Received: 25 August 2017 | Revised: 5 December 2017 | Accepted: 12 January 2018

DOI: 10.1111/gcb.14065

PRIMARY RESEARCH ARTICLE

WILEY Global Change Biology

Adaptive evolution in the coccolithophore *Gephyrocapsa oceanica* following 1,000 generations of selection under elevated CO_2

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Funding information
National Natural Science Foundation, Grant/Award Number: 41430967, 41720104005, 41721005; Joint project of National Natural Science Foundation of China and Shandong province, Grant/Award Number: U1606404; U.S. National Science Foundation grants, Grant/Award Number: OCE 1260490, OCE 1538525, OCE 1657757

Abstract

Coccolithophores are important oceanic primary producers not only in terms of photosynthesis but also because they produce calcite plates called coccoliths. Ongoing ocean acidification associated with changing seawater carbonate chemistry may impair calcification and other metabolic functions in coccolithophores. While short-term ocean acidification effects on calcification and other properties have been examined in a variety of coccolithophore species, long-term adaptive responses have scarcely been documented, other than for the single species *Emiliania huxleyi*. Here, we investigated the effects of ocean acidification on another ecologically important coccolithophore species, *Gephyrocapsa oceanica*, following 1,000 generations of growth under elevated CO_2 conditions (1,000 μatm). High CO_2-selected populations exhibited reduced growth rates and enhanced particulate organic carbon (POC) and nitrogen (PON) production, relative to populations selected under ambient CO_2 (400 μatm). Particulate inorganic carbon (PIC) and PIC/POC ratios decreased progressively throughout the selection period in high CO_2-selected cell lines. All of these trait changes persisted when high CO_2-grown populations were moved back to ambient CO_2 conditions for about 10 generations. The results suggest that the calcification of some coccolithophores may be more heavily impaired by ocean acidification than previously predicted based on short-term studies, with potentially large implications for the ocean's carbon cycle under accelerating anthropogenic influences.

KEYWORDS
calcification, coccolithophore, evolution, *Gephyrocapsa oceanica*, ocean acidification, plasticity

1 | INTRODUCTION

The oceans take up about a quarter of anthropogenic CO_2 emissions, leading to ocean acidification (Qi et al., 2017). Under a business-as-usual emissions scenario, the pH of seawater is predicted to decrease by 0.40 and 0.45 units, respectively, in pelagic and coastal waters by the end of the century (Babila, Rosenthal, Wright, & Miller, 2016; Cai et al., 2011; Gattuso et al., 2015; Lacoue-Labarthe et al., 2016). This change in ocean chemistry properties will profoundly affect many marine organisms (Hutchins & Fu, 2017; Müller, Trull, & Hallegraeff, 2017; Riebesell et al., 2017), especially those that use calcium carbonate to build their cell walls, scales, shells, or skeletons, with likely consequences for marine biogeochemical cycling (Albright et al., 2016; Hofmann et al., 2010; Li, Yang, Li, Xu, & Gao, 2017).

One group of particular interest in this respect are the coccolithophores (Haptophyta, Prymnesiophyceae), characterized by their

ability to form delicate calcite scales (coccoliths) (Paasche, 2002). These calcifying microalgae are estimated to account for more than 50% of the particulate inorganic carbon (PIC) produced in the pelagic ocean each year (Taylor, Brownlee, & Wheeler, 2017). Their calcite plates contribute to export of carbon from the sea surface to the ocean's interior by ballasting organic aggregates and accelerating their sinking to deeper waters. Like other calcifying organisms, most coccolithophores suffer from ocean acidification, with a response pattern of immediately decreased growth rates and calcification (Bolton et al., 2016; Liu et al., 2017; Riebesell et al., 2000). Other traits of coccolithophores such as cell size and elemental composition are also significantly influenced by ocean acidification (Feng et al., 2008; Schlüter et al., 2014). These changes could have effects on their food quality for zooplankton and their sinking rates (Ruan & Giordano, 2017), and could potentially affect nutrient cycling as well as the composition of marine communities in the ocean (Taylor et al., 2017).

Since our knowledge of detrimental ocean acidification effects on coccolithophores is mainly based on short-term studies, there has been general concern about the possibility that adaptive evolution may mitigate the reduction in calcification as the oceans acidify (Collins, Rost, & Rynearson, 2013). This can sometimes occur, as evidenced by well-calcified morphotypes of coccolithophores found in low pH regions of the current ocean (Beaufort et al., 2011). Coccolithophores reproduce quickly and have large population sizes, making them good candidates for experimental evolution studies to detect and measure possible adaptive responses in high CO_2 environments, as well as other environments of interest (Schaum, Rost, & Collins, 2015).

This approach has already been implemented in the last few years with the most cosmopolitan and widely studied coccolithophore species, *Emiliania huxleyi*. For example, Lohbeck, Riebesell, and Reusch (2012) found that after the first year (500 generations) of asexual growth, high CO_2-selected *E. huxleyi* populations showed a 3.3% increase in fitness and partial restoration in calcification rate, relative to ambient CO_2-selected populations tested under elevated CO_2 conditions. Their results demonstrated the possibility for adaptation to ocean acidification in this key phytoplankton species both through de novo mutations in single clone assays, and genotypic selection in multiclone assays (Lohbeck et al., 2012; Schlüter et al., 2014). In the subsequent years, however, no further fitness increases were observed in high CO_2-selected populations, and calcification in particular reverted to a level lower than that of the ambient CO_2-selected populations (Schlüter, Lohbeck, Gröger, Riebesell, & Reusch, 2016).

