

# Perturbing phytoplankton: response and isotopic fractionation with changing carbonate chemistry in two coccolithophore species

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\*\* Invited contribution by R. E. M. Rickaby, recipient of the EGU Arne Richter Award for Outstanding Young Scientists 2008.

Received: 12 February 2010 – Published in Clim. Past Discuss.: 4 March 2010

Revised: 16 November 2010 – Accepted: 19 November 2010 – Published: 1 December 2010

**Abstract.** All species of coccolithophore appear to respond to perturbations of carbonate chemistry in a different way. Here, we show that the degree of malformation, growth rate and stable isotopic composition of organic matter and carbonate produced by two contrasting species of coccolithophore (*Gephyrocapsa oceanica* and *Coccolithus pelagicus ssp. braarudii*) are indicative of differences between their photosynthetic and calcification response to changing DIC levels (ranging from  $\sim 1100$  to  $\sim 7800 \mu\text{mol kg}^{-1}$ ) at constant pH ( $8.13 \pm 0.02$ ). *Gephyrocapsa oceanica* thrived under all conditions of DIC, showing evidence of increased growth rates at higher DIC, but *C. braarudii* was detrimentally affected at high DIC showing signs of malformation, and decreased growth rates. The carbon isotopic fractionation into organic matter and the coccoliths suggests that *C. braarudii* utilises a common internal pool of carbon for calcification and photosynthesis but *G. oceanica* relies on independent supplies for each process. All coccolithophores appear to utilize bicarbonate as their ultimate source of carbon for calcification resulting in the release of a proton. But, we suggest that this proton can be harnessed to enhance the supply of  $\text{CO}_2(\text{aq})$  for photosynthesis either from a large internal  $\text{HCO}_3^-$  pool which acts as a pH buffer (*C. braarudii*), or pumped externally to aid the diffusive supply of  $\text{CO}_2$  across the membrane from the abundant  $\text{HCO}_3^-$  (*G. oceanica*), likely mediated by an internal and external carbonic anhydrase respectively. Our simplified hypothetical spectrum of physiologies may provide a context to understand different species response to changing pH and DIC, the species-specific  $\epsilon_p$  and calcite “vital effects”, as well as accounting for geological trends in coccolithophore cell size.

## 1 Introduction

Whether all marine calcifiers will decrease their rate of calcification under conditions of decreasing saturation state in ocean surface waters remains an important, and unresolved question for constraining future ecological and carbon cycle feedbacks. The likely response of the most prominent group of calcifying phytoplankton, the coccolithophores, in particular has provoked controversy. Coccolithophores are unicellular photosynthesizing and calcifying algae, which precipitate their heterococcolith calcite platelets entirely intracellularly. Evidence suggests that they may not be sensitive to ocean saturation in the same way as organisms which mediate the nucleation of calcification under less physiological control, such as most corals (Kleypas et al., 2006). The species and even strain-specific response (Langer et al., 2009) to changing saturation state is highly complex as shown by a number of culture manipulations (Riebesell et al., 2000; Iglesias-Rodriguez et al., 2008; Langer et al., 2006; Casareto et al., 2009), open ocean mesocosm experiments (Engel et al., 2005), and compilations of coccolith weight data from the modern and past ocean (Beaufort et al., 2010). Notably, rapidly accumulating sediments chart an increase in calcification concomitant with recent anthropogenic change (Halloran et al., 2008; Grelaud et al., 2009). Such an increase in calcification appears at odds with the paradigm view that coccolithophores will reduce the ratio of calcification to photosynthesis rates (PIC/POC ratio) and tend towards malformation with increasing levels of atmospheric carbon dioxide and decreased pH (Riebesell et al., 2000).

A major factor which contributes to the decreasing PIC/POC ratio in previous culture experiments, is that in addition to reduced calcification, there is an increase in carbon fixation with increasing  $p\text{CO}_2$  (Paasche, 1964; Zondervan et al., 2002; Rost et al., 2003; Rost and Riebesell, 2004). The



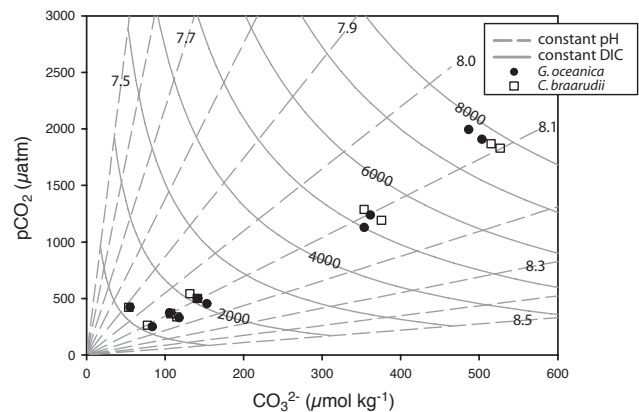
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converse to the calcification paradigm is therefore that coccolithophores may increase their photosynthetic efficiency as  $\text{CO}_2$  increases in the environment (e.g. Riebesell et al., 2007). The enzyme, Ribulose-1-5-bisphosphate carboxylase/oxygenase (RubisCo), indispensable for photosynthesis in all plants and algae, is highly conserved genetically, but is plagued by its historic development under high  $\text{CO}_2$ , low  $\text{O}_2$  conditions and is still slow and inefficient (Tortell, 2000). In the absence of mechanisms that can elevate the  $\text{CO}_2$  at the site of RubisCo action, phytoplankton growth may be limited by  $\text{CO}_2$  availability under a range of oceanic conditions. *Emiliania huxleyi*, the current ubiquitous but one of the smallest sized coccolithophore, may operate at less than 100% photosynthetic efficiency under modern ocean conditions of  $\text{CO}_2(\text{aq})$  (e.g. Rost and Riebesell, 2004; Rost et al., 2003).

To overcome the kinetic hurdle of RubisCo to photosynthesis, most phytoplankton have developed means of concentrating carbon to elevate intracellular  $\text{CO}_2$  (e.g. Giordano et al., 2005) under modern low  $\text{CO}_2$  conditions and drive the forward reaction of RubisCo. Eukaryotic phytoplankton may employ a range of strategies to elevate the internal concentration of  $\text{CO}_2(\text{aq})$  at the site of RubisCo within the chloroplast, involving uptake of  $\text{HCO}_3^-$  and/or  $\text{CO}_2$  (Nimer et al., 1997; Raven, 1997; Tortell et al., 1997; Badger et al., 1998, 2002; Matsuda et al., 1998; Colman et al., 2002; Morel et al., 2002; Giordano et al., 2005). Furthermore, they can catalyse the generation of  $\text{CO}_2$  from accumulated  $\text{HCO}_3^-$  close to the site of RubisCo action, using an internal or chloroplast carbonic anhydrase (CA) (Raven, 1997), or elevate the rate of diffusion of  $\text{CO}_2$  into the cell by speeding up the dehydration of  $\text{CO}_2$  in the external microenvironment by using an external or cell surface CA (Nimer et al., 1997, 1999). Alternatively, the external CA may be employed to “recycle” leaking  $\text{CO}_2$  from the cell (e.g. Trimborn et al., 2008). It must be noted that CA cannot itself change pH, it merely acts to speed up the dehydration/hydration of  $\text{CO}_2(\text{aq})$ .

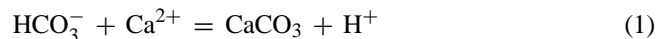
There are widely varying estimates of the extent to which *E. huxleyi* is able to concentrate carbon above ambient seawater levels, ranging from no accumulation (Nimer and Merrett, 1992) such that this species takes up  $\text{CO}_2$  for photosynthesis only via diffusion (Raven and Johnston, 1991); to values approximately 10-fold higher than ambient (Sekino and Shiraiwa, 1994). Rost et al. (2003), also find that *E. huxleyi* operates a rather inefficient yet actively regulated carbon acquisition. But very little is known about the detailed carbon concentrating mechanisms (CCMs) nor the ability or energetic cost for different species and strains of coccolithophore to concentrate carbon, and therefore their photosynthetic success under elevated carbon conditions.

