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

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ABSTRACT

The coccolithophore *Emiliania 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.

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 *Emiliania 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 Ryther, 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 $\mu mol/m^2 * s$ 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 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 $\mu mol\ kg^{-1}$ 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 $\mu mol\ kg^{-1}$ (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 section).

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 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 × 10 ^ 6 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 (Tfin). 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