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# 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- [M-DCHO] 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 short-term response of free and diatom-attached bacteria in terms of abundance and potential β-glucosidase activity (βGlc). 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 βGlc of attached bacteria) and reduced uptake of M-DCHO by P-depleted 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.

KEY WORDS: P limitation · Phytoplankton · Diatom · Bacteria · Mineralization · Carbohydrate · Glucosidase



## 1. INTRODUCTION

Carbohydrates (CHO) are important constituents of living matter, especially in phytoplankton where cellular CHO can account for up to 90% of organic dry matter (Myklestad 1974). The production and excretion of CHO by phytoplankton has received significant attention in the past 2 decades, as excreted CHO strongly influences particle aggregation and ultimately the export of organic material towards the deep sea (Mari et al. 2017). The nutritional condition of micro-algal growth is a key factor in cell CHO accumulation. Nitrogen (N) and phosphorus (P) limitations are known to favor the production and excretion of CHO by diatoms (Myklestad 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 eco-

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systems due to agricultural practices (Grizzetti et al. 2012, Peñuelas 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 bacterial hydrolysis into monosaccharides, subsequent uptake of monosaccharides by bacteria and, consequently, bacterial growth (Middelboe 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 CHO, which has been relatively unexplored (Le Costaouëc et al. 2017). CHO has usually been measured as total dissolved CHO (DCHO) and/or total particulate CHO, without further fractionating between functionally different CHO pools. However, particulate CHO include structural (S; associated with cell wall) and reserve (R; intracellular) polysaccharides (Penna et al. 1999, Staats et al. 2000). S CHO 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). R 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 (Myklestad & Haug 1972, Obata et al. 2013). β-1,3-glucan can account for up to 80% of cellular organic carbon (C) in diatoms (Granum et al. 2002). When the monosaccharide composition of DCHO 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, R 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 2 CHO 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.

In the present study, we investigated how P limitation affects the physiological responses of phytoplankton cells, i.e. *T. weissflogii*, a model diatom, with a particular attention to CHO composition and utilization by bacteria. Particulate CHO were separated into S and R CHO, and DCHO into mono- (M-DCHO) and polysaccharides (P-DCHO). This fractioning enabled us 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 µm) and particle-attached (>3 µm) bacteria was evaluated in terms of biomass and potential βGlc. 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 (Størseth et al. 2005).

## 2. MATERIALS AND METHODS

### 2.1. Culture conditions

#### 2.1.1. Precultures

Non-axenic batch cultures of the diatom *Thalassiosira weissflogii* (AC813; Bay of Veys) were grown in f/2 medium (Guillard & Ryther 1962) in filtered (<0.2 µm) seawater from the Bay of Brest (salinity: 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 and 250 µM, respectively (a N:Si molar ratio of 1.3), and micronutrient concentrations reached f/2 conditions. Cells were grown for 4 d before being harvested, which corresponded to the mid- and end of exponential growth phase for P-replete and P-limiting precultures, respectively.