Such complex long-term dynamics of adaptive evolution are well known in evolutionary model organisms such as yeast and the bacterium *Escherichia coli* (Lang et al., 2013), and have also been found recently in other phytoplankton species (Jin & Gao, 2015; Torstensson, Hedblom, Björk, Chierici, & Wulff, 2015). For example, a noncalcifying strain of another coccolithophore species, *Gephyrocapsa oceanica*, showed increases in growth rate and carbon and nitrogen contents in high CO_2-selected populations after 670 generations (~1 year), while the response pattern was reversed with a prolonged selection time of up to 2,000 generations, accompanied by decreased phenotypic plasticity (Jin & Gao, 2015). These results imply that for some phytoplankton taxa, much longer selection times may be required to examine their evolutionary responses, as responses after only 1 year of adaptation may be transient.

Gephyrocapsa oceanica together with *E. huxleyi* represents the most abundant extant coccolithophore morphospecies. In comparison to the ubiquitous *E. huxleyi* which frequently forms extensive "milky water" blooms in high latitude ecosystems, *G. oceanica* is restricted to tropical and subtropical waters and occasionally forms massive blooms in transitional coastal waters in the Pacific Ocean (Bendif, Probert, Young, & Von Dassow, 2015). *G. oceanica* and many other coccolithophore species (e.g., *Helicosphaera carteri*, *Calcidiscus leptoporus*) have larger cell size, higher cellular calcite content and PIC (particulate inorganic carbon) to POC (particulate organic carbon) ratio relative to *E. huxleyi*, and are known to make large contributions to deep sea calcite fluxes (Daniels, Sheward, & Poulton, 2014; Young & Ziveri, 2000). Despite the important biogeochemical role of *G. oceanica* in the ocean, its calcification and physiological performance under future environmental change is comparatively poorly understood (Tong, Hutchins, Fu, & Gao, 2016), and its possible long-term evolutionary responses to ocean acidification are completely unknown. There is clearly considerable variation in physiological responses to ocean acidification among different coccolithophore species (Beaufort et al., 2011; Iglesias-Rodriguez et al., 2008; Müller, Trull, & Hallegraeff, 2015). This interspecific variation in plastic responses strongly suggests that there may also be variation in evolutionary potential among species, and that it is important to take interspecific diversity into account in evolutionary studies.

In the present study, we have carried out a long-term experiment to investigate the evolutionary responses of *G. oceanica* grown under elevated pCO_2 of 1,000 μatm for nearly 1,000 generations (2 years). At the end of this selection period, reciprocal transfer (shift) experiments were carried out to test for adaptive responses. All the traits measured in the final shift experiment were also examined repeatedly during the long-term adaptive process, in order to monitor phenotypic variability in both high and low CO_2-grown cells over the full selection time. One long-term selection experiment on this species was previously carried out using a strain that had lost calcifying capacity (noncalcifying strain) (Jin, Gao, & Beardall, 2013). Such uncalcified cells could be either mutants, or part of the regular life cycle (Paasche, 2002), but without the complex metabolic interactions between photosynthesis and calcification, they are likely to behave differently in a wide range of physiological properties from the coccolith-forming ones (Thrane, Hessen, & Andersen, 2017; Xu & Gao, 2012). As the calcification process is of particular interest to researchers in a biogeochemical context, it is essential to conduct an evolutionary study using a calcifying *G. oceanic* strain.

2 | MATERIALS AND METHODS

2.1 | Long-term growth under elevated and ambient CO_2

The coccolithophore *G. oceanica* (NIES-1318), originally isolated from the East China Sea, was obtained from the National Institute for

Environmental Studies (NIES, Japan). Cells obtained from stock cultures were inoculated at identical densities of 6×10^4 cells into 600 ml sterile artificial seawater enriched with nutrients and trace metals according to the Aquil recipe (Sunda, Price, & Morel, 2005) under the growth-saturating irradiance of 120 μmol $m^{-2}s^{-1}$ photon flux density (Zhang, Bach, Schulz, & Riebesel, 2015) and a 12:12 light : dark cycle (the same cycle used in the stock culture and in most laboratory studies for coccolithophores and other phytoplankton species) at a constant temperature of 20°C. Three independent replicate cultures were maintained at elevated (1,000 μatm, HC) or ambient (400 μatm, LC) CO_2 concentrations, realized by equilibrating the culture medium prior to inoculations. The triplicate cultures (cell lines) at each CO_2 level were independently diluted every 6 or 7 days by serial transfer of 6×10^4 cells into newly pre-equilibrated seawater (600 ml) to make sure the cell concentrations were below 8×10^4 cells ml^{-1} during growth and pH variations were <0.05 units. The long-term treatments lasted for 712 days, and repeated samplings (cell density, POC, particulate organic nitrogen [PON] and PIC) were carried out at the end of planned batch cycles before dilution (Figure S5) throughout the experiment.

2.2 | Shift experiment

After having been grown in their respective CO_2 levels for 712 days, corresponding to 1,060 generations in the LC treatment and 990 generations in the HC treatment (Figure S4), cells were transferred to the reciprocal CO_2 concentrations to acclimate for another 6 days (>8 generations). The relatively short duration of our shift experiment is comparable to the time used in most other phytoplankton studies (Collins, Sueltemeyer, & Bell, 2006; Hutchins et al., 2015; Lohbeck et al., 2012; Schlüter et al., 2014; Walworth, Lee, Fu, Hutchins, & Webb, 2016), as in order to accurately evaluate evolutionary changes stemming from the selection environment it is important to make sure that adaptation to the shift conditions does not occur. The cell concentrations were kept below 8×10^4 cells ml^{-1}. All the parameters determined in the long-term experiment were also measured in the shift experiment.

