Aquat Ecol
DOI 10.1007/s10452-015-9550-3

Production of exopolymers (EPS) by cyanobacteria: impact on the carbon-to-nutrient ratio of the particulate organic matter

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Received: 3 April 2015 / Accepted: 12 October 2015

Abstract Freshwater cyanobacteria can produce large amount of mucilage, particularly during large blooms. The production of these carbon-rich exopolymers (EPS) should influence the carbon-to-nutrient ratios of the organic matter (OM), which are regularly used as a proxy for the herbivorous food quality. However, little is known about the consequences of EPS production on the carbon-to-nutrient ratio of the OM. Two EPS forms can be distinguished: the free fraction composed of soluble extracellular polymeric substances (S-EPS) and the particulate fraction corresponding to the transparent exopolymer particles (TEP). The aim of the study was to determine whether the TEP and S-EPS productions by cyanobacteria influence the carbon-to-nutrient ratios of the particulate OM (POM). Five cyanobacteria species were grown in batch culture and characterized in terms of photosynthetic activity, EPS production, and C, N, P contents. The variability in EPS production was compared with the variability in stoichiometry of the POM. Most of cyanobacteria live in association with heterotrophic bacteria (HB) within the mucilage. The effect of the presence/absence of HB on EPS production and the carbon-to-nutrient ratios of the POM was also characterized for the cyanobacteria *Microcystis aeruginosa*. We showed that TEP production increased the carbon-to-nutrient ratios of the POM in the absence of HB, while the stoichiometry did not significantly change when HB were present. The C:N ratio of the POM decreased with production of S-EPS by the five species. Lastly, the three colonial species (Chroococcales) tend to produce more TEP than the two filamentous species (Oscillatoriales), with the two picocyanobacteria being the most productive of both TEP and S-EPS.

Keywords Transparent exopolymer particles · Mucilage · Stoichiometry · *Microcystis aeruginosa*

Handling editor: Bas W. Ibelings.

Electronic supplementary material The online version of this article (doi:10.1007/s10452-015-9550-3) contains supplementary material, which is available to authorized users.

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Introduction

Phytoplankton primary production represents one of the basic processes of pelagic ecosystem functioning,

Published online: 17 October 2015

with the synthesis of a major source of organic carbon for heterotrophic communities (Cole et al. 1982; Baines and Pace 1991). The carbon-to-nutrient ratio of phytoplankton varies greatly compared with other aquatic heterotrophic organisms, depending on carbon fixation and nutrient uptake (Van de Waal et al. 2010). The nutritional value of the organic matter (OM) is partly controlled by the carbon-to-nutrient ratios (Sterner and Elser 2002; Urabe et al. 2003; Van de Waal et al. 2010), with food quality for heterotrophic communities decreasing as stoichiometric ratios increase. Exopolymers (EPS) released by phytoplankton are carbohydrate-rich and can thus potentially increase the carbon-to-nutrient ratios of the OM. In marine phytoplankton, it was shown that EPS composition can deviate in C:N far from the Redfield ratio, up to 26 (Engel and Passow 2001). EPS production by phytoplankton is highly variable, from 1 to 99.9 % of the net photosynthetically fixed organic carbon, depending on species and environmental conditions (Bertilsson and Jones 2003). The presence of species producing large amount of EPS should control the elemental ratios (C:N, C:P) of the OM in pelagic ecosystems, with potential repercussions on the trophic network. A better characterization of the link between species, EPS production, and stoichiometry of the POM is thus needed.

Although EPS form a size continuum of organic carbon (Verdugo et al. 2004), they are commonly divided into two forms, one dissolved and one attached. They are rarely simultaneously characterized, so that little is known about this double production and its variability between and among species. A large portion of exudates corresponds to a dissolved fraction, which is called soluble extracellular polymeric substances (S-EPS) (Underwood et al. 1995; Staats et al. 1999; Underwood et al. 2004). Some phytoplankton species, particularly cyanobacteria, produce large amount of cell-bound EPS, which form a mucilaginous matrix in which cells are embedded. These cell-bounded EPS belong to the widely studied 'Transparent Exopolymer Particles' (TEP) in aquatic ecosystems (Passow and Alldredge 1995). Depending on the form of EPS (dissolved or particulate), the influence of their production on the stoichiometry of the particulate organic matter should differ (POM). Production of TEP should increase the C:N and C:P of the POM, while S-EPS should decrease the stoichiometric ratios, owing to a carbon loss. Studies generally focused on one of the two forms of EPS and the associated C:N and C:P ratios of the POM are rarely quantified.

In freshwater ecosystems, cyanobacteria are known to accumulate in dense blooms, with an increasing frequency and intensity due to global changes (Johnk et al. 2008). These blooms lead to high concentrations of TEP at the water surface (Grossart et al. 1997) and one can wonder if such 'TEP events' may induce a change in the carbon-to-nutrient ratios of the particulate OM. These blooms are generally dominated by a few species (Huisman et al. 2005). This raises the question of the species' role in determining the POM stoichiometry. Colonial species, such as Chroococcales, should produce more TEP compared with other pelagic species, such as single-filament species. One can also wonder whether a lower TEP production is counterbalanced by a higher S-EPS production. TEP and S-EPS productions should differ between species depending on their morphological traits, and consequently their impact on the POM stoichiometry.

At the species level, it is already known that nutrient limitation is the predominant controlling factor for both TEP (Passow 2002; Reynolds 2007) and S-EPS (Baines and Pace 1991; Myklestad 1995). When nitrogen (or phosphorus) becomes limiting for growth, phytoplankton still accumulates some carbon during photosynthesis, while storage and metabolic uses (proteins production, growth) are limited (Banse 1974; De Philippis and Vincenzini 1998; Engel et al. 2004). The carbon in excess can be either excreted as polysaccharides, through the EPS (overflow) or stored in the cell through the formation of reserve compounds (De Philippis et al. 1996). However, less is known about the influence of heterotrophic bacteria (HB) on EPS production. Indeed, freshwater cyanobacteria are associated with highly diversified and metabolically active HB embedded in their mucilage (Worm and Søndergaard 1998; Casamatta 2000; Berg et al. 2009). TEP constitute suitable habitat for the microorganisms. HB can modulate the magnitude of the effect of nutrient on EPS production, through mineralization of organic nutrients. HB can also influence directly TEP and S-EPS concentrations through consumption and/or production of dissolved OM (Azam et al. 1994; Gärdes et al. 2012). Lastly, nutrients may modulate the magnitude of the effect of HB on EPS production: It has been demonstrated that nutrient availability influences the type of biological interaction between the

green microalga *Scenedesmus obliquus* and HB (Danger et al. 2007a, b).

