Effect of P depletion on the functional pools of diatom carbohydrates, and their utilization by bacterial communities

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Abstract :

Phosphorus (P) limitation of phytoplankton growth is known to affect the accumulation and release of carbohydrates (CHO) by micro-algae. However, relatively little is known about the fate of algal exudates, notably their bacterial degradation. The CHO chemical characterization is also not exhaustive, especially in 'functional' pools relevant for phytoplankton physiology (particulate reserve [R] or structural [S] CHO) and for bacterial degradation (dissolved mono- [MDCHO] and polysaccharides [P-DCHO]). In this study, we investigated how P depletion and repletion affect the CHO composition in diatom *Thalassiosira weissflogii* cultures, and the shortterm response of free and diatom-attached bacteria in terms of abundance and potential β glucosidase activity (β Glc). The bacterial inoculum was composed of the bacterial consortiums of diatom precultures and a natural bacterial community from the Bay of Brest. P depletion favored CHO accumulation in diatom cells, mainly as R i.e. soluble CHO accumulated in cytoplasm, but also as S, polysaccharides linked to the cell wall. The R:S ratio was high in the present diatom cultures. The high M-DCHO observed in P-deplete cultures (twice that of P-replete cultures) when P-DCHO remained quite similar is explained both by active polysaccharide hydrolysis (very high potential β Glc of attached bacteria) and reduced uptake of M-DCHO by Pdepleted bacteria. P depletion of heterotrophic bacteria favors labile CHO accumulation, which may affect particle potential aggregation. However, the remarkably constant M-DCHO concentration over time for both conditions suggests tight coupling between phytoplankton accumulation, release, polymer hydrolysis and monomer uptake by bacteria.

Keywords : P limitation, Phytoplankton, Diatom, Bacteria, Mineralization, Carbohydrate, Glucosidase

1. Introduction

Carbohydrates (CHO) are important constituents of living matter, especially in phytoplankton where cellular carbohydrates can account for up to 90% of organic dry matter (Mykkestad 1974). The production and excretion of CHO by phytoplankton have received significant attention in the past two decades as excreted CHO strongly influence particle aggregation and ultimately the export of organic material towards the deep sea (Mari et al. 2017). The nutritional condition for micro-algal growth is a key factor in the cell CHO accumulation. Nitrogen (N) and Phosphorus (P) limitations are known to favor the production and excretion of CHO by diatoms (Mykkestad 1995, Urbani et al. 2005, Suroy et al. 2015). However, the effect of P limitation seems to be higher than that of N limitation and this effect could increase with the degree of P depletion (Guerrini et al. 1998). The increasing N:P stoichiometry in aquatic ecosystems, in coastal ecosystem due to agricultural practices (Grizzetti et al. 2012, Penuelas et al. 2013) but also in oligotrophic ecosystems (Karl et al. 2001, Lomas et al. 2010), stresses the need for a better understanding of the effect of P limitation on phytoplankton dynamics.

While the physiological responses of phytoplankton species to nutritional condition has been largely studied, little is known about the fate of excreted phytoplankton CHO. The fate of algal CHO is linked to the bacterial degradation of CHO polymers into monomers. The polysaccharides excreted by algae might actually stimulate the bacterial hydrolysis into monosaccharides, the subsequent uptake of monosaccharides by bacteria and, consequently, bacterial growth (Middleboe et al. 1995). Bacteria can also act as competitors at low phosphate (PO_4) concentrations, which can increase algal P depletion

and their subsequent polysaccharide accumulation (Guerrini et al. 1998). Bacteria may themselves be P limited (Cotner et al. 1997, Thingstad et al. 1998), which slows their growth and, consequently, the hydrolysis of organic matter.

These bacterial responses depend on the bacterial physiological state and on the chemical composition of carbohydrates, which has been relatively unexplored (Le Costaouëc et al. 2017). Carbohydrates have usually been measured as total dissolved CHO and/or total particulate CHO, without further fractionating between functionally different CHO pools. However, particulate CHO include structural (associated with cell wall) and reserve (intracellular) polysaccharides (Penna et al. 1999, Staats et al. 2000). Structural carbohydrates in diatoms are composed of acidic polysaccharides, often in the form of sulphated glucuronomannans (Ford & Percival, 1965), and of heteropolysaccharides (Handa 1969, Hecky et al. 1973). Reserve polysaccharides are mainly glucose polymers (Biersmith & Benner 1998). The most common is chrysolaminarin, a soluble β -1,3-glucan with β -1,6-linked glucose branches (Mykkestad & Haug 1972, Obata et al. 2013). β -1,3-glucan can account for up to 80 % of cellular organic carbon in diatoms (Granum et al. 2002). When the monosaccharide composition of dissolved CHO in P replete and P deplete diatom cultures was detailed, glucose was found to be the dominant monomer (Urbani et al. 2005). Galactose, mannose, xylose and rhamnose are lower contributors (Aluwihare & Repeta 1999). According to species, their contribution could increase in limiting P conditions (Magaletti et al. 2004, Urbani et al. 2005). High turnover rates of dissolved aldoses, notably glucose, found in natural diatom populations (Hama & Yanagi 2001) and in *Thalassiosira weissflogii* cultures (Suroy et al. 2015) and the decreasing contribution of glucose in particulate matter polysaccharides during sinking (Handa &

Tominaga 1969), suggest that glucose and its polymers are bioreactive compounds. Heteropolysaccharides are more resistant to biological degradation than glucan. Thus, reserve polysaccharides and dissolved glucans constitute bioavailable CHO pools. They are degraded by glycoside hydrolases (EC 3.2.1.-), which are endo- and exo-enzymes capable of hydrolyzing the glycosidic bond between two carbohydrate moieties. β -glucosidases (EC 3.2.1.21) are broad-specificity enzymes catalyzing the hydrolysis of terminal β -linked (β -1-2; β -1-3; β -1-4; β -1-6) disaccharides of glucose (Chrost 1989). Since glucans are essentially composed of β -1,3- and β -1,6-linked glucoses, β -glucosidases must be largely used in their bacterial degradation.

