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Seasonal dynamics of transparent exopolymer particles (TEP) and their drivers in the coastal NW Mediterranean Sea

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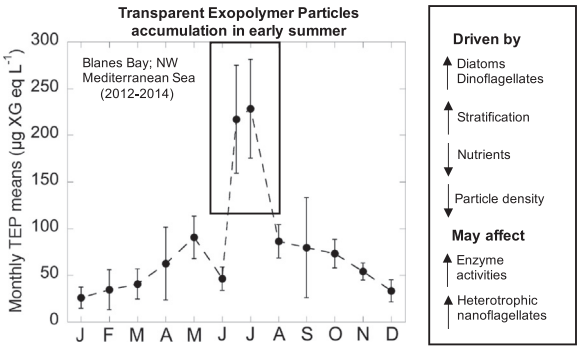
HIGHLIGHTS

- Transparent Exopolymer Particles (TEP) were measured during three years in the NW Mediterranean.
- TEPs peaked in early summer and were temporally disconnected from chlorophyll *a*.
- TEP are released mainly by diatoms and dinoflagellates at low nutrient concentration periods.
- TEP accumulation in stratified waters may enhance prokaryotic enzyme activities.

GRAPHICAL ABSTRACT



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ABSTRACT

Transparent Exopolymer Particles (TEPs) are a subclass of organic particles with high impact in biogeochemical and ecological processes, such as the biological carbon pump, air-sea interactions, or the microbial loop. However, the complexity in production and consumption makes TEP dynamics hardly predictable, calling for the need of descriptive studies about the in situ dynamics of these particles. We followed monthly TEP dynamics and combined them with a dataset of environmental variables during three years in a coastal site of the oligotrophic North Western Mediterranean (Blanes Bay). TEP concentration, ranging from 11.3 to 289.1 µg XG eq L^{-1} (average 81.7 ± 11.7 µg XG eq L^{-1}), showed recurrent peaks in early summer (June–July). TEP were temporally disconnected from chlorophyll *a* maxima, that occurred in late winter and early spring (maxima 1.21 µg L^{-1}), but they were significantly related to the abundance of specific phytoplankton groups (diatoms and dinoflagellates) and also coincided with periods of low nutrient concentrations. The fraction of particulate organic carbon in the form of TEP (the TEP:POC and TEP:PM ratios) were also highest in early summer, indicating that TEP-enriched particles of low density accumulate in surface waters during stratified periods. We hypothesize that the accumulation of these particles affects the microbial food web by enhancing the activity of specific prokaryotic extracellular enzymes (esterase, β-glucosidase and alkaline phosphatase) and promoting the abundance of heterotrophic nanoflagellates.

1. Introduction

Transparent exopolymer particles (TEPs) are operationally defined as acidic polysaccharide particles that are stainable with Alcian Blue

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(Alldredge et al., 1993). TEP self-assemble from precursors in the dissolved fraction (Zhou et al., 1998) to particles up to hundreds of micrometers, thus spanning the whole dissolved to particulate organic matter continuum (Passow, 2002b). Due to their stickiness, TEP promote organic and inorganic particle aggregation forming marine snow, a fundamental process in the biological carbon pump (Passow et al., 1994). However, since TEP have low density, when unballasted they do not sink, and can even ascend in the water column (Azetsu-Scott and Passow, 2004) and accumulate in the sea surface microlayer (Wurl et al., 2016). This enrichment affects sea-air fluxes and can be a substantial source of organic aerosols (Aller et al., 2017; Orellana et al., 2011). TEP can also have important impacts on the microbial foodweb: these particles are usually colonized by heterotrophic prokaryotes (Bar-Zeev et al., 2011; Mari and Kiørboe, 1996), and the presence of TEP can shape the microbial community as they are preferentially consumed by some prokaryotic groups only (Taylor et al., 2014). In the coastal ocean, the prediction of TEP occurrence has also applied interest since they can be precursors of high mucilage events that affect water quality (Radic et al., 2005; Scoullos et al., 2006), and may clog reverse osmosis membranes in the desalination industry (Berman, 2013).

TEP in the ocean are produced mostly by phytoplankton and heterotrophic prokaryotes (Ortega-Retuerta et al., 2010; Passow, 2002a). However, in situ TEP dynamics cannot easily be predicted from bulk microbial abundance or activity rates, since not all microbial species produce TEP equally (Passow, 2002a, 2002b). In addition, TEP production and formation rates are the result of algal and bacterial physiology state, particularly of nutrient stress (Corzo et al., 2000; Mari et al., 2005) and affected as well by environmental factors such as turbulence (Pedrotti et al., 2010). TEP sinks, on the other hand, include degradation by photolysis (Ortega-Retuerta et al., 2009) or by prokaryotic enzymatic activity (Smith et al., 1992). This complexity in production and consumption makes TEP dynamics in the field hardly predictable from one or a few parameters, such as phytoplankton or prokaryote abundance. The lack of simple predictability calls for the collection of data on the dynamics of these particles in situ, particularly throughout full seasonal cycles. Combining TEP observations with a broad suite of physical, chemical and biological measurements should help us understand how these particles are cycled, paving the path for better predictability.

To our knowledge, there are only four previous studies following TEP dynamics over more than one complete seasonal cycle: one in the West coast of India (Bhaskar and Bhosle, 2006), one in a freshwater catchment in Southern Spain (Vicente et al., 2009), one in the Fram Strait, Arctic Ocean (Engel et al., 2017) and the fourth one in the coastal North Western Mediterranean (Iuculano et al., 2017). The few other studies describing TEP temporal dynamics in the ocean spanned less than one year or were sampled only occasionally (i.e. two to three times per year (Parinos et al., 2017)), and the observed seasonal patterns did not follow similar trends. For instance, while in some time series TEP and chlorophyll *a* (chl *a*) co-vary (Beauvais et al., 2003; Engel et al., 2017; Parinos et al., 2017; Scoullos et al., 2006), in some others they do not (Bhaskar and Bhosle, 2006; Taylor et al., 2014), or they only co-vary at certain periods of the year (Dreshchinskii and Engel, 2017).

In the NW Mediterranean sea, chl *a* concentration and particulate primary production are usually highest at the end of the winter (Gasol et al., 2016). During summer, water stratification enhances nutrient limitation of the prokaryote communities, mainly due to phosphorus, in surface waters (Pinhassi et al., 2006; Sala et al., 2002). This nutrient limitation hampers carbon uptake and remineralization (Thingstad et al., 1997) and thus an accumulation of organic matter from spring to the end of summer is frequently observed (Romera-Castillo et al., 2013; Vila-Reixach et al., 2012). Aside from studies done in coastal areas that were eutrophic or heavily influenced by the presence of seagrass meadows, non-representative of the whole Mediterranean basin (Iuculano et al., 2017; Radic et al., 2005; Scoullos et al., 2006), the only published study on Mediterranean TEP dynamics (Beauvais et al., 2003) observed TEP maxima in summer, both at coastal and offshore sites. However, that work followed only one seasonal cycle, and it is thus not known whether these summer peaks are recurrent or episodic. A recent study in the Catalan Sea coastal ocean indicated that horizontal variations of TEP could be predicted from chl *a* concentration only at certain periods of the year, with higher TEP concentrations respect to chl *a* in summer (Ortega-Retuerta et al., 2017). Based on these previous studies, we extended our temporal span to better describe and predict TEP temporal variability. Our research objective was to explore whether TEP, as an organic carbon pool, would accumulate at the end of summer as other organic moieties do in Blanes Bay, or conversely, as a by-product of plankton activity, would match chl *a* or plankton (both eu- and prokaryote) dynamics. We thus followed TEP dynamics combined with a complete dataset of environmental and biological variables during three years in a coastal site of the North Western Mediterranean (Blanes Bay). We aimed at solidly describing the annual TEP cycle, deciphering the main environmental and biological factors that drive TEP variability, and exploring the effect of TEP shaping the microbial foodweb. Elucidating the seasonal and interannual TEP recurrence in Mediterranean coastal areas will help to make predictions about their biogeochemical (aerosol formation, particle fluxes) and economical (desalination industry, mucilage formation) effects.

