

# Effects of long-term high CO<sub>2</sub> exposure on two species of coccolithophores

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**Abstract.** The physiological performance of two coccolithophore species, *Emiliana huxleyi* and *Coccolithus braarudii*, was investigated during long-term exposure to elevated  $p\text{CO}_2$  levels. Mono-specific cultures were grown over 152 (*E. huxleyi*) and 65 (*C. braarudii*) generations while  $p\text{CO}_2$  was gradually increased to maximum levels of 1150  $\mu\text{atm}$  (*E. huxleyi*) and 930  $\mu\text{atm}$  (*C. braarudii*) and kept constant thereafter. Rates of cell growth and cell quotas of particulate organic carbon (POC), particulate inorganic carbon (PIC) and total particulate nitrogen (TPN) were determined repeatedly throughout the incubation period. Increasing  $p\text{CO}_2$  caused a decrease in cell growth rate of 9% and 29% in *E. huxleyi* and *C. braarudii*, respectively. In both species cellular PIC:TPN and PIC:POC ratios decreased in response to rising  $p\text{CO}_2$ , whereas no change was observed in the POC:TPN ratios of *E. huxleyi* and *C. braarudii*. These results are consistent with those obtained in shorter-term high CO<sub>2</sub> exposure experiments following abrupt perturbations of the seawater carbonate system and indicate that for the strains tested here a gradual CO<sub>2</sub> increase does not alleviate CO<sub>2</sub>/pH sensitivity.

reduction in surface ocean pH by 0.12 units over the past 200 years (Alley et al., 2007). This process, termed ocean acidification, seriously alters the physiological performance of pelagic and benthic organisms (Fabry et al., 2008). Due to their ecological and biogeochemical importance and because they are easily grown in cultures, coccolithophores are among the best studied organisms with respect to their response to ocean acidification (reviewed by Rost et al., 2008 and Zondervan, 2007). Results from these studies indicate considerable species- and even strain-specific differences in CO<sub>2</sub> sensitivity.

When exposed to elevated  $p\text{CO}_2$  levels as projected for the course of this century, *Emiliana huxleyi*, *Gephyrocapsa oceanica* and *Calcidiscus quadriperforatus* respond by decreasing calcification to varying degrees, corresponding to a decrease in the particulate inorganic to organic carbon ratio, PIC:POC (Riebesell et al., 2000; Zondervan et al., 2002; Langer et al., 2006; Feng et al., 2008; Langer et al., 2009). *Coccolithus braarudii*, on the other hand, seems to be insensitive to elevated  $p\text{CO}_2$  (Langer et al., 2006) with respect to calcification. Differences in  $p\text{CO}_2$  sensitivity between species were also observed with respect to organic carbon production. While *E. huxleyi* and *G. oceanica* increased POC production by up to 50% under elevated  $p\text{CO}_2$  (Riebesell et al., 2000; Zondervan et al., 2002; Feng et al., 2008; Shi et al., 2009), no change with increasing  $p\text{CO}_2$  was observed in *C. braarudii* and *C. quadriperforatus* (Langer et al., 2006). Recently, Iglesias-Rodriguez et al. (2008) reported an increase of up to 100% in both calcification and organic carbon production per cell in response to elevated  $p\text{CO}_2$ . This trend reverses when accounting for the large difference in cell size between  $p\text{CO}_2$  treatments as observed in previous experiments on *E. huxleyi*, yielding a slight decrease in calcification with increasing  $p\text{CO}_2$  when normalizing the data to cellular carbon content (Riebesell et al., 2008b).

## 1 Introduction

Emissions of anthropogenic CO<sub>2</sub> since the beginning of the industrial revolution have led to an increase in atmospheric carbon dioxide concentration, resulting in a fraction of the anthropogenic CO<sub>2</sub> being absorbed by the ocean. The ongoing oceanic uptake of anthropogenic CO<sub>2</sub> is steadily altering the seawater carbonate chemistry and has led to a



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Most acidification experiments with coccolithophores were done applying an abrupt perturbation of seawater carbonate chemistry, either by bubbling with CO<sub>2</sub> enriched/depleted air (Feng et al., 2008; Iglesias-Rodriguez et al., 2008) or by adding acid/base (Riebesell et al., 2000; Zondervan et al., 2002; Langer et al., 2006), and preacclimating the cultures before starting the experiment (commonly 7–10 generations). After the acclimation time, experimental high CO<sub>2</sub> exposure time ranged between 1–2 (Iglesias-Rodriguez et al., 2008) and 7–10 generations (Riebesell et al., 2000; Zondervan et al., 2002; Langer et al., 2006; Feng et al., 2008). This results in a maximum of 20 cell divisions under high *p*CO<sub>2</sub> conditions. The physiological response to high *p*CO<sub>2</sub> exposure of *E. huxleyi* within one cell generation was recently investigated by Barcelos e Ramos et al. (2010) and was found to be consistent with the observations gained from longer-term experiments. However, it is presently unknown whether the observed CO<sub>2</sub> sensitivities of coccolithophores are persistent over long-term high *p*CO<sub>2</sub> exposure. Here, we report on semi-continuous batch culture experiments with two coccolithophore species (*E. huxleyi* and *C. braarudii*), which were grown over multiple generations (152 and 65 generations, respectively) under gradually increasing *p*CO<sub>2</sub> levels.

