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Phosphorus availability modifies carbon production in *Coccolithus pelagicus* (Haptophyta)

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ABSTRACT

The coccolithophore *Coccolithus pelagicus* (Wallich) Schiller fixes CO_2 into particulate organic carbon (POC) through photosynthesis and into particulate inorganic carbon (PIC) in the form of calcite. To examine the role of phosphorus (P) availability in the production of POC and PIC, *C. pelagicus* subsp. *braarudii* (Gaarder) Geisen et al. was grown in semi-continuous cultures at three initial phosphate concentrations (P-replete, 1, and 0.5 μM [P]). Reduced P-availability (1 and 0.5 μM [P]) decreased POC production, while PIC production only decreased when phosphate concentrations became growth limiting (0.5 μM [P]). This decrease has not been observed previously in batch cultures, highlighting the inadequacy of the batch culture approach with regard to determining carbon production. The reduction in growth rate by 50% at 0.5 μM [P] was accompanied by a doubling in cell volume (and POC). PIC production was halved, resulting in a lowered PIC to POC ratio. The average number of coccoliths per cell (and PIC content) remained the same among treatments, despite the significant change in cell size. Our data suggest that POC production in *C. pelagicus* is more sensitive towards a moderate reduction in phosphorus availability than PIC production. Once phosphorus availability limits cell division, however, phosphorus resources are invested into POC rather than PIC production. This reduces cell density and sinking rates, indicating that coccoliths do not act as ballast for reaching deeper nutrient-rich layers under nutrient limitation.

1. Introduction

Coccolithophores are eukaryotic microalgae that form substantial blooms in the marine environment (Holligan et al., 1983). Their cells are covered with calcite scales, coccoliths, which act as an effective mineral ballast for removing particulate organic carbon (POC) and particulate inorganic carbon (PIC) from surface waters (Ziveri et al., 2007). Ocean–atmosphere CO_2 fluxes are influenced by both the rate of calcification (PIC production) and the rate of CO_2 fixation into organic carbon (POC production). On short time scales, the production of PIC in the form of coccoliths shifts the carbonate system of sea surface waters towards higher CO_2 and lower pH levels, whereas POC production shifts the seawater carbonate system towards lower CO_2 and higher pH levels (Rost and Riebesell, 2004). The ratio of PIC to POC production (PIC/POC ratio) therefore influences whether CO_2 is released or removed from surface waters (Gattuso et al., 1995). This ratio is altered by environmental parameters such as carbonate chemistry (Riebesell et al., 2000), temperature (Gerecht et al., 2014; Paasche, 1968; Watabe and Wilbur, 1966), nutrient availability (Paasche and Brubak, 1994), salinity (Paasche et al., 1996) and light availability (Paasche, 1964, 1999).

Coccolithus pelagicus is a large, heavily calcified coccolithophore that blooms in upwelling areas (Cachão and Moita, 2000). This species is divided into at least two morphotypes (Geisen et al., 2002) of which *C. pelagicus* subsp. *braarudii* occurs in temperate and *C. pelagicus* subsp. *pelagicus* in subarctic oceans (Ziveri et al., 2004). Its PIC/POC ratio is generally >1.5 (Krug et al., 2011; Langer et al., 2006) and its contribution to calcite production may outweigh that of the ubiquitous *Emiliania huxleyi* (Daniels et al., 2014). *C. pelagicus* subsp. *braarudii* is insensitive to short-term IPCC-scenario perturbations of the carbonate system (Langer et al., 2006), but calcification decreases once CO_2-concentrations are high enough to push the calcite saturation state below unity (Krug et al., 2011). Little is known about the effect of nutrient availability on carbon production in this species. A recent study has shown that phosphorus (P) limitation does not affect the PIC/POC ratio of either subspecies of *C. pelagicus* when these are grown in batch culture (Gerecht et al., 2014). However, in batch culture it is impossible to accurately determine production in nutrient-limited cultures using cellular content. The

Abbreviations: A_T, total alkalinity; P, phosphorus; [P], initial phosphate concentrations; PIC, particulate inorganic carbon; POC, particulate organic carbon; POP, particulate organic phosphorus; TPC, total particulate carbon.

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Table 1
Physiological parameters: exponential growth rate (average over 10 dilutions as in Fig. 1A,C), carbon to phosphorus ratio, particulate organic phosphorus, carbon and inorganic carbon content and production as well as inorganic to organic carbon ratio of *Coccolithus pelagicus* subsp. *braarudii* in semi-continuous cultures at three initial phosphate concentrations (P-replete, 1, 0.5 μM). Average ± SD, n = 3.

	P-replete	1 μM	0.5 μM
μ_{exp} [d^{-1}]	0.36 ± 0.017	0.33 ± 0.005	0.16 ± 0.004
POC/POP [mol mol^{-1}]	77 ± 19	100 ± 10	133 ± 7
POP [pg $cell^{-1}$]	9.6 ± 3.6	5.8 ± 1.1	8.8 ± 4.8
POP [pg $cell^{-1}$ d^{-1}]	3.4 ± 0.7	1.9 ± 0.4	1.4 ± 0.2
POC [pg $cell^{-1}$]	276 ± 17	224 ± 19	450 ± 29
POC [pg $cell^{-1}$ d^{-1}]	98.1 ± 3.2	72.7 ± 3.3	72.2 ± 7.0
PIC [pg $cell^{-1}$]	304 ± 25	341 ± 38	357 ± 16
PIC [pg $cell^{-1}$ d^{-1}]	108.4 ± 7.6	110.8 ± 7.1	57.2 ± 1.8
PIC/POC	1.11 ± 0.10	1.52 ± 0.03	0.80 ± 0.08

cellular POC and PIC contents have been measured only at the end of experiments once the growth rate is reduced to zero (Benner, 2008; Gerecht et al., 2014; Langer et al., 2012, 2013). The cellular content is therefore uncoupled from the overall growth rate used to calculate production, which is not constant over the course of the experiment (Langer et al., 2012, 2013). To calculate production in nutrient-limited batch cultures, a different approach is necessary e.g. using a radioactive tracer (Paasche, 1964). Alternatively, cultures can be grown continuously or semi-continuously to ensure constant cell division rates. In contrast to the severe P-limitation experienced only briefly by stationary phase batch cultures, semi-continuous cultures can grow exponentially at quasi-constant cell division rates under reduced P-availability over many generations. Semi-continuous cultures can therefore highlight different acclimation strategies of an algal species to various scenarios of nutrient availability.

