey, no previous study has to date reported the carbon biomass of phytoplankton groups in relation to pigment analysis (from CHEMTAX) with frequent sampling intervals in the Mediterranean.

In previous studies encompassing microscopy, the HPLC-CHEMTAX method has generally been reported to be successful in estimating contributions of large sized diatoms or some other main taxa but poor in respect to assessments of small flagellates, haptophytes, prasinophytes and dinoflagellates to the total biomass (Eker-Develi et al., 2012; Kozlowski et al., 2011; Schlüter et al., 2000). Pico-plankton and nanoplankton may contribute substantially to the total phytoplankton carbon biomass in coastal areas in certain periods (e.g. during stratification in summer; Bosak et al., 2012; Cerino et al., 2012; Rodríguez et al., 2003). These small sized organisms, especially the nanoplankton fraction, are not generally assessed accurately in preserved samples in routine microscopy analyses due to loss of their flagella and deformation of cells (Llewellyn et al., 2005; Cerino and Zingone, 2006; Cerino et al., 2012).

The eastern Mediterranean is an ultra-oligotrophic sea principally because of anti-estuarine circulation in the region (Hamad et al., 2005; Krom et al., 2010) coupled with the scarcity of nutrient input from rivers or below the euphotic zone.

By sampling at weekly intervals over an entire seasonal cycle, our major goals were to evaluate (a) the temporal variations in group-specific pigments as given by the HPLC-CHEMTAX analysis with respect to changing environmental variables, (b) temporal variations in the carbon:Chl *a* ratios for different phytoplankton groups dominating a coastal region with low productivity in the northeastern Mediterranean Sea coast. Additionally, we have attempted to better evaluate the contribution of smaller phytoplankton to C:Chl *a* ratios by the inclusion of live cell counts in unpreserved samples of nanoplanktic groups (i.e. haptophytes, prasinophytes and cryptophytes). According to our literature search, such an attempt has not been previously attempted. Despite the fact that the sampling in our study was carried out from only one station (which is close to the shore for frequent sampling at weekly intervals), it presents new valuable information on coastal phytoplankton dynamics regarding our research goals, irrespective of the station being representative of the region.

2. Material and methods

Samples were collected weekly with a plastic bucket from surface waters of the pier at Erdemli, Turkey (36°36′ N, 34°19′ E) in the northeastern Mediterranean Sea during September 2015–September 2016 (Fig. 1). The Lamas River, at a distance of about 5 km to the sampling point, is one of the main nutrient sources of the sampling area. WTW LF330 model conductivity meter was used for temperature and salinity measurements. Total depth of the water was ~2 m, ebb and tide levels were around half a meter. Suomi National Polar-orbiting Partnership, Visible/Infrared Imager Radiometer Suite (SNPP VIIRS) Photosynthetically active radiation (mol m^{-2} d^{-1}) values during the days of phytoplankton samplings were obtained roughly by checking colour scale of images from https://oceancolor.gsfc.nasa.gov/cgi/l3/ (NASA, 2018).

2.1. Environmental conditions in the sampling region

The sampling region was classified to be in "good environmental condition" based on a variety of Eutrophication Assessment Tools such as the Trophic Index (TRIX), Eutrophication Index (E.I.) or HELCOM Eutrophication Assessment Tool (HEAT) (Tugrul et al., 2018; Akçay et al., 2018). Local rivers and the atmospheric dust coming from the Saharan and Arabian deserts are external sources of nutrients in the sampling region (Doğan-Sağlamtimur and Tuğrul, 2004; Eker-Develi et al., 2006a; Koçak et al., 2010). The eastern Mediterranean Sea is a phosphate limited region and has exclusively high N/P ratios (ranging from 25 to 28) compared to the western Mediterranean (22) and to the Redfield ratio (16) (Koçak et al., 2010; Krom et al., 2010; Yilmaz and Tugrul, 1998).The highest nitrate concentrations (~5–10 μM) are recorded during the winter-spring period in coastal waters (Doğan-Sağlamtimur and Tuğrul, 2004; Uysal and Köksalan, 2006; Boran, 2017). Maximum flow rate of the Lamas River (~7–12 m^3/s) was reported between March–May (Özsoy, 2007). The phytoplankton bloom period usually takes place from February to early March in both coastal and open sea regions of the Mediterranean Sea related to winter mixing prior to stratification (Kideys et al., 1989; Eker-Develi et al., 2006a; Ribera d'Alcalà et al., 2004). Nutrient inflow from the Lamas River and other creeks in the region sustains further phytoplankton growth during spring and summer in the coastal region. Species composition also shows some similarities and differences in the coastal and offshore areas (Eker-

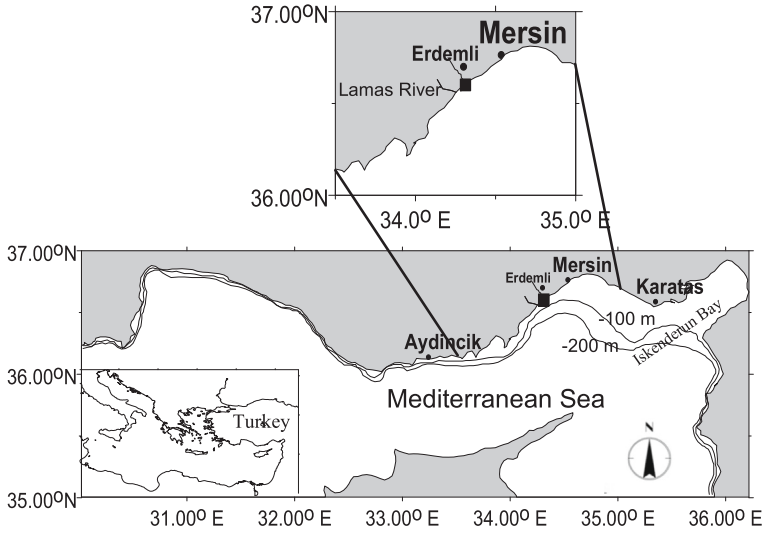

Fig. 1. Sampling location (black square) at Erdemli pier, Mersin from September 2015–September 2016.

Develi, 2004). An increase in nitrate concentrations is also observed during summer months but the concentrations do not reach the levels of winter-spring peaks (Eker-Develi, 2004; Doğan-Sağlamtimur and Tuğrul, 2004; Uysal and Köksalan, 2006; Eker-Develi et al., 2006a). The period during stratification due to seasonal thermocline between May and October (Yücel et al., 2017) is generally dry with very little precipitation in the sampling region (Özsoy and Saydam, 2000; Eker-Develi et al., 2006a, see Fig. 2 for precipitation values).

2.2. Phytoplankton sampling and analysis (including carbon biomass)

Phytoplankton samples were collected weekly from the surface into 1 L amber glass bottles and preserved with 31% formaldehyde buffered with borax to a final concentration of 1.5%. Samples were left to settle for 1–2 weeks after which the supernatant was siphoned off by thin curved tubes down to 15–20 mL. Phytoplankton cells (~400 cells) were counted with a Sedgewick Rafter Cell under a Nikon/eclipse TS100 inverted microscope with 200× and 400× magnification (Karlson et al., 2010).

