

# Significance of Bacterial Activity for the Distribution and Dynamics of Transparent Exopolymer Particles in the Mediterranean Sea

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**Abstract** The study of transparent exopolymer particles (TEP) in the Mediterranean Sea is particularly relevant as they can be promoters of mucilage events, a frequent phenomenon there. We assessed the influence of bacterioplankton on TEP distribution and dynamics across the west–east axis of the Mediterranean Sea. We performed an extensive study of TEP, dissolved carbohydrates, and their relationships with bacterial abundance and bacterial production (BP). A significant and positive relationship was observed between BP and TEP in the study region ( $r^2=0.51$ ,  $p<0.001$ ). The direct release of TEP by bacteria was experimentally corroborated using regrowth cultures where increases in TEP tracked bacterial growth in abundance and production. These TEP increases were positively related to the increases in BP ( $r^2=0.78$ ,  $p<0.05$ ). The consistency

(similar slopes) of the regression lines between BP and TEP in natural conditions and between the increases of BP and TEP in the experiments underlines the relevant role of bacteria in the formation of TEP in this area.

## Introduction

Transparent exopolymer particles (TEP), defined as microscopic sticky particles formed by acidic polysaccharides and stainable with alcian blue [1], are the most ubiquitous particles in the ocean [41]. TEP constitute the interstitial matrix to form large aggregates promoting the vertical export of carbon from surface to deep waters [1, 44]. Due to their low density, unballasted TEP can also migrate upward to the sea surface microlayer [2, 27], affecting sea–air gas exchange [7].

A major process of TEP formation is by spontaneous self-assembly of dissolved precursors [39]. Phytoplankton have been traditionally considered to be a major source of TEP in marine ecosystems [40, 42], either releasing dissolved TEP precursors during exponential growth [1, 5, 40] or excreting TEP directly via sloughing and lysis of senescent cells [4, 6, 21]. Other organisms, such as macroalgae [60] or zooplankton [46], have also been reported as secondary sources of TEP. However, the role of bacterioplankton on TEP distribution and dynamics is complex, as it can operate in contrasting ways. On one hand, bacteria benefit from preexisting TEP as a substrate or a stable microhabitat [29, 42]. Alternatively, bacteria can also release high amounts of polysaccharides and TEP as free exopolymers or as capsular material (up to 25% of the respired carbon [55]), depending on turbulence conditions [40, 56], the presence of phytoplankton [18], the availability of nutrients [47], or viral infection [51]. In addition, the

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presence of bacteria indirectly stimulates the release of TEP by phytoplankton as the more efficient assimilation of nutrients by bacteria [24] may enhance the release of TEP precursors by nutrient-limited phytoplankton [19]. Finally, bacteria can indirectly promote abiotic TEP assembly from dissolved precursors in diverse ways. Brownian motion of bacteria can enhance the collisions of bacteria and larger colloids [23]. Bacteria can also act as nuclei for negatively charged molecules such as TEP precursors [65] or release amphiphilic exopolymers that stabilize aggregates creating hydrophobic bonds [13]. All these processes occur simultaneously in natural systems with different magnitude and timescales. Therefore, the net result of all the processes relating TEP and bacteria will affect TEP dynamics and distribution in marine systems.

In the Mediterranean Sea, the formation of large aggregates including TEP is a frequent and inconvenient phenomenon that often takes extreme manifestations as mucilage events [8]. The accumulation and further aggregation of algal-derived material has been proposed as the main pathway of mucilage formation [34]. However, despite the well-known role of TEP in stabilizing these aggregates in the Adriatic Sea [31], there is little information about their distribution and dynamics in the open Mediterranean Sea [4, 28] and the specific role of bacteria has not been thoroughly explored.

In this study, we describe the vertical distribution of TEP and dissolved carbohydrates across the west–east axis of the Mediterranean Sea and explore empirical relationships between TEP and bacterial abundance (BA) and bacterial production (BP). In addition, using experimentally determined release rates of TEP by bacteria, we estimate the bacterial contribution to TEP dynamics in this ecosystem.

## Material and Methods

### Seawater Sampling

Sampling was performed on the Thresholds 2007 cruise, during May 2007, aboard the Spanish R/V “García del

Cid.” A total of 24 sampling stations were conducted within a transit from Barcelona to Alexandria (Fig. 1). At each station, six depths from the surface to 200 m depth were sampled, including the deep chlorophyll maximum (DCM). Water was collected using a Sea-Bird rosette sampler (12 Niskin bottles, 12 l each) attached to a conductivity/temperature/depth (CTD) system.

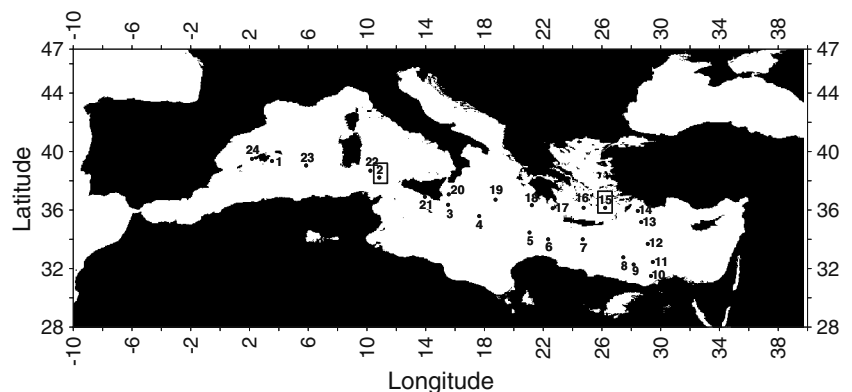
Data were separated into three subsets: data corresponding to waters within the upper mixed layer, data below the upper mixed layer but within the euphotic layer, and data below the euphotic layer. The mixed layer depth was calculated at the depth showing a temperature gradient  $>0.1^{\circ}\text{C m}^{-1}$  after visual inspection of vertical temperature profiles obtained with the CTD system [54]. The euphotic layer depth was estimated as that receiving 1% of photosynthetically active radiation (PAR), as derived from vertical profiles with a PAR sensor. Samples were taken to determine TEP, dissolved monosaccharides (DMCHO), dissolved polysaccharides (DPCHO), total organic carbon (TOC), chlorophyll *a* (chl *a*), BA and BP.