The aim of the current study was to examine the role of P-availability in carbon production in *C. pelagicus* subsp. *braarudii* using an appropriate semi-continuous set-up. This set-up was used to address the question of how resources are allocated to PIC and POC production under non-growth-limiting and growth-limiting phosphate concentrations and allowed us to observe possible acclimation strategies to P-limitation in *C. pelagicus*.

2. Materials & methods

2.1. Cultures

Triplicate semi-continuous cultures of *C. pelagicus* subsp. *braarudii* (Roscoff Culture Collection strain RCC1200) were grown in modified K/2 medium (Gerecht et al., 2014) at three initial phosphate concentrations [P] (10, 1, and 0.5 μM). The cultures were kept at 15 °C in an environmental test chamber (MLR-350, Panasonic, Japan), on a 12:12 h light:dark cycle at an irradiance of ca. 100 μmol photons m^{-2} s^{-1}. The cultures were inoculated at ca. 2000 cells mL^{-1} and cell concentrations as well as coccosphere diameters were determined daily using an electronic particle counter (CASY, Roche Diagnostics, Switzerland). The cultures were diluted with fresh medium down to initial cell concentrations (ca. 2000 cells mL^{-1}) upon reaching a concentration of 4000–5000 cells mL^{-1} to ensure a complete cell cycle between dilutions. Maximum cell concentrations were well below stationary phase

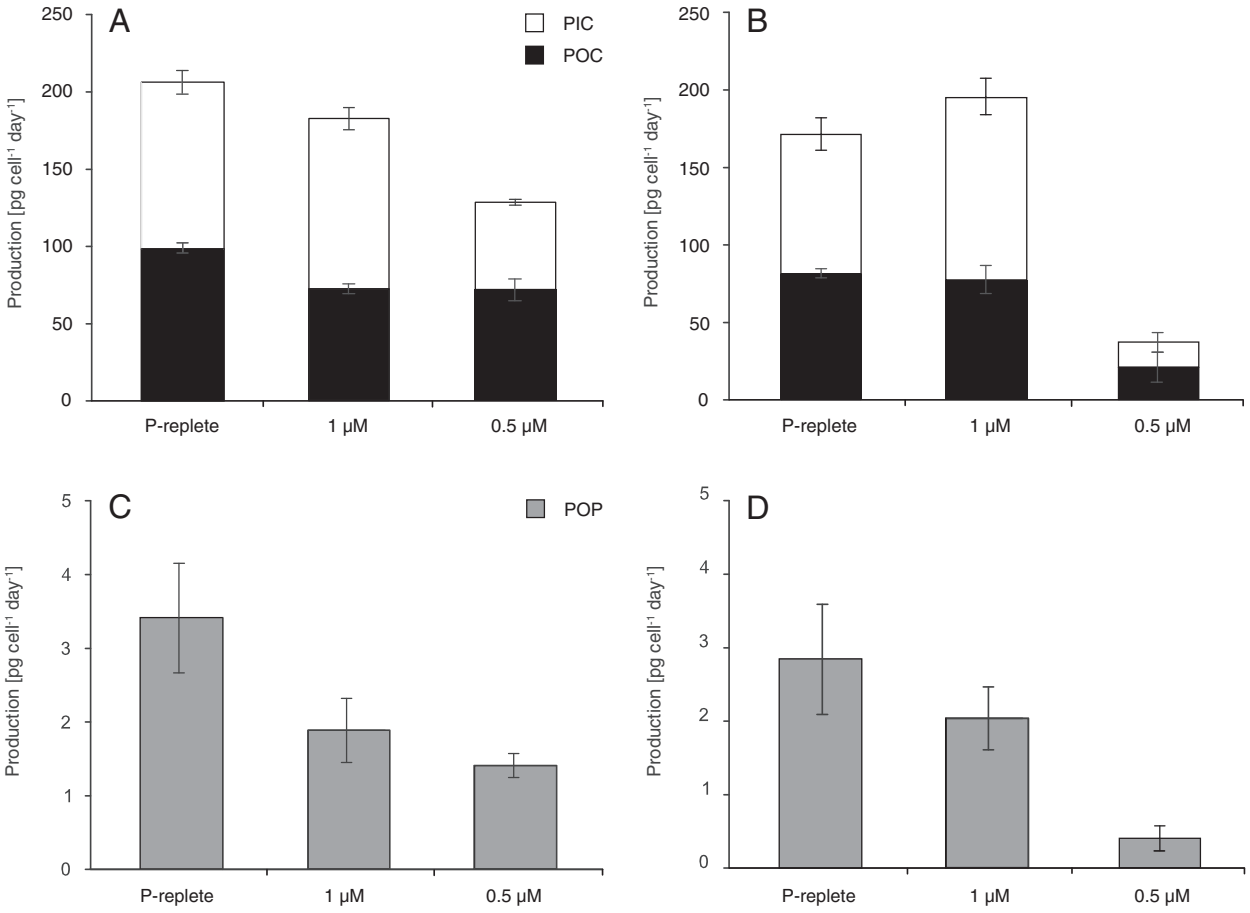

Fig. 1. Production of particulate inorganic and organic carbon (PIC, POC: A, B) and phosphorus (POP: C, D) in semi-continuous cultures of *Coccolithus pelagicus* subsp. *braarudii* at three initial phosphate concentrations (P-replete, 1, 0.5 μM), calculated using the average growth rate over all dilution cycles (A, C) and the growth rate of the last dilution cycle only (B, D). Average ± SD, n = 3.

even for 0.5 μM [P] cultures so that all cultures were kept continuously in exponential growth. Exponential growth rates (μ_{exp}) were calculated by linear regression of log-transformed cell densities over time for each dilution and averaged over the 10 dilution cycles. The cultures were harvested after completing 10 dilution cycles (≈ generations).

