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

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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

Introduction

Phytoplankton primary production represents one of the basic processes of pelagic ecosystem functioning, with the synthesis of a major source of organic carbon for heterotrophic communities (Cole, Likens & Strayer 1982; Baines & 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 & Elser 2002; Urabe, Togari & Elser 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 & 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 & 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.

Despite EPS form a size continuum of organic carbon (Verdugo *et al.* 2004), they are commonly divided in 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, Paterson & Parkes 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.

58 These cell-bounded EPS belong to the widely studied 'Transparent Exopolymer Particles' (TEP) in
59 aquatic ecosystems (Passow & Alldredge 1995). Depending on the form of EPS (dissolved or
60 particulate), the influence of their production on the stoichiometry of the particulate organic matter
61 should differ (POM). Production of TEP should increase the C:N and C:P of the POM, while S-EPS
62 should decrease the stoichiometric ratios, owing to a carbon loss. Studies generally focused on one of
63 the two forms of EPS, and the associated C:N and C:P ratios of the POM are rarely quantified.

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

74 At the species level, it is already known that nutrient limitation is the predominant controlling
75 factor for both TEP (Passow 2002; Reynolds 2007) and S-EPS (Baines & Pace 1991; Myklestad
76 1995). When nitrogen (or phosphorus) become limiting for growth, phytoplankton still accumulate
77 some carbon during photosynthesis, while storage and metabolic uses (proteins production, growth)
78 are limited (Banse 1974; De Philippis & Vincenzini 1998; Engel *et al.* 2004). The carbon in excess
79 can be either excreted as polysaccharides, through the EPS (overflow) or stored in the cell through the
80 formation of reserve compounds (De Philippis *et al*., 1996). However, less is known about the
81 influence of heterotrophic bacteria (HB) on EPS production. Indeed, freshwater cyanobacteria are
82 associated with highly diversified and metabolically active HB embedded in their mucilage
83 (Worm & Søndergaard 1998; Casamatta 2000; Berg *et al.* 2009). TEP constitute suitable habitat for
84 the microorganisms. HB can modulate the magnitude of the effect of nutrient on EPS production,

85 through mineralization of organic nutrients. HB can also influence directly TEP and S-EPS
86 concentrations through consumption and/or production of dissolved OM (Azam *et al.* 1994; Gärdes *et*
87 *al.* 2012). Lastly, nutrients may modulate the magnitude of the effect of HB on EPS production: it has
88 been demonstrated that nutrient availability influence the type of biological interaction between the
89 green microalga *Scenedesmus obliquus* and HB (Danger *et al.* 2007).

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

99

100 **Methods**

101 *Cyanobacteria cultures:*
102 *Aphanothece clathrata* (TCC 4a) and *A. minutissima* (TCC 323) were provided by the INRA - UMR
103 Carrtel (Thonon Culture Collection), while *Oscillatoria sp* (LRP 29) and *Planktothrix agaardhii* are
104 grown in routine in our laboratory. These four strains were all xenic. The axenic strain of *Microcystis*
105 *aeruginosa* (PCC 7806) was provided by the Pasteur Culture collection of Cyanobacteria
106 (http://cyanobacteria.web.pasteur.fr/). The axenic strain was initially checked for bacterial
107 contamination by agar plating, following Briand et al. (2012). *M. aeruginosa* was grown in modified
108 BG11 medium (Rippka 1988), while the four other strains were grown in BG11 medium (Andersen
109 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 (N 48°7'35.465''; W 1°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 two months prior to the experiment, with two inputs of fresh medium (approximately each three 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 to 5 mL sub-samples 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 Erlenmeyers flasks. All the cultures were manually mixed daily. Initial cell density of cyanobacteria was 200 000 cells mL^{-1}. Cultures were sampled every two 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 500mL Erlenmeyers flasks. All the cultures were manually mixed daily. Initial cell density of cyanobacteria was 200 000 cells mL^{-1}. Cultures were sampled every two 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 Fv/Fm was observed.

Cell density and physiological measurements:

Cell density was inferred by the optical density (OD) absorbance following the literature (Svane & Eriksen, 2015 ; Post et al., 1985; Yepremian et al., 2007; Briand et al., 2008; Rorhlack 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. 2007). However, OD measured at 750 nm and 680 nm were highly correlated both in the presence and 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:

□=□□ □□2−□□(□□1)□2−□1

where □□1 and □□2 correspond to the cell density (cells mL^{-1}) at time t1 and t2 (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 two 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. (Shibata, Benson & Calvin 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, Wilhelm & Richter 2000). Y represents the quantum efficiency of the PSII and I the light intensity. The non-linear 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 x 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, two mL of 0.02 % alcian blue in 0.06 % acetic acid were added to the pellets, and samples were centrifuged at 3200 x 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 were then added to the pellets. Absorbance was measured at 787 nm after 2h and converted in equivalent xanthan (Passow & 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 x g for 30 min at 12 °C was performed as for EPS fractionation. Medium and particulate matter were 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 x g for 20 min.

Pellets were then resuspended in five 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 European standard ISO 8245 (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-Analyser (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 content respectively.

The supernatant was divided in two samples, with 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-Analyser (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 were 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 was 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 *versus* Ax) increased significantly (two fold) 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 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 x HB and nitrates x HB), the presence of bacteria increased the effect's magnitude 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.

296

297 *Experiment 2:*

298 *Variability among species in EPS production and stoichiometry:*

299 The five species of cyanobacteria were grown in the presence of HB, with initially high nitrates load.
300 Stationary phases were observed after 15 to 24 days, depending on cultures (Fig. 4). The filamentous
301 cyanobacteria (*Limnothrix* and *Planktothrix*) were the first cultures reaching the stationary phase, but
302 the maximum cell density was two to three times lower than for the other species (Fig. 4). Their
303 growth rate remained low (0.08 ± 0.01 and 0.13 ± 0.01 day^{-1} respectively), while the growth rates of
304 the three Chroococcales was higher than 0.25 day^{-1}. *A. clathrata* showed the highest growth rate, with
305 0.49 ± 0.01 day^{-1},but this occurred only during the seven first days (Fig. 4). *M. aeruginosa* and *A.*
306 *minutissima* showed similar growth rates, with 0.28 ± 0.03 and 0.30 ± 0.03 day^{-1} respectively. The
307 ETRmax measured during the exponential growth phase was also higher for the three Chroococcales,
308 compared with the Oscillatoriales, with the highest ETRmax observed for *M. aeruginosa* (Fig. 5). The
309 ETRmax decreased for all species when they reached the stationary phase (Fig. 5), as well as the dark-
310 adapted photochemical quantum efficiency (Fv/Fm), indicating the onset of the stationary phase (data
311 not shown).

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

319 The five species differ significantly in their molar C:N and C:P ratios, with a low variability among
320 replicates, except for the C:P of *Planktothrix* (Fig. 6C and D). Consequently, variability in the C:N
321 ratio was larger among species than among replicates (Fig. 6C and D). All species together, the C:N

322 ratio was close to the reference value found in the literature (Passow 2002; Thornton 2002; Reynolds
323 2006), with on average 6.5 ± 3.0, a minimum of 1.9 and a maximum of 13.7 (Fig. 6C). The two
324 picocyanobacteria showed the same C:N ratio, with values closed to 6, while *M. aeruginosa* had the
325 highest C:N ratio with 10.7 ± 2.2 (Fig. 6C). *Limnothrix* had the smallest C:N ratio, with less than 4,
326 while *P. agardhii* was closed to 8 (Fig. 6C). The C:P ratio (Fig. 6D) followed the same pattern than
327 the C:N ratio (Fig. 6C). While molar C:N and C:P ratios showed the same pattern among the species,
328 the TEP and the S-EPS production and stoichiometric ratios varied independently between species
329 (Fig. 6). The correlation between TEP production and C:N ratio observed in axenic condition at the
330 specific level (Fig. 3A) was not confirmed at the interspecific level.