To determine the potential drivers of TEP distribution, we explored their relationship with DMCHO, DPCHO, chl *a* concentration, BA, and BP using bivariate and multiple regression analyses. Data were log-transformed when needed to comply with the assumptions of normality and homogeneity of variances.

### Chemical and Biological Analyses

TEP concentration was determined colorimetrically [43]. Triplicate samples (200–250 ml) were filtered onto 0.4  $\mu\text{m}$  polycarbonate filters (Poretics), stained with 500  $\mu\text{l}$  of alcian blue solution, and the filters were stored frozen until further processing in the laboratory (within 1 month). The filters were then thawed, soaked in 80% sulfuric acid for 3 h, and measured spectrophotometrically at 787 nm, using empty, stained filters (stained and frozen in parallel with the samples) as blanks. Alcian blue absorption was calibrated within <1 month using a xanthan gum (XG) solution that was homogenized by tissue grinder and measured by

**Figure 1** Locations of the study stations sampled during the Thresholds 2007 cruise. The stations selected for the experiments of bacterial TEP generation (station no. 2 in the Western Mediterranean and station no. 15 in the Eastern Mediterranean) are marked by squares



weight. The absorbance values of filter blanks did not change substantially between batches of samples, suggesting stability in the staining capacity of the alcian blue solution throughout the cruise. Although the use of a XG solution as a TEP standard appears to yield high variability in TEP determinations [22], it was selected to allow comparison with TEP values in the literature. TEP concentration was, therefore, expressed in micrograms of XG equivalents per liter and in carbon units using the conversion factor of  $0.75 \mu\text{g C } \mu\text{g XG eq L}^{-1}$  [14]. The detection limit of the method was  $2.2 \mu\text{g XG eq L}^{-1}$  and the coefficient of variation was 13%.

Samples for DMCHO and DPCHO were filtered through precombusted ( $>450^\circ\text{C}$ , 3 h) glass fiber filters (Whatman GF/F, nominal pore size  $\approx 0.7 \mu\text{m}$ ) and immediately stored in sterile polypropylene flasks at  $-20^\circ\text{C}$  until analysis. DMCHO and DPCHO were analyzed following the ferricyanide reaction before (DMCHO) or after (DPCHO) hydrolysis by oxidation of the free reduced sugars [33]. Reagents were calibrated using a standard curve of D-glucose, and triplicate reagent blanks in MilliQ water were subtracted. The detection limit of the method was  $0.4 \mu\text{mol C L}^{-1}$ , and the coefficient of variation between samples was 7%. The concentrations were expressed in micromoles of carbon per liter for the in situ samples, but in micrograms of carbon per liter for experiments to compare with changes in TEP-C in the same units.

Samples for TOC analyses were collected into precombusted 10-ml glass ampoules, acidified with phosphoric acid (final pH  $<2$ ), sealed and stored at  $4^\circ\text{C}$  until analysis. TOC was analyzed by high-temperature catalytic oxidation on a Shimadzu TOC-5000A. Standards of 44–45 and  $2 \mu\text{mol C L}^{-1}$ , provided by D.A. Hansell and Wenhao Chen (University of Miami), were used to assess the accuracy of the measurements.

BA was determined by flow cytometry. Subsamples (4 ml) were fixed with a mixture of paraformaldehyde and glutaraldehyde (1%), allowed 30 min to fix in the dark, deep frozen in liquid nitrogen, and then stored frozen at  $-20^\circ\text{C}$  [62]. Analyses were conducted within hours from sample collection. The samples were thawed; a 200- $\mu\text{l}$  sample was stained with  $4 \mu\text{l}$  of  $5 \mu\text{mol L}^{-1}$  SYBR Green (Molecular Probes) for 10 min in the dark, and run through a FACScalibur™ flow cytometer (BD Biosciences) fitted with a laser emitting at 488 nm. Samples were run at a low flow rate and data were acquired in log mode until around 10,000 events were acquired. A stock solution (5  $\mu\text{l}$ ) of yellow-green  $0.92 \mu\text{m}$  Polysciences latex beads was added as an internal standard per 200  $\mu\text{l}$  of sample. The concentration of the fluorescent beads was calibrated every 2 days by direct microscope enumeration. Bacteria were detected by their signature in bivariate plots of side scatter (SSC) vs. green fluorescence (FL1). Data were gated and

counted in the SSC vs. FL1 plot using the Paint-a-Gate software [12, 15]. BA was expressed in cells per liter.

BP was estimated from  $^3\text{H}$ -leucine-protein synthesis following the microcentrifugation technique [53]. Briefly, 5  $\mu\text{l}$  of L-[4,5- $^3\text{H}$ ] leucine was added to 1.5 ml samples, yielding a final concentration of 28.8 nM, and was incubated for 3 h. We used a conversion factor from leucine to carbon incorporation of  $1.5 \text{ kg C mol leu}^{-1}$  [52].

The chl *a* concentration was determined fluorometrically by filtering 50 ml subsamples through 25 mm glass fiber filters (Whatman GF/F), extracted into 10 ml of 90% acetone for approximately 24 h in the dark and at  $4^\circ\text{C}$ . The fluorescence of the extracts was read in a previously calibrated Turner Designs fluorometer [38].