2.2. Medium chemistry

2.2.1. Phosphate concentrations

Phosphate concentrations were determined in the culture media before inoculation and again upon sampling of the cultures. The media were sterile filtered (0.2 μm) into plastic scintillation vials (Kartell, Germany) and stored at −20 °C until analysis. Phosphate concentrations were determined colorimetrically on a spectrophotometer (UV 2550, Shimadzu, Japan) as molybdate reactive phosphate following Murphy and Riley (1962) with a precision of ±4%.

2.2.2. Carbonate chemistry

Total alkalinity (A_T) and pH of the medium were determined before inoculation of the cultures and again upon sampling of the cultures to ensure that the carbonate chemistry was similar among the treatments. Samples for A_T were filtered through GF/F-filters (Whatman, GE Healthcare, UK), stored airtight at 4 °C and analyzed within 24 h. A_T was calculated from Gran plots (Gran, 1952) after duplicate manual titration with a precision of ±50 μmol kg^{-1}. The pH was measured with a combined electrode (Red Rod, Radiometer, Denmark) that was two-point calibrated to NBS scale (precision ± 0.03). The carbonate system was calculated by means of the program CO2sys (version 2.1 developed for MS Excel by D. Pierrot from E. Lewis and D. W. R. Wallace) using A_T and pH as input parameters. The dissociation constants for carbonic acid of Roy et al. (1993) were chosen.

Inferring the carbonate system from A_T is not ideal when varying phosphate concentrations as phosphate itself makes a minor contribution to A_T (Dickson, 1981). However, in the present set-up the aim was to have similar carbonate chemistry among the treatments and to confirm that the treatments were not carbon-limited or undersaturated in calcite ($\Omega_{calcite} < 1$). This was easily achieved because the maximum error due to varying phosphate concentrations in our experimental set-up corresponds to 0.02 pH units or 0.15 in terms of $\Omega_{calcite}$. As we included phosphate concentrations in our carbonate system calculations, the real error is even smaller and therefore does not affect our conclusions.

2.3. Elemental production

2.3.1. Particulate organic phosphorus

Samples for particulate organic phosphorus (POP) were filtered onto precombusted (500 °C, 2 h) GF/C-filters (Whatman) and stored at −20 °C. POP was converted to orthophosphate by oxidative hydrolysis with potassium persulfate under high pressure and temperature in an autoclave (3150EL, Tuttnauer, Netherlands) according to Menzel and Corwin (1965). Converted orthophosphate was then quantified as molybdate reactive phosphate as described in Section 2.2.1.

2.3.2. Particulate organic and inorganic carbon

Samples for total particulate carbon (TPC) and particulate organic carbon (POC) were filtered onto precombusted GF/C-filters, dried at 60 °C overnight in a drying oven and stored in a desiccator until analysis on an elemental analyzer (Flash 1112, Thermo Finnegan, USA; detection limit 2 μg; precision ± 8%). Particulate inorganic carbon (PIC) was removed from POC filters by pipetting 230 μL of 2 M HCl onto the filters before analysis (Langer et al., 2009) and calculated as the difference between TPC and POC.

Average daily production in the treatments was calculated by multiplying TPC with μ_{exp} averaged over all dilution cycles (Table 1; Fig. 1A,C) and compared to those calculated from μ_{exp} during the last dilution cycle (Fig. 1B,D). As TPC by elemental analysis was determined only at the end of the experiment, a proxy for TPC was estimated for each dilution cycle from CASY measurements of coccosphere diameter (Section 2.1) converted to volume (Fig. 3). CASY size measurements are based on the electrical resistance of particles. Therefore, coccosphere volume is underestimated by CASY (unpublished data). This is also known for Coulter Counter measurements, which are based on a similar principle (Oviedo et al., 2014). With CASY we therefore measured a diameter larger than the cell and smaller than the coccosphere diameter, which we used to estimate "TPC" of the cells using the constants of Menden-Deuer and Lessard (2000) (Carbon [pg $cell^{-1}$] = 0.216 × $volume^{0.939}$). Although these constants are applicable only to POC, whereas we had a contribution of PIC (coccosphere) to cell diameter, we here used them to calculate a proxy for carbon production for the purpose of comparison, which we refer to as "total carbon production" in the following.

Table 2a
Initial chemistry (initial phosphate, total alkalinity, pH) of the medium used in semi-continuous cultures of *Coccolithus pelagicus* subsp. *braarudii* at three initial phosphate concentrations (P-replete, 1, 0.5 μM). Average, n = 2.

	P-replete	1 μM	0.5 μM
Initial PO_4 [μM]	7.06	1.11	0.41
A_T [μmol L^{-1}]	2100	2300	2250
pH (NBS)	7.96	7.81	8.00

2.4. Cell geometry

2.4.1. Cell diameter

Cell diameters were measured from light microscopy images (200×) of live cells after dissolving the coccoliths with 0.1 M HCl (19 μL to 1 mL sample). This decreased the pH to just below pH = 4 which led to coccolith dissolution without causing visible changes to the cell membrane. There was no noticeable shrinkage of the cells due to the acid addition. This was confirmed by comparing cell diameters measured within intact coccospheres to cell diameters measured after coccolith removal.