## 2 Methods

### 2.1 Cultures

*Coccolithus braarudii* RCC 1200, formerly known as *C. pelagicus* (Saez et al., 2003), was obtained from the Roscoff Culture Collection. *Emiliania huxleyi* was isolated in 2005 during the PeECE III mesocosm study (Riebesell et al., 2008a) in the Raune Fjord (Norway). Both cultures were grown as asexual diploids at 16 °C in 0.2 µm filtrated North Sea water with a salinity of 33 and f/20 nutrient additions (Guillard, 1975), corresponding to 88 µmol l<sup>-1</sup> nitrate and 3.6 µmol l<sup>-1</sup> phosphate, a sufficient supply of macro- and micronutrients for exponential algal growth under semi-continuous culture conditions (see below), at a photon flux density of 140 µmol photons m<sup>-2</sup> s<sup>-1</sup> (Philips TL-D 90 DeLuxePro, 36W/950) under a 14:10 h light:dark cycle.

### 2.2 Experimental setup

Coccolithophores were grown in duplicates under semi-continuous batch culture conditions (as described above) in 280 ml autoclaved polycarbonate Erlenmeyer flasks. *p*CO<sub>2</sub> was adjusted by additions of HCl or NaOH to the media. Cultures were allowed to grow for about 5–11 generations corresponding to a dissolved inorganic carbon (DIC) and nitrate consumption of maximal 10% and 23%, respectively. At this stage exponentially growing cultures were sampled for DIC, pH, cell number, total particulate and particulate organic carbon (TPC and POC), and total particulate nitrogen

(TPN) before being transferred into fresh medium (f/20 nutrient conditions and the carbonate system already adjusted) to a concentration of 100 and 50 cells ml<sup>-1</sup>, corresponding to a minimum population size of 28 000 and 14 000 cells (*E. huxleyi* and *C. braarudii*, respectively). This culture dilution and sampling protocol was continuously repeated throughout the course of the experiment. The media in the control flasks (low *p*CO<sub>2</sub>) was adjusted to a pH<sub>total</sub> of 8.21 ± 0.05 (±1 SD), resulting in a *p*CO<sub>2</sub> of 260 ± 20 µatm with a corresponding calcite saturation state (Ω) of 5.6 ± 0.4. At the beginning of the experiment all flasks started under conditions described above and then the treatment flasks were slowly acidified over several generations (79 and 28 generations for *E. huxleyi* and *C. braarudii*, respectively) to the target *p*CO<sub>2</sub> values (1150 ± 140 µatm and 930 ± 180 µatm for *E. huxleyi* and *C. braarudii*, respectively) and kept at these levels until the end of the experiment.

### 2.3 TPC, POC, PIC, TPN

Two sub-samples from each flask were filtrated onto precombusted (525 °C for 7 h) GF/F filters and frozen at -20 °C. For POC analysis filters were fumed over HCl for 24 h to remove all inorganic carbon and afterwards all filters were measured on a Euro EA Elemental Analyser (Ehrhardt and Koeve, 1999). PIC (particulate inorganic carbon) was calculated from the difference of TPC and POC.

Cell quotas of carbon and nitrogen increase during the light phase whereas the cell density stays constant because of synchronized cell division in the dark phase (Müller et al., 2008). Because samples were taken at different times of the light phase, normalizing the data on a per cell basis generates a bias in the data due to sampling time. We therefore present the data as cellular ratios of PIC:POC, PIC:TPN and POC:TPN, which show no temporal trend over the course of the light phase (Fig. 1).

### 2.4 Cell counts

One sub-sample from each flask was taken and the cell number was immediately determined with a Coulter Counter (Z Series). Samples were measured three times and the mean was used to calculate the growth rate “μ” (d<sup>-1</sup>) as

$$\mu = \frac{(\ln c_1 - \ln c_0)}{t_1 - t_0} \quad (1)$$

where *c*<sub>0</sub> and *c*<sub>1</sub> are the cell concentrations at the beginning (*t*<sub>0</sub>) and end (*t*<sub>1</sub>) of the incubation period (expressed in days).

### 2.5 Carbonate system

The carbonate system was monitored by DIC and pH measurements. DIC was analyzed after Stoll et al. (2001) using an automated segmented-flow analyzer (Quaatro) equipped with an auto-sampler (±10 µmol kg<sup>-1</sup> accuracy and five µmol kg<sup>-1</sup> precision) and pH was measured using a

“Metrohm 713 pH-Meter”, equipped with pH and reference electrodes and temperature sensor. Sensor and electrodes were stored in filtrated seawater at 16 °C to match the ionic strength of the sampled water. pH measurements were periodically checked by calculating pH from measurements of total alkalinity (Dickson, 1981) and DIC of filtrated seawater using the program CO<sub>2</sub>sys (version 1.05 by E. Lewis and D. W. R. Wallace) with dissociation constants for carbonic acid after Roy et al. (1993). Calculated pH values closely agreed with pH measurements with a maximum deviation of  $\pm 0.02$ . Here we present pH values on the total scale.

## 2.6 Scanning electron microscopy

5 ml samples were taken periodically from the control and high  $p\text{CO}_2$  treatment and fixed with formaldehyde (1% end concentration). Subsequently, the samples were filtered onto polycarbonate filters (0.2  $\mu\text{m}$  pore size), dried at 60 °C for 24 h and then sputter-coated with gold-palladium. Pictures were taken with a CamScan-CS-44 scanning electron microscope at the Institute of Geosciences of the Christian-Albrecht-University in Kiel.

