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Calcification of *Calcidiscus leptoporus* under nitrogen and phosphorus limitation

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ABSTRACT

The coccolithophore *Calcidiscus leptoporus* 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 and coccolith morphology was analysed. While PON production decreased by 70% under N-limitation and POP production decreased by 65% under P-limitation, growth rate decreased by 33% under N- as well as P-limitation. POC as well as PIC production (calcification rate) increased by 27% relative to the control under P-limitation, and did not change under N-limitation. Coccolith morphology did not change in response to either P or N limitation. While these findings, supported by a literature survey, suggest that coccolith morphogenesis is not hampered by either P or N limitation, calcification rate might be. The latter conclusion is in apparent contradiction to our data. We discuss the reasons for this inference.

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1. Introduction

Growth of phytoplankton in sea surface waters is often limited by the macronutrients nitrogen (N) and phosphorus (P) (Beardall et al., 2001). Coccolithophores, unicellular calcite producing algae, are a conspicuous part of this phytoplankton. They are cosmopolitan and often thrive in nutrient poor open ocean waters, e.g. *Calcidiscus leptoporus* which dominates calcite production in the South Atlantic (Baumann et al., 2004). The calcite produced by coccolithophores consists in a sphere of interlocking calcite platelets, the coccoliths. Apart from their biogeochemical importance (Milliman, 1993), the latter represent elaborately crafted biominerals exhibiting a species-specific morphology. Morphogenesis of coccoliths is a highly sophisticated cellular process, which can be disturbed by unfavourable environmental conditions, e.g., relative to normal seawater, acidic carbonate chemistry (Langer et al., 2006). Hampered morphogenesis results in malformations of coccoliths; the latter can be observed in natural samples as well as in cultured specimens (Kleijne, 1990; Langer et al., 2006). The percentage of malformed coccoliths in natural samples varies and it has been hypothesised that nutrient limitation leads to coccolith malformations (Kleijne, 1990; Okada and Honjo, 1975). This hypothesis can only be tested by means of culture experiments. Unfortunately, morphological data in relation to nutrient limitation are rare and, with one exception (Benner, 2008), confined to one single species, namely *Emiliania huxleyi*.

The most informative report was published by Paasche (1998). The latter author performed batch as well as chemostat experiments subjecting the cells to both N and P limitation. He found that N-limited cells produced 10–15% malformed coccoliths, while malformations in P-limited cells were "less apparent". In another chemostat experiment, morphology of N-limited cells did not appear to differ from morphology of non-limited cells (Fritz, 1999). Recently, an increased percentage of incomplete coccoliths, but not malformed ones, was observed in an N-limited semi-continuous culture (Kaffes et al., 2010). In the present study we grew *C. leptoporus* under both N and P limitations in batch culture. Coccolith morphology as well as growth rate, calcification rate, organic carbon (POC), organic nitrogen (PON), and organic phosphorus (POP) production was quantified. To the best of our knowledge this is the first dataset on calcification under nutrient limitation in *C. leptoporus*.

2. Material and methods

Clonal cultures of *C. leptoporus* (strain RCC1135, formerly known as AC365 and NS6-1, isolated in the South Atlantic off South Africa, now residing in the Roscoff Culture Collection, http://www.sb-roscoff.fr/Phyto/RCC/), 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 Ryther, 1962). Initial nitrate and phosphate concentrations varied in dependence of treatment (Table 1). The N-limited treatment featured an initial nitrate concentration of 6 μM and an initial phosphate concentration of ca. 36 μM. The P-limited treatment was characterised by an initial nitrate

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Table 1
Media chemistry measured at the beginning of the experiment (T_0) and at the end of the experiment (T_{fin}).

