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Reduced resilience of a globally distributed coccolithophore to ocean acidification: Confirmed up to 2000 generations

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ABSTRACT

Ocean acidification (OA), induced by rapid anthropogenic CO_2 rise and its dissolution in seawater, is known to have consequences for marine organisms. However, knowledge on the evolutionary responses of phytoplankton to OA has been poorly studied. Here we examined the coccolithophore *Gephyrocapsa oceanica*, while growing it for 2000 generations under ambient and elevated CO_2 levels. While OA stimulated growth in the earlier selection period (from generations ~700 to ~1550), it reduced it in the later selection period up to 2000 generations. Similarly, stimulated production of particulate organic carbon and nitrogen reduced with increasing selection period and decreased under OA up to 2000 generations. The specific adaptation of growth to OA disappeared in generations 1700 to 2000 when compared with that at 1000 generations. Both phenotypic plasticity and fitness decreased within selection time, suggesting that the species' resilience to OA decreased after 2000 generations under high CO_2 selection.

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1. Introduction

The oceans are rapidly taking up anthropogenic CO_2 with increasing atmospheric CO_2 concentrations. Dissolution of CO_2 in seawater increases pCO_2 and decreases pH, leading to ocean acidification (OA, Sabine et al., 2004). OA is known to affect a number of marine organisms, including primary producers (Riebesell and Tortell, 2011; Gao et al., 2012a,b; Beardall et al., 2014), but knowledge on the effects of multiple stressors as well as evolutionary responses has been little documented (Collins and Bell, 2004; Lohbeck et al., 2012; Jin et al., 2013a; Schaum et al., 2013; Riebesell and Gattuso, 2015). Consequently, the possibility of the evolutionary rescue of phytoplankton from OA has been gaining increasing concern (Lohbeck et al., 2012; Schaum et al., 2013; Schlüter et al., 2014; Scheinin et al., 2015).

Coccolithophores, a pan-global group of oceanic phytoplankton, produce platelets of calcium carbonate (called coccoliths) and play fundamental roles in the global carbon cycle through their direct effects on surface ocean chemistry and through ballasting of organic carbon fluxes to the deep sea, having a major feedback effect on global climate (Hutchins, 2011). OA-associated changes in carbonate chemistry can cause physiological, biochemical and photobiological influences on coccolithophores, by altering their calcification (Riebesell et al., 2000; Iglesias-Rodriguez et al., 2008; Gao et al., 2009; Sett et al., 2014), photosynthesis (Feng et al., 2008; Fiorini et al., 2011; Jin et al., 2013b), as well as elemental composition (Müller et al., 2010; Rickaby et al., 2010). The coccolithophore, *Emiliania huxleyi*, is shown to have adapted to long term OA conditions by restoring calcification and growth rates (Lohbeck et al., 2012); its particulate inorganic carbon (PIC) and particulate organic carbon (POC) production becomes significantly higher after a selection of 2000 generations under combined rising CO_2 and temperature conditions (Schlüter et al., 2014). Another cosmopolitan coccolithophore, *Gephyrocapsa oceanica*, after having been grown under OA conditions for ~700 generations, shows a moderate evolutionary response with increased growth, cellular POC and nitrogen contents, but decreased C:N ratios (Jin et al., 2013a).

Coccolithophores, in spite of their energy-driven calcification process, exhibit obvious characteristics similar to other popular phytoplankton groups, such as short generation time and large population sizes (Reusch and Boyd, 2013; Collins et al., 2014), which allow the experimenter to follow evolution in real time in response to global changes such as rising CO_2. The coccolithophores display both plastic and evolutionary responses to OA, with the ability of a genotype to produce distinct phenotypes when exposed to changing environments (Pigliucci, 2005). However, little has been documented as to how these two processes respond to OA along different time scales. Since coccolithophores and other fast growing phytoplankton, such as diatoms, can grow up to 10,000–30,000 generations by the end of this century, more generations might address possible adaptive responses (Lohbeck et al., 2012., Schaum et al., 2013).

Here, we took an experimental evolutionary approach to investigate the responses of the globally distributed coccolithophore, *G. oceanica*, to OA, while growing it for approximately 2000 generations at high

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(1000 μatm, HC) and ambient (390 μatm, LC) CO_2 concentrations, aiming to look into both the phenotypic plasticity and evolutionary responses within different selected time scales.

2. Materials and methods

2.1. Culture conditions

G. oceanica (NIES-1318), originally isolated from the East China Sea, was obtained from the National Institute for Environmental Studies (Japan). This strain was originally calcifying, but had lost its calcifying capacity before we performed the long-term selection experiment as described in Jin et al. (2013a). Part of the rationale of using non-calcifying species is documented in our previous study (Jin et al., 2013a). In addition, naked coccolithophores are normally reported in the sea (Paasche, 2002), and they have been widely used to represent the clones with noncalcifying capacity when compared with the calcifying cells (Paasche, 2002 and references therein). Three independent cultures were run in closed 1 L polycarbonate bottles and were semi-continuously diluted with CO_2-equilibrated seawater at either HC or LC pCO_2. To maintain a stable carbonate chemistry in the cultures (Table 1), the initial cell concentration was set at 100 cells mL^{-1} and the medium was partially renewed every 6 or 7 days (maximal cell densities at the end of each dilution were about 1.4×10^5 cells mL^{-1}) to restore the cell density to its initial level as described previously (Jin et al., 2013a). The cultures were maintained under 100 μmol photons m^{-2} s^{-1} photosynthetically active radiation with a light–dark cycle of 12:12 in a plant growth chamber (GXZ, Ruihua, Wuhan, China) at a constant temperature of 20 °C. For assays, both the HC and LC selected lines under 1000, 1700 and 2000 generations were transferred to media pre-equilibrated to the high or ambient CO_2 concentrations to acclimate for 10 generations which resulted in four treatments: HC selected lines assayed at HC environment (HC × HC), HC selected lines assayed at LC environment (HC × LC), LC selected lines assayed at HC environment (LC × HC) and LC selected lines assayed at LC environment (LC × LC). The initial cell concentration was also 100 cells mL^{-1} as in the long-term selection tests.

2.2. Growth rate determination

Cell concentration was measured every 6 or 7 days before and after the dilution of the medium, using a particle counter (Z2, Beckman instruments, Florida, USA). The specific growth rate (μ) was calculated as $\mu = (\ln C_1 - \ln C_0) / (t_1 - t_0)$, where C_1 and C_0 were the cell densities at time t_1 and t_0 ($t_1 - t_0 = 6$ or 7 days), respectively.