In addition to formaldehyde preserved samples, parallel sampling was also performed for counting live, small, fragile cells of non-calcified haptophytes, cryptophytes and prasinophytes by settling 20 mL seawater directly within a Petri dish (with an area of 4300 mm^2) within 1–3 h under the microscope. Depending on the density of cells present, 45–210 mm^2 of the whole area, (corresponding to 0.2–1 mL seawater was scanned, when between ~50 and 300 nanoflagellate cells were counted. Some of the small and mainly dominant cells were isolated, cultured and identified using 1000× magnification under the light microscope (Nikon Eclipse E100), or using scanning electrone microscope (SEM) and performing DNA sequence analysis as detailed in Sahin and Eker-Develi (2018), Konucu (2018) and Konucu et al. (2019). Fine adjustment was carried out in each microscope field during scanning for living cell counts of these small species for which we had familiarity from culturing. Heterotrophic flagellates were not included in living cell counts (Patterson and Simpson, 1996). Living cell counts of cryptophytes and prasinophytes were used for pigment and microscopy comparisons. For haptophytes, preserved cell counts of calcifying haptophytes and live cell counts of non-calcified haptophytes were considered together for comparsion with pigment results. The biovolume (V) of each cell was calculated by measuring its appropriate morphometric characteristics (i.e. diameter, length and width) (Kovala and Larrance, 1966; Olenina et al., 2006; Eker-Develi et al., 2008). A biovolume (V) of 1 μm^3 was assumed equivalent to 1 pg wet weight (Wasmund et al., 1998; Hillebrand et al., 1999; Gasiunaite et al., 2005). Carbon biomasses were calculated from the volume of each cell throughout the text according to the equations of Menden-Deuer et al. (2000) as below;

for diatoms $log\ C = -0.541 + 0.811\ (logV)$

for diatoms $> 3000\ \mu m^3$ $log\ C = -0.933 + 0.881\ (logV)$

for dinoflagellates $log\ C = -0.353 + 0.864\ (logV)$

for haptophytes $log\ C = -0.642 + 0.899\ (logV)$

for chlorophytes and prasinophytes $log\ C = -1.026 + 1.088\ (logV)$

for small flagellates, cryptophytes and cyanobacteria $log\ C = -0.583 + 0.860\ (logV)$

2.3. Pigment analysis

0.5 to 1 L seawater was filtered through Whatman 25 mm Ø GF/F filters each week depending on the density of microalgae and stored at −20 °C until the analysis within 3 months. A modified version of pigment extraction (Barlow et al., 1997) was used for the analysis with an Agilent 1100 HPLC system. Pigments were extracted using 90% acetone (HPLC grade) and 1 min sonication, kept overnight at −20 °C and centrifuged. Then the samples were transferred to glass vials and placed inside an autosampler. Injections were applied by an autosampler whereby 200 µL of the extract was mixed with 200 µL 1 M ammonium

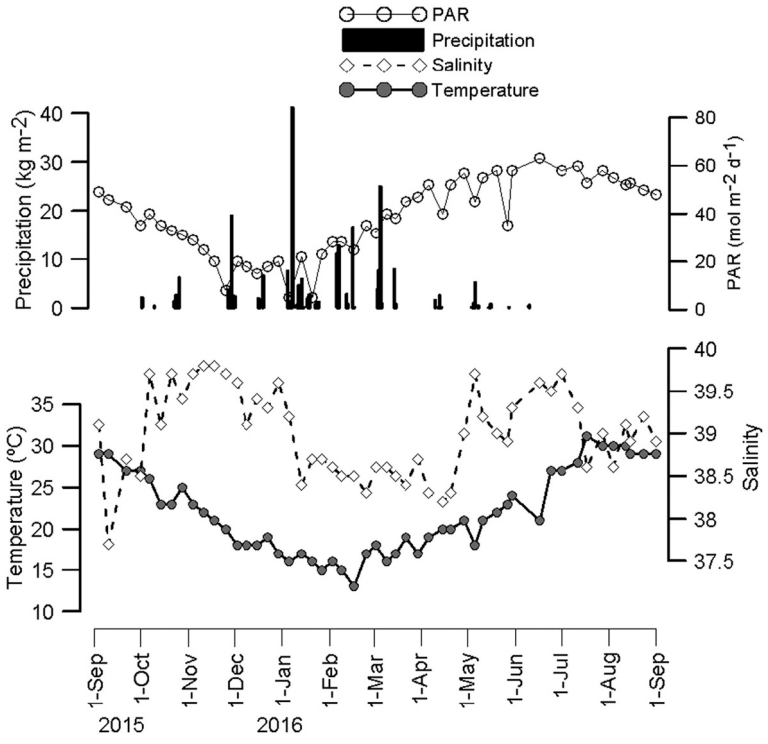

Fig. 2. Temporal changes in temperature, salinity, photosynthetic active radiation (PAR) and precipitation values at the surface layer during sampling.

acetate ion pairing solution (Mantoura and Llewellyn, 1983). Buffered extracts (100 μL) were injected through a 100 μL loop into a Thermo Hypersil MOS-2 C8 column (150 × 4.6 mm, 3 μm particle size, 120 Å pore size and 6.5% carbon loading). Separation of pigments was performed with linear gradient using a binary mobile phase system as reported by Yücel et al. (2017) and Barlow et al. (1997). Thirteen different phytoplankton pigments were detected by absorbance at 440 nm using an Agilent variable wavelength detector (Mantoura and Llewellyn, 1983). Pigment concentrations were calculated by 'external standard' equation (Jeffrey et al., 1997). The thirteen standards used were chlorophyll *a*, chlorophyll *b*, chlorophyll *c*2, peridinin, 19-butanoyloxyfucoxanthin, fucoxanthin, 19-hexanoyloxyfucoxanthin, diadinoxanthin, alloxanthin, lutein, zeaxanthin, divinyl chlorophyll-*a* and β-carotene (DHI company, Denmark). The carotenoids were sub-grouped as photosynthetic carotenoids (PSC) and photoprotective carotenoids (PPC). The PSC comprised fucoxanthin, 19′-butanoyloxyfucoxanthin, 19′-hexanoyloxyfucoxanthin and peridinin, while the PPC included alloxanthin, lutein, diadinoxanthin, β-carotene and zeaxanthin. As a result, three photo-pigment indices were obtained as in Barlow et al. (2008) and Mendes et al. (2015): total chlorophyll *a* to total pigments (TChl*a*:TP), photosynthetic carotenoids to total pigments (PSC:TP) and photoprotective carotenoids to total pigments (PPC:TP). TChl *a* involved chlorophyll *a* and *divinyl chlorophyll a*, TP included TChl *a*, Chl *b*, Chl *c2*, Chl *c3*, PPC and PSC.

2.4. CHEMTAX analysis

CHEMTAX was run by subdividing the dataset into two groups: high and low C:Chl *a* samples, in addition to applying it to the whole dataset. High C:Chl *a* samples corresponded mainly to the large-sized diatoms (*Proboscia alata*, *Trieres mobiliensis*, *Guinardia flaccida* and *Pseudosolenia calcar-avis*). The average C:Chl *a* ratio for the whole sampling period was 9 ± 16 and this value was assumed as the threshold for separating high and low C:Chl *a* ratios.