Sample	Total alkalinity [μmol/kg]	Standard deviation	pH	Standard deviation	DIC [μmol/kg]	Standard deviation	PO_4 [μmol/kg]	Standard deviation	NO_3 [μmol/kg]	Standard deviation
Control										
T_0	2462	3	7.944	0.004	2210	7	32.96	0.29	727.42	5.26
T_{fin}	2208	19	7.866	0.009	2015	18	31.45	0.15	689.74	2.87
PO_4 limited										
T_0	2429	2	7.982	0.012	2194	4	0.25	0.01	726.69	3.39
T_{fin}	1956	40	7.792	0.019	1835	23	0.00	0.00	713.23	5.72
NO_3 limited										
T_0	2462	3	7.982	0.008	2190	8	34.98	0.25	6.00	0.11
T_{fin}	1896	42	7.710	0.026	n/d	n/d	34.42	0.23	0.10	0.09

concentration of ca. 750 μM and an initial phosphate concentration of ca. 0.25 μM. The control contained initially ca. 750 μM nitrate and ca. 34 μM phosphate. The seawater to which the supplements were added was a mixture of 50% natural North Sea seawater and 50% artificial seawater (composition see Table 2). The incident photon flux density was 400 μmol/m^2 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 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} (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 QuaAAtro autoanalyzer (Seal Analytical, 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 CO_2 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} 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.

Table 2
Composition of ASW (not including supplement, see Material and methods).

Salt	Final concentration (mM)
$NaHCO_3$	2.33
NaCl	394
$MgCl_2$	53.6
Na_2SO_4	28.4
KCl	10
$SrCl_2$	0.09
KBr	0.84
$CaCl_2$	10
H_3BO_3	0.4

The carbonate system was calculated from temperature, salinity, TA, pH (total scale) and phosphate concentration using the DOS program CO_2sys (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 Sedgwick Rafter Counting Cell. Cell densities were plotted versus time and growth rate (μ) was calculated from exponential regression including all data-points till harvest day.

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

$$P_{PIC} = \mu * (\text{cellularinorganiccarboncontent}) \quad (1)$$

with cellular inorganic carbon content = pg PIC per cell.

Particulate organic carbon production (P_{POC}, pg POC cell^{-1} d^{-1}) was calculated according to:

$$P_{POC} = \mu * (\text{cellularorganiccarboncontent}) \quad (2)$$

with cellular organic carbon content = pg POC per cell.

Particulate organic nitrogen production (P_{PON}, pg PON cell^{-1} d^{-1}) was calculated according to:

$$P_{PON} = \mu * (\text{cellularorganicnitrogencontent}) \quad (3)$$

with cellular organic nitrogen content = pg PON per cell.

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 potassiumperoxodisulfate–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 Alliance EVOLUTION III Autoanalyser (Alliance Instruments, Austria) (Hansen and Koroleff, 1999).

Particulate organic phosphorus production (P_{POP}, pg POP cell^{-1} d^{-1}) was calculated according to:

$$P_{POP} = \mu * (\text{cellularorganicphosphoruscontent}) \quad (4)$$

with cellular organic phosphorus content = pg POP per cell.

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Nutrient samples (30 ml) were filtered through precombusted (12 h, 500 °C) glass-fibre filters (Whatman GF/F), and nitrate plus nitrite (NOx), and PO_4 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 figures is the mean value of triplicate culture experiments. Standard deviation (SD) is given in tables.

Samples for scanning electron microscope analysis were filtered onto polycarbonate filters (0.8 μm pore size), dried in a drying cabinet at 60 °C for 24 h, then sputter-coated with gold-palladium. Imaging was performed with a Philips XL-30 digital scanning field-emission electron microscope. Four categories were used to describe the morphology of *C. leptoporus*: 'normal', 'malformed', 'incomplete', and 'incomplete and malformed' coccoliths (for reference images for the categories, see Fig. 1). An average of approximately 350 (Langer and Benner, 2009) coccoliths was analysed per sample.

