

# Short-term response of the coccolithophore *Emiliana huxleyi* to an abrupt change in seawater carbon dioxide concentrations

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**Abstract.** The response of the coccolithophore *Emiliana huxleyi* to rising CO<sub>2</sub> concentrations is well documented for acclimated cultures where cells are exposed to the CO<sub>2</sub> treatments for several generations prior to the experiment. The exact number of generations required for acclimation to CO<sub>2</sub>-induced changes in seawater carbonate chemistry, however, is unknown. Here we show that *Emiliana huxleyi*'s short-term response (26 h) after cultures (grown at 500 µatm) were abruptly exposed to changed CO<sub>2</sub> concentrations (~190, 410, 800 and 1500 µatm) is similar to that obtained with acclimated cultures under comparable conditions in earlier studies. Most importantly, from the lower CO<sub>2</sub> levels (190 and 410 µatm) to 750 and 1500 µatm calcification decreased and organic carbon fixation increased within the first 8 to 14 h after exposing the cultures to changes in carbonate chemistry. This suggests that *Emiliana huxleyi* rapidly alters the rates of essential metabolic processes in response to changes in seawater carbonate chemistry, establishing a new physiological “state” (acclimation) within a matter of hours. If this relatively rapid response applies to other phytoplankton species, it may simplify interpretation of studies with natural communities (e.g. mesocosm studies and ship-board incubations), where often it is not feasible to allow for a pre-conditioning phase before starting experimental incubations.

## 1 Introduction

By the year 2100 atmospheric CO<sub>2</sub> concentration is expected, for a “business-as-usual” CO<sub>2</sub> emission scenario, to almost triple from pre-industrial values (IPCC, 2007), with a concomitant 45% decrease of CO<sub>3</sub><sup>2-</sup> ion concentrations and a drop of 0.4 pH units in the surface ocean.

Substantial effort has been undertaken to understand phytoplankton responses to these changes, with different laboratory approaches including incubations with dilute (Burkhardt et al., 1999; Riebesell et al., 2000a; Rost et al., 2003) and dense monoclonal batch cultures (Iglesias-Rodriguez et al., 2008), semi-continuous (Barcelos e Ramos et al., 2007; Fu et al., 2007; Xia and Gao, 2003) and chemostat (Sciandra et al., 2003) cultures, as well as ship-board incubations (Tortell et al., 2002, 2008) and mesocosm field experiments of natural populations (Delille et al., 2005; Engel et al., 2005; Riebesell et al., 2007).

Particular attention has been given to coccolithophores, a group of calcifying marine phytoplankton which was found to exhibit distinct sensitivity to ocean acidification. Indeed, members of this group, which is considered responsible for a significant fraction of the pelagic biogenic carbonate precipitation (Milliman, 1993), responded to CO<sub>2</sub> induced seawater acidification by changing cellular calcification rates. The best studied and probably most productive coccolithophore, *Emiliana huxleyi*, has generally been found to decrease its calcification rate in response to elevated CO<sub>2</sub> concentrations under nutrient and light replete conditions (Feng et al., 2008; Riebesell et al., 2000b; Zondervan et al., 2001).

All laboratory work on CO<sub>2</sub>/pH sensitivity of *Emiliana huxleyi* so far have used cultures pre-exposed (acclimated) to the experimental CO<sub>2</sub> treatment. While a common acclimation period applied in these studies corresponds to about 9 to 12 generations (Riebesell et al., 2000b; Zondervan et al., 2002; Feng et al., 2008), the actual time needed for acclimation to elevated CO<sub>2</sub> is unknown. Acclimation period here refers to the time necessary for individual cells to establish a new physiological “state” in response to a change in the environmental condition.

In cases where an individual's phenotypic plasticity (acclimation) and the population's genotypic variability are insufficient to maintain competitive fitness under changing environmental conditions, a species' survival may depend on its ability to adapt (Bell and Collins, 2008). Projecting a



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species' long-term response to environmental change therefore requires knowledge about both its acclimation and adaptation potential. Phenotypic plasticity responses to a changing environment may delay, favour or even speed up adaptive evolution (Ghalambor et al., 2007), further complicating attempts of predicting pathways of species evolution and ecosystem development under changing environmental conditions. With regard to *Emiliania huxleyi* it is unknown what role the observed phenotypic plasticity will have in its response to changes in the carbonate system, or whether natural populations will have the potential to adapt to the high CO<sub>2</sub> ocean. It is known that this species has high genetic variability, as reported for *Emiliania huxleyi* blooms (Medlin et al., 1996), but no study considering species potential for adaptation to global change has been performed to date.

Studies with natural communities are a valuable approach to address questions related to species interactions in response to climate change. Indeed, shifts in diatom communities in response to elevated CO<sub>2</sub> concentrations were described in phytoplankton assemblages from the Equatorial Pacific (Tortell et al., 2002) and Southern Ocean (Tortell et al., 2008). In recent mesocosm experiments the most pronounced CO<sub>2</sub> related effect was rather on dissolved inorganic carbon uptake and organic carbon loss from the upper water column (Schulz et al., 2008). These types of experiments are often conducted without prior acclimation of the enclosed communities to the CO<sub>2</sub> treatments. The time needed for phytoplankton physiology to respond to abrupt and drastic changes in seawater carbonate chemistry and, therefore, how long cell response mirrors a temporary stress are presently unknown. Considering the importance of studying the potential effects of rising CO<sub>2</sub> on natural communities (e.g. in mesocosm and ship-board incubations) and the relatively limited incubation time in these studies, a better understanding of the relevant time-scales in physiological processes of acclimation is urgently needed.