331

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

333 The C, N, P contents of the POM were plotted for the five species (see symbols), for both growth
334 phases (white versus black symbols), and for the three replicates, as a function of the TEP content
335 (Fig. 7). Some species, like the two *Aphanothece*, showed a great variability in TEP content,
336 associated with their growth phase, while their C, N and P contents changed only little (Fig. 7). All
337 species taken into account, the carbon content of the POM was correlated with the amount of TEP
338 (Fig. 7A), as well as the amount of nitrogen (Fig. 7B) and phosphorus (Fig. 7C). TEP is thus
339 associated with a simultaneous increase of the C,N,P contents of the POM (Fig. 7). On average,
340 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
341 the cell (Fig. 7). The POM increase associated with TEP production thus presented a C:N and a C:P
342 ratio of 11.7 and 140 respectively, which correspond to the highest ratios measured during the study
343 (Fig. 6C and D). Lastly, when comparing C:N ratio of the POM with the concentration of S-EPS in the
344 medium, a decrease of the C:N with larger concentration of S-EPS was observed (Fig. 8), indicating a
345 potential loss of the particulate carbon with S-EPS production. Similarly to the TEP content, some
346 species showed a great variability in EPS production, such as *A. minutissima* and *Limnothrix*, with
347 only few changes in the C:N ratio of the POM (Fig. 8).

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

364

365 **Discussion**

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

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

399 We observed that the influence of TEP production on the stoichiometry of the POM was
400 modulated by the presence of HB (significant statistical interaction). In axenic conditions, the C:N

401 ratio of the POM was increased by TEP production, with a slope of + 6.5 for each added picogram of
402 TEP (in equivalent C) to the cell. TEP, mainly composed of polysaccharides (De Philippis &
403 Vincenzini 1998), are C-enriched compared with living biomass. TEP remaining attached to the POM,
404 their accumulation should increase the C:N ratio of the POM. The C:N ratio of natural TEP from the
405 sea regularly exceeds 20 (Mari *et al.* 2001). However, we observed that the C:N ratio of the POM in
406 the presence of HB was not influenced by the TEP amount, so that the carbon-to-nutrient ratios of the
407 five species did not increase with TEP production. Hence, we conclude that TEP production increased
408 the C:N ratio of the POM, until colonization of the POM by HB. The presence of HB was indeed
409 associated with an increase of N and P contents of the POM, consequently modulating the effect of
410 TEP production on the stoichiometry of the POM. One can also hypothesize that cells of
411 cyanobacteria themselves influenced the C:N:P ratio of the POM through their storage capacity
412 (Kromkamp 1987; Klausmeier, Litchman & Levin 2004). Indeed, cyanobacteria may accumulate P as
413 polyphosphate, as well as N as cyanophycin, both in granules in the cytoplasm (Kromkamp 1987;
414 Marañón *et al.* 2013). However, storage would also have occurred in the absence of HB. Heterotrophic
415 bacteria, through their activity and/or biomass, may have led to a N and P enrichment of the POM,
416 resulting to the simultaneous increase of the C, N and P contents of the POM with TEP, as observed
417 here. The C:N of HB, which is also highly variable (Chrzanowski *et al.* 1996), tend to be slightly
418 lower than the Redfield ratio (C:N:P of 106:16:1) (Redfield, Ketchum & Richards 1963), with about 5,
419 while the C:P is twice to five times smaller, with values going from 50 to 19 depending on bacterial
420 growth rate and nutrient availability (Chrzanowski *et al.* 1996; Fagerbakke, Heldal & Norland 1996).
421 N is mainly associated with proteins and nucleic acids, while P is associated with nucleic acids (20 %
422 of the mass of the cell) and storage through polyphosphate granules (Fagerbakke *et al.* 1996). The C:N
423 and C:P ratios of the POM should thus decrease with bacterial colonization, compensating the increase
424 associated with TEP production. In natural aggregates, HB can represent up to 50% of the total protein
425 of the aggregates (Simon *et al.* 2002).

426 The carbon-to-nutrient ratios of the POM are regularly used to estimate the nutritional quality
427 of the OM for heterotrophic communities (Hessen 1992; Sterner & Elser 2002). Herbivorous

zooplankton can become limited by nitrogen or phosphorus if the C-to-nutrient ratio of their food is too high (Boersma & Kreutzer, 2002). Their growth and reproduction is then affected, but not their life span (Jensen & Verschoor 2004). If the C-to-nutrient ratios are too high, or even too low (Boersma & 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 lyophilisation. 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 & Lopez 1993; Liu & Buskey 2000; Dutz, Breteler & Kramer 2005). Specific allelochemicals might have been produced in association with the EPS, as for instance for 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 & 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 & 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 & 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).

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672

673

673 **Table and Figures legends**

674

675 Table 1: origin and morphological characteristics of the five species of cyanobacteria:

676 Table 2: Parameter estimates for the best model predicting the TEP production in *M. aeruginosa*, as
677 determined by a stepwise selection of the variables using the AIC criterion. The initial model includes
678 the maximum electron transport rate, the concentration of nitrates in the medium, the presence of HB
679 (included as a qualitative factor), and the interaction between nitrates and HB. Result from its
680 ANOVA is also shown. Significance levels are coded as follow: '***' indicates <0.001, '**' <0.01,
681 and '*' <0.05.

682 Table 3: Parameter estimates for the best model predicting the C:N ratio in *M. aeruginosa*, as
683 determined by a stepwise selection of the variables using the AIC criterion. Result from its ANOVA is
684 also shown. Significance levels are coded as follow: '***' indicates <0.001, '**' <0.01, and '*' <0.05.

685

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

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

696 Figure 3: (A) Molar C:N and (B) C:P ratios of *M. aeruginosa* (axenic condition shown by open
697 diamonds) and of the cyanobacteria associated with heterotrophic bacteria (filled circles) depending
698 on the amount of TEP per cyanobacterial cell. Correlations in axenic condition and in the presence of
699 HB (including both +N and –N) are shown.

700 Figure 4: Time series of the biomass absorbance (means of replicate value), expressed as Chlorophyll
701 a concentration, of the cyanobacteria species.

702 Figure 5: The maximum electron transport rate (ETRmax) measured during exponential growth (white
703 area) and the early stationary phase (grey area), depending on cyanobacterial species. Means of
704 replicate value (± standard deviation) are shown. a≠b and c≠d based on Wilcoxon rank-sum test and
705 Tukey post hoc test ($P < 0.05$).

706 Figure 6: Boxplot of (A) the mean concentration of S-EPS in the culture per unit of cell volume (µg
707 ep. C mm^3) depending on cyanobacteria, of (B) the TEP per unit of cell volume (µg ep. C mm^3), of (C)
708 the molar C:N ratio and (D) C:P of the particulate organic matter. C represents the Chroococcales and
709 O the Oscillatoriales. a ≠ b ≠ c based on Wilcoxon rank-sum test and Tukey post hoc test ($P < 0.05$).

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

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

719 Figure 9: Redundancy analysis (RDA) triplots for the molar C:N and N:P ratios, the TEP and S-EPS
720 per cyanobacterial cell (µg eq C $cell^{-1}$) of the five cyanobacteria, explained by the growth rate, the cell
721 ratio surface:volume of the species, and the nitrates and the phosphates concentrations in the medium.
722 Exponential E growth phase (o) and stationary S phase (□) are shown, with the three replicates.
723

723 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

724

725

725 Table 2: Parameter estimates for the best model predicting the TEP production in *M. aeruginosa*, as
726 determined by a stepwise selection of the variables using the AIC criterion. The initial model includes
727 the maximum electron transport rate, the concentration of nitrates in the medium, the presence of HB
728 (included as a qualitative factor), and the interaction between nitrates and HB. Result from its
729 ANOVA is also shown. Significance levels are coded as follow: '***' indicates <0.001, '**' <0.01,
730 and '*' <0.05.