3. Results and discussion

Cells of *C. leptoporus* grown in batch culture typically reach final cell densities of ca. 100,000 cells/ml if growth is not limited by nutrient shortage. In accordance with that frequently made observation the control cultures employed in the present study reached a stationary phase cell density of 113,600 cells/ml (SD 6940 cells/ml). In contrast, maximum cell density in P-limited cultures was 21,116 cells/ml (SD 2512 cells/ml) and in N-limited cultures 33,200 cells/ml (SD 2901 cells/ml). Whereas N- and P-limited cultures were harvested at maximum cell density, the control cultures were, for the sake of comparison, harvested at ca. 24,000 cells/ml. This is particularly important because e.g. coccolith morphology changes with cell density (Langer et al. unpublished results). A sub-sample of the control cultures was kept and growth of the cells was monitored until maximum cell density was reached. The difference in maximum cell densities between control and N- or P-limited cultures respectively clearly demonstrates that growth of the presumably limited cultures (i.e. the cultures supplied with low initial nitrate- or phosphate-concentrations) was indeed limited (Fig. 2.).

The percentage of malformed coccoliths in the control cultures was 53% (Table 4). While this number is high compared to the one obtained from most sea surface water samples, it is commonly observed in cultured specimens (see Langer et al., 2006). The reason for this so called culture artifact is unknown (see also Langer and Benner, 2009), but it is highly unlikely that it is due to the artificial seawater because the phenomenon can also frequently be observed in cells grown in natural seawater. Whatever the cause might be, it applies to both the control and the limited cultures, since the same mixture of artificial and natural seawater was used in both cases. Differences in morphology between the control and the nutrient limited cultures can, therefore, be attributed to nutrient limitation.

However, *C. leptoporus* shows no changes in coccolith morphology in response to either N- or P-limitation (Fig. 3, Table 4). This observation refutes the hypothesis that nutrient limitation results in coccolith malformations (Kleijne, 1990; Okada and Honjo, 1975), at least for this particular strain of *C. leptoporus*. A survey of the literature on coccolith morphology in relation to nutrient limitation provides reasons to suspect that the insensitivity of coccolith shaping-machinery to nutrient limitation is a feature typical for coccolithophores as a group. Unfortunately this inference has to be based on very few and more often than not semi-quantitative or qualitative data. Quantitative data stem from a batch culture experiment with *Coccolithus braarudii* (Benner, 2008) and a semi-continuous culture experiment with *E. huxleyi* (Kaffes et al., 2010). In the latter study the cells were subjected to N-limitation and it was observed that, compared to the control, the percentage of incomplete coccoliths greatly increased whereas the percentage of malformed coccoliths did not change.

