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Effect of elevated nitrate concentration on calcification in *Emiliania huxleyi*

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Abstract A monoclonal culture of *Emiliania huxleyi* (PML92/11) was grown in natural sea-water under varying nitrate concentrations, ranging from 10 to 890μmol L^{-1}. Growth rate, calcification rate, particulate organic carbon production, particulate organic nitrogen production, and the ratio of aberrant coccoliths to normal coccoliths were determined. None of these parameters showed a trend over the range of nitrate concentrations tested. It is concluded that high nitrate concentrations, typical for culture media like f/2, do not affect morphogenesis of coccoliths, nor do they affect the production of particulate organic carbon or nitrogen in this strain.

Keywords *Emiliania huxleyi* culture, nitrate concentration, growth rate, calcification, particulate organic carbon, particulate organic nitrogen, coccolith malformation

1. Intoduction

Coccolithophores are unicellular marine algae, which have evolved the ability to produce minute calcite platelets, the coccoliths. From a biogeochemical point of view, coccolithophores are of special interest, since, besides being important primary producers, they are responsible for approximately half of modern marine pelagic calcium carbonate export production (Baumann *et al*., 2004). The biogeochemical significance of coccolithophores has motivated a number of studies concerned with different aspects of the biogeochemistry, cell biology and physiology of these algae. Besides issues that can be dealt with in field studies, numerous questions can only be answered by means of controlled laboratory experiments using monoclonal cultures. Therefore, the significance of culture banks, such as the Roscoff Culture Collection (www.sb-roscoff.fr/Phyto/RCC), must not be underestimated.

Despite the noted success of standard culturing techniques (Probert & Houdan, 2004), it is well known that the percentage of aberrant coccoliths in cultures is higher than in natural samples (Langer *et al*., 2006). The reason for this hampered morphogenesis of coccoliths in cultured specimens is unknown. Since calcification-related data, such as calcification rate and coccolith morphology, play a major role in coccolithophore research, there is, on one hand, a need to solve this riddle. On the other hand, there are also many possible answers, which is presumably the reason why systematic studies concerned with this question are lacking. Culturing of marine microalgae traditionally includes the usage of sea-water-based media and special additives, including micro- and macronutrients (Probert & Houdan, 2004). For instance, the nitrate concentration of the often-used f/2 medium (Guillard, 1975) is 880μmol L^{-1}, whereas typical nitrate concentrations in the North Atlantic range from 0.2μmol L^{-1} to 30μmol L^{-1} (Garcia *et al*., 2005). ^{14}C uptake experiments, using the most-studied coccolithophore *Emiliania huxleyi*, have revealed a decreased calcification rate under 1000μmol L^{-1} nitrate (Nimer & Merrett, 1993). Since calcification rate and coccolith morphology were shown to be altered in concert by environmental conditions (*e.g*. Langer *et al*., 2006), it is also likely that morphogenesis of *E. huxleyi* coccoliths is impaired by f/2 nitrate concentration. We have addressed this question by growing a monoclonal culture of *E. huxleyi* in natural sea-water enriched with different nitrate concentrations. Calcification rate, coccolith morphology, growth rate, particulate organic carbon production and particulate organic nitrogen production were determined.

2. Material and methods

A monospecific culture of *Emiliania huxleyi* (strain PML92/11) was grown in sterile filtered (0.2μm) sea-water enriched with 7μM phosphate, and with trace metals and vitamins according to f/2 (Guillard, 1975). Sea-water was collected from surface waters off Helgoland (Germany). Nutrient samples (30mL) were filtered through precombusted (12 hours, 500˚C) glass-fibre filters (Whatman GF/F), and nitrate plus nitrite (NOx) was measured on an Alliance EVOLUTION^3 Autoanalyser, according to Hansen & Koroleff (1999). The sea-water off Helgoland had a natural NOx concentration of 10.3μmol L^{-1} (which is considered as nitrate, because the percentage of nitrite is usually negligible). Cells were grown in sea-water with the following nitrate concentrations: 10μmol L^{-1}, natural sea-water without nitrate added (NSW), 98μmol L^{-1}, NSW with 88μmol L^{-1} nitrate added (f/20), 230μmol L^{-1}, NSW with 220μmol L^{-1} nitrate added (f/8), 450μmol L^{-1}, NSW with 440μmol L^{-1} nitrate added (f/4),