### Experiments of Bacterial TEP Generation

To determine bacterial release of TEP and their dissolved precursors (DMCHO and DPCHO), we conducted four experiments using water from a Western Mediterranean Sea station (station no. 2, experiments 1 and 2) and an Eastern Mediterranean Sea station (station no. 15, experiments 3 and 4), and two different depths each (200 m [experiments 1 and 3] and the DCM [situated at 40 and 80 m in experiments 2 and 4, respectively]). Water for each experiment was filtered through precombusted glass fiber GF/F filters to exclude phytoplankton and heterotrophic flagellates. To prevent the growth of a biased and unrepresentative bacterial community due to filtration [16], water was subsequently inoculated with unfiltered water from the same location (10% of final volume). Regrowth cultures were set up in triplicate in 250 ml sterilized glass flasks inside a recirculating water bath set at surface seawater temperature ( $17\text{--}22^\circ\text{C}$ ) and in the dark (flasks were covered with opaque plastic bags). Each experiment consisted of two treatments: +bacteria and -bacteria. In +bacteria treatments, bacteria were allowed to grow, whereas in -bacteria treatments, bacterial growth was suppressed by adding sodium azide (SA, 0.02% final concentration). The addition of SA, which does not interfere with TEP coagulation processes [57], was repeated every 3 days.

Regrowth cultures were incubated for 5 days, sampling at initial time ( $t_0$ ) and after 2.5 days ( $t_1$ ) and 5 days ( $t_2$ ) for BA, BP, TEP, DMCHO, and DPCHO, except in experiments 1 and 3 where TEP, DMCHO, and DPCHO were only sampled at times  $t_0$  and  $t_2$ .

Daily increase rates for all variables were calculated using the expression:

$$\Delta x = \frac{x_{t_f} - x_{t_0}}{t} \quad (1)$$

where  $x$  is the study variable (TEP in micrograms of XG equivalent per liter or in micrograms of carbon per liter,

DMCHO and DPCHO in micrograms of carbon per liter, BA in cells per liter, or BP in micrograms of carbon per liter per hour),  $t_f$  and  $t_0$  are the final and initial times, respectively, and  $t$  is the time elapsed in days.

We examined the relationship between the daily increase rate of TEP ( $\Delta$ TEP) with the daily increase rate in BA ( $\Delta$ BA) or BP ( $\Delta$ BP) using linear regression analysis.

To quantify the bacterial TEP generation rates, we related  $\Delta$ TEP (in micrograms of XG equivalent per liter per day) with time-averaged BA (in cells per liter) and BP (in micrograms of carbon per liter per hour) in all experiments and treatments. The slopes of the regression equations provided experimental estimates of TEP generation rates by bacteria (in micrograms of XG equivalent per liter per day per unit of BA or BP). We used these experimental TEP generation rates to derive in situ TEP generation rates by bacteria using data of BA and BP in the Mediterranean Sea. We also estimated the TEP duplication times attributable to BP by using in situ TEP concentration and TEP generation rate using the following expression:

$$\text{TEP duplication time (days)} = \frac{\text{in situ TEP}}{\text{in situ TEP bacterial generation rate}} \quad (2)$$

## Results

The vertical profiles of temperature and salinity showed similar patterns among stations, with generally shallow mixed layers from 4 m (station nos. 14 and 15) to 29 m (station no. 6). Mean salinity and temperature values generally increased from west to east. Surface temperature ranged from 17.6°C (station no. 1) to 21.8°C (station no. 19). Salinity at the surface ranged from 37.01 PSU in (station no. 23, Northwestern Mediterranean) to 39.16 PSU (station no. 12, Eastern Mediterranean). The influence of the Atlantic jet, with lower salinities in surface waters, was noticeable at the western stations (station nos. 23 and 24).

The chl *a* ranged from undetectable to 1.78  $\mu\text{g L}^{-1}$  (average value, 0.16  $\mu\text{g L}^{-1}$ ). The maxima of chl *a* were located below the upper mixed layer, from 40–50 m (in the western basin) to 140 m (in the eastern basin, close to Alexandria; Fig. 2). BA ranged from 0.54 to  $15.7 \times 10^8$  cells  $\text{L}^{-1}$  and BP ranged from 0.28 to  $60.9 \times 10^{-3} \mu\text{g C L}^{-1} \text{h}^{-1}$  (Table 1). Both BA and BP were highest in the Western Mediterranean and around the Greek Islands. The maximum values of BA and BP over the vertical profiles were generally located shallower than chl *a* peaks, between surface and 90 m depth (Fig. 2).

DMCHO and DPCHO ranged from undetectable to 9.7  $\mu\text{mol C L}^{-1}$ . In general, the total concentration of

dissolved carbohydrates was higher within the euphotic layer than below it (Table 1), but the shapes of the vertical profiles were very variable across stations.