	Estimate	Std.	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 NO3 x bacteria	0.042	0.02	0.92	1	4.58	0.045	*
Residuals			3.81	19			

731 Model statistics: AIC = -34.12, Residual standard error: 0.448, DF = 19, R^2=0.42, p = 0.027.

732

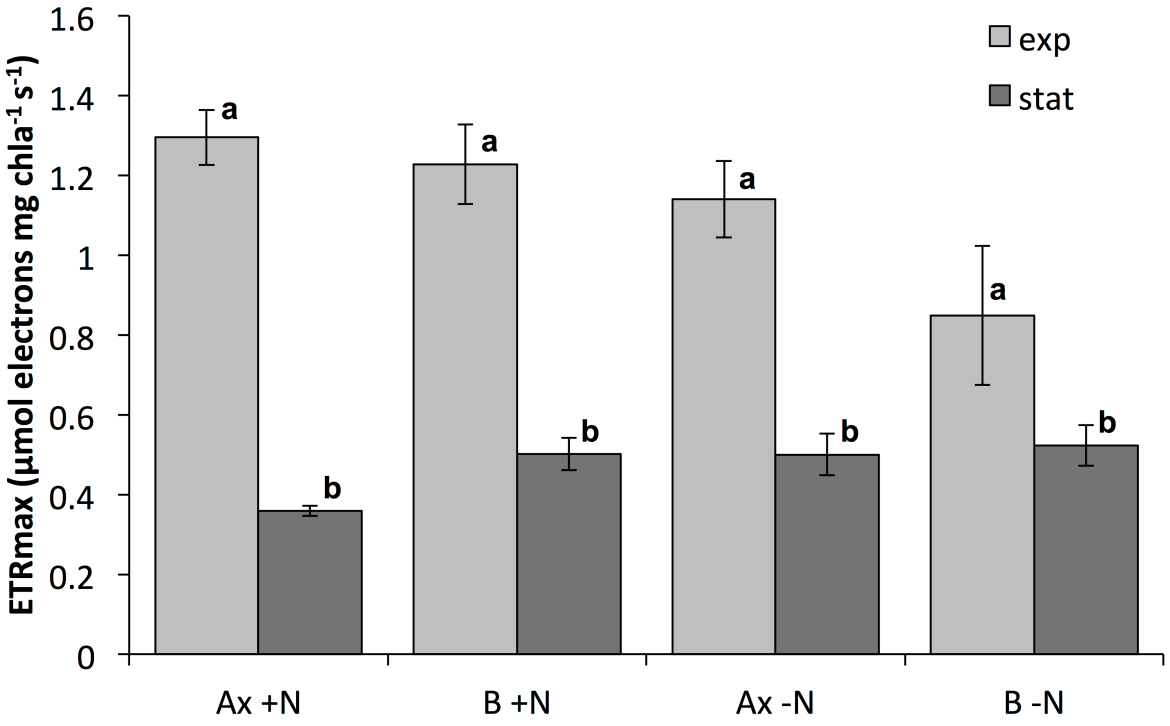
Table 3: Parameter estimates for the best model predicting the C:N ratio in *M. aeruginosa*, as determined by a stepwise selection of the variables using the AIC criterion. Result from its ANOVA is also shown. Significance levels are coded as follow: '***' indicates <0.001, '**' <0.01, and '*' <0.05.

	Estimate	Std.	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 x bacteria	4.44	2.04	25	1	4.74	0.043	*
interaction NO3 x bacteria	-0.27	0.11	35	1	6.67	0.019	*
Residuals			94	18			

Model statistics: AIC = 44.7, Residual standard error: 2.284, DF = 18, R^2=0.84, p < 0.0001.

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

746



747

748

Figure 2: (A) TEP and (B) S-EPS produced by *M. aeruginosa*, during exponential growth (white area) and stationary phase (grey 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 (± standard deviation) are shown. a≠b and c≠d based on Wilcoxon rank-sum test and Tukey post hoc test ($P < 0.05$).