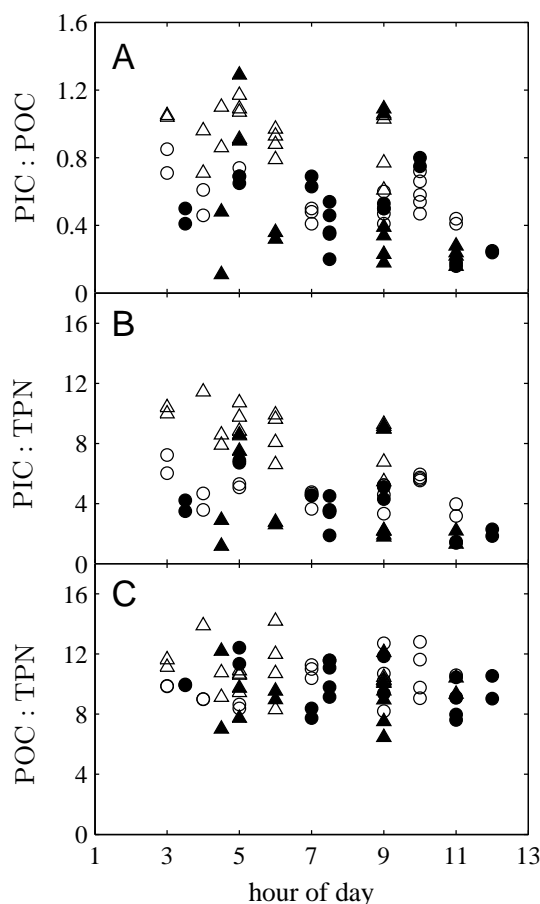
## 2.7 Statistical analysis

As the data set does not allow for parametric statistics, it was analyzed by means of the binomial test. To test the null hypothesis that two categories are observed equally often, mean values of the duplicate samples under continuously high  $p\text{CO}_2$  were compared to the corresponding mean values of the duplicates under low  $p\text{CO}_2$  sampled at similar generations. A mean value under the continuously high  $p\text{CO}_2$  treatment that was greater than the corresponding mean value of the low  $p\text{CO}_2$  treatment constituted the first category (coded as “+”); otherwise it was coded as “-” for the second category. Note that under the null-hypothesis both categories should be observed equally often. Results are given with the  $p$ -value of the binomial test plus the corresponding distribution ratio ( $dr$ ) of “+” to “-”. A note of caution might be required here: the sample size for the binomial test was rather small throughout the experiment (varying between 3 and 8). Thus, non-significant results may be due to a lack of statistical power.

## 3 Results

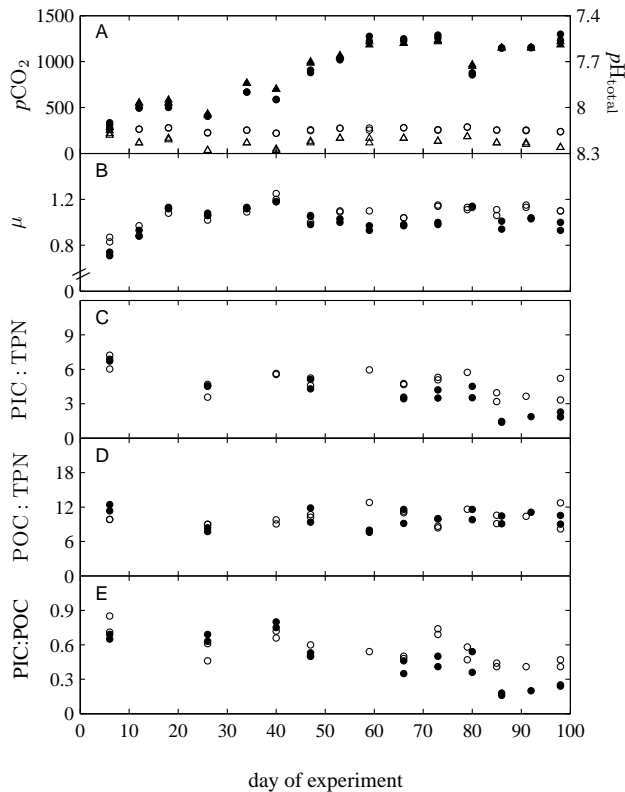
### 3.1 *Emiliania huxleyi*

Cells were cultured for 98 days, corresponding to 152 generations in the control treatment (low  $p\text{CO}_2$ ) and 144 generations in the high  $p\text{CO}_2$  treatment. Cellular division in the control treatment stabilized at a rate of  $\mu = 1.10 \pm 0.06 \text{ d}^{-1}$  ( $\pm 1$  SD) after a couple of weeks into the experiment. During the gradual increase from low to high  $p\text{CO}_2$ ,



**Fig. 1.** Cell ratios over the hours of daily illumination of *E. huxleyi* (circle) and *C. braarudii* (triangle) under low  $p\text{CO}_2$  (open symbols) and high  $p\text{CO}_2$  (closed symbols) from all data points collected during the experiment. (A) PIC:POC ratio (molC:molC). (B) PIC:TPN ratio (molC:molN). (C) POC:TPN ratio (molC:molN).

no change in growth rate was detectable. After reaching the maximum  $p\text{CO}_2$  level of 1150  $\mu\text{atm}$  the growth rate decreased to  $\mu = 1.00 \pm 0.06 \text{ d}^{-1}$  ( $p = 0.07$ ,  $dr = 1:7$ ) and remained at this value until the end of the experiment (Fig. 2a and b). The PIC:TPN ratio was relatively constant at  $4.9 \pm 1.0 \text{ molC molN}^{-1}$  under low  $p\text{CO}_2$  but with the onset of high  $p\text{CO}_2$  the ratio decreased and was consistently lower compared to the control by an average value of  $1.8 \pm 0.7 \text{ molC molN}^{-1}$  (Fig. 2c;  $p = 0.031$ ,  $dr = 0:6$ ). No consistent difference between  $p\text{CO}_2$  treatments was observed in POC:TPN with a mean ratio of  $10.0 \pm 1.4 \text{ molC molN}^{-1}$  (Fig. 2d;  $p = 0.453$ ,  $dr = 2:5$ ). The PIC:POC ratio was considerably lower under constant high  $p\text{CO}_2$  with a mean value of  $0.33 \pm 0.13 \text{ molC molC}^{-1}$  compared to  $0.56 \pm 0.13 \text{ molC molC}^{-1}$  at low  $p\text{CO}_2$  (Fig. 2e;  $p = 0.031$ ,  $dr = 0:6$ ). Coccolith morphology of *E. huxleyi* did not display a visible difference between  $p\text{CO}_2$  treatments (Fig. 4a and b). Between day 73 and 80,  $p\text{CO}_2$  accidentally dropped to