The present work investigates how P limitation affects the physiological responses of phytoplankton cells, i.e. *Thalassiosira weissflogii*, a model diatom, with a particular attention to CHO composition and their utilization by bacteria. Particulate CHO were separated into structural (S) and reserve (R) carbohydrates, and dissolved CHO into mono- (M-DCHO) and polysaccharides (P-DCHO). This fractioning enables to discriminate between constitutive (S) and reactive pools (R), and between β -glucosidase substrates (P-DCHO) and hydrolysis products (M-DCHO). Since transparent exopolymer particles (TEP) are usually involved in the issue of particle aggregation, the TEP concentration was also evaluated. In parallel, the short-term response of free (0.2 - 3 μ m) and particle-attached (> 3 μ m) bacteria was evaluated, in terms of biomass and potential β Glc. In the case of *T. weissflogii*, its chrysolaminarin was isolated, characterized and found to have a low degree of polymerization and no β -1,6 branches, which makes it an easily accessible substrate for β -glucosidases (Storseth et al. 2005).

2. Materials and methods

2.1. Culture conditions

Precultures

Non-axenic batch cultures of the diatom *Thalassiosira weissflogii* (AC813, Bay of Veys, France) were grown in f/2 medium (Guillard & Ryther 1962) in filtered (<0.2 μm) seawater from the Bay of Brest (salinity of 34). Prior to the experiment, cultures were preconditioned in media differing only in the initial PO_4 concentration. The P replete condition contained 18 μM PO_4 with a N:P molar ratio of 18, and the P limiting condition contained 4 μM PO_4 and a N:P molar ratio of 80 (Fig. 1). For both media, nitrate + nitrite and silicate final concentrations were 320 μM and 250 μM , respectively (a N:Si molar ratio of 1.3), and micronutrient concentrations reached f/2 conditions. Cells were grown for 4 days before being harvested, which corresponded to the mid- and end of exponential growth phase for P replete and P limiting precultures, respectively.

Cultures

Seawater was collected in the Bay of Brest (salinity : 34). Initial phosphate, nitrate + nitrite and silicate concentrations in the collected seawater were 0.5, 13.1 and 8.7 μM , respectively. Within a few hours after collection, seawater was gently prefiltered through 0.8 μm in order to preserve the natural bacterial community whilst removing predators and phytoplankton competitors. After filtration, seawater was distributed among six flasks (10 l) and enriched with two media (triplicates for each media) in order to have two different P treatments (Fig. 1): the same P replete condition as in preconditioning (18 μM PO_4) and a P deplete condition (no PO_4 addition). The latter condition creates a gradient of P depletion in time. The two media contained the same concentrations of nitrate +

nitrite (320 μM) and silicate (245 μM). For each P treatment, batches were inoculated with the respective precultures of *T. weissflogii* with an initial concentration of 11700 cells ml^{-1} for both conditions (9-11 % of inoculum volume). The bacterial inoculum was $2.5\text{-}2.8 \times 10^6$ cells ml^{-1} , which is realistic for experiments free of bacterial predators. The bacterial consortium of the experiment was composed both of the natural bacterial community of the Bay of Brest and the bacterial community associated with the diatom precultures. By comparing P deplete to P replete conditions, this experimental set up allows to study the effect of P depletion on the carbohydrate dynamics (micro-algal CHO production, excretion and bacterial degradation). Batches were inoculated in the evening (at 6 pm), preceding the first subsampling (at 10 am the next day, 16 h after). Cultures were grown at 18°C, with a photon irradiance of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in a 12:12 light/dark (L:D) cycle and a constant bubble flow of 0.2 μm filtered air. Incident light was measured with a scalar photosynthetically active radiation (PAR) irradiance sensor (QSL-100, Biospherical Instruments).

To consider diurnal variation in CHO metabolism (Varum et al. 1986), each sampling was carried out exactly 4 hours after the beginning of the light phase, every 24 h. The composition of the dissolved phase in terms of inorganic nutrients and CHO, was determined as well as the composition of the particulate phase i.e. diatom, free and attached bacteria cell number, the elemental composition of particles (C, N, P, Si), the speciation of total particulate phosphorus (TPP) into particulate organic (POP) and inorganic phosphorus (PIP), particulate CHO and hydrolytic enzymatic activities (potential β -glucosidases, alkaline phosphatases).

