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# Inter- and intra-specific responses of coccolithophores to CO<sub>2</sub>-induced ocean acidification

D. S. Wang<sup>1,2,\*</sup>, D. Xu<sup>1,\*</sup>, X. Fan<sup>1</sup>, N. H. Ye<sup>1</sup>, W. Q. Wang<sup>2</sup>, X. W. Zhang<sup>1</sup>, Y. Miao<sup>1</sup>,  
Z. Guan<sup>3</sup>, and S. Wang<sup>3</sup>

<sup>1</sup>Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China

<sup>2</sup>Marine Science and Engineering College, Qingdao Agricultural University, Qingdao, China

<sup>3</sup>College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, China

\*These authors contributed equally to this manuscript.

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Correspondence to: N. H. Ye (yenh@ysfri.ac.cn) and W. Q. Wang (wenqiwang@qau.edu.cn)

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BGD

12, 675–706, 2015

Inter- and  
intra-specific  
responses of  
coccolithophores

D. S. Wang et al.

Title Page

Abstract

Introduction

Conclusions

References

Tables

Figures

◀

▶

◀

▶

Back

Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion



## Abstract

Oceanic uptake of anthropogenic carbon dioxide ( $\text{CO}_2$ ) is altering the seawater chemistry of the world's oceans with consequences for marine bioregions, especially calcareous organisms such as corals, foraminifera and coccolithophores. The coccolithophores, one of the most abundant and widespread groups of calcifying plankton, are responsible for a large proportion of modern oceanic carbonate production. However, culture experiments examining the response of coccolithophores to elevated  $\text{CO}_2$  partial pressure ( $p\text{CO}_2$ ) have mostly been based on investigations of a single strain and have yielded contradictory results from different experiments between and even within species. Here, four strains of the coccolithophores *Emiliana huxleyi* (*E. huxleyi*) and *Gephyrocapsa oceanica* (*G. oceanica*), which contained separately naked and calcifying strains, were investigated simultaneously for the first time in a bubbling batch culture at four  $\text{CO}_2$  grades ranging from approximately 380 to 2000  $\mu\text{atm}$ . We synchronously determined multiple physiological parameters of four coccolithophore strains involving growth, photosynthesis, nitrogen uptake, elemental compositions and calcification efficiency in the process of cultivation. The results did not show a uniform response from different strains to elevated  $p\text{CO}_2$  up to 2000  $\mu\text{atm}$ , and the naked strain *E. huxleyi* (N-E) was seriously suppressed, in sharp contrast to the positive response of the different levels of the other three algae. In addition, we fitted nitrogen uptake rate response curves relative to changing  $p\text{CO}_2$  for the four strains and applied kinetic constants from the response curves to further analyze the hypostatic difference among different strains, which reflected the same variational trend of the four stains above vs. increasing  $\text{CO}_2$ . We determined that the responses of coccolithophores to ocean acidification are inter- and intra-specific, and this variation may cause changes to biodiversity and other ecosystem processes in the future ocean.

## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

◀

▶

◀

▶

[Back](#)

[Close](#)

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)



# 1 Introduction

Phytoplankton plays a crucial role in the ocean's food webs and biogeochemical cycles (Falkowski, 2012). Coccolithophores, unicellular haptophyte algae whose cell surface is covered with small intracellularly produced calcite platelets, are currently one of the most abundant and widespread groups of calcifying plankton in the ocean (Langer et al., 2009; Langer, 2013; Lohbeck et al., 2014; Young et al., 2014). Additionally, they are known to participate in the carbonate pump and play a crucial role in the carbon biogeochemical cycle; coccolithophores are capable of forming great blooms, which are thought to have a feedback effect on global climate change (Hutchins, 2011; Riebesell and Tortell, 2011; Jin et al., 2013; Beaufort et al., 2014). However, ocean acidification generated by the increase in atmospheric  $p\text{CO}_2$  is inducing a decrease in the surface ocean pH (estimated to decrease by 0.5 and 0.7 units by 2100 and 2300, respectively) (Caldeira and Wickett, 2003; IPCC, 2007; Ross et al., 2011), while simultaneously altering the variety of dissolved carbonate (Beaufort et al., 2011). These changes in the surface ocean have massive impacts on coccolithophores and the other calcifying species, which is presumed to affect communities and the overall ecosystem and to damage the marine environment (Turley et al., 2010; Hoppe et al., 2011; Sinutok et al., 2011; Lohbeck et al., 2012; Meier et al., 2014).

Many studies with laboratory cultures and large-scale semi-enclosed field cultures (mesocosms) have intensively studied the response of individual coccolithophores to ocean acidification over the last decade, which has resulted in conflicting reports (Young et al., 2014). As the first widely adopted concept, Riebesell et al. (2000) indicated that there was a clear reduction in calcification of coccolithophores with increasing  $p\text{CO}_2$ , simultaneously accompanied by increasing particulate organic carbon (POC). Subsequent experiments conducted on a single culture strain of *E. huxleyi* also showed a decreasing calcification rate of this species due to ocean acidification but with non-uniform (increasing, decreasing or unaffected) POC production (Zondervan et al., 2002; Sciandra et al., 2003; Delille et al., 2005; Feng et al., 2008; Müller et al., 2010;

## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

◀

▶

◀

▶

[Back](#)

[Close](#)

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)



Hoppe et al., 2011). However, many other studies with strains of *E. huxleyi* continued to reveal conflicting results, which showed an increasing (Iglesias-Rodriguez et al., 2008; Shi et al., 2009; Jones et al., 2013) or insensitive calcification rate (Langer et al., 2009; De Bodt et al., 2010; Fiorini et al., 2011a).

In addition, one culture strain of each of the calcifying coccolithophores (*G. oceanica*, *Calcidiscus leptoporus* and *Coccolithus braarudii*) studied by previous researchers also expressed a different response pattern. For example, the strain *G. oceanica* PC7/1 displayed a comparatively larger decrease in the rate (44.7 %) of calcification (Riebe-sell et al., 2000), while the strain *Coccolithus braarudii* 4762 showed slightly increasing calcification rates (Rickaby et al., 2010), and the strain *Calcidiscus leptoporus* AC370 displayed an insensitive response to elevated  $p\text{CO}_2$  (Fiorini et al., 2011a). Considering this contradictory result, different strains or species of coccolithophores have been simultaneously tested (Langer et al., 2006, 2009; Fiorini et al., 2011a). Langer et al. (2009) noted that the sensitivity of different *E. huxleyi* strains used in every previous study of ocean acidification differed constitutionally, likely because of the existence of a genetic basis to intra-specific responses. However, different calcification or naked strains from different species (as in our research) have not been investigated in previous studies.

With the inconformity in the observed ocean acidification effects on calcification, the accurate prediction of the fate of coccolithophores in an increasingly acidified ocean remains problematic. Additionally, the response of the cosmopolitan dominant species *E. huxleyi* to ocean acidification, based on short-term experiments, has been intensively studied, and the literature is rapidly expanding. Langer et al. (2006) indicated that a bias may be created for species and clones best adapted to modern  $p\text{CO}_2$  and that questions on the universal validity of the observed responses may also be raised. However, due to the reality that non-calcifying strains of coccolithophores play an important ecological role in the marine ecosystem, naked strain should not be ignored and have only recently gained greater attention (Kottmeier et al., 2014). Therefore, simulta-

## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

[◀](#)

[▶](#)

[◀](#)

[▶](#)

[Back](#)

[Close](#)

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)



neously testing a wider range of species, including different strains, and examining the long-term effects of increasing  $p\text{CO}_2$  levels is needed.

