The ongoing ocean acidification associated with a changing carbonate system may impose profound effects on marine planktonic calcifiers. Here, we show that a coccolithophore, *Gephyrocapsa oceanica*, evolved in response to an elevated CO$_2$ concentration of 1000 µatm (pH reduced to 7.8) in a long-term (∼670 generations) selection experiment. The high CO$_2$-selected cells showed increases in photosynthetic carbon fixation, growth rate, cellular particulate organic carbon (POC) or nitrogen (PON) production, and a decrease in C:N elemental ratio, indicating a greater upregulation of PON than of POC production under the ocean acidification condition. Cells from the low CO$_2$ selection process shifted to high CO$_2$ exposure showed an enhanced cellular POC and PON production rates. Our data suggest that the coccolithophorid could adapt to ocean acidification with enhanced assimilations of carbon and nitrogen but decreased C:N ratios.

**KEY WORDS:** CCM, CO$_2$, coccolithophore, *Gephyrocapsa*, pH, selection.
cocolithophores have been found in HC grown cocolithophores, showing increased cellular carbon to nitrogen ratios (Feng et al. 2008; De Bodt et al. 2010). In contrast, a decreased C:N ratio was reported in the cocolithophorid Coccolithus braarudii (Rickaby et al. 2010). Such changes associated with increasing pCO2 may potentially alter trophic structures of the present day marine communities, and ultimately modify the rate at which CO2 is drawn down from the atmosphere into the deep ocean. This in turn would affect marine nutrient biogeochemistry, and the microbes that mediate many nutrient transformations (Hutchins et al. 2009).

Most studies on phytoplankton responses to ocean acidification have been carried out over short time scales, ranging from 1 to 2 generations (Iglesias-Rodriguez et al. 2008; Langer et al. 2009) to ~10 (Riebesell et al. 2000; Langer et al. 2007) and to up to 20 generations (Wu et al. 2010; Yang and Gao 2012). Although important information was obtained from these short-term acclimation experiments, few experiments document how cocolithophores may adapt to HC (Müller et al. 2010; Lobbeck et al. 2012). Consequently, a core issue in ocean acidification studies is to investigate the evolutionary responses of marine organisms to HC (Collin and Bell 2004).

We have carried out a long-term selection experiment to investigate the evolutionary responses of a cocolithophore Gephyrocapsa oceanica Kamptner (noncalcifying strain), which was grown over 670 generations under an elevated CO2 level of 1000 μatm. In contrast to the study with E. huxleyi by Lobbeck et al. 2012, this study looked into the physiological performance of another important cocolithophorid by periodical (every week) examinations, in addition to the tests at the end of the selection period. We show here that after 670 generations at HC, G. oceanica showed only small changes in the specific growth rate but significantly increased its particulate organic carbon (POC) and nitrogen (PON) productions.

Materials and Methods

CULTURE CONDITIONS

Gephyrocapsa oceanica (NIES-1318), originally isolated from the East China Sea, was obtained from the National Institute for Environmental Studies (NIES, Japan). Our cultures were derived from a unialgal population and grown in artificial seawater enriched with Aquil medium (Morel et al. 1979). This strain was originally calcifying, but lost calcifying capacity before we performed the long-term selection experiment as described later. Three independent cultures were run semicontinuously for ~670 generations at ambient (low CO2 [LC], 390 μatm) or elevated (HC, 1000 μatm) CO2 concentrations. To maintain a stable carbonate system, the initial cell concentration was set at 100 cells mL−1 and the medium was partially renewed every 6 or 7 days to restore the cell density to its initial level. Thus, the consumption of dissolved inorganic carbon (DIC) during growth was maintained <5%, with the change of pH < 0.06 units. The culture medium was equilibrated with the ambient or elevated CO2 levels prior to the dilutions. The cultures (without aeration) were maintained under a photon flux density of 100 μmol photons m−2 s−1 (12:12 light:dark period) in a plant growth chamber (GXZ, Ruihua, Wuhan, China) at a constant temperature of 20 °C.