and 890μmol L^{-1}, NSW with 880μmol L^{-1} nitrate added (f/2). The incident photon flux density was 270μmol m^{-2} s^{-1} and a 16/8-hour light/dark cycle was applied. Experiments were carried out at 17˚C. Cells were acclimated to experimental conditions for approximately 10 generations and grown in dilute batch cultures, in duplicate (Langer *et al*., 2006).

Dissolved inorganic carbon (DIC) samples were sterile-filtered (0.2μm) and stored in 13mL borosilicate flasks, free of air bubbles, at 0˚C. DIC was measured photometrically (Stoll *et al*., 2001), in triplicate. Less than 4% DIC consumption (*i.e*. DIC consumed by the cells at the end of the experiment) ensured insignificant changes in carbonate chemistry during growth (Langer *et al*., 2006). Samples for determination of total particulate carbon (TPC) and particulate organic carbon (POC) were filtered on precombusted (12 hours, 500˚C) GF/F filters (Whatman, approx. 0.7μm) and stored at -20˚C. Prior to analysis, 230μL of a HCl solution (5mol L^{-1}) was added on top of the POC filters to remove all inorganic carbon. TPC and POC were subsequently measured on a Euro EA Analyser by 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 directly after sampling using a Coulter Multisizer III. Cell density was plotted against time, and growth rate (μ) was calculated by means of exponential regression and is given in [per day]. Particulate inorganic carbon production (PIC production) was calculated according to:

$$P(PIC) = \mu * (\text{cellular inorganic carbon content}) \text{ [pg PIC cell}^{-1} \text{ day}^{-1}\text{]}$$

Particulate organic carbon production (POC production) was calculated according to:

$$P(POC) = \mu * (\text{cellular organic carbon content}) \text{ [pg POC cell}^{-1} \text{ day}^{-1}\text{]}$$

Particulate organic nitrogen production (PON production) was calculated according to:

$$P(PON) = \mu * (\text{cellular organic nitrogen content}) \text{ [pg PON cell}^{-1} \text{ day}^{-1}\text{]}$$

Particulate inorganic carbon production is termed 'calcification rate' in the following.

Samples for scanning electron microscope (SEM) analysis were filtered on polycarbonate filters (0.2μm pore-size), dried in a drying cabinet at 60˚C for 24 hours, then sputter-coated with gold-palladium. Imaging was performed with a Philips XL-30 digital scanning field-emission electron microscope. Three categories were used to describe the morphology of *E. huxleyi* coccoliths: 'normal', 'malformed' and 'incomplete' (for reference images for the categories, see Figure 1). An average of ~350 coccoliths was analysed per sample.

3. Results and discussion

The coccolith morphology of *Emiliania huxleyi*, grown in natural sea-water with elevated nitrate concentration, was analysed. To quantify morphology, coccoliths were divided into three categories, namely 'normal', 'malformed' and 'incomplete' (Figure 1). The percentages of coccoliths in the three categories did not change significantly with nitrate concentration (Figure 2, Table 1). This shows clearly that the unnaturally high nitrate concentration of the common sea-water supplement f/2 (Guillard, 1975) does not influence coccolith morphology. The percentage of malformed or incomplete coccoliths was ~30% (Figure 2, Table 1). In oceanic samples, this number is, typically, one order of magnitude smaller. However, in the stock culture of the strain used in this study, we have occasionally observed up to 98% of malformed or incomplete coccoliths (cells grown in the f/2 medium). The reason for the highly variable degree of malformation in coccoliths of cultured specimens therefore remains obscure.