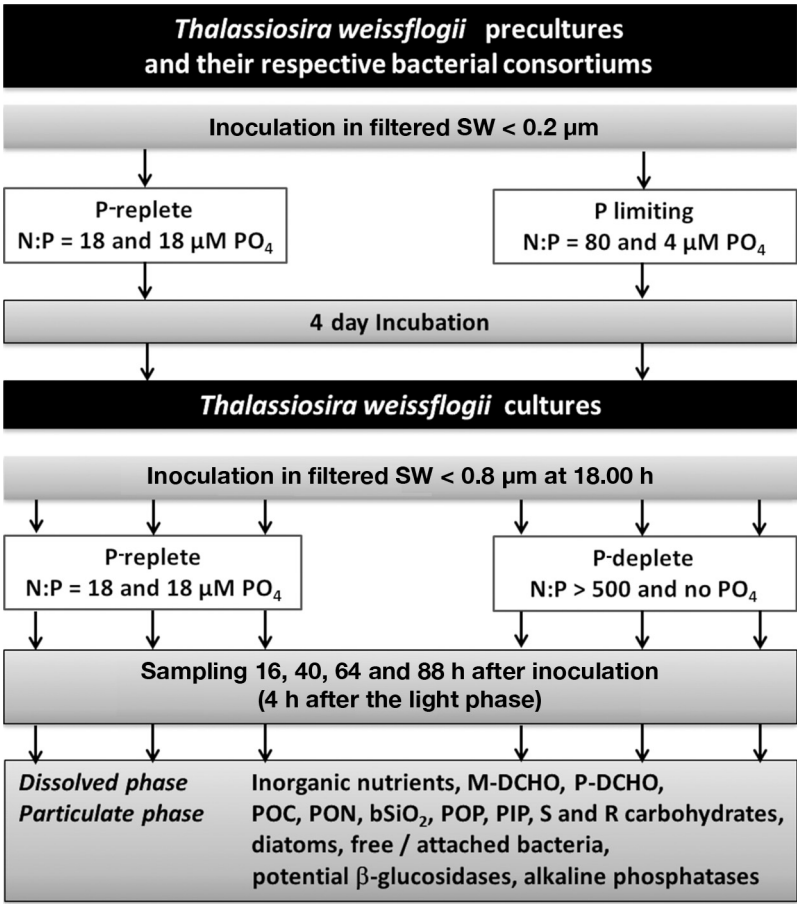


Fig. 1. Experimental setup of *Thalassiosira weissflogii* precultures and cultures. SW: seawater; M-DCHO and P-DCHO: dissolved mono- and polysaccharides; POC, PON, POP: particulate organic C, N and P; PIP: particulate in organic P; S: structural; R: reserve; $bSiO_2$: biogenic silica

### 2.1.2. Cultures

Initial $PO_4$, nitrate + nitrite and silicate concentrations in the collected Bay of Brest seawater were 0.5, 13.1 and 8.7 µM, respectively. Within a few hours after collection, seawater was gently prefiltered (0.8 µm) in order to preserve the natural bacterial community whilst removing predators and phytoplankton competitors. After filtration, seawater was distributed among 6 flasks (10 l) and enriched with 2 media (triplicates for each media) in order to have 2 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 over time. The 2 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 11 700 cells $ml^{-1}$ for both conditions (9–11% of inoculum volume). The bacterial inoculum was 2.5–2.8 × $10^6$ 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 setup allowed us to study the effect of P depletion on the CHO dynamics (micro-algal CHO production, excretion and bacterial degradation). Batches were inoculated in the evening (at 18:00 h), preceding the first subsampling (at 10:00 h the next day, 16 h later). Cultures were grown at 18°C, with a photon irradiance of 150 µmol $m^{-2}$ $s^{-1}$ in a 12 h light:12 h 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 h 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. diatoms, 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 µm) bacterial abundance were preserved with borate buffered formaldehyde (2% final concentration). Samples for free bacteria determination were prepared by prefiltration (3 µm) 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 µm) bacteria was calculated by subtracting the <3 µm 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 3 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 µm 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 ($bSiO_2$) 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 + nitrite, $PO_4$ and silicate were determined according to classic methods using segmented flow analysis (Aminot et al. 2009). POC and PON were analyzed on a CHN analyzer. For $bSiO_2$ determination, after 0.2 M sodium hydroxide digestion was carried out as in Ragueneau et al. (2005), the supernatant was analyzed for silicic acid (Aminot et al. 2009). TPP and PIP were determined as precisely described in Labry et al. (2013), the original methods being that of Solorzano & 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. $PO_4$, pyro- and polyphosphate), a substantial part of di- and triphosphate nucleotides and P adsorbed on cell walls (Labry et al. 2013).

The cellular C, N, P and Si content of *T. weissflogii* ($Q_C$, $Q_N$, $Q_P$, $Q_{Si}$) were obtained by dividing POC, PON, TPP and $bSiO_2$, 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 C:N and C:P atomic ratios equal to 4 and 50, respectively. Total bacterial C, N and P contributed to a maximum of 4 ± 1% (n = 9), 10 ± 2% (n = 12) and 19 ± 4% (n = 12) of POC, PON and TPP, respectively, in P-deplete conditions. In replete conditions, their contributions were 3 ± 0, 5 ± 1 and 3 ± 0% (n = 12) of POC, PON and TPP, respectively.

### 2.4. Dissolved and particulate CHO and TEP analysis

DCHO were analyzed using the 2,4,6-tripyridyl-s-triazine (TPTZ) method of Myklestad et al. (1997). Briefly, M-DCHO were determined by direct reaction without hydrolysis, while total DCHO (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 deviations for all 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 P-DCHO was calculated as the difference between T-DCHO and M-DCHO.