2.3. C and N analysis

Samples (300 mL) taken from each replicate at the middle of the light period were filtered onto pre-combusted (500 °C for 5 h) Whatman GF/F filters (25 mm) and frozen at −20 °C. For particulate organic carbon (POC) analysis, filters were fumed over HCl for 24 h to remove all inorganic carbon. The samples were analyzed using a Perkin Elmer Series II CHNS/O Analyzer 2400 (Perkin Elmer, USA) for the contents of POC and particulate organic nitrogen (PON).

Rates of POC or PON production were calculated as: P = specific growth rate μ (d^{-1}) × cellular POC or PON content (pg $cell^{-1}$).

2.4. Estimation of changes in carbonate chemistry

The pH in the cultures was measured with a pH meter (Benchtop pH 510, Oakton) that was calibrated with National Bureau of Standards (NBS) buffer solution (Hanna). The concentration of DIC was measured before and after the renewal of the culture using a DIC analyzer (AS-C3, Apollo Scitech) that employed an infrared gas detector (Li-Cor 7000, Li-Cor). Other related parameters of the carbonate chemistry were estimated based on known values of pH, DIC, nutrients and pCO_2 using the software CO_2SYS (Lewis and Wallace, 1998). The equilibrium constants (K_1 and K_2) of carbonic acid dissociation (Roy et al., 1993) were used for all calculations.

2.5. Plasticity and fitness responses

Changes in plasticity were calculated from the POC production rate (P) in the cells grown under the low or high CO_2 levels, as follows:

$$\frac{|P(HC) - P(LC)|}{P(LC)}.$$

Although plasticity responses are normally calculated from net photosynthetic oxygen evolution (Schaum et al., 2013), and this trait was not recorded in the present study, the trait of POC content production rate could be the proxy for net photosynthetic oxygen evolution, since it reflects carboxylation efficiency and real primary productivity.

For fitness responses, μ replaced POC production in the above formula. Corresponding measurements were carried out after 1000, 1700 and 2000 generations of selection.

2.6. Direct and correlated responses

Growth rates were used to calculate the direct and correlated responses. Here, the direct responses refer to traits of HC evolved lines measured at 1000 μatm CO_2 relative to growth rates of LC evolved lines measured at 1000 μatm CO_2. Correlated responses refer to traits of HC evolved lines measured at 390 μatm CO_2 relative to phenotypes of LC evolved lines measured at 390 μatm CO_2. Measurements of the related parameters were carried out in 1000, 1700 and 2000 generations of selection.

2.7. Data analysis

A two or three-factor nested random effects model and paired t-tests were used to establish differences among the treatments ($p = 0.05$). The interactive effects of CO_2 treatment and replicate were statistically analyzed using two-way ANOVA with three independent replicates being introduced as a random factor, nested within each selection treatment. The relationship between direct response or fitness and selection generations was fitted using linear regression.

Table 1
Parameters of the seawater carbonate system under high (1000 μatm, HC) and low (390 μatm, LC) pCO_2 levels in *Gephyrocapsa oceanica* cultures at representative 1000, 1700 and 2000 generations. Data are the means ± SD of 3 measurements.

	Treatment	pCO_2 (μatm)	pH_{NBS}	DIC (μmol kg^{-1})	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	Total alkalinity (μmol kg^{-1})
1000 generations	HC	1000	7.80 ± 0.03	2047.2 ± 146.2	1925.0 ± 133.8	89.9 ± 12.3	2144.1 ± 158.4
	LC	390	8.15 ± 0.03	1852.6 ± 124.6	1667.2 ± 103.0	172.8 ± 21.6	2095.5 ± 150.4
1700 generations	HC	1000	7.82 ± 0.03	2129.6 ± 151.4	2000.3 ± 138.1	97.0 ± 13.4	2237.2 ± 167.5
	LC	390	8.17 ± 0.02	1929.5 ± 73.4	1731.0 ± 60.5	185.9 ± 12.9	2188.2 ± 88.8
2000 generations	HC	1000	7.82 ± 0.04	2116.0 ± 205.0	1987.6 ± 186.8	96.1 ± 18.2	2222.3 ± 226.8
	LC	390	8.16 ± 0.03	1916.5 ± 144.8	1719.9 ± 120.0	184.0 ± 25.3	2172.7 ± 174.9

3. Results

3.1. Growth rate during the selection experiment

The growth rates of HC lines in the earlier selection period (from generations 670 to 1564, corresponding to days 470–1077) were significantly higher, by 5.8%, than those in LC lines (paired t-test, t = 8.71, df = 63, $p < 0.001$) (Fig. 1). However, this trend was reversed in the later selection period up to 2000 (corresponding to days 1077–1366); the HC lines grew significantly lower, by 4.6%, than did the LC lines (paired t-test, t = 3.45, df = 30, $p = 0.002$) (Fig. 1). Two-way ANOVA revealed a significant interaction between CO_2 and generations (two-way ANOVA, $F_{2,18} = 17.7$, $p < 0.001$), suggesting that a prolonged selection regime acted antagonistically with high CO_2 to decrease the growth.

3.2. Cellular POC and PON contents and production rates

The contents of POC in the HC-grown cells were 7.8% higher than those in the LC lines during the earlier selection period (from generations 670 to 1564, corresponding to days 470–1077) (paired t-test, t = 2.63, df = 26, $p = 0.014$) (Fig. 2a). However, such an increment of POC decreased in the later selection period from generations 1564 to 2000 (corresponding to days 1077–1366), with no significant differences in POC between the HC and LC lines (paired t-test, t = 1.22, df = 14, $p = 0.242$). Cellular PON showed a similar trend to POC, with values of HC lines increasing by 26.2% compared to those in LC lines in the earlier selection period (from generations 670 to 1564, corresponding to days 470–1077) (Fig. 2b) (paired t-test, t = 4.60, df = 26, $p < 0.001$); but no significant difference in PON was detected between the HC and LC lines in the later selection period (paired t-test, t = 0.06, df = 14, $p = 0.954$) (Fig. 2b). The C:N ratio decreased by 17.8% in the earlier selection period (paired t-test, t = 6.14, df = 26, $p < 0.001$), but showed no significant differences in the later selection period (paired t-test, t = 1.46, df = 14, $p = 0.167$) (Fig. 2c). Similarly to that of growth, the prolonged selection regime acted antagonistically with high CO_2 to decrease the cellular POC and PON content (two-way ANOVA, POC: $F_{2,18} = 8.8$, $p = 0.004$; PON: $F_{2,18} = 16.4$, $p < 0.001$).