Calcification rate did not

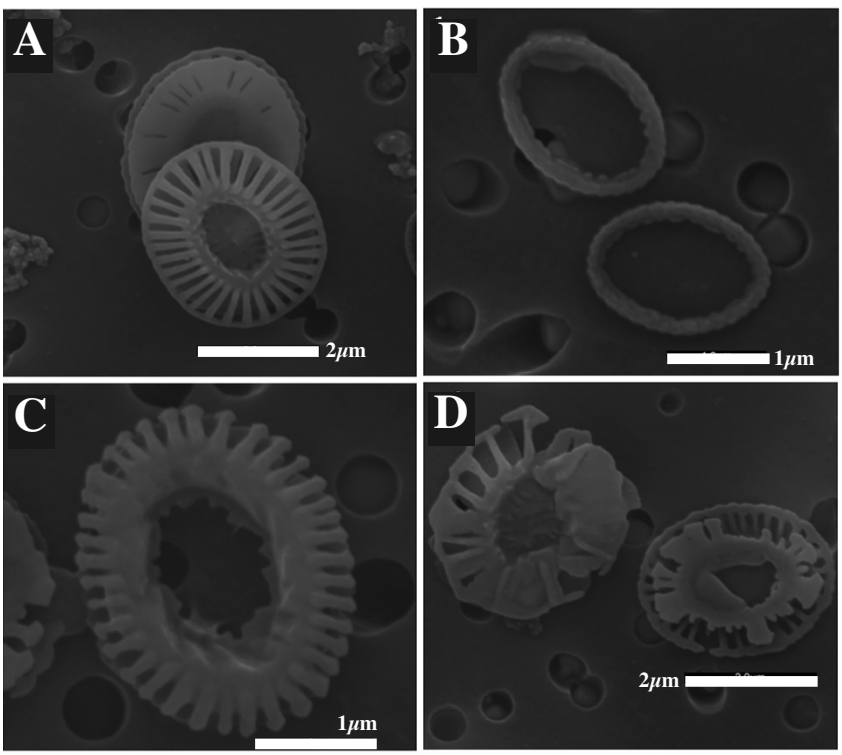

Figure 1: SEM images of *Emiliania huxleyi* coccoliths. **A**) Two normal coccoliths, showing proximal (background) and distal (foreground) shields. **B**) Two incomplete coccoliths, comprising proto-coccolith ring and incipient central area. **C**) Incomplete coccolith, at ~80% growth. **D**) Two malformed coccoliths, showing distal (left) and proximal (right) shields

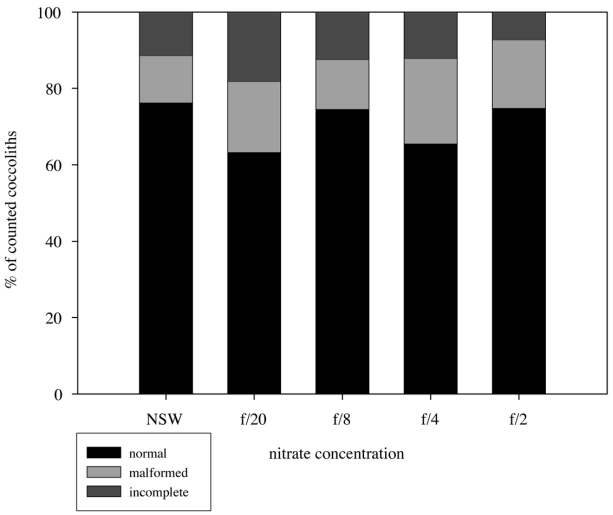

Figure 2: Percentage of normal, malformed and incomplete coccoliths *vs*. nitrate concentration. Values represent an average of duplicate experiments

change with increasing nitrate concentration either (Figure 3, Table 2). However, in an earlier study, decreased calcification rate of *E. huxleyi* under 1000μmol L^{-1} nitrate has been observed (Nimer & Merrett, 1993). The apparent discrepancy between our results and the results of Nimer & Merrett (1993) can be reconciled: firstly, there might be strain-specific responses involved - Nimer & Merrett (1993) used a different strain; and, secondly, the methodologies of the two approaches are quite different. Calcification rate, as measured in this study, is an integrated value over the course of the experiment, that is, over several generations and including light and dark phases (see Material and methods, above). Nimer & Merrett (1993) conducted short-term (minutes) ^{14}C-incorporation measurements, which do not capture lag phases between coccolith production. This latter study, however, showed that there is principally some mechanism by which the nitrate concentration typical for the f/2 medium can affect the process of calcification.

