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Towards a carbon budget of the diazotrophic cyanobacterium *Crocosphaera*: effect of irradiance

Sophie Rabouille[1,*], Georges Semedo Cabral[1,2], Maria Luiza Pedrotti[1]

[1]**Sorbonne Universités, UPMC Univ Paris 06, CNRS, UMR7093, Laboratoire d'Océanographie de Villefranche, 181 Chemin du Lazaret, 06230 Villefranche-sur-mer, France**

[2]***Present address:*** **Université de Moncton, 18 rue Antonine-Maillet, Moncton (NB) E1A 3E9, Canada**

ABSTRACT: We examined changes in carbon fluxes in the marine unicellular diazotrophic cyanobacterium (UCYN) *Crocosphaera watsonii* in response to irradiance. We estimated changes in the total organic carbon and nitrogen contents, incorporation of carbon into reserves, and exudation processes of extracellular exopolymeric substances (EPS), as well as in the formation of transparent exopolymeric particles (TEP). Variability of cellular carbon pools and extracellular EPS and TEP under conditions of different irradiance and the significance of carbon release in the environment is discussed. Our results show a relationship between growth rate, metabolic processes of carbon incorporation in cells, exudation and irradiance. When growth is light-limited, both carbon production and exudation increase with light. No inhibition occurs at growth-saturating irradiances and half of the total particulate organic carbon is then found as intracellular carbohydrates. While the overall growth becomes light saturated, carbon fixation is further stimulated by higher irradiances, leading to an increased EPS exudation. We found that EPS exudation in *C. watsonii* is not only a way for cells to release excess carbon, but also occurs when growth is light-limited, even at the lowest irradiance tested. The formation of TEP increases with irradiance, with TEP-carbon amounting to up to 30 % of the total particulate organic carbon. Our data will help to quantify the role of unicellular diazotrophs in the export of both carbon and nitrogen through and below the euphotic zone in the marine environment.

KEY WORDS: Carbon cycle · Exudation · UCYN · EPS · TEP · Primary production

INTRODUCTION

In the open ocean, organic matter originates from the fixation of atmospheric carbon by photoautotrophic organisms. While the elemental composition of bulk organic material is very consistent in the world's oceans, with molar proportions of C:N:P = 106:16:1 (Redfield 1934, Redfield et al. 1963), there is evidence for anomalous surface dissolved inorganic carbon (DIC) drawdown, exceeding the amounts predicted from nitrate availability and the Redfield C:N ratio (Sambrotto et al. 1993). This apparent overconsumption of DIC in regard to nitrogen in phytoplankton cells suggests that organic matter produced during photosynthesis does not combine carbon and nitrogen in Redfield proportions. While the overall biomass production is limited by nitrogen availability, photo-assimilation of inorganic carbon may persist. The exudation of surplus carbon through the production of extracellular exopolymeric substances (EPS) such as dissolved organic material (DOM) and transparent exopolymeric particles (TEP) (Mari et al. 2001) may validate and partly counterbalance the hypothesis of DIC overconsumption.

TEP are one type of EPS that form abiotically from acidic polysaccharides released as dissolved and colloidal matter from phytoplankton and bacteria (Zhou

*Corresponding author: srabouille@obs-vlfr.fr

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et al. 1998, Passow 2000, 2002a). EPS and TEP participate in bloom formation (Passow et al. 1994) and actively take part in the carbon cycle and biogeochemistry in diverse aquatic systems (Alldredge et al. 1993, Passow et al. 2001, Wotton 2004, Bhaskar & Bhosle 2005). However, the DIC overconsumption hypothesis mentioned above was initially formulated based on NO_3^- consumption data. With the recent discovery of new open-ocean and coastal, unicellular diazotrophic cyanobacteria (UCYN) (Zehr et al. 2001, Montoya et al. 2004, Moisander et al. 2010), additional nitrogen sources for phytoplanktonic growth were found that also participate in the consumption of surface DIC. Diazotrophic cyanobacteria thrive over a wide range of latitudes in the oceans (Stal 2009, Moisander et al. 2010, Monteiro et al. 2010), where they contribute to primary production. Owing to their ability to fix N_2, they play a critical biogeochemical role in nitrogen-depleted oceans, typically in (sub)tropical regions, where they can represent a significant fraction of the plankton biomass (Church et al. 2005). Although the reported contribution of diazotrophs to primary production in these areas is often highly variable, the literature points to a substantial share of the world's ocean's primary production supported by nitrogen fixation (Letelier & Karl 1996, Karl et al. 1997, Carpenter et al. 2004). Diazotrophs would thus represent an important trophic link in sustaining the biological pump in oligotrophic oceans.

Diazotrophic organisms also produce EPS and TEP (Webb et al. 2009; Dron et al. 2012a), which represent a carbon loss for cells, but how important is this process, in regard to the maintenance of cell growth? In non-diazotrophic primary producers, nitrogen limitation can enhance TEP production (Mari et al. 2001), although nitrogen limitation is not a prerequisite for carbon exudation since TEP production also occurs in non-limiting conditions of nitrogen (Claquin et al. 2008, Pedrotti et al. 2010). The aim of the present work was to describe carbon fluxes in *Crocosphaera watsonii*, an open-ocean UCYN. Biomass production rates and growth were monitored in duplicate batch cultures grown at a range of light intensities. In order to assess the exudation potential of cells, we tested whether different irradiances resulted in changes in the amount of carbon stored in cells and exuded. The respective cellular carbon pools (including storage carbohydrates and structural carbohydrates, such as sugars contained in glycolipids) as well as extracellular EPS and TEP were compared in regard to the corresponding light treatment. The importance of carbon release into the environment is discussed.