SHIFT EXPERIMENT

When the cells had been grown for 470 days, corresponding to ~862 generations in the HC and ~670 generations in the LC treatment, they were transferred to media preequilibrated to the high or ambient CO2 concentrations to acclimate for another 10 generations (here termed as “shift experiment” or “reciprocal transplant”). The initial cell concentration was the same (100 cells mL−1) as in the long-term selection. All the parameters determined in the long-term selection were also measured at the end of this shift experiment. For these shift experiments, six independent cultures were run, two from each of the triplicate selection cultures at each CO2 level.

GROWTH RATES, CELL VOLUMES AND CHLOROPHYLL A DETERMINATION

Cell concentration was measured every 6 or 7 days before and after the partial renewal of the medium, using a particle counter (Z2, Beckman Instruments, FL). The specific growth rate (μ) was calculated as μ = (ln C1 − ln C0)/(t1 − t0), where C1 and C0 were the cell densities at time t1 and t0 (t1 − t0 = 6 or 7 days), respectively.

Cell size distribution was derived from the particle counter as mentioned earlier, and the cell volumes were calculated based on the shape of the cells according to the method described by Hillebrand et al. (1999).

Chlorophyll a content was determined by collecting the cells onto Whatman GF/F filters (25 mm) at every dilution, extracting overnight in absolute methanol, and measuring the absorbance of the supernatants over 200–800 nm after centrifugation (10 min at 6000 × g). The calculation for chlorophyll a in the supernatant was carried out according to Porra (2002), where [Chl a] (μg mL−1) = 16.29 × (A665 − A750) − 8.54 × (A652 − A750).

C AND N ANALYSIS

Samples (300 mL) taken from each replicate at the middle of light period from selection day 150 to the end of the experiment at every dilution were filtered onto precombusted (500 °C for 5 h) Whatman GF/F filters (25 mm) and frozen at −20 °C. For POC analysis, filters were fumed over HCl for 24 h to remove all inorganic carbon. The samples were analyzed on a Perkin Elmer Series II CHNS/O Analyzer 2400 (Perkin Elmer Waltham, MA).

Materials and Methods

CULTURE CONDITIONS

Gephyrocapsa oceanica (NIES-1318), originally isolated from the East China Sea, was obtained from the National Institute for Environmental Studies (NIES, Japan). Our cultures were derived from a unialgal population and grown in artificial seawater enriched with Aquil medium (Morel et al. 1979). This strain was originally calcifying, but lost calcifying capacity before we performed the long-term selection experiment as described later. Three independent cultures were run semicontinuously for ~670 generations at ambient (low CO2 [LC], 390 μatm) or elevated (HC, 1000 μatm) CO2 concentrations. To maintain a stable carbonate system, the initial cell concentration was set at 100 cells mL−1 and the medium was partially renewed every 6 or 7 days to restore the cell density to its initial level. Thus, the consumption of dissolved inorganic carbon (DIC) during growth was maintained <5%, with the change of pH < 0.06 units. The culture medium was equilibrated with the ambient or elevated CO2 levels prior to the dilutions. The cultures (without aeration) were maintained under a photon flux density of 100 μmol photons m−2 s−1 (12:12 light:dark period) in a plant growth chamber (GXZ, Ruihua, Wuhan, China) at a constant temperature of 20 °C.
Figure 1. Specific growth rate (A) and chlorophyll a content (B) of Gephyrocapsa oceanica grown at high (1000 µatm HC) and low CO₂ (390 µatm, LC) levels for 470 days. The values are the mean ± SD for triplicate cultures at each treatment.

PIC was calculated from the differences between total particulate carbon (TPC) and POC.

Rates of POC or PON production were calculated as:

\[ P = \text{specific growth rate} \times \text{cellular POC or PON content} \]

DATA ANALYSIS
One- or two-way analysis of variance (ANOVA) or paired t-tests were used to establish differences among the treatments \((P = 0.05)\).

Results
GROWTH RATE AND CHLOROPHYLL A CONTENT DURING THE SELECTION EXPERIMENT
Over 682 generations in the HC and ~670 generations in the LC treatments, specific growth rates (Fig. 1A) stabilized at a value of around 1.0 d⁻¹ after approximately 27 generations. The growth rates averaged over the whole period were significantly higher, by 1.83%, in the HC than in the LC-grown cells (paired t-test, \( t = 2.498 \), degrees of freedom \([df] = 58, P = 0.015\) ), although significant differences were not or only marginally observed at the end the selection experiments (one-way ANOVA, \( F_{1,4} = 6.6, P = 0.062\) ). Further analysis over the adaptation period revealed that, from day 44 to 199, corresponding to generations 58–280 in HC and to generations 60–274 in LC, the growth rate was 3.8% higher in the HC-grown than in the LC-grown cells (paired t-test, \( t = 3.915, P < 0.001, df = 21\) ). The difference appeared to decrease toward the end of selection period.