2.4.2. Coccosphere and coccolith measurements

Coccosphere diameters were measured from scanning electron microscopy images captured with an S-4800 field emission scanning electron microscope (Hitachi, Japan) at 2500× magnification. The number of coccoliths per coccosphere was estimated from the same image by counting visible, forwards facing coccoliths, multiplying by two to account for the coccoliths on the back side of the coccosphere and adding the number of coccoliths lying around the edge of the coccosphere in lateral view. Average coccolith length was calculated from forwards facing coccoliths. Coccolith morphology was classified into the four categories normal, incomplete, malformed, and very malformed following Gerecht et al. (2014).

Table 2b
Medium chemistry (residual phosphate, carbonate chemistry) of *Coccolithus pelagicus* subsp. *braarudii* in semi-continuous cultures at three initial phosphate concentrations (P-replete, 1, 0.5 μM) upon sampling. Average ± SD, n = 3.

	P-replete	1 μM	0.5 μM
Residual PO_4 [μM]	7.21 ± 0.67	0.41 ± 0.09	0.10 ± 0.03
A_T [μmol L^{-1}]	2000 ± 50	2050 ± 50	2150 ± 50
pH (NBS)	7.80 ± 0.02	7.82 ± 0.05	8.00 ± 0.04
DIC [μmol L^{-1}]	1900 ± 50	1950 ± 50	1950 ± 50
HCO_3^- [μmol L^{-1}]	1850 ± 100	1800 ± 50	1800 ± 50
CO_3^{2-} [μmol L^{-1}]	89 ± 5	97 ± 13	146 ± 11
CO_2 [μmol L^{-1}]	25 ± 2	24 ± 3	16 ± 2
pCO_2	650 ± 50	640 ± 80	410 ± 50
$\Omega_{calcite}$	3.7 ± 0.1	2.3 ± 0.3	3.5 ± 0.3

Table 3
Cell geometry (cell and coccosphere diameter, coccolith number and length) as well as cell density and sinking rates in semi-continuous cultures of *Coccolithus pelagicus* subsp. *braarudii* at three initial phosphate concentrations (P-replete, 1, 0.5 μM). Average ± SD.

	P-replete	1 μM	0.5 μM
Cell diameter [μm]	14.9 ± 1.2 (n = 36)	14.9 ± 1.1 (n = 28)	19.1 ± 2.6 (n = 38)
Coccosphere diameter [μm]	18.6 ± 1.9 (n = 256)	19.3 ± 2.1 (n = 266)	21.7 ± 3.7 (n = 177)
Coccolith sphere^{-1}	14 ± 3 (n = 272)	14 ± 4 (n = 272)	14 ± 5 (n = 219)
Coccolith length [μm]	10.5 ± 0.9 (n = 64)	10.5 ± 0.9 (n = 64)	10.7 ± 1.0 (n = 78)
Cell density [g mL^{-1}]	1.48 ± 0.01 (n = 3)	1.46 ± 0.01 (n = 3)	1.33 ± 0.03 (n = 3)
Sinking rates [μm s^{-1}]	70.9 ± 1.8 (n = 3)	73.6 ± 3.9 (n = 3)	64.5 ± 4.6 (n = 3)

2.4.3. Cell density and sinking rates

Cell density was calculated as in Hoffmann et al. (2015) using the cell diameter (Section 2.4.1, Table 3) to calculate cell volume and the constants of Menden-Deuer and Lessard (2000) to infer cellular POC content from cell volume. The cellular PIC content was inferred from the average coccolith length (Section 2.4.2, Table 3), the average coccolith number per coccosphere (Section 2.4.2, Table 3) and a shape factor (Young and Ziveri, 2000). The difference between inferred cellular POC and PIC content and that measured by elemental analysis (Section 2.3.2) was at most 10%. Sinking rates [μm s^{-1}] were calculated from cell density using Stokes' equation: $2 \times (\text{cell density} - \text{seawater density}) \times g \times r^2 / (9 \times V)$ with seawater density = 1.024 g mL^{-1}, g = 9.81 m s^{-2}, r = coccosphere radius (Section 2.4.2), and V = 1.07 g m^{-1} s^{-1} (dynamic viscosity of seawater at 34 ppm salinity and 15 °C).

3. Results

3.1. Phosphorus-limited growth

The low value (ca. 7 μM) reported for [P] of the "10 μM" medium (Table 2a) was likely due to a technical limitation. Importantly, these cultures were at no time limited by the supply of phosphate. To avoid confusion, these control cultures are called "P-replete" in the following. Semi-continuous cultures at 0.5 μM [P] grew about half as fast as semi-continuous cultures supplied with 1 μM [P] or P-replete medium (Table 1). There was no significant difference in growth rate between the latter two cultures when all dilution cycles were considered (t-test: $p > 0.05$). All cultures were sampled before entering stationary phase and there were detectable levels of residual phosphate in all cultures upon sampling (Table 2b). The carbonate chemistry was similar among treatments and all cultures were saturated in calcite ($\Omega_{calcite} > 1$) (Tables 2a, 2b).

3.2. Elemental production

Semi-continuous cultures at 1 μM [P] had a lower cellular POP content, an increased C/P-ratio and a reduced POP production than P-replete cultures (Table 1; Fig. 1C,D). The cellular POP content of 0.5 μM [P] cultures was similar to that of P-replete cultures. However, the C/P-ratio was almost twice as high at 0.5 μM [P] (Table 1) because these cultures contained less POP in respect to POC. The high cellular POP content was therefore due to the large size of the cells (Table 3; Figs. 2,5). In fact, cells of the 0.5 μM [P] cultures contained ca. twice as much POC as those of 1 μM [P] and P-replete cultures (Table 1, Fig. 2A) and POP production was lowest in this treatment (Table 1, Fig. 1C,D).