MATERIALS AND METHODS

Experimental setup

Monocultures of *Crocosphaera watsonii* strain WH8501 (Waterbury et al. 1988, Waterbury & Rippka 1989) were grown in acid-cleaned boro-silicate Erlenmeyer flasks. A teflon tube closed with a clamp was used to ensure aseptic sampling during the experiment. YBCII culture medium (Chen et al. 1996) was prepared from aged Mediterranean Sea surface water collected at the permanent 'Point B' station (43° 41′ 10″ N, 7° 19′ 00″ E), filtered through 1 µm Millipore Polygard filters and stored in the dark at room temperature for 1 mo. Before use, the seawater was filtered (0.1 µm Millipore Polygard filters) and diluted with Milli-Q water up to a final salinity of 35.00 PSU. After sterile addition of dissolved macro- and micronutrients, the medium was transferred to each experimental flask.

The seed culture used for these experiments was kept in a growth chamber at a constant temperature of 27°C and exposed to a 12 h light:12 h dark regime at approximately 120 µmol photons m^{-2} s^{-1}. After inoculation, all cultures were placed in the same growth chamber and were manually and gently swirled twice a day for a few minutes, which was enough to stimulate gas exchanges, prevent wall growth and reach efficient growth rates. Cultures were non-axenic but SYBR-Green-stained samples visualized by flow cytometry pointed to a very low contamination level. As reported in Dron et al. (2012b), bacteria were approximately 0.7 µm in diameter and accounted for 0.5% of the total cell abundance, which corresponded to approximately 0.4% of the total carbon and nitrogen in the cultures. Cultures were exposed to 12 different irradiances ranging from 20 to 500 µmol photons m^{-2} s^{-1} applied on one side only to generate a light gradient within the chamber. Irradiance in the chamber was provided by fluorescent tubes (Sanyo FL40SS W/37) and followed a 12 h light:12 h dark cycle. Flasks were positioned in such a way that (1) no self-shading occurred between the flasks and (2) duplicates received the same incident irradiance. The different levels of irradiance were regularly measured using a US-SQS/LI (WALZ) quantum sensor. A neutral density filter was used to cover a few cultures in order to increase the range of the investigated light gradient.

Flasks were inoculated with exponentially growing culture; the initial concentration in each flask was 9.2×10^4 cells ml^{-1}.

One set of 10 flasks (5 duplicate cultures) containing 500 ml culture was used for the biochemical analyses and the growth rate measurements described below. Another set of 7 flasks containing 250 ml culture was only sampled for additional growth rate estimations. The experiment was limited to duplicate cultures to prevent self-shading and ensure a constant incident light intensity throughout the experiment. Once growing exponentially, cultures were sampled for 3 consecutive days for identification of the different pools detailed below. Given the significant changes in physiological parameters such as cell size, carbon and nitrogen content that occur in cells of *C. watsonii* at the hourly scale, samples in each culture were taken in the late light phase, at the same time every day to prevent any bias that would be due to possible diel fluctuations within each culture. Cultures were initiated at low biomass concentration, and the cell density was still low enough at the time of sampling to prevent light limitation.

Cell abundance and particulate organic carbon (POC) and nitrogen (PON)

Cell abundance and average cell volume were monitored on a daily basis, using a Coulter Counter (Beckman, Multisizer 3). Cell size was estimated as equivalent spherical diameter and used to calculate the cell biovolume. Growth rates, estimated from cell counts, did not show any significant deviations during the sampling period, indicating that cells remained in a similar physiological state over the 3-d monitoring of cellular contents.

The total POC and PON were determined with a CHN analyzer (Perkin Elmer, 2400-II). Samples (5.8 ml) were filtered onto precombusted (4 h at 450°C) GF/F filters (Whatman) and dried at 60°C before analysis. Precombusted, empty filters were used as tare. Carbon and nitrogen contents per cell were estimated using the cell abundance at the time of sampling.

Total cellular carbohydrate and cellular carbohydrate pools

Samples for carbohydrate analyses were taken in the late light phase in order to determine the carbon content when it is highest. The different intracellular carbohydrate pools and soluble extracellular EPS (S-EPS) were analyzed according to Dubois et al. (1956) using glucose as a standard. The concentration of EPS in the fresh culture medium was also measured and subtracted from the results. All fractions obtained were quantified separately and at once, using a dedicated standard curve, to avoid any measurement bias. Immediately before analysis of each fraction, a new standard curve was determined using a range of 9 standard glucose solutions from 0 to 200 mg l^{-1}, prepared freshly and in triplicate from a 5 g l^{-1} stock solution. The linear relationship obtained in the standard curve ($R^2 = 0.99$) was used to convert absorbance values into mg equivalents of glucose per sample. Production rates were then estimated from the 3 consecutive samples (48 h span) using the average biomass concentration during that period to account for possible changes in cell concentration during the sampling period.