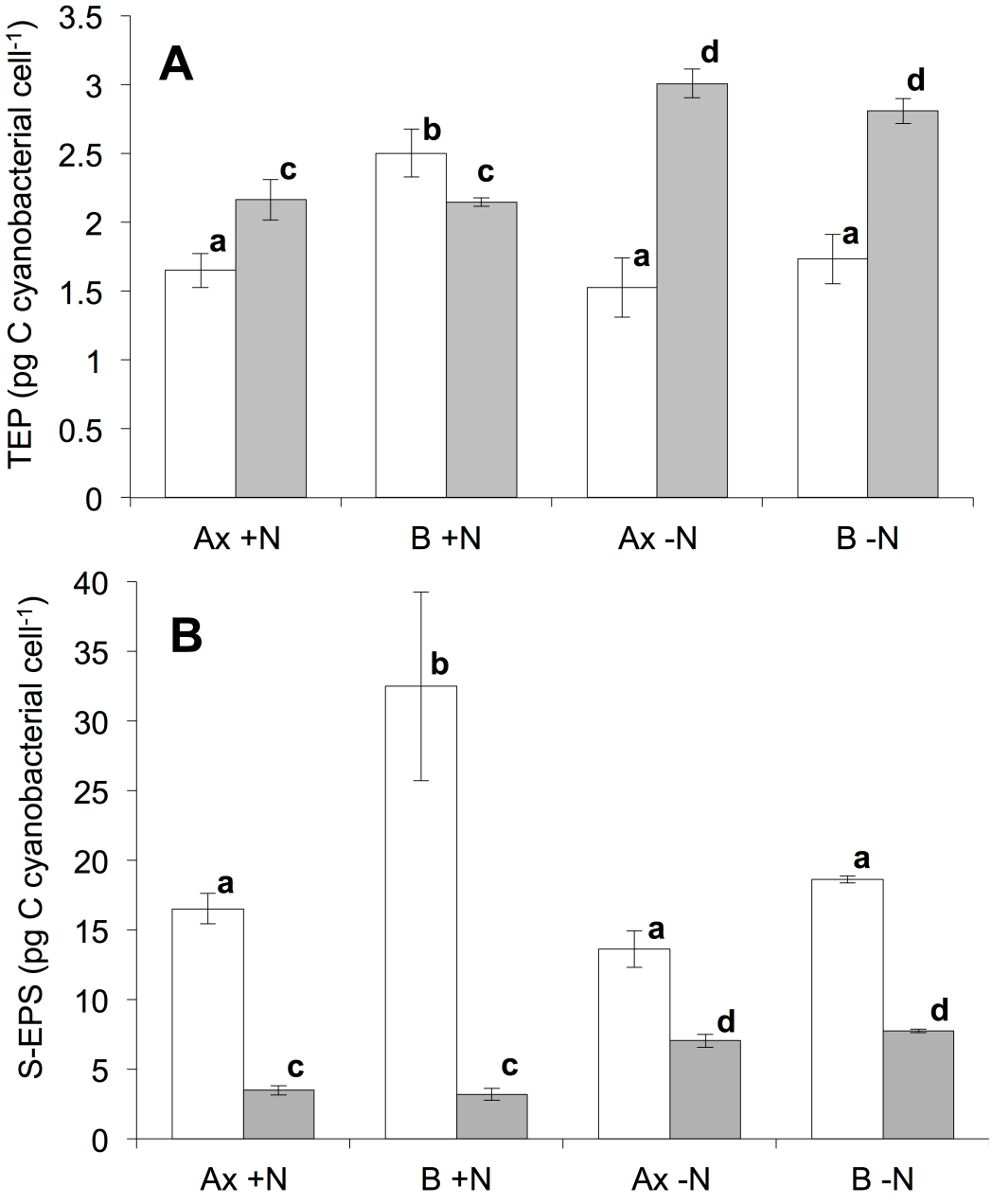
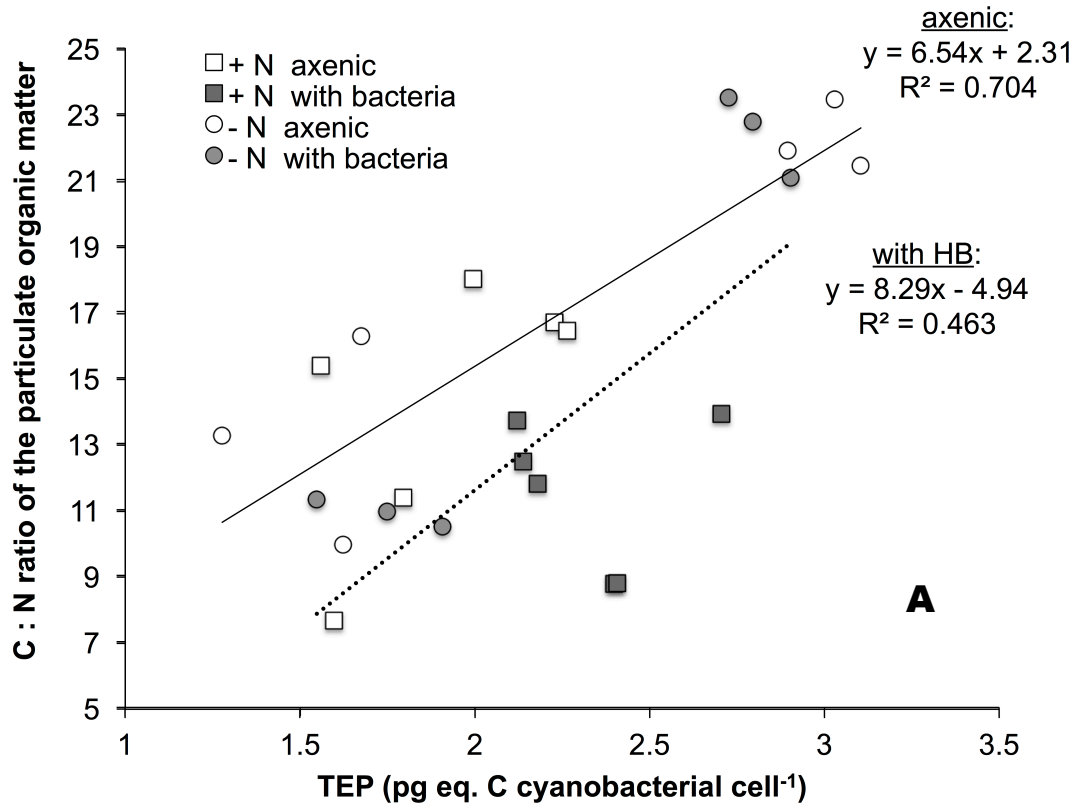
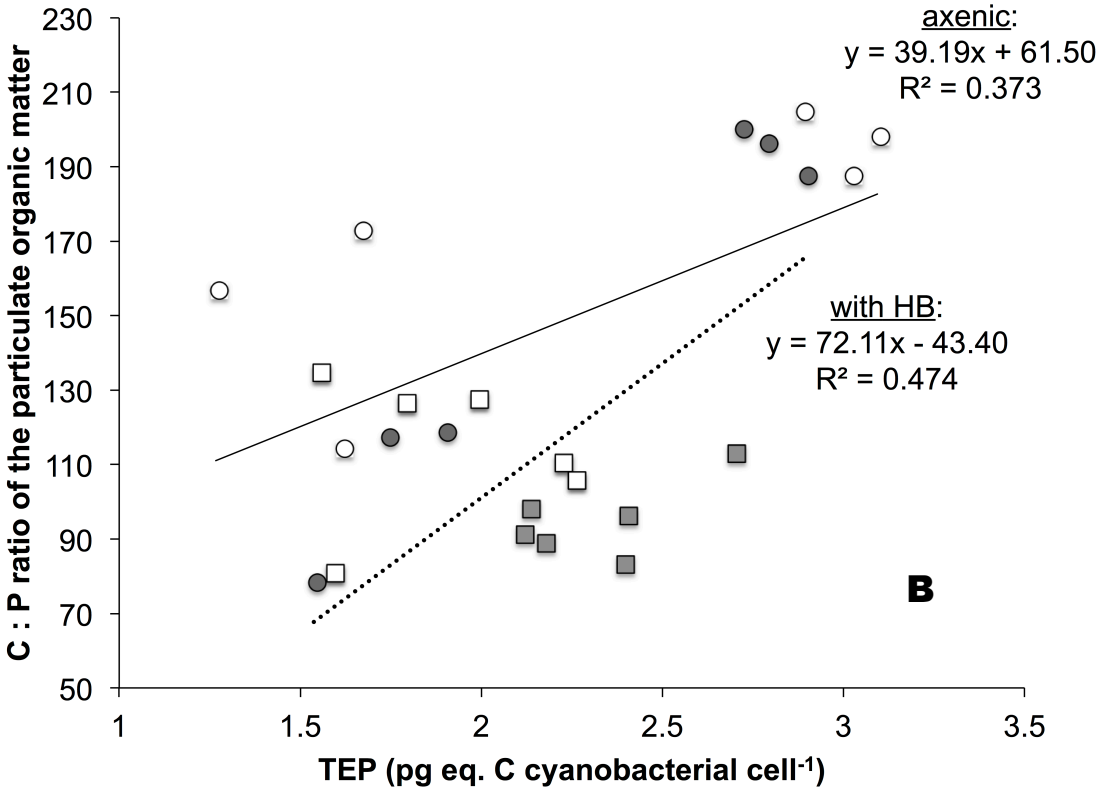