Calcification could represent a cost-efficient alternative to a CCM (Sikes et al., 1980; Anning et al., 1996). Assuming that coccolithophores use primarily the abundant  $\text{HCO}_3^-$  as their carbon source for calcification (Sikes and Wilbur, 1982; Rost et al., 2002), the precipitation of calcite must yield a



**Fig. 1.** The carbonate chemistry conditions ( $\text{CO}_3^{2-}$  in  $\mu\text{mol/kg}$  and  $p\text{CO}_2$  in  $\mu\text{atm}$ ) as measured at the beginning of each of our incubations for *G. oceanica* (black dots) and *C. braarudii* (open squares). Contours of constant pH with increasing DIC are indicated by the dashed grey lines whereby saturation state (proportional to  $\text{CO}_3^{2-}$  since all experiments were run at constant Ca concentration of 10 mmol/kg) and  $p\text{CO}_2$  are positively correlated in our experiments. By contrast, the majority of previous experiments follow the solid grey curves at constant DIC but with varying pH which results in an inverse correlation between  $p\text{CO}_2$  and saturation state.

proton:



It would appear to be cost effective to harness that  $\text{H}^+$  to manipulate the pH and enhance the generation of  $\text{CO}_2$  from the plentiful  $\text{HCO}_3^-$ , potentially accelerated by a CA, either inside or outside the cell (as suggested by Buitenhuis et al., 1999). But whether calcification plays a role in boosting the internal supply of  $\text{CO}_2$  to the coccolithophore is controversial (e.g. Sikes et al., 1980; Young, 1994; Paasche, 2001) and there is increasing evidence that the two processes are not coupled (Paasche, 1964; Balch et al., 1996; Rost and Riebesell, 2004; Trimborn et al., 2007; Leonardos et al., 2009).

In our study, we aim to use the isotopes of both organic matter and calcite generated by two different species of coccolithophores of contrasting cell size to probe how the physiology changes under a range of altered carbon conditions. In most coccolithophore culture experiments to date, the high  $\text{CO}_2$  conditions have been achieved either by titration with acid, or by bubbling with  $\text{CO}_2$ , such that high  $\text{CO}_2$  conditions correlate inversely with pH, carbonate ion, and saturation state (Fig. 1). By decoupling of different parameters, we are able to navigate through the matrices of factors that can affect cell physiology to understand which aspect of carbon chemistry appears to be so detrimental to the coccolithophores. To this end, we have performed culture experiments with the small coccolithophore *Gephyrocapsa oceanica*, and the large coccolithophore *Coccolithus braarudii* at constant pH but with increasing and correlated concentrations of dissolved

inorganic carbon (DIC), aqueous CO<sub>2</sub>, and saturation state (Fig. 1). Such conditions are analogous to the changes in the carbonate system which happen on geological timescales which allow buffering of changes in *p*CO<sub>2</sub> by the carbonate compensation system leading to minimal changes in ocean saturation state (Ridgwell and Zeebe, 2005). Nonetheless, the high experimental DIC conditions with a saturation state of up to 12, are beyond the realm of reconstructed history, where such high DIC conditions are instead accompanied by a lower pH (Tyrrell and Zeebe, 2004).

## 2 Methods

*Gephyrocapsa oceanica* (strain PZ 3.1) and *C. braarudii* (strain 4762) were kindly provided by Ian Probert, Station Biologique de Roscoff. The cultures were grown in North Sea water at 18 °C with manipulated carbonate chemistry and nutrient concentrations. North Sea water was brought to pH 3 with 1 M HCl, filter sterilized (0.2 μm) and bubbled with CO<sub>2</sub>-free air over several days to strip out all dissolved inorganic carbon (DIC). DIC was then added from a 1 M NaHCO<sub>3</sub> stock solution to target concentrations of about 1100, 1600, 2100, 5300 and 7800 μmol kg<sup>-1</sup>, after which pH was adjusted to 8.13 ± 0.02 (see Supplement for complete initial and final carbonate chemistry calculated from CO<sub>2</sub>.sys and DIC, Alkalinity and pH).

Sterile filtered nutrients were added according to K/2 (Keller et al., 1987), with modified final concentrations of EDTA (1/10 of the original concentration) and nitrate and phosphate concentrations of 100 and 6.25 μmol l<sup>-1</sup>, respectively. Also, we did not add the trace metal Cu, instead we added Se and Ni to final concentrations of 0.01 and 0.00627 μmol l<sup>-1</sup>, respectively, since these coccolithophore species tend to grow better in this medium (I. Probert, personal communication, 2007). After inoculation of the cultures in duplicate 2.8-L polycarbonate bottles, the bottles were closed immediately leaving a headspace of only a few ml. The cultures were preacclimated for a week before inoculating the experimental bottles and grown under a 16:8 light:dark cycle at a light intensity of 200 μmol photons m<sup>-2</sup> s<sup>-1</sup> and a temperature of 18 °C. The bottles were shaken every 2 min by means of a pressure pump system. At the end of the experiment, after more than 6 (*C. braarudii*) and 7 (*G. oceanica*) generations, when the drift in DIC and pH was between minus 2.35–9.0% and plus 0.00–0.08 units for *C. braarudii* and minus 2.27–9.0% and plus 0.00–0.13 units for *G. oceanica*, respectively, samples from the dilute cultures (<18 500 cells ml<sup>-1</sup> for *G. oceanica*; <2300 cells ml<sup>-1</sup> for *C. braarudii*) were taken.

Samples (4 ml) for DIC measurements were filtered (0.2 μm pore size), fixed with HgCl<sub>2</sub> and stored free of air bubbles at 4 °C. DIC was measured in duplicate following the method by Stoll et al. (2001). Alkalinity from filtered samples (200 ml) stored at 4 °C was determined by duplicate

potentiometric titration (Bradshaw and Brewer, 1988) and calculated from linear Gran plots (Gran, 1952). The precision of the measurement was ±3 μmol l<sup>-1</sup>. The pH and concentrations of dissolved CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> were calculated from temperature, salinity, and the concentrations of DIC, alkalinity, and phosphate, using equilibrium constants of Mehrbach et al. (1973) as refit by Dickson and Millero (1987). Samples (12 ml) for δ<sup>13</sup>C-DIC were filtered (0.2 μm pore size), fixed with HgCl<sub>2</sub> and stored free of air-bubbles until measurement.

The samples were extracted in a vacuum line, as described by Mackensen et al. (1996). Measurements of δ<sup>13</sup>C<sub>DIC</sub> were performed with a mass spectrometer (Finnigan MAT 252) at a precision of ±0.03‰. The isotopic composition of CO<sub>2</sub> (δ<sup>13</sup>C<sub>CO2</sub>) was calculated from δ<sup>13</sup>C<sub>DIC</sub> using the equation by Rau et al. (1996) based on Mook et al. (1974):

$$\delta^{13}\text{C}_{\text{CO}_2} = \delta^{13}\text{C}_{\text{DIC}} + 23.644 - (9701.5/T_{\text{K}}) \quad (2)$$

Samples for the determination of total particulate carbon (TPC) and particulate organic carbon (POC), as well as their stable carbon isotopic composition, were gently filtered on precombusted (4 h, 500 °C) GF/F filters and stored at -20 °C. Prior to analysis, inorganic C was removed from the POC filters with 200 μl of a 0.2 N HCl solution. After drying for 2 h at 60 °C TPC, POC and δ<sup>13</sup>C values were measured in duplicate on a mass spectrometer (ANCA-SL 20-20), with a precision of ±0.5 μg C and ±0.5‰, respectively. PIC was calculated as the difference between TPC and POC. The isotopic composition is reported relative to the PeeDee belemnite standard (PDB):

$$\delta^{13}\text{C}_{\text{Sample}} = \left[ \frac{(^{13}\text{C}/^{12}\text{C})_{\text{Sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{PDB}}} - 1 \right] \times 1000 \quad (3)$$

Isotope fractionation during POC formation (ε<sub>p</sub>) was calculated relative to the isotopic composition of CO<sub>2</sub> in the medium (Freeman and Hayes, 1992):