The aim of the study was firstly to characterize the influence of HB and nutrient load on EPS production and secondly to characterize the impact of EPS production by cyanobacteria (and their associated HB) on the C:N and C:P ratio of the POM. In a first experiment, we characterize the effect of the presence of HB and nutrient load, on the EPS production by *Microcystis aeruginosa* and the associated C-to-nutrient ratios of the POM. We also test the hypothesis that the variability of the C-to-nutrient ratio of the POM may be explained by the species variability in EPS production. In a second experiment, we characterize the C, N, P content and the TEP and S-EPS productions by cyanobacteria, with three colonial (*Microcystis aeruginosa* and the picocyanobacteria *Aphanothece clathrata* and *A. minutissima*) and two single-filament species (*Limnothrix* sp. and *Planktothrix agardhii*).

Methods

Cyanobacteria cultures

Aphanothece clathrata (TCC 4a) and *A. minutissima* (TCC 323) were provided by the INRA UMR Carrtel (Thonon Culture Collection), while *Oscillatoria sp* (LRP 29) and *Planktothrix agaardhii* are grown in routine in our laboratory (Table 1). These four strains were all xenic. The axenic strain of *Microcystis aeruginosa* (PCC 7806) was provided by the Pasteur Culture collection of Cyanobacteria (http://cyanobacteria.web.pasteur.fr/). The axenic strain was initially checked for bacterial contamination by agar plating, following Briand et al. (2012). *M. aeruginosa* was grown in modified BG11 medium (Rippka 1988), while the four other strains were grown in BG11 medium (Andersen 2005).

To test for the effect of the presence of HB on the EPS production and the C-to-nutrient ratios of the POM, one of the five species, *M. aeruginosa*, was grown in both xenic (B) and axenic (Ax) conditions, at two nitrates loads (+N and −N). Initial nitrogen concentration was 1.76 mmol N L^{-1} in the classical N-replete medium (+N) and was 0.178 mmol N L^{-1} in the N-depleted medium (−N). The xenic culture of *M. aeruginosa* was obtained from the axenic one, after adding HB isolated from a French pond (N48°7′35.465″; W1°38′14.453″), where *M. aeruginosa* is regularly blooming; 2 mL of water from the pond was filtered on sterile 1-µm Poretics polycarbonate membrane filters, and the filtrate was added to 40 mL of *M. aeruginosa* axenic culture. This xenic culture (B) was grown in batch for 2 months prior to the experiment, with two inputs of fresh medium (approximately each 3 weeks). At the beginning of the experiment, the culture reached a total volume of 1.2 L, so that the initial input of pond water represents less than 0.2 % of the total volume. Before and after the experiment, we checked the presence of bacteria in the B culture, and for possible bacterial contamination in the Ax culture, with epifluorescent microscopic observations of 1–5 mL subsamples on 0.2 µm Nuclepore membranes after staining with DAPI (4′6-diamidino-2-phenylindole). Even if we cannot totally exclude a possible contamination by small-sized cyanobacteria, neither picocyanobacteria nor other small unidentified cells have been detected by regular microscopic observations.

Experiment 1 (xenic versus axenic conditions)

M. aeruginosa was tested in the presence (B) and in the absence (Ax) of heterotrophic bacteria, at two levels of nitrate availability (+N and −N). Each treatment (Ax − N, Ax + N, B − N, B + N) was run in triplicate in batch culture in climatic chambers at 25 ± 1 °C, 14:10 light/dark cycle with 30 µmol photons m^{-2} s^{-1} irradiance, in 500-mL Erlenmeyer flasks. All the cultures were manually mixed daily. Initial cell density of cyanobacteria was 200,000 cells mL^{-1}. Cultures were sampled every 2 days until the early stationary phase and characterized in terms of photosynthetic activity and cell density. S-EPS, TEP, and the C:N:P molar ratios of the OM were measured initially, during the exponential growth and as soon as cultures reached the early stationary phase.

Experiment 2 (variability among species)

The five species were grown in triplicate in batch culture in climatic chambers at 25 ± 1 °C, 14:10 light/dark cycle with 30 µmol photons m^{-2} s^{-1} irradiance, in 500-mL Erlenmeyer flasks. All the cultures were manually mixed daily. Initial cell density of cyanobacteria was 200,000 cells mL^{-1}. Cultures were

sampled every 2 days until the early stationary phase and characterized in terms of photosynthetic activity and cell density, as detailed below. S-EPS, TEP and the C:N:P molar ratios of the OM were measured during the exponential growth and the early stationary phases. To limit cyanobacteria cells lysis and release of EPS, the sampling at the early stationary phase was preferred over the advanced stationary phase. The cultures were assumed to be in early stationary phase when cell density remained stable during two successive sampling dates (four consecutive days) and when a decrease in the dark-adapted photochemical quantum efficiency F_v/F_m was observed.

Cell density and physiological measurements

Cell density was inferred by the optical density (OD) absorbance following the literature (Svane and Eriksen 2015; Post et al. 1985; Yéprémian et al. 2007; Briand et al. 2008; Rohrlack et al. 2013).

The 680-nm wavelength (chlorophyll *a*) was preferred over 750 nm (turbidity), which would include both bacteria and cyanobacterial cells (Danger et al. 2007a, b). However, OD measured at 750 and 680 nm was highly correlated both in the presence and in the absence of HB ($R^2 > 0.997$, $N = 105$, $p < 0.001$; Fig. S1). The OD at 680 nm was converted into cell density (cells mL^{-1}) based on the highly significant correlations between the two parameters ($R^2 > 0.99$, $N = 26$, $p < 0.001$; data not shown). We considered as negligible the intraspecific variability in cell size. The absorbance was measured every two days using a spectrophotometer Uvikon XS (Secomam, France).

The maximum growth rate was calculated from the formula:

$$\mu = \frac{\mathrm{Ln}(N_{t2}) - \mathrm{Ln}(N_{t1})}{t2 - t1}$$

where N_{t1} and N_{t2} correspond to the cell density (cells mL^{-1}) at time t_1 and t_2 (day^{-1}), respectively. Some filamentous species tend to form aggregates with time, increasing the daily variability in biomass measurement. The slope of the time series of the neperian logarithm of the cell density (during the exponential growth) was thus preferred over instantaneous growth rate.

To characterize the photosynthetic activity and the physiological state of the cyanobacteria, the electron transport rate (ETR) and the photosynthetic yield were measured every 2 days with a pulse-amplitude-modulated fluorescence monitoring system (PhytoPAM, Walz, Germany), following Schreiber (1998) and Zhang et al. (2011). The phytoPAM is equivalent to 4 separate PAM-fluorometers using light-emitting diodes (LED) with 10 µs light pulses at 4 different excitation wavelengths (470, 520, 645 and 665 nm), with the 645 nm specific to cyanobacteria (due to phycocyanin and allophycocyanin absorption). The phytoPAM was used with only one channel, corresponding to the cyanobacteria. The reference excitation spectrum measured at the factory was used, as it was not significantly different from reference excitation spectra performed on our cyanobacterial cultures. After dark adaptation for 15 min, fluorescence was measured at low measuring light (0.15 µmol photons m^{-2} s^{-1}) and during saturating light pulses (3000 µmol photons m^{-2} s^{-1} for 0.2 s). Fluorescence was measured at 10 different intensities of actinic light from 1 to 1216 µmol photons m^{-2} s^{-1}, with a 20-s time interval. The initial chlorophyll *a* fluorescence was also measured on each sample.

