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Coccolithophores do not increase particulate carbon production under nutrient limitation: A case study using *Emiliana huxleyi* (PML B92/11)

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ABSTRACT

The coccolithophore *Emiliana huxleyi* (PML B92/11) was grown in batch culture under nitrogen (N) as well as phosphorus (P) limitation. Growth rate, particulate inorganic carbon (PIC), particulate organic carbon (POC), particulate organic nitrogen (PON), and particulate organic phosphorus (POP) production were determined. While PON production decreased by 96% under N-limitation and POP production decreased by 85% under P-limitation, growth rate decreased by 31% under N- and by 26% under P-limitation. POC production increased by a factor of 1.5 under N-limitation and by a factor of 3.3 under P-limitation. PIC production increased by a factor of 1.2 under N-limitation and did not change under P-limitation. It is concluded that the decrease in PON production under N-limitation and the decrease in POP production under P-limitation represent a physiological response of the cells while the increase in particulate carbon production represents a methodological artefact. The latter conclusion is based on a direct comparison of this strain's responses to nutrient limitation in different experimental setups, i.e., batch-, semi-continuous-, and continuous cultures.

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1. Introduction

It is generally held that the recent, putatively man-made, increase in sea surface temperature will lead to an enhanced stratification in the oceans. The latter in turn will reduce the input of nutrients into phytoplankton rich surface waters, which will increase the probability of phytoplankton nutrient limitation (Behrenfeld et al., 2006). The response of the biogeochemically important coccolithophores to nutrient (nitrogen, N, and phosphorus, P) limitation is a matter of interest in that context, with special emphasis being put on these algae's production of particulate organic (POC) and inorganic (PIC) carbon (Rost and Riebesell, 2004). By producing POC as well as PIC, coccolithophores, as opposed to e.g., diatoms, contribute to the organic carbon pump as well as the carbonate counter pump (Rost and Riebesell, 2004). The term carbon pump refers to particulate carbon which sinks to depth, thereby transporting carbon from sea surface waters to the deep ocean. The PIC/POC ratio of the material that sinks to depth is an important parameter in the global carbon cycle. A number of recent studies have addressed the question of particulate carbon production in coccolithophores by means of laboratory experiments (Borchard et al., 2011; Kaffes et al., 2010; Langer et al., 2012; Matthiessen et al., 2012). It was suggested that coccolithophores increase PIC production in response to nutrient limitation (McConnaughey and Whelan, 1997). Such a response was indeed shown for *Calcidiscus leptoporus* (Langer et al., 2012), but not for *Emiliana huxleyi* (Borchard et al., 2011; Kaffes

et al., 2010; Paasche, 1998; Riegman et al., 2000). The responses of the latter species moreover varied between different studies, which might hint at strain-specific differences, because a different strain was used in each study (except Borchard et al., 2011; Kaffes et al., 2010, who used the same strain). Species- and strain-specific responses of coccolithophores were shown with respect to e.g., salinity (Brand, 1984) and carbonate chemistry (Langer et al., 2006, 2009, 2011) changes.

Nevertheless, it was argued that coccolithophores do not increase particulate carbon production in response to macro-nutrient limitation, and that the increase in production observed in *C. leptoporus* is a methodological artefact (Langer et al., 2012). The response of coccolithophores to nutrient limitation was studied in batch and (semi)-continuous culture (Benner, 2008; Borchard et al., 2011; Kaffes et al., 2010; Paasche, 1998; Riegman et al., 2000). Langer et al. (2012) argued that there are methodological limitations in determining particulate carbon production in the batch approach, which can lead to apparently increased production under limitation. Briefly, production is the product of growth rate and carbon quota. Both factors are integrated values over the course of the experiment. In batch culture the cells undergo a transition from exponential to stationary growth, entailing a non-constant growth rate. A constant growth rate, by contrast, is a prerequisite for an accurate determination of production by means of this method. The latter is the reason why Langer et al. (2012) hypothesised that production as determined in the batch approach contains a methodological artefact, i.e., a wrong growth rate, which in turn can result in apparently increased production under limitation. This hypothesis can only be tested by comparing the response patterns of a particular culture strain grown in batch as well as (semi)-continuous culture.

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Here we test this hypothesis in a case study using *E. huxleyi* (PML B92/11). The latter strain was recently grown in nitrogen-limited semi-continuous culture (Kaffes et al., 2010) and phosphorus-limited continuous culture (Borchard et al., 2011). In this study we grew *E. huxleyi* (PML B92/11) in nitrogen as well as phosphorus limited batch culture.

2. Material and methods

Clonal cultures of *E. huxleyi* (strain PML B92/11), were grown in sterile filtered (0.2 µm) seawater enriched with trace metals and vitamins according to f/2, a common recipe for culture media additives (Guillard and Rytner, 1962). Initial nitrate and phosphate concentrations varied in dependence of treatment (Table 1). The N-limited treatment featured an initial nitrate concentration of ca. 3 µM and an initial phosphate concentration of ca. 35 µM. The P-limited treatment was characterized by an initial nitrate concentration of ca. 720 µM and an initial phosphate concentration of ca. 0.29 µM. The N-control contained initially ca. 780 µM nitrate and ca. 34 µM phosphate. The P-control contained initially ca. 680 µM nitrate and ca. 32 µM phosphate. The seawater to which the supplements were added was, in the case of the P-experiment, a mixture of 60% natural North Sea seawater and 40% artificial seawater, and in the case of the N-experiment, a mixture of 20% natural North Sea seawater and 80% artificial seawater (composition see Table 2). The incident photon flux density was 400 µmol/m² * s and a 16/8 h light/dark cycle was applied. Experiments were carried out at 15 °C.