Thus, in this study *Emiliania huxleyi*'s response to an abrupt change in CO<sub>2</sub> concentrations was followed during 26 h and the results were compared to those obtained for acclimated cultures in earlier studies. Furthermore, by following short-term cellular responses we investigate the acclimation time necessary for phytoplankton suddenly exposed to elevated CO<sub>2</sub>.

## 2 Material and methods

### 2.1 Experimental setup

Monospecific cultures of the coccolithophore *Emiliania huxleyi* (strain isolated during 2005 mesocosm experiment in Bergen by Marius Müller) were grown at a constant CO<sub>2</sub> concentration (average of 500 µatm, with a corresponding pH<sub>total scale</sub> value of 7.8) for a total of about 20 generations (3 consecutive semi-continuous batch cultures). These pre-

cultures were continuously aerated with 0.2 µm filtered ambient (room) air (Rena Air50 aquarium pump), which allowed to grow the cultures to the cell abundance needed as inocula to start the experiment ( $2.1 \times 10^6$  cell ml<sup>-1</sup>), without major shifts in the CO<sub>2</sub> level. However, while aeration replenishes dissolved inorganic carbon (DIC), calcification reduces total alkalinity (TA), resulting in a decrease in pH and carbonate saturation state (minimum of about 7.7 pH and 0.9 Omega, with a corresponding 495 µatm CO<sub>2</sub>) at constant *p*CO<sub>2</sub>. Thus, the carbonate system from both the last pre-culture and the experiment were monitored through TA and DIC measurements. Both pre-cultures and experimental cultures were grown in 0.2 µm sterile filtered North Sea water, at 15 °C, and a photon flux density of 150 µmol m<sup>-2</sup> s<sup>-1</sup> (supplied from cool white fluorescent bulbs, Philips TLD 36W/54) and a 14/10 h light/dark cycle. Nutrient enrichment followed *f*/2 (Guillard, 1975; Guillard and Ryther, 1962) for the pre-cultures and *f*/20 (88 µmol l<sup>-1</sup> nitrate and 3.6 µmol l<sup>-1</sup> phosphate) for the experiment. The carbonate system of the media was adjusted shortly before the day of the experiment by addition of 1 molar NaOH or HCl. For the experiment, cells were inoculated just before the beginning of the light phase to a starting concentration of about  $3.5 \times 10^4$  cells ml<sup>-1</sup> in each of the 4 CO<sub>2</sub> treatments ranging from minimum values approximately 182 to maximum 1591 µatm. This corresponded to pH<sub>total scale</sub> values ranging from 8.36 to 7.47 with a concomitant 8.5-fold increase in CO<sub>2</sub>, a 1.1-fold increase in bicarbonate (HCO<sub>3</sub><sup>-</sup>), a 7-fold decrease in carbonate ion (CO<sub>3</sub><sup>2-</sup>) concentrations and a calcite saturation state ranging from 7.6 to 1.1 (Table 1). The cell abundance chosen assures that less than 2% of DIC was taken up by the cells during the experiment. After carefully mixing the culture inocula with the manipulated media, each CO<sub>2</sub> treatment was subdivided into smaller bottles for the determination of carbon fixation rate (in duplicate), carbonate chemistry, cell numbers and diameter and *F<sub>v</sub>*/*F<sub>m</sub>*. Additionally, samples were taken for scanning electron microscopy. Sampling occurred 2 h, 4 h, 8 h, 14 h, 24 h and 26 h after the start of the first light phase.

### 2.2 Carbonate system

Carbonate chemistry was calculated from temperature, salinity, phosphate, DIC and TA using CO2sys (Lewis and Wallace, 1998), with the equilibrium constants given in Roy et al. (Roy et al., 1993). DIC was measured photochemically (Stoll et al., 2001) using an automated segmented-flow analyzer (Quattro) equipped with an auto-sampler (+/- 10 µmol kg<sup>-1</sup> accuracy and 5 µmol kg<sup>-1</sup> precision). DIC measurements were calibrated with certified reference material (Dickson standard). Alkalinity was measured according to Dickson et al. (2003) in duplicate (minimum) through potentiometric titration, using a Metrohm Titration 808 with about 24 µmol kg<sup>-1</sup> accuracy (calibration with Dickson standard) and 3.5 µmol kg<sup>-1</sup> precision.

**Table 1.** Carbonate system data determined from total alkalinity (TA) and dissolved inorganic carbon (DIC) at 15 °C, 34 salinity and 3.4  $\mu\text{mol l}^{-1}$  phosphate using CO2sys (Lewis and Wallace, 1998) with the equilibrium constants given in Roy et al. (1993) at different times of the experiment. While all TA values were measured, meaningful values of DIC could only be obtained for the initial water (start) due to storage problems of the remaining samples (calculated DIC in italic).