In this study, we performed a series of laboratory experiments that represent projections of the future ocean acidification and simultaneously used two cosmopolitan species of coccolithophore, *E. huxleyi* and *G. oceanica*, which are naked and calcifying strains, respectively, to examine elevated  $p\text{CO}_2$  from 380 to 2000  $\mu\text{atm}$ . Physiological parameters, such as algal growth, algal photosynthesis parameters, nitrogen assimilation, particulate inorganic carbon (PIC) production, particulate organic carbon (POC) production, particulate organic nitrogen (PON) production and the rates between particulate matter, were examined to assess the potential change of each strain of coccolithophore vs. different  $\text{CO}_2$  concentrations. In addition, we fitted nitrogen uptake rate response curves relative to changing  $p\text{CO}_2$  for four strains and applied kinetic constants from the response curves to further analyze the hypostatic difference among the different strains. These results were intended to provide insight into the different effects of ocean acidification on different coccolithophores under similar conditions and were beneficial for assessing future changes in biodiversity and other ecological processes.

## 2 Materials and methods

### 2.1 Algal materials and growth conditions

Naked and calcifying cells of *E. huxleyi* (strain *E. huxleyi*, abbreviated N-E; strain *E. huxleyi*, CS369, abbreviated C-E) and *G. oceanica* (strain *G. oceanica*, NIES-1318, abbreviated N-G; strain *G. oceanica*, abbreviated C-G) were obtained from the Yellow Sea Fisheries Research Institute Microalgae Culture Center. Taxonomic relationships of the four coccolithophore strains are depicted in Fig. S1. The cells were cultured in filtered ( $0.2\ \mu\text{m}$ ) natural seawater enriched with nitrate and phosphate to concentrations of  $882$  and  $36.2\ \mu\text{mol L}^{-1}$ , respectively, and with metals and vitamins according to f/2 (Guillard and Ryther, 1962) at  $20\ ^\circ\text{C}$  and  $32\ \text{\%}$  salinity. The cells were exposed to

BGD

12, 675–706, 2015

Discussion Paper | Discussion Paper

## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

◀

▶

◀

▶

[Back](#)

[Close](#)

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)



a light:dark cycle (16 : 08 h) and saturating light (300  $\mu\text{mol}$  photons  $\text{m}^{-2} \text{s}^{-1}$ ) provided by white fluorescent lighting in an incubator. The culturing was conducted in sterilized 2 L Erlenmeyer flasks containing 1 L of medium, and algae in the exponential growth phase were used for inoculation in the experiments.

## 5 2.2 Experimental set up

We conducted replicate experiments by bubbling cultures of four species of coccolithophores using air with different  $\text{CO}_2$  partial pressures and adopting a flask and tubing system manipulated in plant growth chambers (GXZ, Ruihua, Wuhan, China) similar to Xu et al. (2014). The experiments were prepared with monospecific cultures of 10 the four species of coccolithophore and reached the exponential growth phase under the above-mentioned conditions. Three replicate cultures of 1000 mL of each coccolithophore were incubated with aeration of different  $\text{CO}_2$  partial pressures in a  $\text{CO}_2$  chamber. All the experiments were generally similar in design. Differences are shown 15 in Table S1, and treatments were bubbled continuously with air containing different partial pressures of  $\text{CO}_2$  to provide 380, 750, 1000 and 2000  $\mu\text{atm}$   $\text{CO}_2$  in the medium. The culture medium typically became stable after 48 h of bubbling and before the start of the experimental inoculation. In each experiment, triplicate flasks for each air- $\text{CO}_2$  mixture 20 were maintained without added cells as a blank. The cell density and pH were monitored, respectively. Triplicate sampling for culture medium, chlorophyll (Chl) content, PIC, POC, DIC and alkalinity measurements were conducted at the sampling point.

## 2.3 Carbonate chemistry analyses

The corresponding pH of the culture medium was monitored with a pH meter (Orion ROSS, Thermo Electron Corp., Beverly, MA, USA). The samples for total alkalinity (TA) 25 measurements were immediately filtered using a Whatman GF/F and stored in sealed 100 mL borosilicate bottles at  $-20^\circ\text{C}$  until measured. The TA value was obtained using the 848 Titrino plus automatic titrator (Metrohm, Riverview, FL, USA). Parameters for

[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

[◀](#)

[▶](#)

[◀](#)

[▶](#)

[Back](#)

[Close](#)

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)



the seawater carbonate system within experiments were calculated using the CO2SYS Package based on the pH, temperature, salinity, and TA according to the method of Pelletier et al. (2007).

BGD

12, 675–706, 2015

## 2.4 Growth determination

5 An accurate cell count was obtained to estimate microalgal growth by manual counting with a hemocytometer under an optical microscope (Nikon, Tokyo, Japan). The specific growth rate ( $\mu$ ) was determined as follows:  $\mu = (\ln C_1 - \ln C)/(t_1 - t)$ , where  $C$  and  $C_1$  are the cell concentrations at initial time  $t$  and subsequent time  $t_1$ , respectively.

## 2.5 Chlorophyll fluorescence parameter and pigment analysis

10 The photosynthetic characteristics of tested coccolithophores were simultaneously determined using the pulse–amplitude modulated method with a Dual-PAM-100 (Walz, Effeltrich, Germany) connected to a PC running WinControl software. Before measurement, the samples were kept in the dark for 15 min, and the original fluorescence ( $F$ ) was determined under low light. A saturation light pulse was applied to obtain maximum fluorescence ( $F_m$ ) in the dark-adapted samples. The  $F_m$  yield in the illuminated samples was denoted as  $F_m'$ , and  $F_t'$  was the real-time fluorescence yield. The maximal PSII quantum yield ( $F_v/F_m$ ) was calculated according to the following equation:  $F_v/F_m = (F_m - F)/F_m$ . The effective PSII quantum yield was calculated as follows:  $Y(II) = (F_m' - F_t')/F_m'$ .

15

20 To determine the pigment content, the cells were collected onto Whatman GF/F filters (25 mm) at every sampling point and then extracted in 10 mL of 90 % acetone for 24 h at 4 °C in darkness. After centrifugation at  $7000 \times g$  for 10 min, the supernatant was used for content analysis at room temperature. The content of the chlorophyll was spectrophotometrically determined using the method of Jeffrey and Humphrey (1975).

Inter- and  
intra-specific  
responses of  
coccolithophores

D. S. Wang et al.

[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

◀

▶

◀

▶

[Back](#)

[Close](#)

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)



## 2.6 Determination of nitrogen uptake

Water samples (5 mL) from the experiment were collected and filtered quickly using acetate cellulose filters and then frozen at  $-80^{\circ}\text{C}$  in polyethylene flasks for storage until analysis. Before analysis, stored culture media samples were thawed to room temperature, and nitrate concentrations were analyzed photometrically using an Auto-Analyzer (BRAN and LUEBBE AA3, Germany). Nitrogen uptake rates were calculated as follows:  $\text{NUR} = (C - C_t)V/N/t$ , where NUR is the nitrogen uptake rate (pmol of nitrate  $\text{cell}^{-1} \text{ day}^{-1}$ );  $C$  and  $C_t$  are the nitrate concentrations ( $\text{pmol L}^{-1}$ ) at the beginning and on the 7th day of the experiment, respectively;  $V$  is the volume of the water (in L);  $N$  is the cell number (ind); and  $t$  is the time interval (in days). Moreover, we used Michaelis–Menten (Michaelis and Menten, 1913; Hutchins et al., 2013) rectangular hyperbolic saturation equation curves to fit  $\text{CO}_2$  response curves for the nitrogen uptake rates in each of the triplicate cultures and for each strain in each  $p\text{CO}_2$  treatment. The work was completed using OriginPro software, including the calculation of kinetic constants and curve correlation coefficients.