Duplicate samples of 5.8 ml culture were taken twice, for determination of the total cellular carbohydrate on the one hand, and of the different cellular carbohydrate pools on the other hand according to Dubois et al. (1956). The latter fraction was obtained following the protocol proposed by Dron et al. (2012a). Two analytical replicates were also performed during the analyses. All samples were filtered through 1 µm polycarbonate filters at gentle vacuum (<150 mm of Hg) so as not to disrupt cells, and filters were placed in 15 ml Falcon® tubes (Corning®) and stored at −80°C until analysis. Total cellular carbohydrate and all cellular carbohydrate fractions were then converted into fmol C $cell^{-1}$ according to Pakulski & Benner (1992), using the cell volume measured at the time of sampling and considering that carbon represents 40 % of the mass of glucose.

Extracellular pools: soluble EPS and TEP

S-EPS comprise an extracellular carbohydrate fraction dissolved in water and can be isolated from particulate material by gentle vacuum filtration (<150 mm of Hg) of the culture through a 0.2 µm polycarbonate filter (Benner et al. 1992, Underwood et al. 1995). The filtrate was collected in a 15 ml Falcon® tube and stored at −80°C until analysis according to Dubois et al. (1956).

TEP are acidic, exo-polysaccharides; this fraction was quantified by spectrophotometry according to the dye-binding assay (Passow & Alldredge 1995) using Alcian Blue as a stain. Duplicate samples of 11.6 ml were filtered through a 0.4 µm polycarbonate filter at low vacuum pressure to preserve cells, and the filter was stored in a 15 ml Falcon® tube at −20°C until analysis. Two analytical replicates were also performed during the analyses. TEP values were

expressed as xanthan gum (XG) weight equivalent (µg XG l^{-1}) calculated by means of a calibration curve using 12 concentrations of xanthan gum (in triplicate) according to Claquin et al. (2008). Linear regressions were fitted to the calibration data with a coefficient of determination of R^2 = 0.98. The carbon content of TEP (TEP-C) was estimated from colorimetric determinations (TEP_{color}) as:

$$TEP\text{-}C = 0.75 \times TEP_{color} \quad (1)$$

where TEP-C is given in µg l^{-1} and TEP_{color} in µg XG l^{-1} (Engel & Passow 2001).

A control measurement was performed in the culture medium prior to inoculation to confirm the absence of EPS in the medium.

Statistical analysis

Average concentrations are provided with their related standard error of the mean (SEM). To determine whether the measured POC, PON and carbon pools show significant differences between the light treatments, an ANOVA with one classification criterion was performed, followed by a post hoc Tukey's HSD test. Data normality of the different data sets was verified using the Rankit test (Bliss et al. 1956). The homogeneity of variance was confirmed using Levene's test (Levene 1960).

RESULTS

Growth dynamics

All cultures showed an exponential growth with good reproducibility between replicates. The exponential growth rates (µ) obtained in the different treatments revealed a saturation relationship with irradiance (Fig. 1). An increase in µ was observed in all cultures up to 150 µmol photons m^{-2} s^{-1}, before tapering off around 0.5 d^{-1} at higher irradiances. Fitting the Monod (1942) model to these data (Fig. 1) predicted a theoretical, maximum growth rate μ_{max} = 0.6 d^{-1} and a half-saturation constant for light utilization K_I = 98.8 µmol photons m^{-2} s^{-1}, with a determination coefficient R^2 = 0.95.

Cultures processed for biomass quantifications were grown at irradiances from 50 to 350 µmol photons m^{-2} s^{-1}. Due to the large number of samples taken for biochemical analyses, not all cultures could be sampled during the exact few days of their respective highest growth rate. In particular, growth had reached a plateau when the set of cultures exposed to the highest irradiance (350 µmol photons m^{-2} s^{-1}) was sampled. Culture replicates showed consistent behavior and results for each light treatment are presented as average values between replicates. When exposed to higher light intensity, cells became larger. The average cell biovolume recorded in each culture over the 3-d sampling period increased with irradiance from 10.71 μm^3 in cultures grown at 50 µmol photons m^{-2} s^{-1} to 13.25 μm^3 in those grown at 350 µmol photons m^{-2} s^{-1}, representing a 24 % biovolume gain (Fig. 1).

Total C and N contents and cellular carbohydrates pools

When expressed per cell, there was a significant linear increase in POC and PON along the range of irradiance up to 275 µmol photons m^{-2} s^{-1} (Fig. 2). POC increased from 264.7 ± 13.8 (SEM) fmol C $cell^{-1}$ at 50 µmol photons m^{-2} s^{-1} to 443.6 ± 12.8 fmol C $cell^{-1}$