Sample	Timing	TA ( $\mu\text{mol kg}^{-1}$ )	DIC ( $\mu\text{mol kg}^{-1}$ )	$p\text{CO}_2$ ( $\mu\text{atm}$ )	pH free scale	pH total scale	$\text{HCO}_3^-$ ( $\mu\text{mol kg}^{-1}$ )	$\text{CO}_3^{2-}$ ( $\mu\text{mol kg}^{-1}$ )	$\text{CO}_2$ ( $\mu\text{mol kg}^{-1}$ )	Omega for calcite
pre-culture	start	2328	2135	502	8.05	7.97	1971	145	19	3.5
pre-culture	end	1084	1029**	495	7.75	7.67	975	36	19	0.9
exp.	0h	2558	2135	203	8.41	8.33	1822	305	8	7.3
exp.	0h	2359	2135	432	8.11	8.04	1954	165	16	4.0
exp.	0h	2228	2135	899	7.81	7.73	2017	85	34	2.0
exp.	0h	2150	2135	1588	7.56	7.49	2027	48	60	1.2
exp.	14h	2511	2071	182	8.44	8.36	1750	315	7	7.6
exp.	14h	2305	2068	386	8.14	8.07	1882	172	15	4.1
exp.	14h	2183	2069	750	7.87	7.79	1946	95	28	2.3
exp.	14h	2106	2081	1435	7.60	7.52	1976	51	54	1.2
exp.	26h	2490	2060	186	8.43	8.35	1746	307	7	7.4
exp.	26h	2263	2047	418	8.12	8.03	1874	157	16	3.8
exp.	26h	2174	2065	772	7.86	7.78	1944	92	29	2.2
exp.	26h	2077	2066	1591	7.55	7.47	1961	45	60	1.1

\*\* Estimated from TA and pH measured through potentiometric titration.

Due to storage problems not all DIC measurements were meaningful. Therefore, we used the DIC measurements ( $2135 \mu\text{mol kg}^{-1}$ ) of the pre-culture media (which was the same media as used in the experiment) to calculate the carbonate system at the start of the experiment (0h). To estimate DIC values 14 h after the label addition (at the end of the light phase), the DIC drawdown (calculated from total particulate carbon, which was measured by the  $^{14}\text{C}$  method, see below) for each  $\text{CO}_2$  concentration was subtracted from the measured starting value. Between 14 and 24 h, during the dark phase, DIC continues to change due to calcification and respiration, but no TPC measurements were done for this interval. The next TPC sampling occurred after 26 h, being DIC then influenced by calcification, respiration and organic carbon fixation (between 24 and 26 h). Hence, the change in DIC by calcification (14 to 26 h) was estimated from changes in total alkalinity, divided by 2, since it decreases both DIC and TA in a 1 to 2 relation. As it was not possible to quantify respiration in the dark phase (14 to 24 h), it was assumed that organic carbon fixation in the first 2 h of the following day (24 to 26 h) decreased DIC with similar magnitude as did the increase in DIC due to respiration.

### 2.3 Carbon fixation

For each data point (time after onset of light: 2 h; 4 h; 8 h; 14 h; and 26 h)  $6 \times 65$  ml culture flasks were spiked with  $100 \mu\text{l}$  of a  $1.85 \times 10^{12}$  Bq  $\text{H}^{14}\text{CO}_3^-$  solution, of which 4 flasks were incubated under experimental conditions and 2 were kept in the dark. Radioactive label was added to all samples just before the light phases of the experiment days (0 and 24 h). Duplicate subsamples for total (25 ml) and organic (40 ml)

particulate carbon, plus the corresponding samples which were kept in the dark were filtered onto cellulose acetate ( $0.45 \mu\text{m}$ ) filters under low pressure (200 mbar). After filtration, 1 ml HCl (0.1 molar) was added to the particulate organic carbon filter (organic carbon fixation) for 30 s, assuring the dissolution of all calcium carbonate (see Müller et al., 2008). Both filters were rinsed with  $0.2 \mu\text{m}$  filtered seawater, removing excess of radioactive dissolved inorganic carbon. Lumagel Plus (Universal LSC cocktail) was then added to the filters in scintillation vials and the radioactivity measured in a Liquid Scintillation Analyser (Tri-Carb 2900TR, Packard). Particulate inorganic carbon fixation (calcification) was calculated as the difference between total carbon (not acidified filters) and organic carbon (acidified filters) fixation.

For the carbon fixation calculation it was assumed that the uptake of  $^{14}\text{C}$  is 5% slower than  $^{12}\text{C}$  and that 6% of the fixed carbon is lost in respiratory process throughout the day (based on Steeman-Nielsen, 1952).

### 2.4 Cell diameter and numbers

Cell abundance and diameter were determined immediately after sampling at each time point by using a Coulter Counter Z series (Beckman Coulter). Cell division rate ( $\mu$ ) was calculated according to:

$$\mu = (\ln C_e - \ln C_i) / \Delta t \quad (1)$$

where  $C_e$  and  $C_i$  refer to end and initial cell concentrations, respectively and  $\Delta t$  to the duration of the experiment in days. For Fig. 4  $C_e$  corresponds to 24 and 26 h and  $C_i$  to 0 and 2 h respectively.

## 2.5 Maximum photochemical quantum yield of photosystem II ( $F_v/F_m$ )

Photosynthetic efficiency was determined as  $F_v/F_m$  by using a PAM (PhytoPAM, Phyto-ED Walz, PPAA0138). Samples were placed in the dark for 20 min (without any previous treatment) before determination of  $F_v/F_m$ .