Particulate CHO were analyzed from the particulate matter collected on filters. R CHO 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 (2 N $H_2SO_4$, 4 h at 100°C) in order to liberate monosaccharides from S (Hecky et al. 1973, Mopper 1977). CHO 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

Ectoenzymatic (0.2–3 and >3 µm) and extracellular (<0.2 µm) activities were measured by the fluorometric method with methylumbelliferyl substrates (Hoppe 1983) on unfiltered (total activities) and 2 prefiltered seawater samples (<0.2 and <3 µm). A saturating concentration of substrate (250 µM 4-methylumbelliferyl phosphate, [MUF-P], 100 µM MUF-β-D-glucopyranoside) was used in order to reach the maximal velocity rate of alkaline phosphatase activity (APA) and βGlc. The complete set of results presented in the present study are thus indicative of potential APA and βGlc. For both activities, 2 ml duplicate samples and respective controls were incubated with 50 µl of substrate solution for 3 h in the dark at the temperature and pH of the cultures. The hydrolysis reaction was halted by the addition of a 4% final concentration of buffered (pH 8) formaldehyde for APA and 200 µl of a buffered (pH 10.5) solution of 0.2 M $NH_4Cl$ / 0.05 M glycine for βGlc. 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, emission at 460 nm). A buffered solution of borate (0.1 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 µm activities were calculated as the difference between total and <3 µm activities, and the 0.2–3 µm activities as the difference between <3 and <0.2 µm activities. The 0.2–3 µm 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 µm βGlc. In addition, no βGlc was found in the <0.2 µm fraction. We are confident that the increase in MUF fluorescence for APA and βGlc was linear over the single incubation time used (3 h), given that 0.02–12% of MUF-P and 0.02–0.08% of MUF-β-D-glucopyranoside were hydrolyzed only at the end of incubation.

The statistical calculations (SD 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 ± 0.01 µM) to 88 h (0.01 ± 0.004 µM), whereas nitrate + nitrite and silicate concentrations remained high (Fig. 2a). By contrast, in P-replete conditions, the fast *Thalassiosira weissflogii*

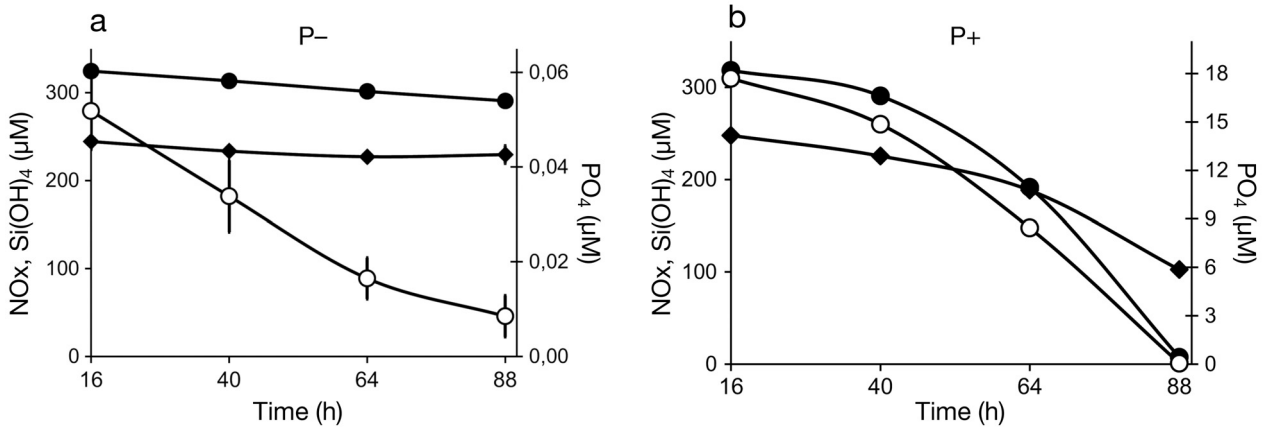


Fig. 2. Nitrate + nitrite (black dots, left scale), silicate (black diamonds, left scale) and phosphate (empty dots, right scale) concentrations in (a) P-deplete (P–) and (b) P-replete (P+) *Thalassiosira weissflogii* cultures. Error bars: SD corresponding to culture triplicates. All error bars are shown

growth exhausted $PO_4$ and nitrate + nitrite after 88 h (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 d of growth are representative of replete conditions and presented in the study. *T. weissflogii* cell number increased 10-fold between 16 and 88 h (Fig. 3) with a cell µ of 0.73 ± 0.16 $d^{-1}$ (mean ± 95% CI). 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. 4). Attached bacteria contributed to 39 ± 8 and 34 ± 4% (n = 12) of the total bacteria abundance in P-deplete and replete conditions, respectively (data not shown).