Although 1 μM [P] and P-replete cultures had the same cell volume (t-test: $p > 0.05$), cells grown at 1 μM [P] contained ca. 20% less POC (Table 1, Fig. 2A). Production of POC was reduced by ca. 25% in both low-P cultures (0.5 and 1 μM [P]) compared to P-replete cultures (Table 1; Fig. 1A). The cellular PIC content on the other hand was similar among treatments (one-way ANOVA: $p = 0.04$) (Table 1, Fig. 2B). This resulted in a low PIC/POC ratio of 0.80 ± 0.08 in 0.5 μM [P] cultures whereas the PIC/POC ratio was highest (1.52 ± 0.03) in 1 μM [P] cultures (Table 1). Production of PIC was the same between 1 μM [P] and P-replete cultures (t-test: $p > 0.05$) and reduced by ca. 50% at 0.5 μM [P] (Table 1, Fig. 1A).

Cell division rates declined in 0.5 μM [P] cultures during the course of the experiment. During the last dilution cycle, the growth rate was only 0.05 d^{-1} and production of POP, POC, and PIC was therefore greatly reduced (Fig. 1B,D). Although there was a high degree of variability over the course of the experiment in 1 μM [P] and P-replete cultures, using a proxy we could show that "total carbon production" was overall similar between these treatments (Fig. 3).

3.3. Cell geometry

The number of coccoliths covering a cell increased with increasing cell size within each treatment (Fig. 4). There was no difference among the treatments in the average number of coccoliths (ca. 14) covering one cell (one-way ANOVA: $p > 0.05$), despite distinct offsets in coccosphere surface area among the treatments (Fig. 4, Table 3). This explains the similar cellular PIC content among all treatments (Table 1) and the looser arrangement of coccoliths around the larger cells of the 0.5 μM [P] treatment (Fig. 5). This led to a difference among the treatments in the density of the cell and the theoretically

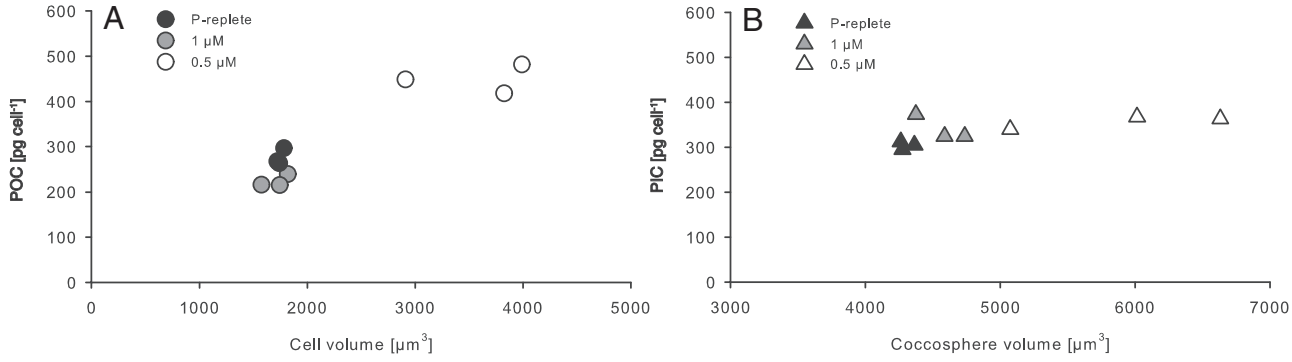

Fig. 2. A) Particulate organic carbon (POC) plotted over mean cell volume and B) particulate inorganic carbon (PIC) plotted over mean coccosphere volume of *Coccolithus pelagicus* subsp. *braarudii* in semi-continuous cultures at three initial phosphate concentrations (P-replete, 1, 0.5 μM). Triplicate semi-continuous cultures are presented individually.

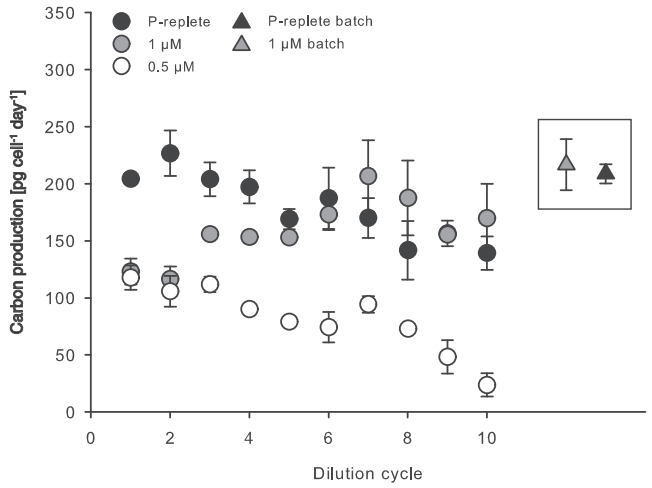

Fig. 3. "Total carbon production" for each dilution cycle of semi-continuous cultures of *Coccolithus pelagicus* subsp. *braarudii* at three initial phosphate concentrations (P-replete, 1, 0.5 µM). The insert shows "total carbon production" for batch cultures of the same strain at 10 and 1 µM [P] from Gerecht et al. (2014). Average ± SD, n = 3.

calculated sinking rate. Cell density and sinking rates were similar in 1 µM [P] and P-replete cultures and reduced in 0.5 µM [P] cultures (one-way ANOVA: $p = 0.054$; Table 3; Fig. 6).