2. Material and methods

2.1. Study site and sampling

Samples were taken from the long-term Blanes Bay Microbial Observatory (BBMO). This is a well-studied temperate oligotrophic coastal site that has relatively little human or riverine influence (Gasol et al., 2016). The observatory is located in a shallow (20 m) open bay (41°39.9′N, 2°48.3′E), around 800 m offshore in the NW Mediterranean (Fig. 1). Sampling was performed once per month plus the June solstice from January 2012 to October 2014. In situ temperature-salinity casts were measured using a calibrated SAIVA/S SD204 sensor. We defined a stratification index as the temperature difference between the surface and near the sea bottom (20 m). Water transparency was measured as the Secchi disk depth. Surface (0.5 m) waters were sampled using a bucket and pre-filtered through a 200-μm mesh net and kept in the dark in 20-L polycarbonate carboys until further processing in the lab (within 2 h).

2.2. Analytical procedures

TEPs were measured following the colorimetric method proposed by Passow and Alldredge (1995). Samples (250 mL) were filtered through 25 mm diameter 0.4 μm pore size polycarbonate filters (DHI). The filters were stained with 500 μL of pre-calibrated (with a xanthan gum solution) Alcian Blue (0.02%, pH 2.5) for 5 s and rinsed with MilliQ water. Subsequently, they were soaked in 80% sulfuric acid for 3 h and the absorbance of the extract was determined at 787 nm in a Varian Cary spectrophotometer. The absorption of every batch of Alcian Blue was calibrated using a xanthan gum (XG) solution that was homogenized with a tissue grinder and measured by weight difference. Duplicates were taken for each sample. Average SD of duplicates was 8.1, and the detection limit was set to 0.024 absorbance units. Duplicate blanks (empty filters stained with Alcian Blue) were also prepared with every batch of filtered samples. We conducted TEP analyses in formalin-fixed (1% final concentration) samples, that were preserved at 4 °C until filtration (within 3 months at most). We decided to conduct TEP analyses on fixed samples, since formalin does not interfere with the measurement (Passow and Alldredge, 1995), in order to optimize the number of samples processed every time a new calibration curve was constructed (one every four months). Anyway, we tested for the effect of formalin addition on Alcian Blue absorption, resulting in non-significant differences between fresh and fixed and stored samples. To

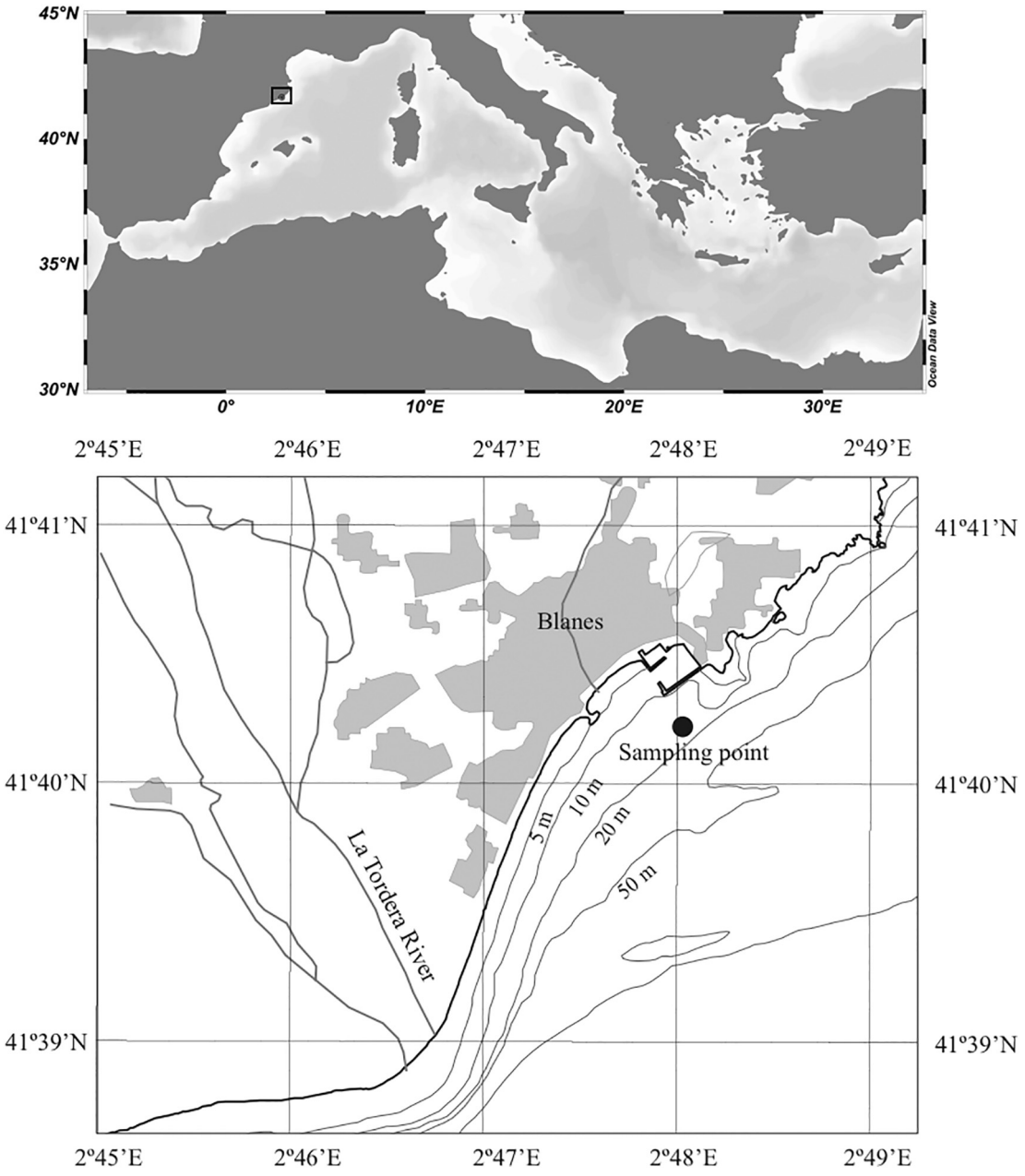


Fig. 1. Map showing the study station.

compare TEP with particulate organic carbon (POC) stocks, we converted TEP into carbon units using the conservative conversion factor of 0.51 μg C μg XG eq^{-1} (Engel and Passow, 2001).

Chlorophyll *a* concentration (chl *a*) was determined by filtering 150 mL of seawater on GF/F filters (Whatman), extracting the pigment in acetone (90% v:v) in the dark at 4 °C for 24 h, and measuring fluorescence with a Turner Designs fluorometer. Particulate primary production was determined measuring incorporation of $^{14}CO_2$ in a light gradient under temperature control. Thirteen 70 mL bottles were inoculated with 10 μCi each and exposed for 2 h in a light gradient (10–1000 μmol photons m^{-2} h^{-1}). The samples were filtered on 0.22 μm cellulose ester filters, exposed to HCl fumes and measured in a liquid scintillation counter. Total in situ production was estimated using the parameters of the P-E curve and the in situ irradiance measured with a Li-Cor sensor.

