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Effects of long-term high CO_2 exposure on two species of coccolithophores

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Abstract

The physiological performance of two coccolithophore species, *Emiliania huxleyi* and *Coccolithus braarudii*, was investigated during long-term exposure to elevated pCO_2 levels. Mono-specific cultures were grown over 152 (*E. huxleyi*) and 65 (*C. braarudii*) generations while pCO_2 was gradually increased to maximum levels of 1150 μatm (*E. huxleyi*) and 930 μatm (*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 pCO_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 pCO_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_2 exposure experiments following abrupt pertubations of the seawater carbonate system, indicating that for the strains tested here i) a gradual CO_2 increase does not alleviate CO_2/pH sensitivity, and ii) observed CO_2 sensitivities are persistent over multiple generations.

1 Introduction

Emissions of anthropogenic CO_2 since the beginning of the industrial revolution have lead to an increase in atmospheric carbon dioxide concentration, resulting in a fraction of the anthropogenic CO_2 being absorbed by the ocean. The ongoing oceanic uptake of anthropogenic CO_2 is steadily altering the seawater carbonate chemistry and has lead to a reduction in surface ocean pH by 0.12 units over the past 200 yr (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; Zondervan, 2007). Results from these studies indicate considerable species- and even strain-specific differences in CO_2 sensitivity.

When exposed to elevated pCO_2 levels as projected for the course of this century, *Emiliania huxleyi*, *Gephyrocapsa oceanica* and *Calcidiscus leptoporus* 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 pCO_2 (Langer et al., 2006) with respect to calcification. Differences in pCO_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 pCO_2 (Riebesell et al., 2000; Zondervan et al., 2002; Feng et al., 2008; Shi et al., 2009), no change with increasing pCO_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 pCO_2. This trend reverses when accounting for the large difference in cell size between pCO_2 treatments as observed in previous experiments on *E. huxleyi*, yielding a slight decrease in calcification with increasing pCO_2 when normalizing the data to cellular carbon content (Riebesell et al., 2008b).

Most acidification experiments with coccolithophores were done applying an abrupt pertubation of seawater carbonate chemistry, either by bubbling with CO_2 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_2 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 pCO_2 conditions. The physiological re-

sponse to high pCO_2 exposure of *E. huxleyi* within one cell generation was recently investigated by Barcelos e Ramos et al. (2009) and was found to be consistent with the observations gained from longer-term experiments. However, it is presently unknown whether the observed CO_2 sensitivities of coccolithophores are persistent over long-term high pCO_2 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 pCO_2 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 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^{-1} nitrate and 3.6 μmol l^{-1} phosphate, at a photon flux density of 140 μmol photons m^{-2} s^{-1} (Philips TL-D 90 DeLuxePro, 36W/950) under a 14:10 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. pCO_2 was adjusted by additions of HCl or NaOH to the media. Cultures were allowed to grow for about 5–10 generations corresponding to a dissolved inorganic carbon (DIC) consumption of 5 to 10%. 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 diluted with fresh media (carbonate system already adjusted) to a concentration of 100 and 50 cells ml^{-1} (*E. huxleyi* and *C. braarudii*, respectively). The media in the control flasks (low pCO_2) was adjusted to a pH_{total} of 8.21±0.05, resulting in a pCO_2 of 260 μatm±20 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 pCO_2 values (1150 μatm±140 and 930 μatm±180 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

Cell number was determined with a Coulter Counter (Z Series). Samples were measured three times and the mean was used to calculate the growth rate (μ) as

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

where c_0 and c_1 are the cell concentrations at the beginning (t_0) and end of the incubation period (t_1).

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^{-1} accuracy and five μmol kg^{-1} 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 CO2sys (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 ±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 CO_2 treatment and fixed with formaldehyde (1% end concentration). Subsequently, the samples were filtered onto polycarbonate filters (0.2 μm 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.