## 2.6 Scanning electron microscopy (SEM)

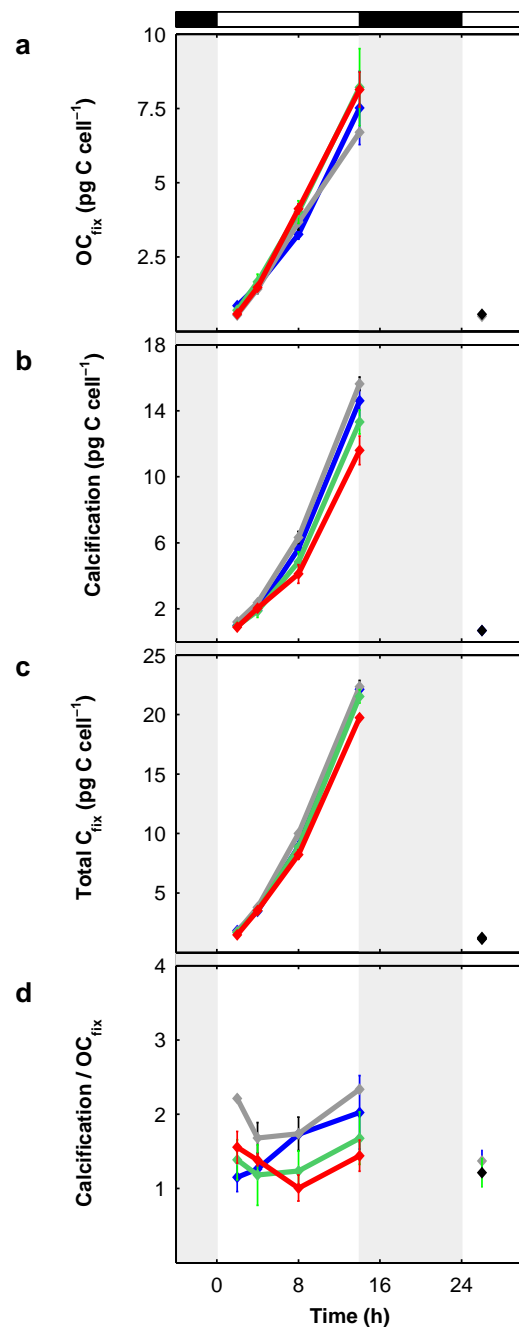
SEM samples were fixed with formaldehyde (1% final concentration) at each time point. Samples were then filtrated onto polycarbonate filters (0.45  $\mu\text{m}$  pore size) under low pressure (<200 mbar), dried for 12 h at 60 °C and glued on aluminium stubs. The filters were coated with gold-palladium and photographs of the most representative specimens taken with a CamScan-CS-44 (Scanning electron microscope) at the Institute of Geosciences of the Christian Albrechts University in Kiel.

## 2.7 Statistic analysis

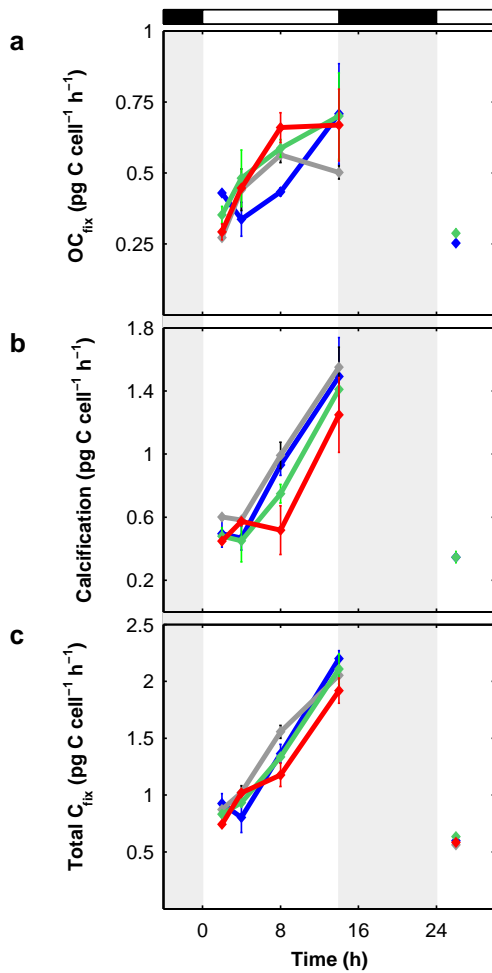
The data was analyzed with a linear correlation test ( $R$ ), which was calculated using a Matlab function.

## 3 Results

After 8 h of exposure to the experimental  $\text{CO}_2$  levels (with 26 h average of each condition being  $\sim 190$ , 410, 800 and 1500  $\mu\text{atm}$ ) cumulative organic carbon fixation in *Emiliana huxleyi* increased with  $\text{CO}_2$  concentration (Fig. 1a), with statistical significance ( $p = 0.0499$ ) and a linear correlation factor ( $R$ ) of 0.7069. After 14 h, however, this increase was not statistically significant ( $R = 0.1941$ ,  $p = 0.6766$ ). The opposite trend, a decrease with increasing  $\text{CO}_2$ , was obtained for cellular calcification (Fig. 1b) ( $R$  of  $-0.7699$  ( $p = 0.0255$ )) 8 h after the addition of the  $^{14}\text{C}$  label and  $-0.7892$  ( $p = 0.0199$ ) after 14 h). Due to a stronger decrease in calcification compared to the increase in organic carbon fixation the cumulative total carbon fixation decreased with rising  $\text{CO}_2$  (Fig. 1c). Carbon fixation rates were also determined for each period between 2 consecutive sampling points. From 4 to 8 h after the inoculation, organic carbon fixation rate increased 35% from the lowest to the highest  $\text{CO}_2$  level, 15% from 410 to 1500  $\mu\text{atm}$  and 27% from 190 to approximately 800  $\mu\text{atm}$  (Fig. 2a). For the same period of time, this corresponded to a 19% decrease in the calcification rate from 190  $\mu\text{atm}$  to approximately 800  $\mu\text{atm}$  and 44% from 190 to 1500  $\mu\text{atm}$  (Fig. 2b). Total carbon fixation and calcification rates increased during the whole light phase. Organic carbon fixation rates increased in two treatments over the course of the day, one treatment (410  $\mu\text{atm}$ ) displayed a decrease and one treatment did not vary between 8 and 14 h. After 26 h, at the beginning of the new light phase, carbon fixation rates were again at the low levels measured at the