## 2.7 Element analysis of tissue carbon and nitrogen content

The samples for element analysis were taken from each replicate, filtered onto pre-combusted ( $500^{\circ}\text{C}$  for 5 h) Whatman GF/F filters (25 mm) and stored at  $-20^{\circ}\text{C}$  until analysis. Prior to analysis, the POC filters were fumed over HCl for 24 h to remove all inorganic carbon and then dried again. The filters were then packed in solvent-rinsed tin sample boats for analysis. The POC, PON and TPC (total particulate carbon) concentrations were determined sequentially with a Vario EL III automatic elemental analyzer (Elementar Analysensysteme Co., Germany). The PIC concentration was calculated by the differences between TPC and POC, and the POC, PIC or PON production was calculated as follows:

$$P = \text{specific growth rate } \mu(\text{d}^{-1}) \times \text{cellular POC, PIC or PON content } (\text{pg cell}^{-1}).$$

[Title Page](#)[Abstract](#)[Introduction](#)[Conclusions](#)[References](#)[Tables](#)[Figures](#)[◀](#)[▶](#)[◀](#)[▶](#)[Back](#)[Close](#)[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)

## 2.8 Data analysis

All statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) 17.0 data processing system software, and the mean  $\pm$  SD was calculated for each experiment. A one-way ANOVA was used to analyze the significance of variance among treatments. The significance level was set at 0.05 for all tests unless otherwise stated.

## 3 Results

### 3.1 Variation of the carbonate system in the experiment

This experiment adopted a gas bubbling method to mimic seawater acidification and monitored the shift in parameters of the seawater-carbonate system, including pH, DIC,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{2-}$ , and  $\text{CO}_2$ , under different treatments. Figure S2 and Table S1 in the Supplement present the changes of seawater pH when culturing different isolates vs. different  $\text{CO}_2$  concentrations. The initial pH values corresponding to elevated  $p\text{CO}_2$  values of 380, 750, 1000 and 2000  $\mu\text{atm}$  were  $8.06 \pm 0.02$ ,  $7.79 \pm 0.01$ ,  $7.65 \pm 0.02$  and  $7.48 \pm 0.02$ , respectively. The experimental conditions set in this study were not exactly the same as those expected in ocean acidification because seawater contains buffers to induce changes in alkalinity. Some measurements required a large biomass; therefore, cell density was also very high, leading to a variation in the carbonate system. Moreover, although these shifts existed, a clear difference in pH among the four gradient cultures was maintained throughout the experiment. Therefore, the results are strongly supported and have significant implications.

### 3.2 Effect of seawater acidification on the growth of coccolithophores

The effect of different  $p\text{CO}_2$  on the growth of four coccolithophores in plant growth chambers is presented in Fig. 1. The cell concentration in all treatments increased

[Title Page](#)[Abstract](#)[Introduction](#)[Conclusions](#)[References](#)[Tables](#)[Figures](#)[◀](#)[▶](#)[◀](#)[▶](#)[Back](#)[Close](#)[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)

gradually over time to a maximum, with a subsequent decline at the end of the experiment, and there was significant effect of  $p\text{CO}_2$  on cell concentration for every species. The C-E, N-E and N-G showed a positive response to gradually increased  $\text{CO}_2$  concentration, while the cell concentration of the N-E was suppressed as  $p\text{CO}_2$  reached 2000  $\mu\text{atm}$ . Algal cells grew exponentially in all treatments during the first 7 days, and the specific growth rate is presented in Fig. 1e–h. Increasing the  $p\text{CO}_2$  by 2000  $\mu\text{atm}$  increases the specific growth rate by approximately 53 % (the C-E), 36 % (the N-G) and 30 % (the C-G), respectively. However, when the bubbling  $\text{CO}_2$  concentration reached 1000  $\mu\text{atm}$ , the specific growth rate increased by approximately 26 % for the N-E; with a continuous rise to 2000  $\mu\text{atm}$ , the ocean acidification decreased the specific growth rate of the N-E by 14 % compared to the control. Moreover, when a comparison was conducted between or within species, the variability of *E. huxleyi* (naked strain to calcifying strain: reducing by 14 % to increasing by 53 %) exceeded that of the species *G. oceanica* (naked strain to calcifying strain: increasing by 36 and 30 %, respectively) under different  $\text{CO}_2$  concentrations.

### 3.3 Effect of ocean acidification on the photosynthetic activity of coccolithophores

Figure 2 shows the effects of ocean acidification by increasing  $p\text{CO}_2$  on the changes in the photosystem activity parameters, including  $\text{Fv}/\text{Fm}$  and  $\text{Y}(\text{II})$ , during the culture of coccolithophores. Both  $\text{Fv}/\text{Fm}$  and  $\text{Y}(\text{II})$  in all treatments showed a negative influence from increasing  $p\text{CO}_2$  by 2000  $\mu\text{atm}$ , but the magnitude was different among the four species. With incubation time, every photosystem activity parameter of the four coccolithophores simultaneously reached a maximum value on the 7th day, before a subsequent decline till the end of experiment, except for the parameter  $\text{Fv}/\text{Fm}$  of C-E (on the 10th day) and the N-E (continuous decline) for the 2000  $\mu\text{atm}$  sample point. As  $p\text{CO}_2$  increased by 2000  $\mu\text{atm}$  in plant growth chambers, significant suppression of the  $\text{Fv}/\text{Fm}$  from ocean acidification occurred on the 7th, 4th, 13th, and 10th day for the N-E, C-E, N-G, and C-G, respectively, and of the  $\text{Y}(\text{II})$  on the 4th, 13th, 13th, and

[Title Page](#)[Abstract](#)[Introduction](#)[Conclusions](#)[References](#)[Tables](#)[Figures](#)[◀](#)[▶](#)[◀](#)[▶](#)[Back](#)[Close](#)[Full Screen / Esc](#)

10th day, respectively. At the end of experiment, the Fv/Fm was repressed by elevated  $p\text{CO}_2$  (2000  $\mu\text{atm}$ ) and decreased by 60, 45, 55, and 46 % and Y(II) also declined by 75, 69, 76, and 39 %, respectively, compared to the control. In addition, by comparison to the maximal Y(II) of N-E and N-G (under 380  $\mu\text{atm}$ ;  $0.32 \pm 1.9\%$  and  $0.34 \pm 2.6\%$ , respectively), the maximal Y(II) of the C-E and C-G (under 380  $\mu\text{atm}$ ;  $0.39 \pm 3.1\%$  and  $0.48 \pm 2.3\%$ , respectively) were significantly higher, while differences in Fv/Fm were not obvious among them.