Samples for total alkalinity (TA) measurements were filtered through glass-fibre filters (0.6 µm nominal pore size) and stored in 150 mL borosilicate bottles at 3 °C. TA was determined by duplicate potentiometric titrations (Brewer et al., 1986) using a TitroLine alpha plus autosampler (Schott Instruments, Mainz, Germany), and a calculation from linear Gran plots (Gran, 1952). Certified Reference Materials (CRMs, Batch No. 54) supplied by A. Dickson (Scripps Institution of Oceanography, USA) were used to correct the measurements. The average reproducibility was ± 5 µmol kg⁻¹ seawater (n = 10).

Dissolved inorganic carbon (DIC) samples were filtered through 0.2 µm cellulose-acetate syringe-filters and stored head-space free in 5 mL gas-tight borosilicate bottles at 3 °C. This procedure ensures that no gas exchange occurs during sampling. DIC was measured photometrically in triplicate (Stoll et al., 2001) using a QuAAtro autoanalyzer (Seal Analytical Inc., Mequon, USA) with an average reproducibility of ± 5 µmol kg⁻¹ (n = 20). CRMs (Batch No. 54) were used to correct the measurements. Shifts in DIC concentrations due to CO₂ exchange were prevented by opening the storage vials less than 1 min prior to each measurement.

Seawater pH was determined potentiometrically using a glass electrode/reference electrode cell (Schott Instruments, Mainz, Germany), which included a temperature sensor and was two-point calibrated with NBS buffers prior to every set of measurements. Average repeatability was found to be ± 0.02 pH units (n = 30). The measured pH_{NBS}

Table 2

Composition of ASW (not including supplement, see Material and methods section).

Salt	Final concentration (mM)
NaHCO ₃	2.33
NaCl	394
MgCl ₂	53.6
Na ₂ SO ₄	28.4
KCl	10
SrCl ₂	0.09
KBr	0.84
CaCl ₂	10
H ₃ BO ₃	0.4

values were converted to the total scale using respective Certified Reference Materials (Tris-based pH reference material, Batch No. 2, Scripps Institution of Oceanography, USA), see also Dickson (2010). All pH values are reported on the total scale. Salinity, measured with a conductivity metre (WTW Multi 340i) combined with a TetraCon 325 sensor, was 32.

The carbonate system was calculated from temperature, salinity, TA, pH (total scale) and phosphate concentration using the DOS program CO₂sys (Lewis and Wallace, 1998). The equilibrium constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987) were used.

Samples for determination of total particulate carbon (TPC), particulate organic carbon (POC), and particulate organic nitrogen (PON) were filtered onto pre-combusted (12 h, 500 °C) 0.6 µm nominal pore-size glass fibre filters (Whatman GF/F) and stored at -20 °C. Prior to analysis, 230 µL of an HCl solution (5 mol L⁻¹) was added on top of the POC filters in order to remove all inorganic carbon. TPC, POC, and PON were subsequently measured on a Euro EA Analyser (Euro Vector). Particulate inorganic carbon (PIC) was calculated as the difference between TPC and POC. For determination of cell density, samples were taken daily and counted immediately after sampling using a Coulter Multisizer III (Beckmann Coulter). Cell densities were plotted versus time and growth rate (µ) was calculated from exponential regression including all data-points till harvest day, i.e., day 8 in case of the limited cultures (Fig. 1). The control cultures reached the cell densities which the limited cultures reached on day 8, on day 5 already and were consequently harvested on day 5 (Langer et al., 2012). After harvest, a sample of the control cultures was kept under experimental conditions and the growth of the cells was monitored till they reached stationary phase at a cell density of ca. 2 × 10⁶ cells per mL, which is a typical value for *E. huxleyi* (Langer et al., in press).

Particulate inorganic carbon production, i.e., calcification rate (P_{PIC}, pg PIC cell⁻¹ d⁻¹) was calculated according to:

$$P_{PIC} = \mu * (\text{cellular inorganic carbon content}) \quad (1)$$

with cellular inorganic carbon content = pg PIC per cell.

Table 1

Media chemistry measured at the beginning of the experiment (T₀) and at the end of the experiment (T_{fin}). Concentrations are given in µmol/kg seawater, abbreviated as µmol/kg.

Sample	Total alkalinity [µmol/kg]	Standard deviation	pH [total scale]	Standard deviation	DIC [µmol/kg]	Standard deviation	PO ₄ [µmol/kg]	Standard deviation	NO ₃ [µmol/kg]	Standard deviation
Control PO ₄										
T ₀	2516	4	8.159	0.002	2225	8	31.82	0.48	682.90	3.85
T _{fin}	2383	4	8.206	0.007	2085	8	31.81	0.08	670.90	3.09
PO ₄ limited										
T ₀	2484	1	8.074	0.003	2243	1	0.29	0.00	718.98	5.43
T _{fin}	1872	11	8.137	0.008	1660	7	0.00	0.00	734.19	3.31
control NO ₃										
T ₀	2651	4	8.057	0.002	2309	6	33.87	0.52	782.83	5.59
T _{fin}	2452	12	8.115	0.007	2101	13	32.69	0.49	770.07	2.40
NO ₃ limited										
T ₀	2657	1	8.189	0.005	2287	4	35.21	0.14	2.69	0.05
T _{fin}	2350	9	8.138	0.006	2041	1	32.22	0.05	0.00	0.00