During the exponential growth and the stationary phase, the chlorophyll-specific absorption cross section a^* (m^2 mg chla^{-1}) was measured from in vivo absorption spectra of the cyanobacteria between 400 and 750 nm and from the chlorophyll *a* concentration, following Shibata et al. (1954). The ETR (µmol electron mg chla^{-1} s^{-1}) was then calculated for each light intensity I following Kromkamp and Forster (2003):

$$\mathrm{ETR} = 0.5 Y\, I\, a*$$

with 0.5 corresponding to the 50 % of photons intercepted by the PSII of the chlorophyll *a* (Gilbert et al. 2000). Y represents the quantum efficiency of the PSII and I the light intensity. The nonlinear least squares regression model of Eilers and Peeters (1988) was used to fit the ETR irradiance curves and to estimate the physiological parameters, such as the light-saturated maximum electron transport rate (ETRmax).

EPS measurements

To separate cells from the supernatant, centrifugation at $3200 \times g$ for 30 min at 12 °C was performed following Claquin et al. (2008). TEP and S-EPS were then analyzed separately.

The method of Passow and Alldredge (1995) modified by Claquin et al. (2008) was used to quantify the TEP fraction in 10 mL of culture. Briefly, 2 mL of 0.02 % alcian blue in 0.06 % acetic acid was added to the pellets, and samples were centrifuged at 3200×*g* at 4 °C for 20 min. Pellets were rinsed with 2 mL of distilled water and centrifuged again until the supernatant remained clear, in order to evacuate the excess of alcian blue; 4 mL of 80 % sulfuric acid was then added to the pellets. Absorbance was measured at 787 nm after 2 h and converted in equivalent xanthan (Passow and Alldredge 1995). A calibration curve was performed using xanthan gum following the same protocol. Xanthan was then converted in equivalent carbon using the factor of 0.75 observed by Engel and Passow (2001).

S-EPS were quantified using the method of Dubois et al. (1956). Briefly, 0.5 mL of supernatant was placed in a glass tube with 1 mL of 5 % phenol solution and 5 mL of 80 % sulfuric acid. After 30 min, absorption was measured at 485 nm and converted in equivalent glucose, using a standard calibration of glucose. Glucose was also converted in carbon, using the factor of 0.4 as for hexoses.

C, N, P measurements

To separate cells from the medium, centrifugation at 3200×*g* for 30 min at 12 °C was performed as for EPS fractionation. Medium and particulate matter was then analyzed separately. To remove the excess of surface-adsorbed C, N and P, pellets were briefly rinsed with distilled water and centrifuged a second time at 3200×*g* for 20 min.

Pellets were then resuspended in 5 mL of deionized water and analyzed for C, N, P content. Total particulate organic carbon was measured with an high-temperature persulfate oxidation technology using an OI Analytical carbon analyzer (model 1010 with a 1051 auto-sampler; Bioritech, France) following the International Organization of Standardization ISO 8245:1999 (1999). Total particulate nitrogen and total particulate phosphorus were measured, after an acidic digestion with potassium persulfate at 120 °C, using a continuous flow auto-analyzer (Brann and Luebbe, Axflow, France), based on colorimetric methods according to Aminot and Chaussepied (1983). Molar stoichiometric ratios of the POM (C:N and C:P) were than calculated, by dividing C content by N and P contents, respectively.

The supernatant was divided into two samples: one analyzed for nitrates and phosphates and the second one for total dissolved nitrogen and total dissolved phosphorus after mineralization through a potassium persulfate digestion at 120 °C. N and P concentrations were then measured using a continuous flow auto-analyzer (Brann and Luebbe, Axflow, France), based on colorimetric methods according to Aminot and Chaussepied (1983).

Statistical analysis

All statistical analyses (boxplot, correlation, ordination, linear model) were carried out using R studio software (R Development Core Team 2011). Wilcoxon rank-sum test followed by a post hoc Tukey test was used to detect differences between species and treatments, with significance threshold set at 0.05.

Linear models were used to examine the best set of predictor variables affecting the EPS production and the molar stoichiometric ratios of the POM produced by *M. aeruginosa*. One can expect nutrient load to modulate the effect of the presence of HB on EPS production and vice versa. We thus test for statistical interactions between nutrient load and HB in the models. As models for C:N and C:P revealed the same set of explanatory parameters, only the C:N model will be presented here (see supplementary data for the C:P model). Before analysis, data were checked to meet the assumptions of normality and homoscedasticity. A stepwise selection of the variables, which combines backward elimination and forward selection, was used to build the model, using the function 'stepAIC' (package MASS version 7.3-31 for R). The 'best' final model showed the lowest Akaike information criterion (Sugiura 1978). The significance of the model was tested using an ANOVA, while a Shapiro–Wilk normality test was performed on the residuals of the model.

To highlight controlling factors of the EPS production and C:N ratio in the five species of cyanobacteria, multivariate approach has been used on centered and scaled data. Explanatory variables were first reduced using forward selection of constraints with the *forward.sel* function of the 'packfor' library developed by S. Dray, as advised by Blanchet et al. (2008). Monte Carlo permutations tests retained only explanatory variables with probability value lower than 0.05. Redundancy analysis (RDA) was then performed with

significant explanatory variables using 'vegan' library (Oksanen 2013). This constrained multivariate analysis detects and quantifies the modifications in the biological response (TEP, S-EPS and C:N and N:P ratios of the POM), which can be explained by biological parameters of the species (surface/volume ratio and growth rate) and the availability of the resource (nitrates and phosphates concentration in the medium), through a multiple regression. While the canonical analysis requires a unimodal relationship between the environmental parameters and the biological response (typically environmental gradient analysis), the RDA underlies a linear relationship. The significance of the RDA was tested through a permutation test.