Figure 3 presents the effects of elevated  $\text{CO}_2$  conditions from 380 to 2000  $\mu\text{atm}$  on the intracellular chlorophyll content of different strains. After an initial value showing slight variation, the chlorophyll *a* content of cells increased to maximum values on the 20th (N-E, C-E, N-G) and 7th day (C-G), with a subsequent declining tendency until the final experiment (Fig. 3a–d). However, chlorophyll *c* displayed a steady overall decline over time (Fig. 3e–f). However, as the  $\text{CO}_2$  concentration increased from 380 to 1000  $\mu\text{atm}$ , the chlorophyll content of every coccolithophore decreased in an orderly pattern (except for individual points in time that were presented differently) compared to 380  $\mu\text{atm}$  at each sampling time. Nevertheless, the results determined under 2000  $\mu\text{atm}$  conditions did not exhibit the same responses. During the 2000  $\mu\text{atm}$   $\text{CO}_2$  enrichment, the chlorophyll *a* and *c* content of the N-E with respect to cultivation time presented lower values than the 1000  $\mu\text{atm}$   $\text{CO}_2$  concentration at each sampling point. However, for the C-E, N-G and C-G strains, as the  $\text{CO}_2$  concentration in the culture increased above 1000  $\mu\text{atm}$ , the chlorophyll content tended to increase at each sampling point.

### 3.4 Effect of ocean acidification on the nitrogen uptake rate of coccolithophores

The effect of ocean acidification on the nitrogen uptake rate of the four coccolithophores is displayed in Fig. 5. With increasing  $\text{CO}_2$  concentration from 380 to 2000  $\mu\text{atm}$ , the nitrogen uptake rate of strains all increased, by 48.2 % (N-E), 33.9 % (C-E), 41.6 % (N-G) and 34.3 % (C-G). The maximum variation value (48.2 %, N-E) of the nitrogen uptake rate was nearly 1.5-fold larger than the minimum variation value (33.9 %, C-E). More-

over, the response of nitrogen uptake rates to increasing  $p\text{CO}_2$  in all the strains were closely described by a Michaelis–Menten curve fitting approach, and both half saturation constants ( $\text{Km}$ ,  $\mu\text{atm CO}_2$ ) and maximum  $\text{CO}_2$ -saturated rates ( $\text{Vmax}$ ,  $\text{pmol N cell}^{-1} \text{ day}^{-1}$ ) were obtained from the response curves. The maximum  $\text{Km}$  and  $\text{Vmax}$  value were  $307.2 \mu\text{atm}$  and  $24.1 \text{ pmol N cell}^{-1} \text{ day}^{-1}$  from strain N-E, respectively, which were approximately 1.7-fold and 1.3-fold larger than the minimum values presented by strain C-E, respectively.

### 3.5 Effect of ocean acidification on element composition and the calcification rate of coccolithophores

Figure 5 shows the production of POC and PON of the four species of coccolithophore on the 7th day in elevated  $p\text{CO}_2$  conditions. Acidification by  $\text{CO}_2$  enrichment up to  $2000 \mu\text{atm}$  distinctly stimulated the production of POC and PON of coccolithophores except for N-E, whose increasing POC production under  $1000 \mu\text{atm}$   $p\text{CO}_2$  conditions exhibited a significant decline at the  $2000 \mu\text{atm}$  sample point, and the variations were enormous. For the C-E, N-G and C-G species, the POC production increased by approximately 101, 35 and 49 %, respectively, compared with the control, and the variation of the former reached 2.9 and 2.1-fold of the N-G and C-G, respectively. Moreover, over this range, PON production had a comparatively larger increase of 233 % (N-E), 289 % (C-E), 148 % (N-G) and 129 % (C-G) than controls. However, the changes of particle carbon and nitrogen production led to a decrease in the POC : PON for the four species by 76.6 % (N-E), 48.3 % (C-E), 45.7 % (N-G) and 34.9 % (C-G), with the  $p\text{CO}_2$  variation between 380 and  $2000 \mu\text{atm}$ . In addition, the seawater acidification under  $2000 \mu\text{atm}$  also had a negative effect on PIC production, which decreased by 35.4 and 68.9 % for two calcification species, C-E and C-G, respectively, in comparison with the production value at  $380 \mu\text{atm}$  (Fig. 6). The C-G species presented a higher reduction ratio (approximately 1.9-fold) of PIC than the C-E when  $\text{CO}_2$  concentrations increased by  $2000 \mu\text{atm}$ . Simultaneously, the ratio of PIC : POC for the two species also declined by 67.9 and 79.2 %.

[Title Page](#)[Abstract](#)[Introduction](#)[Conclusions](#)[References](#)[Tables](#)[Figures](#)[◀](#)[▶](#)[◀](#)[▶](#)[Back](#)[Close](#)[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)

## 4 Discussion

In this study, two species of the coccolithophores were used in the first experiment involving naked and calcifying strains, and their responses to elevated  $\text{CO}_2$  concentration were investigated. Simultaneously, kinetic constants from the fitted  $\text{CO}_2$  response curves regarding the nitrogen uptake rate were also applied to analyze and display the inherited variation of different coccolithophores. The N-E was seriously suppressed in comparison with the different levels of the positive response the other three strains when the  $p\text{CO}_2$  was elevated to 2000  $\mu\text{atm}$ . We concluded that the sensitivity of coccolithophores to ocean acidification was strain- and species-specific, which may potentially affect future species distributions and abundances. We also speculated that ocean acidification may result in changes to biodiversity, trophic interactions, and other ecosystem processes in the future ocean.

### 4.1 Effect of the ocean acidification on the growth and photosynthetic performance of different strains of coccolithophores

This study showed that a high  $\text{CO}_2$  concentration (increasing to 2000  $\mu\text{atm}$ ) led to an increase in growth, except for N-E, whose growth declined at the 2000  $\mu\text{atm}$  sample point and whose specific growth rates were significantly reduced by approximately 7.61 % on the 7th day in comparison to the control (Fig. 1). However, before  $p\text{CO}_2$  was elevated to 1000  $\mu\text{atm}$ , the growth of N-E maintained an increasing state (Fig. 1a and e). Recently, Fiorini et al. (2011a) displayed similar results and showed an increasing growth trend of coccolithophores in  $\text{CO}_2$ -rich water up to approximately 750  $\mu\text{atm}$ .

Previous studies suggested that coccolithophores were carbon limited because of a comparatively less-efficient  $\text{CO}_2$  concentrating mechanism (CCM) caused by low activity for extracellular carbonic anhydrase in the present ocean (Herfort et al., 2002; Trimborn et al., 2007; Rokitta and Rost, 2012; Jin et al., 2013). Therefore, an increase in available  $\text{CO}_2$  by ocean acidification in seawater may accelerate the accumulation of  $\text{CO}_2$  in the vicinity of Rubisco to offset the short supply and further promote coccol-

BGD

12, 675–706, 2015

Discussion Paper | Discussion Paper

Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

◀

▶

◀

▶

[Back](#)

[Close](#)

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)



ithophore carboxylation and growth (along with an increase in POC) (Barcelose Ramos et al., 2010; Reinfelder, 2011; Jin et al., 2013; Kottmeier et al., 2014). In this study, we observed the increased growth of four strains including N-E (1000  $\mu$ atm; 23 %), C-E (2000  $\mu$ atm; 53 %), N-G (2000  $\mu$ atm; 36 %) and C-G (2000  $\mu$ atm; 30 %) (Fig. 1). However, every strain revealed different response capacities and strategies to the increasing  $p\text{CO}_2$ . In addition, N-E was also significantly and negatively affected, which tended to lead to bleaching as the  $p\text{CO}_2$  value reached 2000  $\mu$ atm (Fig. 1).