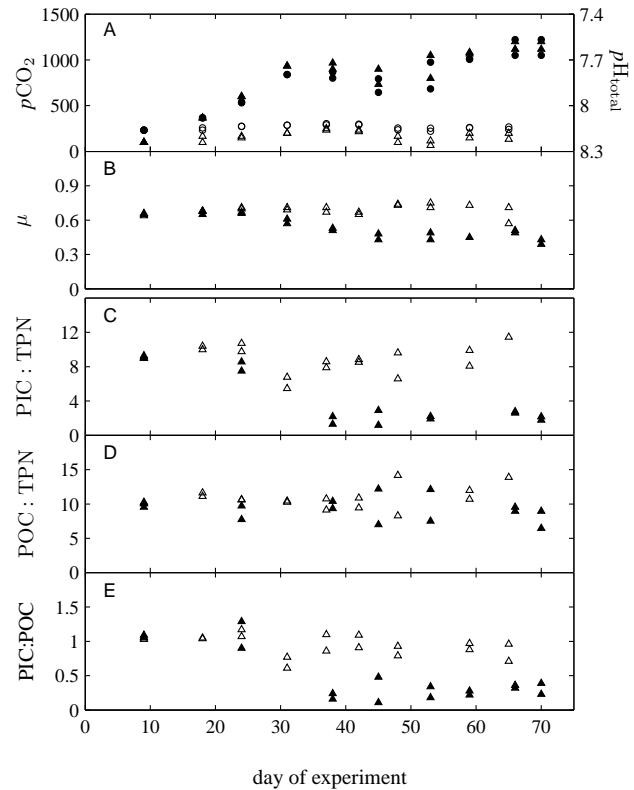


**Fig. 2.** Physiological responses of *Emiliana huxleyi* to elevated  $p\text{CO}_2$  over the course of the experiment (open and closed symbols represent the low  $p\text{CO}_2$  and high  $p\text{CO}_2$  treatments, respectively). (A)  $p\text{CO}_2$  (circles,  $\mu\text{atm}$ ) and pH (triangle) over experimental time. (B) growth rate ( $\text{d}^{-1}$ ). (C) PIC:TPN (molC:molN). (D) POC:TPN (molC:molN). (E) PIC:POC (molC:molC).

870  $\mu\text{atm}$ , which was followed by an immediate increase in cell growth. As soon as  $p\text{CO}_2$  was elevated above 1000  $\mu\text{atm}$  again, the cell growth rate decreased back to the previous level.

### 3.2 *Coccolithus braarudii*

Cells were cultured for 66 days corresponding to 65 generations in the control treatment and 51 generations in the high  $p\text{CO}_2$  treatment. After transition to constant high  $p\text{CO}_2$  on day 31 the growth rate decreased from initially  $0.69 \pm 0.04$  to  $0.49 \pm 0.06 \text{ d}^{-1}$  ( $p=0.031$ ,  $dr=0:6$ ), whereas it remained at the initial level in the control treatment (Fig. 3a and b). Both PIC:TPN and POC:TPN displayed similar trends as seen in *E. huxleyi*. PIC:TPN decreased from  $8.9 \pm 1.6$  to  $2.1 \pm 0.6 \text{ molC molN}^{-1}$  under high  $p\text{CO}_2$  ( $p=0.063$ ,  $dr=0:5$ ), whereas no change was detected in the POC:TPN ratio (Fig. 3c and d;  $p=0.25$ ,  $dr=0:3$ ). Mean values of the POC:TPN ratio under low and high  $p\text{CO}_2$  were calculated as  $10.9 \pm 1.5$  and  $9.3 \pm 2.0 \text{ molC molN}^{-1}$ , respectively. The PIC:POC ratio was reduced by  $\approx 70\%$  to a mean value of



**Fig. 3.** Physiological responses of *Coccolithus braarudii* to elevated  $p\text{CO}_2$  over the course of the experiment. Labels and symbols as in Fig. 2.

$0.28 \pm 0.11 \text{ molC molC}^{-1}$  (Fig. 3e;  $p=0.125$ ,  $dr=0:4$ ) and clear signs of malformation were observed on individual coccoliths under constant high  $p\text{CO}_2$  conditions (Fig. 4d).