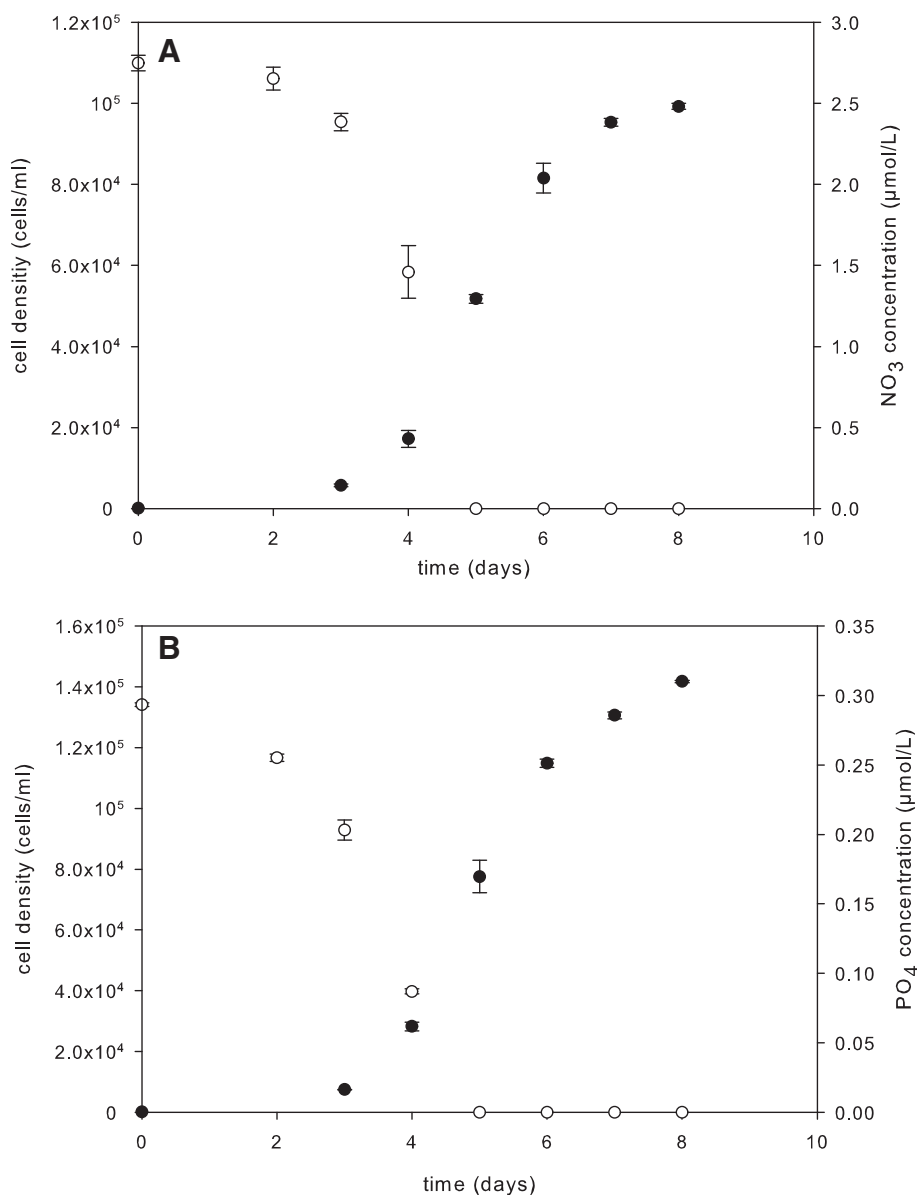


Fig. 1. Nutrient concentrations of culture media (open circles) and cell densities (closed circles) vs. time. A) N-limited cultures. B) P-limited cultures. Error bars represent standard deviation of triplicates.

Particulate organic carbon production (P_{POC} , pg POC cell⁻¹ d⁻¹), particulate organic nitrogen production (P_{PON} , pg PON cell⁻¹ d⁻¹), and particulate organic phosphorus production (P_{POP} , pg POP cell⁻¹ d⁻¹) was calculated accordingly.

Samples for determination of particulate organic phosphorus (POP) were filtered onto pre-combusted (12 h, 500 °C) 0.6 μm nominal pore-size glass fibre filters (Whatman GF/F) and stored at -20 °C. Prior to measurement the samples were dissolved in a potassium peroxodisulfate–water-mixture and autoclaved overnight. After the addition of ascorbic acid and a mixed-reagent (sulphuric acid, ammoniumheptamolybdate-tetrahydrate, potassiumantimoyltartrate and distilled water) samples were measured photometrically using an Optizen 2120 UV photometer (Hansen and Koroleff, 1999).

Nutrient samples (30 mL) were filtered through precombusted (12 h, 500 °C) glass-fibre filters (Whatman GF/F), and nitrate plus nitrite (NO_x), and PO₄ was measured using an Alliance Evolution III Autoanalyser (Alliance Instruments, Austria), according to Hansen and Koroleff (1999).

Each data point presented in the tables and figure is the mean value of triplicate culture experiments. Standard deviation (SD) is given in Tables 1, 3, and 4.

Please note that all numbers ascribed to the study of Borchard et al. (2011) are calculated by us on the basis of the treatment “300-14”, which features experimental conditions similar to the ones employed by us, i.e., CO₂ of ca. 300 μatm (Table 2, Borchard et al., 2011) and a temperature of 14 °C (we used 15 °C, see above, Borchard et al., 2011). We calculated production on the basis of data given in Table 2 of Borchard et al. (2011) applying the method described here.