Results

Experiment 1: The influence of HB

To highlight the influence of HB and nutrient load on the stoichiometry of the POM, EPS productions and stoichiometric ratios were first measured in *M. aeruginosa* in the presence and in the absence of bacteria, at two nitrates loads. Dissolved inorganic phosphorus (DIP) in the medium was always higher than 96.9 µmol P L^{-1} throughout the experiment, indicating that phosphorus was never limiting in our experiment. N-NO_3^- concentration in the medium remained higher than 570 µmol N L^{-1} in nutrient-replete condition (+N), while the concentration was lower than 3.5 µmol N L^{-1} during the stationary phase in nutrient depleted conditions (−N) (data not shown). Neither the bacterial presence nor the two levels of nitrates availability induced a significant effect on the photosynthetic activity of *M. aeruginosa*, measured through the ETRmax (Fig. 1). Only growth phase changed significantly the ETRmax, in accordance with the decrease in photosynthetic activity when reaching the stationary phase (Fig. 1). TEP and S-EPS productions were also influenced by growth phase (Fig. 2): TEP tend to increase during the stationary phase (Fig. 2a), while S-EPS were at least three times higher during the exponential growth phase than during the stationary phase (Fig. 2b). The productions of TEP and S-EPS showed a similar pattern, in response to nutrient availability and the presence of HB, with a predominating effect of bacteria during the exponential growth and a predominating effect of nutrient during the stationary phase (Fig. 2). Low nitrate availability (−N; nitrate concentrations <15 µmol N L^{-1}; data not shown) increased both TEP and S-EPS concentrations, during the stationary phase (Fig. 2). The presence of HB increased TEP during the exponential phase when associated with high nitrate availability (Fig. 2a), leading to a significant interaction between HB and nitrates as shown by the linear model (Table 2). The presence of HB (B vs. Ax) increased significantly (twofold) the S-EPS concentrations during the exponential phase (Fig. 2b).

TEP and S-EPS productions were then compared with modifications of the molar stoichiometric ratios of the POM. The C:N ratio of the POM was highly correlated with TEP in axenic conditions (Fig. 3a). There was no significant correlation in the case of the C:P ratio (Fig. 3b). POM associated with bacteria (+B) showed more variable stoichiometric ratios, with lower values compared with axenic condition (Fig. 3).

Linear models were used to examine the best set of predictor variables for the molar stoichiometric ratios in *M. aeruginosa*. The initial model includes the amount of TEP and S-EPS per cell, the concentration

Table 1 Origin and morphological characteristics of the five species of cyanobacteria

Genus	Species	Origin	Form	V (µm^3)	S/V
Microcystis	*aeruginosa PCC7806*	Pasteur institute	Sphere	33.5	1500
Aphanothece	*clathrata (TCC 4a)*	INRA UMR CARRTEL	Prolate spheroid	8.4	633
Aphanothece	*minutissima (TCC 323)*	INRA UMR CARRTEL	Prolate spheroid	8.4	633
Limnothrix	*(LRP29)*	UMR 6553	Filamentous	8.2	2
Planktothrix	*agardhii*	UMR 6553	Filamentous	84.8	2

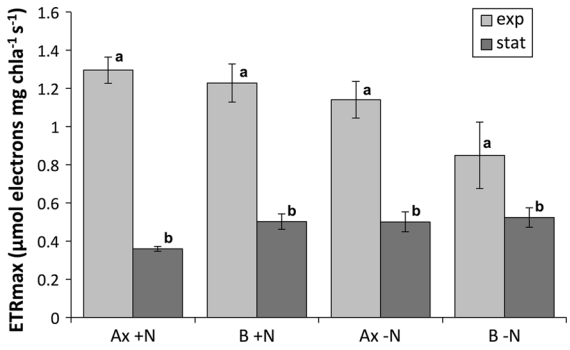

Fig. 1 Maximum electron transport rate (ETRmax) of *M. aeruginosa*, during exponential growth (*white area*) and stationary phase (*gray area*), depending on the presence of heterotrophic bacteria and nitrate availability: *Ax* Axenic, *B* associated with bacteria, $+N$ high level of N availability, $-N$ low level of N availability. Means of replicate value (±SD) are shown. No statistical difference based on Wilcoxon rank-sum test and Tukey post hoc test ($p > 0.05$)

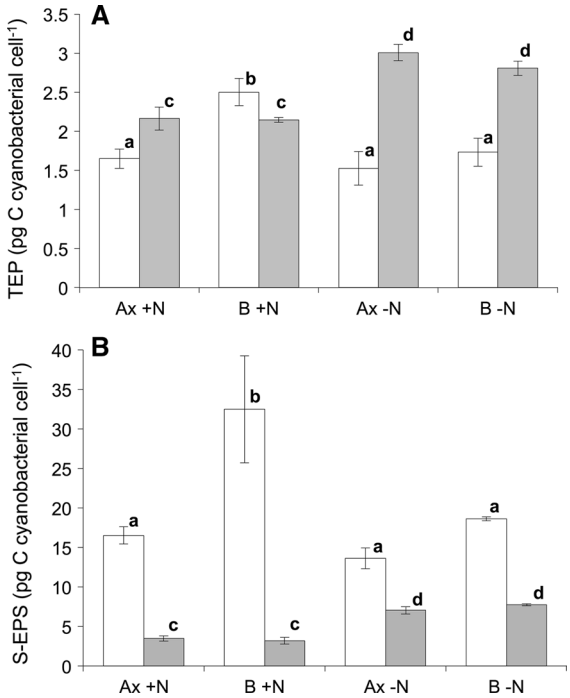

Fig. 2 **a** TEP and **b** S-EPS produced by *M. aeruginosa*, during exponential growth (*white area*) and stationary phase (*gray area*), depending on the presence of heterotrophic bacteria and nitrate availability: *Ax* Axenic, *B* associated with bacteria, $+N$ high level of N availability, $-N$ low level of N availability. Means of replicate value (±SD) are shown. a ≠ b and c ≠ d based on Wilcoxon rank-sum test and Tukey post hoc test ($p < 0.05$)

of nitrates and phosphates in the medium, the presence of HB (included as a qualitative factor), and the ETRmax. Interactions between bacteria and TEP and between bacteria and nutrients were also included in the initial model, bacterial activities being able to influence both TEP and nutrients. Regression slopes significantly differed in the presence and in the absence of HB, indicating that the magnitude of the effect of TEP and nitrates on the C:N ratio depends on the presence/absence of bacteria. The best model for the C:N ratio (Table 3), determined by a stepwise selection of the variables using the AIC criterion, selected the amount of TEP per cell, the nitrate concentration in the medium, the presence/absence of HB and two interactions, both with bacteria. In *M. aeruginosa*, TEP increased C:N, while the presence of bacteria and the availability of nitrate decreased them (Table 3). For both interactions (TEP × HB and nitrates × HB), the presence of bacteria increased the effect of the factor (TEP or nitrates) on C:N ratio. In the presence of HB, the C:N increased faster with TEP and decreased faster with nitrate availability, compared with axenic conditions.