The production rates of POC and PON showed similar trends with that of the POC and PON quota (Fig. 3). High CO_2 selection enhanced the POC production rate by 15.4% (paired t-test, t = 6.15, df = 26, $p < 0.001$) (Fig. 3a) and that of PON production by about 35% (paired t-test, t = 5.79, df = 26, $p < 0.001$) (Fig. 3b) in the earlier selection period, but the rates declined toward the end of the selection period (Fig. 3), so that POC production was significantly lowered by 27.0% in the HC lines than that in the LC lines at 2000 generations (two-way ANOVA, $F_{1,4} = 86.4$, $p < 0.001$) (Fig. 3a). However, no significant difference of PON production was detected between the HC and LC evolved cells (two-way ANOVA, $F_{1,4} = 0.2$, $p = 0.209$) (Fig. 3b). Prolonged selection generations acted antagonistically with high CO_2 to decrease the POC production (two-way ANOVA, $F_{2,18} = 24.5$, $p < 0.001$), but not the PON production (two-way ANOVA, $F_{2,18} = 3.2$, $p = 0.075$).

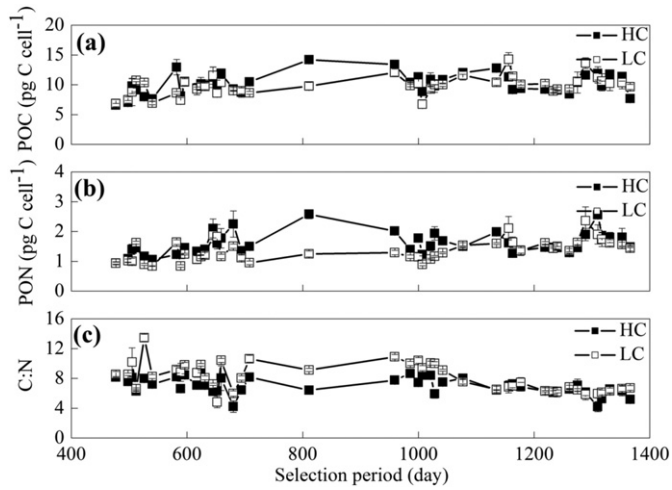

Fig. 2. Cellular POC (a), PON (b) and C:N ratio (c) of *Gephyrocapsa oceanica* under high (1000 μatm) and low (390 μatm) CO_2 levels during the long-term selection period. Panel (c) was derived from panels (a) and (b). The values are the means ± SD for triplicate cultures at each treatment.

3.3. Growth responses in the assay experiments

In the 1000 generation assay experiment (Fig. 4a), growth rates of the HC lines under high CO_2 assay were significantly higher by 9.7% than that of LC lines under ambient CO_2 assay (two-way ANOVA, $F_{1,4} = 100.5$, $p < 0.001$). However, in the 1700 generation assay test (Fig. 4b), no significant difference was found in the growth rates assayed as above (two-way ANOVA, $F_{1,4} = 1.4$, $p = 0.300$). In the 2000 generation assay experiment (Fig. 4c), growth rates of the HC evolved lines under high CO_2 assay were significantly lower than those of the LC evolved lines under ambient CO_2 assay (two-way ANOVA, $F_{1,4} = 42.1$, $p < 0.003$). There was a positive direct response to high CO_2 selection, where the growth rates of HC lines were significantly higher, by 17.1% than those of LC lines under high CO_2 assay in 1000 generations (two-way ANOVA, $F_{1,4} = 666.6$, $p < 0.001$) (Fig. 4a). It appeared that the direct response disappeared after 1700 generations (two-way ANOVA, $F_{1,4} = 0.1$, $p = 0.752$) (Fig. 4b), and a negative direct response showed up after 2000 generations, resulting in significantly lower growth rate by 5% in the HC evolved lines than that in the LC evolved lines under high CO_2 assay (two-way ANOVA, $F_{1,4} = 21.4$, $p < 0.010$) (Fig. 4c). The correlated responses, measured as growth rate of high CO_2 evolved lines assayed in the ancestral ambient CO_2 environment, decreased by 11.3% (two-way ANOVA, $F_{1,4} = 108.9$, $p < 0.001$) and 7.4% (two-way ANOVA, $F_{1,4} = 20.2$, $p = 0.011$) relative

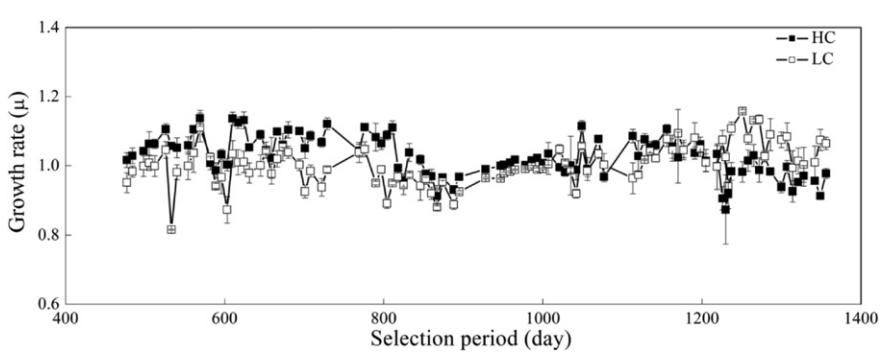

Fig. 1. Specific growth rate of *Gephyrocapsa oceanica* grown at high (1000 μatm HC) and low (390 μatm, LC) CO_2 levels for the selection period of days 470–1356. The values are the means ± SD for triplicate cultures at each treatment.