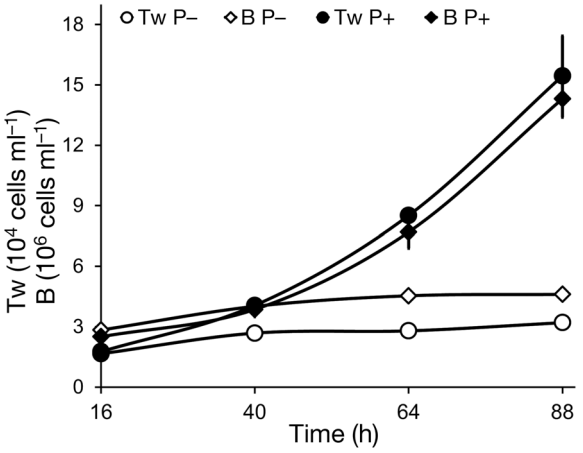


Fig. 3. *Thalassiosira weissflogii* (Tw, dots) and bacterial abundance (B, diamonds) in P-deplete (P–) and P-replete (P+) cultures. Error bars: SD corresponding to culture triplicates. All error bars are shown

### 3.2. Physiological indicators of 2 different cell P states

Several physiological parameters indicate that *T. weissflogii* and bacteria actually developed 2 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 inversely related to growth rate under non-Si 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 Si occurs; Claquin et al. 2002), resulting in a higher Si:C ratio in P-depleted cells compared to P-repleted cells. The low Si:C ratio at 88 h 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 4 d. 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 2 treatments from 16–88 h. P-repleted cells stocked more P as $PO_4$, pyro- and polyphosphate forms (luxury uptake) and used reserve P to support diatom growth. This re-

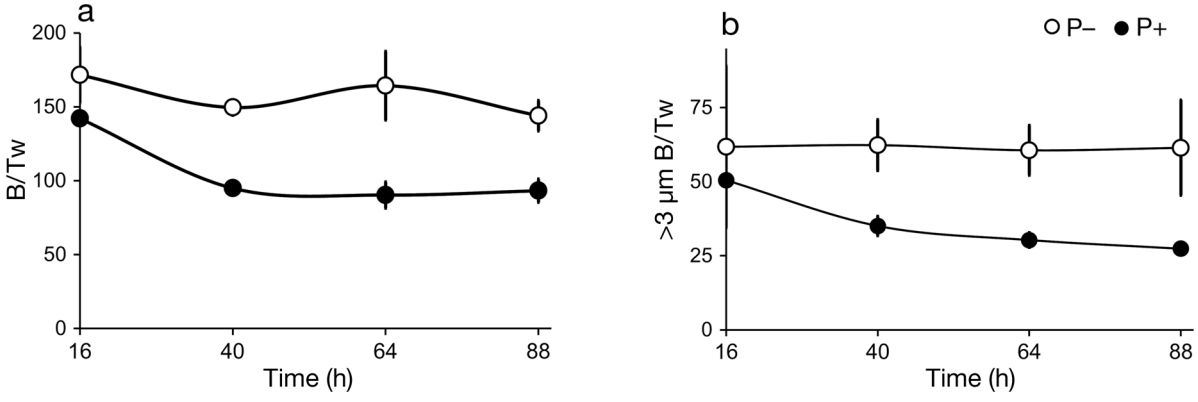


Fig. 4. Ratio of (a) total and (b) attached (>3 µm) bacteria (B) to *Thalassiosira weissflogii* (Tw) in P-deplete (P–) and P-replete (P+) cultures. Error bars: SD corresponding to culture triplicates. All error bars are shown