## 4 Discussion

### 4.1 Carbonate system manipulation

Manipulation of the carbonate system by acid/base addition changes the total alkalinity (TA) at a constant dissolved inorganic carbon (DIC) concentration, whereas “ongoing ocean acidification” changes the DIC concentration at constant TA. However, biologically important parameters ( $[\text{CO}_{2(\text{aq})}]$ ,  $[\text{HCO}_3^-]$ ,  $[\text{CO}_3^{2-}]$  and  $[\text{H}^+]$ ) undergo similar changes by manipulating TA at constant DIC compared to manipulating DIC at constant TA in the  $p\text{CO}_2$  range applied here (Schulz et al., 2009). For example, manipulating seawater with a salinity of 35 at 15 °C,  $p\text{CO}_2$  of 380  $\mu\text{atm}$  and a DIC concentration of 2100  $\mu\text{mol kg}^{-1}$  by i) aeration with  $\text{CO}_2$  enriched air (TA constant) or ii) acid addition (DIC constant) to a  $p\text{CO}_2$  of 1000  $\mu\text{atm}$  would result in the following percentage changes of biologically relevant parameters.  $[\text{CO}_{2(\text{aq})}]$ : +164% (i and ii);  $[\text{HCO}_3^-]$ : +12% (i) and +4% (ii);  $[\text{CO}_3^{2-}]$ :

–52% (i) and –59% (ii); [H<sup>+</sup>]: +135% (i) and +152% (ii). Calculations were done using the program CO<sub>2</sub>sys (version 1.05 by E. Lewis and D. W. R. Wallace) using dissociation constants for carbonic acid after Roy et al. (1993).

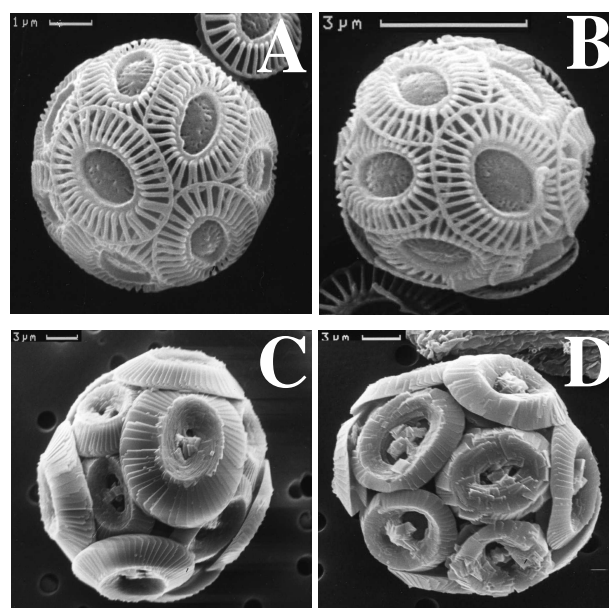
#### 4.2 Growth rate

An average decrease of 9% was observed in *E. huxleyi*'s growth rate in response to increasing *p*CO<sub>2</sub> from 260 to 1150 μatm. This reduction in growth rate under continuous high *p*CO<sub>2</sub> was tested to be different from the rate at low *p*CO<sub>2</sub> with a 93% probability (*p*=0.07); even though it is marginally non-significant, we consider this decrease as biologically relevant. Previous studies detected no change in growth rates after exposure to elevated *p*CO<sub>2</sub> (Riebesell et al., 2000; Zondervan et al., 2002; Feng et al., 2008). While this difference may be due to the fact that the maximum *p*CO<sub>2</sub> of 900 μatm in these earlier studies was somewhat lower than in the present experiment, the small difference in growth rate of 9% may also have been below the detection limit in the single end-point sampling approach used in previous studies. Recent results by Barcelos e Ramos et al. (2010) and Langer et al. (2009) indicate a reduced growth rate at a *p*CO<sub>2</sub> >1000 μatm in short-term experiments. Interestingly, between day 73 and day 80, when *p*CO<sub>2</sub> accidentally dropped to 870 μatm, the growth rate increased to control values (Fig. 2b) and immediately returned to the lower value after *p*CO<sub>2</sub> was raised again to 1150 μatm. A similar instantaneous effect of *p*CO<sub>2</sub> on the growth rate of *E. huxleyi* was reported to occur within one cell generation (Barcelos e Ramos et al., 2010).

Under constant high *p*CO<sub>2</sub> the growth rate of *C. braarudii* was reduced by 29%. At comparable *p*CO<sub>2</sub> values, Langer et al. (2006) observed no significant reduction in the growth rate of *C. braarudii*. This difference might be induced by the long-term culturing under constant high *p*CO<sub>2</sub>, but other potential factors such as differences in the experimental temperature and the light intensity cannot be excluded as being responsible. Recent results by S. Krug (personal communication, 2009) indicate a reduced growth rate of *C. braarudii* when exposed to *p*CO<sub>2</sub> >1400 μatm for 15 generations.

#### 4.3 PIC:TPN and PIC:POC

Under constant high *p*CO<sub>2</sub> the ratios of PIC:TPN (and equally PIC:POC) in *E. huxleyi* and *C. braarudii* were reduced by ≈42% and ≈70%, respectively. The reduction of the PIC:POC ratio in *E. huxleyi* is a commonly observed response under high *p*CO<sub>2</sub> which is driven by the decrease of the cellular PIC quota and increase in POC quota and ranges between 10 and 60% depending on *p*CO<sub>2</sub> level, temperature and light intensity (Riebesell et al., 2000; Zondervan et al., 2002; Feng et al., 2008; Langer et al., 2009; Barcelos e Ramos et al., 2010). Since the POC:TPN ratio remains constant under different *p*CO<sub>2</sub> values (as discussed in the