3 Results

3.1 *Emiliania huxleyi*

Cells were cultured for 98 d, corresponding to 152 generations in the control treatment (low pCO_2) and 144 generations in the high pCO_2 treatment. Cellular division in the control treatment stabilized at a rate of μ=1.10±0.06 d^{-1} after a couple of weeks into the experiment. During the gradual increase from low to high pCO_2, no change in growth rate was detectable. After reaching the maximum pCO_2 level of 1150 μatm the growth rate decreased to μ=1.00±0.06 d^{-1} and remained at this value until the end of the experiment (Fig. 2a, b). The PIC:TPN ratio was relatively constant at 4.9±1.0 mol C mol N^{-1} under low pCO_2 but with the onset of high pCO_2 the ratio decreased and was consistently lower compared to the control by an average value of 1.8±0.7 mol C mol N^{-1} (Fig. 2c). No consistent difference between pCO_2 treatments was observed in POC:TPN with a mean ratio of 10.0±1.4 mol C mol N^{-1} (Fig. 2d). The PIC:POC ratio was considerably lower under constant high pCO_2 with a mean value of 0.33±1.3 mol C mol C^{-1} compared to 0.56±1.3 mol C mol C^{-1} at low pCO_2 (Fig. 2e). Coccolith morphology of *E. huxleyi* did not display a visible difference between pCO_2 treatments (Fig. 4a, b). Between day 73 and 80, pCO_2 accidentally dropped to 870 μatm, which was followed by an immediate increase in cell growth. As soon as pCO_2 was elevated above 1000 μatm, the cell growth rate decreased again to the previous level.

3.2 *Coccolithus braarudii*

Cells were cultured for 66 d corresponding to 65 generations in the control treatment and 51 generations in the high pCO_2 treatment. After transition to constant high pCO_2 on day 31 the growth rate decreased from initially 0.69±0.04 to 0.49±0.06 d^{-1}, whereas it remained at the initial level in the control treatment (Fig. 3a, b). Both PIC:TPN and POC:TPN displayed similar trends as seen in *E. huxleyi*. PIC:TPN decreased from 8.9±1.6 to 2.1±0.6 mol C mol N^{-1} under high pCO_2, whereas no change was detected in the POC:TPN ratio (Fig. 3c,d). Mean values of the POC:TPN ratio under low and high pCO_2 were calculated as 10.9±1.5 and 9.3±2.0 mol C mol N^{-1}, respectively. The PIC:POC ratio was reduced by ≈70% to a mean value of 0.28±0.11 mol C mol C^{-1} (Fig. 3e) and clear signs of malformation were observed on individual coccoliths under constant high pCO_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 ($[CO_{2(aq)}]$, $[HCO_3^-]$, $[CO_3^{2-}]$ and $[H^+]$) undergo similar changes by manipulating TA at constant DIC compared to manipulating DIC at constant TA in the pCO_2 range applied here (Schulz et al., 2009). For example, manipulating seawater with a salinity of 35 at 15°C, pCO_2 of 380 μatm and a DIC concentration of 2100 μmol kg^{-1} by i) aeration with CO_2 enriched air (TA constant) or ii) acid addition (DIC constant) to a pCO_2 of 1000 μatm would result in the following percentage changes of biologically relevant parameters. $[CO_{2(aq)}]$: +164% (i and ii); $[HCO_3^-]$: +12% (i) and +4% (ii); $[CO_3^{2-}]$: −52% (i) and −59% (ii); $[H^+]$: +135% (i) and +152%

(ii). Calculations were done using the program CO2sys (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 pCO_2 from 260 to 1150 μatm. Previous studies detected no change in growth rates after exposure to elevated pCO_2 (Riebesell et al., 2000; Zondervan et al., 2002; Feng et al., 2008). While this difference may be due to the fact that the maximum pCO_2 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. (2009) and Langer et al. (2009) indicate a reduced growth rate at a pCO_2>1000 μatm in short-term experiments. Interestingly, between day 73 and day 80, when pCO_2 accidently dropped to 870 μatm, the growth rate increased to control values (Fig. 3b) and immediately returned to the lower value after pCO_2 was raised to 1150 μatm. A similar instantaneous effect of pCO_2 on the growth rate of *E. huxleyi* was reported to occur within one cell generation (Barcelos e Ramos et al., 2009).