2.2. Microalgae and bacteria cell count

For *T. weissflogii* enumerations, samples were fixed with a few drops of Lugol's iodine and cells were counted with an inverse optical microscope (Utermöhl method). Samples for total and free ($< 3 \mu\text{m}$) bacterial abundance were preserved with borate buffered formaldehyde (2% final concentration). Samples for free bacteria determination were prepared by prefiltration ($3 \mu\text{m}$) of culture samples. Total bacteria were determined after pyrophosphate treatment (Velji & Albright 1986), then free and total bacteria were enumerated by epifluorescence microscopy after staining with 4',6-diamidino-2-phenylindole (DAPI; Porter & Feig 1980). The number of particle-attached ($> 3 \mu\text{m}$) bacteria was calculated by subtracting $< 3 \mu\text{m}$ cell number from the total bacteria. The cell division rate of phytoplankton and bacteria was used as the reference for the specific growth rate (μ) in the rest of the manuscript i.e. measured as increase in cell densities. It was calculated by plotting the logarithm of cells against time and determining the slope according to the least square's criterion. A minimum of three sampling points was included in the calculation.

2.3. Inorganic nutrients and particulate matter analysis

Samples for the determination of dissolved (PO_4 , nitrate + nitrite and CHO) and particulate (POC, PON, TPP, POP, PIP, CHO) compounds were very carefully filtered (20 ml for each particulate analysis) on precombusted (12 h at 400°C) 25 mm Whatman® GF/F. Filtrates and filters were deep frozen (-20°C). Samples for the determination of silicate were filtered on $0.45 \mu\text{m}$ cellulose acetate filters with a syringe filtration system.

Filtrates were stored at 4°C. For TEP analysis, samples (10 ml) were gently filtered onto 0.4 µm polycarbonate membranes and stained with 0.5 ml of a 0.02% aqueous solution of Alcian blue in 0.06% acetic acid (pH 2.5) according to the method of Passow & Alldredge (1995). Filters were kept frozen at -20°C. Samples for biogenic silica (bSiO₂) determination were filtered on 0.6 µm polycarbonate membranes, rinsed with artificial seawater, then dried at 60°C for 24 h and stored at room temperature until digestion and analysis.

Nitrate plus nitrite, PO₄ and silicate were determined according to classic methods using segmented flow analysis (Aminot et al. 2009). Particulate organic C and N (POC, PON) were analysed on a CHN analyser. For biogenic silica (bSiO₂) determination, after 0.2 M sodium hydroxide digestion carried out as in Ragueneau et al. (2005), the supernatant was analyzed for silicic acid (Aminot et al. 2009). Total particulate phosphorus (TPP) and PIP were determined as precisely described in Labry et al. (2013), the original methods being that of Solorzano and Sharp (1980) for TPP and Aspila et al. (1976) for PIP. POP was calculated as the difference between TPP and PIP. The PIP comprises intracellular storage P forms (i.e. phosphate, pyro- and polyphosphate), a substantial part of di and triphosphate nucleotides and P adsorbed on cell wall (Labry et al. 2013).

The cellular C, N, P, Si content of *T. weissflogii* (Q_C, Q_N, Q_P, Q_{Si}) were obtained by dividing POC, PON, TPP and bSiO₂, respectively, by the corresponding cell number considering filtered volumes. The total bacterial C, N and P were minor contributors to POC, PON and TPP, respectively. They could be estimated assuming a cell content of 20 and 50 fg C for free and fixed bacteria, respectively (Lee & Fuhrman 1987, Simon et al. 1990), and a C:N and C:P atomic ratios equal to 4 and 50, respectively. Total bacterial C,

N, P contributed to a maximum of $4 \pm 1\%$ ($n = 9$), $10 \pm 2\%$ ($n = 12$) and $19 \pm 4\%$ ($n = 12$) of POC, PON and TPP, respectively, in P deplete conditions. In replete conditions, their contributions were $3 \pm 0\%$, $5 \pm 1\%$, and $3 \pm 0\%$ ($n = 12$) of POC, PON and TPP, respectively.

2.4. Dissolved, particulate carbohydrates and TEP analysis

Dissolved carbohydrates were analyzed using the 2,4,6-tri-pyridyl-s-triazine (TPTZ) method of Myklestad et al. (1997). Briefly, monosaccharides (M-DCHO) were determined by direct reaction without hydrolysis, while total dissolved carbohydrates (T-DCHO) were analyzed by TPTZ after 20 h hydrolysis by 0.1 M HCl at 100°C and neutralization (Burney & Sieburth 1977). All measurements were conducted in triplicates, the relative mean standard deviation for all the analyzed samples were 1.2% for M-DCHO (range 0.16 - 3.4%) and 2.7% (range 0.6 - 7.5%) for T-DCHO. The polymeric dissolved carbohydrates (P-DCHO) were calculated as the difference between T-DCHO and M-DCHO.

Particulate CHO were analyzed from the particulate matter collected on filters. Reserve carbohydrates (R) were first extracted with milliQ water for 30 min at 100°C (Handa 1969). After centrifugation, the supernatant was kept for CHO analysis. The filter and the residual pellets were then hydrolyzed (2N H₂SO₄, 4 h at 100°C) in order to liberate monosaccharides from structural carbohydrates (S, Hecky et al. 1973, Mopper 1977). Carbohydrates in both fractions were then analyzed by the phenol sulfuric acid method (Dubois et al. 1956). Filters for TEP were then analyzed by the colorimetric method of Passow & Alldredge (1995) and expressed as Gum Xanthan equivalent (Xeq).