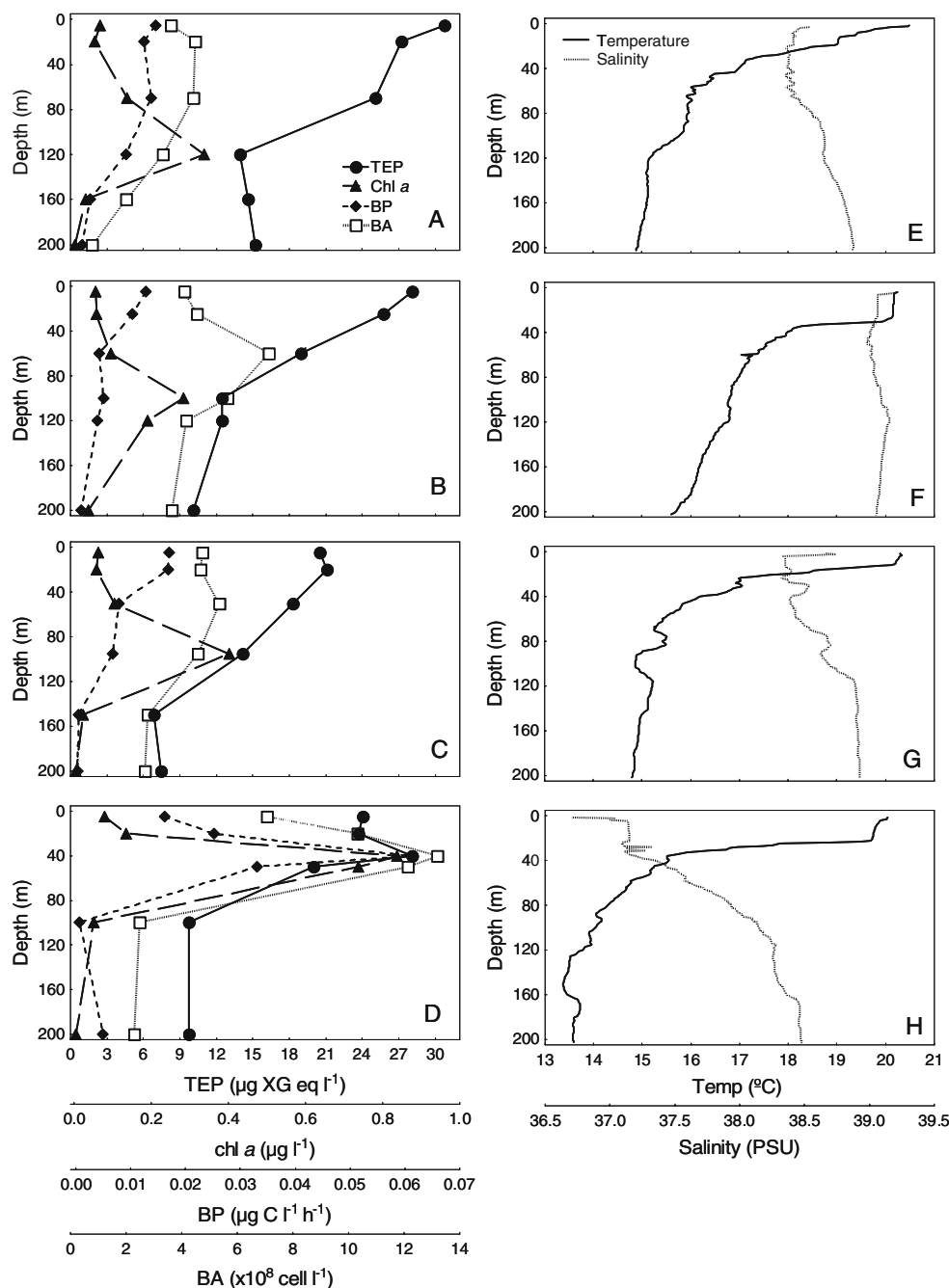
TEP concentration ranged from 4.5 to 94.3  $\mu\text{g XG eq L}^{-1}$  (Table 1). TEP concentrations decreased with depth in about half of the stations (11 out of 24 stations, e.g., station no. 4 in the central Mediterranean and station nos. 8 to 13 near Alexandria; Fig. 2a, b), while at others, TEP maxima coincided with the peaks of BP (e.g., station no. 2 or no. 17, near Sicily; Fig. 2c) or peaks of both chl *a* and bacteria (e.g., station no. 23 in the Western Mediterranean; Fig. 2d), in agreement with the reported influence of the Atlantic jet on biological productivity [64]. TEP concentrations were minimal at the surface layer. TEP concentrations did not vary consistently from the western to the eastern basin. The highest TEP concentration was observed within the euphotic layer of station no. 13 to no. 17 at the Aegean Sea near the Greek Islands, at station no. 20 in the central Mediterranean, and below the euphotic layer of station nos. 4 and 5. TEP carbon represented, on average, 1.7% of TOC.

TEP concentration was significantly and positively related with DMCHO + DPCHO concentration, chl *a*, BA, and BP (Table 2). However, the variance explained by these relationships was lower than 50% in all cases, except for the TEP–BP relationship (Table 2). The calculation of partial correlation coefficients, to examine the particular contribution of each factor, confirmed that BP was the best predictor of TEP concentration in the Mediterranean Sea (partial coefficient=0.66,  $p < 0.001$ ).

## Experiments to Determine Bacterial TEP Generation

BA and BP increased in all experiments and treatments both in the Western (Fig. 3) and Eastern (Fig. 4) Mediterranean Sea. The addition of SA (–bacteria treatments) did not completely prevent bacterial growth, but inhibited it partially in three experiments yielding lower BA (63% below the +bacteria treatment, on average) and BP (73% below the +bacteria treatment, on average). These differences were significant in three out of four experiments for BA and in two out of four experiments for BP (Table 3). Daily increases in BA ranged from 0.06 to  $2.49 \times 10^8$  cells  $\text{L}^{-1} \text{day}^{-1}$  and in BP from 0.02 to  $0.31 \mu\text{g C L}^{-1} \text{h}^{-1}$  (Table 4). TEP concentration increased in all experiments and treatments (Figs. 3 and 4), particularly in those without SA additions (+bacteria treatments). The differences of TEP concentration between treatments (+bacteria vs. –bacteria) were significant in experiments 2 (analysis of variance [ANOVA],  $p < 0.01$ ) and 4 ( $p < 0.05$ ). Daily increases in TEP ranged from  $4.8 \pm 0.9$  to  $19 \pm 0.4 \mu\text{g XG eq L}^{-1} \text{day}^{-1}$ . The TEP/BA ratios also increased over incubation time in seven of eight regrowth cultures (Table 4).