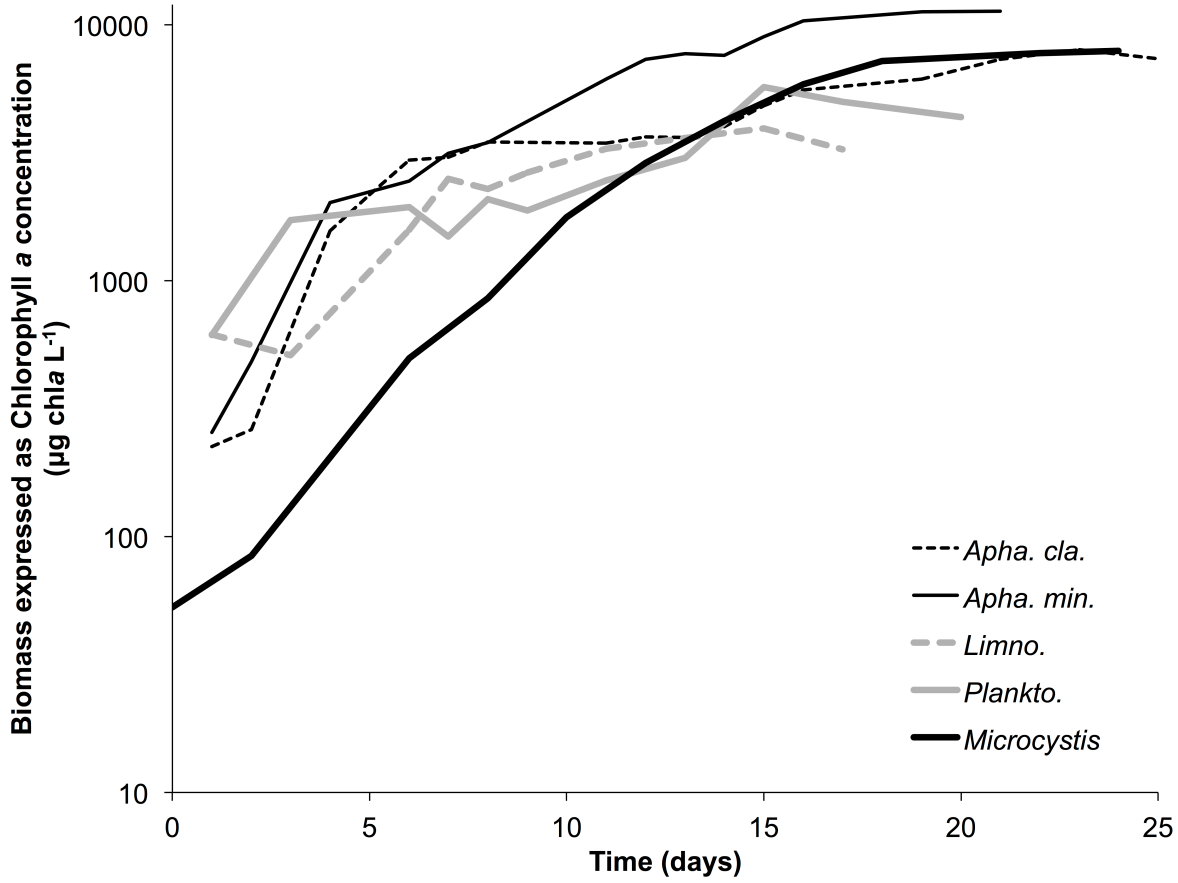
Figure 3: (A) Molar C:N and (B) C:P ratios of *M. aeruginosa* (axenic condition shown by open diamonds) and of the cyanobacteria associated with heterotrophic bacteria (filled circles) depending on the amount of TEP per cyanobacterial cell. Correlations in axenic condition and in the presence of HB (including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





763 Figure 4: Time series of the biomass absorbance (means of replicate value), expressed as Chlorophyll
764 a concentration, of the cyanobacteria species.

765



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768

Figure 5: The maximum electron transport rate (ETRmax) measured during exponential growth (white area) and the early stationary phase (grey area), depending on cyanobacterial species. Means of replicate value (± standard deviation) are shown. a≠b and c≠d based on Wilcoxon rank-sum test and Tukey post hoc test ($P < 0.05$).

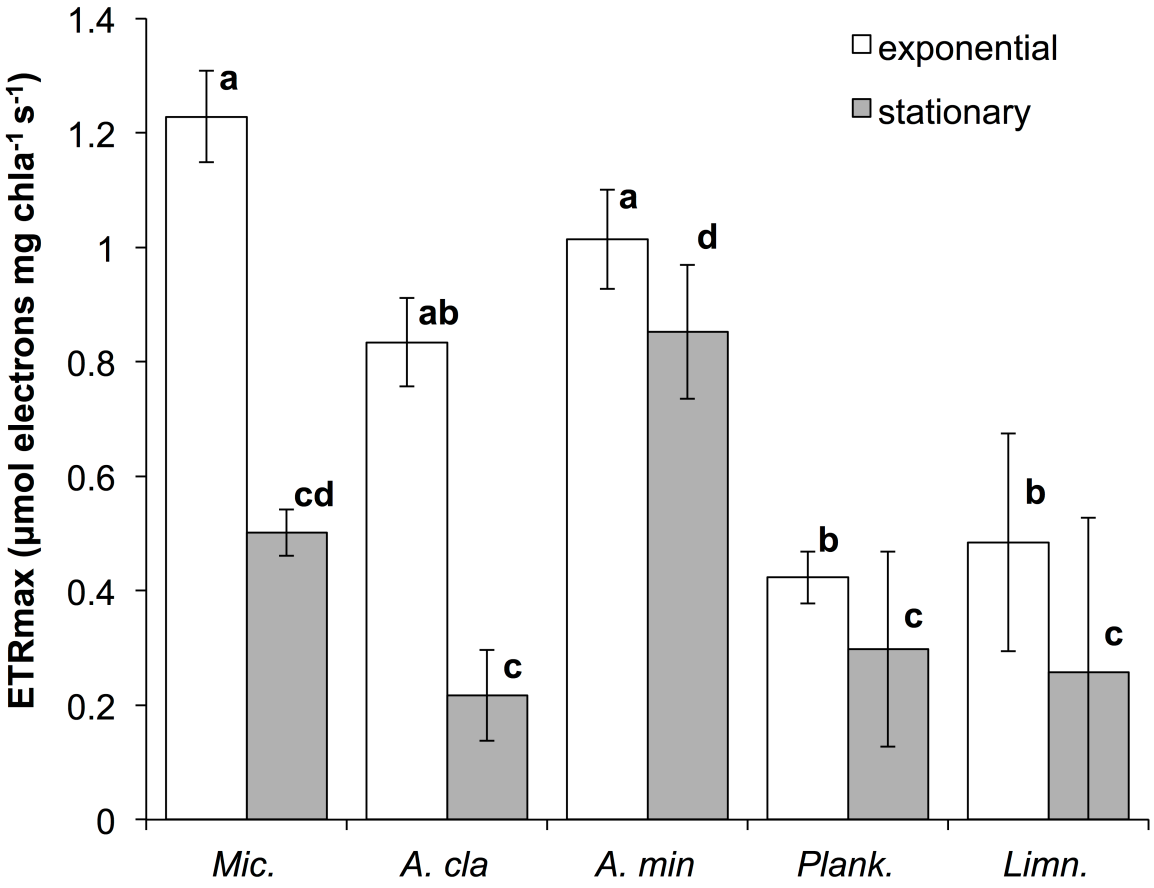


Figure 6: Boxplot of (A) the mean concentration of S-EPS in the culture per unit of cell volume (µg ep. C mm^3) depending on cyanobacteria, of (B) the TEP per unit of cell volume (µg ep. C mm^3), of (C) the molar C:N ratio and (D) C:P of the particulate organic matter. C represents the Chroococcales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