2.3 | Growth rate determination

Cell concentration was measured at the beginning (t_0) and end (t_1) of every culturing cycle using a particle counter (Z2, Beckman Instruments) without cell size shift. The specific growth rate (μ) was calculated using the equation

$$\mu = (\ln C_1 - \ln C_0)/(t_1 - t_0)$$

where C_0 and C_1 were the cell concentrations at time t_0 and t_1, respectively.

2.4 | C and N analysis

Duplicate samples of the same volume (200 ml) from each culture line were collected onto precombusted (500°C for 6 hr) Whatman GF/F filters and frozen at −20°C. One of the duplicate filters was exposed to fuming HCl for 12 hr to remove inorganic carbon, and dried overnight at 60°C for POC and PON analysis. The other filters were dried directly overnight for TPC (total particulate carbon) analysis. PIC was calculated as the difference between TPC and POC (Riebesell et al., 2000). All the filters were analyzed on a Perkin Elmer Series II CHNS/O Analyzer 2,400 (Perkin Elmer Waltham, MA). The production rates of POC, PIC, or PON were calculated as: P (pg $cell^{-1}$ d^{-1}) = μ (d^{-1}) × cellular POC, PIC, or PON content (pg $cell^{-1}$).

2.5 | Carbonate chemistry

The desired pCO_2 levels (LC, HC) were achieved by aerating the culture medium using ambient (outdoor air) and CO_2-enriched air from a commercial CO_2 Enrichlor (CE-100B, Wuhan Ruihua Instrument & Equipment Ltd, China) for 16 hr before inoculation, which was long enough to bring about the equilibrium between the air and the water. The CO_2 Enrichlor is reliable and has been used extensively in previous studies (Gao, Helbling, Häder, & Hutchins, 2012; Liu et al., 2017). CO_2 partial pressure output of the Enrichlor was stable as evidenced by continuous monitoring with a CO_2 detector (M170, Vaisala Oyj). The pH was measured with a pH meter (Orion 2 STAR, Thermo Scientific) that was calibrated with standard National Bureau of Standards (NBS). Carbonate chemistry parameters were calculated with the CO2SYS software using pCO_2 and pH (Lewis, Wallace, & Allison, 1998). The pH during the culture experiments was stable, with variations <0.05 units. The carbonate chemistry parameters of the cultures are shown in Table S1.

2.6 | Data analysis

One-way analysis of variance (ANOVA) was used to establish differences among treatments in the shift experiment with 95% confidence intervals. Paired *t* tests were used to establish differences between treatments in the long-term process with 95% confidence intervals.

3 | RESULTS

3.1 | Growth rates in the selection experiment

The HC-selected populations consistently grew more slowly than the LC-selected ones throughout the selection period. Overall, the growth rates were 6.6% lower in the HC compared to the LC treatment (paired *t* test, $t = 11.143$, $df = 59$, $p < .001$, Figure 1). Further analysis revealed that, from the beginning until Day 174 (220 and 237 generations in the HC and LC treatments, respectively), the growth rates were 7.3% lower in HC-selected populations than in LC-selected ones (paired *t* test, $t = 6.267$, $df = 15$, $p < .001$). Similarly, the growth rates were 7.4% lower in HC-selected populations than in LC-selected ones (paired *t* test, $t = 5.703$, $df = 16$, $p < .001$) in the later selection period from Day 558 to Day 712,

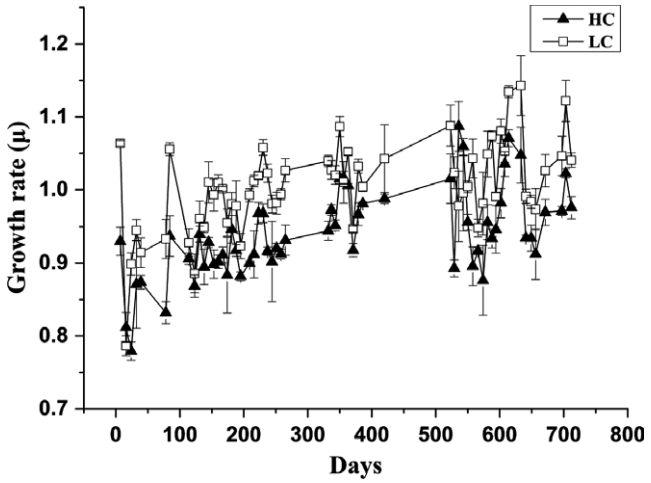

FIGURE 1 Growth rates of *Gephyrocapsa oceanica* grown under elevated (1,000 μatm, HC) and ambient (400 μatm, LC) CO_2 in the long-term selection experiment. The values are the means and error bars are standard deviations of triplicate cultures

corresponding to generations 771–990 in the HC treatment and generations 828–1,060 in the LC treatment. However, in the middle part of the selection period, corresponding to generations 230–761 in the HC treatment and generations 247–816 in the LC treatment, the difference in growth rates was less, being 5.4% lower in HC-selected populations than in LC-selected ones (paired *t* test, $t = 5.703$, $df = 26$, $p < .001$).

3.2 | Cellular POC and PON contents in the selection experiment

Cellular POC was on average 21.1% higher in the HC than in the LC treatment throughout the experiment (paired *t* test, $t = 7.97$, $df = 19$, $p < .001$, Figure 2a). The difference in POC content between HC and LC cultures also varied over time. In the early phase from the beginning until Day 123, cellular POC content was 24.7% higher in HC-selected populations compared to LC-selected ones (paired *t* test, $t = 5.29$, $df = 5$, $p = .003$), while it was only 8.9% higher in the HC than in the LC treatment during the middle phase from Day 145 to Day 258 (paired *t* test, $t = 3.51$, $df = 6$, $p < .013$). This difference increased again in the latter phase from Day 343 to Day 712, where cellular POC was 31.9% higher in the HC than in the LC treatment (paired *t* test, $t = 16.41$, $df = 6$, $p < .001$).