The average coccolith length was the same among treatments (one-way ANOVA: $p > 0.05$; Table 3). There was a significant decrease in the proportion of normal coccoliths with a concomitant increase in the proportion of malformed coccoliths, especially of coccoliths classified as "very malformed", from P-replete to 0.5 µM [P] cultures (X^2-test: $p < 0.05$, df = 2; Fig. 7).

4. Discussion

4.1. Semi-continuous vs. batch culture

Using semi-continuous cultures we could show that carbon production in *C. pelagicus* is reduced at growth-rate limiting P-availability (0.5 µM [P]). This has not been observed in batch cultures (Gerecht et al., 2014). On the contrary, carbon production was the same in exponentially growing non-limited cultures and cultures that entered stationary phase because of P-limitation (see insert, Fig. 3). As already highlighted by Langer et al. (2012), production cannot be calculated in nutrient-limited batch cultures using the final cellular carbon content and the overall growth rate. If this is nevertheless done, the result is, more often than not, an overestimation of production. This is the case in *E. huxleyi* (strain B92/11; Langer et al., 2013) and, as shown here, also in *C. pelagicus* subsp. *braarudii*. The same strain of *C. pelagicus* subsp. *braarudii* (RCC1200) was shown to retain its PIC production under nitrogen limitation in batch culture (Benner, 2008). Considering our data on P-limitation (Figs. 1,3) together with the data presented in Gerecht et al. (2014), we conclude that the apparently unabated PIC production under nitrogen limitation (Benner, 2008) is most likely an artifact of the batch approach. With the semi-continuous set-up used here, it was possible to calculate a proxy for total carbon production for each dilution cycle, showing that sustained P-limitation reduces carbon production.

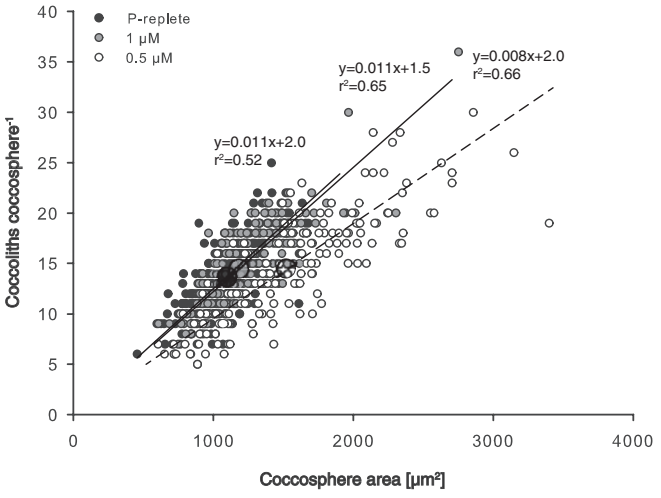

Fig. 4. Scatter plot of coccolith number per coccosphere over the surface area of that coccosphere in semi-continuous cultures of *Coccolithus pelagicus* subsp. *braarudii* at three initial phosphate concentrations: P-replete (n = 255), 1 µM (n = 266), and 0.5 µM [P] (n = 173). Large symbols represent average values. Linear regressions illustrate the offset in coccosphere allometry between the growth-limited (0.5 µM) and non-limited (P-replete, 1 µM) treatments.

The difference between semi-continuous and batch culture may be related to the different P-limitation experienced by the cultures. Whereas batch cultures are exposed briefly to a strong P-limitation in stationary phase, semi-continuous cultures are exposed to growth-limiting phosphate concentrations over multiple generations, allowing the cultures to acclimate to the decreased P-availability. Fig. 3 might serve as an illustration of this hypothesis. The "total carbon production" of the 1 µM [P] culture increased over the course of the experiment, indicating an acclimation of the P-uptake machinery. Contrarily, the 0.5 µM [P] culture decreased "total carbon production", which might point to the depletion of internal P-stores and an acclimation to P-limited growth.

At reduced phosphate concentrations that are not limiting to growth (1 µM [P]), POC production is reduced compared to PIC production, increasing the PIC/POC ratio (Table 1). This decreased production of POC under reduced P-availability (Fig. 1) may be related to the energy demands of POC production and a reduced availability of the P-rich energy carrier ATP (Beardall et al., 2005). The present data reveals two acclimation strategies of *C. pelagicus* to reduced P-availability. At moderately reduced P-availability (1 µM [P]), resources are invested into maintaining exponential growth rates, but POC production is reduced (Fig. 1A). This indicates that POC production is more sensitive to a reduction in P-availability than PIC production. Similarly, Paasche and Brubak (1994) observed a reduction in POC production in P-limited chemostats of *E. huxleyi* growing at 50% of the maximal growth rate, which raised the PIC/POC ratio by ca. 60%. Müller et al. (2008) also measured a strong increase in the PIC/POC ratio in P-limited batch cultures of *E. huxleyi*. These authors explained this increase by the different stages of the cellular cycle in which POC and PIC production take place. Once cell division stops due to a lack of phosphorus, cells accumulate in the G1 phase (assimilation phase). In this phase calcification continues and in the absence of cell division, coccoliths accumulate in multiple layers around the cell (Müller et al., 2008). However, this explanation can only be part of the picture, because the response of the PIC/POC ratio to P-limitation is highly strain-specific. In five out of six Mediterranean *E. huxleyi* strains the PIC/POC ratio decreased in response to P-limitation, while one strain showed no change (Oviedo et al., 2014). Although these authors used the batch approach, other studies show that strain-specificity can also be observed in continuous cultures (Borchard et al., 2011; Riegman et al., 2000).