The CHEMTAX 1.95 software (Mackey et al., 1996), Microsoft Excel version, as used in Wright et al. (2009) was implemented in order to estimate phytoplankton classes based on pigments in the sampling region.

The pigments measured and their abbreviations (along with phytoplankton groups) are shown in Table 1. Eight phytoplankton classes; diatoms, dinoflagellates, haptophytes, cryptophytes, chlorophytes, prasinophytes, prochlorophytes and cyanobacteria and their respective marker pigments Fuco, Peri, Hex-fuco, Allo, Chl *b*, lutein, DVChl *a* as well as Chl *c2,* Chl *c3* and But-fuco, were chosen for CHEMTAX analysis based on microscopy and pigment data (Tables 1, 2).

60 different ratio matrices were formed from randomized copies of the initial ratio matrices (F_0) to find the best optimized accessory pigment:Chl *a* ratio matrix for each subgroup (Higgins et al., 2011; Wright et al., 2009; Simmons et al., 2016). 10% of the generated ratios having the minimum root mean square (RMS) was averaged and output ratios were used as a new input ratio matrix repeatedly until the ratios become stable for each of the two datasets (Table 2).

The parameters set for the calculations were as follows: ratio limits were set to 500, weighting was 'bounded relative error by pigment', iteration limit = 200, epsilon limit = 0.0001, initial step size = 10, step ratio = 1.3, cutoff step = 200, elements varied = 5, subiterations = 1, weight bound = 30 (Mackey et al., 1996).

CHEMTAX was run separately for all diatoms (Table 2) and for two different diatom groups (as Chl *c3* containing and lacking, Table S1, Fig. S1).

2.5. Statistical analyses

Model I and II Linear regression analyses (using Microsoft Excel 2013) were performed in order to assess numerical relationships between HPLC based parameters (CHEMTAX assigned Chl *a* values of each phytoplankton taxa) and microscopy based parameters (abundance and carbon biomasses of corresponding phytoplankton groups). For the statistical significance, a *P* value of 0.05 was used. For Spearman rank correlation analysis, SPSS statistical package version 22 was used.

Table 1
List of abbreviations used in this article (according to SCOR terminology, Jeffrey et al., 2011, Higgins et al., 2011) for phytoplankton classes, abundance, carbon biomass and pigments. Combined abbreviations are sometimes used in the text; e.g. Dino-A denotes dinoflagellate abundance or Prasino-C means Prasinophyte carbon biomass.

Phytoplankton classes	Abbreviation
Diatoms	Diatom
Dinoflagellates	Dino
Cyanobacteria	Cyano
Prochlorococcus sp.	Prochloro
Chlorophytes	Chloro
Prasinophytes in live samples	Prasino
Calcified Haptophytes	Cal-Hapto
Noncalcified Haptophytes in unpreserved samples	NonCal-Hapto
Small flagellates	sFlag
Cryptophytes in unpreserved samples	Crypto
Raphidophytes, Silicoflagellates, Euglenophytes	Others
Carbon biomass	C
Abundance	A
Pigments	
Chlorophyll *a*	Chl *a*
Fucoxanthin	Fuco
Chlorophyll *c2*	Chl *c2*
Chlorophyll *c3*	Chl *c3*
Diadinoxanthin	Diadino
Zeaxanthin	Zea
19′-hexanoyloxyfucoxanthin	Hex-fuco
Alloxanthin	Allo
Chlorophyll *b*	Chl *b*
Divinyl chlorophyll a	DVChl *a*
Lutein	Lut
Peridinin	Peri
19′-butanoyloxyfucoxanthin	But-fuco
β-carotene	β-carotene

3. Results

3.1. Physicochemical parameters

Low salinity values (38.3–38.5) were usually recorded during winter-spring and in autumn periods (37.7) showing freshwater input via rain and rivers which may trigger phytoplankton growth (Fig. 2). July–August was the warmest period with 30–31 °C temperature and February was the coldest month (13 °C). Photosythetically Active Radiation (PAR) values (5–63 mol m^{-2} d^{-1}) showed a similar seasonal pattern to that of temperature. During warm seasons, the rainfall was minimal with highest cumulative precipitation occurring between December and April.

3.2. Pigment indices

TChl*a*:TP ratio varied between 0.4 and 0.6 during September 2015–September 2016 period (Fig. 3). PSC:TP ratios were higher during January–May 2016 than the other months while PPC:TP showed an opposite trend displaying higher values during warmer June–August period.

3.3. Phytoplankton composition, microscopy and pigment results

The species number was relatively high during the study period. A total of 219 species were identified. 117 of these species were diatoms. Species identified were listed as: 67 dinoflagellates, 17 haptophytes, 3 dictyochophytes (*Dictyocha*, *Pedinella* sp., *Hermesinum adriaticum*), 4 cryptophytes (*Hemiselmis*, *Storeatula*, *Plagioselmis*, *Teleaulax*), 7

Table 2
Input ratios before the run (derived from Schlüter et al., 2000, Mackey et al., 1996, Gibb et al., 2001; Wright et al., 2009) and output ratios after optimization for marker pigments to Chl *a* for the selected phytoplankton classes. Abbreviations defined in Table 1. The output root mean square (RMS) error values were 0.259 and 0.261 for high and low C:Chl *a* ratios, respectively.

	Fuco	Peri	Hex-fuco	Allo	Chl *b*	Zea	Chl *c2*	Chl *c3*	But-fuco	Lut	DVChl *a*	Chl *a*
Diatom	0.81	0	0	0	0	0	0.3	0	0	0	0	1
Dino	0	0.55	0	0	0	0	0.28	0	0	0	0	1
Hapto	0.25	0	0.35	0	0	0	0.18	0.18	0.036	0	0	1
Crypto	0	0	0	0.35	0	0	0.13	0	0	0	0	1
Chloro	0	0	0	0	0.285	0.06	0	0	0	0.176	0	1
Prasino	0	0	0	0	0.623	0	0	0	0	0.035	0	1
Cyano	0	0	0	0	0	0.22	0	0	0	0	0	1
Prochloro	0	0	0	0	0	0	0	0	0	0	1	0
	Output ratios for high C:Chl *a* ratios (≥9)											
Diatom	0.64	0	0	0	0	0	0.22	0	0	0	0	1
Dino	0	0.52	0	0	0	0	0.26	0	0	0	0	1
Hapto	0.21	0	0.43	0	0	0	0.18	3.39	1.82	0	0	1
Crypto	0	0	0	0.3	0	0	0.12	0	0	0	0	1
Chloro	0	0	0	0	0.28	0.06	0	0	0	0.20	0	1
Prasino	0	0	0	0	0.73	0	0	0	0	0.03	0	1
Cyano	0	0	0	0	0	0.4	0	0	0	0	0	1
Prochloro	0	0	0	0	0	0	0	0	0	0	1	0
	Output ratios for low C:Chl a ratios (<9)											
Diatom	0.85	0	0	0	0	0	0.14	0	0	0	0	1
Dino	0	0.51	0	0	0	0	0.28	0	0	0	0	1
Hapto	0.26	0	0.35	0	0	0	0.18	0.17	0.04	0	0	1
Crypto	0	0	0	0.31	0	0	0.13	0	0	0	0	1
Chloro	0	0	0	0	0.23	0.06	0	0	0	0.16	0	1
Prasino	0	0	0	0	0.62	0	0	0	0	0.03	0	1
Cyano	0	0	0	0	0	0.43	0	0	0	0	0	1
Prochloro	0	0	0	0	0	0	0	0	0	0	1	0