Cellular PON content was also significantly higher in the HC than in the LC treatment, but showed a different trend from cellular POC in the long-term process. PON content was increased by 27.3% in the HC-selected populations relative to the LC-selected ones from the beginning until Day 529 (paired *t* test, $t = 16.419.18$, $df = 15$, $p < .001$, Figure 2b). However, in the later phase (Days 602–712), cellular PON content was only 19.7% higher in the HC than in the LC treatment (paired *t* test, $t = 6.03$, $df = 3$, $p = .009$). On average, PON was 25.7% higher in HC-selected populations than in

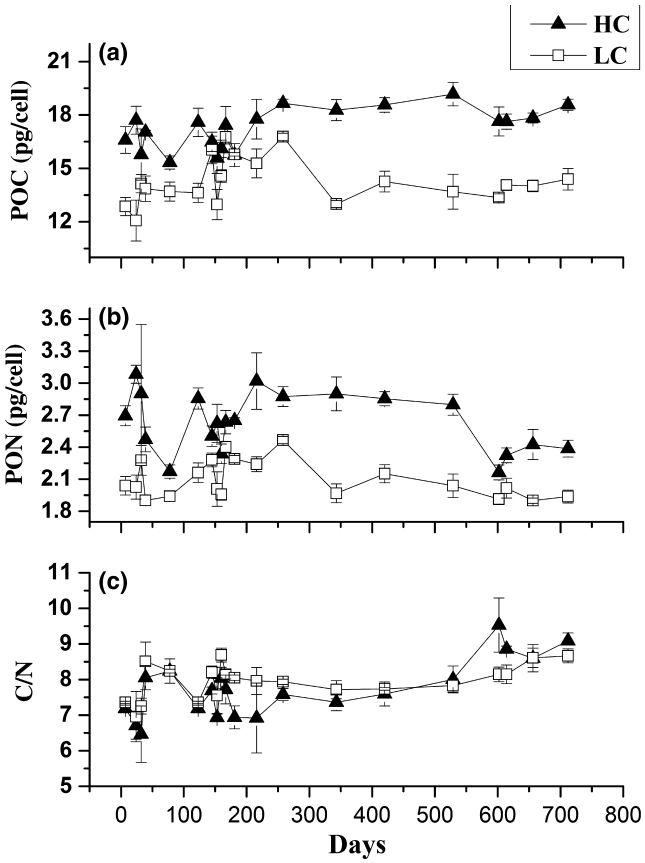

FIGURE 2 Cellular particulate organic carbon (POC) (a), particulate organic nitrogen (PON) (b), and carbon to nitrogen (C/N) ratio (c) of *Gephyrocapsa oceanica* grown under elevated (1,000 μatm, HC) and ambient (400 μatm, LC) CO_2 in the long-term selection experiment. The values are the means and error bars are standard deviations of triplicate cultures

LC-selected ones over the whole selection period (paired *t* test, $t = 9.92$, $df = 19$, $p < .001$).

Although both cellular PON and POC quotas increased in the HC treatment, the larger increase in PON from the beginning until Day 420 resulted in a 6% lower POC to PON ratio (C/N ratio) in the HC than in the LC treatment (paired *t* test, $t = 5.62$, $df = 14$, $p < .001$, Figure 2c), while there was no significant difference for C/N ratios during the latter part of the experiment (Days 529 to 712, paired *t* test, $t = 2.18$, $df = 4$, $p = .094$), due to the decreased PON in HC cultures during that period.

3.3 | Cellular PIC and PIC to POC ratio (PIC/POC ratio) in the selection experiment

Cellular PIC content fluctuated during the long-term selection process, but showed the same pattern between HC and LC treatments. HC- and LC-selected populations had the same cellular PIC contents at the first time of sampling (one-way ANOVA, $p = .62$, Figure 3a). At the second (Day 24) and third time (Day 39) we sampled, the HC-selected populations appeared to have lower PIC contents compared to the LC-selected ones, but the differences were not