3. Results

E. huxleyi (strain PML B92/11) was grown in both N-limited and P-limited dilute batch cultures. The evolution of seawater phosphate concentrations in the P-limited treatment and seawater nitrate concentrations in the N-limited treatment in relation to cell density is depicted in Fig. 1. It can be seen that phosphate and nitrate concentrations in the

Table 3
Cellular element quotas and production.

Sample	Growth rate [μ]	Standard deviation	POC [pg/cell]	Standard deviation	POC rate [pg/cell*day]	Standard deviation	PIC [pg/cell]	Standard deviation	PIC rate [pg/cell*day]	Standard deviation	POP [pg/cell]	Standard deviation	POP rate [pg/cell*day]	Standard deviation	PON [pg/cell]	Standard deviation	PON rate [pg/cell*day]	Standard deviation	
Control PO ₄																			
T _{fin}	1.34	0.01	8.31	0.11	11.12	0.20	8.40	0.24	11.23	0.29	0.10	0.00	0.13	0.01	1.62	0.03	2.17	0.10	
PO ₄ limited																			
T _{fin}	0.92	0.00	39.86	0.37	36.51	0.44	11.77	0.99	10.78	0.91	0.02	0.00	0.02	0.00	2.62	0.13	2.40	0.12	
control NO ₃																			
T _{fin}	1.19	0.02	8.30	0.28	9.86	0.34	9.43	0.50	11.16	0.48	0.09	0.00	0.11	0.00	1.45	0.03	1.72	0.06	
NO ₃ limited																			
T _{fin}	0.88	0.00	16.21	0.30	14.33	0.17	15.44	0.19	13.54	0.22	0.13	0.00	0.12	0.00	0.08	0.00	0.07	0.00	

P- and N-limited treatments respectively fell below the detection limit on day 5. By that time ca. 50% of the final cell density had been produced. Growth rate decreased under both P- and N-limitations (Table 3). P-limitation led to a marked decrease in cellular POP quota, and a marked increase in cellular POC quota (Table 3, Fig. 2). Cellular PON and PIC quotas also increased under P-limitation but to a lesser extent (Table 3, Fig. 2). The calculated PON production under P-limitation increased slightly, whereas there was no change in PIC production (Table 3, Fig. 2). Substantial changes occurred in POC production (increase, Table 3, Fig. 2) and POP production (decrease, Table 3, Fig. 2).

N-limitation resulted in a pronounced decrease in cellular N quota, while cellular POC, PIC, and POP quotas increased (Table 3, Fig. 3). The calculated PON production decreased markedly under N-limitation, while POP production increased slightly (Table 3, Fig. 3). An increase was observed as well in POC and PIC productions in response to N-limitation (Table 3, Fig. 3). Carbonate chemistry remained quasi-constant over the course of the experiments, with the P-limited treatment featuring the biggest change due to growth of cells (Table 4).

4. Discussion

Phosphorus limitation did apparently not affect PIC production of *E. huxleyi* (PML B92/11), whereas POC production increased by a factor of 3.3 under P-limitation (Table 3, Fig. 2). While the former observation tallies with data of Borchard et al. (2011), the latter observation is in stark contrast to the results of Borchard et al. (2011). The latter authors performed a chemostat (i.e., continuous culture) experiment on that very same strain including two different levels of P-limitation characterized by different growth rates of the cells. The cells featuring the lower growth rate displayed a lower (factor of 2.1) POC production (Borchard et al., 2011). This comparison of POC productions (we will discuss PIC production below) clearly shows that the response of *E. huxleyi* (PML B92/11) to P-limitation in batch culture is qualitatively different from the one in continuous culture.

From now on we will call a qualitative difference (i.e., increase as opposed to e.g., no change) a difference in the response pattern. This stark difference in response pattern is clear evidence in favour of the hypothesis that production cannot be calculated according to the batch approach (Langer et al., 2012). By entailment this means that the production response pattern as determined in the batch approach does not represent the physiological performance of the cells. A response pattern which does represent the physiological performance of the cells will be called “true”. Following the reasoning of the latter authors, we propose that the response pattern as reported in Borchard et al. (2011) represents the “true” response pattern of *E. huxleyi* (PML B92/11), whereas the one reported here is merely due to an inapplicable method of calculating production. We would like to stress that this conclusion also holds for PIC production, not only for POC production. The reason for this conclusion is that the PIC quota is higher in the P-limited cells than in the control cells (Table 3). According to the reasoning of Langer et al. (2012) a response pattern of production as determined in the batch approach can only safely be regarded as the “true” response pattern if the respective quota of the limited cells is equal to or lower than the quota of the control cells. The reason for the latter is that a constant production over the course of a batch experiment would result in an increased quota relative to the control. A quota of the limited cells equal to (or lower than) the one of the control cells therefore can only have been brought about by a decreasing production. The latter production would then necessarily be lower than the one of the control. On the one hand, it consequently remains highly uncertain whether the constancy of PIC production in response to N-limitation in *Coccolithus braarudii* (Benner, 2008) reflects the “true” response pattern of this species, because the PIC quota of the limited cells is ca. by a factor of 4 higher than the PIC quota of the control cells. On the other hand, the decrease in POP production in response to P-limitation (Table 3) can be regarded as “true”, because it was accompanied by a decrease in POP quota (Table 3). The latter inference tallies well