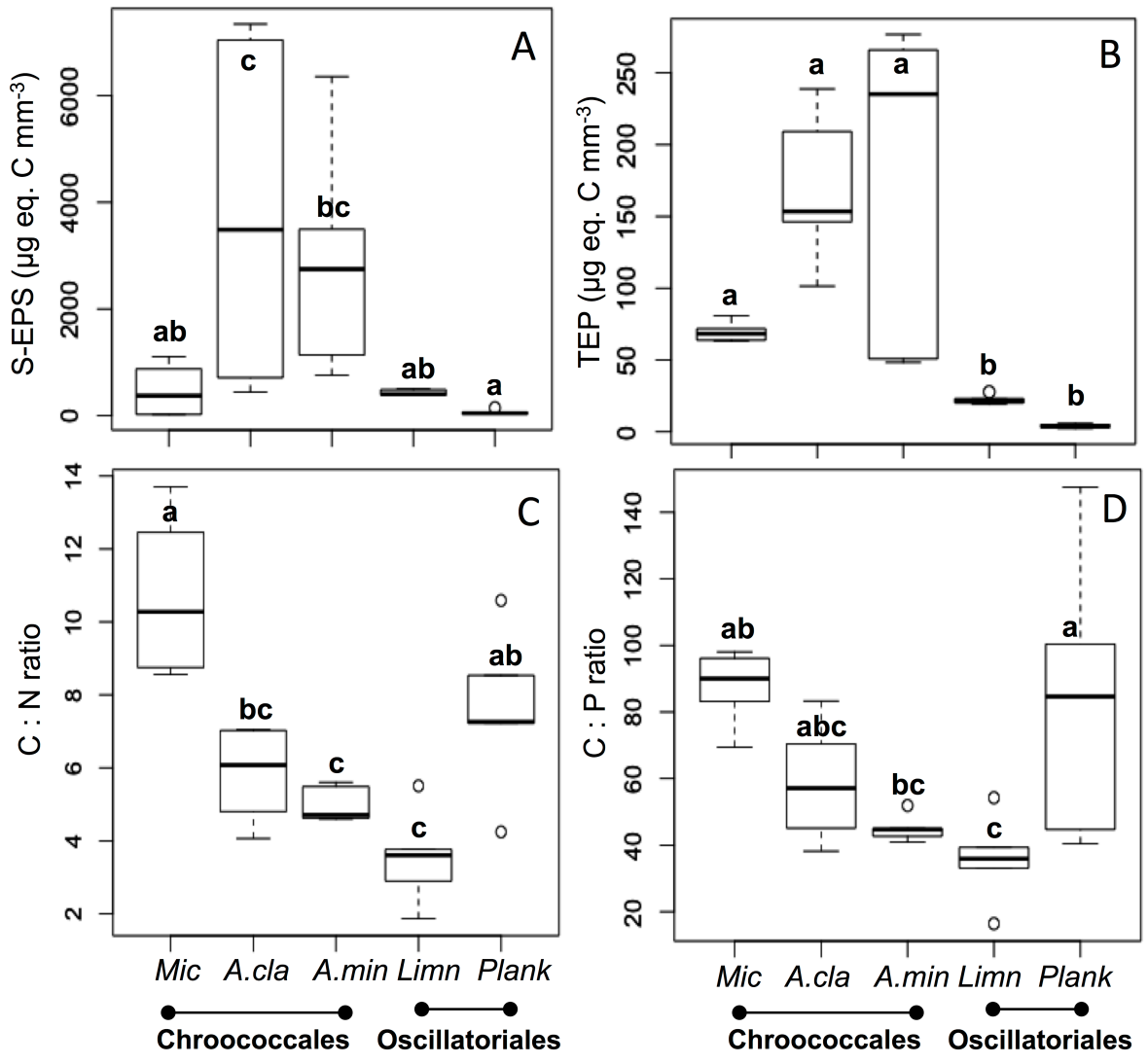
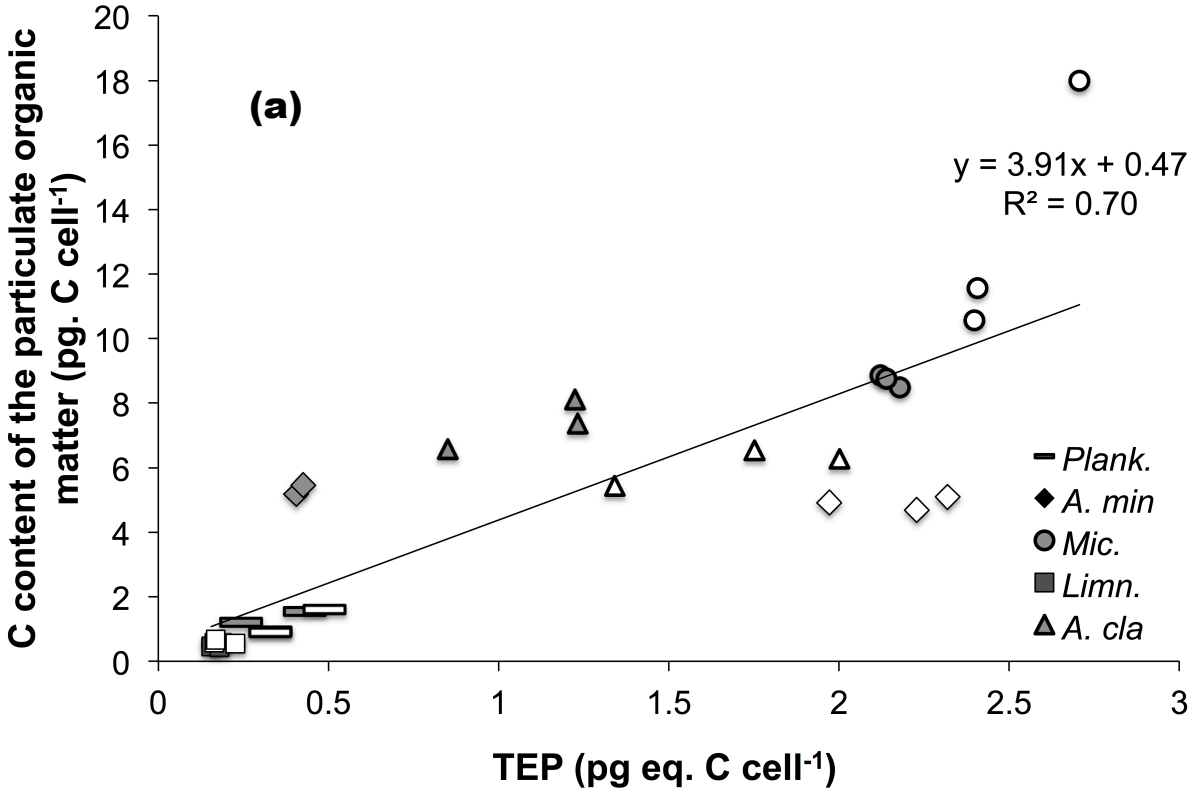
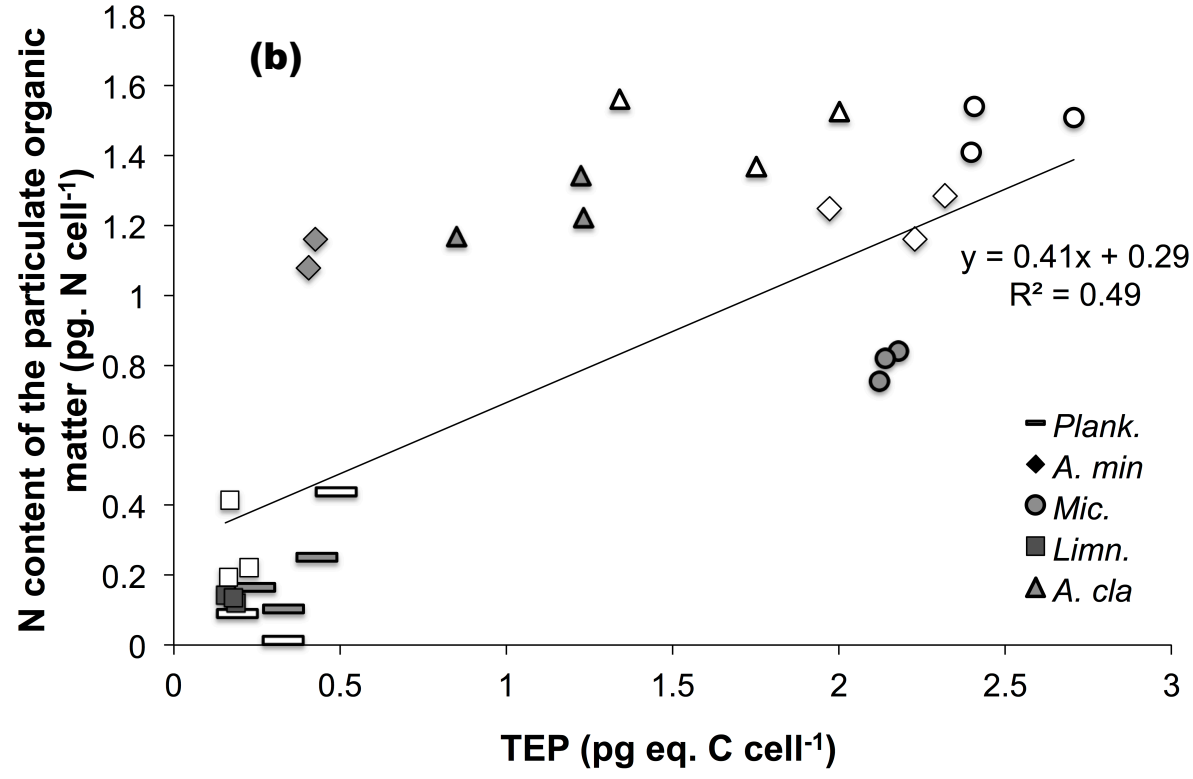
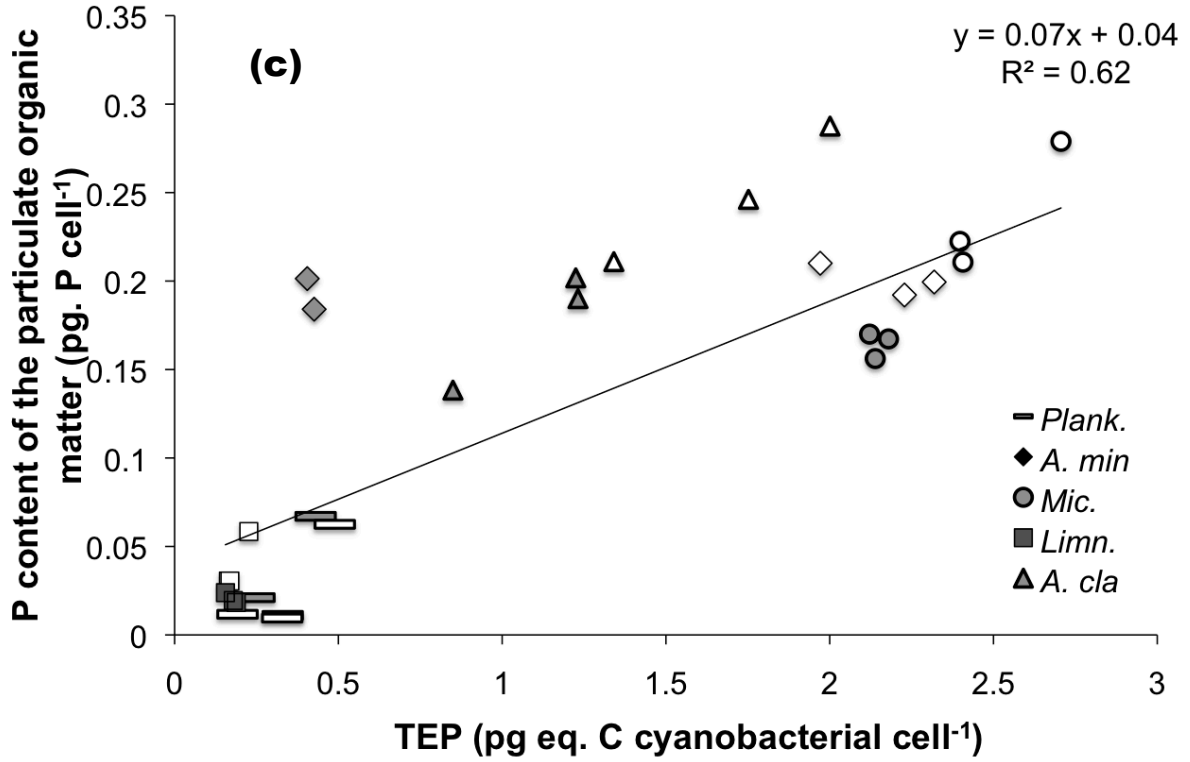