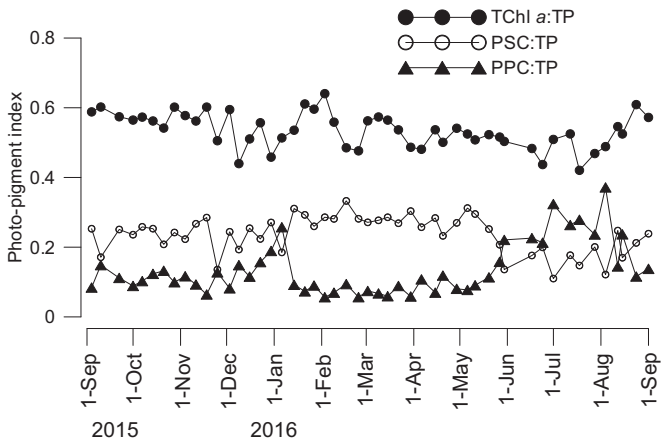

Fig. 3. Variations in photo-pigment indices at the sampling station in the northeastern Mediterranean Sea coast during 2015–2016.

prasinophytes (*Micromonas, Pseudoscourfieldia, Nephroselmis, Pyramimonas* spp., *Pterosperma* sp.), 3 cyanobacteria (*Oscillatoria, Merismopedia* and *Spirulina*), 1 euglenophyte (*Eutreptia viridis*), 1 raphidophyte (*Chattonella subsalsa*) and 1 chlorophyte (*Microspora*) species.

Diatoms were the most important group with respect to annual averages of CHEMTAX assigned Chl *a* values (40% of total) and microscopy-based carbon biomass values (50%) in the study area (Fig. 4a, b). Zeaxantin containing phytoplankton groups (i.e. mainly picocyanobacteria) were also among the major contributors to the CHEMTAX assigned Chl *a* values (27%, including *Prochlorococcus* and *Synechococcus*). Unfortunately picocyanobacteria was not counted with microscopy. Contribution of dinoflagellates to the total biomass seemed to be underestimated with the pigment based method. Contribution of main nanoplanktic groups, namely cryptophytes, prasinophytes and haptophytes to the total Chl *a* was 29% according to the results of CHEMTAX, while their share within the carbon biomass was 19% (Fig. 4a, b).

3.4. Weekly variations in chlorophyll a, phytoplankton carbon biomass, and C:Chl a ratios for total phytoplankton

Total Chl *a* values were < 1 μg L^{-1} in half of the samples with an average concentration of 1.2 ± 1.5 μg L^{-1} ($n = 50$) during the sampling period. The two highest total Chl *a* values (10.2 and 3.7 μg L^{-1}) were observed on 3 and 25 Feb 2016 respectively (Fig. 5a). Carbon biomass of phytoplankton was disproportionally high on these dates (i.e. 9 μg L^{-1} on the former and 50 μg L^{-1} on the latter date, Fig. 5b). The dinoflagellates, which generally have high C:Chl *a* ratios, were dominant on the latter date. In addition, *Asterionella glacialis*, which was the dominant diatom species on both dates, could be at the stationary growth phase on the latter date.

Other peaks in total carbon biomass between January and May 2016 period were mainly due to the diatoms.

An increase in Chl *a* on 1 July 2016 was due to picoplanktic cyanobacteria which was inferred from the rise in Cyano-Chl *a* concentration in the results of HPLC-CHEMTAX analysis (Fig. 5a).

Among the environmental parameters, total carbon biomasses correlated well ($p < 0.05$) with low salinity, and high PAR values ($p < 0.01$) but not with temperature or rainfall (Table 3).

Both carbon biomass of diatoms and dinoflagellates are positively correlated with PAR values (Table 3). A significant positive correlation between PAR values and photoprotective pigments were also observed (Table 3). Carbon biomass of cryptophytes was positively correlated with PAR values. Chl *a* of cyanophytes were positively correlated with both PAR and temperature. Hapto-Chl *a* is negatively correlated with both temperature and salinity (Table 3).

While there was no correlation between total carbon biomass and total Chl *a* concentrations ($p > 0.05$) for the whole dataset (Fig. 6a, Fig. S2), a significant correlation between these parameters did appear when the data was categorised as high and low C:Chl *a* samples (Fig. 6b, c, $p < 0.001$ for both cases, see Fig. S2).

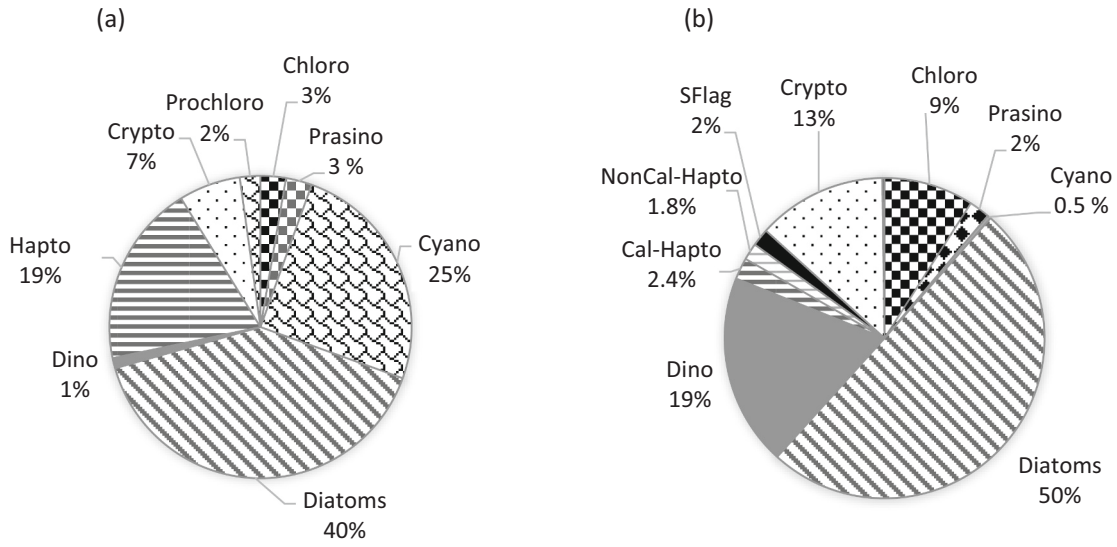

Fig. 4. Annual average percentage contributions of (a) different phytoplankton classes tothe total carbon biomass (b) CHEMTAX derived phytoplankton classes to chlorophyll *a* from September 2015–September 2016 at the sampling location.