There is a fundamental difference between *E. huxleyi* and *C. pelagicus* in that the former can overcalcify, producing multiple layers of coccoliths which are shed into the surrounding waters (Holligan et al., 1983; Paasche, 1998), whereas *C. pelagicus* covers its cell with a single coccolith layer. In *C. pelagicus* coccolith production therefore does not continue once cells stop dividing (Gerecht et al., 2014) and no difference was observed in the PIC/POC ratio between P-replete, exponentially growing cells and P-limited non-dividing cells in stationary phase batch cultures (Gerecht et al., 2014). As shown here, when *C. pelagicus* subsp. *braarudii* is grown under sustained P-limitation (0.5 µM [P]),

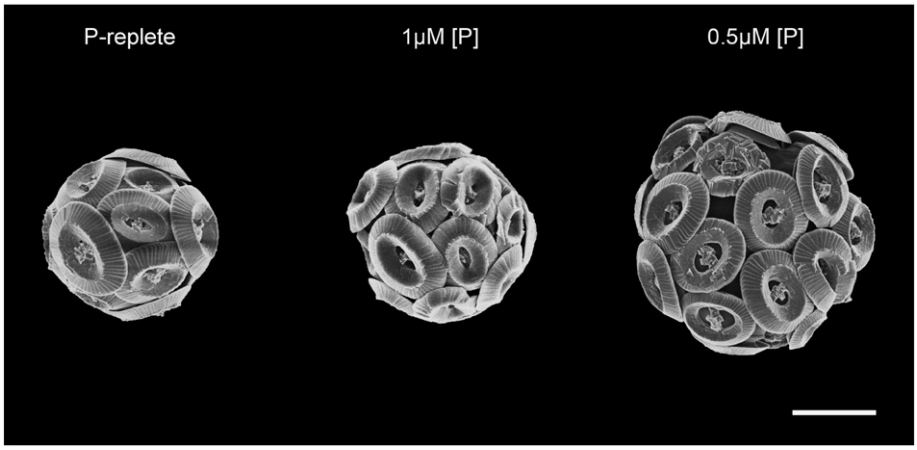

Fig. 5. Scanning electron microscopic images of individual *Coccolithus pelagicus* subsp. *braarudii* coccospheres representative for each experimental treatment (P-replete, 1, 0.5 µM) at the time of sampling. Scale bar = 10 µm.

PIC production decreases drastically leading to a low PIC/POC ratio in these cultures. This indicates a priority for using phosphorus resources to fix organic, rather than inorganic carbon under growth-limiting P-supply. This may be due to a necessary usage of photosynthetic energy and reducing equivalents that may be more efficient in POC than in PIC production.

4.2. Coccolith malformations

The increase in the proportion of malformed coccoliths in the P-limited cells indicates that P-limitation imposed a significant physiological stress on this species (Fig. 7). As the same response has been observed previously in batch culture (Gerecht et al., 2014), our experiments show that the batch approach is sufficient to analyze the response patterns of coccolith morphology (Langer et al., 2012, 2013). Furthermore, it is now clear that the effects of P-limitation on coccolith morphogenesis are species-specific, because increased malformations under P-limitation have not been observed in *Calcidiscus leptoporus* (Langer et al., 2012) or *E. huxleyi* (Oviedo et al., 2014). The lack of increase in malformations in these two species is likely related to their lower P-requirements (the cellular phosphorus content is one order of magnitude lower in *C. leptoporus* (Langer et al., 2012) and two orders of magnitude in *E. huxleyi* (Oviedo et al., 2014)) and their overall better performance in low-P environments (Zondervan, 2007). Nevertheless, the available data for *C. pelagicus* (Gerecht et al., 2014; this study), show for the first time, that coccolith malformations in field samples could be caused by nutrient limitation as hypothesized decades ago (Kleijne, 1990; Okada and Honjo, 1975).

4.3. Cell size and sinking rates

In 0.5 µM [P] cultures, there was a decrease in cell division rate over each dilution cycle with very low growth rates towards the end of the experiment (0.05 d^{-1}). This coincided with a doubling in cell volume and the accumulation of POC in the cells (Fig. 2A). A similar increase in cell size and/or POC content accompanying a reduction in growth rate has also been described for the well-studied coccolithophore *E. huxleyi* growing under P-limitation (Müller et al., 2008; Paasche and Brubak, 1994; Riegman et al., 2000). Cell size is an important factor in both diffusive nutrient uptake (Chisholm, 1992) and the PIC/POC ratio. Cell volume determines both nutrient requirements and the POC content of the cell. Surface area, on the other hand, influences diffusive nutrient uptake and determines the area available for coccoliths (PIC). Besides the differential impact of P-availability on POC and PIC production, the PIC/POC ratio is therefore also linked to coccolithophore cell size. The cell volume and cellular POC content, dependent on (cell radius)^3, increase faster with an increase in cell size than the surface area available for PIC, dependent on (cell radius)^2. Therefore, within a strain or species, large cells will have a higher POC over PIC content and conversely a lower PIC/POC ratio than small cells. This is only true for species such as *C. pelagicus* that do not accumulate coccoliths as *E. huxleyi* does. These considerations are nicely illustrated by our data set, revealing distinct cell size differences between fast and slow-growing cultures of the same strain (Figs. 4,5). Under growth-limiting conditions (0.5 µM [P]), the cell volume was doubled (Fig. 2A), increasing the area of the cell by ca. 65%. However, this increase in surface area was not paralleled by an increase in average coccolith number (Fig. 4),