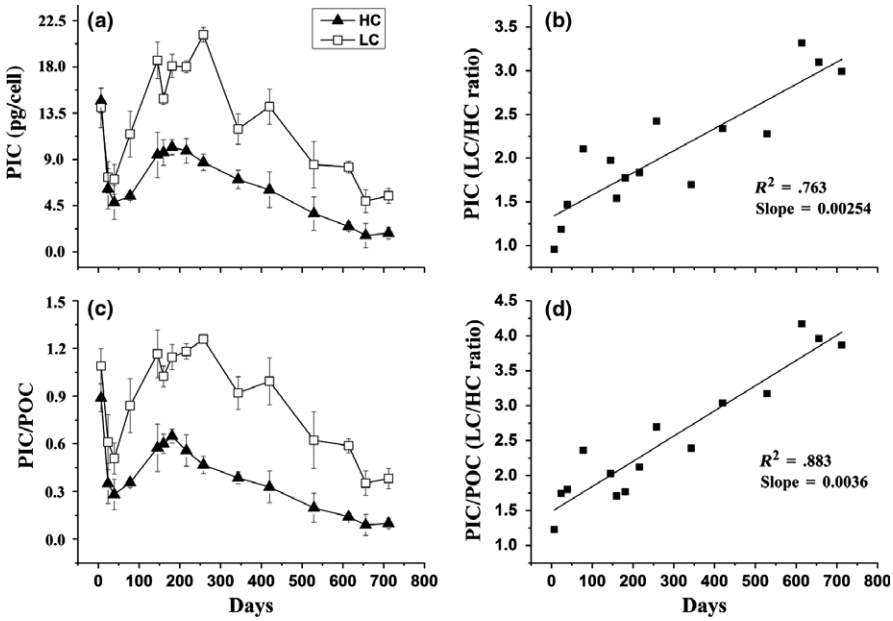

FIGURE 3 Cellular particulate inorganic carbon (PIC) of *Gephyrocapsa oceanica* grown under elevated (1,000 μatm, HC) and ambient (400 μatm, LC) CO_2 (a), the ratio of cellular PIC in LC to cellular PIC in HC (b), PIC/particulate organic carbon (POC) of *G. oceanica* grown under HC and LC (c), and the ratio of PIC/POC in LC to PIC/POC in HC (d) in the long-term selection experiment. The values of cellular PIC (a) and PIC/POC (c) are the means and error bars are standard deviations of triplicate cultures. The LC to HC ratios of PIC (b) and PIC/POC (d) are the means of values in LC to those in HC

TABLE 1 Specific growth rates and the corresponding generations of *Gephyrocapsa oceanica* populations in the shift experiment

Populations	LC-selected LC-assayed	LC-selected HC-assayed	HC-selected LC-assayed	HC-selected HC-assayed
Growth rates (μ)	1.04 ± 0.01[a]	0.99 ± 0.02[b]	0.97 ± 0.02[b]	0.98 ± 0.01[b]
Generations	9.0 ± 0.08[a]	8.6 ± 0.16[b]	8.4 ± 0.16[b]	8.5 ± 0.13[b]

The values are the means with standard deviations for triplicate cultures. The different letters after the values indicate significant differences among the treatments ($p < .05$).

statistically significant (one-way ANOVA, $p = .47$ and 0.12, respectively). By the fourth time of sampling on Day 78, PIC was 52.51% lower in the HC than in the LC treatment (one-way ANOVA, $p = .01$). Subsequently, cellular PIC was permanently lower in HC-selected populations compared to in LC-selected ones, with the difference getting larger over time, that is, the ratio between the low CO_2-selected populations and the high CO_2-selected ones (LC/HC ratio) of cellular PIC increased linearly with time (Figure 3b).

The PIC/POC ratio showed the same trend as cellular PIC. On average PIC/POC was 53% lower in the HC than in the LC treatment throughout the experiment (paired t test, $t = 9.84$, $df = 14$, $p < .001$, Figure 3c). The extent to which PIC/POC ratio in HC-selected populations was lowered compared to LC-selected ones also increased linearly with time (Figure 3d).

3.4 | Growth rates in the shift experiment

After the LC-selected populations were acclimated in HC for 6 days, their growth rates decreased by 4.2% compared to the populations continuously grown in LC conditions (one-way ANOVA, $p = .024$, Table 1). The growth rates of the LC-selected populations that were shifted to HC were the same as those of the long-term HC-grown populations (one-way ANOVA, $p = .192$). When the HC-selected populations were acclimated under LC for 6 days, however, their growth rates did not change compared to the HC-selected populations (one-way ANOVA, $p = .845$).

3.5 | Cellular POC and PON in the shift experiment

Cellular POC and PON content showed the same pattern among the four populations in the shift experiment. POC and PON were increased by 16.9% (one-way ANOVA, $p = .002$, Figure 4a) and 11.9% (one-way ANOVA, $p = .06$, Figure 4b), respectively, in LC-selected populations that were assayed in HC relative to LC-selected populations, but were 9.4% (one-way ANOVA, $p < .001$) and 9.1% (one-way ANOVA, $p = .01$) lower compared to HC-selected populations. Similar to the growth rates, the HC-selected populations did not change their POC or PON content when they were switched to the LC environment for short-term acclimation (one-way ANOVA, $p = .81$ and 0.53, respectively). The C/N ratio showed no significant difference among any of the four populations in the shift experiment (one-way ANOVA, $p > .05$, Figure 4c), with values around 9.

3.6 | Cellular PIC and PIC/POC ratio in the shift experiment

After the LC-selected populations were shifted to HC to acclimate for 6 days, their immediate physiological response to elevated CO_2

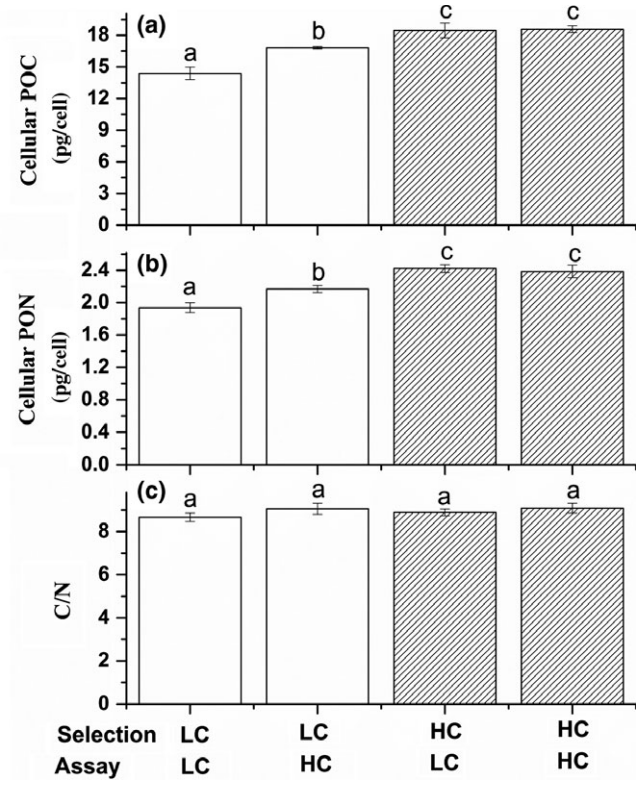

FIGURE 4 Cellular particulate organic carbon (POC) (a), particulate organic nitrogen (PON) (b), and C/N ratio (c) of *Gephyrocapsa oceanica* populations in the shift experiment. The values are the means and error bars are standard deviations of triplicate cultures. The different letters above the bars indicate significant differences among the treatments while the same letters indicate no differences ($p < .05$)