3.5. Weekly variations in carbon biomass, chlorophyll a, and C:Chl a ratios for different phytoplankton groups

Carbon biomasses and Chl *a* values of diatoms were high in the winter-spring period (Figs. 4a, b, 7). The highest dinoflagellate carbon biomass was also observed in the winter period simultaneous with the diatom peak on 25 February 2016 (Fig. 4b). Nanoplanktic groups (cryptophytes, prasinophytes and haptophytes) dominated during the autumn, early winter and the summer period when diatoms were not abundant (Fig. 4a, b) and these periods corresponded to low C:Chl *a* ratios. Contribution of cyanobacteria to the total Chl *a* increased between June–September period (Fig. 5a). Notable observations on species compositions for different phytoplankton classes and major species throughout the sampling period are detailed below;

3.5.1. Diatoms

There was a significant correlation ($p < 0.001$) between CHEMTAX derived Diatom-Chl *a* and Diatom-C for the low C:Chl *a* case when the data was sub-grouped with respect to C:Chl *a* (Figs. S2, S3).

Upon dividing diatoms into two parts (as Diatom1 and Diatom2 for *Pseudo-nitzschia* spp.) CHEMTAX analysis did not provide a better relation between microscopy and pigment based results ($p > 0.05$, Fig. S1).

Variations in C:Chl *a* ratios were mainly due to the dominance of different size groups of diatoms. However, in one instance this ratio was observed to change substantially due to the distinct growth phase of a phytoplankton species during our sampling: High fucoxanthin concentration (4.4 μg L^{-1}) but low carbon biomass of the dominant diatom species *A. glacialis* (6.4 μg L^{-1}) on 3 February 2016 compared to low fucoxanthin levels (2.2 μg L^{-1}) but high carbon biomass of the same dominant species (20 μg L^{-1}) on 25 February 2016 could be attributed to exponential and stationary growth phases of this species, respectively (Figs. 7, S3).

3.5.2. Haptophytes

While calcified haptophytes, mainly *Emiliania huxleyi* sp. were dominant during the winter-spring period, noncalcified haptophytes, mainly *Chrysochromulina* spp. (counted in unpreserved samples), were abundant during spring-summer (Figs. 5b, S3).There were significant correlations between total Hapto-C and Hapto-Chl *a* values ($p < 0.05$, Figs. S2, S3).

3.5.3. Cryptophytes

Cryptophytes were widespread during the sampling period in the study area (Figs. 5, S3). Abundance of this group in live samples was ~1000 times higher than in preserved samples as annual average.

In addition to *Storeatula* cf. *major, Hemiselmis* sp., *Teleaulax* sp. and *Plagioselmis prolonga* a few cells of *Myrionecta rubra* were recorded during May–August in living cell counts.

However, Crypto-Chl *a* values allocated by CHEMTAX did not show a significant correlation with Crypto-C ($p > 0.05$, Figs. 7).

3.5.4. Chlorophytes and Prasinophytes

Chlorophytes, in the studied region, were generally represented by the subphylum Prasinophytina (referred to as prasinophytes in the text) rather than Chlorophytina. The majority of prasinophytes in the samples belonged to the genera *Pyramimonas, Nephroselmis* and *Pseudoscourfieldia*. In addition, the flagellated stage of *Pterosperma* sp. was occasionally observed. Cell counts of living and preserved prasinophytes, mainly *Pyramimonas* spp., were statistically correlated with each other ($r^2 = 0.99$, $p < 0.05$), however, unpreserved samples produced 5–6 times higher cell counts than preserved samples underlining the importance of live cell counts for Prasinophytina.

The correlation between carbon biomass of prasinophytes and Prasino-Chl *a* was significant in the low C:Chl *a* ratio samples, while no correlation was observed within the samples having high C:Chl *a* ratios (Fig. S2). Prasinophyte abundance increased during summer-autumn months in general (Fig. S3).

3.5.5. Dinoflagellates

There was no statistically significant correlation between microscopy and pigment based results for dinoflagellates (Fig. 5, S3). A major peak in dinoflagellate carbon biomass was observed on 25 February 2016 (Fig. 5b, S3), where the dominant phytoplankton species on this date was *Alexandrium* sp. Other dominant species during the year were *Gonyaulax* spp., *Protoperidinium* spp. and heterotrophic *Gyrodinium spirale*.

3.5.6. Cyanobacteria

No correlation was found between the abundance of preserved filamentous cyanobacteria and CHEMTAX derived Chl *a* of cyanobacteria ($p > 0.05$). According to CHEMTAX results cyanobacteria abundance increased at the end of May and remained high until the beginning of August (Figs. 5a, S3).