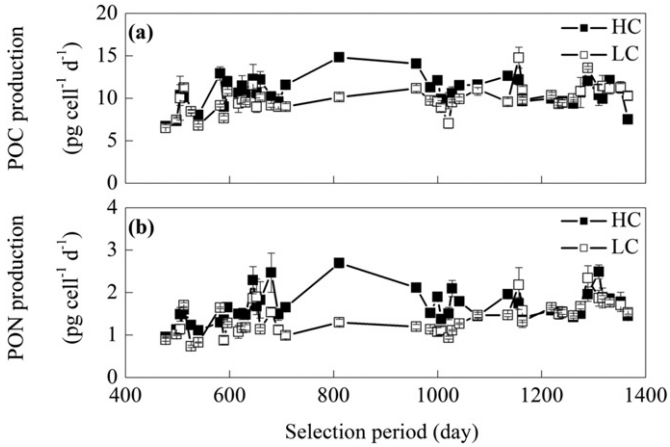

Fig. 3. Cellular POC (a) and PON (b) production rates of *Gephyrocapsa oceanica* under high (1000 μatm) and low (390 μatm) CO_2 levels during the long-term selection period. The values are the means ± SD for triplicate cultures at each treatment.

to ambient CO_2 evolved lines, after 1000 and 2000 generations, respectively (Fig. 4a, c). No correlated responses were found at generation 1700 (two-way ANOVA, $F_{1,4} = 0.2$, $p = 0.666$) (Fig. 4b). A mixed model (three-way ANOVA, selection regime (HC and LC) × assay condition (HC and LC) with three independent replicates as the random factor) showed significant interactions between selection regimes and assay conditions ($F_{1,8} = 352.7$, $p < 0.001$) at 1000 generations, indicating that *G. oceanica* showed specific adaptation to high CO_2 in terms of growth. However, no specific adaptation to high CO_2 was detected either in 1700 ($F_{1,8} = 0.001$, $p = 0.975$) (Fig. 4b) or 2000 generations ($F_{1,8} = 1.430$, $p = 0.298$) (Fig. 4c).

3.4. POC, PON and C:N responses in the assay experiments

Different from that of growth, POC of the HC lines under high CO_2 assay was insignificantly different from that of LC lines under ambient CO_2 assay at 1000 generations (two-way ANOVA, $F_{1,4} = 0.2$, $p = 0.670$) (Fig. 5a). However, in the 2000 generation assay test, it was significantly lower by 25.7% in the POC assayed as above (two-way ANOVA, $F_{1,4} = 50.2$, $p = 0.002$) (Fig. 5b). There was a positive direct response to high CO_2 selection, where the POC of HC lines was significantly higher, by 29.9% than that of LC lines under high CO_2 assay in the 1000 generation (two-way ANOVA, $F_{1,4} = 10.9$, $p = 0.030$) (Fig. 5a), while a negative direct response was detected at 2000 generations, where the POC of HC lines was significantly lower, by 14.4% than that of LC lines under high CO_2 assay (two-way ANOVA, $F_{1,4} = 98.6$, $p < 0.001$) (Fig. 5b). The correlated responses, measured as POC of high CO_2 evolved lines assayed in the ancestral ambient CO_2 environment, decreased by 22.3% (two-way ANOVA, $F_{1,4} = 24.8$, $p = 0.008$) relative to ambient CO_2 evolved lines, after 1000 generations (Fig. 5a). No correlated responses were found at 2000 generations (two-way ANOVA, $F_{1,4} = 1.7$, $p = 0.263$) (Fig. 5b).

The PON of HC lines under high CO_2 assay was insignificantly different from that of LC lines under ambient CO_2 assay either at 1000 (two-way ANOVA, $F_{1,4} = 6.6$, $p = 0.062$) (Fig. 5c) or 2000 generations (two-way ANOVA, $F_{1,4} = 0.7$, $p = 0.460$) (Fig. 5d). A positive direct response to high CO_2 selection for PON was detected at 1000 generations ([HC-selection × HC-assay] > [LC-selection × HC-assay], by 51.0%) (two-way ANOVA, $F_{1,4} = 34.5$, $p = 0.004$) (Fig. 5c), while it disappeared at 2000 generations, where the PON of HC evolved lines differed insignificantly from that of LC evolved lines under high CO_2 assay (two-way ANOVA, $F_{1,4} = 0.8$, $p = 0.427$) (Fig. 5d). There was a positive correlated response to high CO_2 selection in PON at 1000 generations, where the PON in HC evolved lines was significantly higher by 35.3% than in LC

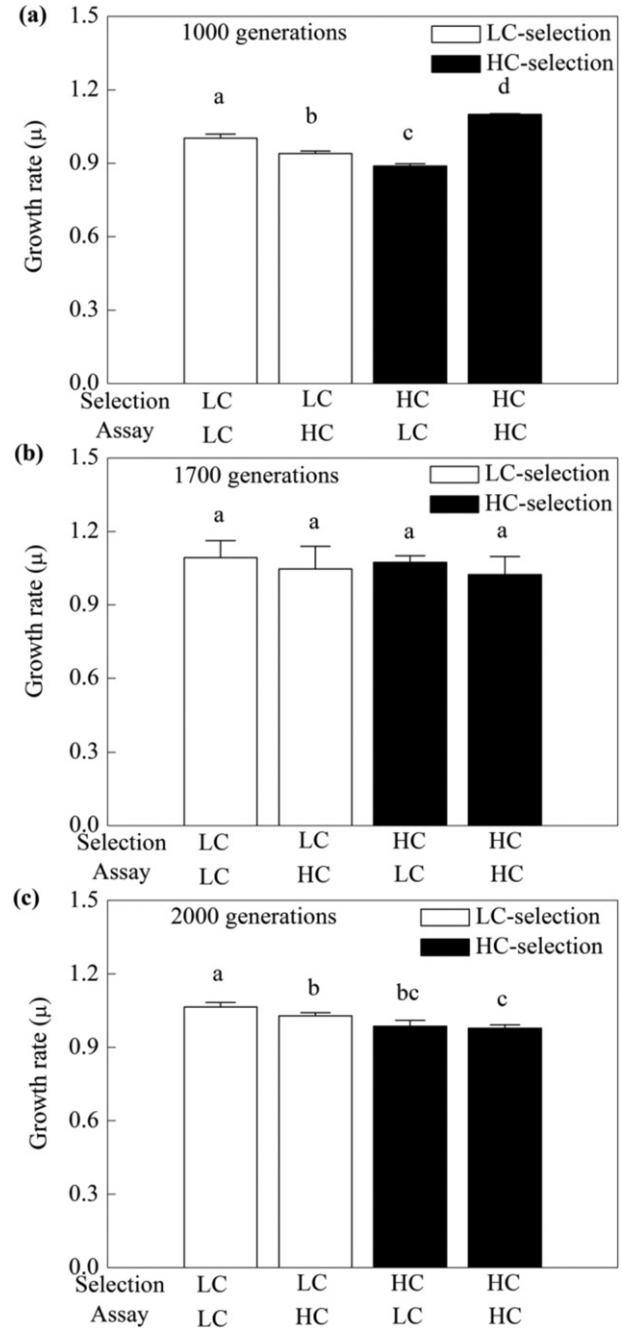

Fig. 4. Growth responses following selection at elevated CO_2 conditions and at different selection generations. a: 1000 generations; b: 1700 generations; c: 2000 generations. The values are the means ± SD for triplicate cultures at each treatment, and the different letters indicate significant differences.

evolved lines when assayed in an ambient CO_2 environment (two-way ANOVA, $F_{1,4} = 19.2$, $p = 0.012$) (Fig. 5c). However, the correlated response was not observed after 2000 generations (two-way ANOVA, $F_{1,4} = 3.4$, $p = 0.138$) (Fig. 5d).