Analyses of dissolved inorganic nutrient concentrations (nitrate (NO_3), nitrite (NO_2), ammonium (NH_4) phosphate (PO_4), silicate (SiO_2)), were done by standard segmented flow analyses with colorimetric detection (Hansen and Grasshoff, 1983) using an SEAL Auto Analyzer AA3 HR. The detection limits were: 0.0100 μM for NO_3; 0.0015 μM for NO_2; 0.0030 μM for NH_4 (except for 2012, which was 0.0370 μM); 0.0248 μM for PO_4 and 0.0160 μM for SiO_4. We express dissolved inorganic nitrogen (DIN) as the sum of NO_3 + NO_2+ NH_4 concentrations. Samples for particulate matter concentration (PM) were taken only from January 2013 to July 2014 (21 samples in total) and PM was measured by dry mass weight of 250 mL samples that were filtered through polycarbonate filters (25 mm diameter, 0.4 μm pore size, DHI) that had been previously desiccated and weighted. After sample filtration, the filters were dried for 24 h and weighted again. PM concentration was calculated as the difference in weight after-before filtration divided by the filtered volume. POC was measured by filtering 1000 mL of seawater on pre-combusted GF/F glass fiber filters (4 h, 450 °C). The filters were frozen at −20 °C until analysis. To remove inorganic compounds, prior to analysis, the filters were thawed in an HCl-saturated atmosphere for 24 h. Then the filters were dried again and analyzed with a C:H:N autoanalyser (Perkin-Elmer 240). The mean values of N and C values for pre-combusted (450 °C) GF/F filters were 0.07 μmol and

0.53 μmol respectively. These values were substracted from the values obtained for each of the samples.

Extracellular enzyme activities were quantified with the use of fluorogenic substrates (Hoppe, 1983) according to Sala and Güde (1999) with the modifications for plate readers described in Sala et al. (2016). Each sample (350 μL) was pipetted in quadruplicate into 96 black well plates, with 50 μL of the following substrates: 4-methylumbelliferyl ß-D-glucopyranoside (for ß-glucosidase), 4-methylumbelliferyl phosphate (for alkaline phosphatase), 4-methylumbelliferyl butyrate (for esterase), and L-leucine-7-amido-4-methyl coumarin (for leu-aminopeptidase). Fluorescence was measured immediately after addition of the substrate and after incubations of 15 min, 30 min, 1 h, 3 h and 5 h, in the dark at in situ temperature. Measurements were done with a Modulus Microplate (DISMED, Turner BioSystems) at 365 nm emission and 450 nm excitation wavelengths. The increase of fluorescence units during the period of incubation was converted into enzymatic activity with a standard curve prepared with the end products of the reactions, 7-amido-4-methylcoumarin for leu-aminopeptidase and 4-methylumbelliferone (MUF) for the rest of enzymes.

Between 70 mL and 100 mL of glutaraldehyde (1–5% final)-fixed water were filtered through a 0.6-μm polycarbonate black filter (DHI) and stained with DAPI (Porter and Feig, 1980) to a final concentration of 5 mg mL^{-1} to enumerate nanoflagellates by epifluorescence microscopy (Olympus BX40, 1000×). In DAPI stained samples, flagellates presented a bright blue fluorescence under ultraviolet excitation. To distinguish pigmented and non-pigmented cells we use blue light excitation, under which Chlorophyll exhibit red fluorescence. Nanoflagellates showing red fluorescence were assumed to be autotrophic and colorless flagellates to be heterotrophic. At least 20 random fields or 30 cells were counted in each filter. Heterotrophic prokaryotes and the different picophytoplankton organisms were enumerated by flow cytometry following standard methods after fixation with paraformaldehyde 1% and glutaraldehyde 0.05% (Gasol and Morán, 2016). Prokaryotic heterotrophic production was estimated from tritiated leucine incorporation in quadruplicate aliquotes and 2 TCA-killed controls to which 40 nM leucine was added. Prokaryotic heterotrophic production (PHP) data are provided as leucine incorporation (pmol leu l^{-1} h^{-1}). Microphytoplankton were identified and counted with an inverted microscope after sedimentation in Utermöhl chambers of formalin-hexamine (0.4% final concentration) samples kept at 4 °C until analysis (Guadayol et al., 2009).

2.3. Statistical analysis

We checked for statistical differences of the different environmental variables among seasons using Mann Whitney tests after Bonferroni corrections. The seasons were separated by the winter/summer solstices and the spring/fall equinoxes. To assess co-variability between environmental and biological variables in the Blanes dataset, pairwise Spearman Rank correlation analyses were performed, and seasonality was tested by autocorrelation analysis using the PAST software (Hammer et al., 2001). The level of significance (p) was set at 0.05. Principal component analysis (*Stats* and *ggfortify* packages in R), was applied to all samples after centering and scaling a total number of 25 physical, chemical and biological variables.

3. Results

3.1. Variation of the main physical and chemical parameters in Blanes Bay

As the heat flux and seawater temperature rise in summer, the establishment of a stratified layer is frequently observed, as illustrated by significant increases in the stratification index (Table 1). Consequently, surface nutrients normally exhibit lower concentrations in summer (Guadayol et al., 2009). In the studied three-year period, dissolved phosphate (DIP) concentration varied between 0.03 and 0.22 μmol L^{-1}, dissolved inorganic nitrogen (DIN) ranged from 0.29 to 5.35 μmol L^{-1} and silicate varied between 0.46 and 2.49 μmol L^{-1}(Table 1). DIN and silicate were significantly lower in summer than in winter (Table 1), but no significant differences in DIP between seasons were observed. Water transparency, measured as Secchi disk depths, ranged between 8 and 20 m (Table 1).

3.2. Seasonal variability of TEP and other particle stocks in Blanes Bay

In that three-year period, TEP ranged from 11.3 to 289.1 μg XG eq L^{-1} (average 81.7 ± 11.7 μg XG eq L^{-1}). The highest concentrations of TEP were recurrently observed in early summer (June solstice and July, average 224.0 ± 7.9 μg XG eq L^{-1}, Fig. 2a). After the early summer peak, TEP concentrations decreased again, reaching minimum values in the fall and winter (Fig. 2a). This pattern was interannually recurrent with a lag = 13, corresponding to thirteen samples per year (two sampling times in June; autocorrelation analysis, $r = 0.79$, $p < 0.05$). However, the early summer peak magnitude was highest in 2012 (>250 μg XG eq L^{-1} in the June solstice and July, Fig. 2a). By contrast, TEP in late summer (August and September) were highest in 2014 (192.0 μg XG eq L^{-1}, Fig. 2a).

Particulate matter concentration (PM) ranged from 0.162 to 0.852 mg L^{-1} (average 0.417 ± 0.199 mg L^{-1}). Maxima were recorded in the winter months (December to March) and the June solstice (Fig. 2b). Particulate organic carbon (POC) concentrations ranged from 5.43 to 23.95 μmol L^{-1} (average 9.73 ± 4.61 μmol L−1). POC concentrations did not follow the same seasonal pattern throughout the three study years (Fig. 2b), hence they did not show significant differences over the year (Table 1). TEP, PM and POC dynamics were not significantly coupled (Table 2). However, PM, as expected, was negatively correlated to water transparency ($r = -0.53$, $p < 0.02$, $n = 19$). The TEP/PM and TEP/POC ratios progressively increased from winter to summer, decreasing again in the fall (Fig. 2c).