In another study using *E. huxleyi* (strain 92-a), it was observed that cells produced no coccoliths under 1800μmol L^{-1} nitrate, whereas they did under 200μmol L^{-1} (Wilbur & Watabe, 1963). Another strain of *E. huxleyi* (strain BT-6), however, developed coccoliths at both 200μmol L^{-1} and 1800μmol L^{-1} (Wilbur & Watabe, 1963). This observation suggests that strain-specific effects play a considerable role in the physiological response to high nitrate concentrations. However, we did not test 1800μmol L^{-1}, because there is no common sea-water-supplement recipe including such a high nitrate concentration (Probert & Houdan, 2004).

Hymenomonas sp. was grown under even higher nitrate concentrations, namely 6mmol L^{-1} and 24mmol L^{-1} (Baumann *et al*., 1978). A decrease in the particulate inorganic calcium to particulate nitrogen ratio was observed with increasing nitrate concentration, indicating either a hampered calcification or an increased nitrogen assimilation under high nitrate concentration. Since the authors reported neither particulate calcium nor particulate nitrogen normalised to cell nor growth rates, the two processes, that is, calcification and nitrogen assimilation, cannot be distinguished. For *E. huxleyi*, we measured growth rate, cellular PIC, POC and PON content, and PIC, POC and PON production (Figures 3 and 4, Table 2). None of these parameters showed a trend over the range of nitrogen concentrations tested. The difference compared with the results of Baumann *et al*. (1978) might be explained by either species-specific effects or the higher nitrate concentrations tested by Baumann *et al*. (1978). The fact that POC production did not change (Figure 4A, Table 2) is in apparent contradiction to the findings of Nimer & Merrett (1993), who described diminished ^{14}C uptake rates, with respect to organic carbon. This apparent contradiction can be reconciled by applying the same reasoning as we did with respect to calcification rate (see above).

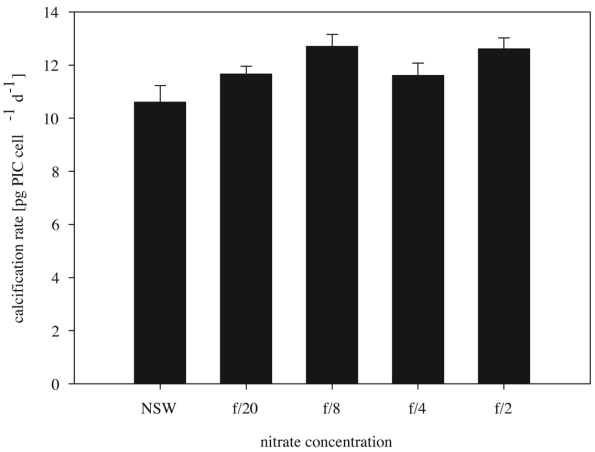

Figure 3: Calcification rate *vs*. nitrate concentration. Values represent an average of duplicate experiments

To summarise, we have shown that the nitrate concentration of f/2 affects neither organic carbon or organic nitrogen production, nor calcification rate or coccolith morphology of *E. huxleyi* (PML92/11). It

nitrate concentration	normal %	malformed %	incomplete %
NSW A	82	10	8
NSW B	71	15	15
f/20 A	64	18	18
f/20 B	63	19	18
f/8 A	77	14	10
f/8 B	73	12	15
f/4 A	64	25	11
f/4 B	67	20	13
f/2 A	72	21	7
f/2 B	77	15	8