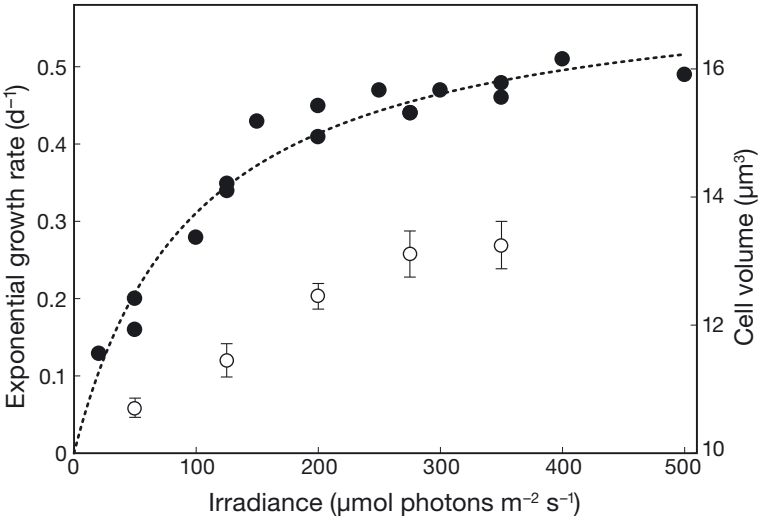

Fig. 1. Exponential growth rate µ (d^{-1}) observed in cultures of *Crocosphaera watsonii* as a function of irradiance (closed circles; left axis) and average (±SEM) cell biovolume (open circles; μm^3, right axis). Note the scale on the secondary *y*-axis. A Monod relationship (dotted line) was fitted to the growth rate data, which predicts a maximum growth rate μ_{max} of 0.6 d^{-1} and a half saturation constant K_I = 98.8 µmol photons m^{-2} s^{-1}. The quality of the fit was assessed by estimating the determination coefficient of this non-linear relationship: $R^2 = 1-(SS_e/SS_t)$, where SS_e is the sum of the square differences between data *y* and model estimations $\hat{y}$: $SS_e = \sum(y-\hat{y})^2$ and SS_t is the sum of the squares of the distances between data *y* and their average $\bar{y}$: $SS_t = \sum(y-\bar{y})^2$

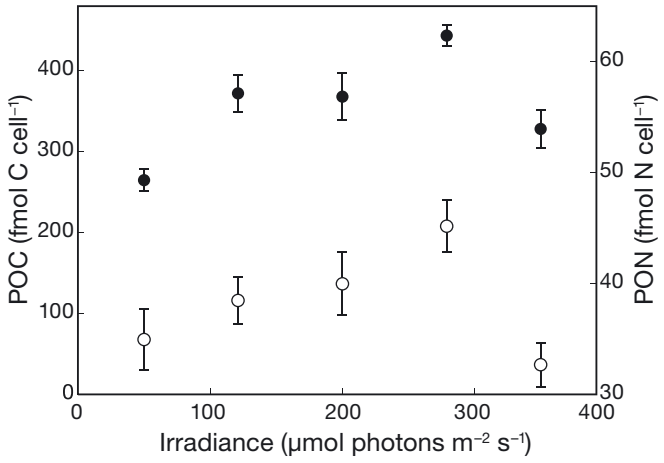

Fig. 2. Average total particulate organic carbon (POC; closed circles; fmol C cell^{-1}, left axis) and nitrogen (PON; open circles; fmol N cell^{-1}, right axis) per cell in cultures exposed to increasing irradiances. Each data point is the average of 6 time points (samples taken for 3 consecutive days in duplicate cultures). Error bars are ±SEM (n = 6). A linear regression is observed over the first 4 irradiances for both POC and PON, whose respective determination coefficients (R^2) are 0.87 and 0.96

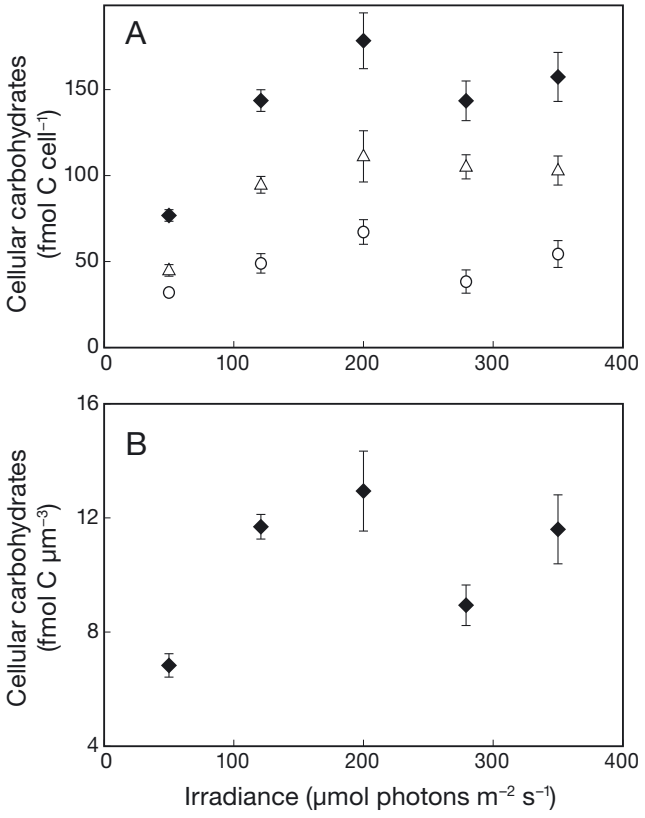

Fig. 3. Average reserve (open circles), structure (open triangles) and total (reserve + structure; diamonds) carbohydrate content in cultures grown at different light intensities, expressed (A) per cell (fmol C cell^{-1}) and (B) per biovolume (fmol C µm^{-3}). Error bars are ±SEM (n = 6)

at 275 µmol photons m^{-2} s^{-1} (R^2 = 0.87; $p < 0.01$), while PON evolved from 34.9 ± 2.8 to 45.2 ± 2.3 fmol N cell^{-1} (R^2 = 0.95; $p < 0.01$). Cultures grown at 350 µmol photons m^{-2} s^{-1} were an exception and stand out from the increasing trend, with a smaller cell volume and cellular content.