3.3. Seasonal variability of biological parameters in Blanes Bay

Chlorophyll *a* (chl *a*) ranged from 0.15 to 1.21 μg L^{-1}. Maxima of chl *a* occurred in March 2012 and 2013; but in 2014 the maximum was in January–February (Fig. 3a). The dynamics of chl *a* were not significantly correlated to variations of single phytoplankton groups (diatoms, coccolitophorids, cryptophytes, cyanobacteria). *Synechococcus* presented peaks of high abundance in April 2012 and 2013, but not in 2014, when the highest abundances were recorded in late summer (Fig.3b). The abundances of *Prochlorococcus* cells were always highest in fall (Fig. 3b). Pico- and nanoplankton abundances were highest in the first 4 months of every year, although a second period of relatively high nanoeukaryote abundance was also apparent in May–June 2014 (Fig. 3c). Dinoflagellates and diatoms showed abundance peaks in June–July 2012 and 2013, and in August, only for dinoflagellates, in 2014 (Fig. 3d). The abundance of coccolithophorids and other microplankton groups did not exhibit clear seasonal variations in the form of recurrent peaks (Fig. 3e). Primary production, determined once per season, ranged from 0.58 to 1.63 mg m^{-3} h^{-1} and was highest, although not significant, in spring (1.16 mg m^{-3} h^{-1}), but the maximum value was recorded in September 2013.

Prokaryotic heterotrophic abundance (PHA) ranged from 4.4 to 14.4 × 10^5 cell mL^{-1} (Fig. 4a). Prokaryotic heterotrophic production (PHP) ranged from 3.6 to 364.4 pmol leu L^{-1} h^{-1} (equivalent to 0.13 to 13.56 μgC L^{-1} d^{-1}, Fig. 4a). PHA was significantly lower in the winter than in the rest of the seasons, while PHP was significantly higher in summer than in winter (Table 1). The activities of the exoenzymes alkaline phosphatase and esterase were significantly higher in summer (Table 1, Fig. 4b-c). The activity of β-glucosidase was highest in summer (Fig. 4d), yet not statistically significant, and Leucine aminopeptidase activity was highest in spring (Table 1). The abundances of heterotrophic

Table 1
Averages of the main physical and biological variables at each season measured in the Blanes Bay time series between 2012 and 2014. Mann Whitney pairwise tests with Bonferroni corrections are used to test for significant differences among seasons. Significantly different groups ($p < 0.05$) are labeled with different letters (a, b, c). DIN: Dissolved inorganic nitrogen. PP: Primary production. POC: Particulate organic carbon. TEP: Transparent exopolymer particles. PM: Particulate matter. PHA: Prokaryote heterotrophic abundance. PHP: Prokaryote heterotrophic production. HNF: Heterotrophic nanoflagellates.

	Winter	Spring	Summer	Fall
Variable	Average (±SD)	Average (±SD)	Average (±SD)	Average (±SD)
Temperature (°C)	13.4 ± 0.7 a	15.6 ± 1.4 b	22.4 ± 1.7 c	17.7 ± 2.6 d
Salinity	38.15 ± 0.10 a	37.90 ± 0.22 a	37.99 ± 0.15 a	38.01 ± 0.12 a
Stratification Index (°C)	0.05 ± 0.07 a	0.45 ± 0.34 b	2.80 ± 1.54 c	0.32 ± 0.95 ab
Water transparency (m)	14.0 ± 3.9 a	16.1 ± 2.7 a	17.3 ± 2.2 a	13.7 ± 4.3 a
DIN (μM)	2.39 ± 1.01 a	1.52 ± 0.69 ab	0.77 ± 0.46 b	1.61 ± 1.17 ab
PO_4 (μM)	0.11 ± 0.03 a	0.07 ± 0.03 a	0.08 ± 0.02 a	0.09 ± 0.06 a
N/P	21.7 ± 5.6 a	22.3 ± 6.6 a	12.2 ± 8.3 a	16.7 ± 10.6 a
SiO_4 (μM)	1.67 ± 0.67 a	1.19 ± 0.37 a	0.73 ± 0.21 b	1.27 ± 0.55 ab
Chl *a* (μg L^{-1})	0.72 ± 0.23 a	0.50 ± 0.33 a	0.32 ± 0.18 b	0.39 ± 0.12 b
PP (μg L^{-1} h^{-1})	0.45 ± 0.05 a	1.16 ± 0.11 a	0.86 ± 0.55 a	0.58 ± 0.36 a
POC (μM)	9.2 ± 2.0 a	9.9 ± 6.7 a	11.9 ± 5.1 a	7.3 ± 2.1 a
TEP (μg XG eq L^{-1})	33.7 ± 15.9 a	65.9 ± 30.9 ab	147.0 ± 83.4 b	56.2 ± 20.9 ab
PM (mg L^{-1})	0.50 ± 0.25 a	0.36 ± 0.11 a	0.41 ± 0.19 a	0.38 ± 0.24 a
PHA ($\times 10^5$ cell mL^{-1})	6.64 ± 1.49 a	9.45 ± 2.80 b	8.24 ± 1.37 b	9.21 ± 1.74 b
PHP (pmol leu L^{-1} h^{-1})	20.8 ± 18.3 a	59.8 ± 35.2 ab	95.5 ± 93.5 b	76.6 ± 64.2 ab
HNF ($\times 10^3$ cell mL^{-1})	0.59 ± 0.18 a	1.13 ± 0.64 ab	1.39 ± 0.55 b	0.76 ± 0.28 a
ß-Glucosidase (nmol L^{-1} h^{-1})	0.37 ± 0.39 a	0.80 ± 0.31 a	1.26 ± 0.91 a	0.73 ± 0.38 a
Leucine-Aminopeptidase (nmol L^{-1} h^{-1})	9.86 ± 11.92 a	30.35 ± 10.47 a	20.54 ± 17.08 a	13.17 ± 11.10 a
Alkaline Phosphatase (nmol L^{-1} h^{-1})	12.71 ± 7.55 a	32.01 ± 11.57 b	47.27 ± 15.97 b	32.26 ± 15.46 b
Esterase (nmol L^{-1} h^{-1})	383.9 ± 216.6 a	783.3 ± 348.0 ab	1146.5 ± 470.3 b	796.6 ± 402.4 ab

flagellates, ranging from 210 to 2718 cells mL^{-1}, were significantly higher in summer than in the rest of seasons (Table 1).

3.4. Environmental and biological variables determining TEP dynamics in Blanes Bay

Higher TEP concentrations were found in early summer. Therefore, TEP co-varied with sea surface temperature and the stratification index (Table 2). The dynamics of TEP and chl *a* were not significantly coupled (Table 2). Rather, cross-correlation analyses showed a lag-time of 4 months between chl *a* and TEP peaks ($r = 0.48$, $p < 0.04$). TEP were significantly related to the abundance of specific phytoplankton groups, such as diatoms ($r = 0.63$ $p < 0.001$, $n = 33$) and dinoflagellates ($r = 0.65$ $p < 0.001$, $n = 33$). No significant correlations were found between TEP and other eukaryotic (coccolithophores, cryptophytes) nor prokaryotic (*Synechococcus* and *Prochlorococcus*) groups (Table 2).

Non-significant covariations were observed between TEP and PHA and PHP (Fig. 4a–b). Particle-attached prokaryotes, that represented <10% of total abundance (Mestre et al., 2017), were also uncorrelated to TEP. Noticeably, increases in TEP coincided with increases in some extracellular enzyme activities: Esterase ($r = 0.60$ $p < 0.001$, $n = 34$), alkaline phosphatase ($r = 0.66$ $p < 0.001$, $n = 34$), ß-glucosidase ($r = 0.69$ $p < 0.001$, $n = 34$, Table 2). TEP were also significantly correlated to the abundance of heterotrophic nanoflagellates (Table 2).