2.5. Potential β -glucosidase and alkaline phosphatase activities

Ecto-enzymatic ($0.2\text{--}3\ \mu\text{m}$ and $> 3\ \mu\text{m}$) and extracellular ($< 0.2\ \mu\text{m}$) activities were measured by the fluorometric method with methylumbelliferyl (MUF) substrates (Hoppe 1983) on unfiltered (total activities) and two prefiltered seawater samples ($< 0.2\ \mu\text{m}$ and $< 3\ \mu\text{m}$). A saturating concentration of substrate ($250\ \mu\text{M}$ 4-Methylumbelliferyl phosphate MUF-P, $100\ \mu\text{M}$ MUF- β -D-glucopyranoside) was used in order to reach the maximal velocity rate of alkaline phosphatase activity (APA) and β Glc. The complete set of results presented in the present study are thus indicative of potential APA and β Glc. For both activities, 2 ml duplicate samples and respective controls were incubated with $50\ \mu\text{l}$ of substrate solution for 3 h in the dark at temperature and pH of the cultures. The hydrolysis reaction was halted by the addition of a 4% final concentration of formaldehyde for APA and $200\ \mu\text{l}$ of a buffered (pH 10.5) solution of $0.2\ \text{M}$ NH_4Cl / $0.05\ \text{M}$ glycine for β Glc. These inhibitors were added before the addition of substrate for controls. Both samples and controls were deep frozen. Fluorescence, produced by the 4-Methylumbelliferone (MUF) released, was measured by flow injection analysis coupled with a fluorescence spectrometer (Kontron SFM 25, excitation at 364 nm and emission at 460 nm). A buffered solution of borate ($0.1\ \text{M}$, pH 10.5) was used as the carrier fluid, providing maximum MUF fluorescence (Hoppe 1983, Chrost & Krambeck 1986). The system was previously calibrated with MUF solutions. The $> 3\ \mu\text{m}$ activities were calculated as the difference between total and $< 3\ \mu\text{m}$ activities, and the $0.2\text{--}3\ \mu\text{m}$ activities as the difference between $< 3\ \mu\text{m}$ and $< 0.2\ \mu\text{m}$ activities. The $0.2\text{--}3\ \mu\text{m}$ APA is due to free bacteria while attached bacteria and *T. weissflogii* both contribute to the $>$

3 μm APA and cannot be separated by size fractionation. Since phytoplankton is not known to produce β -glucosidase ectoenzymes (Chrost 1989), only attached bacteria contribute to the $> 3 \mu\text{m}$ βGlc . In addition, no βGlc was found in the $< 0.2 \mu\text{m}$ fraction. We are confident that the increase in MUF fluorescence for APA and βGlc was linear over the single incubation time used (3h) given that 0.02 to 12% of MUF-P and 0.02 to 0.08% of MUF- β -D-glucopyranoside were hydrolyzed only at the end of incubation. The statistical calculations (standard deviation from culture triplicates) and tests (Mann & Whitney, Spearman correlation) were performed with Excel software (Microsoft) using computing utilities from <https://www.anastats.fr>

3. Results

3.1. Nutrient culture conditions, diatom and bacteria growth curves

Low PO_4 concentrations were measured in P deplete conditions from 16 h ($0.05 \pm 0.01 \mu\text{M}$) to 88 h ($0.01 \pm 0.004 \mu\text{M}$), whereas nitrate plus nitrite and silicate concentrations remained high (Fig. 2a). By contrast, in P replete conditions, the fast *Thalassiosira weissflogii* growth exhausted PO_4 and nitrate plus nitrite after 88h (Fig. 2b) while silicate remained high. Since *T. weissflogii* growth could have induced N, P and C co-limitation in P replete treatment after 88 h, only results from the first 4 days of growth are representative of replete conditions and presented in the study. *T. weissflogii* cell number increased tenfold between 16 h and 88 h (Fig. 3) with a cell specific growth rate of $0.73 \pm 0.16 \text{ d}^{-1}$ (mean $\pm$ 95% confidence interval). By contrast, diatom growth was initially low in P deplete conditions (0.48 d^{-1}), before ceasing altogether after 40 h. Total bacteria

cell growth was very closely linked to that of *T. weissflogii* for both P treatments (Fig. 3). Total bacteria and particle-attached bacteria abundances normalized to that of *T. weissflogii* were higher in P deplete than in P replete conditions throughout the time of experience (Fig. 4a, b). Attached bacteria contributed to $39 \pm 8\%$ and $34 \pm 4\%$ ($n = 12$) of the total bacteria abundance in P deplete and replete conditions, respectively (not shown).

3.2. Physiological indicators of two different cell P states

Several physiological parameters indicate that *T. weissflogii* and bacteria actually developed two different physiological states according to the P treatment (Table 1). Q_P was 4 times lower in P deplete than in P replete treatments. By contrast, Q_C , and Q_N were only slightly lower in P deplete than in P replete conditions. Q_{Si} was approximately 50% lower in P replete than in P deplete conditions at 88 h whereas it was quite similar at 16 h. Q_{Si} is usually found to be inversely related to growth rate under non-silicon limitation (Martin-Jézéquel et al. 2000). At low growth rate, the silicification of the cells may be increased (elongation of the G2+M phase of the cell cycle during which major uptake of silicon occurs, Claquin et al. 2002), resulting in higher Si:C ratio in P depleted cells compared to P repleted cells. The low Si:C ratio at 88h in P repleted cells could indicate the imminent onset of C limitation. The C:P and N:P atomic ratios in particulate matter were far greater than the Redfield ratio (106 and 16) in P deplete cultures, whereas they were approximately half the Redfield ratio in P replete conditions. For both cultures, the C:N atomic ratio increased after four days. This ratio was higher in P depleted than in P repleted cells, which could be an indicator of cells being less healthy after 64 h of severe