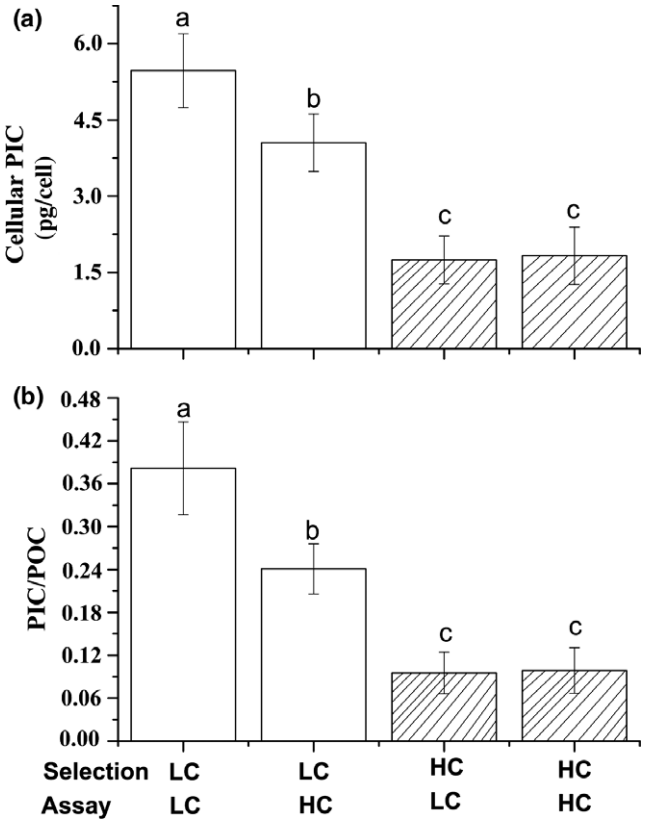

FIGURE 5 Cellular particulate inorganic carbon (PIC) (a) and PIC/particulate organic carbon (POC) ratio (b) of *Gephyrocapsa oceanica* populations in the shift experiment. The values are the means and error bars are standard deviations of triplicate cultures. The different letters above the bars indicate significant differences among the treatments while the same letters indicate no differences ($p < .05$)

was a 25.9% decrease in cellular PIC (one-way ANOVA, $p = .046$, Figure 5a). This decrease in cellular PIC is consistent with results from previous short-term OA studies (Feng et al., 2008; Riebesell et al., 2000). In contrast, the cellular PIC content was sharply decreased by 66.6% in the HC-selected populations compared to the LC-selected ones (one-way ANOVA, $p = .002$). After the HC-selected populations were returned to LC for 6 days of acclimation, the cellular PIC remained virtually identical to that of the populations maintained continuously in HC (one-way ANOVA, $p = .855$).

The PIC/POC ratio of the HC-selected populations that were assayed in LC was identical to that of the HC-selected ones (one-way ANOVA, $p = .896$, Figure 5b), and was 75% lower compared to that of the LC-selected populations (one-way ANOVA, $p < .002$). In contrast, the PIC/POC ratio was only 36.9% lower in LC-selected populations that were assayed in HC, compared to the LC-selected populations (one-way ANOVA, $p = .03$).

4 | DISCUSSION

In this study, replicate populations of the ecologically and biogeochemically important coccolithophore species *G. oceanica* were grown for nearly 1,000 generations under elevated and ambient CO_2 conditions to test for possible adaptive responses. Similar to the findings of previous short-term studies, by the end of the selection period the high CO_2-selected populations showed reduced growth rates, increased cellular POC and PON content and production rates, and decreased cellular PIC and PIC/POC ratios, compared to the ambient CO_2-selected populations. Thus, in the long run, the initial physiological responses persisted with time, and this constitutes the specific adaptation of *G. oceanica* to elevated CO_2.

In the past decade, the long-term responses to selection by elevated CO_2 have been investigated in several phytoplankton species (Collins et al., 2006; Schlüter et al., 2014), including the coccolithophore *E. huxleyi*. As calcifying organisms, they suffer from ocean acidification and an immediate response pattern of decreased growth rates and calcification has been revealed by a wealth of experiments wherein *E. huxleyi* cells were exposed to OA environment for several generations (Riebesell et al., 2017; Zhang et al., 2015). In contrast, in a long-term experiment conducted for ~500 asexual generations, *E. huxleyi* populations selected at elevated CO_2 exhibited higher calcification and growth rates (evolutionary responses) compared with those kept at ambient CO_2 when tested under OA conditions (physiological responses) (Lohbeck et al., 2012). In another noncalcifying strain of the same coccolithophore species investigated here,

G. oceanica, the evolutionary response of growth rates to OA also went in the opposite direction from the physiological response (Jin et al., 2013), in that the high CO_2-selected populations showed an increase in growth rate. This difference could be due to the effects of CO_2 selection on faster growing cells in the absence of the energy-consuming calcification process (Jin et al., 2013).