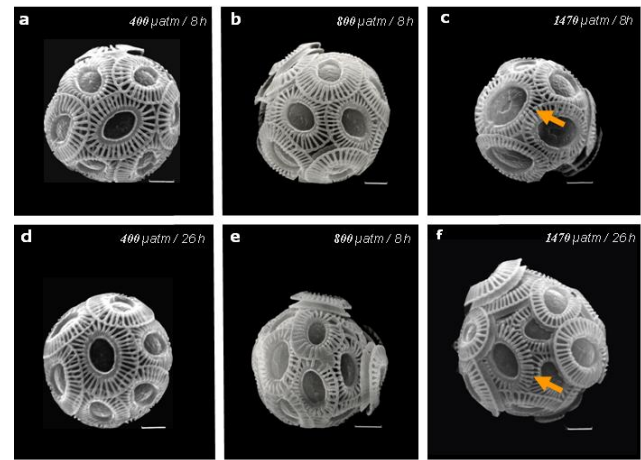
**Fig. 1.** Cumulative carbon fixation of *Emiliana huxleyi* through time. (a) organic carbon fixation per cell, (b) calcification per cell, (c) total carbon fixation per cell and (d) ratio between calcification and organic carbon fixation. 190  $\mu\text{atm}$   $\text{CO}_2$  (blue), 410  $\mu\text{atm}$   $\text{CO}_2$  (grey), 800  $\mu\text{atm}$   $\text{CO}_2$  (green), 1500  $\mu\text{atm}$   $\text{CO}_2$  (red). Data from the 26 h considers only a 2 h incubation period. Each  $\text{CO}_2$  level has duplicate measurements. Vertical error bars represent the range of the data and the lines connect the averages of each time point. The white/black bar on top represents the light/dark diel cycle, vertical grey bars denote the dark phase.



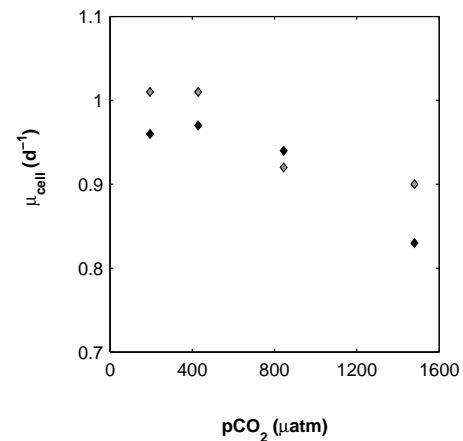
**Fig. 2.** Carbon fixation rates of *Emiliania huxleyi* determined for each period of time between consecutive sampling points. (a) organic carbon fixation per cell per h, (b) calcification per cell per h and (c) total carbon fixation per cell per h. For each period of time the data point marks the end of the incubation. Each CO<sub>2</sub> level has duplicate measurements. Vertical error bars represent the range of the data and the lines connect the averages of each time point. Line and color coding as in Fig. 1.

start of the experiment (Fig. 2). At this point, organic carbon fixation rates slightly increased and calcification rates slightly decreased from 190 to 1500 µatm (difficult to spot at the y axes range in Fig. 2).

The decrease in the ratio of calcification to organic carbon fixation (Calcification/OC<sub>fix</sub>) with rising CO<sub>2</sub> (Fig. 1d) became evident about 8 h after the inoculation. This trend is maintained even after the start of the next light phase, even though with a smaller slope of the linear regression and absolute values. Scanning electron microscopy after 8 h and 26 h reveals some under-calcified coccoliths on cell's exposed to high CO<sub>2</sub> concentration (Fig. 3). The under-calcified coccoliths are mostly in the layer closest to the cells surface, as is expected for newly produced coccoliths. After 8 h