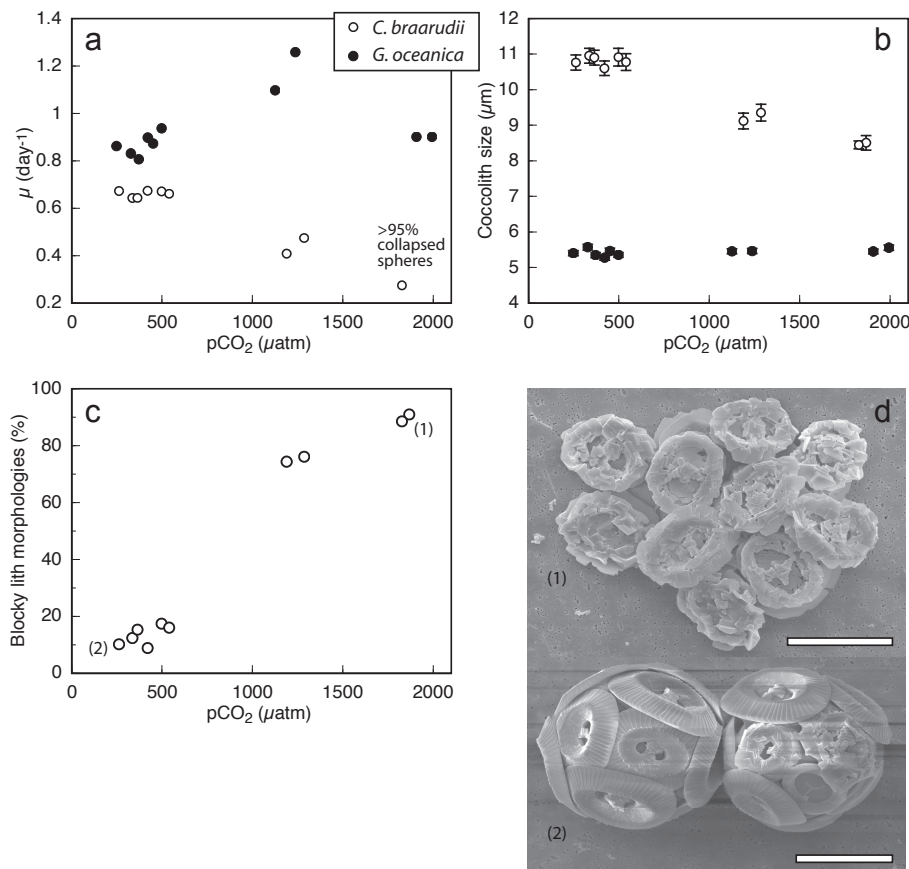
$$\varepsilon_p = \frac{\delta^{13}\text{C}_{\text{CO}_2} - \delta^{13}\text{C}_{\text{POC}}}{1 + \frac{\delta^{13}\text{C}_{\text{POC}}}{1000}} \quad (4)$$

Samples for cell counts were fixed with 400 μl/20 ml sample of a 20% formaldehyde solution buffered with hexamethylenetetramine, and counted by means of an inverted microscope (Zeiss Axiovert 200M) in a counting cell (Sedgewick Rafter Cell), and checked via a Beckman Coulter Counter. The growth rate (μ) was calculated as:

$$\mu = \frac{(\ln c_1 - \ln c_0)}{\Delta t} \quad (5)$$

where *c*<sub>0</sub> and *c*<sub>1</sub> are the cell concentrations at the beginning and the end of the experiment, respectively, and Δ*t* is the duration of the incubation in days.

Scanning electron microscope samples (10 ml) were taken by gentle filtration on polycarbonate filters (Nuclepore



**Fig. 2.** (a) Growth rates ( $\mu$ , day<sup>-1</sup>) and (b) coccolith size for *G. oceanica* (black circles) and *C. braarudii* (open circles) with correlated and increasing DIC,  $\text{CO}_3^{2-}$ , and  $p\text{CO}_2$ . Under the high DIC conditions, *C. braarudii* was severely detrimentally affected (c) showing signs of significant malformation as shown by comparison under the SEM (d) and >95% of the coccospheres were collapsed at the highest DIC treatment. The scale bars indicate 10  $\mu\text{m}$ .

0.4  $\mu\text{m}$  pore size) and rinsing with 10 ml of distilled water. Filters were then dried at 60 °C and stored dry at room temperature prior to analysis on a Philips XL-30-SEM at Stockholm University. In each sample, the maximum diameter (size, in  $\mu\text{m}$ ) of 100–160 coccoliths was measured on 50–80 individual spheres using calibrated SEM images at 2000x (*C. braarudii*) and 5000x (*G. oceanica*) magnification (pixel resolutions of 0.082  $\mu\text{m}$  and 0.041  $\mu\text{m}$ , respectively). Quantification of collapsed vs. complete coccospheres was carried out in each *C. braarudii* sample by counting at least 300 coccospheres. Degree of malformation in *C. braarudii* was derived from a tally of various coccolith morphologies, e.g. “normal”, “incompletely grown” and “rhombic/blocky”, of the visible liths on each coccosphere.

Samples for  $\delta^{13}\text{C}_{\text{PIC}}$ ,  $\delta^{18}\text{O}_{\text{PIC}}$  and trace metals (100 ml) were centrifuged (Heraeus Megafuge 1.0R; 4000 rpm), the pellets transferred to 2 ml eppendorf cups, rinsed 3 times with distilled water (Hettich Mikro 22R; 14 000 rpm) and dried at 60 °C. They were then stored at -20 °C prior to analysis at the University of Oxford. The stable isotopes were analyzed online using a VG Isocarb device and a Prism mass

spectrometer with a precision of 0.1‰. A Carrera marble standard was run and used to calculate  $\delta^{18}\text{O}_{\text{PIC}}$  and  $\delta^{13}\text{C}_{\text{PIC}}$  relative to Peedee Belemnite (PDB).

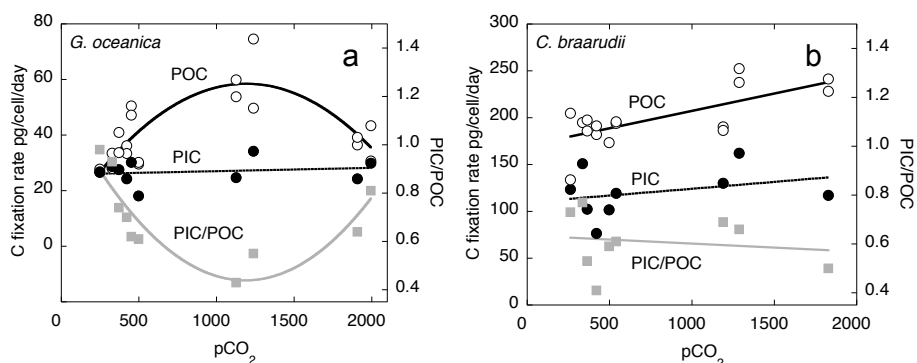
### 3 Results

All results can be found in the Supplement.

#### 3.1 Carbon fixation and growth rates

*Gephyrocapsa oceanica* grew well under all DIC conditions with little or no sign of malformed coccoliths, and even thrived with elevated growth rates by 50% at ~1200  $\mu\text{atm}$   $\text{CO}_2$ . *C. braarudii* was severely affected by high DIC conditions with growth rates reduced by a third, decreased coccolith size, and obvious malformation of the coccoliths (Fig. 2).

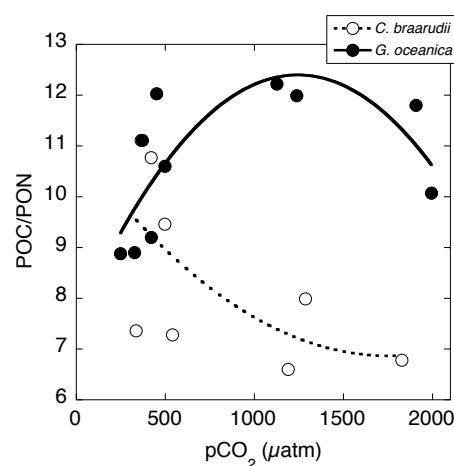
Calcification rates and photosynthetic carbon fixation rates increased very slightly with increasing DIC in *C. braarudii*, despite the poor visual nature of the cells, but since these were parallel in trend and magnitude, there was no change



**Fig. 3.** POC (open circles), PIC (black circles) C fixation rates in pg/cell/day and PIC/POC ratio (grey squares) for *G. oceanica* with increasing  $p\text{CO}_2$ . Like the earlier experiments of changing pH by Riebesell et al. (2000), there is a decrease in PIC/POC for *G. oceanica* with increasing  $p\text{CO}_2$  but this is driven by the greater increase in POC relative to the nominal change in calcite production rates. We have plotted a 2nd order polynomial curve through the data where appropriate to highlight the trends.