Experiment 2

Variability among species in EPS production and stoichiometry

The five species of cyanobacteria were grown in the presence of HB, with initially high nitrates load. Stationary phases were observed after 15–24 days, depending on cultures (Fig. 4). The filamentous cyanobacteria (*Limnothrix* and *Planktothrix*) were the first cultures reaching the stationary phase, but the maximum cell density was two to three times lower than for the other species (Fig. 4). Their growth rate remained low (0.08 ± 0.01 and 0.13 ± 0.01 day^{-1}, respectively), while the growth rates of the three Chroococcales were higher than 0.25 day^{-1}. *A. clathrata* showed the highest growth rate, with 0.49 ± 0.01 day^{-1}, but this occurred only during the first seven days (Fig. 4). *M. aeruginosa* and *A. minutissima* showed similar growth rates, with 0.28 ± 0.03 and 0.30 ± 0.03 day^{-1}, respectively. The ETRmax measured during the exponential growth phase was also higher for the three Chroococcales,

Table 2 Parameter estimates for the best model predicting the TEP production in *M. aeruginosa*, as determined by a stepwise selection of the variables using the AIC

	Estimate	SE	Sum of sq	*df*	*F* value	Proba (>*F*)
(Intercept)	2.86	0.26				
NO3	−0.01	0.01	0.087	1	0.43	0.52
bacteria	−0.16	0.24	0.19	1	0.97	0.34
ETRmax	−0.84	0.30	1.59	1	7.93	0.011*
interaction NO_3 × bacteria	0.042	0.02	0.92	1	4.58	0.045*
Residuals			3.81	19		

The initial model includes the maximum electron transport rate, the concentration of nitrates in the medium, the presence of HB (included as a qualitative factor) and the interaction between nitrates and HB. Result from its ANOVA is also shown

Model statistics: AIC = −34.12, residual SE: 0.448, $df = 19$, $R^2 = 0.42$, $p = 0.027$

Significance levels are coded as follows: *** <0.001, ** <0.01, and * <0.05

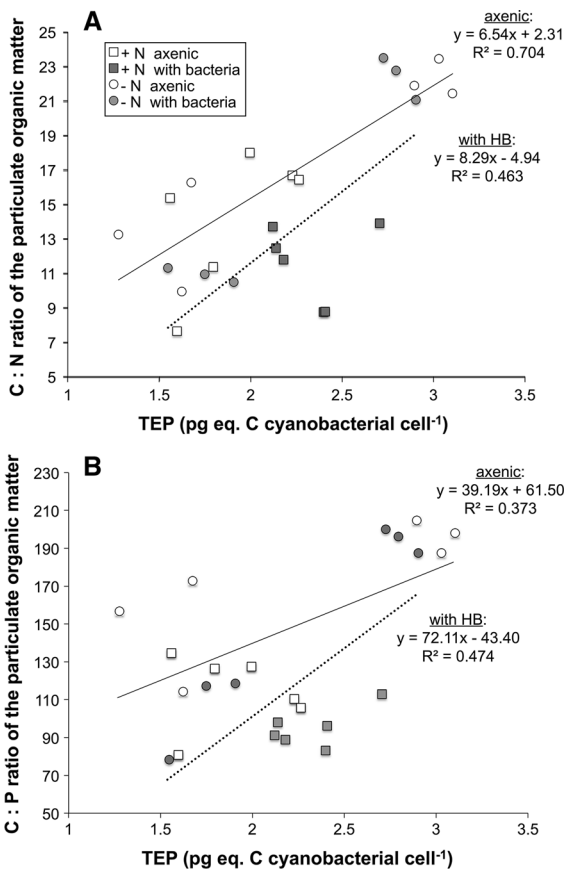

Fig. 3 **a** Molar C:N and **b** C:P ratios of *M. aeruginosa* (axenic condition shown *by open diamonds*) and of the cyanobacteria associated with heterotrophic bacteria (*filled circles*) depending on the amount of TEP per cyanobacterial cell. Correlations in axenic condition and in the presence of HB (including both +N and −N) are shown

compared with the Oscillatoriales, with the highest ETRmax observed for *M. aeruginosa* (Fig. 5). The ETRmax decreased for all species when they reached the stationary phase (Fig. 5), as well as the dark-adapted photochemical quantum efficiency (F_v/F_m), indicating the onset of the stationary phase (data not shown).

While Chroococcales and Oscillatoriales differed in their growth rate during the exponential phase, they also differed in their TEP production (Fig. 6a), contrary to the S-EPS production and POM's stoichiometry (Fig. 6b–d). EPS productions by the five cyanobacteria species, and particularly the picocyanobacteria, showed a high variability among replicates, larger than the variability among species (Fig. 6a, b). However, species producing large amounts of S-EPS (Fig. 6a) tended to produce large quantities of TEP (Fig. 6b). For the three Chroococcales, S-EPS production decreased with growth phase (data not shown), as observed in the previous experiment (Fig. 2b).

The five species differ significantly in their molar C:N and C:P ratios, with a low variability among replicates, except for the C:P of *Planktothrix* (Fig. 6c, d). Consequently, variability in the C:N ratio was larger among species than among replicates (Fig. 6c, d). All species together, the C:N ratio was close to the reference value found in the literature (Passow 2002; Thornton 2002; Reynolds 2006), with an average of 6.5 ± 3.0, a minimum of 1.9 and a maximum of 13.7 (Fig. 6c). The two picocyanobacteria showed the same C:N ratio, with values close to 6, while *M. aeruginosa* had the highest C:N ratio with 10.7 ± 2.2 (Fig. 6c).

Table 3 Parameter estimates for the best model predicting the C:N ratio in *M. aeruginosa*, as determined by a stepwise selection of the variables using the AIC

	Estimate	SE	Sum of sq	*df*	*F* value	Proba (>*F*)
(Intercept)	4.96	2.98				
TEP	5.73	1.23	292	1	55.99	<0.0001***
NO3	−0.11	0.07	114	1	21.77	0.0002***
bacteria	−11.28	4.76	67	1	12.76	0.002**
interaction TEP × bacteria	4.44	2.04	25	1	4.74	0.043*
interaction NO_3 × bacteria	−0.27	0.11	35	1	6.67	0.019*
Residuals			94	18		

Result from its ANOVA is also shown

Model statistics: AIC = 44.7, residual SE: 2.284, $df = 18$, $R^2 = 0.84$, $p < 0.0001$

Significance levels are coded as follows: *** <0.001, ** <0.01, and * <0.05

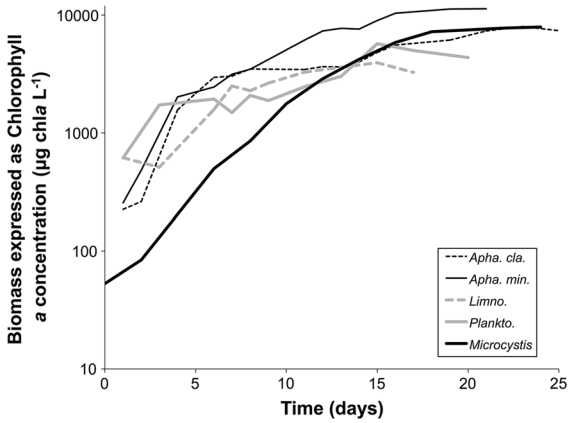

Fig. 4 Time series of the biomass absorbance (means of replicate value), expressed as chlorophyll *a* concentration, of the cyanobacteria species