Recent studies reported that changes in extracellular pH could affect the activity of sensitive algal cells by upsetting the constant membrane potential balance and physiological parameters (Langer et al., 2006; Taylor et al., 2011; Rokitta et al., 2012). Hence, the more sensitive alga N-E, relative to the other three stains, showed a negative response because of the overstepping of its tolerance ability and declined pH (7.48, 2000  $\mu$ atm, Table S1). Because coccolithophores are an abundant formal species, which have been characterized with a unique physiology and morphology (Westbroek et al., 1993; Winter and Siesser, 1994; Raven and Crawford, 2012), we propose that the observed differences in the response of coccolithophores to increasing  $p\text{CO}_2$  have a genetic basis. Recently, Read et al. (2013) reported the first haptophyte reference genome (from *E. huxleyi* CCMP1516) and assumed that genome variability within this species complex seems to underpin its capacity to thrive in different habitats. The inherent difference in adaptive capacity from coccolithophores would contribute to an inherent discrepancy reaction in response to ocean acidification, similar to the specific response results in this study.

However, algal photosynthesis (which is closely related to growth) that is evaluated by the activities of the photosystems and pigment content exerted a variable negative response with elevated  $p\text{CO}_2$ , especially for N-E, which tended to bleach early in the experiment (Figs. 2 and 3). These results involving the activities of the photosystems revealed that cells remained “photosynthetically unhealthy” due to the effect on cell ion balance from declining pH (elevated  $p\text{CO}_2$ ) (Langer et al., 2006), which induced a decreasing in chlorophyll content (Fig. 3). In particular, N-E was seriously affected in com-

Title Page

Abstract

Introduction

Conclusions

References

Tables

Figures

◀

▶

◀

▶

Back

Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion



parison to the other coccolithophores when ocean acidification reached the 2000  $\mu\text{atm}$  sample point, which showed exceeding maladjustment (Figs. 2a and 3a). Similar to the different responses in growth results with elevated  $p\text{CO}_2$ , algal photosynthesis also reflected an inherent difference in its reaction. For the whole ecosystem, for which a slight change in one part may affect the whole situation, the specific reaction of coccolithophores to ocean acidification would likely affect biodiversity and other ecological process (Orr et al., 2005; Hendriks et al., 2010).

#### 4.2 Effect of ocean acidification on the nitrogen uptake rate of different strains of coccolithophores

Considering the effect of ocean acidification on nutrient uptake, we tested the nitrogen uptake efficiency of each strain under different  $p\text{CO}_2$  levels and fitted nitrogen uptake rate response curves to changing  $p\text{CO}_2$  for further analysis at the 7th day sampling point. The results showed that the nitrogen uptake rate of all strains increased at the 2000  $\mu\text{atm}$   $\text{CO}_2$  concentration (Fig. 4). The naked strains N-E and N-G exhibited a greater increase of 48.2 and 41.6 % (Fig. 4a and c), respectively, while the nitrogen uptake rate increased by 33.9 and 34.3 % in the calcifying strain C-E and C-G, respectively (Fig. 4b and d). Jin et al. (2013) reported that inorganic nitrogen uptake was enhanced in coccolithophores and that the gene for nitrate reductase in a diatom was upregulated under elevated  $\text{CO}_2$  levels. In addition, disparities between strains also existed.

Previous studies suggested that coccolithophore cells exerted a process of energy redistribution under ocean acidification conditions (Raven, 2011; Beaufort et al., 2011), resulting in the release of extra ATP to preferentially support additional N uptake for the synthesis of more proteins (Jin et al., 2013). However, calcifying cells, which need more energy input for  $\text{H}^+$  from calcification, because there is an outward transport to acidic seawater or neutralization (Suffrian et al., 2011; Taylor et al., 2011; Beaufort et al., 2011; Stojkovic et al., 2013), absorbed substantially less nitrogen than naked strains for elevated  $p\text{CO}_2$  at 2000  $\mu\text{atm}$ . In addition, the increasing PON production with ele-

BGD

12, 675–706, 2015

[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

◀

▶

◀

▶

[Back](#)

[Close](#)

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)



vated  $p\text{CO}_2$  determined in this study also indirectly confirmed the energy redistribution preference for nitrogen uptake (Fig. 5b).

The response curves of nitrogen uptake efficiency to changing  $p\text{CO}_2$  confirmed the influencing behavior of  $\text{CO}_2$  for each of these coccolithophores. Kinetic rate constants (Km value), which were derived from curves, represented the enzyme affinity to the substrate, and when the Km value increased, the affinity became smaller (Michaelis and Menten, 1913). Recently, Hutchins et al. (2013) documented strain-specific differences in the relationship between nitrogen fixation and  $\text{CO}_2$  concentration through different kinetic constant analysis. In this study, N-E showed the maximum Km value, 5 namely, the minimum affinity, compared with other three strains (differences also existed among them) (Fig. 4). This result reflected the same trend with the other parametric measurements, such as growth rate, photosynthetic activity, etc., which indicated that N-E was seriously affected by elevated  $\text{CO}_2$  concentrations, and inter- and intra-specific responses were also displayed. In addition, the principal coordinate analysis (PCoA) and hierarchical clustering multi-variate statistical analyses for the replicates of each of the four coccolithophore strains displayed obvious differences in  $\text{CO}_2$  responses (Fig. S3). Replicates of each of the four strains formed well-defined clusters through PCoA (Fig. S3a). Moreover, independent hierarchical clustering of each replicate also showed the same serious groups, and this analysis revealed that the two 10 calcifying strains were more similar to one another than they were to the two naked strains (Fig. S3b). These data analyses further highlighted the inter- and intra-specific variability in coccolithophore response to  $\text{CO}_2$ -induced acidification.

#### 4.3 Effect of ocean acidification on the elemental composition of different strains of coccolithophores

25 Variations in the elemental stoichiometry of phytoplankton are known to have an effect on trophic interactions and ultimately exert an influence on marine nutrient biogeochemistry because the dietary value of prey for marine zooplankton varied with the POC to PON (C:N) ratio (Hutchins et al., 2009; Anderson et al., 2013). In this study,

[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

◀

▶

◀

▶

[Back](#)

[Close](#)

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)



we measured the ratios of C : N to assess whether the elemental composition of the organic material was additionally affected by changing  $p\text{CO}_2$ , and we also explored the extent of the impact. According to the results, changes in the elemental composition of coccolithophores grown at 2000  $\mu\text{atm}$   $p\text{CO}_2$  have been found (N-E, C-E, N-G and C-G), revealing a reduced C : N ratio (by approximately 76, 48, 45 and 35 %, respectively) (Fig. 5c). The ratio of N-E was affected greatly with respect to the other three isolates, and C-G was the least affected. In previous studies, similar variable ratios for coccolithophores based on an enhanced  $\text{CO}_2$  concentration were reported by Fiorini et al. (2011a, b) and Rickaby et al. (2010). These changes occurred with increasing POC (except for N-E at 2000  $\mu\text{atm}$  of  $\text{CO}_2$ ) and PON production and under rising  $p\text{CO}_2$  concentration between 380 and 2000  $\mu\text{atm}$  (Fig. 5).

It was also discovered that the incremental ratios of PON were higher than those of POC compared to the control groups with the ultimate proportion. We have discussed in the above section that the redistribution of energy resulted in more nitrogen absorption and increasing PON production with elevated  $p\text{CO}_2$ . This preference advantage made the PON production exceed the POC production, even though differences existed in different strains, leading to different decreasing C : N ratios (Fig. 4c). Similar to previous research by Riebesell and Tortell (2011), the changes with which high  $p\text{CO}_2$  levels were associated with C : N ratios were highly species specific. Additionally, a variable proportion was also the embodiment of the inherited differences among different strains. In contrast, differences from current findings also exist, which show increased cellular C : N ratios with elevated  $p\text{CO}_2$  (Feng et al., 2008; De Bodt et al., 2010; Kottmeier et al., 2014).