Chl a content showed a more fluctuating pattern in both HC- and LC-grown cells than did growth rate (Fig. 1B). Overall, analysis through all of the generations (>600), HC-grown cells had a slight but statistically significant lower chl a content (by 4.14%; mean value of 0.117 ± 0.02 pg chl a cell⁻¹) than that under LC treatments (paired t-test, \( t = 2.03, df = 51, P = 0.048\) ).

CELLULAR PIC, POC, AND PON CONTENTS DURING THE SELECTION PERIOD
After grown for over 200 generations (209 in HC and 204 generations in LC; Fig. 2), no significant cellular PIC was detected in the noncalcifying strain either under the HC or LC conditions (Fig. 2A). Cellular POC quota varied from to 6.7 to 14.0 pg cell⁻¹ in HC-grown cells and from 6.3 to 12.0 pg cell⁻¹ in LC treatments (Fig. 2B). Overall POC was about 12% higher in the HC-grown than in LC-grown cells (paired t-test, \( t = 4.54, df = 35, P < 0.001\) ). However, as with growth rate, differences in POC were larger earlier on (days 150–312, corresponding to generations 209–444 in HC and generations 204–434) than in the latter period (days 319–470, corresponding to generations 454–682 in HC and generations 445–670 in LC). In the earlier period, POC was 13.8% higher in HC than in LC cells (paired t-test, \( t = 3.882, P = 0.001, df = 19\) ), whereas in the later period the mean difference between HC and LC cells was about 9.6% and highly significant (paired t-test, \( t = 2.417, P = 0.029, df = 15\) ).

Cellular PON showed a similar trend to cellular POC, with values under the elevated CO₂ selection condition increased by 23.7% compared to those in the LC condition (Fig. 2C; paired t-test, \( t = 5.85, df = 35, P < 0.001\) ). In contrast to growth rate and POC changes, the difference in PON between HC and
LC conditions was sustained throughout the selection period. Although both cellular POC and PON quota increased in the HC-grown cells, the larger increment in PON resulted in a decline of C:N ratio by 13.3% throughout the whole selection period (Fig. 2D; paired t-test, $t = 6.98$, df = 35, $P < 0.001$).

The production rates of POC and PON showed similar trends with that of POC and PON quota (Fig. 3). High CO$_2$ selection conditions enhanced the POC production rate by 12% (paired t-test, $t = 4.03$, df = 35, $P < 0.001$) (Fig. 3A) and that of PON by about 24% (paired t-test, $t = 6.05$, df = 35, $P < 0.001$) during the whole selection period (Fig. 3B).

**GROWTH RATE, CHLOROPHYLL A CONTENT, AND CELL VOLUME AT THE END OF THE SHIFT EXPERIMENT**

The expected short-term effect of an increase in CO$_2$ is an increase in growth rate in this species. Following evolution, this response is maintained ($F_{1,23} = 70.6$, $P < 0.001$) (Fig. 4A). A mixed model (two-way ANOVA, selection regime [HC and LC] × assay condition [HC and LC]) shows no significant interaction between selection regime and assay condition ($F_{1,23} = 1.832$, $P = 0.191$), indicating that CO$_2$ enrichment over the short-term affects all populations the same way—increases growth rate regardless of the environment they evolved in (Fig. 4A). However, there was a direct response to selection ($F_{1,23} = 271.9$, $P < 0.001$), where populations selected under HC grew faster than control populations in HC conditions (one-way ANOVA, $F_{1,10} = 215.5$, $P < 0.001$). On average, HC-selected populations grew 17.4% faster than LC-selected populations when cultured in HC (Fig. 4A).