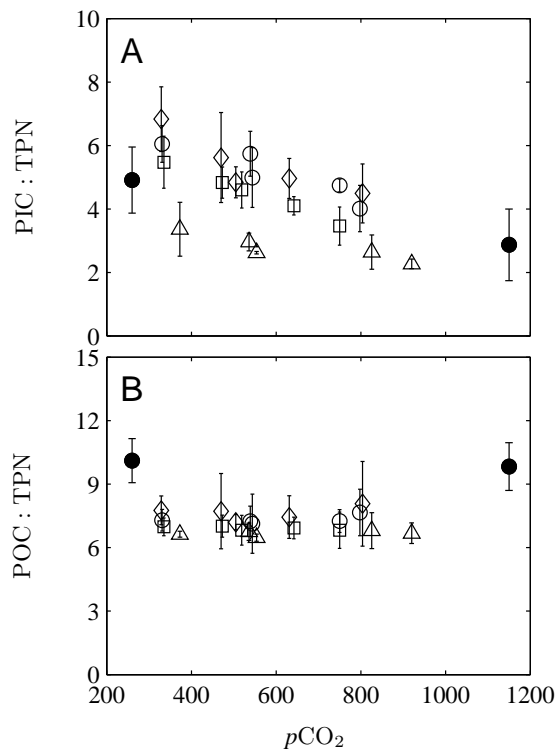


**Fig. 4.** Representative SEM photographs of the two coccolithophore species. Cells of *E. huxleyi* grown in the control treatment (A) and under high *p*CO<sub>2</sub> at day 73 (B). Cells of *C. braarudii* grown in the control treatment (C) and under high *p*CO<sub>2</sub> at day 66 (D).

next paragraph) the PIC:TPN ratio should display a similar behaviour as the PIC:POC ratio. Indeed, the PIC:TPN ratios are decreasing in response to elevated *p*CO<sub>2</sub> values in both species under long-term high *p*CO<sub>2</sub> cultivation (Figs. 2c and 3c), which is also seen in short-term experiments under different light intensities for *E. huxleyi* (Fig. 5a, data from Zondervan et al., 2002). *Coccolithus braarudii*, on the other hand, is reported to maintain constant PIC:POC and PIC:TPN ratios within *p*CO<sub>2</sub> values ranging from 345 to 915 μatm (Langer et al., 2006). However, more recently S. Krug (personal communication, 2009) measured in short-term experiments a significant decrease in both the PIC:POC and the PIC:TPN ratio when *C. braarudii* was exposed to *p*CO<sub>2</sub> values above 1000 μatm.

#### 4.4 POC:TPN

Particulate organic carbon production was observed to slightly increase under high *p*CO<sub>2</sub> and nutrient replete conditions in *E. huxleyi* (Zondervan et al., 2002; Feng et al., 2008; Barcelos e Ramos et al., 2010), whereas *C. braarudii* maintains a constant rate (Langer et al., 2006). The POC:TPN ratio, however, remains unchanged under short-term high *p*CO<sub>2</sub> exposure in *E. huxleyi* (Feng et al., 2008) and *C. braarudii* (G. Langer, personal communication, 2009). These observations from short-term studies are well in agreement with those revealed in the current long-term study. Figure 5b displays the POC:TPN ratios of *E. huxleyi* (data from



**Fig. 5.** Particulate carbon to nitrogen ratios of *E. huxleyi* as a function of  $p\text{CO}_2$  ( $\mu\text{atm}$ ) at a 24:0 light:dark cycle under various light intensities: 15 (triangle), 30 (square), 80 (circle) and 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (diamond). Open symbols represent data from Zondervan et al. (2002) (error bars represent 1 SD,  $n = 3$ ) and closed symbols indicate mean values of the present study with according standard deviations. (A) PIC:TPN (molC:molN). (B) POC:TPN (molC:molN).

Zondervan et al., 2002) in comparison to the present long-term data. The measured ratio of  $10.0 \pm 1.4 \text{ molC molN}^{-1}$  under long-term cultivation is higher than ratios reported from short-term and mesocosms experiments, which vary between 6 and 7  $\text{molC molN}^{-1}$  (Engel et al., 2005; Feng et al., 2008). Previous studies reported POC:TPN ratios of  $\approx 10$  in *E. huxleyi* only under nitrogen limitation (Engel et al., 2004; Sciandra et al., 2003). However, we can exclude nitrogen limitation of *E. huxleyi* in the present study since i) an initial nitrate concentration of  $88 \mu\text{mol l}^{-1}$  would be sufficient to support exponential growth up to a cell density of  $5 \times 10^8 \text{ l}^{-1}$  (calculated with a TPN content of  $2.6 \text{ pgN cell}^{-1}$ ) which was never reached during this study, and ii) the measured growth rate of  $1.10 \pm 0.06 \text{ d}^{-1}$  corresponds to maximal growth rates under nutrient replete conditions for the temperature and light levels applied in our study (Buitenhuis et al., 2008). Therefore, we can rule out nitrogen limitation to be responsible for the higher POC:TPN ratio in the present study.

In general, we can confirm the observed trend in the POC:TPN ratios of short-term experiments and conclude that

*E. huxleyi* increases the POC production per cell under long-term high  $p\text{CO}_2$  exposure within the tested range. However, since the POC:TPN ratio stays constant the bulk organic matter per available nitrogen of an exponentially growing *E. huxleyi* or *C. braarudii* population will be equal under high and low  $p\text{CO}_2$ .

#### 4.5 From short- to long-term $p\text{CO}_2$ response of coccolithophores

Results from short-term  $p\text{CO}_2$  perturbation experiments, typically involving 7–10 cell generations, were found to be consistent with those under long-term incubation (up to 150 generations). Additionally, Barcelos e Ramos et al. (2010) demonstrated that the  $p\text{CO}_2$  response observed in short-term studies is even detectable within one generation ( $\approx 14 \text{ h}$  after perturbation of the seawater carbonate chemistry). Considering the short “generation time” of coccolithophores (the average time between two cell divisions;  $\approx 1\text{--}2$  days for coccolithophores), evolutionary change and adaptation may occur during long-term incubations. However, the experimental setup of the current long-term study was not suited for the detection of genetic changes. In comparison to natural populations of *E. huxleyi*, where a high genetic variation can be found and sexual reproduction by a haploid stage occurs (Medlin et al., 1996; Iglesias-Rodriguez et al., 2006; Morin, 2008), the strains used in this study were grown asexual as vegetative diploids, what reduces the mutational supply rate and imposes a speed limit on adaptive evolution (de Visser et al., 1999).