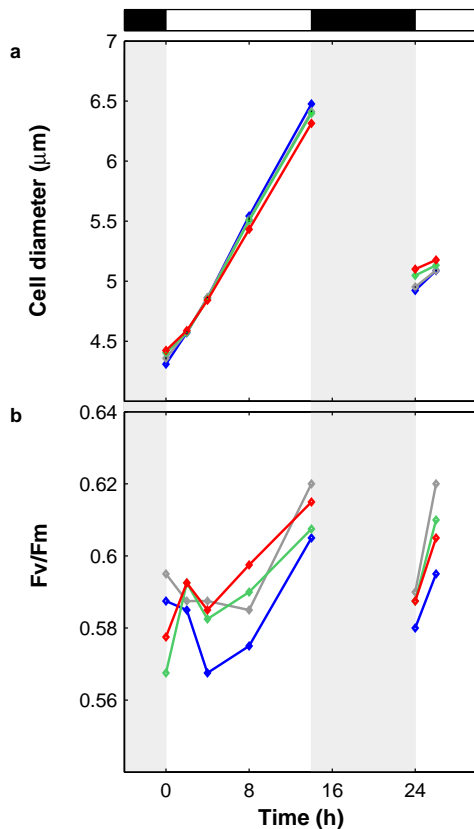


**Fig. 3.** Scanning electron microscope pictures of *Emiliania huxleyi* grown under different CO<sub>2</sub> concentrations after 8 h of exposure to (a) 410 µatm, (b and e) 800 µatm and (c) 1500 µatm, and after 26 h of exposure to (d) 410 µatm and (f) 1500 µatm. The photos chosen are representative of the trend observed. Note the presence of under-calcified coccoliths under enhanced CO<sub>2</sub> conditions, especially visible in the connections between the elements forming the “outer ring” (orange arrows) and in the frequent enlargement of the central area. For the 800 µatm treatment both photographs correspond to cells exposed to the increase on CO<sub>2</sub> concentrations for 8 h, because no differences were found within the time considered (8 and 26 h) and the photographs taken after 26 h were not well focused. Scale bars correspond to 1 µm.



**Fig. 4.** Cell division rate ( $\mu$ ) based on cell counts of *Emiliania huxleyi* in relation to CO<sub>2</sub> levels ( $p\text{CO}_2$ ). Black diamonds correspond to calculations based on cell counts conducted at the beginning of the light phase (0 h) and after 24 h and grey diamonds to those done 2 h after the start of the light phase and after 26 h.

the under-calcified coccoliths of the 1500 µatm CO<sub>2</sub> treatment were mostly observed in smaller cells, because it is on those that the most recently formed layer becomes visible. The 800 µatm CO<sub>2</sub> treatment showed only slight under-calcification both 8 and 26 h after the manipulation.



**Fig. 5.** (a) Cell diameter and (b)  $F_v/F_m$  of *Emiliana huxleyi* through time. In Fig. 5a each  $\text{CO}_2$  level has 4 measurements from each bottle with vertical error bars representing standard errors. In Fig. 5b each data point results from the average of measurements done at 470, 520, 645 and 665 nm in one bottle. 190  $\mu\text{atm}$   $\text{CO}_2$  (blue), 410  $\mu\text{atm}$   $\text{CO}_2$  (grey), 800  $\mu\text{atm}$   $\text{CO}_2$  (green), 1500  $\mu\text{atm}$   $\text{CO}_2$  (red). The white/black bar on top represents the light/dark diel cycle, vertical grey bars denote the dark phase.

Cell division rate decreased from 1.01 at  $\sim 190 \mu\text{atm}$  to 0.90 at  $\sim 1500 \mu\text{atm}$ , having a  $R$  value of  $-0.8827$  and  $p = 0.0037$  (Fig. 4). Cell size increased during the light phase, with a weak trend of decreasing cell diameters with increasing  $\text{CO}_2$  concentrations at the end of the light phase (Fig. 5a). This trend was reversed after cell division.  $F_v/F_m$  increased during the light phase and was lower in the lowest  $\text{CO}_2$  treatment between 2 and 26 h after changing the  $\text{CO}_2$  levels (although no significant trend was observed across the 4  $\text{CO}_2$  treatments), maintaining the same trend at the beginning of the following light phase (Fig. 5b).

## 4 Discussion

### 4.1 From short-term to acclimated response

The effect of increasing  $\text{CO}_2$  concentrations in the ocean has generally been assessed by the physiological response of acclimated phytoplankton cultures (from days to weeks). However, virtually nothing is known about their short (within 24 h) and long-term (months to years) response.

### 4.2 Calcification

Our results showed that within hours after the high  $\text{CO}_2$  exposure the calcification response of non-acclimated *Emiliana huxleyi* is similar to that observed in acclimated cultures (Riebesell et al., 2000b), under the same light irradiance ( $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), temperature ( $15^\circ\text{C}$ ), similar  $\text{CO}_2$  range ( $\sim 190$  to  $800 \mu\text{atm}$ ) and L/D cycle (in this study 14/10 while others 16/8). In fact, after 8 h we found a 19% decrease in calcification with rising  $\text{CO}_2$  concentrations which compares well with the 15.7% found by Riebesell et al. (2000b). In terms of the absolute values, calcification was slightly higher in this compared to the previous study.

Remarkably the decrease in calcification could be seen with scanning electron microscopy already after short-term exposure to high  $\text{CO}_2$  (1500  $\mu\text{atm}$ ). Cells grown under elevated  $\text{CO}_2$  levels showed increased numbers of incomplete or under-calcified coccoliths. However, because newly formed coccoliths are positioned at the cell surface and were therefore hidden by a second layer of coccoliths formed under pre-experimental conditions (approx. 1 coccolith per hour, Paasche, 2002), a systematic analysis of the degree of calcification and the frequency of malformations was not possible in this short-term incubation.

#### 4.2.1 Organic carbon fixation and $F_v/F_m$

As previously reported, elevated  $\text{CO}_2$  stimulated organic carbon fixation, although the effect was almost 3-fold higher (8 h after changing  $\text{CO}_2$  concentrations) than observed in an earlier study (Riebesell et al., 2000b).