Table 1. Mean (SD) values (n = 3), after 16 and 88 h, of the cellular C, N, P and Si content of *Thalassiosira weissflogii* ($Q_C$, $Q_N$, $Q_P$, $Q_{Si}$), particulate C:N, Si:C, C:P, and N:P atomic ratios (at:at), the particulate inorganic phosphorus (PIP) contribution to total particulate phosphorus (TPP), specific particulate alkaline phosphatase activity (APA) per TPP and specific 0.2–3 µm APA per bacterial cell for the P-deplete (P–) and replete (P+) cultures

| Physiological parameters | P– | | P+ | |
|---|---|---|---|---|
| | 16 h | 88 h | 16 h | 88 h |
| $Q_C$ (pmol C cell$^{-1}$) | 11 (2) | 11 (1)[a] | 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)[a] | 7.0 (0.5) | 7.9 (0.01) |
| Si:C at:at | 0.5 (0.1) | 0.4 (0.1)[a] | 0.3 (0.0) | 0.1 (0.0) |
| C:P at:at | 167 (19) | 266 (24)[a] | 54 (8) | 89 (2) |
| N:P at:at | 22 (1) | 33 (4) | 8 (2) | 11 (0.3) |
| PIP (%) | 41 (4) | 31 (2)[a] | 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) |

[a]Values after 64 h rather than 88 h

serve of inorganic P may have been used for diatom survival in P-depleted cells, as shown by the low PIP contribution to TPP at the beginning of 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. 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 5–44 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 DCHO

The total particulate R and S CHO 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 5 times higher in P-replete conditions at 88 h 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 ± 20 pg cell$^{-1}$) was comparable with reserve β-glucan concentrations previously reported for *T.*

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

| 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 |

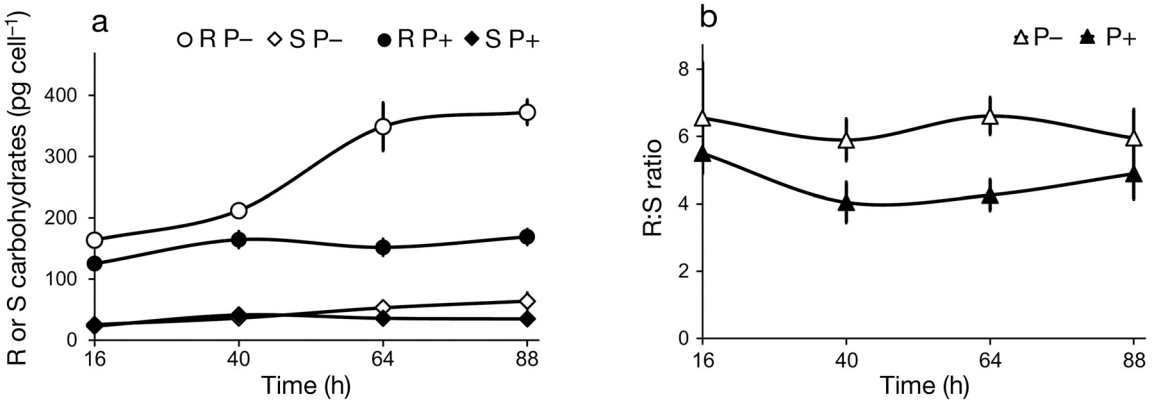


Fig. 5. Reserve (R) and structural (S) particulate carbohydrates (a) normalized to *Thalassiosira weissflogii* cells and (b) the R:S ratio in P-deplete (P–) and P-replete (P+) cultures. Error bars: SD corresponding to culture triplicates. All error bars are shown

*weissflogii* (from 364–412 pg cell$^{-1}$; Størseth et al. 2005). In both treatments, R largely dominated over S (Fig. 5b). The R:S ratios were greater ($p < 0.001$, Mann-Whitney test) in P-depleted (range: 5.9–6.6) than in P-repleted cells (range: 4.0–5.5). R CHO stayed constant in P-replete, while it increased in P-deplete between 40 and 64 h before stabilizing until 88 h. Simultaneously, the concentrations of TEP increased in P-replete from 40–88 h and were higher than in P-deplete cultures where the TEP concentrations started to increase when R CHO stabilized (Fig. 6a). They showed very similar values to S with which a significant correlation was found (Spearman correlation, $r = 0.61$, $n = 11$, $p < 0.05$ for P-deplete cultures; $r = 0.92$, $n = 10$, $p < 0.001$ for P-replete cultures; Fig. 6b).