Results from cultures of *Chlamydomonas reinhardtii*, grown over 1000 generations under elevated  $p\text{CO}_2$ , indicated no change in growth rate and cells failed to evolve specific adaptation to high  $p\text{CO}_2$  even though the phenotype of the evolved population was attributed to genetic change (Collins and Bell, 2004). Both investigated coccolithophorid species decreased growth rates in response to elevated  $p\text{CO}_2$  and did not recover over the time frame of the experiment. The potential to re-establish previous growth rates through adaptation to a new environment might be increased by long-term experiments using a higher mutational supply rate (by larger populations and multiple strains culturing) and induced sexual reproduction (Colegrave, 2002).

## 5 Conclusions

Since the studies of Riebesell et al. (2000) and Langer et al. (2006, 2009) species- and strain-specific performance of coccolithophores under elevated  $p\text{CO}_2$  levels is known from short-term experiments, typically involving 7–10 cell generations. Here, we discussed data from a multiple-generation experiment using two coccolithophore species which generally confirm the observed CO<sub>2</sub> sensitivities obtained in short-term experiments, though based on our data it can not be

distinguished whether this is the result of a sustained acclimation response alone, or involved genetic change. A gradual CO<sub>2</sub> increase did not alleviate the CO<sub>2</sub>/pH sensitivity under the experimental conditions. In contrast to earlier studies we observed reduced growth rates in response to elevated pCO<sub>2</sub>.

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## References

- Alley, R. B., Berntsen, T., Bindoff, N. L., Chen, Z., Chidthaisong, A., Friedlingstein, P., Gregory, J. M., Hegerl, G. C., Heimann, M., Hewitson, B., Hoskins, B. J., Joos, F., Jouzel, J., Kattsov, V., Lohmann, U., Manning, M., Matsuno, T., Molina, M., Nicholls, N., Overpeck, J., Qin, D., Raga, G., Ramaswamy, V., Ren, J., Rusticucci, M., Solomon, S., Somerville, R., Stocker, T. F., Stott, P. A., Stouffer, R. J., Whetton, P., Wood, R. A., and Wratt, D.: Summary for Policymakers. In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, Elsevier Inc., Cambridge and New York, 2007.
- Barcelos e Ramos, J., Müller, M. N., and Riebesell, U.: Short-term response of the coccolithophore *Emiliana huxleyi* to an abrupt change in seawater carbon dioxide concentrations, *Biogeosciences*, 7, 177–186, 2010, <http://www.biogeosciences.net/7/177/2010/>.
- Buitenhuis, E. T., Pangerc, T., Franklin, D. J., Le Quere, C., and Malin, G.: Growth rates of six coccolithophorid strains as a function of temperature, *Limnol. Oceanogr.*, 53, 1181–1185, 2008.
- Colegrave, N.: Sex releases the speed limit on evolution, *Nature*, 420, 664–666, 2002.
- Collins, S. and Bell, G.: Phenotypic consequences of 1,000 generations of selection at elevated CO<sub>2</sub> in a green alga, *Nature*, 431, 566–569, 2004.
- de Visser, J. A. G. M., Zeyl, C. W., Gerrish, P. J., Blanchard, J. L., and Lenski, R. E.: Diminishing returns from mutation supply rate in asexual populations, *Science*, 283, 404–406, 1999.
- Dickson, A.: An exact definition of total alkalinity and a procedure for the estimation of alkalinity and total inorganic carbon from titration data, *Deep Sea Res.*, 28, 609–623, 1981.
- Ehrhardt, M. and Koeve, W.: Determination of particulate organic carbon and nitrogen, in: *Methods of seawater analysis*, edited by: Grasshoff, K., Kremling, K., and Erhardt, M., 3rd ed., WILEY-VCH, 1999.
- Engel, A., Delille, B., Jacquet, S., Riebesell, U., Rochelle-Newall, E., Terbrüggen, A., and Zondervan, I.: Transparent exopolymer particles and dissolved organic carbon production by *Emiliana huxleyi* exposed to different CO<sub>2</sub> concentrations: a mesocosm experiment, *Aquat. Microb. Ecol.*, 34, 93–104, 2004.
- Engel, A., Zondervan, I., Aerts, K., Beaufort, L., Benthien, A., Chou, L., Delille, B., Gattuso, J.-P., Harley, J., Heemann, C., Hoffmann, L., Jacquet, S., Nejstgaard, J., Pizay, M.-D., Rochelle-Newall, E., Schneider, U., Terbrüggen, A., and Riebesell, U.: Testing the direct effect of CO<sub>2</sub> concentration on a bloom of the coccolithophorid *Emiliana huxleyi* in mesocosm experiments, *Limnol. Oceanogr.*, 50, 493–504, 2005.
- Fabry, V., Seibel, B. S., Feely, R. A., and Orr, J. C.: Impacts of ocean acidification on marine fauna and ecosystem processes, *ICES J. Mar. Science*, 65, 414–432, 2008.
- Feng, Y., Warner, M., Zhang, Y., Sun, J., Fu, F.-X., Rose, J., and Hutchins, D.: Interactive effects of increased pCO<sub>2</sub>, temperature and irradiance on the marine coccolithophore *Emiliana huxleyi* (Prymnesiophyceae), *Eur. J. Phycol.*, 43, 87–98, 2008.
- Guillard, R.: Culture of phytoplankton for feeding marine invertebrates, in: *Culture of marine invertebrates*, edited by: Smith, W. and Chanley, M., Plenum, 1975.
- Iglesias-Rodriguez, M. D., Schofield, O. M., Batley, J., Medlin, L. K., and Hayes, P. K.: Intraspecific genetic diversity in the marine coccolithophore *Emiliana huxleyi* (Prymnesiophyceae): The use of microsatellite analysis in marine phytoplankton population studies, *J. Phycol.*, 42, 526–536, 2006.
- Iglesias-Rodriguez, M. D., Halloran, P. R., Rickaby, R. E. M., Hall, I. R., Colmenero-Hidalgo, E., Gittins, J. R., Green, D. R. H., Tyrell, T., Gibbs, S. J., von Dassow, P., Rehm, E., Armbrust, E. V., and Boessenkool, K. P.: Phytoplankton Calcification in a High-CO<sub>2</sub> World, *Science*, 320, 336–340, 2008.
- Langer, G., Geisen, M., Baumann, K.-H., Kls, J., Riebesell, U., Thoms, S., and Young, J. R.: Species-specific responses of calcifying algae to changing seawater carbonate chemistry, *G-cubed*, 7, Q09006, doi:10.1029/2005GC001227, 2006.
- Langer, G., Nehrke, G., Probert, I., Ly, J., and Ziveri, P.: Strain-specific responses of *Emiliana huxleyi* to changing seawater carbonate chemistry, *Biogeosciences*, 6, 2637–2646, 2009, <http://www.biogeosciences.net/6/2637/2009/>.
- Medlin, L., Barker, G., Campbell, L., Green, J., Hayes, P., Marie, D., Wrieden, S., and Vaultot, D.: Genetic characterisation of *Emiliana huxleyi* (Haptophyta), *J. Mar. Syst.*, 9, 13–31, 1996.
- Morin, P. J.: Sex as an algal antiviral strategy, *P. Natl. Acad. Sci.*, 105, 15639–15640, 2008.
- Müller, M. N., Antia, A. N., and LaRoche, J.: Influence of cell cycle phase on calcification in the coccolithophore *Emiliana huxleyi*, *Limnol. Oceanogr.*, 53, 506–512, 2008.
- Riebesell, U., Zondervan, I., Rost, B., Tortell, P. D., Zeebe, R. E.,