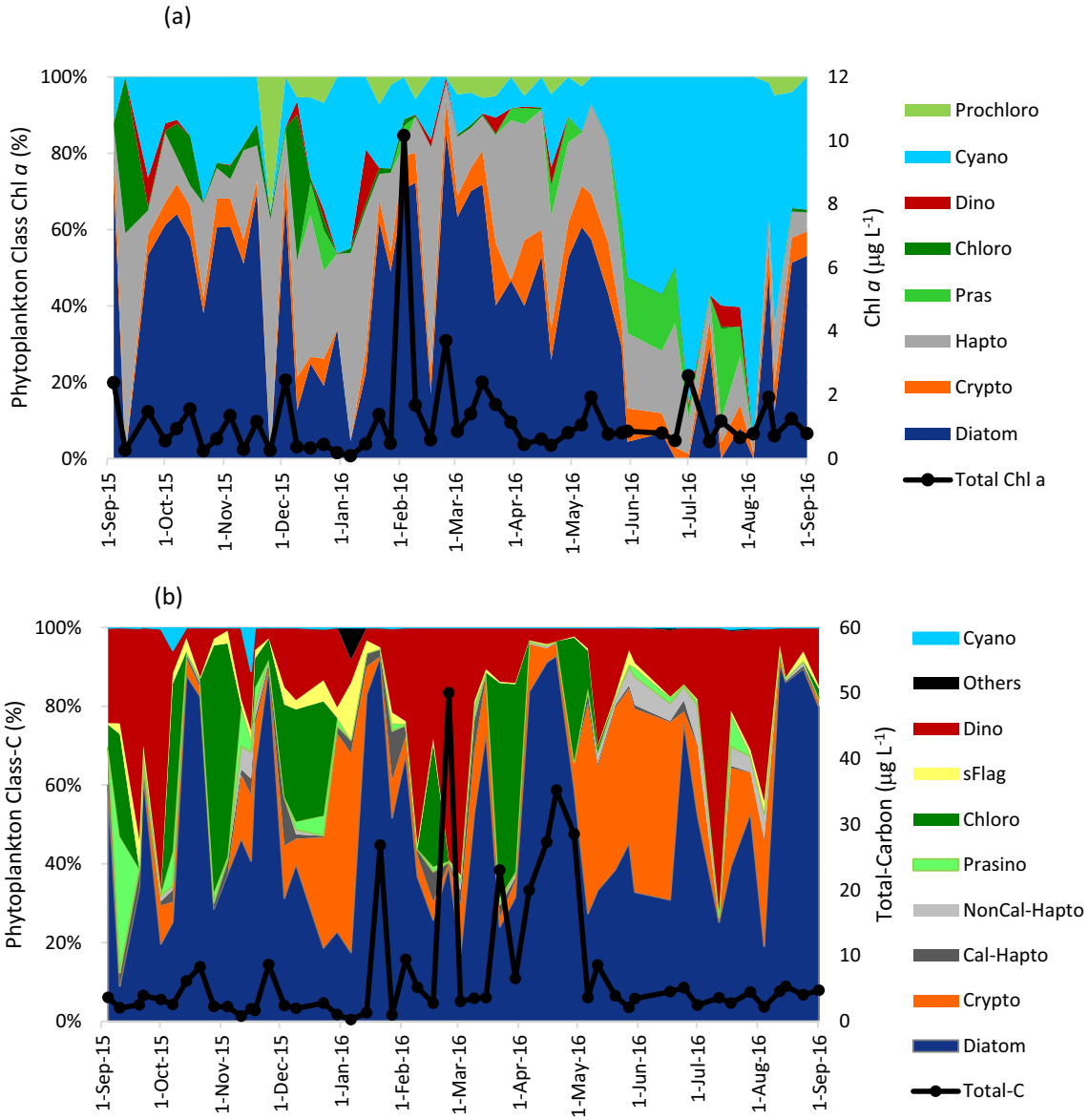

Fig. 5. Percentage contributions of (a) phytoplankton class-Chl *a*, (b) carbon biomass during 2015–2016.

4. Discussion

4.1. Pigment indices

Higher PPC:TP ratios during warm summer months (June–August) observed in the present study is expected due to the dominance of small species such as zeaxanthin and β-carotene containing cyanobacteria and prasinophytes and partially alloxanthin containing cryptophytes during the warm period. PSC:TP ratios were high during September–November 2015 and January–May 2016 (Figs. 3, 5) when the contribution of diatoms to the total carbon biomass and Chl *a* were high similar to observations in distinct latitudes of the Southern Ocean and South Africa coast (Mendes et al., 2015; Barlow et al., 2008).

4.2. Phytoplankton composition

When the present phytoplankton sampling approach was compared with a similar weekly study performed during December 2000–April 2002 (Eker-Develi, 2004; Eker-Develi et al., 2006a) near to the sampling region, more or less similar species were observed to dominate with diatoms were being the dominant class in terms of biomass in both studies. The species displaying the highest contribution to the diatom carbon biomass were *P. alata*, *Pseudo-nitzschia* sp. *Cerataulina pelagica* and *Pseudosolenia calcar-avis* (Eker-Develi, 2004).

In addition to aforamentioned diatom genera, *Chaetoceros*, *Leptocylindrus*, *Guinardia*, *Skeletonema*, *Thalassionema* and *Hemiaulus* also reach high abundances in the Mediterranean Sea (Polat and Işık, 2002; Ribera d'Alcalà et al., 2004; Siokou-Frangou et al., 2010; Yılmaz, 2006).

4.3. Impact of environmental parameters on phytoplankton carbon biomass and pigments

There was a significant positive correlation ($p < 0.01$) between total carbon biomass and PAR values showing an increase in carbon biomass with increasing light intensities (Table 3). The reverse of this situation was evident by the lowest levels of phytoplankton carbon in December and January, possibly due to overgrazing by zooplankton (Gaudy et al., 2003; Siokou-Frangou et al., 2010). Mesozooplankton grazing was estimated to remove 47% and 50% of primary production in winter and spring respectively (Gaudy et al., 2003).

Table 3
Spearman rank correlation between phytoplankton carbon biomasses and environmental parameters (only significant correlations are shown) (weekly cumulative precipitation values were analysed).

	Temperature (°C)	Salinity	PAR (mol m^{-2} d^{-1})	Precipitation (kg m^{-2})
Total-C	n	−0.32*	0.373**	n
Total-Chl *a*	n	n	n	n
Diatom-C	n	n	0.287*	n
Diatom-Chl *a*	n	n	n	n
Dino-C	n	−0,475**	0.331*	n
Dino-Chl *a*	n	−0.282*	n	n
Hapto-C	n	n	n	n
Hapto-Chl *a*	−0.402**	−0.322*	n	n
Crypto-C	n	n	0.347*	n
Crypto-Chl *a*	n	n	n	n
Prasino-C	n	n	n	n
Prasino-Chl *a*	n	n	n	n
Cyano-Chl *a*	0.419**	n	0.372**	n
PPC	0.368**	n	0.528**	n
PSC	n	n	n	n

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Significant positive correlation (at $p < 0.01$ level) between PPC and PAR indicates increased abundance of nano- and pico-plankton such as cryptophytes and cyanobacteria and/or rising in their cellular content of photoprotective pigments, with increasing light intensities (Schlüter et al., 2006).

4.4. Relationship between microscopy and HPLC-CHEMTAX approaches

Prior to this study, HPLC-CHEMTAX derived Chl *a* results had not been compared with carbon biomasses of different phytoplankton classes in a frequent time series investigation in the Mediterranean Sea. On a global scale, there are limited parallel studies for comparison of our results other than Llewellyn et al. (2005) and Rodríguez et al. (2006) which were carried out in the English Channel and Atlantic coast of Spain. Previous CHEMTAX studies focus on the impact of nutrients on phytoplankton composition and abundance disregarding phytoplankton carbon biomass (Sebastiá and Rodilla, 2013; Yücel et al., 2017;

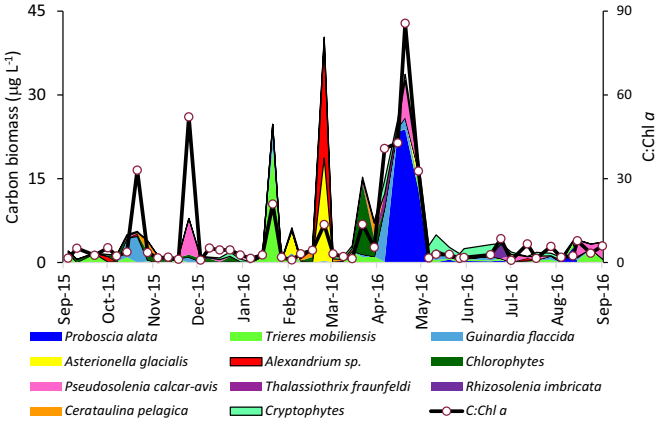

Fig. 7. Phytoplankton species or groups displaying the highest carbon biomasses (exceeding annual average values of 0.05 µg L^{-1}) during 2015–2016 Carbon biomasses and C:Chl *a* ratios.