in the PIC/POC ratio (Fig. 3). This lack of variation in PIC/POC is a robust feature but we note that there is some uncertainty regarding the rate of carbon fixation at the highest  $p\text{CO}_2$  conditions in *C. braarudii*. Under these extreme conditions, we found a mismatch between the counts by eye and the Coulter Counter. We deemed the Coulter Counter to overestimate the healthy growing population of coccolithophores, and favoured population estimates based on microscope counts which could distinguish between healthy growing cells, and collapsed spheres. Since our population counts at the highest  $p\text{CO}_2$  were lower by up to 50% than the measurements made by Coulter Counter, but also yielded slower growth rates, the two effects approximately cancel in the calculation of carbon fixation rates, but overall this Coulter Counter approach to population measurement yields a very slight decrease in the PIC and POC production rates at the highest  $p\text{CO}_2$ , but with no net change in the PIC/POC. Our PIC/POC results are consistent with Langer et al. (2006) who reported no change in PIC/POC of *C. pelagicus* (*spp. braarudii*) over a range of  $p\text{CO}_2$  from 149–914  $\mu\text{atm}$ , which was achieved by altering pH (8.56–7.81). However, in contrast to our results under constant pH, in these experiments *C. pelagicus* was not adversely affected under any of the treatments, with no change in malformation or changing growth and carbon fixation rates.

Whilst *G. oceanica* shows a peak in its photosynthetic carbon fixation rate with a doubling at  $\sim 1200 \mu\text{atm}$ , there is only a statistically insignificant increase in calcification rate, hence the PIC/POC ratio decreases with increasing  $p\text{CO}_2$  to a minimum at  $\sim 1200 \mu\text{atm}$  and then increases again. This observation is consistent with data from a number of pH manipulation studies (Riebesell et al., 2000), but crucially here, this decrease is only driven by the increasing photosynthetic carbon fixation rate (also seen in Rost et al., 2002; Zondervan et al., 2002) and there is no evidence of any decrease in calcification. Further evidence for elevated C fixation rates



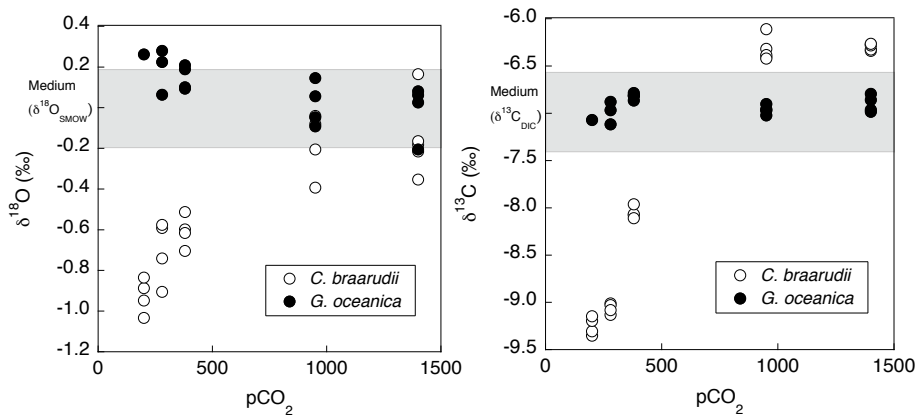
**Fig. 4.** Evidence for elevated C fixation rates with increasing  $p\text{CO}_2$  in *G. oceanica* (black circles) arises from elevated POC/PON ratios with increasing  $p\text{CO}_2$ . By contrast, *C. braarudii* (open circles) appears detrimentally affected, showing a decrease in POC/PON with increasing  $p\text{CO}_2$ .

with increasing  $p\text{CO}_2$  arises from elevated POC/PON ratios with increasing DIC (Fig. 4).

### 3.2 Malformation of the coccoliths

We find little to no evidence for any change in the degree of malformation of *G. oceanica* with increasing DIC, but *C. braarudii* is increasingly adversely affected (Fig. 2c) in terms of the degree of malformation of its liths. We note that malformed *C. braarudii* coccoliths with rhombic, “blocky” calcite features (Fig. 2d) were found incorporated within cocspheres grown under all experimental conditions (compare also Langer et al., 2006), indicating that malformation occurs occasionally even under normal and lower DIC levels





**Fig. 5.** The (left)  $\delta^{18}\text{O}$  and (right)  $\delta^{13}\text{C}$  in ‰PDB of the calcite liths of *G. oceanica* (black circles) and *C. braarudii* (open circles) compared to the  $\delta^{18}\text{O}_{\text{SMOW}}$  composition estimated from average North Sea waters (Harwood et al., 2008), and the  $\delta^{13}\text{C}_{\text{DIC}}$  as measured in the medium respectively with increasing  $p\text{CO}_2$ . The grey box indicates the range in the estimated  $\delta^{18}\text{O}$  isotopic value of the North Sea Waters, and also the range in the  $\delta^{13}\text{C}_{\text{DIC}}$  of the medium as measured at the beginning and end of each culture experiment.

and that this likely is an intracellular process. *Coccolithus braarudii* produces one coccolith at the time, and only on maturation does the single coccolith vesicle migrate away from the nucleus before the coccolith is extruded and incorporated into the coccosphere (Taylor et al., 2007). Indeed, our SEM analyses revealed that each (collapsed) coccosphere contains multiple (malformed or incomplete) coccoliths indicative of an intact cellular mechanism for initiating coccolithogenesis and expulsion, despite the significant malformation and slower growth rates under high DIC. The blocky character of these liths speaks for a major change in the interaction between the coccolith associated polysaccharide (CAP) template and the growing calcite (Langer et al., 2006; Henriksen and Stipp, 2009), either as a result of a change in chemical composition or pH in the calcification vesicle.

### 3.3 Stable isotopes of the coccoliths ( $\delta^{18}\text{O}_{\text{PIC}}$ and $\delta^{13}\text{C}_{\text{PIC}}$ )

The isotopic fractionation between the coccoliths and the medium provides an indication of which carbon species is used during calcification and the calcifying vesicle conditions such as pH,  $\text{CO}_3^{2-}/\text{HCO}_3^-$  ratio during these culture experiments (Fig. 5), assuming little isotopic fractionation during uptake. At equilibrium, there is a  $-2\text{‰}$  fractionation between the  $\text{CO}_3^{2-}$  ion and  $\text{HCO}_3^-$  (Zeebe and Wolf-Gladrow, 2001) in  $^{13}\text{C}$ .

The  $\delta^{18}\text{O}$  of the North Sea Water was not directly measured during these experiments but we can constrain the composition to lie within the range found for surface waters from the North Sea as  $-0.2$  to  $+0.2\text{‰}$ . The  $\delta^{18}\text{O}_{\text{PIC}} - \delta^{18}\text{O}_{\text{medium}}$  of *G. oceanica* is within error of  $0\text{‰}$ , i.e. at equilibrium with the medium but  $\sim 1\text{‰}$  lighter than expected from Ziveri et al. (2003) who obtain a value of between  $1\text{--}2\text{‰}$  at  $18^\circ\text{C}$ .

The  $\delta^{13}\text{C}_{\text{PIC}}$  for *G. oceanica* are within error the same as the  $\delta^{13}\text{C}_{\text{DIC}}$  in all experiments, in agreement with Ziveri et al. (2003). This indicates either that the pH of the calcification vesicle is very similar to that of the media, or that the pH in the vesicle is in the range where  $\text{HCO}_3^-$  is the dominant ion, and the primary source of the carbon for calcification as determined elsewhere (Sikes and Wilbur, 1982; Rost et al., 2002). Furthermore, the constancy of the isotopes in *G. oceanica* is suggestive of no major physiological perturbations under the range of our experimental conditions. The  $\delta^{18}\text{O}_{\text{PIC}}$  and  $\delta^{13}\text{C}_{\text{PIC}}$  of *C. braarudii*, at low DIC and normal growth rates, are  $1\text{‰}$  and  $2\text{‰}$  lighter than the media respectively, and consistent with previous interspecific variations and offsets (Ziveri et al., 2003). But there is a large change in the isotopic composition under the high DIC conditions as  $\delta^{18}\text{O}_{\text{PIC}}$  and  $\delta^{13}\text{C}_{\text{PIC}}$  record more positive values, within error of calcite equilibrium with the composition of the DIC in the medium.