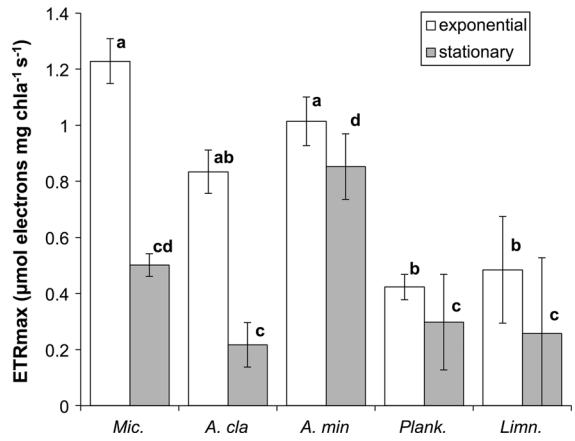

Fig. 5 Maximum electron transport rate (ETRmax) measured during exponential growth (*white area*) and the early stationary phase (*gray area*), depending on cyanobacterial species. Means of replicate value (±SD) are shown. a ≠ b and c ≠ d based on Wilcoxon rank-sum test and Tukey post hoc test ($p < 0.05$)

Limnothrix had the smallest C:N ratio, with less than 4, while *P. agardhii* was close to 8 (Fig. 6c). The C:P ratio (Fig. 6d) followed the same pattern than the C:N ratio (Fig. 6c). While molar C:N and C:P ratios showed the same pattern among the species, the TEP and the S-EPS production and stoichiometric ratios varied independently between species (Fig. 6). The correlation between TEP production and C:N ratio observed in axenic condition at the specific level (Fig. 3a) was not confirmed at the interspecific level.

Influence of EPS production on C, N, P contents and stoichiometry of cyanobacteria

The C, N, P contents of the POM were plotted for the five species (see symbols), for both growth phases (white versus black symbols), and for the three replicates, as a function of the TEP content (Fig. 7). Some species, like the two *Aphanothece*, showed a great variability in TEP content, associated with their growth phase, while their C, N and P contents changed only little (Fig. 7). All species taken into account, the carbon content of the POM was correlated with the amount of TEP (Fig. 7a), as well as the amount of nitrogen (Fig. 7b) and phosphorus (Fig. 7c). TEP is thus associated with a simultaneous increase in the C, N, P contents of the POM (Fig. 7). On average, increasing TEP content of 1 pg eq. C per cell led to an increase of 4 pg C, 0.4 pg N and 0.07 pg P of the cell (Fig. 7). The POM increase associated with TEP

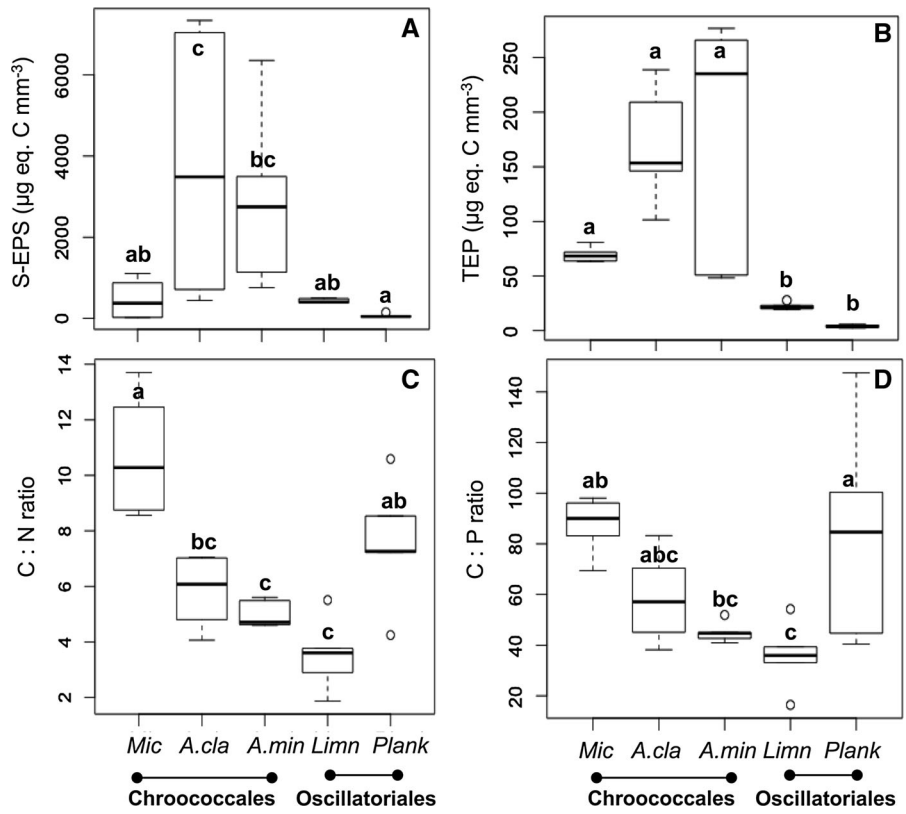

Fig. 6 Boxplot of **a** the mean concentration of S-EPS in the culture per unit of cell volume (µg ep. C mm^3) depending on cyanobacteria, of **b** the TEP per unit of cell volume (µg ep. C mm^3), of **c** the molar C:N ratio and **d** C:P of the particulate organic matter. C represents the Chroococcales and O the Oscillatoriales. a $\neq$ b $\neq$ c based on Wilcoxon rank-sum test and Tukey post hoc test ($p < 0.05$)

production thus presented a C:N and a C:P ratio of 11.7 and 140, respectively, which correspond to the highest ratios measured during the study (Fig. 6c, d). Lastly, when comparing C:N ratio of the POM with the concentration of S-EPS in the medium, a decrease in the C:N with larger concentration of S-EPS was observed (Fig. 8), indicating a potential loss of the particulate carbon with S-EPS production. Similarly to the TEP content, some species showed a great variability in EPS production, such as *A. minutissima* and *Limnothrix*, with only few changes in the C:N ratio of the POM (Fig. 8).

A redundancy analysis was performed to explain the C:N and N:P ratios and the EPS productions with species parameters and nutrients availability. The N:P ratio was preferred over the C:P ratio in the RDA analysis, because of the strong correlation between C:N and C:P ($R^2 = 0.77$, $p < 0.0001$). The RDA triplot showed that species and growth phases are well separated in the ordination space (Fig. 9). The filamentous cyanobacteria are grouped together on the left part of the triplot, whatever their growth rate, with the picocyanobacteria in stationary phase. The second axis separated *M. aeruginosa* depending on its growth phase from the picocyanobacteria in exponential phase. The first axis (38 % of the total variance) of the ordination was mainly described by TEP and in a lower extent by S-EPS and C:N. The explanatory variables of the first axis were the phosphates concentration and the cellular surface-to-volume ratio (S:V ratio), which were opposite to nitrates concentration. Species in the right part of the graph thus presented a higher C:N ratio and higher EPS productions, associated with high phosphates availability and low nitrates one in the medium. These species also presented a higher cellular S:V ratio. The second axis (25 % of the total variance) was mainly described by S-EPS and C:N, with species presenting a high C:N producing low S-EPS. The nitrates concentration and the growth rate explained the second axis. Species with high C:N ratio showed a low growth rate in a nitrate-depleted medium.