A principal component analysis (PCA) was performed to visualize which subset of variables could better explain the TEP seasonal patterns in Blanes Bay. Principal components 1 and 2 explained 29.0 and 13.2% of the variability respectively. Component 1 separated the data between summer and winter samples (Fig. 5) and the major loadings included temperature (0.32), stratification index (0.30), TEP concentration (0.31) and (negatively related) dissolved inorganic silica (Si; −0.32) and nitrogen (DIN; −0.29). Component 2 separated the samples along a productivity gradient, where the major loadings included chl *a* (−0.33), diatoms (−0.33) and dinoflagellates (−0.35) and prokaryotic heterotrophic production (PHP, 0.33). Early summer samples clustered together since they had relatively high stratification indices, TEP and POC concentrations, heterotrophic nanoflagellates (HNF), and abundance of diatoms, dinoflagellates and other microphytoplankton groups (Fig. 5).

4. Discussion

To date, and as we are aware, there are only three published seasonal studies on TEP variability in the Mediterranean Sea (Beauvais et al., 2003; Iuculano et al., 2017; Mari et al., 2001). In Mari et al. (2001) and Beauvais et al. (2003), that present the same 12- month TEP data series, TEP accumulation was observed in June in offshore and coastal sites. However, absolute values cannot be compared since TEP were quantified using different methods (microscopic enumeration in their study vs. colorimetric assay in ours). Chl *a* was also highest in June at the deep chlorophyll maxima in the offshore site, yielding a significant correlation with TEP abundance. Conversely, chl *a* in their coastal site was highest in March but TEP peaks occurred in early summer, thus TEP and chl *a* seemed to be decoupled as in our study. In the Balearic Sea, TEP ranges were similar to our study (from 4.6 to 90.6 μg XG eq L^{-1}) in a rocky shore coastal site (Iuculano et al., 2017), while higher TEP concentrations were found in a coastal site accumulating *Posidonia oceanica* leaf litter (from 26.8 to 1878.4 μg XG eq L^{-1}). Our detailed three-year seasonal study showed, in accordance with these previous studies, that TEP accumulation in surface waters of the coastal NW Mediterranean Sea is a recurring pattern in early summer, but not in late summer as occurs with other organic matter pools (Romera-Castillo et al., 2013; Vila-Reixach et al., 2012). Other temporal studies in the Mediterranean Sea (Parinos et al., 2017; Radic et al., 2005; Scoullos et al., 2006) have lower temporal resolution (i.e. 3 times per year, (Parinos et al., 2017)) or had been performed in quite more eutrophic areas (i.e. the Aegean Sea and the Northern Adriatic Sea at the Po River delta). Parinos et al. (2017) showed in the Aegean Sea surface, contrary to our results, higher TEP in March (101 ± 32.7 μg XG eq L^{-1}) than in July (88.6 ± 26.5 μg XG eq L^{-1}). Radic et al. (2005); Scoullos et al. (2006) show high interannual TEP variations in eutrophic coastal areas in the Adriatic and Aegean Seas. The few other time series studies in marine ecosystems (i.e. Engel et al. (2017) in the Arctic Ocean, Klein et al. (2011) and Taylor et al. (2014) in the English Channel, Bhaskar and Bhosle (2006) in the West coast of India) are climatically and oceanographically very different from, hence hardly comparable to, our temperate oligotrophic coastal system.

Ocean particles often contain a variable proportion of TEP or gel-like structures. The proportion of TEP vs. POC or vs. solid particles determines their export efficiency (Azetsu-Scott and Passow, 2004; Mari

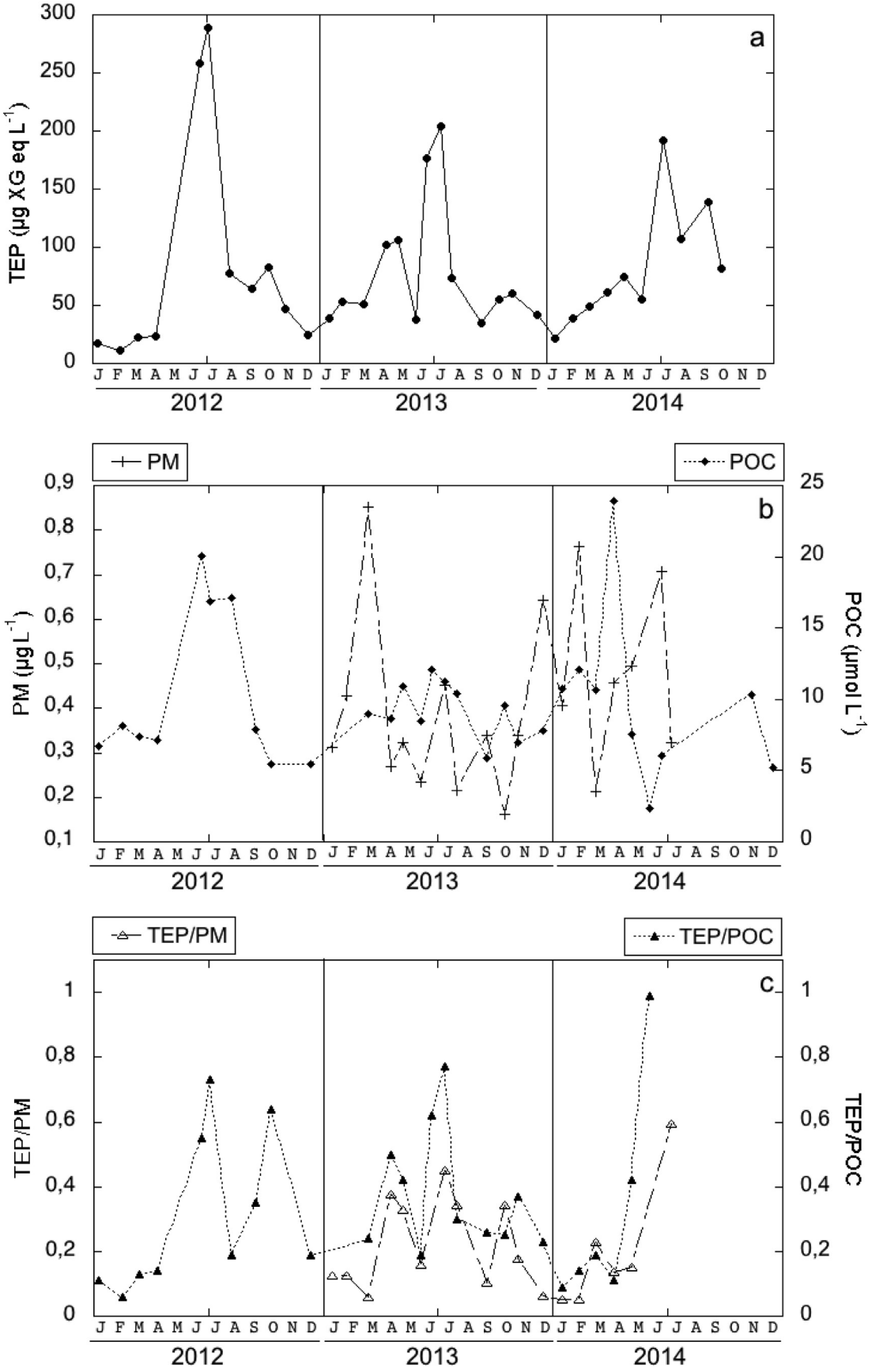


Fig. 2. Monthly average values of transparent exopolymer particles (TEP, a), particulate matter (PM, b), and particulate organic carbon concentrations (POC, c) and TEP/PM and TEP/POC (in g/g) ratios (d) over the three-year study.