This isotopic shift towards heavier isotopes under high DIC could reflect a decreasing kinetic fractionation as the growth rates also decrease at these apparently unfavourable conditions. But the trend in isotopic values (Fig. 5) under stable growth rates at the lower DIC levels implies that the isotopic shift cannot be due to decreased growth rate at high DIC. Furthermore, the rate of calcification that should have the greatest influence on the kinetic isotopic fractionation, is unchanged by the DIC conditions. The magnitude of the isotopic change (by  $1\text{--}2\text{‰}$ ) is also in line with a change in the dominant carbon containing ion used during calcification, from preferential use of  $\text{CO}_3^{2-}$  for calcification at low DIC, to calcification from  $\text{HCO}_3^-$  at high DIC (Zeebe and Wolf-Gladrow, 2001). The use of  $\text{CO}_3^{2-}$  for calcification implies that the coccolith vesicle of *C. braarudii* normally has a pH higher than that of the media, which is likely achieved by efficient transport of protons out of this vesicle. Indeed,

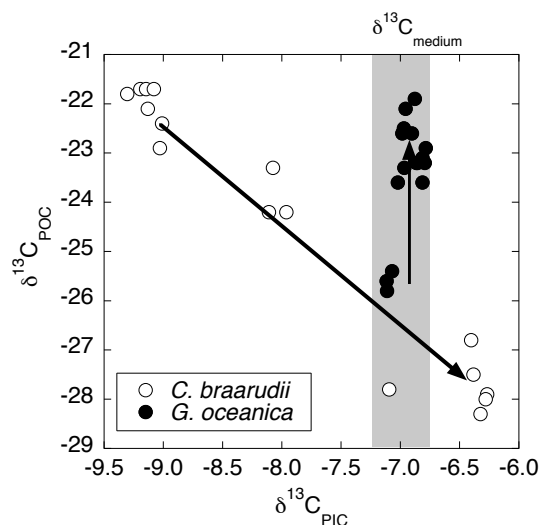
coccolith vesicle pH in *Coccolithus pelagicus* was up to 8.3 while the pH in the cytosol was only 7.0 (Anning et al., 1996). Such efficient maintenance of elevated pH may be essential to maintain the  $\sim 4$  times faster rates of calcification of *C. braarudii* over the smaller coccolithophore, *G. oceanica*. Alternatively, *C. braarudii* may actively transport  $\text{CO}_3^{2-}$  and elevate the  $\text{CO}_3^{2-}/\text{HCO}_3^-$  at the site of calcification.

High DIC conditions appear to invoke a lower pH, or a higher  $\text{HCO}_3^-/\text{CO}_3^{2-}$ , at the site of calcification, perhaps through diminished proton expulsion from the vesicle, which yields a greater proportion of  $\text{HCO}_3^-$  within the vesicle. An alternative possibility is that active transport of  $\text{CO}_3^{2-}$  to the site of calcification becomes less selective as the media  $\text{HCO}_3^-$  increases so dramatically. Such changes in the chemistry of the calcifying vesicle could induce the malformation seen under these conditions by altering the interaction of CAP and the growing lith (Henriksen and Stipp, 2009).

Our stable isotopic data suggest that different species of coccolithophore could maintain different pHs within the calcification vesicle, perhaps due to varied efficiency of proton management from this vesicle linked with the rate of calcification. Calcification within the vesicle will utilize the carbon ions in proportion to their availability inside the vesicle, using a greater proportion of  $\text{HCO}_3^-$  at lower pH and a greater proportion of  $\text{CO}_3^{2-}$  at higher pH. We infer that the mechanism of malformation of coccoliths observed in culture arises from accumulation of protons and acidification within the coccolith vesicle under extreme culture manipulations, as evidenced by the trend in isotopic data. We note that *G. oceanica* buffers its internal pH and physiology under all our manipulations of DIC, but we infer that it decreases internal pH under decreased media pH leading to malformation (Riebesell et al., 2000). By contrast, *C. braarudii* appears to be effective at buffering external changes in pH (Langer et al., 2006) from its internal chemistry, but is detrimentally affected at high DIC.

### 3.4 Carbon isotopic composition of organic matter ( $\delta^{13}\text{C}_{\text{POC}}$ )

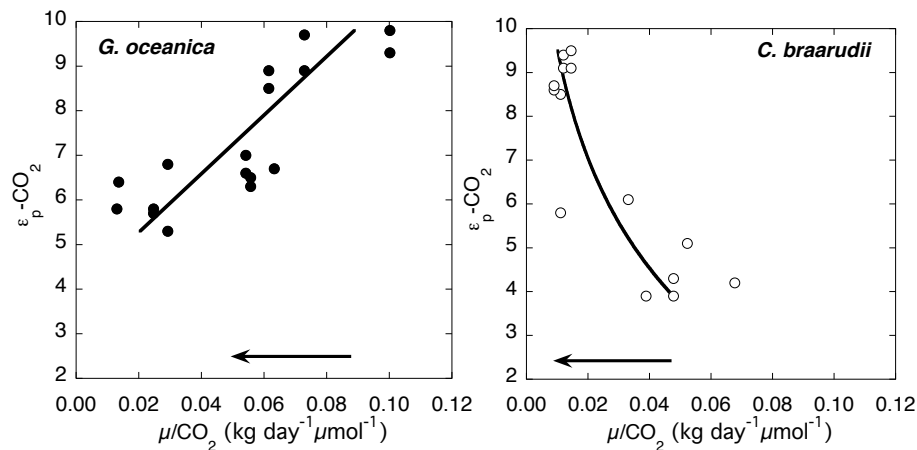
A simple plot of  $\delta^{13}\text{C}_{\text{POC}}$  and  $\delta^{13}\text{C}_{\text{PIC}}$  of the two species immediately reveals their contrasting physiologies under changing DIC conditions (Fig. 6). As discussed previously, *G. oceanica* shows little significant change in calcite  $\delta^{13}\text{C}_{\text{PIC}}$  but  $\delta^{13}\text{C}_{\text{POC}}$  tends from  $-26\text{‰}$  towards heavier values of  $-22\text{‰}$  with increasing DIC. The isotopic difference between the calcite and the organic matter diminishes with more DIC in the environment from  $19\text{‰}$  to  $15\text{‰}$ . By contrast in *C. braarudii*,  $\delta^{13}\text{C}_{\text{PIC}}$  tends from  $-9\text{‰}$  towards heavier values of  $-6.5\text{‰}$ , but the  $\delta^{13}\text{C}_{\text{POC}}$  changes in the opposite direction from  $-22\text{‰}$  towards even lighter values of  $-28\text{‰}$  indicating a greater isotopic difference between the calcite and the organic matter from  $13\text{‰}$  to  $21.5\text{‰}$  with increasing DIC. The linear correlation between  $\delta^{13}\text{C}_{\text{POC}}$  and  $\delta^{13}\text{C}_{\text{PIC}}$  implies



**Fig. 6.** A simple plot of  $\delta^{13}\text{C}_{\text{Org}}$  versus  $\delta^{13}\text{C}_{\text{Cocco}}$  for *G. oceanica* (black circles) and *C. braarudii* (open circles) with the arrows pointing towards the conditions of increasing DIC etc during each experiment. The contrasting physiologies of the two species under these manipulations become immediately apparent. The grey box highlights the range at the beginning and end of the culture experiments.

that their response is coupled and their changes in fractionation are driven or at least related to a common physiological mechanism.