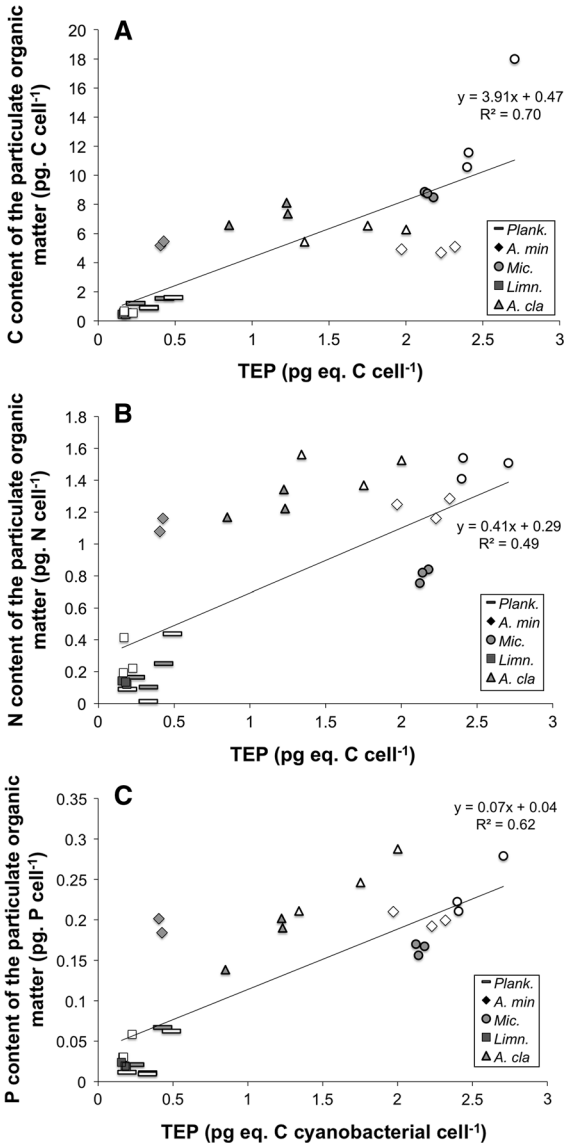

Fig. 7 **a** C content, **b** N content and **c** P content per cyanobacterial cell of the particulate organic matter depending on the TEP concentration per cyanobacterial cell, considering the five species. Data correspond to the three replicates measured during the exponential phase (*open symbols*) and during the early stationary phase (*black symbols*). Regressions refer to the entire set of data points

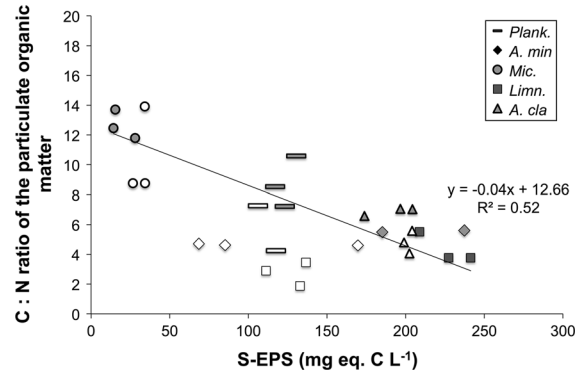

Fig. 8 Molar C:N ratio of the particulate organic matter depending on the S-EPS concentration in the medium, considering the five species. Data correspond to the three replicates measured during the exponential phase (*open symbols*) and during the early stationary phase (*black symbols*). Regression refers to the entire set of points

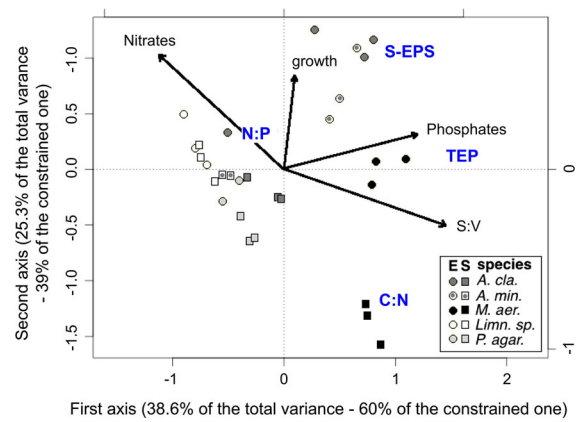

Fig. 9 Redundancy analysis (RDA) triplots for the molar C:N and N:P ratios, the TEP and S-EPS per cyanobacterial cell (μg eq C cell^{-1}) of the five cyanobacteria, explained by the growth rate, the cellular surface-to-volume ratio of the species, and the nitrates and the phosphates concentrations in the medium. Exponential E growth phase (*white circle*) and stationary S phase (*white square*) are shown, with the three replicates

Discussion

The highest concentrations of TEP in natural environment are regularly observed during and at the end of phytoplankton blooms (Grossart et al. 1997; Passow 2002; Vieira et al. 2008). These POM are rapidly colonized by heterotrophic bacteria (Mari and Kiørboe 1996), forming hotspots with elevated microbial activity and nutrient cycling, particularly as cells become senescent. But HB also colonize healthy phytoplankton. For instance, numerous specific bacteria are embedded in the colonies of *Microcystis* (Brunberg 1999; Casamatta 2000). It has been shown that many bloom-associated bacteria can enhance the cyanobacterial growth (Berg et al. 2009). Moreover,

there is increasing evidence of mutualistic relationships between phytoplankton and attached bacteria (Passow 2002; Croft et al. 2005). This may be a reason why axenic strains can be more difficult to maintain for long periods in algal culture banks. In our study, the presence of heterotrophic bacteria did not affect significantly the growth of *M. aeruginosa*, neither positively nor negatively. The maximum photosynthetic activity, the mean growth rate and the final cell density of the cyanobacteria were indeed similar in the presence and in the absence of HB. No significant cost neither benefit for the cyanobacteria could be identified from this biotic interaction. However, the presence of HB was associated with a higher EPS production, of both TEP and S-EPS, during the exponential growth phase of *M. aeruginosa*. HB can have produced these additional TEP, even if previous studies showed that HB associated with the mucilage of *M. aeruginosa* produced negligible TEP and S-EPS amounts (Yallop et al. 2000; Shen et al. 2011). Recent studies on HB–phytoplankton interactions also showed that HB can stimulate TEP release by marine diatoms (Bruckner et al. 2008; Gärdes et al. 2012). Increasing TEP production by phytoplankton means higher C-rich organic matter available for heterotrophic bacteria, which can in turn mineralize organic nitrogen and phosphorus. The higher EPS production observed in our experiment occurred under nutrient-replete conditions, when mineralization of OM was not essential to support the growth of cyanobacteria. The stimulation is thus not expected here. The additional TEP observed here were thus produced either by HB themselves or by the cyanobacteria after a stimulation of release induced by the HB. The higher S-EPS concentration observed in the medium can be explained by the hydrolytic activity of HB on TEP. The relationship between HB and EPS is complex, as bacteria are involved in production, modification and degradation of EPS (Passow 2002). As we have no abundance estimates of HB, neither any measure of their diversity and biological activity, we are limited to these hypotheses. Coupling isotopic tracers with imaging mass spectrometry analysis (NanoSIMS) would be a powerful approach to highlight C and N transfers from the cyanobacteria to the heterotrophic bacteria, as performed with earthworms in their burrow-lining (Gicquel et al. 2012) or in N transfer within single filament of cyanobacteria (Ploug et al. 2010).