Table 2
Correlations between transparent exopolymer particle (TEP) concentration and other environmental and biological variables during the 2012–2014 period in Blanes Bay.

Variable		r	p	n
TEP	Temperature (°C)	0.64	<0.001	34
	Salinity	−0.44	0.013	34
	Stratification index (°C)	0.60	<0.001	34
	Water transparency (m)		ns	
	DIN (μM)		ns	
	PO_4 (μM)		ns	
	SiO_4 (μM)	−0.55	<0.001	34
	Chl *a* (μg L^{-1})	−0.45	0.007	34
	PP (μg L^{-1} h^{-1})		ns	
	Synechococcus (cell mL^{-1})		ns	
	Prochlorococcus (cell mL^{-1})		ns	
	Diatoms (cell mL^{-1})	0.63	<0.001	33
	Dinoflagellates (cell mL^{-1})	0.65	<0.001	33
	Coccolithophorids (cell mL^{-1})		ns	
	Other microplankton (cell mL^{-1})		ns	
	Nanoplankton (cell mL^{-1})		ns	
	Picoplankton (cell mL^{-1})		ns	
	POC (μM)		ns	
	PM (mg L^{-1})		ns	
	PHA ($\times 10^5$ cell mL^{-1})		ns	
	PHP (pmol leu L^{-1} h^{-1})		ns	
	HNF ($\times 10^3$ cell mL^{-1})	0.59	<0.001	34
	ß-Glucosidase (nmol L^{-1} h^{-1})	0.69	<0.001	33
	Leucine-aminopeptidase (nmol L^{-1} h^{-1})		ns	
	Alkaline phosphatase (nmol L^{-1} h^{-1})	0.66	<0.001	34
	Esterase (nmol L^{-1} h^{-1})	0.60	<0.001	32

et al., 2017). Particles with high TEP content have lower density than those not enriched in TEP (Mari et al., 2017), and thus have higher retention times in the water column. Comparison between ours and previous studies that calculated the proportion of TEP to PM is not possible, since different methodologies (and thus particle fractions) have been employed (dry weight onto 0.4 μm filters in our case, particle volume fractions determined by Coulter counter in Engel (2004) and Prieto et al. (2006)). In our study, TEP and other particles, either POC or total particle mass (PM), were decoupled. Particles in early summer showed the highest TEP:PM ratio, indicating that the particle stocks were mainly composed by low density particles that are less efficiently exported, thus weakening organic carbon sinking fluxes. Comparing the TEP and POC pools in our dataset, we observed that in early summer TEP represented on average 77% of POC (Fig. 2c). We used the most conservative conversion factor, this is, 0.51 μg C μg XG eq^{-1}, from those proposed by Engel and Passow (2001), which would correspond to diatom-dominated ecosystems. While it could be a good proxy for our early summer data, we may be over- or underestimating the TEP-C pool in other periods of the year. Also, previous studies in the Mediterranean Sea (Bar-Zeev et al., 2011; Ortega-Retuerta et al., 2010) using the published conversion factors show higher TEP-C than POC, which is obviously inaccurate and claim for the need to determine specific conversion factors to convert TEP in C units in this area. We are aware about the shortcomings of conversion factors, however we consider that for the purpose of our study, the interannual variability of TEP throughout the years, the Engel and Passow (2001) conversion factor, used here, is appropriate to estimate such variability. TEP can accumulate in the water column during several months (Mari et al., 2017), and get trapped and enriched near the surface by strengthening stratification. Although no previous studies have considered TEP when looking at particle dynamics in Blanes, López-Fernández et al. (2013) observed that the organic carbon content of the particles in the nearby Blanes canyon was also highest in summer, although particle fluxes were higher in winter.

The comparison of TEP dynamics with a complete suite of environmental and biological variables highlighted the complexity of predicting TEP occurrence based on few parameters. In fact, correlation analyses revealed that only up to 48% of the variance of TEP can be explained

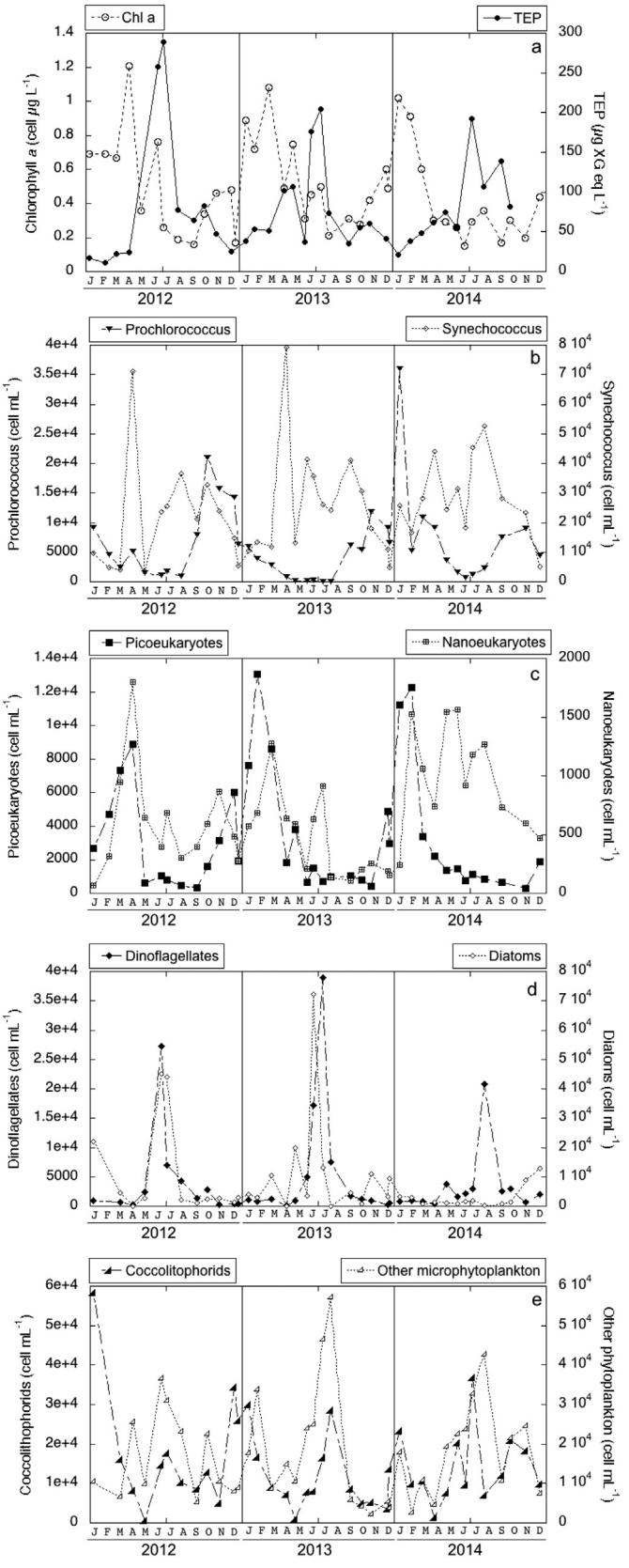


Fig. 3. Dynamics of Chlorophyll *a* and TEP concentration (a); *Prochlorococcus* and *Synechococcus* abundances (b); pico- and nanoeukaryote abundances (c); dinoflagellate and diatom abundances (d); and coccolitophore and other microplankton groups abundances (e) in Blanes Bay during the 2012–2014 period.

by a single parameter (Table 2). In addition, only 40% of the overall variation in Blanes Bay samples was explained by the available data as indicated from the PCA. Based on our observation that TEP did not correlate to prokaryote abundance nor production, we suggest that phytoplankton rather than prokaryotes are the main TEP source in Blanes Bay. Indeed, TEP were significantly correlated to the abundance of diatoms and dinoflagellates, that are known to produce TEP (Passow, 2002b) and references therein. If TEP in Blanes Bay are a direct result of the presence of these groups, we can predict that TEP maxima will normally occur in spring, when these phytoplankton groups usually peak (Nunes et al., in press). Longer time series would be necessary to explore this hypothesis further. However, we observed summer TEP accumulation also in July 2014, when relevant increases in diatoms or dinoflagellates were not detected (only a peak of dinoflagellates was detected in August, Fig. 2d). Therefore, we argue that release by phytoplankton cannot be the only factor determining the presence of TEP peaks in Blanes Bay.