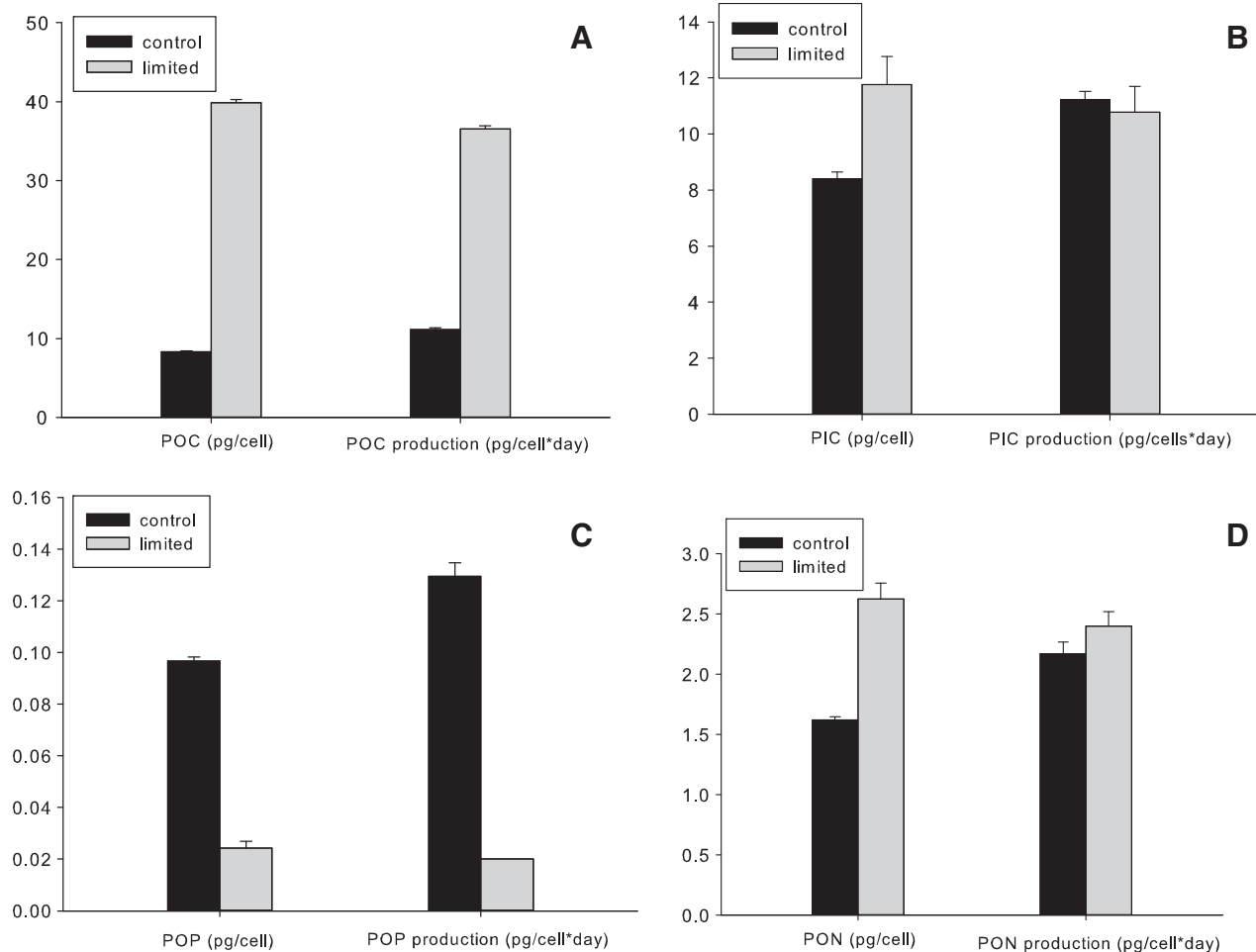
Table 4
Carbonate chemistry calculated from TA and pH.

Sample	Total alkalinity [$\mu\text{mol/kg}$]	Standard deviation	pH [total scale]	Standard deviation	DIC [$\mu\text{mol/kg}$]	Standard deviation	pCO_2 [μatm]	Standard deviation	HCO_3^- [$\mu\text{mol/kg}$]	Standard deviation	CO_3^{2-} [$\mu\text{mol/kg}$]	Standard deviation	Ω_{Ca}	Standard deviation
Control PO_4														
T_0	2516	4	8.159	0.002	2181	4.73	288	1.73	1955	5.51	215	0.58	4.39	0.01
T_{fin}	2383	4	8.206	0.007	2031	4.36	238	4.58	1801	5.57	221	2.65	4.51	0.06
PO_4 limited														
T_0	2484	1	8.074	0.003	2232	2.65	364	3.00	2034	3.21	184	1.00	3.75	0.02
T_{fin}	1872	11	8.137	0.008	1636	12.12	228	6.24	1473	12.49	154	2.65	3.14	0.04
Control NO_3														
T_0	2651	4	8.057	0.002	2355	4.62	399	2.65	2140	4.36	191	1.00	3.88	0.02
T_{fin}	2452	12	8.115	0.007	2139	8.19	313	4.51	1931	5.00	197	3.21	3.99	0.07
NO_3 limited														
T_0	2657	1	8.189	0.005	2279	4.58	278	4.16	2025	7.09	244	2.00	4.94	0.04
T_{fin}	2350	9	8.138	0.006	2034	9.24	282	4.73	1828	9.45	196	2.00	3.97	0.04

with the decreased POP production reported by Borchard et al. (2011). From the latter study we infer that *E. huxleyi* (PML B92/11) decreases POC production in response to P-limitation while PIC production remains unchanged. These response patterns were also described for another strain of the same species grown in a chemostat (Paasche, 1998). However, it cannot be assumed that there are no strain-specific differences, because Riegman et al. (2000) observed a decrease in both POC- and PIC-productions.