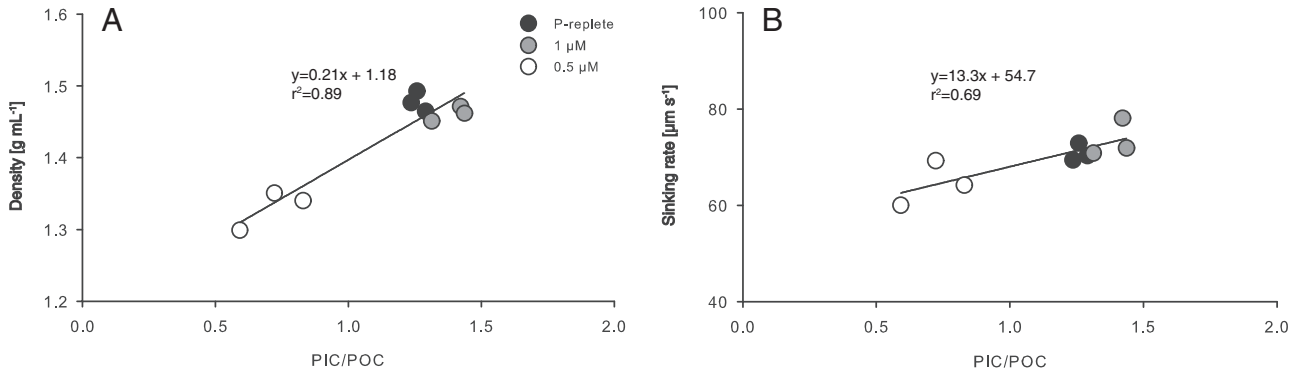

Fig. 6. A) Cell density and B) sinking rate plotted over the PIC/POC ratio in semi-continuous cultures of *Coccolithus pelagicus* subsp. *braarudii* at three initial phosphate concentrations (P-replete, 1, 0.5 µM). Triplicate semi-continuous cultures are presented individually.

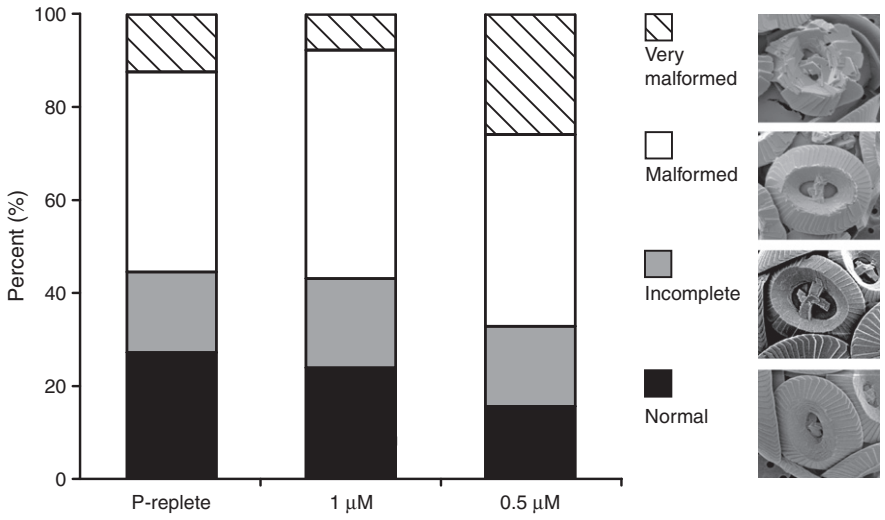

Fig. 7. Proportion of coccoliths classified as normal (black), incomplete (gray), malformed (white) and very malformed (hatched) in semi-continuous cultures of *Coccolithus pelagicus* subsp. *braarudii* at three initial phosphate concentrations: P-replete (n = 1640), 1 µM (n = 1691), and 0.5 µM [P] (n = 1622). 95% C.I. for all values 1.3–2.4%.

resulting in loosely interlocking coccospheres (Fig. 5). In consequence, the overall density of these cells was reduced leading to a reduced sinking rate of cells grown at 0.5 µM [P] (Fig. 6). Several authors have suggested that the tendency of senescent or nutrient-limited cells to acquire additional coccolith layers may be a method for increasing sinking rates (Lecourt et al., 1996; Linschooten et al., 1991; Young, 1994). However, the present data refutes this hypothesis, at least for *C. pelagicus*, as coccolith production decreased under P-limited growth. As sinking rate is positively correlated to the PIC/POC ratio (Fig. 6B; Hoffmann et al., 2015), and the PIC/POC ratio of *E. huxleyi* under P-limitation decreases in a substantial percentage of all strains investigated to date (Oviedo et al., 2014), the ballasting effect of coccoliths might also be irrelevant for *E. huxleyi*.

A similar reduction in PIC production, PIC/POC ratio, and coccolith coverage of the cells has been previously observed in *C. pelagicus* subsp. *pelagicus* grown under high temperature stress (Gerecht et al., 2014). Both studies point towards a reduction in PIC production under either high temperature stress and/or growth-limiting P-availability. In *E. huxleyi*, on the other hand, both P-limitation and a temperature increase have been suggested as a trigger for coccolith production (Kayano and Shiraiwa, 2009; Shiraiwa, 2003; Sorrosa et al., 2005). These differences in species' physiology leading to different responses to environmental stressors need to be taken into account when trying to model the effect of environmental change on carbon production of coccolithophores as a group.

4.4. Concluding remarks

Using semi-continuous cultures we could show that carbon production is reduced under P-limitation in *C. pelagicus*. This effect has not been previously observed in batch cultures (Gerecht et al., 2014), illustrating the inadequacy of the batch approach with respect to determining production. POC production was more sensitive than PIC production to a moderate reduction in P-availability. Once P-availability became growth limiting, however, phosphorus resources were invested into POC rather than PIC production, leading to a decrease in cell density and sinking rate in P-limited cells. The present results suggest that sustained P-limited growth can reduce PIC over POC production in *C. pelagicus*, favoring CO_2-uptake by coccolithopore growth and CO_2-removal from surface waters. The present data also provide evidence that coccoliths do not act as ballast for sinking out of nutrient-poor into nutrient-rich deeper water layers.

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