- and Morel, F. M. M.: Reduced calcification of marine plankton in response to increased atmospheric CO<sub>2</sub>, *Nature*, 407, 364–367, 2000.
- Riebesell, U., Bellerby, R. G. J., Grossart, H.-P., and Thingstad, F.: Mesocosm CO<sub>2</sub> perturbation studies: from organism to community level, *Biogeosciences*, 5, 1157–1164, 2008a.
- Riebesell, U., Bellerby, R. G. J., Engel, A., Fabry, V. J., Reusch, T. B. H., Schulz, K. G., and Morel, F. M. M.: Phytoplankton Calcification in a High CO<sub>2</sub> World (technical comment), *Science*, 322, 1466b, doi:10.1126/science.1161096, 2008b.
- Rost, B., Zondervan, I., and Wolf-Gladrow, D.: Sensitivity of phytoplankton to future changes in ocean carbonate chemistry: current knowledge, contradictions and research directions, *Mar. Ecol. Prog. Ser.*, 373, 227–237, 2008.
- Roy, R., Roy, L., Vogel, K., Porter-Moore, C., Pearson, T., Good, C., Millero, F., and Campbell, D.: The dissociation constants of carbonic acid in seawater at salinities 5 to 45 and temperatures 0 to 45 °C, *Mar. Chem.*, 44, 249–267, 1993.
- Saez, A., Probert, I., Geisen, M., Quinn, P., Young, J., and Medlin, L.: Pseudo-cryptic speciation in coccolithophores, *P. Natl. Acad. Sci.*, 100, 7163–7168, 2003.
- Schulz, K. G., Barcelos e Ramos, J., Zeebe, R. E., and Riebesell, U.: CO<sub>2</sub> perturbation experiments: similarities and differences between dissolved inorganic carbon and total alkalinity manipulations, *Biogeosciences*, 6, 2145–2153, 2009, <http://www.biogeosciences.net/6/2145/2009/>.
- Sciandra, A., Harlay, J., Lefvre, D., Leme, R., Rimmelin, P., Denis, M., and Gattuso, J.-P.: Response of coccolithophorid *Emiliania huxleyi* to elevated partial pressure of CO<sub>2</sub> under nitrogen limitation, *Mar. Ecol. Prog. Ser.*, 261, 111–122, 2003.
- Shi, D., Xu, Y., and Morel, F. M. M.: Effects of the pH/pCO<sub>2</sub> control method on medium chemistry and phytoplankton growth, *Biogeosciences*, 6, 1199–1207, 2009, <http://www.biogeosciences.net/6/1199/2009/>.
- Stoll, M., Bakker, K., Nobbe, G., and Haese, R.: Continuous-flow analysis of dissolved inorganic carbon content in seawater, *Anal. Chem.*, 73, 4111–4116, 2001.
- Zondervan, I.: The effect of light, macronutrients, trace metals and CO<sub>2</sub> on the production of calcium carbonate and organic carbon in coccolithophores – A review, *Deep Sea Res. II*, 41, 521–537, 2007.
- Zondervan, I., Rost, B., and Riebesell, U.: Effect of CO<sub>2</sub> concentration on the PIC/POC ratio in the coccolithophore *Emiliania huxleyi* grown under light-limiting conditions and different daylengths, *J. Exp. Mar. Biol. Ecol.*, 272, 55–70, 2002.