Some other taxa examined thus far including dinoflagellates, diatoms, and chlorophytes, which are generally insensitive or increase their physiological responses to high CO_2 over short exposures, exhibited little if any adaptive responses to CO_2 enrichment in long-term experiments. For example, in the unicellular green alga *Chlamydomonas reinhardtii*, the physiological response to elevated CO_2 is an increase in photosynthetic and growth rates, but this species failed to evolve specific adaptation to the elevated CO_2 after 1000 generations of selection (Collins & Bell, 2004). In the model diatom species *Phaeodactylum tricornutum*, which physiologically benefits from high CO_2, the phenotypic changes of long-term high CO_2-grown populations showed a different direction in a wide range of features (smaller cells with lower photosynthesis and respiratory activities) from those seen in the population that was only grown at high CO_2 for 20 generations (Li, Beardall, Collins, & Gao, 2016).

Recently, Hutchins et al. (2015) showed that the evolutionary responses of the marine nitrogen-fixing cyanobacterium *Trichodesmium* following long-term selection by high CO_2 differed markedly from those prior results. *Trichodesmium* growth and nitrogen fixation rates increased immediately when cultures were moved to elevated CO_2, but then remained unchanged despite the subsequent 4 years (~850 generations) of selection at this CO_2 level. Our *G. oceanica* results were analogous to those of *Trichodesmium*, in which the evolutionary and physiological responses of all traits examined to elevated CO_2 went in the same direction. The difference from *Trichodesmium* was that the extent of changes in traits including cellular POC, PON, PIC, and PIC/POC ratio was larger in high CO_2-selected populations compared to low CO_2-selected ones when both were assayed at elevated CO_2 conditions. These results indicated that for the coccolithophore species tested here, the phenotypic changes originally acquired due to a plasticity response can scale up over time in the high CO_2-selected populations, differing markedly from those prior results. This is most obviously demonstrated in the changes of PIC, PIC production rate (Figure S1, S2), and PIC/POC ratio in the long-term selection process, in which the ratio between the low CO_2-selected populations and the high CO_2-selected ones (LC/HC ratio) of these traits increased linearly with time. These unprecedented long-term dynamics will have consequences for the adaptive evolution of those ecologically relevant traits, and could greatly impact the ocean's biological carbon cycle in an acidified ocean.

Another interesting result was that, in the shift experiment, when the high CO_2-selected populations were shifted back to their ancestral ambient CO_2 environment for 6 days, all the traits examined remained at virtually identical levels to those of the high CO_2-selected populations maintained continuously at elevated CO_2. This trend held for growth rates as well as other ecologically relevant parameters, including cellular POC, PON, PIC, and PIC/POC ratio. This indicates that those traits of the high CO_2-evolved populations appeared to have lost their phenotypic plasticity, the ability to adjust phenotypic values of genotypes depending on the environment without genetic changes (Schlichting & Pigliucci, 1995). However, all these traits still retained their initial plasticity in the low CO_2-selected populations when assayed in high CO_2 condition.

Loss of trait plasticity in stable high CO_2 environments has also been previously reported for other photosynthetic microbes. For example, Collins and Bell (2004) found that populations of *C. reinhardtii* selected at elevated CO_2 for 1,000 generations degraded their CCM activity and when transferred back to ambient CO_2, the high-affinity uptake for CO_2 could no longer be induced. Recently, Hutchins et al. (2015) reported that *Trichodesmium* lineages grown at 750 ppm CO_2 exhibited large increases in growth and N_2 fixation rates compared to populations grown for the same amount of time (4.5 years) at ambient CO_2 levels. When the high CO_2-selected cell lines were switched back to ambient CO_2 growing for 2 weeks or even 2 years, their growth and N_2 fixation rates persisted at high levels, while remaining plastic in the control-selected (380 ppm) cell lines. They found that this "genetic assimilation" of a plastic response into an evolutionary one was mediated by upstream genetic regulatory mechanisms such as expression of several specific sigma factors, and possibly by alterations through transposition or other regulatory mechanisms that control a variety of metabolic pathways (Walworth et al., 2016). In our study, the traits of the high CO_2 selected *G. oceanica* populations have two obvious aspects in common with those of the *Trichodesmium* populations, that is, the loss of phenotypic plasticity and the long-term responses to elevated CO_2 going the same direction with the short-term physiological responses, they may share some similar underlying mechanisms. However, it is worth nothing that 6 days was not long enough to see if the unchanged physiological responses would persist longer as they did in *Trichodesmium* (in which they appear to be permanent, Hutchins et al., 2015), and molecular-level regulatory responses remain to be determined in our *G. oceanica* populations.

Gephyrocapsa oceanica's growth rates, a proxy for microbial reproductive fitness, decreased in the high CO_2-selected populations and remained unchanged when transferred back to ambient CO_2. This might impose a potential competitive disadvantage on *G. oceanica* and could have major ecological implications for this biogeochemically critical coccolithophore in the future high CO_2 ocean. In contrast, in the extensively studied *E. huxleyi*, the populations selected at increased CO_2 level exhibited higher growth rates after 500 asexual generations, and in the subsequent 4 years of selection, the growth rate adaptation continually increased (Schlüter et al., 2016). In these two closely related coccolithophore species, both of their growth rates decreased when exposed to elevated CO_2 for several generations, while long-term exposure brought about completely opposite responses between them. This suggests that evolutionary responses are more complex and difficult to predict. Different genotypes that were chosen to start the experiment may be responsible for the discrepancy. Even if the genetic starting material is

completely identical, idiosyncratic outcomes may be produced by historical chance events when "rewinding the evolutionary tape" (Blount, Borland, & Lenski, 2008). The decrease in growth rate was accompanied by increases in cellular POC throughout the experiment. Such a relationship under high CO_2 has been reported previously in *G. oceanica* and *E. huxleyi* (Feng et al., 2008; Jin et al., 2013). In the present study, this relationship persisted in our selection experiment, indicating the absence of adaptation that can change this relationship.