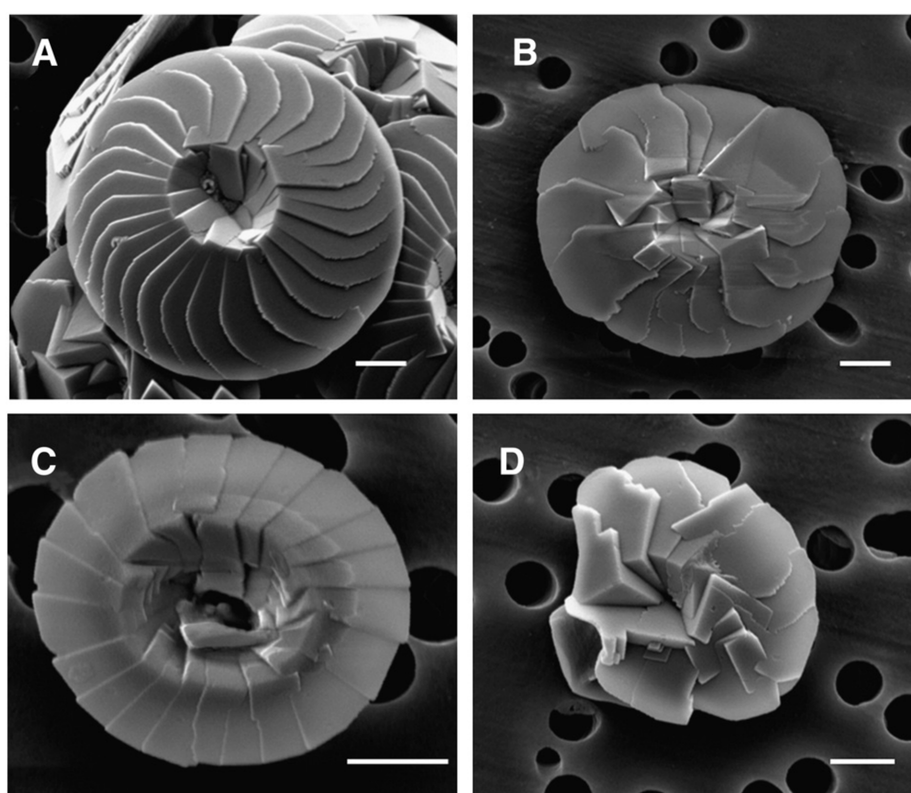

Fig. 1. Scanning electron micrographs of *Calcidiscus leptoporus* coccoliths. A) Normal, B) malformed, C) incomplete, and D) malformed and incomplete. All coccoliths in distal view. All scale bars are 1 μm.

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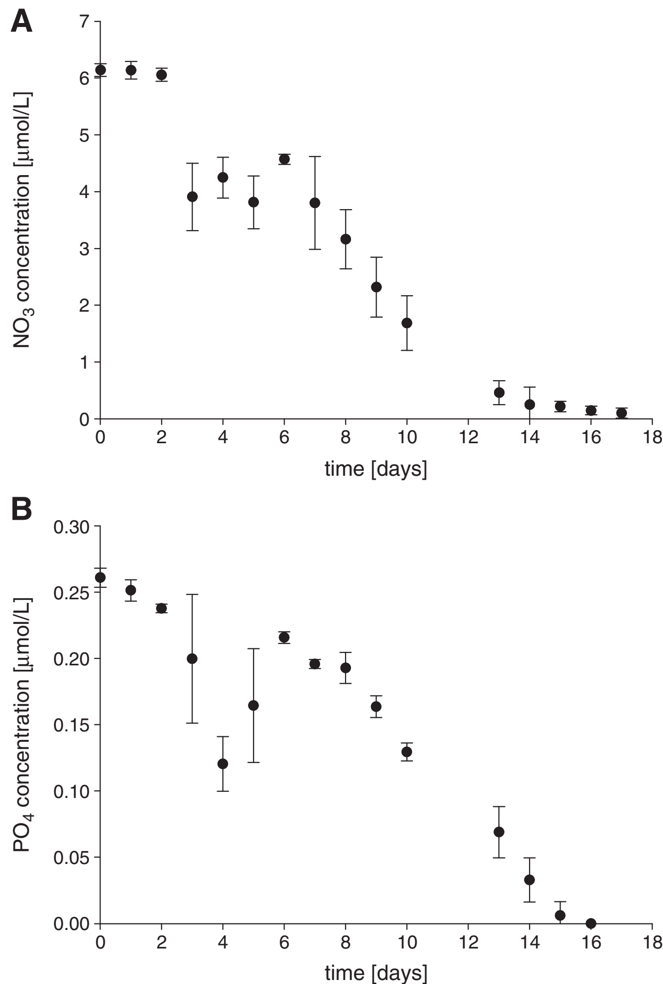

Fig. 2. Nutrient concentrations of culture media vs. time. A) Nitrate concentration in N-limited cultures. B) Phosphate concentration in P-limited cultures.

From this observation it was inferred that the coccolith-shaping machinery as such is not affected by N-limitation, whereas the processes producing the “stop-signal” for coccolith growth are affected.

The latter conclusion cannot be drawn for *C. leptoporus*, because the percentage of incomplete coccoliths did not change (Fig. 3, Table 4). Hence the effect of N-limitation on the “stop-signal” for coccolith growth appears to be species-specific. Moreover, comparison of the data in Kaffes et al. (2010) with observations made by Paasche (1998) and Fritz (1999) suggests that it is even strain-specific. Fritz (1999) stated that *E. huxleyi* cells grown under N-limitation in a chemostat did not exhibit altered coccolith morphology compared to the control. Unfortunately this statement was not based on quantification of coccolith morphology, but it should nevertheless suffice to exclude obvious changes in morphology as observed by Kaffes et al. (2010). Paasche (1998) reported that *E. huxleyi* grown in batch as well as chemostat under N-limitation showed 10–15% malformed coccoliths. Unfortunately neither the standard deviation nor the value for the control is reported. As a rule the maximum percentage of normal coccoliths in cultured specimens is 98%. Assuming the latter value for the control cultures of Paasche (1998), the difference between control and N-limited would be 8–13%. Although the author interprets this difference as an effect on morphology, we do not regard a difference of less than 10% as meaningful, because the latter lies within the range of normal variability. Please note that this statement is not based on a single dataset (e.g. the one presented here), but is based on observations made over the last ten years. It is a conservative interpretation which aims at avoiding over-interpretation of small differences.