LC evolved lines showed significantly higher POC under ambient CO_2 assay than under high CO_2 assay, by 33.6% (two-way ANOVA, $F_{1,4} = 20.9$, $p = 0.010$) and 7.6% (two-way ANOVA, $F_{1,4} = 7.5$, $p = 0.05$) in the 1000 and 2000 generations, respectively (Fig. 5a, b). The same trend was also detected for PON after 1000 generations (by 24.8% higher, two-way ANOVA, $F_{1,4} = 12.7$, $p = 0.023$) (Fig. 5c), but not for that of 2000 generations (two-way ANOVA, $F_{1,4} = 0.03$, $p = 0.863$) (Fig. 5d). Furthermore, when the HC evolved lines were transferred to

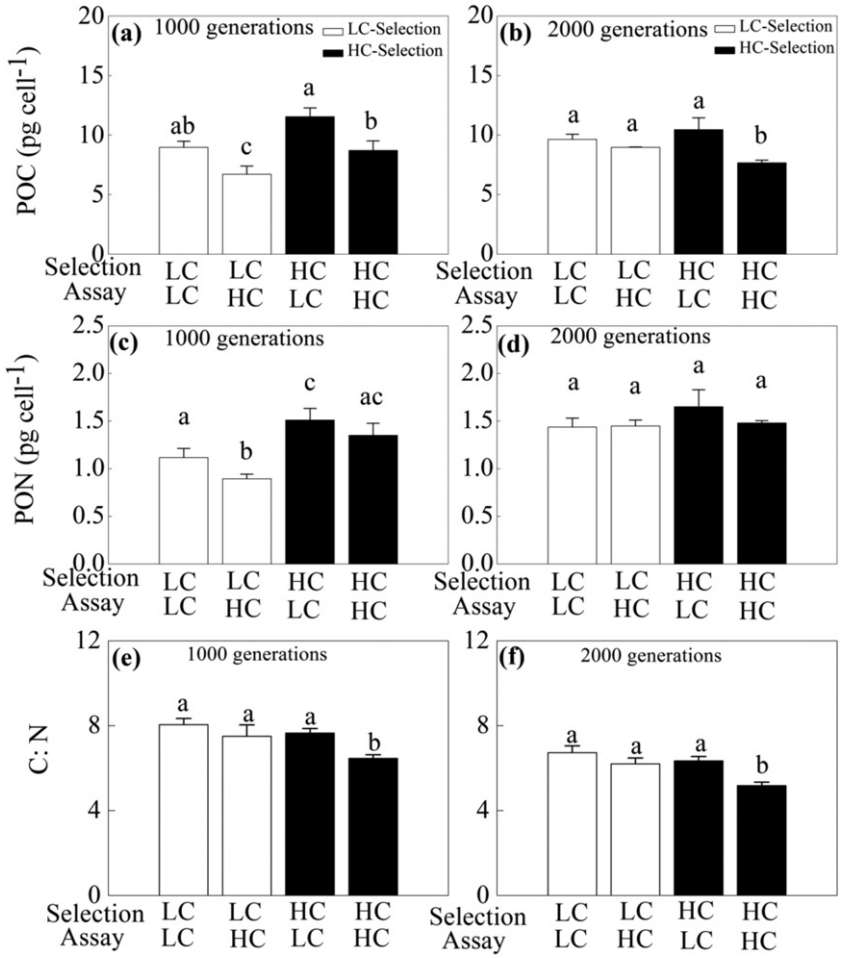

Fig. 5. Cellular POC (a, b), PON (c, d) and C:N ratio (e, f) responses following selection at elevated CO_2 conditions and at different selection generations. a, c, e: 1000 generations; b, d, f: 2000 generations. The values are the means ± SD for triplicate cultures at each treatment, and the different letters indicate significant differences.

the ancestral ambient CO_2 environment, they showed an increase of POC by 32.4% (two-way ANOVA, $F_{1,4} = 20.3$, $p = 0.011$) (Fig. 5a) and 36.3% (two-way ANOVA, $F_{1,4} = 20.4$, $p = 0.009$) (Fig. 5b) in the 1000 and 2000 generations, respectively. However, this trend was not so obvious in PON (Fig. 5c, d). These data implied that long-term high CO_2 selection might be a stressor for *G. oceanica*, since it decreased both POC and PON in this species, while a short-term decrease in CO_2 favored its POC and PON (Fig. 5).

The C:N ratios in the HC selected lines under HC assay were significantly lower, by 19.8 and 23.0% than those of LC selected lines under ambient CO_2 assay at 1000 (two-way ANOVA, $F_{1,4} = 65.3$, $p = 0.001$) (Fig. 5e) and 2000 generations (two-way ANOVA, $F_{1,4} = 54.5$, $p = 0.002$) (Fig. 5f), respectively. A direct response of C:N to high CO_2 was found both at 1000 (two-way ANOVA, $F_{1,4} = 10.2$, $p = 0.033$) (Fig. 5e) and 2000 generations (two-way ANOVA, $F_{1,4} = 29.9$, $p = 0.005$) (Fig. 5f). However, no correlated response was detected either at 1000 (two-way ANOVA, $F_{1,4} = 3.6$, $p = 0.132$) (Fig. 5e) or 2000 generations (two-way ANOVA, $F_{1,4} = 2.9$, $p = 0.162$) (Fig. 5f).

The mixed model showed no significant interaction of selection regime and assay condition on the POC and PON in the assay experiment after 1000 and 2000 generations (1000 generations: POC: $F_{1,8} = 0.4$, $p = 0.554$; PON: $F_{1,8} = 0.4$, $p = 0.579$; 2000 generations: PON: $F_{1,8} = 3.4$, $p = 0.139$) (Fig. 5), showing no specific responses to changes in high CO_2, except for the POC in the 2000 generation ($F_{1,8} = 9.7$, $p = 0.036$) (Fig. 5b). However, specific responses of C:N to high CO_2 selection were detected both at 1000 ($F_{1,8} = 11.8$, $p = 0.026$) (Fig. 5e) and 2000 generations ($F_{1,8} = 12.1$, $p = 0.025$) (Fig. 5f).