Similar to *E. huxleyi*, we were particularly interested in how cellular PIC would change during selection. In *E. huxleyi*, Lohbeck et al. (2012) found the restoration of calcification rate in elevated CO_2-adapted populations, compared to ambient CO_2-selected ones when assayed under high CO_2 conditions after 500 generations of asexual growth. However, the response pattern was later reversed. By the end of the 4 years of selection, high CO_2-adapted populations displayed more than 20% lower PIC compared to the immediate physiological decline of PIC in ambient CO_2-selected populations (Schlüter et al., 2016). In *G. oceanica*, the long-term exposure to high CO_2 resulted in a further 55% decrease in cellular PIC compared to the physiological decline, and outside the range of responses reported for both *G. oceanica* and *E. huxleyi* in previous short-term studies (Beardall & Raven, 2013; Bodt, Oostende, Harlay, Sabbe, & Chou, 2010; Borchard, Borges, Händel, & Engel, 2011). Although the adaptive dynamics in the long-term process and the direct as well as correlated responses in the shift experiment differed greatly between *E. huxleyi* and *G. oceanica*, both of their immediate physiological declines in calcification were exacerbated over long-term evolution.

This trend is consistent with the assumption that calcification is an energy-consuming process, leading to its inhibition under OA conditions and gradual reduction with time (Raven & Crawfurd, 2012). The inhibitory effects are usually attributed to electrochemical gradient changes and the associated costs of removing H^+ (Suffrian, Schulz, Gutowska, Riebesell, & Bleich, 2011; Taylor, Chrachri, Wheeler, Goddard, & Brownlee, 2011). In the present work, it appears that lowered PIC/POC ratio correlated with higher growth rates at the elevated CO_2 concentration, but with lower growth rates at the ambient CO_2 level, in *G. oceanica* (Figure S3). Increased availability of CO_2 or saved energy due to reduced calcification could enhance the growth when the cells were grown under the elevated CO_2, although increased H^+ concentration showed obvious impacts. Increased light supply to *E. huxleyi* has recently shown to decrease the inhibitory effects of OA on its calcification (Jin et al. 2017).

It should be noted that cellular PIC content and PIC/POC ratio also decreased in low CO_2-selected populations after 200 days. By the end of the selection experiment, cellular PIC was only 19% of that at Day 200, and had the potential to decrease further. Spontaneous appearance of noncalcifying cells (naked cells) from initially heavily calcified ones is a frequent event in laboratory cultures of both *E. huxleyi* (Lecourt, Muggli, & Harrison, 1996; Paasche, 2002) and *G. oceanica* (Jin et al., 2013). Possible reasons for this are unknown to date. Laboratory constant conditions are very different from natural dynamic environments. For example, the presence of UV radiation stimulates production of coccoliths in *E. huxleyi* (Guan & Gao, 2009), so long-term maintenance of the cultures in the laboratory without exposure to UV might be one of the reasons for gradual loss of calcification capacity. Our study is the first one to record the gradual changes in calcification over a relatively long time scale under HC and LC, respectively, showing that loss of calcifying ability occurs faster under HC. Changes in the capacity to calcify can influence energy allocations among different metabolic pathways, resulting in variability of growth rate and POC production in the long-term selection process.

It should also be noted that the difference in traits (growth rate, POC, PON, etc.) between HC- and LC-grown cells varied over time, a phenomenon also observed in other phytoplankton species (Li et al., 2016; Schlüter et al., 2016). Thus, the evolutionary outcome to some extent depends upon when the experiment is terminated. In contrast to some previous studies which only presented the parameters in the shift experiment (Hutchins et al., 2015; Lohbeck et al., 2012), all the traits were also examined continually during the long-term adaptation process in the present study. Subsequently, we conclude that cellular PIC and the PIC/POC ratio under the high CO_2 condition are lower than previously predicted based on short-term studies. However, we can only conclude the general direction of changes in growth rate and cellular POC and PON in high CO_2-selected relative to low CO_2-selected populations, while the extent may depend on when we stop the experiment.

To sum up, compared to short-term physiological responses, adaptive consequences were much more divergent between *G. oceanica* and *E. huxleyi*, both in the long-term dynamics and the ultimate results of shift experiments. This suggests that it is necessary to take interspecific diversity into account in marine microbial evolutionary studies, even when dealing with related taxa. In contrast, both species showed exacerbated calcification performance in high CO_2-selected populations, which will potentially have profound consequences for the ocean's carbon cycle under accelerating anthropogenic change. In the future oceans, increased CO_2 will be accompanied by higher irradiance, lower nutrient inputs, and warmer temperatures. Experiments considering the integrated effects of these changing environmental variables under more realistic conditions (and including predators, competitors and pathogens) may be necessary to determine the full range of evolutionary responses of these biogeochemically important organisms to an altered future ocean.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Sinéad Collins for her critical advice and comments on evolutionary experiments during her 1 month visit to Xiamen University. This study was supported by National Natural Science Foundation (41430967, 41720104005, 41721005), and Joint project of National Natural Science Foundation of China and Shandong province (No. U1606404) to KS.G, and by U.S. National

Science Foundation grants OCE 1260490, OCE 1538525 and OCE 1657757 to D.A.H. D.A.H's visit to Xiamen University was supported by the MEL visiting scientist program.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Tong S, Gao K, Hutchins DA. Adaptive evolution in the coccolithophore *Gephyrocapsa oceanica* following 1,000 generations of selection under elevated CO_2. *Glob Change Biol*. 2018;00:1–10. https://doi.org/10.1111/gcb.14065