Under constant high pCO_2 the growth rate of *C. braarudii* was reduced by 29%. At comparable pCO_2 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 pCO_2, but other potential factors such as differences in the experimental temperature and the light intensity cannot be excluded as being responsible. Recent results by Krug (personal communication) indicate a reduced growth rate of *C. braarudii* when exposed to pCO_2>1400 μatm for 15 generations.

4.3 PIC:TPN and PIC:POC

Under constant high pCO_2 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 pCO_2 which is driven by the decrease of the cellular PIC quota and increase in POC quota and ranges between 10 and 60% depending on pCO_2 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., 2009). Since the POC:TPN ratio remains constant under different pCO_2 values (as discussed in the 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 pCO_2 values in both species under long-term high pCO_2 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 pCO_2 values ranging from 345 to 915 μatm (Langer et al., 2006). However, more recently Krug (personal communication) measured in short-term experiments a significant decrease in both the PIC:POC and the PIC:TPN ratio when *C. braarudii* was exposed to pCO_2 values above 1000 μatm.

4.4 POC:TPN

Particulate organic carbon production was observed to slightly increase under high pCO_2 and nutrient replete conditions in *E. huxleyi* (Zondervan et al., 2002; Feng et al., 2008; Barcelos e Ramos et al., 2009), whereas *C. braarudii* maintains a constant rate (Langer et al., 2006). The POC:TPN ratio, however, remains unchanged under short-term high pCO_2 exposure in *E. huxleyi* (Feng et al., 2008) and *C. braarudii* (Langer, personal communication). These observations from short-term studies are well in agreement with those revealed from the current long-term study. Figure 5b displays the POC:TPN ratios of *E. huxleyi* (data from Zondervan et al., 2002) in comparison to

the present long-term data. The measured ratio of 10.0 ± 1.4 mol C mol N^{-1} under long-term cultivation is slightly higher than ratios reported from short-term and mesocosms experiments, which vary between 6 and 7 mol C mol N^{-1} (Engel et al., 2005; Feng et al., 2008). POC:TPN ratios of ≈10 and higher were observed in *E. huxleyi* under nitrogen limitation (Engel et al., 2004; Sciandra et al., 2003). However, we can assure that in the present study *E. huxleyi* was not nitrogen limited since i) an initial nitrate concentration of 88 μmol l^{-1} would be sufficient to supply exponential growth up to a cell density of $5x10^8$ l^{-1} which was never reached during this study, and ii) the measured growth rate of 1.10 ± 0.06 d^{-1} is well in agreement with maximal growth rates under nutrient replete, similar temperature and light conditions (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 pCO_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* population will be equal under high and low pCO_2.

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 pCO_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_2 sensitivities obtained in short-term experiments. A gradual CO_2 increase did not to alleviate the CO_2/pH sensitivity under the experimental conditions. In contrast to earlier studies we observed reduced growth rates in response to elevated pCO_2.

Coccolithophores and other phytoplankton groups will face a changing environment

in the future ocean. The question of genetic/physiological adaptation to changing environmental conditions is a challenge that needs to be addressed in future investigations. Experiments with a higher genetic variation induced by multiclonal culturing and sexual reproduction will probably provide a suitable tool to test for adaptation potential in the lab (Colegrave, 2002).