The reasons for the opposite result may be a difference between the experimental settings, such as temperature, illumination intensity, and/or choice of algae strains. In addition, the final results exhibited a very interesting phenomenon, that is, that the reduced C : N ratios of the lost calcification strain were higher than those of the calcification strain in each species. The reason for the phenomenon is still unclear, but it was most likely associated with shell calcification. This part of the observed results

<a href="#">Title Page</a>	
<a href="#">Abstract</a>	<a href="#">Introduction</a>
<a href="#">Conclusions</a>	<a href="#">References</a>
<a href="#">Tables</a>	<a href="#">Figures</a>
<a href="#">◀</a>	<a href="#">▶</a>
<a href="#">◀</a>	<a href="#">▶</a>
<a href="#">Back</a>	<a href="#">Close</a>
<a href="#">Full Screen / Esc</a>	
<a href="#">Printer-friendly Version</a>	
<a href="#">Interactive Discussion</a>	



indicated that the increased  $p\text{CO}_2$  affected algal C:N ratios and produced a diversity of effects on different coccolithophores. In addition, it will further influence grazing-selection pressure on phytoplankton and has many biogeochemical consequences (in particular implications for the export flux of carbon) (Iglesias-Rodriguez et al., 2008).

#### 5 4.4 Effect of the ocean acidification on the calcification efficiency of different strains of coccolithophores

In the past few years, PIC production (calcification efficiency) has been widely investigated in coccolithophores to predict the impact of ocean acidification, and results show that the predominant response was a decreased rate of calcification when cells were 10 grown at  $\text{CO}_2$  levels higher than those found today (380  $\mu\text{atm}$ ) or at least a decrease in PIC:POC (e.g., Zon-dervan et al., 2001; Langer et al., 2006; Muller et al., 2010; Hoppe et al., 2011). In this research, the response regarding PIC was in agreement with the 15 response already described in the above-mentioned tendency. Namely, calcification species *E. huxleyi* and *G. oceanica* experienced a comparatively larger decrease in the rate of calcification of 35.4 and 68.9 %, respectively, between 380 and 2000  $\mu\text{atm}$  at the 7th day sampling point (Fig. 6). In comparison, *G. oceanica* was more vulnerable than *E. huxleyi* under ocean acidification, leading to an unbalanced ecological factor. However, regarding lost calcification species in the experiment, the PIC production was 20 nearly negligible because of the lost calcification ability to cover shells (not show).

Recent studies with various organisms showed calcification to be largely controlled 25 by  $\Omega$ -cal rather than by pH alone (Langer et al., 2006; Trimbom, 2007), and  $\Omega$ -cal was controlled by both [DIC] and pH (Iglesias-Rodriguez et al., 2008). When supersaturated surface seawater (i.e.,  $\Omega > 1$ ) of calcium carbonate minerals (which was considered generally less than current ocean conditions, Feely et al., 2009) declined in saturation ( $\Omega = 1$ ) because of ocean acidification or other natural processes, carbonate biominerals in shells and skeletons may begin to dissolve (Feely et al., 2010). The data in this study showed that  $\Omega$ -cal ranged from 7.76 (C-E) and 8.79 (C-G) at 380  $\mu\text{atm}$  of  $p\text{CO}_2$  to 1.58 and 1.57 at 2000  $\mu\text{atm}$  of  $p\text{CO}_2$  (Fig. S4), which were above the threshold at

[Title Page](#)[Abstract](#)[Introduction](#)[Conclusions](#)[References](#)[Tables](#)[Figures](#)[◀](#)[▶](#)[◀](#)[▶](#)[Back](#)[Close](#)[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)

which dissolution occurs. Nevertheless, the results clearly revealed that PIC production (calcification efficiency) finally had declined in comparison with the control group (Fig. 6).

From the results reported by Langer et al. (2009), the same phenomenon was also observed when  $\Omega$ -cal was above 1, and dissolution still occurred. To explain this phenomenon, we found that the pH values of the cultures incubated at 380 and 2000  $\mu\text{atm}$  of  $\text{CO}_2$  ranged between 8.81 and 7.62 on the 7th day (Table S1 and Fig. S2), and a shift in pH was caused by comparing blanks with cell physiology. These changes affected the photosynthetic health of cells, as discussed above, which implies that our pH conditions were not completely within the tolerance levels of cells. Bach et al. (2011) suggested that calcification is specifically responsive to the associated decrease in pH. Under elevated  $p\text{CO}_2$  circumstances, coccolithophore calcification, which is a stringently controlled biological process (Mackinder et al., 2010), would be affected by a decreasing pH level.

Recently, substantial physiological and molecular evidence has indicated that when  $\text{HCO}_3^-$  is used for calcification (which produces  $\text{H}^+$  and also maintains a cytosolic pH homeostasis near neutrality), the  $\text{H}^+$  must be removed across the plasmalemma  $\text{H}^+$  channel (Suffrian et al., 2011; Taylor et al., 2011) or neutralized in coccolithophores cells (Fabry et al., 2008; Rokitta and Rost, 2012; Stojkovic et al., 2013). To maintain the appropriate transplasmalemma electrical potential difference and the  $\text{H}^+$  efflux, energy input is needed in the process of  $\text{H}^+$  transport (Raven, 2011). When elevated  $p\text{CO}_2$  resulted in a low pH and/or  $[\text{CO}_3^{2-}]$  level in waters, this input was likely greater per unit calcification and led to decreased calcification rates (Raven et al., 2011; Beaufort et al., 2011). Based on research of the adverse impact caused by increasing  $p\text{CO}_2$  on coccolithophore calcification, which was associated closely with biogeochemical cycles, although we give a reasonable explanation for decreasing calcification, this explanation is only a small fraction of the truth. Currently, the reasons for the impacts of ocean acidification on calcified coccolithophores still need further exploration.

[Title Page](#)[Abstract](#)[Introduction](#)[Conclusions](#)[References](#)[Tables](#)[Figures](#)[◀](#)[▶](#)[◀](#)[▶](#)[Back](#)[Close](#)[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)

Title Page

Abstract

Introduction

Conclusions

References

Tables

Figures

◀

▶

◀

▶

Back

Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion



Through our research, different coccolithophores revealed synthetically inter- and intra-specific variability to cope with the threat from ocean acidification and showed a significantly different response derived from inherent heredity. With the CO<sub>2</sub> concentration gradual increasing, the original state maintained steadily by different coccolithophores would be changed on different levels. Thereby, potential changes in species distributions and abundances could propagate through multiple trophic levels of marine food webs. Although research into the long-term ecosystem impacts of ocean acidification is in its infancy, these results may indicate possible changes in biodiversity, trophic interactions, biogeochemical cycles, and other ecosystem processes.

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## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

◀

▶

◀

▶

[Back](#)

[Close](#)

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)



## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

Feng, Y., Warner, M. E., Zhang, Y., Sun, J., Fu, F.-X., Rose, J. M., and Hutchins, D. A.: Interactive effects of increased  $p\text{CO}_2$ , temperature and irradiance on the marine coccolithophore *Emiliania huxleyi* (*Prymnesiophyceae*), *Eur. J. Phycol.*, 43, 87–98, 2008.