Different from that of growth rate at the end of the shift experiment, the mixed model shows significant interaction between selection regime and assay condition in chl a content, indicating that selection changes the direction of the reaction norm of chl a content in response to changes in CO$_2$ ($F_{1,11} = 10.8$, $P = 0.011$; Fig. 4B). However, there was no direct response to selection ($F_{1,11} = 0.198$, $P = 0.668$), where no significant differences were found between the populations selected under HC with that under control CO$_2$ in HC conditions (one-way ANOVA, $F_{1,4} = 4.2$, $P = 0.110$; Fig. 4B). Significant decrease of 17.6% in chl a content were found in the HC-selected cells when shifted to the LC level (one-way ANOVA, $F_{1,4} = 12.7$, $P = 0.023$; Fig. 4B).

As for the cell volume, significant interactive effects of selection regime and assay condition were found in this study ($F_{1,23} = 10.2$, $P = 0.005$), indicating that selection also changes the direction of the reaction norm of cell volumes in response to changes in CO$_2$ (Fig. 4C), which was similar to that found in chl a content. Also there was a direct response of cell volumes to HC selection ($F_{1,23} = 11.7$, $P = 0.003$), where the cell volumes of populations selected under HC were significantly smaller than that of control populations under HC were significantly smaller than that of control populations in HC conditions (one-way ANOVA, $F_{1,10} = 19.8$, $P = 0.001$; Fig. 4C). When the HC-selected cells were transferred to the ambient CO$_2$ level, they showed a significantly smaller cell volume than those under HC assay.
Figure 3. Cellular POC (A) and PON (B) production rate of *Gephyrocapsa oceanica* under high (1000 µatm) and low CO₂ (390 µatm) during the long-term selection period. The values are the mean ± SD for triplicate cultures at each treatment.

When considering the C:N ratio at the end of the shift experiment, the lowest C:N ratio (6.06 ± 0.11) was found under the LC selection–HC assay conditions, whereas the highest value (8.63 ± 0.29) was observed under the LC selection–LC assay (Fig. 5C). As for values of this ratio during the selection period, HC selection–HC assay cells had a significantly lower C:N ratio than that of LC selection–LC assay (one-way ANOVA, F_{1,10} = 14.2, P = 0.004). Furthermore, a significant increase in C:N ratio was detected in the cells from the HC selection transferred to the LC when compared to those HC cells maintained under HC for the shift assay (one-way ANOVA, F_{1,10} = 23.3, P < 0.001; Fig. 5C).

Significant interactions of selection regime and assay conditions were also found in POC and PON production rates (POC production: F_{1,23} = 114.6, P < 0.001; PON production: F_{1,23} = 182.7, P < 0.001), which was in a good agreement with findings for POC and PON (Fig. 6A and B). There was a direct response to selection (POC production: F_{1,23} = 190.5, P < 0.001; PON production: F_{1,23} = 235.5, P < 0.001), where populations selected under HC produced less organic carbon or nitrogen than control populations in HC conditions (one-way ANOVA, POC production: F_{1,10} = 187.5, P < 0.001; PON production: F_{1,10} = 222.4, P < 0.001). Similar to that of cellular POC quota, no differences in the production rates of POC (Fig. 6A) or PON (Fig. 6B) were found when comparing the HC-selected cells under the HC shift assay with the cells of LC selection under the LC shift assay (one-way ANOVA, POC production: F_{1,10} = 0.2, P = 0.632; PON production: F_{1,10} = 4.8, P = 0.052). However, significant increases in the production rates of POC (Fig. 6A) and PON (Fig. 6B) were observed when the LC-selected cells were shifted to the HC condition (one-way ANOVA, POC production: F_{1,23} = 190.5, P < 0.001; PON production: F_{1,23} = 235.5, P < 0.001), where populations selected under HC produced less organic carbon or nitrogen than control populations in HC conditions (one-way ANOVA, POC production: F_{1,10} = 187.5, P < 0.001; PON production: F_{1,10} = 222.4, P < 0.001).
Figure 4. Phenotypic responses following selection at elevated CO$_2$ conditions. (A) Specific growth rate, (B) chlorophyll $a$ content, and (C) cell volume. Data are the mean ± SD of six measurements, and the different letters indicate significant differences between treatments.