3.5. Evolutionary responses and phenotypic plasticity

When the growth rates were used to calculate the direct and correlated responses, and then plotted against the selection generations as shown in Fig. 6a and b (the data of 670 generations were derived from our previous work described in Jin et al., 2013a), the direct responses decreased within the selection generations: from a positive direct response in 670 generations (mean value of 0.175) to a negative direct response in 2000 generations (mean value of −0.050) (Fig. 6a). Similarly, there was a positive correlated response in 670 generations (mean value of 0.136), but it showed negative correlated responses at longer selection generations (e.g. mean value of −0.074 in generations 2000) (Fig. 6b).

The same as that of direct responses to high CO_2 selection, fitness decreased almost linearly within the selection generations ($R^2 = 0.97$, $p = 0.013$) (Fig. 6c). As for the phenotypic plasticity (calculated from POC production data), we also found a tendency that the evolved plasticity decreased within selection time regimes (Fig. 6e). For instance, evolved plasticity in generation 2000 (mean value 0.10) was significantly lower than that of generation 670 (mean value 1.17) (two-way ANOVA, $F_{1,7} = 182.8$, $p < 0.001$) (Fig. 6d).

4. Discussion

The coccolithophore, *G. oceanica*, after having adapted to OA for 2000 generations, decreased its growth, cellular POC and PON content under high CO_2 conditions, suggesting a negative effect of the high-

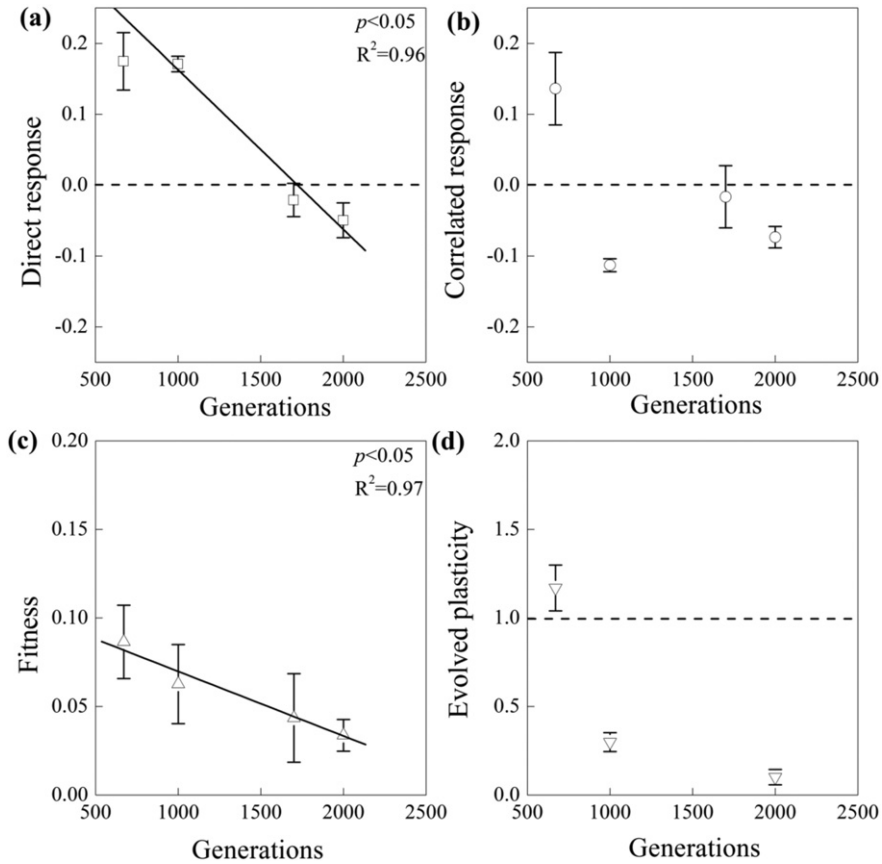

Fig. 6. Direct (a) and correlated responses (b), fitness (c) and evolved plasticity (d) of *Gephyrocapsa oceanica* as a function of selection generations. The dashed line indicates no response to selection (a, b) or no change in plasticity after 2000 generations of evolution. The values are the means ± SD for triplicate cultures at each treatment.

CO_2 altered seawater chemistry when compared with that in the earlier selection period. OA decreased the C:N ratios in the earlier selection period, while this effect was absent in the later selection period up to 2000 generations, and also its specific adaptation of growth to OA disappeared. Since phenotypic plasticity and fitness decreased within the selection time, the cells appeared to have adapted to increased acidity associated with increased pCO_2, so that plastic acclimation to the changes in seawater carbonate chemistry became weak.

Since CO_2 is considered to be an important factor that can kinetically limit phytoplankton photosynthetic carbon fixation (Raven, 1993; Riebesell et al., 1993), the growth of phytoplankton species can, in turn, be limited by the availability of CO_2 (Riebesell et al., 1993). Therefore, phytoplankton growth may benefit from rising atmospheric CO_2 concentration (Hein and Sand-Jensen, 1997; Schippers et al., 2004). However, our present study demonstrated a decrease in growth after 2000 generations under high CO_2 selection compared to that under ambient CO_2 selection (Fig. 1). Such a result was contrary to our previously published work with 670 generation in *G. oceanica* (Jin et al., 2013a) and the work by Lohbeck et al. (2012) with 500 generations in *E. huxleyi*. Such a discrepancy may be due to the accumulation of deleterious mutations along with the elevated CO_2 selection over 2000 generations (Low-Décarie et al., 2013). It is widely reported that elevated CO_2 leads to down-regulation of carbon concentration mechanisms (CCMs) in microalga (Giordano et al., 2005; Collins et al., 2006; Spijkerman, 2008), and increased availability of CO_2 in seawater might weaken the selective pressure on the maintenance of CCMs. On the other hand, increased acidity associated with carbonate chemistry changes under elevated CO_2 could have affected the organism's protonic pumps: such an influence could have stressed the cells to spend extra energy to maintain intracellular acid base balance. Therefore, the balance between the beneficial (increased pCO_2) and harmful (increased acidity) effects could have been vitiated, so that the cells appeared to have adapted to the stress at the sacrifice of their growth and assimilation rates. Thus conditionally deleterious mutations regulating carbon uptake and protonic pumps would have accumulated under the OA condition, an evolutionary response we detected in our reciprocal transplant assays in the 1000 and 2000 generations: HC evolved lines had significantly lower growth relative to LC evolved lines, when assayed in ambient CO_2 (Fig. 4a and c). The specific adaptation of growth to elevated CO_2 detected in 1000 generations (Fig. 4a) could be a trade-off in adapting to elevated CO_2 (Low-Décarie et al., 2013). Metabolic pathways related to CO_2 acquisition and acid–base regulation could have evolved differentially with selection time, so that divergence between the 1000 and 2000 generations occurred. Therefore, at 2000 generations, both HC and LC lines adapted to their own carbonate chemistry (μ [HC-selection × HC-assay] > μ [LC-selection × HC-assay]; μ [LC-selection × LC-assay] > μ [HC-selection × LC-assay]) (Fig. 4a). The conditionally deleterious mutation overwhelmed the specific responses with increasing selection time, consequently leading to no specific adaptation to elevated CO_2 after 2000 generations (Fig. 4c).