Table 3
Cellular element quotas and production. The values for each parameter differ significantly between control, P-limited, and N-limited, with the exception of growth rate between P- and N-limited, POP quota between control and N-limited, POC production between control and N-limited, calcification rate between control and N-limited, POP production between control and N-limited. Two datasets were regarded as significantly different if the p-value of a *t*-test was smaller than 0.02.

Sample	Growth rate [µ]	Standard deviation	POC [pg/cell]	Standard deviation	POC rate [pg/cell day]	Standard deviation	PIC [pg/cell]	Standard deviation	Calcification 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																		
T_0	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
T_{fin}	0.54	0.02	30.53	1.79	16.53	0.57	70.81	4.50	38.33	1.64	0.39	0.04	0.21	0.03	10.45	1.38	5.65	0.62
PO_4 limited																		
T_0	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
T_{fin}	0.36	0.01	61.82	8.51	22.63	2.46	144.71	12.39	52.31	3.83	0.17	0.10	0.07	0.04	12.97	1.35	4.75	0.44
NO_3 limited																		
T_0	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
T_{fin}	0.36	0.01	42.37	3.16	15.24	0.95	107.13	5.26	38.35	2.01	0.52	0.07	0.19	0.04	4.79	1.00	1.72	0.32

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Table 4
Coccolith morphology.

Sample	Normal [%]	Standard deviation	Malformed [%]	Standard deviation	Incomplete [%]	Standard deviation	Incompete and malformed [%]	Standard deviation
Control								
T_0	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
T_{fin}	43.83	2.75	20.66	3.24	2.88	1.42	32.63	5.27
PO_4 limited								
T_0	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
T_{fin}	47.20	1.34	26.59	0.94	1.90	0.55	24.41	0.87
NO_3 limited								
T_0	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
T_{fin}	48.25	1.40	20.55	3.31	1.25	0.42	29.95	4.24

We conclude that in the studies of Paasche (1998) and Fritz (1999) the effect of N-limitation on the percentage of coccolith malformations, if any, is very small. The study of Benner (2008) on the effect of N-limitation on *C. braarudii* morphology does not suggest an increase in malformations due to N-limitation either. On the contrary, the percentage of malformed coccoliths is lower under N-limitation. However, we refrain from considering this observation as evidence in favour of a positive effect of N-limitation on coccolith morphogenesis, because the harvest cell densities in the control cultures were ca. 10,000 cells per ml, as opposed to ca. 3000 cells per ml in the N-limited cultures. Since coccolith malformations increase with increasing cell density (Langer et al. unpublished results), the data of Benner (2008) can be explained solely in terms of a cell density effect. Anyhow, there is, as detailed above, no evidence for a detrimental effect of N-limitation on the coccolith-shaping machinery per se. Please note that the data of Kaffes et al. (2010) do not contradict this conclusion (for details see above).

Furthermore there is no evidence for a detrimental effect of P-limitation on coccolith morphogenesis. Although this inference has to be based on fewer observations, namely a dataset on *E. huxleyi* (Paasche, 1998), and our own data on *C. leptoporus*, it leads us to conclude that neither N- nor P-limitation disturbs the regulatory processes involved in coccolith shaping per se, i.e. disregarding any effects on the cellular “stop-signal” for coccolith growth (see the discussion of Kaffes et al. (2010) above).