Figure 7: (a) C content, (b) N content and (c) P content per cyanobacterial cell of the particulate organic matter depending on the TEP concentration per cyanobacterial cell, considering the five species. Data correspond to the three replicates measured during the exponential phase (open symbols) and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






(c)
y = 0.07x + 0.04
R² = 0.62
P content of the particulate organic matter (pg. P cell-1)
TEP (pg eq. C cyanobacterial cell-1)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
2.5
3
Plank.
A. min
Mic.
Limn.
A. cla

790
791

Figure 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.

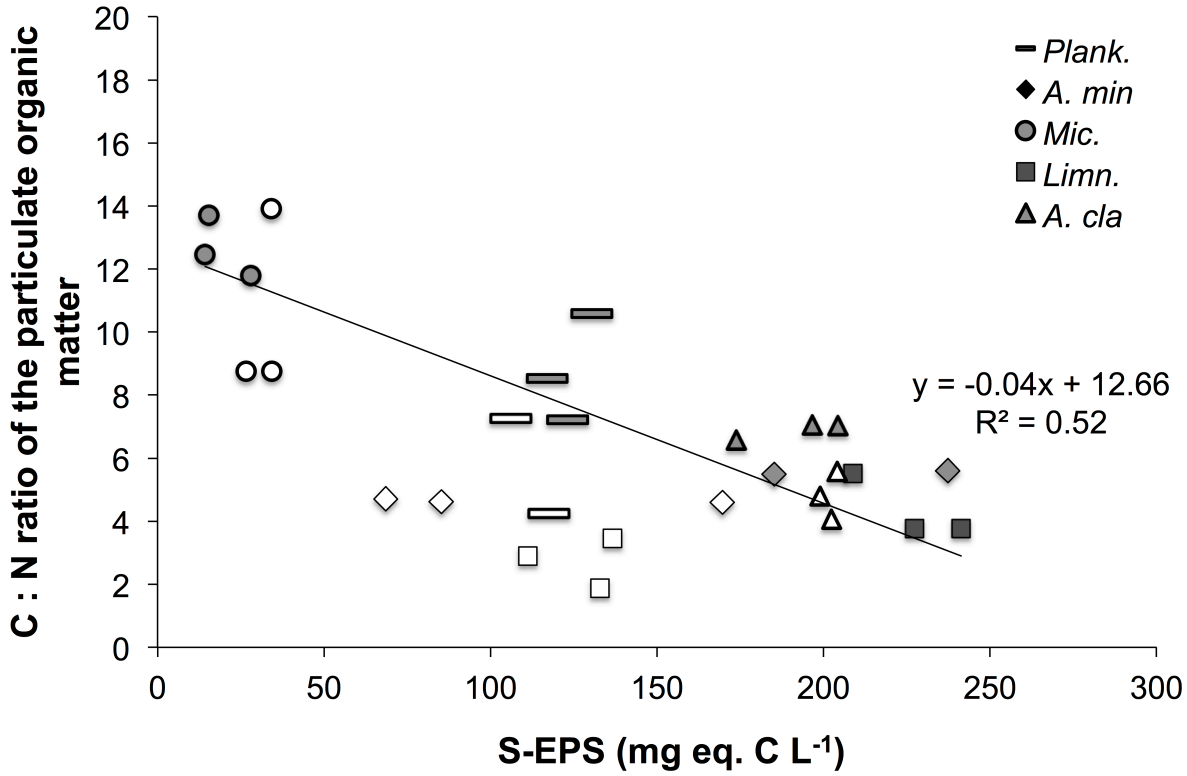
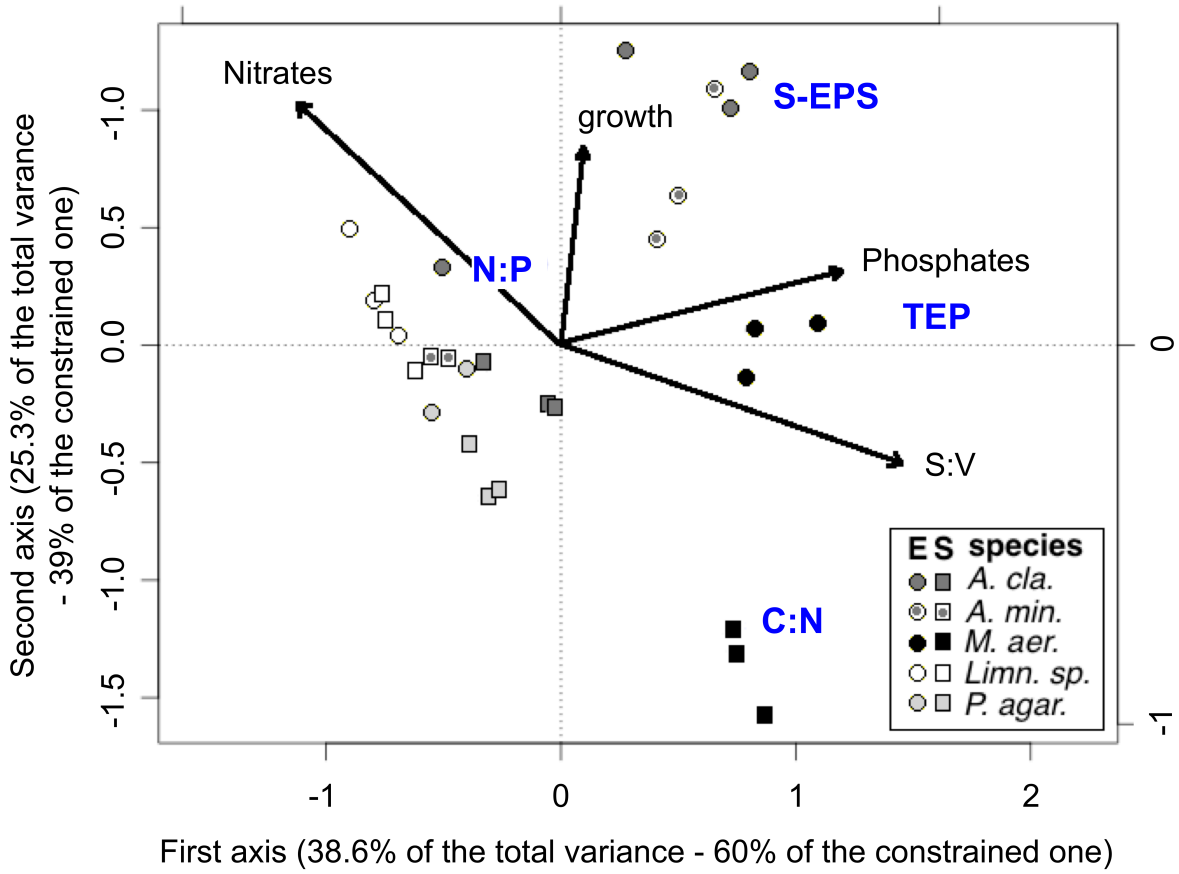