depletion. The PIP contributions to TPP were higher in P replete than in P deplete treatments and these values decreased in the two treatments from 16 h to 88 h. P repleted cells stocked more P as phosphate, pyro- and polyphosphate forms (luxury uptake) and used reserve P to support diatom growth. This reserve of inorganic P may have been used for diatom survival in P depleted cells as shown by the low PIP contribution to the TPP at the beginning of the growth. These results concur with previous studies on P limited cells compared with non-limited or N limited cells (See review in Labry et al. 2013, their Table 5).

Both diatom and bacterial PO_4 uptake were limited in P deplete conditions. The PO_4 concentration (0.009 - 0.052 μM) was close to or lower than the values of the half saturation constant (K_t) + the natural substrate concentration (S_n) for PO_4 uptake, as already reported in severely P limited environments for phytoplankton (0.056 - 0.090 μM) and bacteria (0.002 - 0.038 μM , Labry et al. 2002). The PO_4 concentration at 88 h in P replete cultures (0.063 μM) was consequently limiting for diatom PO_4 uptake. Alkaline phosphatase activity (APA) is sensitive to PO_4 concentration and as such, is commonly used as an indicator of bacterio- and phytoplankton P limitation (Labry et al. 2005). Specific particulate APA (i.e. particulate APA normalized to TPP), and specific free bacteria APA were 500 times and 5 to 44 times higher in P deplete than in P replete conditions, respectively (Table 1). In the P deplete conditions, bacteria P limitation was indicated by the high level of specific free bacteria APA, i.e. in a range of values (17 - 143 pmol per 10^6 cells h^{-1}) already reported for severely P-limited free bacteria in coastal waters (1 - 99 pmol per 10^6 cells h^{-1} , Labry et al. 2005).

3.3. Particulate and dissolved carbohydrates

The total particulate reserve and structural CHO (R+S) concentrations increased for both cultures and were more than twice as high in P replete than in P deplete conditions at 88 h (Table 2). However, the cell numbers differed between P treatments due to different growth rates, being five times higher in P replete conditions at 88h than in P deplete conditions (Fig. 3). When normalized to diatom cell numbers, R and S, were twice as high under P deplete than under P replete conditions (Fig. 5a). The maximum value obtained for R ($372 \pm 20 \text{ pg cell}^{-1}$) was comparable with reserve β -glucan concentrations previously reported for *T. weissflogii* (from 364 to 412 pg cell^{-1} , Storseth et al. 2005). In both treatments, R largely dominated over the S (Fig. 5b). The R / S ratios were greater ($p < 0.001$, Mann & Whitney test) in P depleted (range from 5.9 to 6.6) than in P repleted cells (range from 4.0 to 5.5). Reserve CHO staid constant in P replete, while it increased in P deplete between 40 and 64h before stabilizing until 88h. Simultaneously, the concentrations of TEP increased in P replete from 40h to 88h and were higher than in P deplete cultures where the TEP concentrations start to increase when reserve CHO stabilized (Fig. 6a). They showed very similar values to S with which a significant correlation was found (Spearman correlation coefficient $r = 0.61$, $n = 11$, $p < 0.05$ for P deplete cultures; $r = 0.92$, $n = 10$, $p < 0.001$ for P replete cultures Fig. 6b).

Regarding the dissolved compartment, the T-DCHO concentrations were always higher under P deplete than under P replete conditions and displayed a slight increase over time (Table 2). The P-DCHO concentrations were similar for both cultures, while M-DCHO concentrations were approximately twice as high in P deplete than in P replete cultures

(Table 2). It should be noted that, for both conditions, the M-DCHO concentrations remained remarkably constant over time while the P-DCHO concentrations increased. The ratio of P-DCHO to M-DCHO was far lower in P deplete (0.15 - 0.30) than in P replete conditions (0.37 - 1.09).

3.4. β -glucosidase activities

Total potential β Glc showed higher values in P deplete than in P replete treatment (Fig. 7a). Attached bacteria dominated potential β Glc throughout the experiment both in P deplete and P replete conditions, with cell count contributions of $78 \pm 3\%$ and $65 \pm 6\%$ ($n = 12$), respectively (not shown). They developed a greater enzymatic potential to hydrolyze polysaccharides than their free-living counterparts, as suggested by the higher specific β Glc calculated separately for attached and free bacteria (Fig. 7b). This pattern is even more pronounced under P deplete conditions: specific β Glc of attached bacteria were 4.6 to 6.8 times greater than that of free bacteria in this condition whereas they were only 2.6 to 4.6 times higher in P replete treatments (Fig 7b).