The conclusions drawn from the comparison of our data with the data of Borchard et al. (2011) are confirmed when considering the case of N-limitation. Under N-limitation POC and PIC productions increased by factors of ca. 1.5 and 1.2 respectively (Table 3, Fig. 3). Grown in

semi-continuous culture the same strain, *E. huxleyi* (PML B92/11), decreased POC and PIC productions by 39 and 30% respectively, as calculated by us on the basis of the data in Kaffes et al. (2010). Again, we propose that the response pattern reported by Kaffes et al. (2010) represents the “true” pattern. This proposition is, by comparison, not as straightforward as in the case of P-limitation, because Borchard et al. (2011) used continuous cultures, whereas Kaffes et al. (2010) used semi-continuous cultures. The latter feature the same kind of problem as batch cultures, namely a change in growth rate over the course of the experiment. In semi-continuous cultures this change is comparatively small and therefore the problem with respect to calculating production should be less serious. In support of the latter suggestion,

**Fig. 2.** Element quotas and production under P-limitation. A) POC B) PIC C) POP D) PON. Error bars represent standard deviation of triplicates.

decreasing POC- and PIC-productions due to nutrient limitation was described for three additional *E. huxleyi* strains grown in continuous culture (Fritz, 1999; Paasche, 1998; Riegman et al., 2000).

As in the case of P-limitation, the production of the limiting element, i.e., N, decreased in the batch (Table 3) as well as in the semi-continuous approach (Kaffes et al., 2010) in response to limitation. And again, the decrease in PON production was accompanied by a decrease in PON quota (Table 3). However, we will point out that the comparison of our data and the data of Kaffes et al. (2010) and Borchard et al. (2011) is not completely straightforward, because the limitation imposed on the cells by Kaffes et al. (2010) is weak, presumably much weaker than the one imposed by us. In the case of Borchard et al. (2011) the opposite is true, i.e., the latter authors imposed a limitation on the cells which is probably more severe than the one we inflicted. This poses the question whether these three studies are comparable. We argue that they actually are, because it was shown that the response to limitation gradually becomes more obvious when the limitation is stronger (Fritz, 1999; Riegman et al., 2000). Hence there is no change in the response pattern between weak and severe limitations. This renders the comparison of our data with the data by Kaffes et al. (2010) and Borchard et al. (2011) feasible.

On the whole, the data on *E. huxleyi* (PML B92/11) confirm the proposition by Langer et al. (2012) that there is no evidence of increased particulate carbon production in response to macro-nutrient limitation in coccolithophores. An increase in production observed in the batch approach stems almost with certainty from the fact that growth rate cannot be determined with sufficient accuracy. The reason for this is the fast change in growth rate (on a daily basis) which cannot be accounted for (see also below).

Is the batch approach useless with regard to determining production response patterns? Not entirely, because, as stated above, if the accompanying quota of the limited cells is equal to or lower than the one of the control cells, the response pattern, i.e., a decrease in production under limitation, can be regarded as “true”. Langer et al. (2012) suggested that the daily sampling for quota data alongside cell density data and the calculation of incremental production could help better in constraining production under limitation in the batch approach. While it is doubtlessly true, this method is also not capable of providing production data as reliable as the ones obtained in the (semi)-continuous approach, because in the batch culture growth rate and quotas are constantly changing, rendering it impossible to measure with certainty truly matching values (i.e., growth rate and quota). As discussed above, the semi-continuous approach suffers in principle from the same problem as the batch approach, but with less detrimental consequences. A second problem is that an estimate of the growth rate on a daily basis becomes less accurate if the growth rate falls well below one division per day, which is obviously the case in the batch culture. To sum it up, if the primary research question centres on production under limitation, the continuous approach (and to some extent the semi-continuous approach) is clearly to be favoured.

However, the batch approach is not entirely useless when the primary question centres on the comparison between response patterns, e.g., the comparison between different strains or the comparison of effects of N and P limitations on one particular strain. This, however, requires a very similar experimental setup, i.e., it is probably confined to comparisons within one single study and might be problematic when comparisons between different studies are concerned. The batch

