

ORIGINAL ARTICLE

Environmental stability affects phenotypic evolution in a globally distributed marine picoplankton

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Marine phytoplankton can evolve rapidly when confronted with aspects of climate change because of their large population sizes and fast generation times. Despite this, the importance of environment fluctuations, a key feature of climate change, has received little attention—selection experiments with marine phytoplankton are usually carried out in stable environments and use single or few representatives of a species, genus or functional group. Here we investigate whether and by how much environmental fluctuations contribute to changes in ecologically important phytoplankton traits such as C:N ratios and cell size, and test the variability of changes in these traits within the globally distributed species *Ostreococcus*. We have evolved 16 physiologically distinct lineages of *Ostreococcus* at stable high CO₂ (1031 ± 87 µatm CO₂, SH) and fluctuating high CO₂ (1012 ± 244 µatm CO₂, FH) for 400 generations. We find that although both fluctuation and high CO₂ drive evolution, FH-evolved lineages are smaller, have reduced C:N ratios and respond more strongly to further increases in CO₂ than do SH-evolved lineages. This indicates that environmental fluctuations are an important factor to consider when predicting how the characteristics of future phytoplankton populations will have an impact on biogeochemical cycles and higher trophic levels in marine food webs.

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Introduction

The function of marine ecosystems hinges on their smallest primary producers. Marine phytoplankton are responsible for roughly 50% of global primary production (Falkowski *et al.*, 1998), are major drivers of the carbon pump (Beardall and Raven, 2004) and form the base of marine food webs (Rossoll *et al.*, 2012; Thomsen *et al.*, 2013). Marine phytoplankton with large population sizes and short generation times will have ample scope for evolution in a changing ocean (Collins, 2011), where CO₂ levels are predicted to have doubled by the end of the century (IPCC report, 2007) and to become less stable (Egleston *et al.*, 2010; Flynn *et al.*, 2012). Thus, phytoplankton are and will be evolving in unstable CO₂ environments. Natural fluctuations in CO₂ can range from diurnal changes to annual fluctuations and changes on geological timescales, for example, due to microbial activity, severe weather and seasonal vertical mixing (Joint *et al.*, 2011; Gilbert *et al.*, 2008; Li *et al.*, 2009). We have previously shown that average annual fluctuations in CO₂ at the

sampling location affect the growth responses of marine picoplankton to short-term increases in CO₂ (Schaum *et al.*, 2013).

As buffering capacity decreases at lower pH values, any changes in dissolved CO₂ are expected to become more pronounced and less predictable in the future (Egleston *et al.*, 2010; Flynn *et al.*, 2012), making it necessary for phytoplankton to not only evolve mechanisms to deal with CO₂ levels that are on average higher than today's but also to cope with larger and more frequent deviations from that average. It has not been tested empirically whether fluctuations modulate responses to further environmental changes in marine phytoplankton, although theory and experiments on other model organisms such as *Escherichia coli* suggests that it should (Ghalambor *et al.*, 2007; Draghi and Whitlock, 2012; Chevin *et al.*, 2013; Karve *et al.*, 2014). This is particularly important, as even under optimistic scenarios, CO₂ levels in the ocean are going to increase long after the 2100 cutoff applied to most climate models (IPCC report, 2007). Yet, despite the likely influence of environmental variability on evolution, selection experiments that aim to characterize phenotypes of marine phytoplankton in a changing ocean are usually carried out in stable environments (for example, Lohbeck *et al.*, 2012; Crawford *et al.*, 2011; Tatters *et al.*, 2013; Schlüter *et al.*, 2014).

Most previous studies have used few representatives of a species (or, where species are cryptic,

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species complex), with no test of whether genotypically or physiologically distinct lineages of the same species differ in the phenotypes they evolve in response to elevated CO₂ levels. However, this information is crucial to make predictions about how structures of phytoplankton communities and their function in the ecosystem will be affected by increased CO₂ levels or climate change in a broader sense. We need two main pieces of information: who will be present in future phytoplankton communities and what they will be doing. The evolution of lineage growth rates in response to environmental change informs our predictions of how frequencies of lineages within a population are expected to change (Schaum and Collins, 2014), and thus who is likely to be there. The evolution of other aspects of the phenotype, such as size and cellular stoichiometry, allow us to understand what they will be doing and how this will affect higher trophic levels and nutrient cycling. Often, phenotypic characteristics of marine microbial communities to climate change are examined through observation and short-term experiments; however, recent years have seen an increase in studies showing that evolutionary processes need to be considered when predicting effects of climate change on marine microbial communities and the expected phenotypes (see Collins *et al.*, 2013 for a review). In stable environments, these include decreased cell size and a partial restoration of the calcification process in the coccolithophore *Emiliania huxleyi* (Lohbeck *et al.*, 2012; Schlüter *et al.*, 2014) and lowered C:N ratios in diatoms (Crawford *et al.*, 2011). However, it is not known how environmental fluctuations affect phenotypic evolution.