4. Discussion

Phytoplankton and specifically diatoms are known to be important producers of polysaccharides and exopolymers (Hoagland et al. 1993). Bacterioplankton may also release polysaccharides (Passow 2002a, Panhota et al. 2007). However, bacteria cannot be considered a net producer of particulate and dissolved CHO. Bacteria are heterotrophs and between 50 and 90% of the Carbon taken up by bacteria in aquatic ecosystems is

mineralized (Del Giorgio & Cole 1998). Through a decay experiment using glucose as Carbon source, Panhota et al. (2007) showed that the production of polysaccharides by a natural bacterial community represented less than 2% of the glucose degradation products; 94.4 % being mineralized. Gärdes et al. (2011) demonstrated that the major source of polysaccharides in *Thalassiosira weissflogii* and bacteria co-cultures was photosynthetically active *T. weissflogii*. In our cultures, bacterial C contributed to 3% of POC only, while particulate carbohydrate Carbon constituted 65 and 79% of POC in P replete and P deplete cultures, respectively. Bacteria were only minor contributors of particulate and dissolved polysaccharides in our cultures. However, their enzymatic equipment for polymer hydrolysis could have modified the composition of polysaccharides.

4.1. Speciation of particulate carbohydrates

The present study highlights that P deplete conditions promote accumulation of carbohydrates in diatom cells, mainly as reserve polysaccharides: the ratio of Reserve to Structural polysaccharides, R / S, is higher (5.9 to 6.6) than in P repleted cells (4.0 to 5.5). This may explain the high C:N ratio found at the end of incubation in P deplete cultures whereas it remained quite constant in P replete conditions (Suroy et al. 2015). There are very few data on R versus S partition of carbohydrates in phytoplankton. Granum & Mykkestad (2002) found a similar R / S ratio in the late exponential phase of axenic *Skeletonema costatum* cultures (3.8) and a far higher ratio in the N limited stationary phase (15.5). By contrast, they reported lower R / S values (0.34 to 0.84) for natural nutrient sufficient phytoplankton communities, with an equal composition of diatoms and

haptophytes. Haptophyte cell walls are composed of several layers of cellulose and complex polysaccharides, which explains the higher S contribution. In the same way, natural communities substantially comprising dinoflagellates whose cell walls are made up of insoluble glucan as cellulose, lead to low R / S ratio (< 0.5 , Haug et al. 1973). The partition between R and S actually depends on the relative composition of plankton species. However, according to the present study and that of Granum & Mykkestad (2002), a higher contribution of diatoms in microalgal communities could lead to a higher R polysaccharide proportion in particulate matter. This proportion is increased in P deplete conditions. Since R in diatoms are essentially composed of glucose polymers (Mykkestad & Haug 1972, Granum et al. 2002), the consequence may be a higher bioavailable pool of carbohydrates in the dissolved environment when cells become senescent, die and lyse.

TEP are operationally defined as discrete exopolymer particles that can be stained with alcian blue due to the presence of anionic carboxyl and sulfate half ester groups (Passow 2002b, Passow & Alldredge 1995). By definition, TEP should not include cell coatings. For both culture conditions, concentrations of particulate S and TEP were quantitatively very similar and were significantly correlated. It is well known that diatom S polysaccharides are essentially composed of mannose, glucuronic acid and sulfate groups (Ford & Percival 1965, Chiovitti et al. 2005, Tesson & Hildebrand 2013). These chemical groups represent the key factors for the staining of polysaccharides with alcian blue (Ramus 1977). Passow (2002b) as well as Engel et al. (2004) indicated that the colorimetric method may overestimate TEP if organisms with stainable coatings are abundant. Thus, we suspect that the acidic S polysaccharides associated with the frustules

constitute the major part of the analyzed TEP in our diatom cultures. Real TEP were of unknown importance.

4.2. Speciation of dissolved carbohydrates

Dissolved CHO in the form of mono- and polysaccharides correspond to “functional pools” with respect to bacterial nutrition dynamics. Indeed, M-DCHO can be directly taken up by bacteria, whereas P-DCHO need to be hydrolyzed by glucosidases before being taken up as monosaccharides (Fig. 8). As in the present study, Borsheim et al. (2005) found low fluctuations of M-DCHO over time in mesocosms receiving contrasted nutrient supplies and supporting a diatom bloom and its decline. They assumed a tight coupling between the production of M-DCHO and their uptake by heterotrophic bacteria. In addition, their M-DCHO concentrations did not differ over a gradient of daily nutrient supply. In the present study, the balance between production and uptake occurs at two different levels according to P condition, as illustrated in Fig. 8. The difference comes from either a higher hydrolysis of P-DCHO or a lower uptake of M-DCHO in P deplete conditions compared to P replete conditions, or both. The very high level of potential β Glc found as early as 16 h after inoculation in P deplete conditions is an indicator of a high P-DCHO hydrolysis, even if these activities are not actual in situ rates (see Discussion below, Section 4.3). Micro-organisms have a high enzymatic capacity for polymer hydrolysis.