Nutrient limitation enhances TEP production rates (Corzo et al., 2000), making TEP concentrations higher in the summer months when nutrients are scarcer. The lag-phase observed between chl *a* and TEP peaks in Blanes Bay would concur with previous results that suggest that TEP production is normally higher at the end of phytoplankton blooms when nutrients have been exhausted rather than at maximum chl *a* levels (Engel, 2000; Hong et al., 1997). After the chl *a* peak, TEP would accumulate in early summer assisted by the increasing stratification, that prevents vertical export and further injections of nutrients from below (higher nutrient concentration increases with depth in summer (Aparicio et al., 2017)). In other words, TEP would result from the combination of high chl *a* values in late winter, evolving nutrient limitation from spring on, and stronger stratification in summer, when significantly lower nitrogen concentrations were found (Table 1). This is particularly noticeable in 2014, when TEP peaks were not concurrent with high abundance of diatoms but DIN and silicate concentration showed low values (0.39 μmol L^{-1}, the lowest value for the entire period), which would enhance per cell TEP production by phytoplankton. The joint effects of these processes would explain TEP dynamics in the area. More studies (e.g., with controlled incubations) to quantify TEP production and consumption in different seasons and/or nutrient levels are needed to validate this hypothesis.

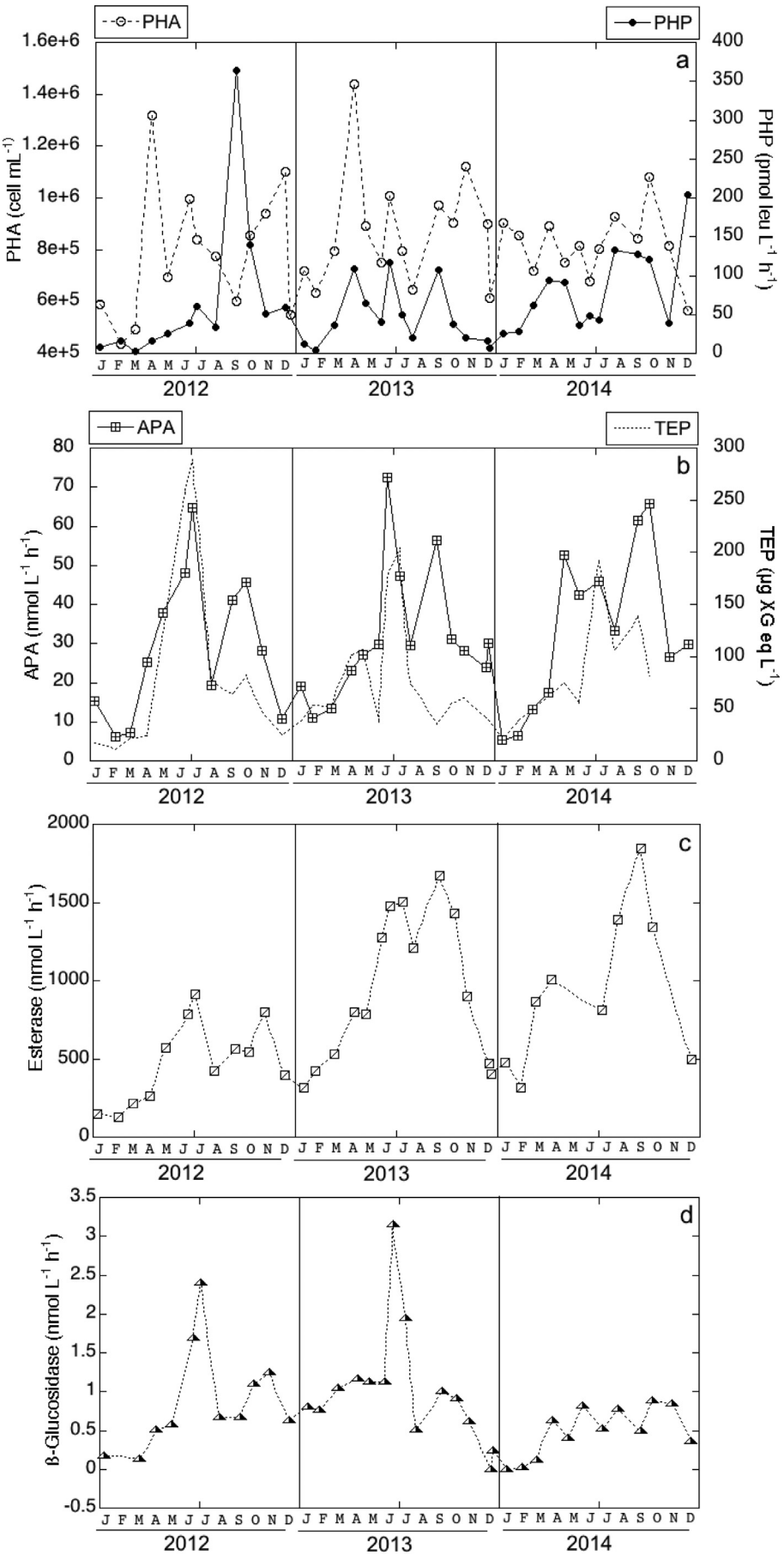
Once TEP have accumulated at the surface in early summer, they can serve as a substrate for further processing by the microbial communities. In our dataset, TEP were not related to heterotrophic prokaryote abundance nor production, so here we discard a major source of TEP from prokaryotes. However, TEP were correlated to the activities of specific enzymes (ß-glucosidase, esterase and alkaline phosphatase); thus, TEP likely shaped prokaryote functional diversity although not bulk abundances nor biomass production. High enzymatic activity associated to marine aggregates has been previously shown (Smith et al., 1992). As TEP are mainly composed of acidic polysaccharide (Passow, 2002b), we could hypothesize that TEP maxima would be followed by higher increases in enzymes involved in the degradation of these substances (i.e. β-glucosidase and esterase) than in aminopeptidase or phosphatase enzymes. This was confirmed by the correlation between TEP and ß-glucosidase and esterase. Looking back in the Blanes Bay time series, we observed that ß-glucosidase activities recurrently peak in summer (Alonso-Sáez et al., 2008). Although this relation is not surprising since TEP are carbon-rich substances, the effect of TEP accumulation on specific microbial metabolisms is worthy to note. Similar positive correlations between TEP and ß-glucosidase had previously been observed in controlled incubations using Eastern Mediterranean waters (Rahav et al., 2016) but not in a time series in the west coast of India (Bhaskar and Bhosle, 2006). No previous studies have compared TEP concentration and esterase activities, but TEP are made of polysaccharides enriched in sulfate in the form of half-ester groups (Passow and Alldredge, 1995) being thus likely the coincidence between TEP peaks and high esterase activity. Increases in TEP were also concurrent with increases in the activities of alkaline phosphatase. This has previously been shown in the Eastern Mediterranean basin (Bar-Zeev et al., 2011) and in controlled experiments (Berman-Frank et al., 2016). Prokaryotic abundance and activity in Blanes Bay in summer is controlled by the availability of inorganic phosphorus (Pinhassi et al., 2006), although we did not observe significant decreases in P in summer in our study years (Table 1). Previous studies have suggested that under phosphorus limitation, prokaryotes could use alkaline phosphatase to access phosphorus from alternative sources such as TEP (Berman-Frank et al., 2016). However, to our knowledge, TEP are not directly enriched in phosphorus (although phosphorus compounds could be adsorbed into the gel-like TEP structure). In addition, lower N/P ratios in summer suggest, contrary to previous knowledge (Sala et al., 2002; Pinhassi et al., 2006), that P was not the limiting nutrient for prokaryotes in Blanes Bay. We cannot rule out with our approach the possibility that increases in enzyme activities are not a direct consequence of the presence of TEP but both factors increase concurrently due to the release of specific DOM compounds by phytoplankton during periods of high phosphorus limitation. Experimental studies with manipulations of the environmental conditions or time series studies with higher resolution would be needed to sort this out.