The discrimination and quantification of internal carbon pools, carried out in parallel, indicate a carbohydrate buildup in cells with increased irradiances up to 200 µmol photons m^{-2} s^{-1}. Fig. 3A represents cellular contents in structure, reserve and total (i.e. structural + reserve) carbohydrates; both reserve (R^2 = 0.99) and structure (R^2 = 0.92) pools showed a saturation relationship with irradiance. Reserve carbohydrates doubled from an average of 32 fmol C cell^{-1} in cultures exposed to 50 µmol photons m^{-2} s^{-1}, to 67 fmol C cell^{-1} at 200 µmol photons m^{-2} s^{-1}. Structural carbohydrates also showed a near 2-fold increase, from 17 fmol C cell^{-1} to 31 fmol C cell^{-1} in the same range of irradiances (Fig. 3A). Accumulation of cellular reserve and structure carbohydrates saturated above 200 µmol photons m^{-2} s^{-1} and cultures even showed sub-maximal contents. Total carbon (POC) expressed per biovolume did not change significantly with irradiance. On the contrary, carbohydrate contents, whether expressed per biovolume or per carbon unit, did increase with irradiance up to 200 µmol photons m^{-2} s^{-1} ($p < 0.01$), pointing to a reserve accumulation in cells. This increase in carbohydrate storage saturated beyond 200 µmol photons m^{-2} s^{-1} (Fig. 3B). These results indicate that carbon accumulation is not to be related to the increase in cell size only; at higher light levels there is an increase in total carbon with larger proportions of POC found as carbohydrates: 50% at 200 µmol photons m^{-2} s^{-1} versus 29% at 50 µmol photons m^{-2} s^{-1}.

EPS and TEP production rates

Production rates can be reasonably estimated from 2 closely related time points, between which little change in biomass can be assumed. In the present study, the time interval between 2 samples is 1 d and production rates were calculated as follows:

$$\text{Production rate} = \left(\text{Pool}_{t+\delta t} - \text{Pool}_t\right) / \left(\overline{B} \times \delta t\right) \quad (2)$$

where $\text{Pool}_{t+\delta t} - \text{Pool}_t$ is the concentration of the element formed or consumed during the time interval δt, expressed as fmol C l^{-1}, and $\overline{B}$ is the average biomass concentration during the same time interval: $\overline{B} = 0.5 \times (B_{t+\delta t} + B_t)$, expressed as cells l^{-1}.

At the time of sampling, the highest absolute EPS concentration expressed relative to cell abundance

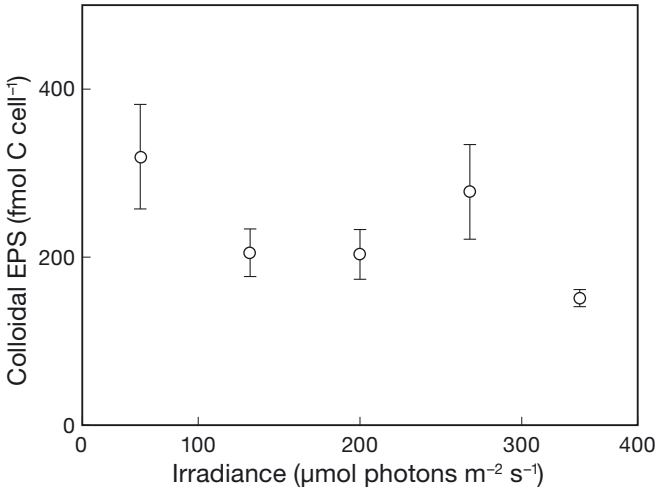

Fig. 4. Average colloidal extracellular exopolymeric substance (EPS) concentration (fmol C cell^{-1}) in cultures exposed to different light intensities, and expressed relative to cell abundance. Error bars are ±SEM (n = 6)

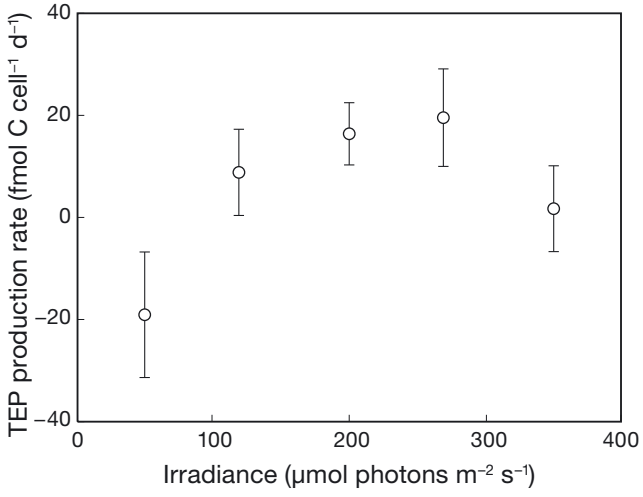

Fig. 5. Average net transparent exopolymeric particle (TEP) production rate in duplicate cultures of *C. watsonii* exposed to different irradiances. Error bars are ±SEM (n = 6).

(319.6 ± 62.2 fmol C cell^{-1}) was observed in cultures exposed to the lowest light intensity, and the lowest EPS concentration (151.2 ± 10.2 fmol C cell^{-1}) was observed in cultures exposed to the highest light intensity. The cultures grown at intermediate irradiances showed an overall decreasing trend in EPS concentration (Fig. 4). Average EPS production rates, calculated over the 3 d of sampling, yielded mostly negative values, indicative of a disappearance of EPS rather than a production. Rates ranged from −25 fmol C cell^{-1} d^{-1} at 50 µmol photons m^{-2} s^{-1} to 4.22 fmol C cell^{-1} d^{-1} at 350 µmol photons m^{-2} s^{-1}.