**Figure 2** Vertical profiles of (*left panels*) TEP concentration (in micrograms of XG equivalent per liter), chl *a* (in micrograms per liter), BP (in micrograms of carbon per liter per hour), and BA ( $\times 10^8$  cells per liter) and (*right panels*) temperature (in degrees Celsius) and salinity (in practical salinity unit) in four contrasting stations in the Mediterranean Sea: **a** and **e** station no. 4 (passed Sicily), **b** and **f** station no. 11 (near Alexandria), **c** and **g** station no. 17 (near Greece), and **d** and **h** station no. 23 (between Sardinia and Balearic Islands)



DMCHO concentration increased in seven out of eight regrowth cultures, ranging from a decrease of  $3.8 \mu\text{g C L}^{-1} \text{day}^{-1}$  to an increase of  $19.6 \mu\text{g C L}^{-1} \text{day}^{-1}$  (Table 4). In contrast, DPCHO concentration decreased with time in five out of eight regrowth cultures and ranged from a decrease of  $10.9 \mu\text{g C L}^{-1} \text{day}^{-1}$  to an increase of  $4.2 \mu\text{g C L}^{-1} \text{day}^{-1}$  (Table 4). Although DPCHO generally decreased with time, these daily decreases were smaller (average daily decrease,  $-2.7 \mu\text{g C L}^{-1} \text{day}^{-1}$ ) than the corresponding daily increases in TEP-C (average daily increase,  $8.0 \mu\text{g C L}^{-1} \text{day}^{-1}$ ; Table 4).

Independent of the initial conditions, daily TEP increases were strongly and positively related to daily increases in

BA ( $r^2=0.78$ ,  $p<0.01$ ) and BP ( $r^2=0.78$ ,  $p<0.01$ ), as described by the fitted regression equations:

$$\Delta\text{TEP}(\mu\text{g XG eq l}^{-1} \text{day}^{-1}) = 5.73 + 5.37\Delta\text{BA}(\times 10^8 \text{ cells l}^{-1} \text{day}^{-1}),$$

$$\Delta\text{TEP}(\mu\text{g XG eq l}^{-1} \text{day}^{-1}) = 5.40 + 44.21\Delta\text{BP}(\mu\text{g C l}^{-1} \text{h}^{-1} \text{day}^{-1}).$$

These experimental results corroborated the robustness of the relationship between bacteria and TEP in the Mediterranean Sea. Interestingly, the relationship between increases in TEP and increases in BP, when expressed as a



**Table 1** Average and ranges of BA ( $\times 10^{-8}$  cells  $L^{-1}$ ), BP ( $\times 10^{-3}$   $\mu g C L^{-1} h^{-1}$ ), DMCHO ( $\mu mol C L^{-1}$ ), DPCHO ( $\mu mol C L^{-1}$ ), TEP ( $\mu g XG eq L^{-1}$ ), carbon content of TEP ( $\mu g TEP-C L^{-1}$ ), TEP/chl *a* ( $\mu g XG eq \mu g chl a^{-1}$ ), TEP/BA ( $fg XG cell^{-1}$ ), and %TEP-C/TOC in the upper mixed layer, below the mixed layer but within the photic layer, and below the photic layer

	Total		Upper mixed layer		Below mixed layer photic layer		Below mixed layer below photic layer	
	Mean (ranges)	Number	Mean (ranges)	Number	Mean (ranges)	Number	Mean (ranges)	Number
BA	5.04 (0.54–15.7)	145	4.92 (0.53–12.4)	31	6.29 (3.20–15.7)	65	3.42 (0.76–7.88)	49
BP	12.1 (0.28–60.9)	145	14.3 (1.63–26.3)	31	16.9 (3.32–60.9)	65	4.11 (0.28–8.55)	49
DMCHO	2.7 (0–9.6)	125	2.7 (0–8.9)	30	3.1 (0–9.6)	53	2.2 (0–7.7)	42
DPCHO	2.4 (0–9.7)	124	3.0 (0–9.5)	30	2.5 (0–9.7)	52	1.7 (0–4.4)	42
TEP	21.4 (4.5–94.3)	123	29.2 (19.4–53.1)	30	26.1 (9.1–94.3)	53	9.4 (4.5–23.5)	40
TEP-C	16.0 (3.4–70.7)	123	21.9 (14.6–31.8)	30	19.5 (6.8–70.7)	53	7.1 (3.4–17.6)	40
%TEP/TOC	1.7 (0.7–8.0)	119	2.2 (1.2–4.0)	29	2.0 (0.7–8.0)	51	6.2 (1.2–14.7)	39
TEP/chl <i>a</i>	506 (12.6–12386)	118	484 (178–1293)	29	190 (12.6–1329)	51	947 (41.4–12386)	38
TEP/BA	50.9 (10.2–404)	123	80.9 (23.0–404)	30	44.5 (14.9–164)	53	37.0 (10.2–199)	40

log–log relationship ( $\log_{10}\Delta TEP=1.44+0.43(\log_{10}(\Delta BP))$ ), shows a similar slope ( $0.43\pm 0.14$ ) than that for the TEP–BP relationship in the field ( $0.39\pm 0.03$ ; Fig. 5).