The  $\delta^{13}\text{C}_{\text{POC}}$  and  $\epsilon_p$  can provide information on modes of carbon uptake in marine phytoplankton (Raven and Johnston, 1991; Rost et al., 2002). But the interpretation of such data often remains complicated because of a lack of knowledge on different carbon uptake mechanisms and their efficiency in different species, as well as the processes involved in fractionation. Simplistically, the degree to which the maximum fractionation associated with the carboxylation by RubisCo ( $\epsilon_f$ ) is expressed in the resulting cellular matter relative to the media ( $\epsilon_p$ ) reflects the degree of Rayleigh distillation of the internal carbon pool. Initially, models to account for  $\epsilon_p$  assumed only a diffusive supply of  $\text{CO}_2$  into the cell, such that the net carbon isotopic fractionation should show a negative relationship between  $\epsilon_p$  and  $\mu/\text{CO}_2(\text{aq})$  (where  $\mu$  is growth rate: for details of this theoretical approach see Laws et al., 2002; Keller and Morel, 1999; Cassar et al., 2006) but needs to account for such variables as growth rate, cell geometry, and other factors limiting growth. In this model,  $\mu/\text{CO}_2(\text{aq})$  represents the degree of utilization by growth rate, relative to supply rate which occurs in proportion to  $\text{CO}_2(\text{aq})$  such that this ratio reflects the degree of utilization of an internal pool hence the Rayleigh distillation factor. Such a model provides the basis for the application of  $\epsilon_p$  to reconstruct past atmospheric  $\text{CO}_2$  (Jasper et al., 1994; Pagani et al., 2005; Hendriks and Pagani, 2008).



**Fig. 7.** The isotopic composition ( $\delta^{13}\text{C}_{\text{POC}}$ ) of organic matter calculated as  $\varepsilon_p$  relative to  $\text{CO}_2$  for *G. oceanica* (black circles) and *C. braarudii* (open circles) and plotted versus  $\mu/\text{CO}_2$ . The arrows indicate the direction of increasing carbon in the experiments to highlight the inverse nature of the isotopic fractionation response between the two species.

The  $\varepsilon_p$  calculated for both *G. oceanica* and *C. braarudii* relative to  $\text{CO}_2(\text{aq})$ , assumed to be the substrate for photosynthesis, and plotted versus  $\mu/\text{CO}_2$  shows that  $\varepsilon_p$  is small (Fig. 7), varying between 4‰ and a maximum value of 10‰. Using this formulation higher positive values of  $\varepsilon_p$  indicate more negative values of  $\delta^{13}\text{C}_{\text{POC}}$ . Such small isotopic fractionations relative to  $\text{CO}_2(\text{aq})$  could arise because it is more appropriate to measure  $\varepsilon_p$  relative to the medium  $\text{HCO}_3^-$ , which these coccolithophore species are accessing as their primary source of carbon for photosynthesis and calcification, and which is  $\sim 10\text{‰}$  heavier than  $\text{CO}_2(\text{aq})$ . These coccolithophores, therefore, cannot be dependent on a diffusive supply of  $\text{CO}_2$  only, and are employing some form of CCM to transform  $\text{HCO}_3^-$  and boost  $\text{CO}_2$  for RubisCo carboxylation. Additionally, our data reveal strong indications for species-specific physiologies under similar carbon conditions. Whilst *C. braarudii* adheres to the expectation of a negative slope of  $\varepsilon_p$  versus  $\mu/\text{CO}_2$  according to the diffusive model, the  $\varepsilon_p$  for *G. oceanica* shows the opposite trend, decreasing by  $\sim 6\text{‰}$  with increasing DIC, suggestive of increased Rayleigh distillation under higher DIC conditions.

A more realistic model to capture the biologically controlled modes of carbon acquisition in different species, proposed by Sharkey and Berry (1985), and refined by Burkhardt et al. (1999), shows that variations in  $\varepsilon_p$  are mainly determined by the carbon ion used and the “leakage” ( $L$ ), defined as the ratio of  $\text{CO}_2$  efflux ( $F_{\text{out}}$ ) to the total carbon influx ( $F_{\text{in}}$ ):

$$\varepsilon_p = a \times \varepsilon_{\text{b/d}} + \varepsilon_f \times \frac{F_{\text{out}}}{F_{\text{in}}} \quad (6)$$

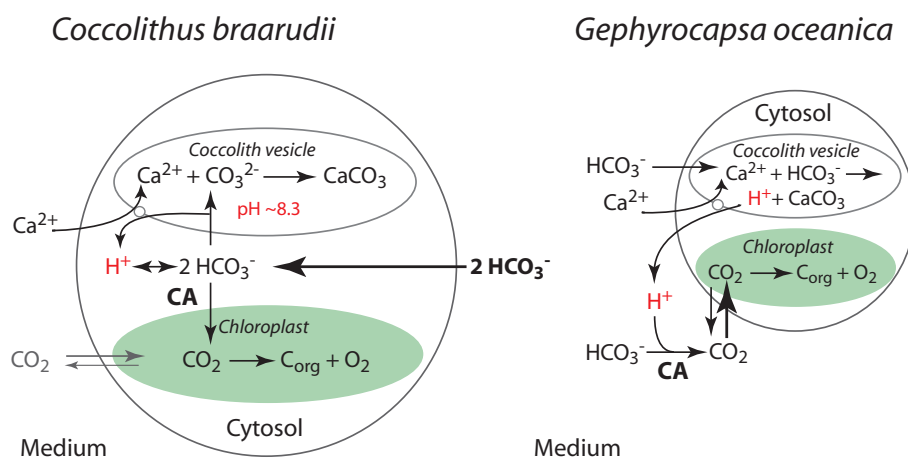
In this equation,  $a$  represents the fraction of  $\text{HCO}_3^-$  to total carbon uptake and  $\varepsilon_{\text{b/d}}$  is the equilibrium discrimination between  $\text{CO}_2$  and  $\text{HCO}_3^-$  (approximately  $-10\text{‰}$ ). The fractionation of the carbon-fixing enzyme RubisCo ( $\varepsilon_f$ ) is

assumed to be 29‰ (for eukaryotic phytoplankton  $\varepsilon_f$  is estimated to be in range of 25–28‰; Popp et al., 1998). As  $\text{HCO}_3^-$  is about 10‰ enriched in  $^{13}\text{C}$  compared with  $\text{CO}_2$  (Zeebe and Wolf-Gladrow, 2001), an increasing proportion of  $\text{HCO}_3^-$  uptake diminishes  $\varepsilon_p$ , which is defined relative to  $\text{CO}_2$  as the carbon source. If there is no change in carbon source,  $\varepsilon_p$  decreases with decreasing leakage. With the increasing DIC of our experiments, we would expect the leakiness of the cells to decrease since the high DIC creates a gradient able to drive carbon into the cell. So *C. braarudii* likely utilizes a greater proportion of  $\text{CO}_2$  for photosynthesis at high DIC, because there are such high concentrations in the media, the gas diffuses rapidly across the membrane to the chloroplast and is available, whilst *G. oceanica* likely experiences less leakage at high DIC.

*Coccolithus braarudii* has a smaller positive  $\varepsilon_p$  than *G. oceanica* under modern conditions. The implication is that the larger *C. braarudii* utilizes a greater proportion of  $\text{HCO}_3^-$  and/or is less leaky than the smaller *G. oceanica*, while *G. oceanica* is less photosynthetically efficient under modern conditions and more reliant on a diffusive supply of  $\text{CO}_2(\text{aq})$ . Indeed similar inverse trends in  $\varepsilon_p$  have been observed in other culture experiments and are consistent with cells operating at less than their critical  $\text{CO}_2$  concentration i.e. in cells limited by the rate of diffusion (Laws et al., 1997; Burkhardt et al., 1999). *Gephyrocapsa oceanica* certainly increases both its growth rate and its rate of photosynthetic carbon fixation as DIC increases in the environment suggestive that it is partly limited by the diffusive supply of  $\text{CO}_2$  at low carbon conditions. Extra energy may be allocated to the expression of carbon concentrating mechanisms under low carbon conditions which is then available for growth as DIC becomes more plentiful.

For *C. braarudii*, there is the additional observation that the shift towards lighter values in  $\delta^{13}\text{C}_{\text{POC}}$  is linearly related





**Fig. 8.** A schematic to illustrate our conceptual model which proposes that the C source for photosynthesis dictates what happens to the  $H^+$  from calcification may to account for differing responses of species of coccolithophore to changing pH and  $CO_2$  availability. We show two different extreme endmembers for our conceptual model and it is most likely that species and even strains consist of a proportional mixture of the two processes but lie along a gradient between them. The curved arrows at the membrane of the calcification vesicle represent the  $Ca^{2+}/H^+$  ATP-ase (Arako and Gonzalez, 1998).

to a shift towards heavier values in the  $\delta^{13}C_{PIC}$ . These potentially related changes in isotopic fractionation imply that the carbon for both calcification and photosynthesis in *C. braarudii* is derived from a common internal pool of  $HCO_3^-$ , under low DIC conditions. However, a closed system internal pool must be overprinted by an external source of carbon under the high DIC conditions. Under a closed system, a decreased degree of utilization of this common pool by calcification or photosynthesis under the high carbon conditions, would result in less internal Rayleigh distillation in that common pool, while both  $\delta^{13}C_{POC}$  and  $\delta^{13}C_{PIC}$  should tend to lighter values. Instead the divergence between these isotopic values implies that a new, unfractionated source presumably a diffusive supply of  $CO_2$  from the exterior becomes a significant source, as the DIC increases in the external media.