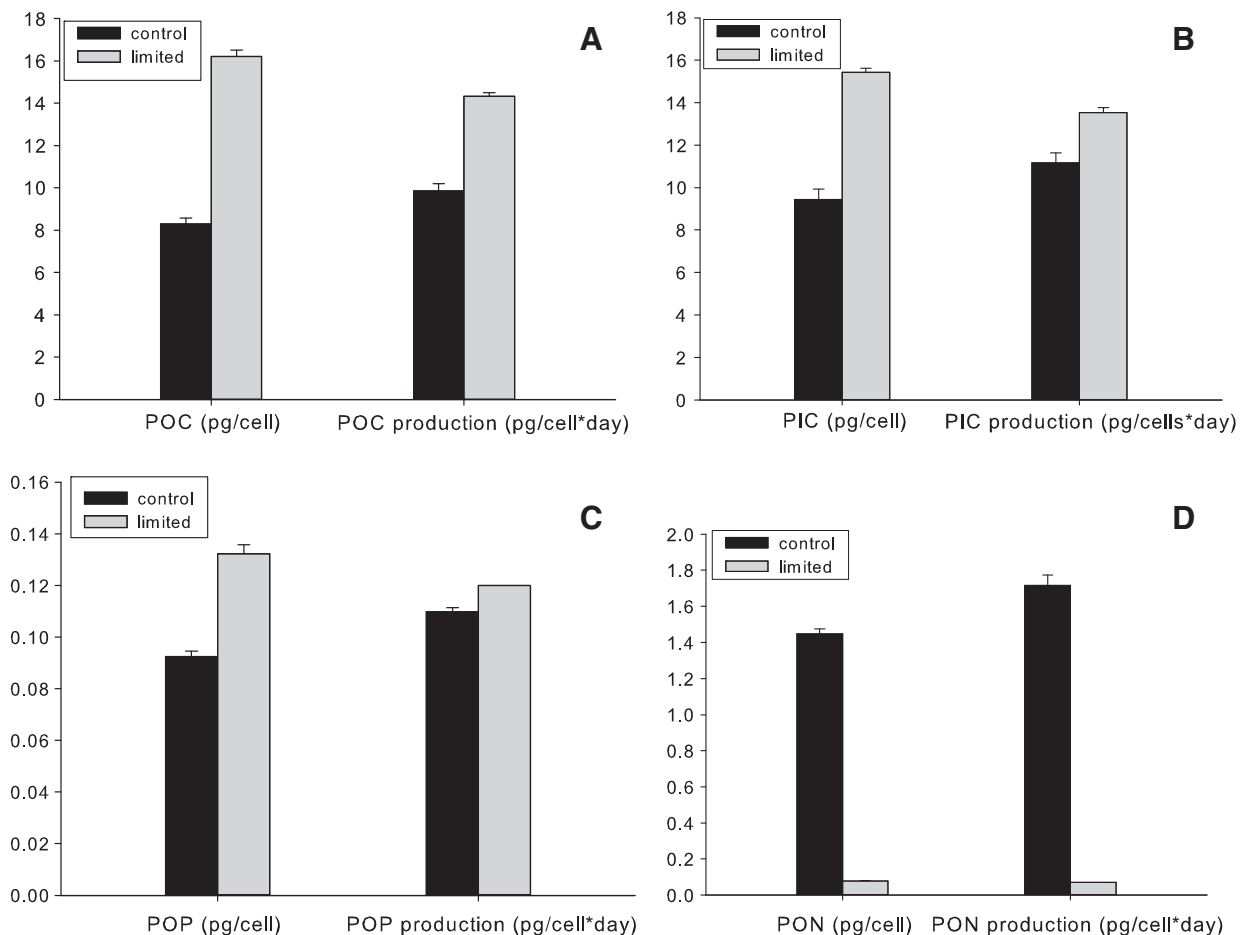


Fig. 3. Element quotas and production under N-limitation. A) POC B) PIC C) POP D) PON. Error bars represent standard deviation of triplicates.

approach should be as useful as the (semi)-continuous approach when ratio data such as coccolith morphology (Langer et al., 2012) or Sr/Ca ratios of coccoliths (Rickaby et al., 2002; Stoll et al., 2007) are concerned. The reason for this is that the bulk of the harvested material was produced under limitation so that the “contamination” due to the material produced under non-limiting conditions should be negligible. In the case of *C. leptoporus* (Langer et al., 2012) it was calculated that ca. 75% of the cells are produced under limitation as indicated by a decreased growth rate. In the case of *E. huxleyi* (PML B92/11) the value is very similar, i.e., ca. 80%. The latter value was calculated assuming that day 4 (Fig. 1) was the last non-limited day. This assumption, in turn, is based on the observation that the cell density on day 5 already clearly deviates from the one expected from exponential growth. Please note that a decrease in growth rate is a relatively late-appearing, and therewith unmistakable, sign of limitation (Kaffes et al., 2010). Moreover, nutrient limited cells produced later during the course of the experiment often contain more PIC or POC than cells produced in the early growth phase (see discussion in Langer et al. (2012) and Table 3) and therefore, in terms of PIC, the calculated 80% produced under limitation have to be regarded as a lower limit. Besides the applicability to questions concerning ratio data, the batch approach also has advantages compared to the (semi)-continuous approach. Firstly, it is far easier to keep the carbonate chemistry quasi-constant (compare Table 4 and data plus discussion in Borchard et al., 2011). Secondly, it is relatively straightforward in terms of experimental setup. Especially, a chemostat is, by comparison, very expensive and might be unaffordable in certain situations.

To conclude, there is no evidence of increased particulate carbon production under N or P limitation in coccolithophores. Reported increased production in batch cultures are a methodological artefact resulting from a wrong determination of growth rate. From a practical point of view, the (semi)-continuous approach should be used if the research question centres on production under limitation. The batch approach is useful if ratio data are concerned, especially if a chemostat is unaffordable.