Although daily TEP increases were related to the daily changes in both BP and BA, these TEP increases were only positively related to time-averaged BP in the cultures ( $r^2=0.78$ ,  $p<0.01$ ) and independent of time-averaged BA ( $r^2=0.25$ ,  $p>0.05$ ). Therefore, the estimation of bacterial TEP generation rate for in situ conditions was only possible from BP measurements [ $\Delta TEP$  ( $\mu g XG eq L^{-1}$ )= $5.24+11.94 BP$  ( $\mu g C L^{-1} h^{-1}$ )]. The use of these experimental rates ( $\Delta TEP$  per unit of BP) to predict TEP generation rates from average BP in situ values yielded TEP rates of  $5.4 \mu g XG eq L^{-1} day^{-1}$ , leading to a TEP duplication time from 0.8 to 17.5 days (average value, 4 days).

## Discussion

This research provides empirical evidences of a significant influence of bacterial activity on TEP distribution and dynamics in the open Mediterranean Sea. The TEP concentrations reported here are at the lowest threshold of

those reported in marine environments [3, 20, 36], including also different areas of the Mediterranean Sea [4, 28]. This discrepancy is probably attributable to the fact that these previous studies were performed in more eutrophic areas (e.g., coastal areas of the Western Mediterranean [4, 28] or during bloom events [3]). Indeed, despite the low TEP concentrations reported in this study, the TEP/chl *a* and TEP/BA ratios obtained were at the highest threshold of those reported in other marine systems [36, 45], but similar to those observed in oligotrophic areas [20, 48]. These high ratios are consistent with the extreme oligotrophic condition of the Mediterranean Sea. Indeed, the BP rates encountered in our study coincide with the lowest reported in the area [58, 63]. Acute limitation by inorganic nutrients appears to enhance TEP production by algae and bacteria [19, 30, 47]. Specifically, high N/P ratios in the Adriatic Sea have been identified as an ultimate cause for the formation of large organic matter aggregates through high rates of extracellular release of polymeric substances by phytoplankton [34]. In contrast, the concentrations of DMCHO and DPCHO observed in this study are in the range of those previously reported for the Mediterranean Sea [32] and for other areas of the ocean [33, 37].

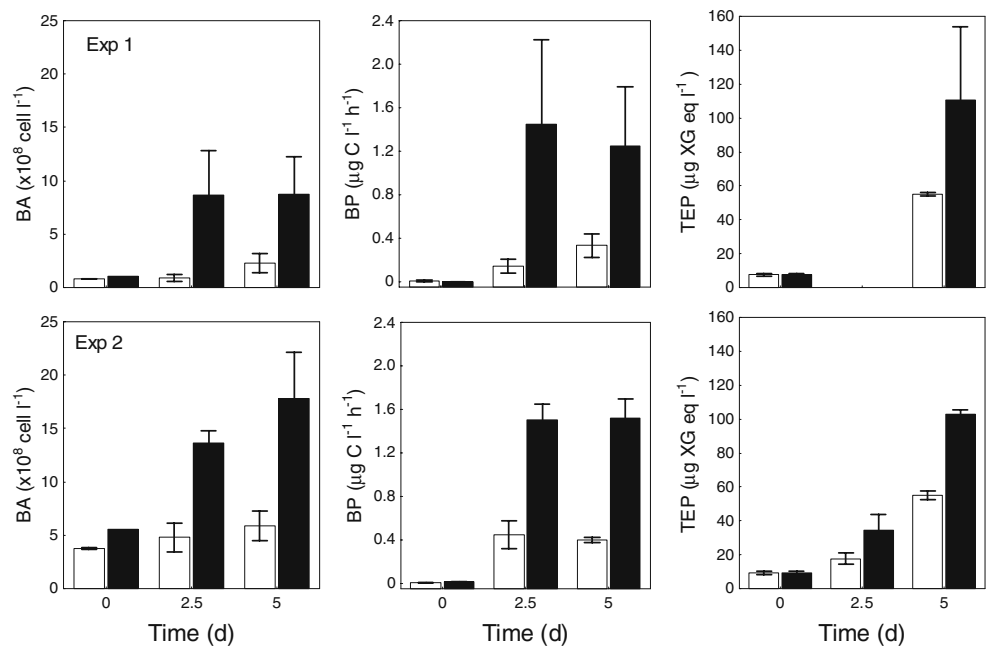
**Table 2** Regression equations and statistics describing the relationship between TEP and the sum of DMCHO and DPCHO concentration, chl *a* concentration, BP and BA

Dependent variable	Independent variables	Intercept	Slope $\pm$ SE	$r^2$	<i>n</i>	<i>p</i> level
TEP ( $\mu g XG eq L^{-1}$ )	DMCHO + DPCHO ( $\mu mol C L^{-1}$ )	0.35	$0.44\pm 0.12$	0.10	119	<0.001
	Chl <i>a</i> ( $\mu g L^{-1}$ )	1.43	$0.16\pm 0.04$	0.11	118	<0.001
	BP ( $\mu g C L^{-1} h^{-1}$ )	2.07	$0.39\pm 0.03$	0.51	122	<0.001
	BA (cells $L^{-1}$ )	-2.90	$0.48\pm 0.10$	0.15	123	<0.001

All variables were  $\log_{10}$ -transformed

SE standard error,  $r^2$  explained variance, *n* sample size, *p* level level of significance