Under normal conditions therefore, we infer that *C. braarudii* contains a common internal pool of carbon. Calcification appears unlikely to provide a direct supply of  $CO_2$  for photosynthesis – but through pH regulation of the coccolith vesicle, provides protons that aid conversion of  $HCO_3^-$  into  $CO_2$  near the chloroplast. We propose that *C. braarudii* actively transports  $HCO_3^-$  into the cell which then provides  $CO_3^{2-}$  for calcification and  $CO_2$  for photosynthesis, perhaps catalysed by a chloroplast carbonic anhydrase and proton transfer between the two equilibria. Such a scenario is consistent with the observation that the addition of  $HCO_3^-$  to carbon-starved cells resulted in an increase in pH, while its removal resulted in the cytosolic acidification, suggesting a role of  $HCO_3^-$  in buffering cytosolic pH (Brownlee and Taylor, 2004) and the internal proton transfer from calcification to photosynthesis.  $HCO_3^-$  influx (Sikes and Wilbur, 1982; Nimer and Merrett, 1992) was shown previously to

be very rapid (Nimer and Merrett, 1992). The conversion of  $HCO_3^-$  to  $CO_2$  by the enzyme carbonic anhydrase discriminates against  $^{13}C$  by the same sort of value as the inorganic equilibration of  $CO_2(aq)$  with  $HCO_3^-$  of  $\sim 9\%$  (Paneth and O'Leary, 1985). This means that a chloroplast envelope carbonic anhydrase inside the cell enables organic matter to become isotopically heavy because the cellular  $HCO_3^-$  would completely convert to  $CO_2$ . There is strong evidence to support a chloroplast carbonic anhydrase within the coccolithophores (Nimer et al., 1994; Quiroga and Gonzalez, 1993).

We propose that the carbon uptake mechanism within the large cells of *C. braarudii* provides an internal buffering carbon pool between calcification and photosynthesis which makes this species insensitive to changes in external pH (Langer et al., 2006), and that this species is largely dependent on uptake of  $HCO_3^-$  for photosynthesis. In contrast, we propose that *G. oceanica* can be limited by diffusive supply of  $CO_2$  and acts similarly to its close relation *E. huxleyi*, and uptakes  $CO_2$  mainly across the membrane for photosynthesis (Fig. 8). We hypothesise that the  $CO_2$  supply may be at times facilitated by a carbon concentrating mechanism such as an external CA which is found in some strains of *E. huxleyi* (Elzenga et al., 2000).

#### 4 Discussion and a working hypothesis

Our culture experiments reveal two key observations to develop a conceptual model of the mechanism behind the response of coccolithophores to elevated carbon in the environment (Fig. 8). In order to simplify the model, we present the two species as contrasting end-members, although it is most

likely they are at different points on a spectrum. *Coccolithus braarudii* likely generates  $\text{CO}_3^{2-}$  for calcification, and  $\text{CO}_2$  for photosynthesis from a common internal pool of actively pumped  $\text{HCO}_3^-$  which acts to buffer these two processes, and maintain the cytosol at pH 7.0 (Dixon et al., 1989; Nimer et al., 1994). The transformation of  $\text{HCO}_3^-$  to  $\text{CO}_2$  may be catalysed by an internal chloroplast carbonic anhydrase. By contrast, *G. oceanica* utilizes  $\text{HCO}_3^-$ , at a lower pH within the calcifying vesicle, for calcification but has a largely separate diffusive supply of  $\text{CO}_2$  across the membrane for photosynthesis, which, we hypothesise may be enhanced by activation of an external CA.

All coccolithophores transport calcium ( $\text{Ca}^{2+}$ ) and  $\text{HCO}_3^-$  into the cell for calcification, which results in the release of a proton. We will use a charge balance approach to consider the fate of this proton. We acknowledge that all transports across membranes will be charge balanced but we try to simplify in this context the crucial ion transports across the membrane for photosynthesis and calcification. This proton may be used to aid photosynthesis as it will drive  $\text{HCO}_3^-$  to form  $\text{CO}_2$ . From the perspective of cellular charge balance, if the diffusion of  $\text{CO}_2$  across the cell membrane is the primary source of carbon for photosynthesis, then the proton must be pumped out of the cell. Here, the proton can acidify the external microenvironment enhancing the conversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$  and boosting the diffusive flux of  $\text{CO}_2$  back into the cell. Hence acidifying seawater for e.g. *G. oceanica*, makes the efflux of protons from calcification more energetically consuming such that the site of calcification may become more acidic and induce malformation (Riebesell et al., 2000). Hence calcification in this species is largely sensitive to pH.

From a charge balance perspective if a larger cell such as *C. braarudii*, which has a smaller diffusive influx of  $\text{CO}_2$ , relies primarily on the active transport of  $\text{HCO}_3^-$  across the cell membrane as the main source of carbon for photosynthesis then the proton must be pumped intracellularly. Indeed a strong intracellular transport of protons from the site of calcification may elevate the pH to enhance calcification rates in larger coccolithophores and allow  $\text{CO}_3^{2-}$  as the source for calcification. The proton would be likely transported to the internal pool of  $\text{HCO}_3^-$  to aid the intracellular conversion to  $\text{CO}_2$  and its diffusion into the chloroplast. The internal buffering of this larger cell to pH means its calcification is insensitive to changing pH (Langer et al., 2006). Under high DIC conditions there is an additional and large diffusive flux of the  $\text{CO}_2$  into the cell, which reduces the equilibrium reaction of  $\text{HCO}_3^-$ , and potentially drives the cytosol to more acidic conditions. The intracellular pump of protons from coccolith vesicle into cytosol would become more energy consuming, the site of calcification more acidic and again induce malformation as found in this study.

## 5 Implications

### 5.1 Evolution of coccolithophore cell size

The Cenozoic ancestors of *G. oceanica* and *E. huxleyi* are identified within the *Reticulofenestra* genus (family *Noelaerhabdaceae*) (Marlowe et al., 1990; Young et al., 1992; Henderiks and Pagani, 2008). The *Reticulofenestra* genus displays large size variability during the Cenozoic, with a robust trend towards smaller cells since the earliest Oligocene, when atmospheric  $\text{CO}_2$  declined (Henderiks and Pagani, 2008; Lowenstein and Demicco, 2006; Pearson et al., 2009). By comparison, the *Coccolithus* genus reveals an overall constancy in size and coccolith morphology since its first occurrence 63 Ma (Henderiks and Rickaby, 2007). These observations support our hypothesis of contrasting ways of carbon acquisition: (a) Lineages that depend more on diffusive uptake of  $\text{CO}_2$  will select for smaller cells under  $\text{CO}_2$  limitation and tend towards the emergence of small, prolific bloom forming species (as evidenced by the Pleistocene success of *Gephyrocapsa* spp. and prominent bloom forming *E. huxleyi* which originated  $\sim 268$  kyrs ago and rose to dominance during the last 78 kyrs; Thierstein et al., 1977), (b) Lineages that primarily use  $\text{HCO}_3^-$  can sustain larger cells under lower levels of  $\text{CO}_2$ , and indeed may need to remain large to maintain a large enough buffering internal pool of  $\text{HCO}_3^-$ , but have a greater resource requirement for optimal growth. We add the caveat that we extrapolate our culture results to the higher taxonomic level only tentatively since strain-specific responses are well documented within *E. huxleyi* (Langer et al., 2009).