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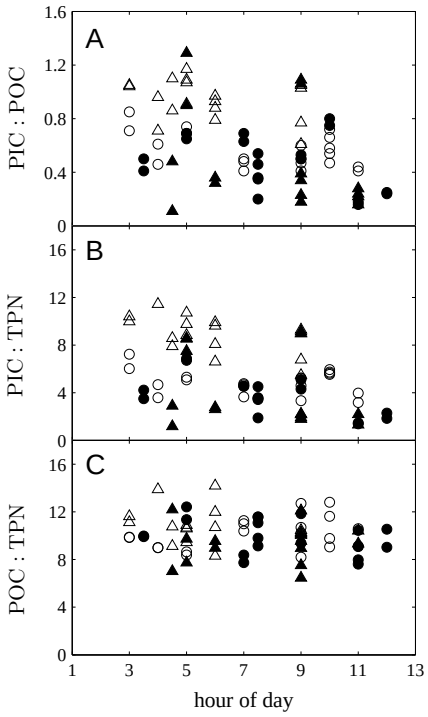

Fig. 1. Cell ratios over the hours of daily illumination of *E. huxleyi* (circle) and *C. braarudii* (triangle) under low pCO_2 (open symbols) and high pCO_2 (closed symbols) from all data points collected during the experiment. **(A)** PIC:POC ratio (mol C:mol C). **(B)** PIC:TPN ratio (mol C:mol N). **(C)** POC:TPN ratio (mol C:mol N).

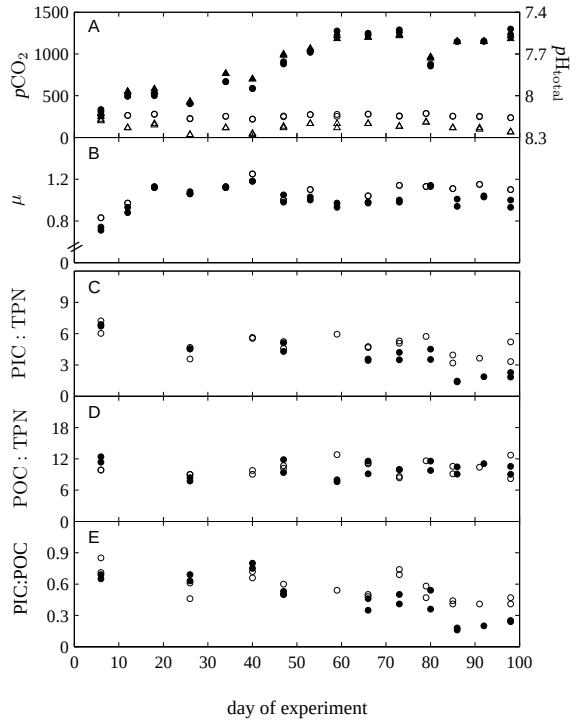

Fig. 2. Physiological responses of *Emiliania huxleyi* to elevated pCO_2 over the course of the experiment (open and closed symbols represent the low pCO_2 and high pCO_2 treatments, respectively). **(A)** pCO_2 (circles, μatm) and pH (triangle) over experimental time. **(B)** growth rate (d^{-1}). **(C)** PIC:TPN (mol C:mol N). **(D)** POC:TPN (mol C:mol N). **(E)** PIC:POC (mol C:mol C).

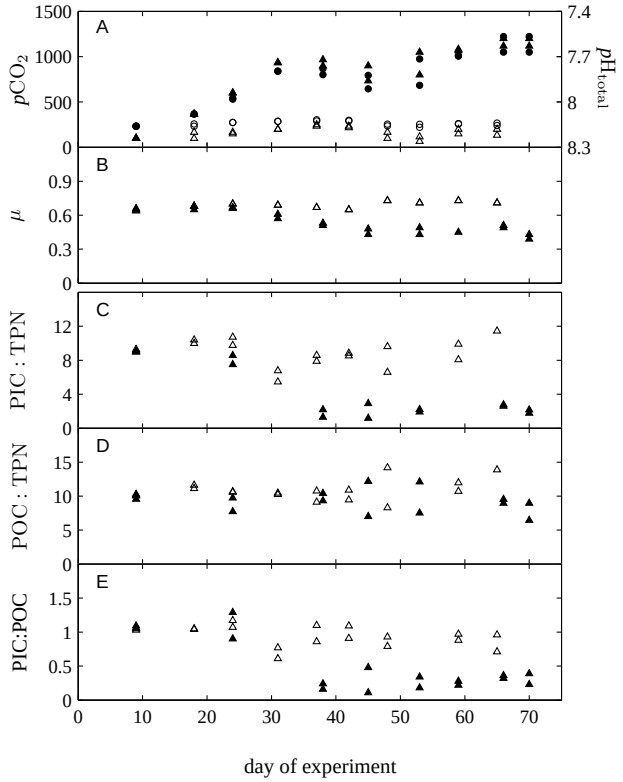

Fig. 3. Physiological responses of *Coccolithus braarudii* to elevated pCO_2 over the course of the experiment. Labels and symbols as in Fig. 2.