Although an abiotic process, TEP formation is affected by cell exudation and so is likely to depend on cell density. Therefore, we normalized the concentration of TEP relative to cell abundance, which also allows for comparison with other carbon pools. Importantly, normalization by cell abundance did not affect the dynamics described in the 'Discussion', which remained true when considering absolute concentrations per volume (mg C l^{-1} for instance). TEP were detected in all cultures and their concentration varied from 77 fmol C cell^{-1} at 50 µmol photons m^{-2} s^{-1} to 123.5 fmol C cell^{-1} at 275 µmol photons m^{-2} s^{-1}, thus representing a 60% increase. Accordingly, the net TEP production rate increased with light intensity, although not linearly (Fig. 5). The order of magnitude of this process was similar to that of EPS production, with a maximum rate of 19.5 ± 9.5 fmol C cell^{-1} d^{-1} at 275 µmol photons m^{-2} s^{-1}. However, an exception is observed in cultures exposed to 350 µmol photons m^{-2} s^{-1}, which stand apart from the trend and show a lower production rate.

DISCUSSION

Among the different lineages of diazotrophs, unicellular cyanobacteria have been given increasing attention in the last 2 decades (Zehr et al. 2000). These include UCYN-B, a group of phototrophic cyanobacteria, whose cultured representatives are strains from the genus *Crocosphaera* (Zehr et al. 2001), which are widely distributed in the world's (sub)tropical oceans (Church et al. 2005, Hewson et al. 2007, Zehr et al. 2007).

The ecophysiology of *Crocosphaera* still shows a number of puzzling features. For instance, the cell cycle (Dron et al. 2013) as well as diel cycles of gene expression (Mohr et al. 2010, Shi et al. 2010) tightly control the different metabolic processes, which participate in maintaining a stable elemental stoichiometry at the daily scale (Dron et al. 2012b). But there is evidence for discrepancies between the net metabolic rates, deduced from the net growth rate, and actual activities of carbon and nitrogen acquisition, that seem to largely exceed the metabolic costs for these activities. For instance, gross nitrogen fixation in *Crocosphaera watsonii* WH8501 would exceed up to 3-fold the nitrogen requirements for growth under conditions of exponential, balanced growth (Dron et al. 2012b). Such luxury growth dynamics associated with a release of compounds into the environment has also been observed in a number of other phototrophic diazotrophs, such as *Cyanothece* BG 43511, which ex-

udes up to 89% of its recently fixed nitrogen (Agawin et al. 2007), or *Trichodesmium*, reported to release 50 to 90% of the fixed nitrogen (Capone et al. 1994, Glibert & Bronk 1994, Mulholland & Bernhardt 2005).

Carbon exudation by phytoplanktonic or cyanobacterial cells occurs through the release of EPS, which promotes the formation of TEP. These have been related to the photosynthetic rate (Mague et al. 1980, Claquin et al. 2008) or the growth rate (Waite et al. 1995). While the ability of diazotrophs to exude carbon compounds is documented in the literature (Berman-Frank et al. 2007, Webb et al. 2009, Sohm et al. 2011, Dron et al. 2012a), it is still unclear how significant these fluxes are for the biogeochemistry of their environment. In unicellular strains, such as *C. watsonii*, one could wonder whether the energy-demanding diazotrophic growth would permit significant carbon losses. In the following text, we show that it actually does.

Irradiance–growth relationship

The growth kinetics data reveal a saturation in the growth response to irradiance, accurately fitted using the Monod (1942) model, with a saturation threshold around 200 µmol photons m^{-2} s^{-1} (Fig. 1). The absence of any reduced growth rate at the highest irradiances could suggest that none of the light levels applied here were inhibiting. However, a markedly reduced POC and PON content (Fig. 2), as well as a reduced EPS concentration and TEP production (Figs. 4, 5), at the highest irradiance may instead reflect the initiation of photoinhibition. We believe that the absolute threshold value for light saturation observed here is not an unalterable, physiological feature of *C. watsonii* but rather reflects the long-term light-acclimation state of this strain at the time at which this experiment was conducted. This strain features a certain resistance to strong lights (Rabouille & Claquin 2016). It is likely that, had cultures been pre-acclimated at higher irradiances, the saturation relationship would have remained, but with a threshold possibly shifted towards higher light levels (e.g. see Mortain-Bertrand et al. 1988). In the following subsections, we show that such a saturation relationship also characterizes the different processes of carbon and nitrogen acquisition.

All samples for biochemical analyses were taken in the second half of the light period. As expected, the molar C:N ratios exceeded the Redfield value, indicating that cells were accumulating carbon in all cultures. C:N ranged from 7.45 and 7.72 at the lowest irradiance to 9.87 and 10.21 at the highest irradiance. This range consistently brackets values previously reported for this strain: when considering the same time window, this ratio varied from 8.5 to 9.5 in continuous cultures of *C. watsonii* grown in similar conditions and exposed to a bell shape, 12 h light:12 h dark regime with a maximum of 130 µmol photons m^{-2} s^{-1} (Dron et al. 2012b).