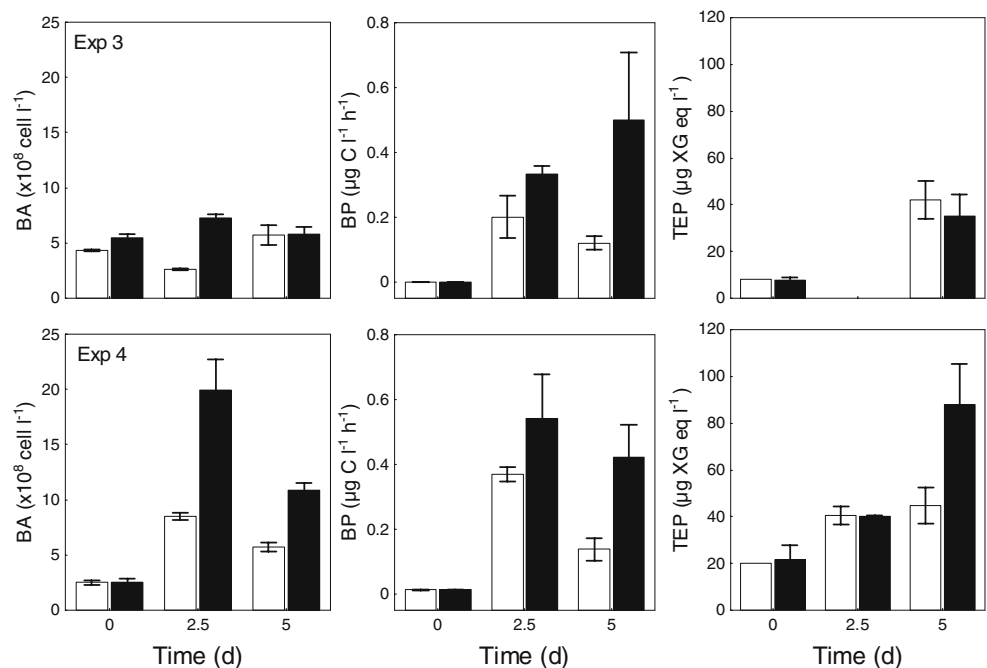
**Figure 3** Changes in BA, BP, and TEP concentration in the experiments performed in the Western Mediterranean (experiments 1 and 2). *White bars* -bacteria treatments, *black bars* +bacteria treatments, *whiskers* standard errors



Traditionally, the major source of TEP and their precursors are phytoplankton cells, particularly under bloom conditions [21, 41, 42]. In particular, the formation of marine mucilage in the Mediterranean Sea is thought to be ultimately of phytoplankton origin [11]. However, in this study, the relationship between chl *a* and TEP explained less variance than the TEP–BP relationship, suggesting a weaker link when phytoplankton are under nonbloom conditions. This weaker relationship could be also attribut-

ed to the selection of chl *a* as a surrogate of phytoplankton activity instead of primary production, to geographical differences in phytoplankton community structure, or to the extent of mineral nutrient limitation. Indeed, we observed the lowest or undetectable phosphate concentrations in the areas with highest chl *a* values (data not shown), in agreement with the well-known phosphorus limitation of this ecosystem [59]. Under these conditions, cell lysis causes an increase in TEP, while chl *a* concentrations

**Figure 4** Changes in BA, BP, and TEP concentration in the experiments performed in the Eastern Mediterranean (experiments 3 and 4). *White bars* -bacteria treatments, *black bars* +bacteria treatments, *whiskers* standard errors



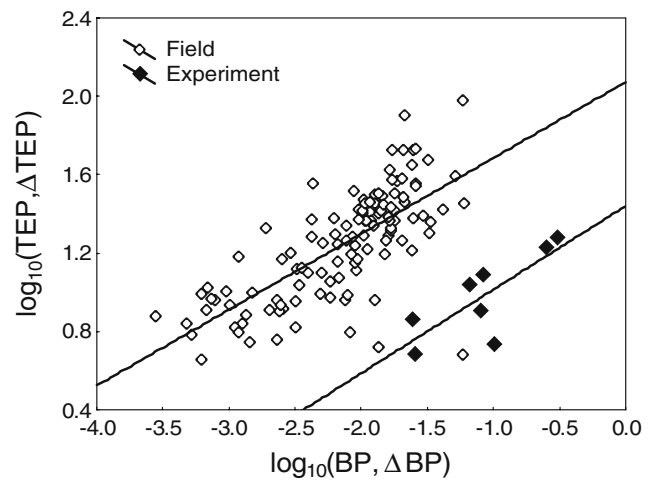
**Table 3** Results of the repeated-measures ANOVA to study differences in BA and BP over the incubation time in both treatments (+bacteria vs. -bacteria) in the experiments

Variable	Experiment no.	Effect	F	p level
BA	1	SA	68.02	<0.05
		Time×SA	0.95	ns
	2	SA	17.11	ns (=0.05)
		Time×SA	2.50	ns
	3	SA	17.22	ns (=0.05)
		Time×SA	29.70	<0.01
	4	SA	32.91	<0.05
		Time×SA	30.00	<0.05
BP	1	SA	10.06	<0.05
		Time×SA	1.20	ns
	2	SA	130.73	<0.01
		Time×SA	15.25	<0.01
	3	SA	2.27	ns
		Time×SA	1.78	ns
	4	SA	1.08	ns
		Time×SA	1.40	ns

ns not significant

decrease [41]. Moreover, the link between TEP, chl *a*, and BP may vary throughout the year. Thus, different patterns could be observed in winter, when primary production is usually higher in the Mediterranean Sea.

The higher slope and explained variance observed for the relationship between TEP concentration and BP (Fig. 5) suggest that this link appears to be more immediate and robust than the relationship with phytoplankton. This result contrasts with previous studies that attributed a minor role of bacteria on TEP formation in the sea [42, 50], although a positive relationship between BP and TEP has also been recently observed in the Gulf of Aqaba [3]. This positive relationship in the natural conditions could be explained by



**Figure 5** Relationship between TEP concentration and BP relationship in the natural conditions (*open diamonds*) and the relationship between daily TEP increases ( $\Delta$ TEP) and the daily BP increases ( $\Delta$ BP) in the regrowth experiments (*closed diamonds*). The fitted regression equations are  $\log_{10} \text{TEP} = 2.07 + 0.39 \log_{10} \text{BP}$  ( $r^2 = 0.51$ ,  $p < 0.001$ ) and  $\log_{10} \Delta \text{TEP} = 1.44 + 0.43 \log_{10} \Delta \text{BP}$  ( $r^2 = 0.78$ ,  $p < 0.05$ ), respectively

various mechanisms besides bacterial TEP generation. TEP in marine environments are frequently colonized by active bacteria and bacteria also appear to affect TEP assembly.