In Riebesell et al. (2000b) carbon fixation rates are integrated over several days representing, therefore, net carbon fixation. Here  $^{14}\text{C}$  samples taken after a few hours would measure gross carbon fixation since during this period most of the organic matter being respired was produced prior to  $^{14}\text{C}$  label addition. For that reason, all calculations of  $^{14}\text{C}$  incorporation assumed that 6% of the fixed carbon was lost in the respiratory process, giving this way also net carbon fixation (Steeman-Nielsen, 1952). Longer incubations, including respiration in the dark phase, result in net carbon fixation rates. Thus the use of 6% in the calculations might slightly underestimate the values obtained at 8 and 14 h, but this small bias does not influence our conclusions.

In agreement with the increase of organic carbon fixation rates under enhanced CO<sub>2</sub> conditions there was an increase of the maximum photochemical quantum yield of photosystem II ( $F_v/F_m$ ) during the first 14 h. Interestingly, cells subjected to a decrease in the CO<sub>2</sub> concentration (from average ~500  $\mu\text{atm}$  in the pre-culture to 190  $\mu\text{atm}$ ) had the lowest  $F_v/F_m$  between 2 and 26 h after changing the CO<sub>2</sub> levels.  $F_v/F_m$  is lower when the electrons can not be transported as fast as their production. In this case, a decrease in organic carbon fixation rate due to a change in CO<sub>2</sub> supply might be faster than the re-organization of the Calvin-Benson Cycle substrates, with consequent “clogging” of the electron transport chain.

#### 4.2.2 Calcification/OC<sub>fix</sub>

There was a decrease in the calcification to OC<sub>fix</sub> ratio (already 8 h after the beginning of the light phase) like in previous studies with acclimated cultures within a similar CO<sub>2</sub> range (Riebesell et al., 2000b; Zondervan et al., 2001). However, there was an overall higher Calcification/OC<sub>fix</sub> which can be explained by higher calcification rates in this study, since the organic carbon fixation was quite similar to that in a previous study (Riebesell et al., 2000b). Interestingly, the decrease of the Calcification/OC<sub>fix</sub> ratio after the start of the next light phase had a less pronounced slope of the linear regression with rising CO<sub>2</sub>.

#### 4.2.3 Diel cycle

The diurnal variation of cellular calcification and organic carbon fixation was higher than the differences encountered between the CO<sub>2</sub> levels ranging from ~190 to 1500  $\mu\text{atm}$ . This highlights the importance of the timing of sampling during experiments. For most of the time considered, both our study and Zondervan et al. (2002) show higher organic carbon fixation and lower calcification at enhanced CO<sub>2</sub> concentrations.

#### 4.2.4 Cell division rate and diameter

While cell division of *Emiliana huxleyi* was not found to be affected by elevated CO<sub>2</sub> concentrations in previous studies (Buitenhuis et al., 1999; Clark and Flynn, 2000; Rost et al., 2002) a slight decrease in cell division rate with rising CO<sub>2</sub> was observed in this investigation. This difference may be due to the broader range of CO<sub>2</sub> levels applied here. We do not expect the CO<sub>2</sub> effect on cell division rate to be a short-term stress response caused by changing the CO<sub>2</sub> manipulation procedure (aeration in the pre-cultures and non-aeration in the experiment) or other factors derived from the experimental procedure because cell division rate of the 410  $\mu\text{atm}$  treatment ( $1.01 \text{ d}^{-1}$ ) was very similar to that of the pre-cultures ( $1.02 \text{ d}^{-1} \pm 0.09$ , 4 replicates) exposed to similar CO<sub>2</sub> conditions. Moreover, a similar effect on cell division rate was also found during a long-term (>100 generations) high CO<sub>2</sub> exposure by Müller et al. (personal com-

munication, 2009), indicating that the observed response was unrelated to the abrupt change in CO<sub>2</sub> concentrations applied in this approach. The opposite trend in cell division rate with rising CO<sub>2</sub> concentration has been observed in other phytoplankton groups, such as diatoms (Riebesell et al., 1993) and the cyanobacterium *Trichodesmium* (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Levitan et al., 2007). The apparent difference in specific growth rate responses between various taxonomic groups may be related to the process of calcification, but further investigation is needed to clarify this.

In this study, the cell diameter decreased with increasing CO<sub>2</sub> concentration during the first 14 h. This is most likely due to a more pronounced decrease in calcification than the increase of organic carbon fixation with a consequent decrease in the cellular total carbon. After the dark period, when most cells had divided, on average cells exposed to elevated CO<sub>2</sub> levels had a larger cell diameter. This may be due to the slightly lower cell division rate of high CO<sub>2</sub> exposed cells resulting in a larger number of cells which had not yet undergone cell division. Lower cell diameters at the beginning of the experiment in all treatments may have resulted from higher coccolith detachment due to aeration of the pre-culture.