Coccolithogenesis in *C. leptoporus* is sensitive to high CO_2 concentrations (Langer and Bode, 2011; Langer et al., 2006). Due to the higher DIC/TA consumption in the limited cultures, the CO_2 level on harvest day was lowest in the control and highest in the N-limited culture (Table 5). The difference of 160 μatm CO_2 between the control and the N-limited culture should have caused an 11% (Langer and Bode, 2011) or even 14% (Langer et al., 2006) reduction in the percentage of normal coccoliths. This was not observed (Table 4, Fig. 3). The reason for that is, probably, the fact that the N-limited culture in the present experiment did experience, relative to the control, the high CO_2 concentration only at the end of the experiment as opposed to the cultures used in the earlier studies (Langer and Bode, 2011; Langer et al., 2006) which were grown under the different CO_2 levels for more than 10 generations.

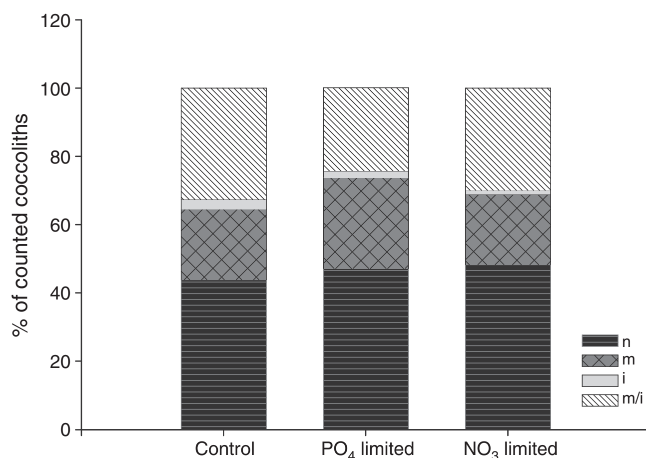

Fig. 3. Percentages of normal (n), malformed (m), incomplete (i), and malformed and incomplete (m/i) coccoliths vs. nutrient treatment.

In *C. leptoporus*, calcification rate increased by 27% in response to P-limitation (Table 3). Such a marked increase has never been described before for a coccolithophore. For *E. huxleyi* it was found that the calcification rate remained unchanged (Paasche, 1998) with a slight tendency towards either increase (Müller et al., 2008; Paasche and Brubak, 1994) or decrease (Riegman et al., 2000).

In *C. leptoporus* N-limitation caused no change in calcification rate (Table 3). This holds also for *C. braarudii* (Benner, 2008). On the one hand N-limitation had no effect on calcification rate of *E. huxleyi* grown in batch (Müller et al., 2008) culture. On the other hand a decrease in calcification rate was observed in the very same species grown in continuous, i.e. chemostat (Fritz, 1999; Paasche, 1998; Riegman et al., 2000), and semi-continuous (Kaffes et al., 2010) culture. Although a different strain was used in each of these studies (and strain-specific effects can therefore not be ruled out), it can be hypothesised that the effect of N-limitation on calcification rate is influenced by the experimental setup, i.e. batch versus (semi-) continuous culture. When grown in batch culture three species, namely *C. leptoporus*, *C. braarudii*, and *E. huxleyi*, display no change in calcification rate in response to N-limitation. By contrast, *E. huxleyi* grown in (semi-) continuous culture, decreases calcification rate due to N-limitation. Why should the effect of N-limitation on calcification rate be more detrimental in (semi-) continuous culture than in batch culture? We propose that the answer to this question must partly be given in terms of cell physiology and partly in terms of methodology as detailed in the following. Concerning cell physiology it has to be noted that cells grown in N-limited batch culture undergo a transition from an initial unlimited physiological state, characterised by normal cell division rate, to a final severely limited state in which cell division is no longer possible. In (semi-) continuous culture, by contrast, the cells are in the same physiological (N-limited) state over the entire period of observation as indicated by a constant (and lower than normal) cell division rate. Hence the physiological state of cells grown in (semi-) continuous culture is characteristic of cells in batch culture only for a short period of time. What does this mean for the determination of calcification rate? In this study as well as in the studies cited above, calcification rate was calculated from overall growth rate and harvest day PIC quota (see Material and methods). This method relies on exponential growth with a constant growth rate. In nutrient limited cultures, the growth rate changes reaching zero at the end of the experimental period. Nevertheless, the growth rate is calculated by exponential regression (see Material and methods) ignoring the fact that only the first part of the curve follows exponential growth. This means that calcification rate (and POC, PON, POP production) cannot be calculated accurately using overall growth rate and harvest day quotas. To illustrate this,

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Table 5
Carbonate chemistry calculated from TA and pH.