Regarding the dissolved compartment, 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: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 βGlc showed higher values in P-deplete than in P-replete treatments (Fig. 7a). Attached bacteria dominated potential βGlc throughout the experiment both in P-deplete and P-replete conditions, with cell count contributions of 78 ± 3 and 65 ± 6 % (n = 12), respectively (data not shown). They developed a greater enzymatic potential to hydrolyze

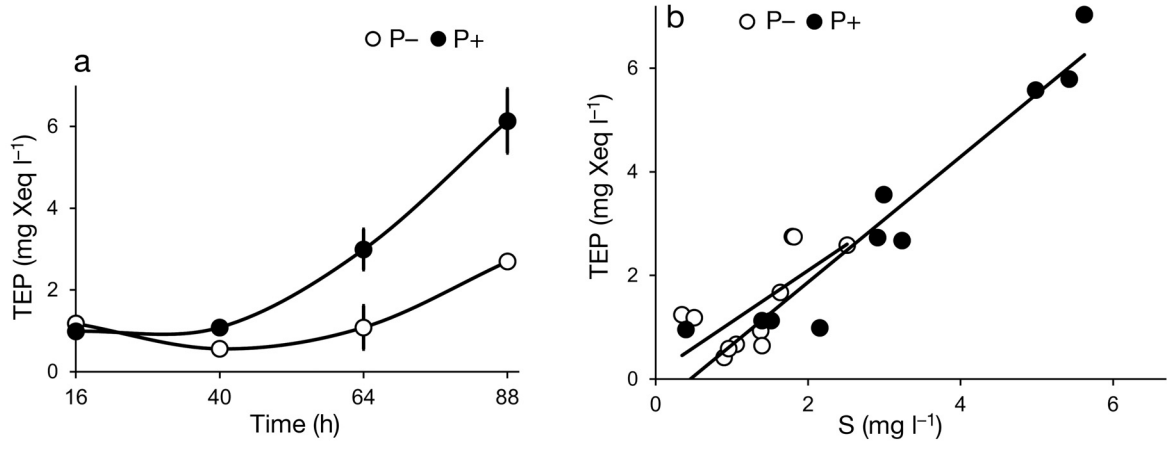


Fig. 6. (a) Total transparent exopolymer particles (TEP) in P-deplete (P–) and P-replete (P+) cultures and (b) their relationship with structural particulate polysaccharides (S). Error bars: SD corresponding to culture triplicates. All error bars are shown

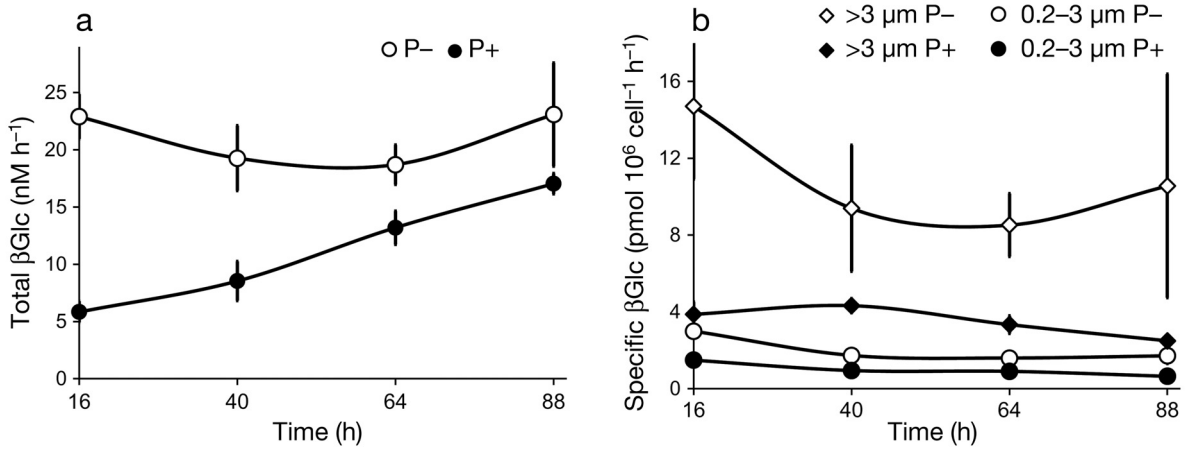


Fig. 7. (a) Total potential β-glucosidase activities (βGlc) and (b) specific βGlc normalized to bacterial cells for attached (>3 µm) and free (0.2–3 µm) bacteria in P-deplete (P–) and P-replete (P+) cultures. Error bars: SD corresponding to culture triplicates. All error bars are shown

polysaccharides than their free-living counterparts, as suggested by the higher specific βGlc calculated separately for attached and free bacteria (Fig. 7b). This pattern is even more pronounced under P-deplete conditions: specific βGlc of attached bacteria were 4.6–6.8 times greater than that of free bacteria in this condition, whereas they were only 2.6–4.6 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 C taken up by bacteria in aquatic ecosystems is mineralized (Del Giorgio & Cole 1998). Through a decay experiment using glucose as a C source, Panhota et al. (2007) showed that the production of polysaccharides by a natural bacterial community represented <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 CHO C 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 CHO