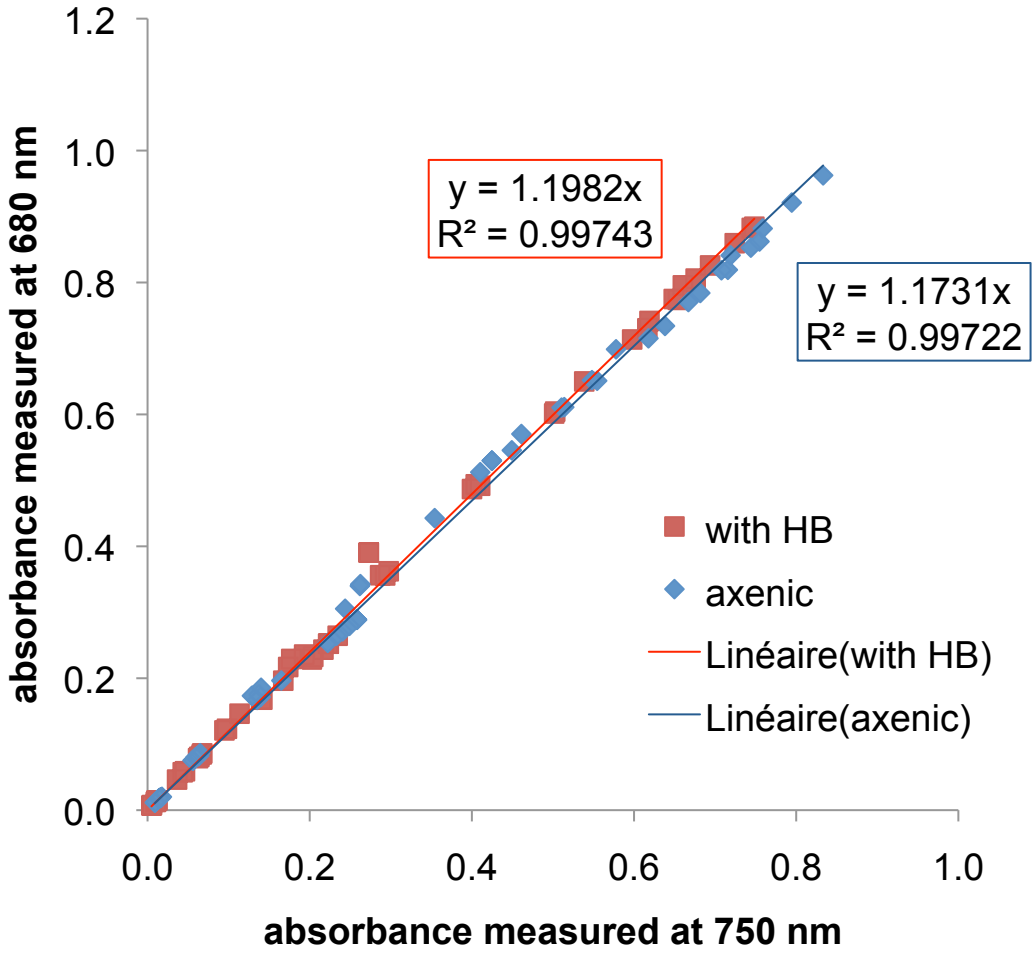
Figure 9: Redundancy analysis (RDA) triplots for the molar C:N and N:P ratios, the TEP and S-EPS per cyanobacterial cell (µg eq C $cell^{-1}$) of the five cyanobacteria, explained by the growth rate, the cell ratio surface:volume of the species, and the nitrates and the phosphates concentrations in the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



801 Supporting Information

802 Additional Supporting Information may be found in the online version of this article:

803 **Fig. S1.** Absorbance measured at 680 nm as a function of the absorbance measured at 750 nm
804 for Microcystis cultures, in axenic condition (blue diamonds) and in the presence of
805 heterotrophic bacteria (red squares).



806

807 **Table S1:** Parameter estimates for the best model predicting the C:P ratio in *M. aeruginosa*,
808 as determined by a stepwise selection of the variables using the AIC criterion. Result from its
809 ANOVA is also shown. Significance levels are coded as follow: '***' indicates <0.001, '**'
810 <0.01, and '*' <0.05.

	Estimate	Std.	Sum of sq	Df	F value	Proba (>F)	
(Intercept)	66.79	65.09					
TEP	61.76	22.30	32482	1	27.94	<0.0001	***
NO3	-2.99	1.13	30351	1	26.11	<0.0001	***
bacteria	-176.4	74.1	16159	1	13.90	0.0017	**
interaction tep x bacteria	69.1	31.5	5605	1	4.82	0,042	*
interaction NO3 x bacteria	-4.18	1.67	7262	1	6.25	0.023	*
Residuals			19761	18			

811