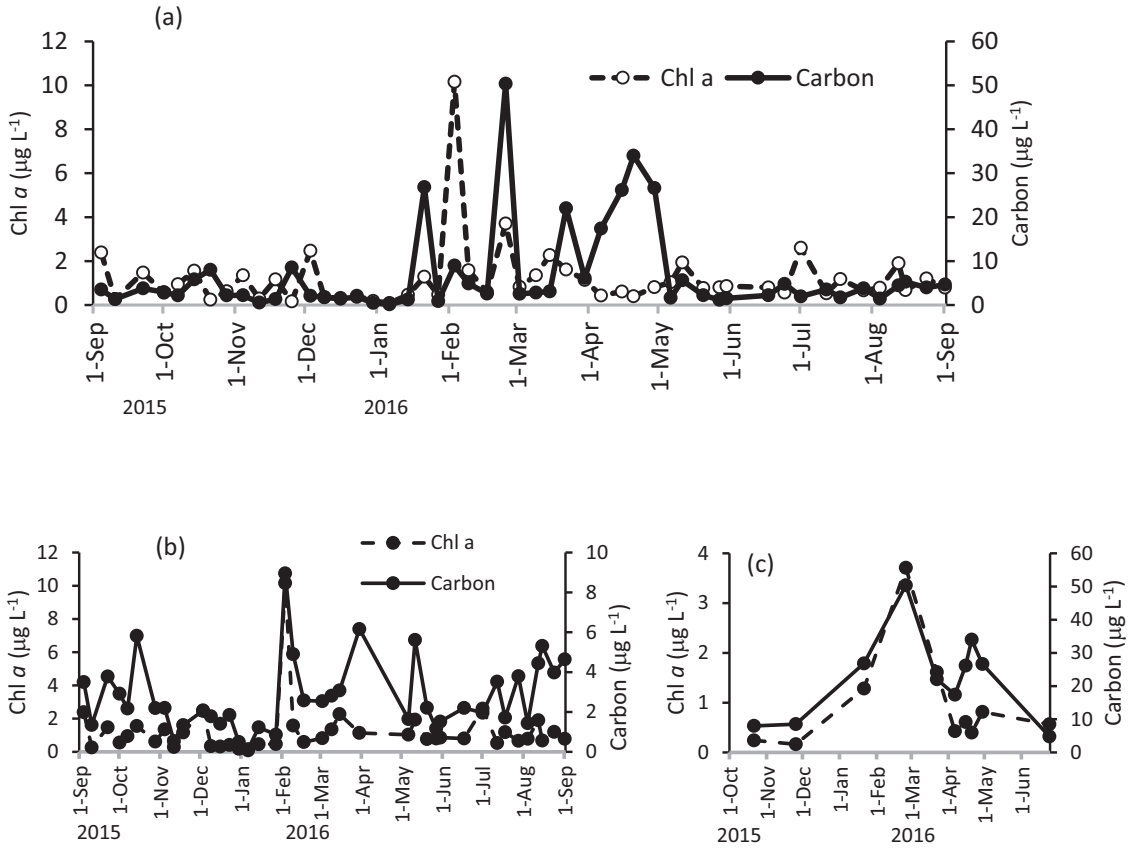

Fig. 6. Variations in (a) carbon and Chl *a* values in ungrouped samples and in samples with (b) low C:Chl *a* ratios of <9 (c) high C:Chl *a* ratios of ≥9.

Krivokapić et al., 2018) in the Mediterranean Sea.

Our results show that CHEMTAX assigned chlorophyll *a* values of phytoplankton classes should not be assumed as a proxy to carbon biomasses of these classes unless they are further sub-grouped. The data was sub-grouped based on C:Chl *a* ratios since changes in this ratio are mainly related to the size differences of phytoplankton (mainly of diatoms) here (Figs. 7, S2). Most probably for the same reason, removal of very large diatoms from the analysis was reported to improve correlation of pigments with cell biomass in previous studies (Schlüter and Møhlenberg, 2003; Havskum et al., 2004; Wright and Jeffrey, 2006).

Although nutrients are not measured in this study, it is well known that the limiting nutrients are often used up rapidly as soon as they are available in the euphotic zone (Eker-Develi et al., 2006a). Therefore, subgrouping phytoplankton based on nutrient concentrations does not seem reasonable for the present dataset. Splitting the data based on PAR values did not provide a better correlation between microscopy and pigment based results of phytoplankton classes either.

In previous studies, pigment data were split into groups based on depth layers, seasons, microscopic analysis, physical, chemical and light conditions for CHEMTAX analysis (Descy et al., 2003; Descy et al., 2009; Goela et al., 2014). In a four-year time series study performed in the English Channel, pigment data was separated into two parts based on C:Chl *a* ratios $<$ and $>$ 25, with low ratios approximated to samples collected during winter months (Llewellyn et al., 2005). In addition, Rodríguez et al. (2006) divided only their diatom dataset into two parts based on C:Chl *a* ratios. In large diatom cells, not only Chl *a* content of cells relative to carbon decreases, but also the amount of light absorbed per unit pigment is reduced due to the package effect (intracellular self-shading, Maranon, 2009). In contrast to the increase in this ratio with rising cell size in diatoms, some strains of smaller species belonging to distinct taxonomic classes (e.g. cyanobacteria such as *Prochlorococcus* sp. and *Synechococcus* sp.) were reported to have much higher C:Chl *a* ratios than seen in diatoms (Finenko et al., 2003; Sathyendranath et al., 2009). Thus, seasonal variations in local community composition of phytoplankton and their cell sizes should be taken into account while estimating carbon biomass from optical properties of seawater using either in situ measurements (Behrenfeld et al., 2005) or via satellite observations.

Knowledge about taxonomic structure of microalgae and usage of size-fractionated pigment measurements in CHEMTAX analysis could help to solve the mentioned problems. Diatoms were the most important taxonomic class in terms of carbon biomass during the majority of the sampling period, as revealed by microscopy. However, pigment data displayed the importance of cyanobacteria and haptophytes in addition to diatoms within total Chl *a* concentrations in the present study. In a previous study performed in a nearby location, (5 km distance to ours), CHEMTAX analysis showed the importance of diatoms and dinoflagellates in the coastal area and the prominence of cyanobacteria and diatoms in the offshore region (Yılmaz, 2006).

Much higher share of dinoflagellates within total carbon biomass (19%) than within CHEMTAX derived Chl *a* (1%) in the present study could be related to dinoflagellates containing 19'Hexanoyloxyfucoxanthin (Zapata et al., 2012). Apart from 19'Hexanoyloxyfucoxanthin, some dinoflagellates also contain unusual pigments of their endosymbionts, such as fucoxanthin, alloxanthin and chlorophyll *b* rather than their marker pigment peridinin (Higgins et al., 2011) and hence, underestimation of this group by HPLC-CHEMTAX method is possible (Irigoien et al., 2004; Lewitus et al., 2005). It is also well known that several dinoflagellates species completely lacks photosynthetic pigments (Jeong et al., 2010).

For prasinophytes, when high abundances were reached during the May–September 2016 period, a significant correlation appeared between Chl *a* and carbon biomass for this class (Fig. S2, S3).

4.5. Contribution of smaller cells

It was striking that utilizing living cell counts of prasinophytes and noncalcified haptophytes rather than preserved cells alone under the light microscope produced better correlations in terms of the pigment based approach in the present study. Abundances of these nanoplanktic groups are either underestimated or cannot be differentiated taxonomically within preserved samples. 96.4% of phytoflagellates could not be attributed to any certain taxonomic class in preserved samples in a study performed in the southern Adriatic Sea (Cerino et al., 2012). These small and mainly nanoplanktic groups dominated the sampling region especially during warm and dry periods (May–September, Fig. 5, Fig. S3) similar to other investigations performed in the Mediterranean Sea (Unrein et al., 2014; Cerino and Zingone, 2006; Krivokapić et al., 2018).