The experimental results presented here corroborated the direct generation of TEP by bacteria. Since most phytoplankton were initially removed in the experiments, we can rule out an increase of TEP production by phytoplankton induced by competition for mineral nutrients with bacteria. Similarly, TEP initial concentration was low due to the GF/F filtration; therefore, bacterial colonization of preexisting TEP, although feasible in the natural environment, was likely a minor process in our experiments. In fact, the percentage of bacterial colonization is related to TEP size [29, 42], which was  $< 0.7 \mu\text{m}$  at the initial time in our

**Table 4** Daily increments in BA, BP, TEP, TEP/BA ratios, DMCHO and DPCHO in all experiments and treatments

Experiment no.	Treatment	$\Delta \text{BA}$ ( $\times 10^8$ cells $\text{L}^{-1} \text{day}^{-1}$ )	$\Delta \text{BP}$ ( $\mu\text{g C L}^{-1} \text{h}^{-1} \text{day}^{-1}$ )	$\Delta \text{TEP}$ ( $\mu\text{g XG eq L}^{-1} \text{day}^{-1}$ )	$\Delta(\text{TEP}/\text{BA})$ (fg XG $\text{eq}^{-1} \text{cell}^{-1} \text{day}^{-1}$ )	$\Delta \text{TEP-C}$ ( $\mu\text{g C L}^{-1} \text{day}^{-1}$ )	$\Delta \text{DMCHO}$ ( $\mu\text{g C L}^{-1} \text{day}^{-1}$ )	$\Delta \text{DPCHO}$ ( $\mu\text{g C L}^{-1} \text{day}^{-1}$ )
1	-Bact	0.30	0.07	10.9	36.2	8.2	14.3	4.2
	+Bact	1.56	0.25	17.0	6.5	12.8	16.1	-2.4
2	-Bact	0.43	0.08	8.1	12.0	6.1	12.8	-10.3
	+Bact	2.49	0.31	19.0	8.4	14.3	19.6	-10.9
3	-Bact	0.26	0.02	7.3	12.0	5.5	7.9	2.2
	+Bact	0.06	0.10	5.4	9.3	4.1	-3.8	-2.4
4	-Bact	0.60	0.03	4.8	0.3	3.6	9.7	0.0
	+Bact	1.56	0.08	12.3	-1.0	9.2	13.1	-1.9

-Bact bacteria growth suppressed, +Bact bacteria growth allowed



experiments. Increases in TEP tracked bacterial increases in abundance and production, suggesting that TEP accumulation was a direct consequence of bacterial growth (Figs. 3 and 4), consistent with previous studies [40, 55, 56]. The bacterial TEP generation rates determined in the experiments yielded a mean duplication time of 4 days, similar to that obtained by Sugimoto et al. [57] when monitoring maximum TEP generation rates. However, it is worth to mention that, in the field, TEP also come from sources other than bacteria, so TEP concentration would have shorter duplication times aside from loss mechanisms.

Bacterial capsules are also stainable with alcian blue and cannot be discriminated from TEP with the colorimetric analysis used here. However, the proportion of capsulated bacteria in the regrowth cultures is expected to be lower than in the natural conditions [10] as grazing pressure is substantially reduced with the dilution procedure used in this study [25].

In most of the regrowth cultures, an increase in TEP/BA ratios was detected over time. This is comparable to observed TEP dynamics coupled to phytoplankton growth where increases in TEP formation have been frequently described at the end of algal blooms [9, 26]. The higher TEP accumulation at the end of growth stages of microorganisms may be due to the increase in TEP as a result of cell lysis, possibly due to viral infection or nutrient depletion, as these particles have a high C/N ratio. Indeed, bacterioplankton in the Mediterranean Sea are, like phytoplankton, extremely limited by inorganic nutrients [49, 59].

To elucidate if bacteria generate TEP directly or, alternatively, enhance physically the abiotic TEP formation from dissolved precursors, we compared the increases in TEP-C with decreases in DPCHO in the cultures. DPCHO is a bulk measurement of dissolved neutral and acidic polysaccharide. TEP precursors (a specific group of acidic polysaccharides) may be included in the whole pool of DPCHO. Increases in TEP were not counterbalanced by a decrease of the same magnitude in DPCHO (Table 4), supporting the idea that most TEP accumulated during the experiments were not formed directly by abiotic assembly of dissolved precursors. Hence, the most likely pathway for TEP increase is an active release of TEP by bacteria, which is also in agreement with the stronger TEP-BP relationship in comparison to TEP-BA relationship observed in the field (Fig. 5).

In marine systems, changes in TEP concentrations result from a balance between sources (production by bacteria, algae, and possibly other organisms) and sinks (particle sedimentation [44], consumption by organisms [61], or photolysis by UV radiation [35]). In this environment, bacteria not only generate or degrade TEP but can also modify the properties and the aggregation patterns of TEP released by algae [17–19]. The strong relationship between BP and TEP concentration, showing relationships with

similar slopes both in natural conditions and in experiments (Fig. 5), emphasizes the role of bacteria as a source of TEP, with further consequences on the production of macroaggregates in the Mediterranean Sea.

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