What could cause such differences in carbon management systems for these two lineages and dictate their relative success in the modern ocean from an evolutionary perspective? The surface ocean has evolved from low pH high  $p\text{CO}_2$ , to higher pH, lower  $p\text{CO}_2$  conditions (Pearson and Palmer, 2000). The *Coccolithus* lineage, with larger cells, has always accessed the most abundant form of carbon  $\text{HCO}_3^-$ , with an internal buffering between  $\text{HCO}_3^-$  and photosynthesis and calcification, and so has never undergone evolutionary pressure to diminish in size (Henderiks and Rickaby, 2007). By contrast, the *Reticulofenestra-Gephyrocapsa-Emiliana* lineage which today appear more reliant on a diffusive supply of  $\text{CO}_2$  for photosynthesis would have thrived in the high  $\text{CO}_2(\text{aq})$ , and more acidic conditions of the Paleogene. But as the  $\text{CO}_2$  dropped and the ocean became more alkaline, they adapted by evolving towards smaller size to increase their diffusive supply of  $\text{CO}_2$  (Henderiks and Pagani, 2008). As the  $\text{CO}_2$  levels dropped even further, they were able to evolve and express an external CA to further boost their diffusive  $\text{CO}_2$  supply. The enzyme, CA, appears to have evolved in nature several times independently, due to its essential mechanism, providing an excellent example of convergent evolution (Badger and Price, 1994). Intriguingly, one of the novel and unique sequences of the external  $\delta\text{-CA}$

identified from the reticulofenestrid *E. huxleyi* ( $\delta$ -EhCA1) shows that it hosts a large transmembrane N-terminal region (Soto et al., 2006). Such a genetic motif is more characteristic of a transmembrane transporter, and it has been speculated that this CA had a transporter function related to carbon acquisition and/or ion transport processes so may have evolved from a  $\text{HCO}_3^-$ -transporter, since there is a common requirement to bind  $\text{HCO}_3^-$ . Therefore the increasingly small reticulofenestrads were able to thrive in low  $\text{CO}_2$  conditions alongside the increasingly nutrient limited conditions during the Cenozoic as the water column cooled, the thermocline developed and the upper ocean became more stratified with only a sporadic nutrient supply from mixing (Falkowski et al., 2004). Indeed *E. huxleyi* is best adapted to and calcifies heavily under low  $\text{PO}_4^{3-}$  conditions (Anderson, 1981). Meanwhile the *Coccolithus* genus has been biogeographically marginalized to only a few niches in the modern ocean, largely characterized by high nutrients, after its cosmopolitan distribution and dominance during the Paleogene and most of the Miocene (Henderiks and Rickaby, 2007).

## 5.2 Application of $\epsilon_p$ for reconstruction of $p\text{CO}_2$

Our results have implications for the use of the stable isotopic composition of coccolithophores and their specific organic compounds (alkenones) for the reconstruction of  $p\text{CO}_2$ . The  $\text{CO}_2$  dependence of the carbon isotopic fractionation between alkenones and DIC has been used as a proxy for levels of atmospheric  $\text{CO}_2$  over the Cenozoic (Pagani et al., 2005). Of the two major producers of alkenones in the modern ocean, *E. huxleyi* and *G. oceanica* (Conte et al., 1995), almost all calibrations of alkenone isotopes have focused on *E. huxleyi* which has only emerged since 268 ka. Importantly, all known alkenone-producing haptophytes group within the order Isochrysidales, and their ability to produce alkenones most likely evolved only once. Recent molecular clock studies (Medlin et al., 2008) place the divergence between the Isochrysidales (including modern *G. oceanica*) and other coccolithophores (such as *C. braarudii*) at  $\sim 195$  Ma, before the first sedimentary evidence of alkenones in Cretaceous black shales at  $\sim 120$  Ma (Farrimond et al., 1986; Brassell et al., 2004). As a primary candidate for alkenone production in the pre-Pleistocene world, however, the *Reticulofenestra* genus has no dedicated calibration.

This study reveals two cautionary aspects to consider. First, it supports that  $\epsilon_p$  cannot be related to diffusive  $\text{CO}_2$  supply for any modern coccolithophore species, and that a model accounting for different modes of carbon acquisition is more applicable (cf. Sharkey and Berry, 1985; Burkhardt et al., 1999). Second, it appears that the more recent relatives of *Reticulofenestra* are limited by a supply of  $\text{CO}_2$  up to levels of  $1200 \mu\text{atm}$ . Thus, if we do consider a relationship to  $\mu/\text{CO}_2$ , any calibration would only adhere to the theoretical relationship above this level. Furthermore, the Early Cenozoic larger *Reticulofenestra* may have been limited by even

higher levels of  $\text{CO}_2(\text{aq})$  than  $1200 \mu\text{atm}$  suggesting that the theoretical predictions for the relationship between  $\epsilon_p$  and  $\mu/\text{CO}_2$  only comes into play above those higher thresholds. Nonetheless, our approach suggests that offsets between organic matter and calcite produced by the same species of coccolithophore are related to the ambient carbonate chemistry, but must be interpreted in the context of physiology and calibrations of closely related extant species.

## 5.3 Implications for “vital effects”

Our hypothesis poses a mechanism to explain the species-specific isotopic composition of coccoliths. The  $\delta^{18}\text{O}$  isotopic composition of coccoliths displays a  $\sim 5\%$  array of disequilibrium or “vital effects” across eight different species but those species tend to fall into two distinct groups (Dudley et al., 1986; Ziveri et al., 2003; Minoletti et al., 2009). The disequilibrium offsets of  $\delta^{18}\text{O}_{\text{PIC}}$  remain relatively constant in each group with changing conditions for cell growth, and the variance of  $\delta^{18}\text{O}_{\text{PIC}}$  with temperature parallels that expected for the equilibrium precipitation of inorganic calcite. The size of the disequilibrium offset correlates with the size of the cell: larger, slower growing coccolithophores such as *C. pelagicus* and *Helicosphaera carteri* are offset to lighter isotopic values, and smaller faster growing species such as *G. oceanica* (and *E. huxleyi*) are offset to heavier values, a trend matched in the  $\delta^{13}\text{C}_{\text{PIC}}$ . Assuming, as we propose, that these offsets between species are due to different internal management of the carbonate system, there is the potential to obtain information regarding the carbonate system of surface waters from the isotopic compositions of mono-specific coccolithophore samples through the geological record, given knowledge of species ecology.

## 6 Conclusions

The physiologies of small and large coccolithophore species differ in terms of their control on internal pH and carbon acquisition as evidenced by the sensitivity of the isotopes in organic matter and calcite to changing DIC at constant pH in *G. oceanica* and *C. braarudii*. We propose a hypothetical model which accounts for the sensitivity, or not, of the calcification of different species to changing external pH and DIC. Briefly, the smaller species (*Gephyrocapsa*) largely rely on diffusive supply of  $\text{CO}_2$  for photosynthesis and uptake of  $\text{HCO}_3^-$  for calcification, which releases a proton to the extracellular microenvironment and catalyses the production of  $\text{CO}_2$  from  $\text{HCO}_3^-$ . The larger species (*Coccolithus*), generally insensitive to pH (Langer et al., 2006), actively takes up  $\text{HCO}_3^-$  to a common internal pool that provides a pH buffer between the processes of photosynthesis and calcification. Our proposed model has implications for the use of  $\epsilon_p$  for reconstructing past atmospheric  $\text{CO}_2$ , and starts to provide a framework for understanding the geological record

of coccolithophore-specific isotopes and cell size quantitatively.

**Supplementary material related to this article is available online at:**

**<http://www.clim-past.net/6/771/2010/cp-6-771-2010-supplement.zip>**

*Acknowledgements.* R. R. is grateful to the ERC Starting Grant (SP2-GA-2008-200915) and to the Leverhulme Trust for support. J. H. is grateful to the Swedish Research Council (grant VR621-2003-3614) and the Royal Swedish Academy of Sciences for support through a grant financed by the Knut and Alice Wallenberg foundation. We both thank the Alfred Wegener Institute for Polar and Marine Research for hosting us for the period of laboratory work. We are indebted to Ingrid Zondervan whose expertise and help was pivotal to the experiments which were carried out with partial support by the German Research Foundation (DFG) under Grant No. BI 432/4-2 (“PaleoSalt”), under Grant No. BI 432/6-2 (“BioCalc”), and by the European Science Foundation (ESF) under the EUROCORES Programmes EuroCLIMATE and EuroMinSci, respectively, through Contract No. ERAS-CT-2003-980409 of the European Commission, DG Research, FP6. We also owe thanks to Norman Charnley for help with analyses and Beate Müller for help with experiments.

Edited by: T. Kiefer



The publication of this article was sponsored by PAGES.

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