Poor preservation of cryptophytes in fixatives have been reported in a previous study (Llewellyn et al., 2005). Although live counting of cells under microscope allowed to observe all cells as intact and facilitated identification, there may be some inaccuracies in counts of rapidly moving specimens such as cryptophytes in the samples. Inconsistencies between HPLC-CHEMTAX and microscopy based results for cryptophytes (Fig. S2, S3) could be related to this reason, in addition to light dependent variation of the photoprotective pigment alloxanthin in cryptophytes (Henriksen et al., 2002; Tamm et al., 2015). Moreover, the alloxanthin containing ciliate *Myrionecta rubra* might have been overlooked by microscopy in some samples.

Cyanobacteria was the second most important class contributing to total Chl *a* based on CHEMTAX analysis (25%, Prochloro-Chl *a* + Cyano-Chl *a* together 27%, Fig. S3) and their share was especially important during the warm July–August period, consistent with flow cytometer results from a nearby location (Boran, 2017). In the Adriatic Sea, the picophytoplankton group was the most important fraction forming 49% of average biomass (Cerino et al., 2012). Haptophytes exhibited relatively high CHEMTAX derived Chl *a* following cyanobacteria (Fig. 4).

In this study, the average contribution of mainly nanoplanktic classes, cryptophytes, prasinophytes and haptophytes based on CHEMTAX was 29%. Based on microscopy, the nanoplankton (haptophytes, prasinophytes, cryptophytes and small flagellates) percentage share within the total carbon biomass was 21%.

4.6. Carbon: chlorophyl a ratio

Total phytoplankton carbon biomass and Chl *a* changed between $<$1–50 μg C L^{-1} (mean: 7 ± 10 μg C L^{-1}) and 0.1–10 μg Chl *a* L^{-1} (mean: 1.2 ± 1.5 μg Chl *a* L^{-1}) resulting in an overall average C:Chl *a* ratio of 9 ± 16, which seems far too low in the present investigation. This ratio varies between $<$10 and $>$ 200 among different phytoplankton classes and under varying light, nutrient and temperature conditions (Geider, 1987; Laws and Bannister, 1980; Llewellyn et al., 2005; Sathyendranath et al., 2009)". The average of Chl *a* was higher in our study compared to that of Eker-Develi (2004), Uysal and Köksalan (2006) and Yılmaz (2006) for 2001–2002, 1998–1999 and 2001–2003 (0.7–0.8 μg L^{-1}) in a close distance to our sampling station. The range of total carbon biomass in this study seems reasonable when compared to total phytoplankton biomass observed in the southern Adriatic Sea (range 8.5 and 80.7 μg C L^{-1} during 2006–2008, Cerino et al., 2012), which is a more eutrophic region. The reason for the very low C:Chl *a* ratio found in our study is not entirely clear. However, this ratios was often reported as low ($<$10) in a study carried out in an estuary of Belgium as well (Lionard et al., 2008). Furthermore, in the study of Rodríguez et al. (2006), in one-third of the samples, pigments of diatoms did not correspond to any microscopic counts, which cause low C:Chl *a* ratios for this group. It is also worthy to note that C:Chl *a* ratios during low carbon biomass periods in this study appear to be more error prone. Low carbon biomass relative to Chl *a* observed could be partly contributed by a methodological bias in this study: It is possible that some of large and rare cells or particles of macroalgae could not have been inspected in the limited volume of

subsample under microscope; whilst their Chl *a* were included in the pigment analysis. In addition, in the present investigation, sporadic increases in nutrient concentrations might have caused an increase in Chl *a* content of cells leading to low C:Chl *a* ratios during the periods of low carbon biomass which might be under pressure of zooplankton grazing.

C:Chl *a* ratios based on fixed carbon values obtained from the results of primary production and total Chl *a* were also less than 10 at a coastal station near to the present sampling station during monthly sampling performed in 2010–2011 (Yücel, 2013). This ratio is known to decrease under nutrient replete and low light conditions (Geider, 1987; Schlüter et al., 2000; Eker-Develi et al., 2006b). In addition, phytoplankton composition, their cell size and growth phase also play a role in variations in C:Chl *a* ratio (Finkel, 2001; Sathyendranath et al., 2009; Stelmakh and Gorbunova, 2018).

Higher average carbon biomass (14 ± 15 μg C L^{-1}, range < 1–39 μg C L^{-1}) and C:Chl *a* ratios (55 ± 18) were observed during December 2000–February 2002 in a nearby sampling station (36° 33′N and 34°15, Eker-Develi, 2004), which did neither include picoplanktic cyanobacteria, similar to this study. The average of C:Chl *a* ratio was also higher in the Baltic Sea (20 ± 7, Eker-Develi et al., 2008) and in the Black Sea (124 ± 50, Eker-Develi et al., 2012). While calculating C:Chl *a* ratio, same methodology and equations have been used here and aforamentioned studies, except more detailed nanoflagellate analysis in the present study (Eker-Develi, 2004; Eker-Develi et al., 2008, 2012).

The highest C:Chl *a* ratios were observed when large size diatoms (*P. alata, G. flaccida, Trieres mobilienis* and *Pseudosolenia calcar-avis*) were present in October, November, January, April and during the stationary growth phase of the diatom *A. glacialis* in February in the present study (Fig. 7). Changes in the C:Chl *a* ratio were also associated with variation in cell size and taxonomic composition of phytoplankton during the summer period in the Black Sea (Stelmakh and Gorbunova, 2018). Similar to our observation, the C:Chl *a* ratio was reported to increase with cell volume in diatom cultures (Finkel, 2001).

5. Conclusions

The HPLC-CHEMTAX method was deemed successful in estimation of the dominant phytoplankton classes: diatoms, haptophytes and partially prasinophytes in the study area when the data set was split into two parts based on carbon biomass:chlorophyll *a* ratios. Variations in this ratio were mainly due to the dominance of different size groups of diatoms. Microscopy and pigment results for cryptophytes did not correlate with each other either due to possible errors in live cell counts or due to variation in the cellular content of photoprotective pigment alloxanthin. Although dinoflagellates were the second most important group in terms of carbon biomass in the sampling region, their contribution to total chlorophyll *a* was underestimated by the HPLC-CHEMTAX approach, similar to previous investigations (Lewitus et al., 2005; Taylor et al., 2016).

The share of the nano and pico-phytoplankton fractions to the Chl *a* was greater than 56% in the present investigation. Low C:Chl *a* ratios found in this study should be further investigated with field and laboratory studies. We have shown for the first time that live cell counts of nanoflagellates provide a better correlation between microscopy and pigment based approaches for this size group in the study region.

Author contributions

MK: Phytoplankton analysis, Methodology, Writing—review. EED: Phytoplankton analysis, Writing—original draft, Conceptualisation, Analysis and Investigation, Methodology, Data curation, Validation. HO: Methodology, Writing—review, editing. SB: HPLC analysis, Methodology, review, AEK: Conceptualisation, Analysis and Investigation, Methodology, Data curation, Validation, Review and Editing.

Declaration of Competing Interest

The authors declare that they have no competing interests that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jembe.2022.151718.

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