## 5 CO<sub>2</sub> and pH, a combined effect

Rising CO<sub>2</sub> concentration in the ocean also changes pH, [HCO<sub>3</sub><sup>-</sup>] and [CO<sub>3</sub><sup>2-</sup>], so it is hard to separate the potential effect of each parameter individually. Maintaining a high concentration of CO<sub>2</sub> at the site of carboxylation to ensure efficient operation of the CO<sub>2</sub> fixing enzyme ribulose-1, 5-biphosphate carboxylase/oxygenase (RuBisCO) is an energy demanding process. A CO<sub>2</sub> increase in the surrounding environment of a cell is likely to decrease the net diffusive efflux of CO<sub>2</sub>, reducing the energy needed to maintain high CO<sub>2</sub> inside the cell. The lower energetic cost may be used to increase organic carbon fixation. As for calcification, the decrease in the calcite and aragonite saturation states has been connected to the observed decrease in calcification in foraminifera (Bijma et al., 1999) and corals (Langdon et al., 2000; Leclercq and Gattuso, 2002; Leclercq et al., 2000). As coccolithophore calcification occurs intracellularly and there is no evidence of CO<sub>3</sub><sup>2-</sup> utilization or any known CO<sub>3</sub><sup>2-</sup> transporters, the observed response may rather reflect sensitivity to a decrease in pH, associated with increased energetic costs of transporting protons generated during calcification outside the cell.

Based on the observed increase of organic carbon production at high CO<sub>2</sub> concentration one might expect a concomitant increase in cell division rate, but a slight decrease was observed instead. This effect on cell division rate could be a direct consequence of changing seawater pH, affecting cellular acid-base regulation. In a study on 3 red-tide dinoflagellates Hansen et al. (2007) concluded that growth is mostly

affected by pH and that inorganic carbon only plays a minor role under low initial dissolved inorganic carbon concentrations and high pH.

Whatever parameter or combination of parameters influences the different cellular rates in this study the cells showed a fast physiological adjustment (probably with the exception of calcification) potentially at the expense of intracellular regulation of DIC content and pH. This possibly happened at the regulation level of both transporters in the membrane and electron chain, and/or enzymes.

### 5.1 Short (acclimated) to long-term experiments, stepping stones in the understanding of the effect of future climate change

Experiments done with acclimated cells often looked at how the individuals of a clonal phytoplankton culture respond to the projected changes in CO<sub>2</sub> concentrations (acclimation referred to the individual's phenotypic plasticity). *Emiliana huxleyi* acclimated (already after hours) to increasing CO<sub>2</sub> concentrations by decreased calcification and increased organic carbon fixation rates in several studies (this study; Feng et al., 2008; Riebesell et al., 2000b; Zondervan et al., 2001). However, the term acclimation is probably not applicable to the changes observed in calcification, since its decrease with increasing CO<sub>2</sub> concentrations might simply reflect the differences in the carbonate system (possibly pH). Indeed, coccolith formation might be dictated by a biological clock more than by a perfect final product, explaining malformed coccoliths under high CO<sub>2</sub> concentrations. The organic carbon fixation increase under high CO<sub>2</sub> concentrations, on the other hand, might result in a physiological readjustment by reallocating excess energy saved in dissolved inorganic carbon transport to increase Calvin-Benson Cycle turnover rate and/or substrates production.

While evolutionary adaptation to increasing CO<sub>2</sub> concentrations has so far not been addressed in *Emiliana huxleyi*, helpful information can be obtained from work done with the plant *Arabidopsis thaliana* (Lau et al., 2007), the alga *Chlamydomonas* (Collins and Bell, 2004) and natural populations from CO<sub>2</sub> springs (Collins and Bell, 2006). Both these species and natural populations from CO<sub>2</sub> springs showed phenotypic changes with increased CO<sub>2</sub> treatments, but no adaptation (e.g. correlations between CO<sub>2</sub> treatment and genetic variations, mostly due to mutation, which might give competitive advantage as well as heritability, or the transfer of that CO<sub>2</sub>-induced information to the descendents). Still, these phenotypic changes might favour or even speed up adaptive evolution. The lack of indications for adaptation reinforces, on the one hand, the importance to further study phenotypic plasticity changes with rising CO<sub>2</sub> and, on the other hand, to re-evaluate the long-term experimental designs. Some long-term experiments consider that after 1000 generations there is enough genetic variability so that the culture is not clonal anymore and, therefore, can be treated as a

population (Collins et al., 2006). Nevertheless, future long-term experiments could allow for more genetic variability by using several clones, preferentially freshly isolated from the same location, and/or inducing sexual reproduction. It is also important to include some CO<sub>2</sub> variability in these experimental setups, since phytoplankton in its natural environment will not evolve under constant CO<sub>2</sub> concentration, but to an average higher concentration with changes through time. Even more so because the daily and seasonal changes of CO<sub>2</sub> concentration will be even more pronounced in the future, due to decreasing ocean buffer capacity. Finally, one has to start considering in both acclimated and long term experiments, that phytoplankton will be exposed to a combined CO<sub>2</sub>, temperature and potentially nutrient composition/availability change.

In summary, short/acclimated and long-term experiments provide complementary information about the phytoplankton response to increasing CO<sub>2</sub> and other changes in the carbonate system.

## 6 Conclusions

With this work we were able to show that the response of acclimated cultures to rising CO<sub>2</sub> apparently correspond to establishing a new physiological "equilibrium" through the change of rates of various essential processes, which *Emiliana huxleyi* cells appear to achieve in less than 24 h. This implies that the cellular adjustment to increasing CO<sub>2</sub> concentrations is independent of cell division. If this relatively rapid response applies to other phytoplankton species, it might simplify the interpretation of studies with natural communities (e.g. mesocosm studies and ship-board incubations), where often it is not feasible to allow for a pre-conditioning phase before starting experimental incubations.

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