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References

- Behrenfeld, M.J., O'Malley, R.T., Siegel, D.A., McClain, C.R., Sarmiento, J.L., Feldman, G.C., Milligan, A.J., Falkowski, P.G., Letelier, R.M., Boss, E.S., 2006. Climate-driven trends in contemporary ocean productivity. *Nature* 444, 752–755.
- Benner, I., 2008. The utilization of organic nutrients in marine phytoplankton with emphasis on coccolithophores, PhD thesis, Universität Bremen.
- Borchard, C., Borges, A.V., Händel, N., Engel, A., 2011. Biogeochemical response of *Emiliania huxleyi* (PML B92/11) to elevated CO₂ and temperature under phosphorous limitation: a chemostat study. *J. Exp. Mar. Biol. Ecol.* 410, 61–71.
- Brand, L.E., 1984. The salinity tolerance of forty-six marine phytoplankton isolates. *Estuarine Coastal Shelf Sci.* 18, 543–556.
- Brewer, P.G., Bradshaw, A.L., Williams, R.T., 1986. Measurement of total carbon dioxide and alkalinity in the North Atlantic ocean in 1981. In: Trabalka, J.R., Reichle, D.E. (Eds.), *The Changing Carbon Cycle – A Global Analysis*. Springer Verlag, New York, pp. 358–381.
- Dickson, A.G., 2010. The carbon dioxide system in sea water: equilibrium chemistry and measurements. In: Riebesell, U., Fabry, V.J., Hansson, L., Gattuso, J.-P. (Eds.), *Guide for Best Practices in Ocean Acidification Research and Data Reporting*. Office for Official Publications of the European Union, Luxembourg, pp. 17–40.
- Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Res.* 34, 1733–1743.
- Fritz, J.J., 1999. Carbon fixation and coccolith detachment in the coccolithophore *Emiliania huxleyi* in nitrate-limited cyclostats. *Mar. Biol.* 133, 509–518.
- Gran, G., 1952. Determination of the equivalence point in potentiometric titrations of seawater with hydrochloric acid. *Oceanol Acta.* 5, 209–218.
- Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms, I, *Cyclotella nana* (Hustedt) and *Detonula convervacea* (Cleve). *Can. J. Microbiol.* 8, 229–239.
- Hansen, H.P., Koroleff, F., 1999. Determination of nutrients. In: Grasshoff, K., Kremling, K., Ehrhardt, M. (Eds.), *Methods of Seawater Analysis*. Wiley-VCH, Weinheim, pp. 159–228.
- Kaffes, A., Thoms, S., Trimborn, S., Rost, B., Langer, G., Richter, K.-U., Köhler, A., Norici, A., Giordano, M., 2010. Carbon and nitrogen fluxes in the marine coccolithophore *Emiliania huxleyi* grown under different nitrate concentrations. *J. Exp. Mar. Biol. Ecol.* 393, 1–8.
- Langer, G., Oetjen, K., Brenneis, T., in press. On culture artefacts in coccolith morphology. *Helgoland Marine Research*. <http://dx.doi.org/10.1007/s10152-012-0328-x>.
- Langer, G., Geisen, M., Baumann, K.-H., Kläs, J., Riebesell, U., Thoms, S., Young, J.R., 2006. Species-specific responses of calcifying algae to changing seawater carbonate chemistry. *Geochem. Geophys. Geosyst.* 7, Q09006. <http://dx.doi.org/10.1029/2005GC001227>.
- Langer, G., Nehrke, G., Probert, I., Ly, J., Ziveri, P., 2009. Strain-specific responses of *Emiliania huxleyi* to changing seawater carbonate chemistry. *Biogeosciences* 6, 2637–2646 (J1 – BG).
- Langer, G., Probert, I., Nehrke, G., Ziveri, P., 2011. The morphological response of *Emiliania huxleyi* to seawater carbonate chemistry changes: an inter-strain comparison. *J. Nanoplankton Res.* 32, 27–32.
- Langer, G., Oetjen, K., Brenneis, T., 2012. Calcification of *Calcidiscus leptoporus* under nitrogen and phosphorus limitation. *J. Exp. Mar. Biol. Ecol.* 413, 131–137.
- Lewis, E., Wallace, D.W.R., 1998. Program Developed for CO₂ System Calculations ORNL/CDIAC-105. Carbon Dioxide Information Analysis Centre, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.
- Matthiessen, B., Eggers, S.L., Krug, S.A., 2012. High nitrate to phosphorus regime attenuates negative effects of rising pCO₂ on total population carbon accumulation. *Biogeosciences* 9, 1195–1203 (J1 – BG).
- McConnaughey, T.A., Whelan, J.F., 1997. Calcification generates protons for nutrient and bicarbonate uptake. *Earth Sci. Rev.* 42, 95–117.
- Mehrbach, C., Culbertson, C.H., Hawley, J.E., Pytkoviz, R.M., 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.* 18, 897–907.
- Paasche, E., 1998. Roles of nitrogen and phosphorus in coccolith formation in *Emiliania huxleyi* (Prynesiophyceae). *Eur. J. Phycol.* 33, 33–42.
- Rickaby, R.E.M., Schrag, D.P., Zondervan, I., Riebesell, U., 2002. Growth rate dependence of Sr incorporation during calcification of *Emiliania huxleyi*. *Glob. Biogeochem. Cycles* 16, 1006. <http://dx.doi.org/10.1029/2001GB001408>.
- Riegman, R., Stolte, W., Noordeloos, A.A.M., Slezak, D., 2000. Nutrient uptake and alkaline phosphatase (EC 3:1:3:1) activity of *Emiliania huxleyi* (Prynesiophyceae) during growth under n and p limitation in continuous cultures. *J. Phycol.* 36, 87–96.
- Rost, B., Riebesell, U., 2004. Coccolithophore and the biological pump: responses to environmental changes. In: Thierstein, H.R., Young, J.R. (Eds.), *Coccolithophores – From Molecular Processes to Global Impact*. Springer-Verlag, Berlin-Heidelberg, pp. 99–125.
- Stoll, M.H.C., Bakker, K., Nobbe, G.H., Haese, R.R., 2001. Continuous-flow analysis of dissolved inorganic carbon content in seawater. *Anal. Chem.* 73, 4111–4116.
- Stoll, H.M., Ziveri, P., Shimizu, N., Conte, M., Theroux, S., 2007. Relationship between coccolith Sr/Ca ratios and coccolithophore production and export in the Arabian Sea and Sargasso Sea. *Deep-Sea Res. II Top. Stud. Oceanogr.* 54, 581–600.