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<a href="#">Title Page</a>	
<a href="#">Abstract</a>	<a href="#">Introduction</a>
<a href="#">Conclusions</a>	<a href="#">References</a>
<a href="#">Tables</a>	<a href="#">Figures</a>
<a href="#">◀</a>	<a href="#">▶</a>
<a href="#">◀</a>	<a href="#">▶</a>
<a href="#">Back</a>	<a href="#">Close</a>
<a href="#">Full Screen / Esc</a>	
<a href="#">Printer-friendly Version</a>	
<a href="#">Interactive Discussion</a>	



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## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

Title Page

Abstract

Introduction

Conclusions

References

Tables

Figures

◀

▶

◀

▶

Back

Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion



## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

Title Page

Abstract

Introduction

Conclusions

References

Tables

Figures

◀

▶

◀

▶

Back

Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion



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[Title Page](#)[Abstract](#)[Introduction](#)[Conclusions](#)[References](#)[Tables](#)[Figures](#)[◀](#)[▶](#)[◀](#)[▶](#)[Back](#)[Close](#)[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)

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## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

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[Title Page](#)

[Abstract](#)

[Introduction](#)

[Conclusions](#)

[References](#)

[Tables](#)

[Figures](#)

◀

▶

[Back](#)

[Close](#)

[Full Screen / Esc](#)

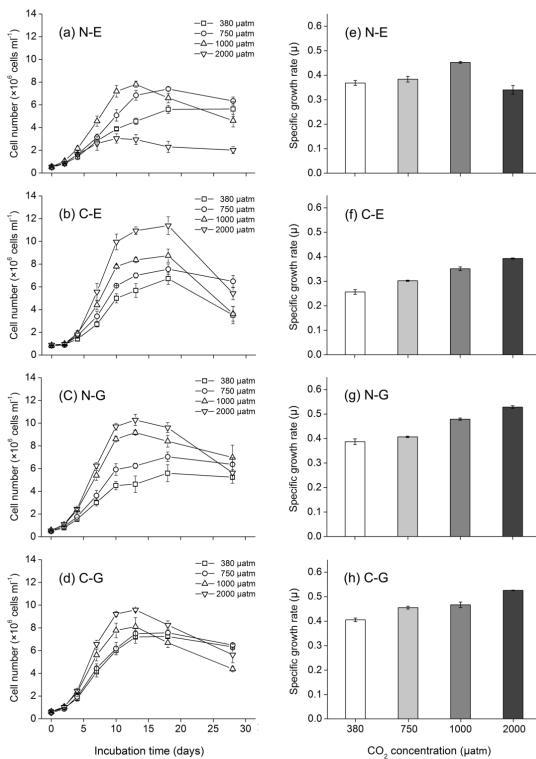
[Printer-friendly Version](#)

[Interactive Discussion](#)



## Inter- and intra-specific responses of coccolithophores

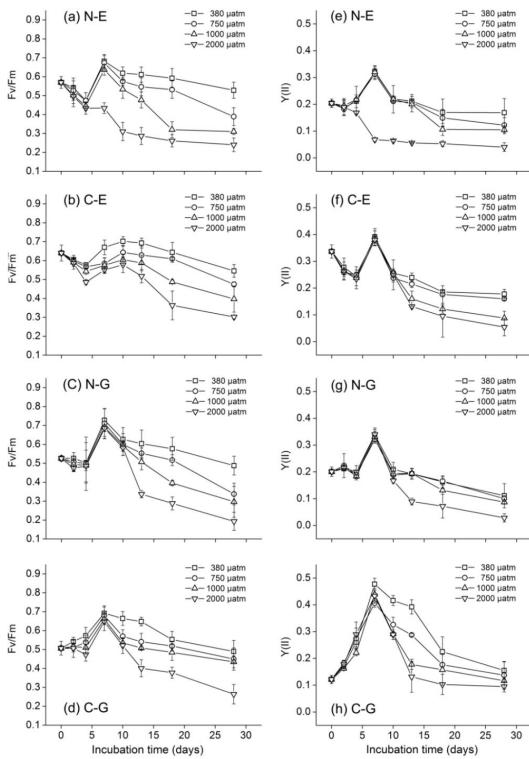
D. S. Wang et al.



**Figure 1.** The growth curves (a–d) and specific growth rates (e–h) of the four species *E. huxleyi* (a, e), *E. huxleyi* CS369 (b, f), *G. oceanica* NIES-1318 (c, g), and *G. oceanica* (d, h) for different  $\text{CO}_2$  concentrations (380, 750, 1000, and 2000  $\mu\text{atm}$ ). The vertical bars represent the SD ( $n = 3$ ). (N-E: naked strain *E. huxleyi*; C-E: calcifying strain *E. huxleyi* CS369; N-G: naked strain *G. oceanica* NIES-1318; C-G: calcifying strain *G. oceanica*.)

## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.



**Figure 2.** The effect of ocean acidification by increasing  $p\text{CO}_2$  on the changes of  $\text{Fv/Fm}$  (a–d) and  $\text{Y(II)}$  (e–f) in the photosystem activity parameter during growth of the four species *E. huxleyi* (a, e), *E. huxleyi* CS369 (b, f), *G. oceanica* NIES-1318 (c, g), and *G. oceanica* (d, h) for different  $\text{CO}_2$  concentrations (380, 750, 1000, and 2000  $\mu\text{atm}$ ). The vertical bars represent the SD ( $n = 3$ ). (N-E: naked strain *E. huxleyi*; C-E: calcifying strain *E. huxleyi* CS369; N-G: naked strain *G. oceanica* NIES-1318; C-G: calcifying strain *G. oceanica*.)