Concerning the bacterial uptake of M-DCHO, it seems not to have been limited by the M-DCHO concentrations in our cultures (4.6 - 10.3 μ M). These concentrations were far higher than the range of $K_t + S_n$ for glucose uptake, as already reported in brackish (0.009

– 0.038 μM Glucose, Gocke et al. 1981) and oligotrophic waters (0.043 – 0.220 μM , Ayo et al. 2001). In addition, there is no evidence in the literature that P depletion can change the M-DCHO composition into aldoses that are more resistant to bacterial degradation. On the contrary, a higher contribution of glucose, one of the most bioavailable aldoses (Hama & Yanagi 2001), was found in P depleted diatom exudates compared to P replete cell exudates (Urbani et al. 2005). Alternatively, bacteria were found to be PO_4 limited in the P deplete conditions, as highlighted by their reduced growth rate, the PO_4 concentration close to reported $K_t + S_n$ values for bacterial PO_4 uptake, and the very high level of specific APA for free bacteria. As a consequence of reduced growth rate, the uptake of M-DCHO must have also been reduced. Thus, high specific βGlc and reduced uptake of M-DCHO can explain the high M-DCHO concentration observed in P deplete cultures (Fig.8). As in Borsheim et al. (2005), the increase in P-DCHO with time in our cultures suggests that their excretion by phytoplankton became higher with time compared to their hydrolysis by bacteria (Fig. 8).

4.3. Enzymatic responses of free and attached bacteria

Enzymatic measurements provide potential activities (enzymatic equipment) since there is no data on actual hydrolysis rates which are not biased by the competitive inhibition between natural and artificial substrates necessary for their measurement. In our study, the ratio between saturating artificial (100 μM) and natural substrate (1.5 to 5.5 μM P-DCHO) is high enough to avoid this competitive inhibition. If bacteria are not net CHO producers in the two P treatments, they could have modified the CHO composition excreted by *T. weissflogii* via their enzymatic equipment and, ultimately, have changed

their ability to aggregate particles. The very high values of potential specific β Glc in P deplete treatment as early as 16 h after inoculation have resulted in the observed high M-DCHO concentrations found in these cultures (see Discussion above, Section 4.2). These very high activities can be explained both by an induction of β -glucosidases synthesis a few hours after the beginning of incubation, and by the bacteria present, those coming from the diatom precultures, which may have already been equipped with a significant amount of glucosidases. Whatever their origin, the major contributors to these high potential β Glc are bacteria attached to $> 3 \mu\text{m}$ particles. In term of bacterial cell numbers, they are the lowest contributors. Such differences in specific β Glc between attached and free bacteria have already been observed on natural and artificial phytoplankton aggregates (Karner & Herndl 1992, Agis et al. 1998) and during the development and decline of a phytoplankton bloom (Middelboe et al. 1995). The present study emphasizes the fact that P limitation promotes free and attached bacteria having higher cell-specific β Glc than in P replete conditions and with a higher ratio of attached to free bacterial activity. P limitation may increase the difference between free and attached bacterial enzymatic equipment for polysaccharide hydrolysis.

4.4. Consequences for labile DOC accumulation

Saad et al. (2016) found that dissolved CHO represented 30% of the dissolved organic carbon (DOC) production in both low P and P replete axenic cultures of *T. pseudonana*. Similar values were observed for *T. weissflogii* cultures (28%; Aluwihare & Repeta 1999), and from mesocosm experiments with natural planktonic population (18-21%; Meon & Kirchman 2001). Using an average value of 24%, we can roughly estimate DOC

concentrations in our P deplete (from 290 to 330 μM) and P replete conditions (from 160 to 260 μM). These values are in the range generally found in such experiments ($> 200 \mu\text{M}$). DOC accumulation may have represented up to 100% of POC in P deplete conditions. It decreased from 70 to 15% of POC in P replete experiments as diatom biomass increased. Such difference between replete conditions (i.e. blooming phase), and nutrient deplete conditions (post bloom) concurs with the accumulation of carbon rich dissolved organic matter observed in surface marine environments (Thingstad et al. 1997, Williams 1995). This accumulation was attributed to the imbalance between excretion and lysis processes consecutive to the decline of blooms, and reduction of bacterial uptake caused by nutrient depletion. As we observed in our experiments, P depletion of heterotrophic bacteria favors labile DOC accumulation (Fig. 8; Vadstein et al. 2003).

5. Conclusions

Our results show that P depletion favors firstly diatom carbohydrate accumulation, mainly in the form of R CHO, and their subsequent cell release. The R / S ratio is high in diatom cultures and increases with P depletion. A high contribution of diatoms in microalgal communities could lead to high proportion of polysaccharides as reserve pools in particulate matter. In addition, the fact that bacteria were also P depleted might have stressed this accumulation of reserve polysaccharides, by competing for phosphate. This promoted an active degradation (polysaccharide hydrolysis by β -glucosidases) by the bacterial communities, notably by attached bacteria. However bacterial growth and metabolism were slowed down by P depletion, and consequently the uptake of M-DCHO was reduced. P depletion of heterotrophic bacteria favored M-DCHO accumulation. This

is in accordance with the accumulation of degradable DOC observed in surface marine waters. The P status of bacteria must be considered to evaluate the effect of P depletion on carbohydrate dynamics and subsequent consequences for particle potential aggregation.