Phenotypic plasticity, which is defined to describe the different phenotypes produced by the same genotype as a function of the environment (Schlichting and Pigliucci, 1995), decreased within the selection time regime in the present study (Fig. 6d), differing from some previous studies (Schaum and Collins, 2014). Species-specific evolutionary responses to increased pCO_2 and water acidity might be possible. Since the increased acidity under elevated CO_2 is a stressor for most organisms (Kroeker et al., 2010), *G. oceanica* grown under elevated CO_2 might have evolved lines that decreased phenotypic plasticity to diminish energetic cost, because of phenotypic buffering (Reusch, 2013). At the same time, the HC evolved lines need to respond in a truly plastic way to accommodate the external stress of elevated CO_2 and maintain

homeostasis (Schlichting and Pigliucci, 1998). Hence, the elevated CO_2 evolved lines lowered their growth, POC and PON in the later selection phase (Figs. 1 and 2), reflecting a cost of plasticity. It is also demonstrated that the cost of plasticity is coupled with the reduction of fitness (Dewitt et al., 1998), which was evidenced in the coccolithophore in the present work (Fig. 6c). Holistically, the decreases in plasticity and fitness in *G. oceanica* in 2000 generations under elevated CO_2 could reflect the alga's strategies to cope with OA.

CCMs are found in a number of species of prokaryotic and eukaryotic photosynthetic organisms to cope with limited availability of CO_2 in seawater (Giordano et al., 2005). Our previous study demonstrates that CCMs are present in *G. oceanica*, but do not cause complete saturation of inorganic carbon acquisition in the long-term (670 generations) high CO_2 selection (Jin et al., 2013a, 2013b). As reported in a previous study, elevated CO_2 leads to downregulation of genes governing the CCMs (Spijkerman, 2008), and the increased availability of CO_2 may relax or eliminate the selective pressure on the maintenance of CCMs (Low-Décarie et al., 2013). Then, conditionally deleterious mutations in genes regulating carbon acquisition and utilization will accumulate at elevated CO_2 (Low-Décarie et al., 2013). The results from our study (Fig. 4a and c) implied that the CCMs in *G. oceanica* under high CO_2 selection in 2000 generations were still present, but became downregulated. Nevertheless, related gene expressions governing the CCMs both in HC- and LC-grown cells need to be explored in future studies.

Our results implied that much longer selection experiments may be required to investigate evolutionary responses of phytoplankton species to OA, since both evolutionary responses and phenotypic plasticity changed within different selection time spans in *G. oceanica* (Jin et al., 2013a, the present work), although different phytoplankton taxa may respond differentially to long-term OA treatment.

Our results have important implications for projected changes in marine primary production and biogeochemical cycles in response to anthropogenic increases in atmospheric CO_2. Based on the results that growth and POC production decreased after 2000 generations under OA, marine production and biological pump efficiency are likely to decrease in response to rising CO_2 levels in the future ocean. OA is suggested to influence the phytoplankton's biochemical composition (Rossoll et al., 2012; Jin et al., 2015), and we observed that the relatively increased C:N ratio in *G. oceanica* grown under high CO_2 selection after 2000 generations resulted in decreased nutritional value. Considering the effects of multiple stressors (Riebesell and Gattuso, 2015), it is essential to assess how OA and other global environmental alternations such as warming and de-oxygenation (Doney et al., 2012) would affect food quality and trophic energy transfer in the context of evolution.