We observed that the influence of TEP production on the stoichiometry of the POM was modulated by the presence of HB (significant statistical interaction). In axenic conditions, the C:N ratio of the POM was increased by TEP production, with a slope of +6.5 for each added picogram of TEP (in equivalent C) to the cell. TEP, mainly composed of polysaccharides (De Philippis and Vincenzini 1998), are C-enriched compared with living biomass. TEP remaining attached to the POM, their accumulation should increase the C:N ratio of the POM. The C:N ratio of natural TEP from the sea regularly exceeds 20 (Mari et al. 2001). However, we observed that the C:N ratio of the POM in the presence of HB was not influenced by the TEP amount, so that the carbon-to-nutrient ratios of the five species did not increase with TEP production. Hence, we conclude that TEP production increased the C:N ratio of the POM, until colonization of the POM by HB. The presence of HB was indeed associated with an increase in N and P contents of the POM, consequently modulating the effect of TEP production on the stoichiometry of the POM. One can also hypothesize that cells of cyanobacteria themselves influenced the C:N:P ratio of the POM through their storage capacity (Kromkamp 1987; Klausmeier et al. 2004). Indeed, cyanobacteria may accumulate P as polyphosphate and N as cyanophycin, both in granules in the cytoplasm (Kromkamp 1987; Marañón et al. 2013). However, storage would also have occurred in the absence of HB. Heterotrophic bacteria, through their activity and/or biomass, may have led to a N and P enrichment of the POM, resulting to the simultaneous increase in the C, N and P contents of the POM with TEP, as observed here. The C:N ratio of HB, which is also highly variable (Chrzanowski et al. 1996), tends to be slightly lower than the Redfield ratio (C:N:P of 106:16:1) (Redfield et al. 1963), with about 5, while the C:P ratio is twice to five times smaller, with values going from 50 to 19 depending on bacterial growth rate and nutrient availability (Chrzanowski et al. 1996; Fagerbakke et al. 1996). N is mainly associated with proteins and nucleic acids, while P is associated with nucleic acids (20 % of the mass of the cell) and storage through polyphosphate granules (Fagerbakke et al. 1996). The C:N and C:P ratios of the POM should thus decrease with bacterial colonization, compensating the increase associated with TEP production. In natural aggregates, HB can

represent up to 50 % of the total protein of the aggregates (Simon et al. 2002).

The carbon-to-nutrient ratios of the POM are regularly used to estimate the nutritional quality of the OM for heterotrophic communities (Hessen 1992; Sterner and Elser 2002). Herbivorous zooplankton can become limited by nitrogen or phosphorus if the C:N ratio of their food is too high (Boersma and Kreutzer 2002). Their growth and reproduction are then affected, but not their life span (Jensen and Verschoor 2004). If the C-to-nutrient ratios are too high, or even too low (Boersma and Elser 2006), heterotrophic grazers must eliminate the molecule in excess, as many organisms are strongly homeostatic in their elemental composition. EPS production associated with the presence of HB did not change here the carbon-to-nutrient ratios of the POM and probably the nutritional quality of the food. This is in accordance with previous studies. For example, a cladocera *Ceriodaphnia cornuta* fed with TEP released from the cyanobacteria *Anabaena spiroides* presented a higher growth rate and a higher fitness compared with the cladocera fed on seston at natural concentration (Choueri et al. 2007). TEP were obtained from filtrate of cultures in stationary phase, after evaporation, dialyze and lyophilization. However, the nutritional quality of the TEP is controversial in the literature, as several studies reported a negative impact of TEP on zooplankton grazing, hypothesizing either an allelochemical activity or an inhibitory effect of the EPS or protection against digestion (Decho and Lopez 1993; Liu and Buskey 2000; Dutz et al. 2005). Specific allelochemicals might have been produced in association with the EPS, as for instance the toxic species *Phaeocystis* (Dutz et al. 2005), in response to grazing pressure. While the nutritional quality may not have been affected, its quantity was, as TEP and HB increased the C, N, P contents of the POM. HB attached to aggregates become available as food for larger organisms (Passow and Alldredge 1999). Ling and Alldredge (2003) hypothesized that the consumption of TEP partly shunts organic carbon from the microbial loop to higher trophic levels. A higher size structure of the herbivorous community may be expected in the presence of TEP-producing species.

Contrary to our expectation, no trade-off between the free fraction and the particulate form has been observed. Species producing more S-EPS also tend to produce more TEP, compared with the other species. Size controlling the S:V ratio and colony formation requesting TEP to embedded cells, a higher TEP production was expecting for small cell size and colonial species. The smallest species (picocyanobacteria) thus showed the highest production of both forms of EPS, but also the greatest variability. Marine studies, including a greater number of species, revealed no relationship between size and EPS production (López-Sandoval et al. 2013). The most probable hypothesis is that life form is the predominant factor controlling EPS production. The three colonial species Chroococcales, *M. aeruginosa* and the picocyanobacteria tend to produce more TEP than our two filamentous cyanobacteria. The large production of TEP may correspond to the functional trait 'colonial mucilaginous species': *M. aeruginosa* aggregates can contain millions of cells, while the picocyanobacteria remained in small aggregates with generally less than 50 cells (Callieri and Stockner 2002; Costas et al. 2008), but with a large proportion of mucilage relative to cell volume. Colony-forming species may be seen as 'suspended biofilm,' with mucilage filling functions of cohesion, protection, retention or exchange (Flemming and Wingender 2010). EPS production, and particularly TEP, constitutes a functional trait, whose ecological roles are still discussed probably due to a multiplicity of its functions (Reynolds 2007).

Acknowledgments This research was funded by a grant from CNRS-UMR 6553 Ecobio and research funds from the University of Rennes ('Action incitative, Projets scientifiques émergents 2011') and from the french INSU-EC2CO program ('Microflux' 2012). We thank Nathalie Josselin-Le Bris and Marie-Paule Briand for laboratory assistance. Bio-chemical analysis, microscopy and experimental chambers were supported by the common technical centers from the UMR Ecobio: the Analysis Biogeochemical center (ABGC), the optical and digital imaging center (COIN) and the Ecology experimental center (ECOLEX). We thank the two anonymous referees for helpful comments on an earlier version of the manuscript.

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