Title Page

Abstract

Introduction

Conclusions

References

Tables

Figures

◀

▶

◀

▶

Back

Close

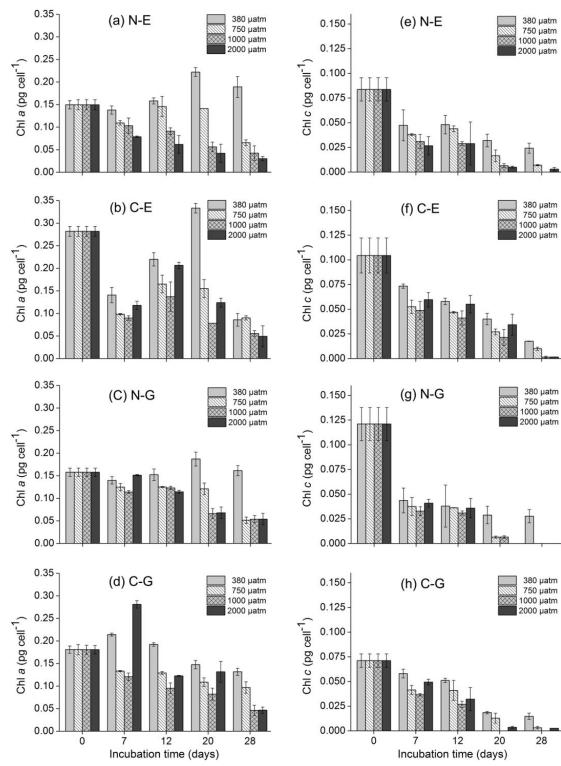
Full Screen / Esc

Printer-friendly Version

Interactive Discussion

## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

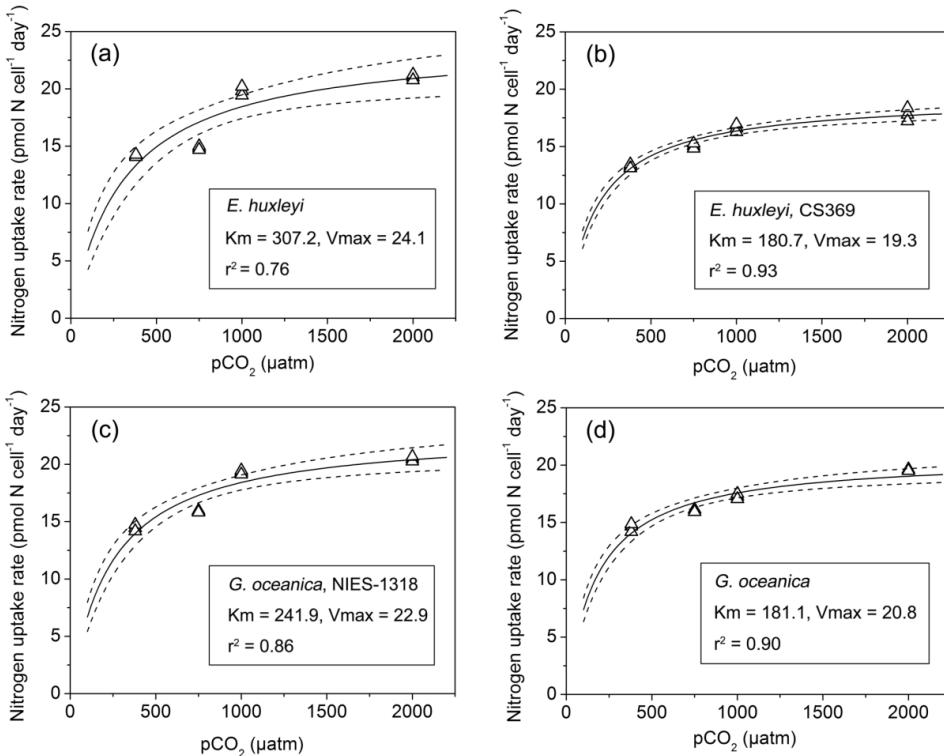


**Figure 3.** The change of chlorophyll content of the four species (*E. huxleyi* (a, e), *E. huxleyi* CS369 (b, f), *G. oceanica* NIES-1318 (c, g), *G. oceanica* (d, h)) at elevated CO<sub>2</sub> conditions (380, 750, 1000, and 2000 μatm). The vertical bars represent the SD ( $n = 3$ ). (N-E: naked strain *E. huxleyi*; C-E: calcifying strain *E. huxleyi* CS369; N-G: naked strain *G. oceanica* NIES-1318; C-G: calcifying strain *G. oceanica*.)

## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.

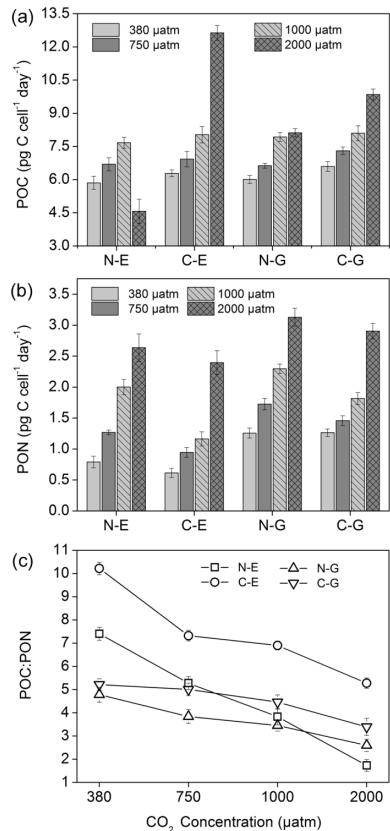
<a href="#">Title Page</a>	
<a href="#">Abstract</a>	<a href="#">Introduction</a>
<a href="#">Conclusions</a>	<a href="#">References</a>
<a href="#">Tables</a>	<a href="#">Figures</a>
<a href="#">◀</a>	<a href="#">▶</a>
<a href="#">◀</a>	<a href="#">▶</a>
<a href="#">Back</a>	<a href="#">Close</a>
<a href="#">Full Screen / Esc</a>	
<a href="#">Printer-friendly Version</a>	
<a href="#">Interactive Discussion</a>	



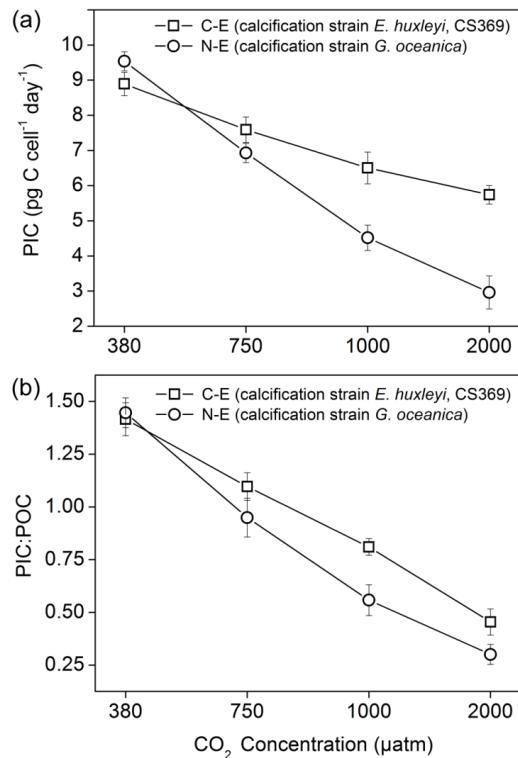
**Figure 4.** Nitrogen uptake rate response curves relative to changing  $p\text{CO}_2$  for four *E. huxleyi* and *G. oceanica* strains. The best-fit hyperbolic saturation curves (solid lines) with 95 % confidence limits (dashed lines) for *E. huxleyi* (N-E; **(a)**), *E. CS369* (C-E; **(b)**), *G. NIES-1318* (N-G; **(c)**) and *G. oceanica* (C-G; **(d)**) are also presented. The text on each board depicts the strain name, half-saturation constants (Km,  $\mu\text{atm CO}_2$ ), maximum  $\text{CO}_2$ -saturated nitrogen uptake rate (Vmax,  $\text{pmol N cell}^{-1} \text{ day}^{-1}$ ), and curve fitting correlation coefficients ( $r^2$ ). The symbols represent triplicate values.

## Inter- and intra-specific responses of coccolithophores

D. S. Wang et al.



**Figure 5.** Effect of seawater acidification by elevated  $p\text{CO}_2$  conditions (380, 750, 1000, and 2000  $\mu\text{atm}$ ) on POC (a), PON production (b) and POC:PON (c) of the four species (*E. huxleyi*, *E. huxleyi* CS369, *G. oceanica* NIES-1318, and *G. oceanica*). The vertical bars represent the SD ( $n = 3$ ). (N-E: naked strain *E. huxleyi*; C-E: calcifying strain *E. huxleyi* CS369; N-G: naked strain *G. oceanica* NIES-1318; C-G: calcifying strain *G. oceanica*.)



**Figure 6.** Effect of seawater acidification by elevated  $p\text{CO}_2$  conditions (380, 750, 1000, and 2000  $\mu\text{atm}$ ) on PIC production **(a)** and PIC : POC **(b)** of the calcifying species (*E. huxleyi* CS369 and *G. oceanica*) on the 7th day. The vertical bars represent the SD ( $n = 3$ ).