Table 1: Percentages of normal *vs*. malformed *vs*. incomplete coccoliths derived from the morphology analysis. Data from duplicate experiments (A, B) listed separately

nitrate concentration		NSW	f/20	f/8	f/4	f/2
PIC content	pg PIC*cell^{-1}	7.5	8.1	8.8	8.0	8.9
	st. dev.	0.4	0.2	0.3	0.3	0.3
POC content	pg POC*cell^{-1}	10.5	11.3	10.8	10.2	11.0
	st. dev.	0.2	0.2	0.1	0.1	0.3
PON content	pg PON*cell^{-1}	1.7	2.0	1.6	1.6	1.8
	st. dev.	0.1	0.2	0.1	0.1	0.1
PIC production	pg PIC*cell^{-1}*d^{-1}	10.6	11.7	12.7	11.6	12.6
	st. dev.	0.6	0.3	0.4	0.5	0.4
POC production	pg POC*cell^{-1}*d^{-1}	14.9	16.2	15.7	14.8	15.6
	st. dev.	0.3	0.3	0.2	0.2	0.5
PON production	pg PON*cell^{-1}*d^{-1}	2.3	2.8	2.3	2.2	2.4
	st. dev.	0.1	0.1	0.1	0.0	0.1
growth rate μ	d^{-1}	1.41	1.44	1.45	1.45	1.41
	st. dev.	0.05	0.02	0.00	0.01	0.00
PIC/POC	mol*mol^{-1}	0.7	0.7	0.8	0.8	0.8
	st. dev.	0.0	0.0	0.0	0.0	0.0
PIC/PON	mol*mol^{-1}	5.1	4.7	6.2	5.8	5.8
	st. dev.	0.4	0.4	0.4	0.3	0.3

Table 2: Dataset derived from the cell count, particulate carbon and particulate nitrogen analyses. Values represent average and standard deviation of duplicate experiments

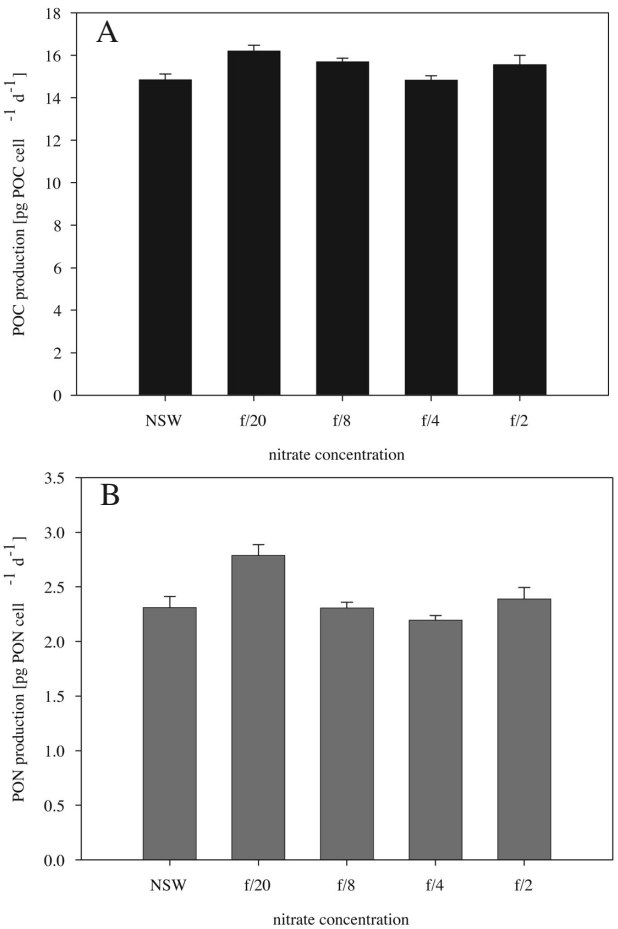

Figure 4: POC production (**A**) and PON production (**B**) *vs*. nitrate concentration. Values represent an average of duplicate experiments

remains to be tested whether this result holds true for other clones of *E. huxleyi*, or even other species.

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