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References

Beardall, J., Stojkovic, S., Gao, K., 2014. Interactive effects of nutrient supply and other environmental factors on the sensitivity of marine primary producers to ultraviolet radiation: implications for the impacts of global change. Aquat. Biol. 22, 5–23.
Collins, S., Bell, G., 2004. Phenotypic consequences of 1000 generations of selection at elevated CO_2 in a green alga. Nature 431, 566–569.
Collins, S., Sültemeyer, D., Bell, G., 2006. Changes in C uptake in populations of *Chlamydomonas reinhardtii* selected at high CO_2. Plant Cell Environ. 29, 1812–1819.
Collins, S., Rost, B., Rynearson, T.A., 2014. Evolutionary potential of marine phytoplankton under ocean acidification. Evol. Appl. 7, 140–155.
Dewitt, T.J., Sih, A., Wilson, D.S., 1998. Costs and limits of phenotypic plasticity. Trends Ecol. Evol. 13, 77–81.
Doney, S.C., Ruckelshaus, M., Duffy, J.E., et al., 2012. Climate change impacts on marine ecosystems. Ann. Rev. Mar. Sci. 4, 11–37.
Feng, Y., Warner, M.E., Zhang, Y., Sun, J., Fu, F.-X., Rose, J.M., Hutchins, D.A., 2008. Interactive effects of increased pCO_2, temperature and irradiance on the marine coccolithophore *Emiliania huxleyi* (Prymnesiophyceae). Eur. J. Phycol. 43, 87–98.
Fiorini, S., Middelburg, J.J., Gattuso, J.P., 2011. Effects of elevated CO_2 partial pressure and temperature on the coccolithophore *Syracosphaera pulchra*. Aquat. Microb. Ecol. 64, 221–232.
Gao, K., Ruan, Z., Villafañe, V.E., Gattuso, J.P., Helbling, E.W., 2009. Ocean acidification exacerbates the effect of UV radiation on the calcifying phytoplankter *Emiliania huxleyi*. Limnol. Oceanogr. 54, 1855–1862.
Gao, K.S., Helbling, E.W., Häder, D.P., Hutchins, D.A., 2012a. Responses of marine primary producers to interactions between ocean acidification, solar radiation, and warming. Mar. Ecol. Prog. Ser. 470, 167–189.
Gao, K.S., Xu, J., Gao, G., et al., 2012b. Rising CO_2 and increased light exposure synergistically reduce marine primary productivity. Nat. Clim. Chang. 2, 519–523.
Giordano, M., Beardall, J., Raven, J.A., 2005. CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. Annu. Rev. Plant Biol. 56, 99–131.
Hein, M., Sand-Jensen, K., 1997. CO_2 increases oceanic primary production. Nature 388, 526–527.
Hutchins, D.A., 2011. Oceanography: forecasting the rain ratio. Nature 476, 41–42.
Iglesias-Rodriguez, M.D., Halloran, P.R., Rickaby, R.E.M., et al., 2008. Phytoplankton calcification in a high-CO_2 world. Science 320, 336.
Jin, P., Gao, K., Beardall, J., 2013a. Evolutionary responses of a coccolithophorid *Gephyrocapsa oceanica* to ocean acidification. Evolution 67, 1869–1878.
Jin, P., Gao, K., Villafañe, V.E., Campbell, D.A., Helbling, W., 2013b. Ocean acidification alters the photosynthetic responses of a coccolithophorid to fluctuating UV and visible radiation. Plant Physiol. 162, 2084–2094.
Jin, P., Wang, T., Liu, N., Dupont, S., Beardall, J., Boyd, P., Riebesell, U., Gao, K., 2015. Ocean acidification increases the accumulation of the toxic phenolic compounds across trophic levels. Nat. Commun. 6, 8714. http://dx.doi.org/10.1038/ncomms9714.
Kroeker, K.J., Kordas, R.L., Crim, R.N., Singh, G.G., 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol. Lett. 13, 1419–1434.
Lewis, E., Wallace, D., 1998. Program developed for CO_2 system calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee.
Lohbeck, K.T., Riebesell, U., Reusch, T.B.H., 2012. Adaptive evolution of a key phytoplankton species to ocean acidification. Nat. Geosci. 5, 346–351.
Low-Décarie, E., Jewell, M.D., Fussmann, G.F., Bell, G., 2013. Long-term culture at elevated atmospheric CO_2 fails to evoke specific adaptation in seven freshwater phytoplankton species. Proc. R. Soc. B Biol. Sci. 280, 20122598.
Müller, M., Schulz, K., Riebesell, U., 2010. Effects of long-term high CO_2 exposure on two species of coccolithophores. Biogeosciences 7, 1109–1116.
Paasche, E., 2002. A review of the coccolithophorid *Emiliania huxleyi* (prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions. Phycologia 40, 503–529.
Pigliucci, M., 2005. Evolution of phenotypic plasticity: where are we going now? Trends Ecol. Evol. 20, 481–486.
Raven, J., 1993. Limits on growth rates. Nature 361, 209–210.
Reusch, T.B., 2013. Climate change in the oceans: evolutionary versus phenotypically plastic responses of marine animals and plants. Evol. Appl. 7, 104–122.
Reusch, T.B., Boyd, P.W., 2013. Experimental evolution meets marine phytoplankton. Evolution 67, 1849–1859.
Rickaby, R., Henderiks, J., Young, J., 2010. Perturbing phytoplankton: response and isotopic fractionation with changing carbonate chemistry in two coccolithophore species. Clim. Past 6, 771–785.
Riebesell, U., Gattuso, J.-P., 2015. Lessons learned from ocean acidification research. Nat. Clim. Chang. 5, 12–14.
Riebesell, U., Tortell, P.D., 2011. In: Gattuso, J.P., Hanson, L. (Eds.), Effects of Ocean Acidification on Pelagic Organisms and Ecosystems. Oxford University Press, Oxford, pp. 99–121.
Riebesell, U., Wolf-Gladrow, D., Smetacek, V., 1993. Carbon dioxide limitation of marine phytoplankton growth rates. Nature 361, 249–251.
Riebesell, U., Zondervan, I., Rost, B., Tortell, P.D., Zeebe, R.E., Morel, F.M.M., 2000. Reduced calcification of marine plankton in response to increased atmospheric CO_2. Nature 407, 364–367.
Rossoll, D., Bermúdez, R., Hauss, H., Schulz, K.G., Riebesell, U., Sommer, U., Winder, M., 2012. Ocean acidification-induced food quality deterioration constrains trophic transfer. PLoS One 7, e34737.
Roy, R.N., Roy, L.N., Vogel, K.M., et al., 1993. The dissociation constants of carbonic acid in seawater at salinities 5 to 45 and temperatures 0 to 45 °C. Mar. Chem. 44, 249–267.
Sabine, C.L., Feely, R.A., Gruber, N., et al., 2004. The oceanic sink for anthropogenic CO_2. Science 305, 367–371.
Schaum, E., Collins, S., 2014. Plasticity predicts evolution in a marine alga. Proc. R. Soc. B Biol. Sci. 281, 20141486.
Schaum, E., Rost, B., Millar, A.J., Collins, S., 2013. Variation in plastic responses of a globally distributed picoplankton species to ocean acidification. Nat. Clim. Chang. 3, 298–302.
Scheinin, M., Riebesell, U., Rynearson, T., Lohbeck, K., Collins, S., 2015. Experimental evolution gone wild. J. R. Soc. Interface 12, 20150056.
Schippers, P., Lurling, M., Scheffer, M., 2004. Increase of atmospheric CO_2 promotes phytoplankton productivity. Ecol. Lett. 7, 446–451.
Schlichting, C.D., Pigliucci, M., 1995. Gene regulation, quantitative genetics and the evolution of reaction norms. Evol. Ecol. 9, 154–168.

Schlichting, C.D., Pigliucci, M., 1998. Phenotypic Evolution: A Reaction Norm Perspective. Sinauer Associates, Sunderland, MA.
Schlüter, L., Lohbeck, K.T., Gutowska, M.A., Gröger, J.P., Riebesell, U., Reusch, T.B., 2014. Adaptation of a globally important coccolithophore to ocean warming and acidification. Nat. Clim. Chang. 4, 1024–1030.
Sett, S., Bach, L.T., Schulz, K.G., Koch-Klavsen, S., Lebrato, M., Riebesell, U., 2014. Temperature modulates coccolithophorid sensitivity of growth, photosynthesis and calcification to increasing seawater pCO_2. PLoS One 9, e88308.
Spijkerman, E., 2008. What physiological acclimation supports increased growth at high CO_2 conditions? Physiol. Plant. 133, 41–48.