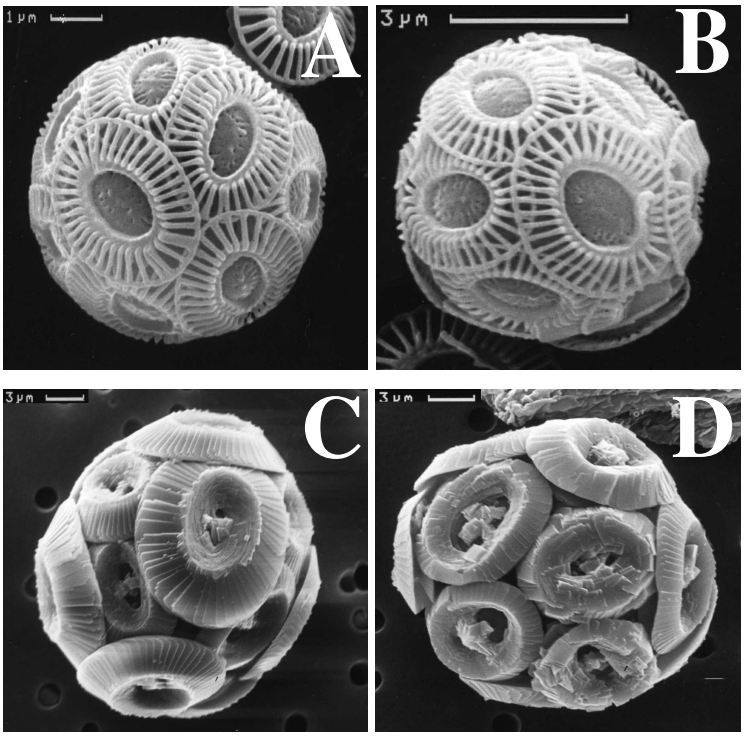

Fig. 4. Representative SEM photographs of the two coccolithophore species. Cells of *E. huxleyi* grown in the control treatment **(A)** and under high pCO_2 at day 73 **(B)**. Cells of *C. braarudii* grown in the control treatment **(C)** and under high pCO_2 at day 66 **(D)**.

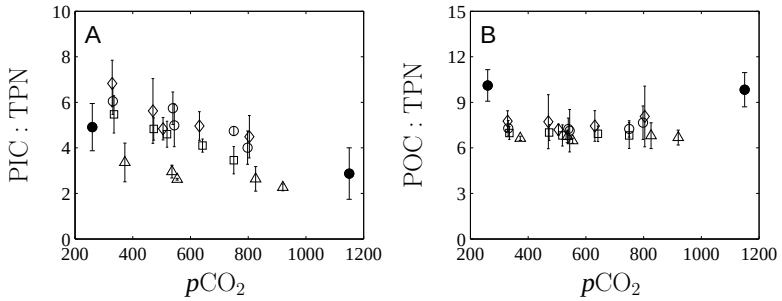

Fig. 5. Particulate carbon to nitrogen ratios of *E. huxleyi* as a function of pCO_2 (μatm) at a 24:0 light:dark cycle under various light intensities: 15 (triangle), 30 (square), 80 (circle) and 150 μmol photons m^{-2} s^{-1} (diamond). Open symbols represent data from Zondervan et al. (2002) (error bars represent 1SD, n=3) and closed symbols indicate mean values of the present study with according standard deviations. **(A)** PIC:TPN (mol C:mol N). **(B)** POC:TPN (mol C:mol N).