$F_{1,10} = 170.7$, $P < 0.001$; $F_{1,10} = 234.8$, PON production: $P < 0.001$). Furthermore, same as that of cellular POC and PON content, when the HC-selected cells were transferred to the LC level, POC (Fig. 6A) and PON (Fig. 6B) production rates decreased significantly (one-way ANOVA, POC production: $F_{1,10} = 35.4$, $P < 0.001$; PON production: $F_{1,10} = 54.1$, $P < 0.001$). Taken together, our growth rate and phenotypic data show that G. oceanica evolve generally in response to elevated CO$_2$ in that they show a direct response to selection, but show little specific adaptation in terms of differences between treatments.

Discussion

Coccolithophores with different levels of calcification either due to elevated CO$_2$ (Gao et al. 2009) or reduced availability of calcium ions (Xu et al. 2011) could show differences in their photosynthetic performance due to a shielding role played by the coccolith cover. In addition, the energy-demanding calcification process can play a photoprotective role (Xu and Gao 2012). Therefore, it is essential to use a noncalcifying strain to look into the other metabolic/physiological processes of coccolithophores without complications from calcification. In this study, we used a noncalcifying strain of the coccolithophore G. oceanica and investigated its capacity to show evolutionary responses to elevated CO$_2$ concentration. Gephyrocapsa oceanica showed only low-level adaptations to HC conditions, which suggests that HC would bring about significant changes in photosynthetic production and elemental stoichiometry of this species in a scenario of ocean acidification projected for the end of this century. Effects on chl $a$ content, cell volume, POC, PON content, and POC, PON production rates indicated specific adaptation to the elevated CO$_2$ level (Figs. 4B, C, 5, 6). Although HC lines grew faster at the end of the adaptation period than LC lines, but they also grew faster when they were shifted back to LC conditions (Fig. 4A).
might have been some low-level selection for faster growing cells, but this was not a specific adaptation to the higher CO\textsubscript{2} environment. The reasons for such a phenomenon are to be explored.

An increase in photosynthetic carbon acquisition (detected as a rise in chl \textit{a} content, was found in HC-selected cells during the selection period (Figs. 1, 2). The PON also increased in cells under HC selection, with a decreased C:N ratio, indicating a more upregulated production of PON than of POC under the ocean acidification conditions (Fig. 2). Cells from the LC selection process shifted to HC exposure showed enhanced cellular POC/POC production rate and PON/PON production rate (Figs. 5, 6). In contrast, long-term HC selection showed a significant increase in C:N ratio when shifted to LC (Fig. 5). Enhanced inorganic nitrogen uptake under the elevated CO\textsubscript{2} level could be responsible for the observed increase of PON quota and decreased C:N ratio (Fig. 2). In support of this is the observation that the gene for nitrate reductase in a diatom was upregulated under ocean acidification conditions (Li et al., unpubl. data). Decreased in POC:PON (= C:N ratio of cells) under elevated CO\textsubscript{2} have been reported for another coccolithophorid, \textit{C. braarudii} (Rickaby et al. 2010). Changes in C:N ratio associated with pCO\textsubscript{2} may potentially alter nutritional quality of herbivores and thus affect the trophic structures of the present day marine communities. In turn this could alter the rate of CO\textsubscript{2} draw-down from the atmosphere and thereby have an impact on marine nutrient biogeochemistry (Hutchins et al. 2009).

Long-term HC selection can lead to loss of capacity for induction of CO\textsubscript{2} concentrating mechanisms (CCMs; Collins et al. 2006), causing a decrease of cellular POC and POC production rate when these HC-selected cells were subsequently acclimated at LC conditions (Figs. 5, 6). This is contradictory to the observed increased growth rates in the HC-selected cells transferred to the LC (Fig. 4) in our experiments. Under the HC/low pH conditions, mitochondrial respiration is known to be stimulated by more than 30% in diatoms (Wu et al. 2010; Gao et al. 2012; Yang and Gao 2012); at the same time, photorespiration in diatoms is also enhanced when CCM activity is downregulated (Gao et al. 2012). These respiratory carbon losses and photosynthetic carbon fixation are both enhanced under ocean acidification conditions and may lead to unbalanced carbon gain or loss. Therefore, when the HC-selected cells were transferred to the LC level, respiratory carbon loss could be muted, which could contribute to the observed increase of growth rate during the acclimation.