Light-limited growth processes

At increasing sub-saturating irradiances, cells not only grow faster, they are bigger (Fig. 1) and contain more cellular compounds (Fig. 2). POC per cell increased by 68% between 50 and 275 µmol photons m^{-2} s^{-1}. This trend is mirrored in the PON cell content, pointing to a light stimulation of nitrogen fixation, but with a lower rate of change (29% increase). As a result, a carbon surplus accumulates in cells, shown by the increase in the C:N ratio with irradiance. POC accumulation reflects the dynamics of the cellular carbon pools; when exposed to higher irradiances, cells build up more structural carbon but they also increase their storage reserves (Fig. 3). The proportion of reserve carbohydrates increases from 12.2% of POC at 50 µmol photons m^{-2} s^{-1} to 19% at 200 µmol photons m^{-2} s^{-1}. Altogether, cellular carbohydrates then account for 29% (at 50 µmol photons m^{-2} s^{-1}) to 50% (at 200 µmol photons m^{-2} s^{-1}) of the total POC in the cultures. The present data assert that the commonly observed, widely fluctuating pools of cellular carbohydrates are a signature in cyanobacteria and an important component of their ecology, whether in diazotrophic strains (Schneegurt et al. 1994, Rabouille et al. 2006, Dron et al. 2012b) or non-diazotrophic strains, such as *Microcystis* sp. (Rabouille & Salencon 2005, Rabouille et al. 2005).

The absolute concentration of any element or pool results from processes of production and losses (such as possible degradation, consumption or transformation) that occur between the inoculation and the moment of sampling. At any time, the instantaneous net production rate is directly dependent on the biomass concentration as well as on the growth status of cells. Therefore, for a relevant production rate to be estimated, it is critical to relate the measured compound concentrations to the actual cell abundance responsible for production or loss. Following the stimulation of POC production by light in *C. watsonii*, one might reasonably expect that carbon exudation increases as well, as already reported for several non-diazotrophic phytoplankton strains (Zlotnik & Dubinski 1989). The fast transient dynamics of the

EPS pool, together with the negative values of EPS production rates observed up to 200 µmol photons m^{-2} s^{-1}, point to a net decrease in the EPS fraction, even though the rate of EPS production appears to increase with the irradiance. In parallel, absolute concentrations measured in the cultures indicate that the residual EPS is inversely related to the irradiance level (Fig. 4). These 2 observations suggest that the application of higher light levels triggered increases in EPS production, while their aggregation into TEP (see below) is responsible for an even higher decrease in the concentration of EPS. Overall, results point to fast dynamics of the extracellular carbon pools, whose kinetics prove non-trivial to describe. The actual dynamics of the EPS pool may not be seen at time scales longer than those of metabolic processes. It is also still unclear whether EPS exudation is a continuous process or, on the contrary, whether EPS are actively released only at certain phases of the growth cycle. We wonder, for instance, to what extent the exudation process is correlated with the cell division cycle, with increased exudation following carbon incorporation (a possible consequence of excess carbon incorporation) and/or decreased exudation during the cell division phase (when cells need cellular material to make new daughter cells). Fluorescent probes would probably help to further characterize this phenomenon. Comparison of Figs. 3 and 4 indicates that, although exuded EPS are rapidly removed from the dissolved pool, the remaining EPS concentration in cultures is of the same order of magnitude as the total carbohydrates in cells (and even 4 times more at the lowest irradiance), demonstrating that, in exponentially growing cultures, a significant fraction of carbon incorporated daily through photosynthesis is actually released into the environment. We believe that EPS exudation is much more than just an outlet for excess fixed carbon. The magnitude of EPS exudation, especially at low irradiances, is strong evidence of its constitutive character: carbon release would be part of the growth process, whether it is light limited or light saturated. Nutrients and cell exudates trapped in the EPS matrix (Flemming & Wingender 2001) remain available to cells in the colony. In the natural environment, such a constantly enriched, extracellular matrix would even constitute a possible attachment surface and substrate for symbiotic consortia to set up.

Despite fluctuating production rates between consecutive days, the averaged TEP production rates showed reproducibility between duplicates, and the data showed evidence of a very clear, positive stimulation of TEP production by increasing irradiances (Fig. 5). We believe the culture experiment did not introduce artifacts in TEP formation, as a similar relationship between incident irradiance and TEP formation was also reported in natural phytoplankton assemblages (Hong et al. 1997). The formation of TEP per se is an abiotic process; therefore, it is most certainly not directly affected by the ambient light intensity, but instead depends on the amount of dissolved and colloidal material in the medium. Following the observations of Passow (2000), data suggest that the accumulation of EPS in the present cultures concomitantly stimulated the formation of TEP. The present data suggest that the more EPS are exuded, the more they collate into TEP, and the lesser EPS remain (as observed in cultures exposed to higher irradiances). The non-linearity in the relationship between irradiance and TEP production is further caused by the fact that TEP production also relies on turbulence (Klein et al. 2011), as well as on bacterial degradation and grazing (Mari & Burd 1998, Penna et al. 1999, Kiorbøe 2000, Garcia et al. 2002), although the exact role of these processes in TEP formation in the natural environment may still be debated.