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1 **Table 1**

2

Physiological parameters	P-		P+	
	16 h	88 h	16 h	88 h
Q _C (pmol C cell ⁻¹)	11 (2)	11 (1)*	13 (1)	13 (1)
Q _N (pmol N cell ⁻¹)	1.5 (0.1)	1.2 (0.2)	1.9 (0.3)	1.6 (0.1)
Q _P (pmol P cell ⁻¹)	0.068 (0.007)	0.035 (0.001)	0.244 (0.009)	0.143 (0.015)
Q _{Si} (pmol Si cell ⁻¹)	4.8 (0.7)	3.4 (0.3)	4.2 (0.1)	1.5 (0.1)
C:N at:at	7.6 (0.9)	10.5 (0.6)*	7.0 (0.5)	7.9 (0.01)
Si:C at:at	0.5 (0.1)	0.4 (0.1)*	0.3 (0.0)	0.1 (0.0)
C:P at:at	167 (19)	266 (24)*	54 (8)	89 (2)
N:P at:at	22 (1)	33 (4)	8 (2)	11 (0.3)
PIP (%)	41 (4)	31 (2)*	56 (2)	34 (1)
APA > 0.2 μm (nmol P μmol TPP ⁻¹ h ⁻¹)	1487 (242)	10110 (796)	2 (1)	18 (2)
APA 0.2-3 μm (pmol P 10 ⁶ cells ⁻¹ h ⁻¹)	17 (3)	143 (24)	3 (1)	3 (1)

3

Table 2

Time (h)	P-				P+			
	R+S	M-DCHO	P-DCHO	T-DCHO	R+S	M-DCHO	P-DCHO	T-DCHO
16	17 ± 1	10.3 ± 0.1	1.5 ± 0.1	11.8 ± 0.1	14	4.7 ± 0.1	1.7 ± 0.2	6.4 ± 0.3
40	37 ± 1	10.3 ± 0.0	2.0 ± 0.1	12.4 ± 0.1	47 ± 6	4.6 ± 0.1	2.2 ± 0.3	6.8 ± 0.2
64	62 ± 2	10.3 ± 0.1	3.1 ± 0.3	13.4 ± 0.2	89 ± 6	4.7 ± 0.0	3.3 ± 0.1	8.0 ± 0.1
88	78 ± 6	10.3 ± 0.0	2.9 ± 0.5	13.2 ± 0.5	176 ± 31	5.0 ± 0.3	5.5 ± 0.1	10.5 ± 0.2

1 Fig. 1

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1 **Fig. 2**

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5 **Fig. 3**

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Fig. 4

Fig. 5

Fig. 6

Fig. 7

1 Fig. 8

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Tables and Figures

Table 1. Mean values (standard deviation, $n = 3$) on 16 h and 88 h of the cellular C, N, P and Si content of *Thalassiosira weissflogii* (Q_C , Q_N , Q_P , Q_{Si}), the particulate C:N, Si:C, C:P, and N:P atomic ratios, the PIP contribution to TPP (%), specific particulate APA per TPP and specific 0.2-3 μm APA per bacterial cell for the P deplete (P-) and replete (P+) cultures. * indicates values on 64 h rather than 88 h.

Table 2. Mean values of particulate carbohydrates (reserve plus structural, R+S), dissolved mono- (M-DCHO), polysaccharides (P-DCHO) and total dissolved carbohydrates (T-DCHO) concentrations (μM) for the P deplete (P-) and replete (P+) cultures of *Thalassiosira weissflogii*. The standard deviations correspond to triplicate cultures ($n = 3$) for particulate carbohydrate analysis and triplicate measurements on triplicate cultures ($n = 9$) for all dissolved carbohydrate forms.

Fig. 1. Experimental set-up of *Thalassiosira weissflogii* precultures and cultures

Fig. 2. Nitrate plus nitrite (black dots, left scale), silicate (black diamonds, left scale) and phosphate (empty dots, right scale) concentrations (μM) in P deplete (P-, a) and P replete (P+, b) *Thalassiosira weissflogii* cultures. Error bars illustrate the standard deviation corresponding to culture triplicates. All error bars are shown.

Fig. 3. *Thalassiosira weissflogii* (Tw, dots) and bacterial abundance (B, diamonds) in P deplete (P-, empty symbols) and P replete (P+, full symbols) cultures. Error bars illustrate the standard deviation corresponding to culture triplicates. All error bars are shown.

Fig. 4. The ratio of (a) total and (b) attached ($> 3 \mu\text{m}$) bacteria to *Thalassiosira weissflogii* in P deplete (P-) and P replete (P+) cultures. Error bars illustrate the standard deviation corresponding to culture triplicates. All error bars are shown.

Fig. 5. Reserve (R) and structural (S) particulate carbohydrates normalized to *Thalassiosira weissflogii* cells (a) and the R / S ratio (b) in P deplete (P-) and P replete (P+) cultures. Error bars illustrate the standard deviation corresponding to culture triplicates. All error bars are shown.

Fig. 6. Total transparent exopolymer particles (TEP) in P deplete (P-) and P replete (P+) cultures (a) and their relationship with structural particulate polysaccharides (b). Error bars illustrate the standard deviation corresponding to culture triplicates. All error bars are shown.

Fig. 7. Total potential β -glucosidase activities (βGlc , nM h^{-1} , a) and specific βGlc normalized to bacterial cells for attached ($> 3 \mu\text{m}$) and free ($0.2 - 3 \mu\text{m}$) bacteria ($\text{pmol } 10^6 \text{ cells}^{-1} \text{ h}^{-1}$, b) in P deplete (P-) and P replete (P+) cultures. Error bars illustrate the standard deviation corresponding to culture triplicates. All error bars are shown.

- 1 Fig. 8. Representation of the dissolved mono- (M-DCHO) and polysaccharides (P-
- 2 DCHO) and particulate reserve (R) and structural (S) carbohydrate pools and cell specific
- 3 fluxes in P deplete and P replete culture conditions for diatoms and bacteria.
- 4