The present study highlights that P-deplete conditions promote accumulation of CHO in diatom cells, mainly as R polysaccharides: the ratio of R:S polysaccharides 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 CHO in phytoplankton. Granum & Myklestad (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 (0.34–0.84) 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 ratios (<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 & Myklestad (2002), a higher contribution of diatoms in microalgal communities could lead to a higher R polysaccharide proportion in particulate matter. This proportion increases in P-

deplete conditions. Since R in diatoms are essentially composed of glucose polymers (Myklestad & Haug 1972, Granum et al. 2002), the consequence may be a higher bioavailable pool of CHO 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 DCHO

DCHO 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 occurred at 2 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 βGlc found as early as 16 h after inoculation in P-deplete conditions is an indicator of a high P-DCHO

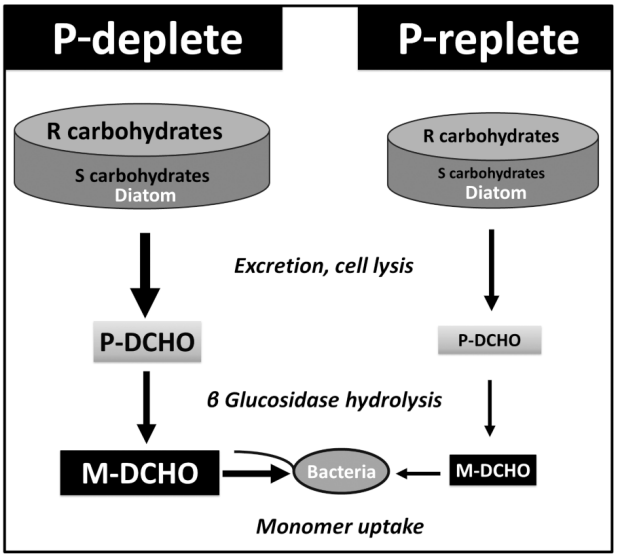


Fig. 8. Representation of the dissolved mono- (M-DCHO) and polysaccharides (P-DCHO) and particulate reserve (R) and structural (S) carbohydrate pools and cell specific fluxes in P-deplete and P-replete culture conditions for diatoms and bacteria

hydrolysis, even if these activities are not actual *in situ* rates (see discussion below, Section 4.3). Microorganisms 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 µM). 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 (1.5–5.5 µM P-DCHO) is high enough to avoid this competitive inhibition. If bacteria are not net CHO producers in the 2 P treatments, they could have modified the CHO composition excreted by *T. weissflogii* via their enzymatic equipment and, ultimately, changed their ability to aggregate particles. The very high values of potential specific βGlc in P-deplete treatment as early as 16 h after inoculation 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-glucosidase 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 β-glucosidases. Whatever their origin, the major contributors to these high potential βGlc are bacteria attached to >3 µm particles. In terms of bacterial cell numbers, they are the lowest contributors. Such differences in specific βGlc 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 βGlc 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 dissolved organic carbon accumulation

Saad et al. (2016) found that DCHO 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 a 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 290–330 µM) and P-replete conditions (160–260 µM). These values are in the range generally found in such experiments (>200 µM). DOC accumulation may have represented up to 100% of POC in P-deplete conditions. It decreased from 70–15% of POC in P-replete experiments as diatom biomass increased. Such a difference between replete conditions (i.e. blooming phase), and nutrient-deplete conditions (post bloom) concurs with the accumulation of C-rich dissolved organic matter observed in surface marine environments (Williams 1995, Thingstad et al. 1997). This accumulation was attributed to the imbalance between excretion and lysis processes consecutive with 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 CHO 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 a high proportion of polysaccharides as R pools in particulate matter. In addition, the fact that bacteria were also P-depleted might have stressed this accumulation of R polysaccharides, by competing for $PO_4$. 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 when evaluating the effect of P depletion on CHO dynamics and subsequent consequences for particle potential aggregation.

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