Sample	Total alkalinity [μmol/kg]	Standard deviation	pH	Standard deviation	DIC [μmol/kg]	Standard deviation	pCO_2 [μatm]	Standard deviation	HCO_3^- [μmol/kg]	Standard deviation	CO_3^{2-} [μmol/kg]	Standard deviation	Ω_{Ca}	Standard deviation
Control														
T_0	2462	3	7.944	0.004	2263	1	565	6	2113	0	129	1	3.14	0.03
T_{fin}	2208	18	7.866	0.009	2052	15	619	10	1930	13	99	3	2.40	0.06
PO_4 limited														
T_0	2429	2	7.982	0.012	2249	4	513	15	2090	7	140	3	3.39	0.08
T_{fin}	1956	39	7.792	0.019	1865	32	670	18	1763	28	76	5	1.85	0.11
NO_3 limited														
T_0	2462	3	7.982	0.008	2245	6	512	11	2086	7	139	2	3.38	0.05
T_{fin}	1896	41	7.710	0.026	1796	33	782	32	1705	29	61	5	1.48	0.12

assume for argument's sake that production does not change over the entire experimental period while growth rate decreases. This would lead to an increased harvest day quota. The relative change of growth rate and production during the non-exponential phase of the growth curve will determine the harvest day quota. As a result the production of the limited culture calculated as described in Material and methods can be lower than, equal to, or higher than the one calculated for the control culture. What does this mean for the interpretation of production data? Firstly, it can explain why production can apparently be higher in limited cultures. This observation is otherwise hard to explain in terms of cell physiology. Secondly, it reconciles the discrepancy between results from batch and (semi-) continuous culture studies. In the latter growth rate is constant (and so are nutrient concentrations) and the method of calculating production (see Material and methods) should be applicable. Please note that an increase in PIC or POC production has never been reported in a (semi-) continuous culture study (references see above). Consequently, we conclude that N or P limitation does not lead to an increase in PIC or POC production. If an increase of production is seen in a batch culture experiment (e.g. Table 3), this is due to the method of calculating production. Thirdly, it means that if a harvest day quota in a nutrient limited batch culture is equal to or lower than the respective quota in the control culture, the production has certainly gone down. Two particularly striking examples are the PON production under N-limitation and the POP production under P-limitation (Table 3). Hence the decrease in these two numbers can safely be regarded as an indicator for nutrient limitation.

To overcome the difficulties in estimating production in batch culture experiments, incremental samples of quota could be taken daily (alongside the daily cell density samples). This would allow for calculating incremental production. However, this procedure requires more volume of culture and is therefore more difficult to realise in practise.

Do the considerations concerning production also have impact on the interpretation of the morphology data? Only in the sense that there could be an attenuation of a possible effect due to the non-limited growth phase at the beginning of the growth curve. Since ca. 75% of the cells were produced under nutrient limitation, as indicated by a decreased growth rate, a detrimental effect of limitation on morphology should be detectable. This conclusion is supported by the fact that even in (semi-) continuous culture studies no increase in coccolith malformations could be observed (for details and references see above).

To summarise, we conclude that N- and P-limitations are not detrimental to the coccolith shaping machinery per se. There is no evidence of an increased calcification rate or POC production under N- or P-limitation. An apparent increase as observed in this study is due to an inapplicable method of calculating production. If the reasoning leading to this conclusion is correct, it should not be possible to observe an increase in calcification rate or POC production in a continuous culture experiment. A direct comparison between the batch culture and the continuous culture approach to studying nutrient limitation is needed to test some of the conclusions drawn here.

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