Light-saturated growth processes

At the highest irradiances investigated, cultures showed a light-saturated growth with no further accumulation of cellular compounds (Fig. 3). Cells most probably reached their maximal average size. Given the saturation trend in the growth response depicted in Fig. 1, we expected the different cellular parameters to reach a plateau in cultures exposed to irradiances above 200 µmol photons m^{-2} s^{-1}. Yet, at 275 µmol photons m^{-2} s^{-1}, the cell biovolume, POC and PON kept increasing even though the intracellular carbohydrates pools showed the expected saturation. Considering that GF/F filters can actually collect some TEP (Engel & Passow 2001) and given the much higher concentration of TEP observed in these cultures, we suspect that the presence of TEP could be the reason for the high POC measured. Importantly, we bring up again some methodological issues that may not have resounded through the literature: Moran et al. (1999) showed that when assessing primary production, the measured ^{14}C incorporation rates vary depending on whether a GF/F or polycarbonate filter is used to collect the biomass. They point to higher carbon incorporation on GF/F filters, which they attribute to the retention of labelled DOC on the filter. Given the risk of DOC adsorption on GF/F filters, we acknowledge that there is scope for improve-

ment in the present experimental protocol; yet, our protocol rigorously follows methods used in the literature, which was required to make our study comparable with all the data published so far. Our results also remain valid when comparing the magnitude of exudation between treatments, which was the main purpose of the present work.

The POC, PON and carbohydrates contents measured in cultures exposed to 350 µmol photons m^{-2} s^{-1} are lower than expected in regard to the saturation relationship. Cells also appear smaller. We believe these measurements are reliable because the analyses, performed on distinct pools in different samples from 2 cultures, show congruent results. Sampling was done in the late growth phase in these cultures, which then showed a reduced growth rate; these actually showed an altered dynamic. In our experiments, we explored the exponential growth capacity and not the behavior of likely pre-senescent cells. Because they are not representative of the exponential growth dynamics expected at this light level, these cultures are not further considered in this study. From a methodological point of view, this observation stresses how much a correct analysis of the growth dynamics in response to environmental conditions is bound to an accurate comprehension of the growth status of cells at the time of sampling.

As cell concentration varies exponentially in time, the inaccuracy of estimated production increases with the time interval. In our study, we followed cell dynamics on a daily basis in our cultures, and pool concentrations were measured over 3 d to derive 2 consecutive production rates over the period of sampling, with a 24 h interval. In 2 cultures growing at 0.5 and 0.15 d^{-1}, cells will on average take 1.4 and 4.6 d to divide, respectively. The estimated production rates will be strongly affected by the occurrence, or not, of cell division within the considered time interval. This fact at least partly explains why our cultures showed fluctuating net EPS production rates from day to day. Bacterial contamination in the cultures could be a partial sink for released EPS, but due to the extremely low contamination observed in our cultures, the related consumption is considered negligible. Importantly, because EPS participate in the formation of TEP, the measured EPS production is a net apparent rate, which does not reflect the actual gross EPS exudation, should EPS collate into TEP at a fast rate.

Wotton (2004) suggested that EPS production was a way for cells to release excess carbon. If EPS exudation was exclusively consequent to an over-accumulation of carbon, then such release should only be observed once cell growth becomes light saturated and cells have reached their maximum storage capacity. Instead, the present results evidence a sustained increase in EPS exudation following a stimulation of photosynthesis by increasing irradiance. When the growth rate is light saturated, then releasing EPS does appear as a way out for excess carbon, which, as discussed by Otero & Vincenzini (2004), compensates for excess reducing power when carbon and nitrogen metabolism become too unbalanced.

Probable impacts in the natural environment

We expect the permanent release of EPS and formation of TEP under different light conditions by diazotrophic cells to represent a steady supply to other communities in the natural environment. Such provision of organic molecules will benefit bacteria and the microbial carbon pump. Given the possible mixotrophy in a number of phytoplankton groups, it is likely that blooms of diazotrophs also create conducive conditions for the immediate development of phototrophic communities in oligotrophic areas. Released as dissolved material, EPS may remain as such and be used locally. However, the present data suggest that a significant fraction actually enters the particulate pool when aggregating into TEP, where they add to the export flux.

TEP formation is related to carbon overproduction by cells. TEP are carbon-rich exopolymers, composed of various polysaccharides that can aggregate nitrogen molecules such as amino acids, even though they have a C:N ratio above Redfield (Engel & Passow 2001). As emphasized by Passow (2002b), the sedimentation of carbon-rich TEP is thought to decouple the cycles of carbon and nitrogen in the marine environment. But in blooms of diazotrophs, the potentially high nitrogen excretion rates would move the TEP composition closer to the Redfield balance. As a consequence, sedimentation processes following the development of blooms of nitrogen fixers can represent an export of both carbon and nitrogen towards deeper layers, if these have not benefitted other communities within the euphotic zone.

CONCLUSIONS

In line with observations in the eukaryote *Emiliania huxleyi* (Engel et al. 2004), we found that carbon exudation as EPS in the prokaryote *C. watsonii* WH8501 is constitutive of growth and increases with the rate of carbon acquisition. While carbon storage

and cell growth rate saturate at high light with up to 50% of the POC found as intracellular carbohydrates, both EPS exudation rates and the subsequent formation of TEP further increase with irradiance. Exudation is not an outlet for carbon fixed in excess but instead takes part in the overall growth strategy of this organism. As a consequence, diazotrophic cyanobacteria show a much wider impact on the environment than solely a stimulation of primary production. Their gross activities generate direct fluxes of carbon and nitrogen into their environment, which, when contributing to the dissolved compartment, are excluded from primary production estimates. Our results highlight a significant exudation capacity in *C. watsonii*, with EPS concentrations amounting to 1- to 2-fold the cellular carbohydrates and TEP representing up to 30% of the biomass standing stock (expressed as carbon), illustrating the impact of these organisms on the carbon cycle in the current upper ocean. We believe that UCYN both stimulate the bacterial pump and exert a non-negligible contribution to export in oligotrophic oceans.

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