After TEP maxima in early summer, TEP peaks decreased to reach lower values at the end of summer. Based on our results, TEP decreases after summer peaks could be a direct consequence of these extracellular enzyme activities. Other non-exclusive sinks that likely accounted for TEP decreases is photolysis, which can degrade TEP stocks in few days (Ortega-Retuerta et al., 2009), as TEP maxima in our study, coincide with longer days and highest solar radiation (Ruiz-González et al., 2012). Finally, we cannot rule out the possibility that TEP are exported to deeper layers at the end of summer when waters become less stratified. Also, the high stickiness of TEP-rich material (Engel, 2000) favours the particle aggregation thus facilitating its export.

TEP dynamics in Blanes Bay also co-varied with the abundance of heterotrophic nanoflagellates. These covariations were also observed by Arnous et al. (2010) in a lake, where TEP and PHA were not correlated either. Since the prokaryote to heterotrophic nanoflagellate ratios in Blanes Bay are lowest in early summer (data not shown), these organisms could benefit from feeding on TEP-attached prokaryotic communities, which are believed to occur at higher densities than their free-living counterparts. Although prokaryote colonization of TEP was not directly measured in our study, higher densities of prokaryotic cells on TEP than in the surrounding water have been repeatedly observed (Bar-Zeev et al., 2011; Mari and Kiørboe, 1996). An alternative non-excluding explanation is that heterotrophic nanoflagellates embedded in the TEP matrix are protected against predation by ciliates and microzooplankton (Arnous et al., 2010).

5. Conclusions

We have confirmed the seasonal recurrence of TEP in the oligotrophic North Western Mediterranean Sea and the temporal disconnection between TEP and chlorophyll *a* in a three-year dataset. We present evidence that the TEP dynamics result from a combination of factors rather than from the variation of a single predictor variable. The presence of specific phytoplankton groups (diatoms and dinoflagellates), the possible enhancement of TEP production under nutrient limitation, the TEP accumulation at surface due to positive buoyancy (low density) and the water stratification would give rise to TEP maxima in early summer. We hypothesize that the presence of these particles has an effect in the microbial food web by enhancing the activity of specific prokaryotic


PHA
PHP
a
PHA (cell mL-1)
PHP (pmol leu L-1 h-1)
1.6e+6
1.4e+6
1.2e+6
1e+6
8e+5
6e+5
4e+5
400
350
300
250
200
150
100
50
0
J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
2012
2013
2014
APA
TEP
b
APA (nmol L-1 h-1)
TEP (µg XG eq L-1)
80
70
60
50
40
30
20
10
0
300
250
200
150
100
50
0
J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
2012
2013
2014
c
Esterase (nmol L-1 h-1)
2000
1500
1000
500
0
J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
2012
2013
2014
d
ß-Glucosidase (nmol L-1 h-1)
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
2012
2013
2014

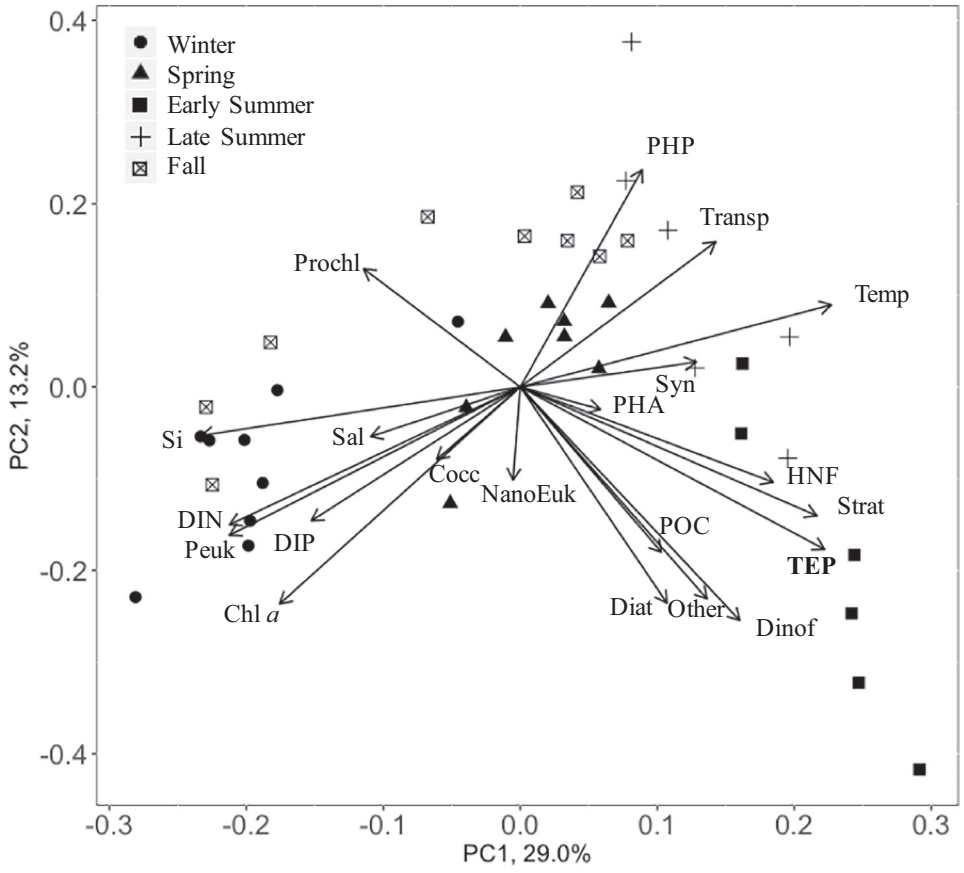


Fig. 5. Principal component analysis (PCA) of all samples from Blanes Bay. Each Principal component (PC) is accompanied by its explained variation (%). The major loading on PC1 include Temperature (Temp), transparent exopolymer particles (TEP) and stratification index (Strat). The major loadings on PC2 include chlorophyll *a* (Chl *a*), diatoms (diat), dinoflagellates (Dinof) and prokaryotic heterotrophic production (PHP).

extracellular enzymes and likely promoting the abundance of heterotrophic nanoflagellates.

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Fig. 4. Dynamics of prokaryotic heterotrophic abundance (PHA, a) and prokaryotic heterotrophic activity (PHP, a); alkaline phosphatase activity (APA, b), esterase activity (c); and ß-glucosidase activity (d) in Blanes Bay during the 2012–2014 period. TEP dynamics are presented in panel 4b to facilitate comparisons.

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