If phytoplankton photosynthesis was reliant solely on the diffusional entry of CO\textsubscript{2} to the active site of the CO\textsubscript{2} fixing enzyme Rubisco, then carbon acquisition and carboxylation would be severely limited under present-day levels of CO\textsubscript{2} in seawater. To cope with the limited availability of CO\textsubscript{2}, phytoplankton species have evolved an inducible CCM (Badger et al., 1998; Raven et al. 2011) whose function is to actively transport inorganic carbon (either CO\textsubscript{2} or bicarbonate) into cells, effectively enhancing CO\textsubscript{2} levels at the active site of Rubisco. CCMs have been found in a number of species of prokaryotic and eukaryotic photosynthetic organisms, although only a small proportion of the \textasciitilde 1500 described species of cyanobacteria, or \textasciitilde 53,000 described species of eukaryotic algae have been directly examined for CCM activity (Giordano et al. 2005). A small but significantly higher growth rate was found in the long-term HC selection compared to that under ambient treatment (Fig. 1), leading us to the conclusion that the CCM in \textit{G. oceanica} is present, but does not cause complete saturation of inorganic carbon acquisition. As discussed in some previous studies (Sikes and Wheeler 1982; Nielsen 1995), coccolithophores, unlike other dominant phytoplankton groups such as diatoms and cyanobacteria, have been suggested to operate comparatively less-efficient CCMs. However, recent work
in Beardall’s laboratory (S. Larsen, unpubl. data) showed that a calcifying strain of G. oceanica growing in LC conditions had an inorganic carbon affinity consistent with operation of a CCM and resulting in photosynthetic rates at air equilibrium which were around 80–85% of DIC-saturated values. Such values would be consistent with the magnitude of the increases in POC found in the first half of the selection period (Fig. 2).

The data presented in this study provide evidence that HC led to an increase of growth, POC/POC production rate, and PON/PON production rate during the adaptation period or at the end of the shift experiment (Figs. 1, 3, 4–6). This is in good agreement with a recent study in which E. huxleyi positively adapted to increased CO₂ levels (Lobbeck et al. 2012). Collins et al. (2006) indicated that growth for 1000 generations led to loss of CCM induction in a green alga Chlamydomonas reinhardtii. However, E. huxleyi was found to concentrate inorganic carbon within its cells to a level about 13–16 times higher than the ambient (Sekino and Shiraiwa 1994). It appears possible for G. oceanica to downregulate its CCM at elevated CO₂ concentrations and to result in the loss of CCM induction after adaptation, therefore, a decreased chl a (Fig. 4) under HC selection and the LC assay condition could reflect a lower energy demand (and hence a reduced need for light harvesting) associated with the less or inactive CCM. The slight decrease of POC and POC production rate in the HC selection–LC assay data suggest that the ability to express CCM activity became lost in the long-term HC selection (Figs. 5, 6), and then CCM activity could not be fully expressed when cells were subsequently exposed to low-ambient CO₂ levels. The work of Collins et al. (2006) documented that adaptation over 1000 generations led to loss of CCM induction in a green alga Chlamydomonas reinhardtii. However, E. huxleyi was found to concentrate inorganic carbon within its cells to a level about 13–16 times higher than the ambient (Sekino and Shiraiwa 1994). It appears possible for G. oceanica to downregulate its CCM at elevated CO₂ concentrations and to result in the loss of CCM induction after adaptation, therefore, a decreased chl a (Fig. 4) under HC selection and the LC assay condition could reflect a lower energy demand (and hence a reduced need for light harvesting) associated with the less or inactive CCM.

Conclusions

The coccolithophore, G. oceanica tested in this study, showed increased POC and PON production but decreased C:N ratio along with enhanced growth rate under the ocean acidification condition. Downregulated operation of CCM could be responsible for enhanced POC and PON production due to saved energy, which can be used for additional assimilation of the elements. Increased acidity might also have played a role in upregulating nitrate uptake, leading to decreased C:N ratio. Taken together, our growth rate and phenotypic data show that G. oceanica evolve generally in response to elevated CO₂ in that they show a direct response to selection, but show little specific adaptation in terms of differences in phenotype.

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