The goal of this study is to measure whether evolving in fluctuating high CO₂ (1012 ± 244 µatm CO₂, FH) versus stable high CO₂ (1031 ± 87 µatm CO₂, SH) results in differences in the evolution of key characteristics of a marine phytoplankton. Using 16 genetically distinct lineages of the species complex *Ostreococcus* (Courties *et al.*, 1994; Subirana *et al.*, 2013) from different habitat types (for example, lineages from coasts and the open ocean, surface waters and near the deep chlorophyll maximum), we can estimate whether trait evolution in the face of elevated CO₂ is conserved within a species or genus (Schaum *et al.*, 2013; Zhang *et al.*, 2014). As part of the phenotypic characterization, we investigate how responsive lineages evolved in the SH and FH CO₂ environments are to further increases in CO₂, that is, how well they are able to adjust their growth rates in response to further CO₂ enrichment. The rationale for this is that the ability to adjust growth rates in response to environmental fluctuations is likely an important trait in marine picoplankton when marine environments become more variable. It is not feasible to measure all possible phenotypic traits; hence, here we focus on key traits that determine the role of phytoplankton in biogeochemical cycles, food webs or both.

For example, the ratio of oxygen evolution and consumption, combined with size and C:N ratios, data can inform future studies or models on carbon export to the deeper ocean. In addition, changes in size and elemental composition can affect the quality of phytoplankton as a food source for grazers so that changes in the elemental composition of phytoplankton can affect grazer population size and life history (Rossoll *et al.*, 2012; Thomsen *et al.*, 2013).

Materials and methods

Cultures and experimental setup

We used 16 lineages of *Ostreococcus* from 9 habitat types (see Schaum *et al.*, 2013 and Supplementary Table S1). Lineages were made clonal by dilution at the beginning of the experiment (Schaum and Collins, 2014). Three biological replicates of each lineage were grown in each of the four CO₂ selection environments: stable ambient (SA 444 ± 43 µatm CO₂), fluctuating ambient (FA 490 ± 97 µatm CO₂), SH (1031 ± 87 µatm CO₂) and FH (1012 ± 244 µatm CO₂). This is a total of 192 populations in the experiment (16 lineages × 3 biological replicates × 4 selection environments; see Figure 1 for experimental setup). Temperature (18 °C), salinity (31) and light intensity (160 µE) were kept stable, while pCO₂ was varied. In the fluctuating treatments, pCO₂ was set to change every 7 days (in the SA environment, *Ostreococcus* divide about once a day), to a random value between 430 and 630 µatm CO₂ for FA, and 700 and 1300 µatm CO₂ for FH environments. Samples were maintained in a semi-continuous batch culture with a transfer approximately every seven generations (see also Schaum and Collins, 2014).

Before evolution in any of the selection environments (t₀) and also following 100 (t₁₀₀) and 400 (t₄₀₀) generations of evolution in the selection environments, populations were grown at 430 and 1000 µatm CO₂ for 7–14 generations and then assayed for the investigated phenotypic traits (see Supplementary Figure S1B for FH and SH at 430 µatm CO₂). At t₆₀₀, samples from all selection environments were assayed for growth rates and other phenotypic traits at 2000 µatm CO₂ (see Figure 1). To account for the additional generations passed, growth rates, size and chlorophyll content were also re-measured for all populations evolved in SA, FA, SH and FH.

As this study focuses on trait evolution, we report three response types for lineages after they have evolved in their respective selection environments: ambient, plastic and evolved responses. Here the ambient responses are phenotypic traits of lineages evolved at SA or FA that have been measured at 430 µatm CO₂. The respective trait values function as a reference to which the plastic and evolved responses are compared. Plastic responses are changes in phenotype that do not require any

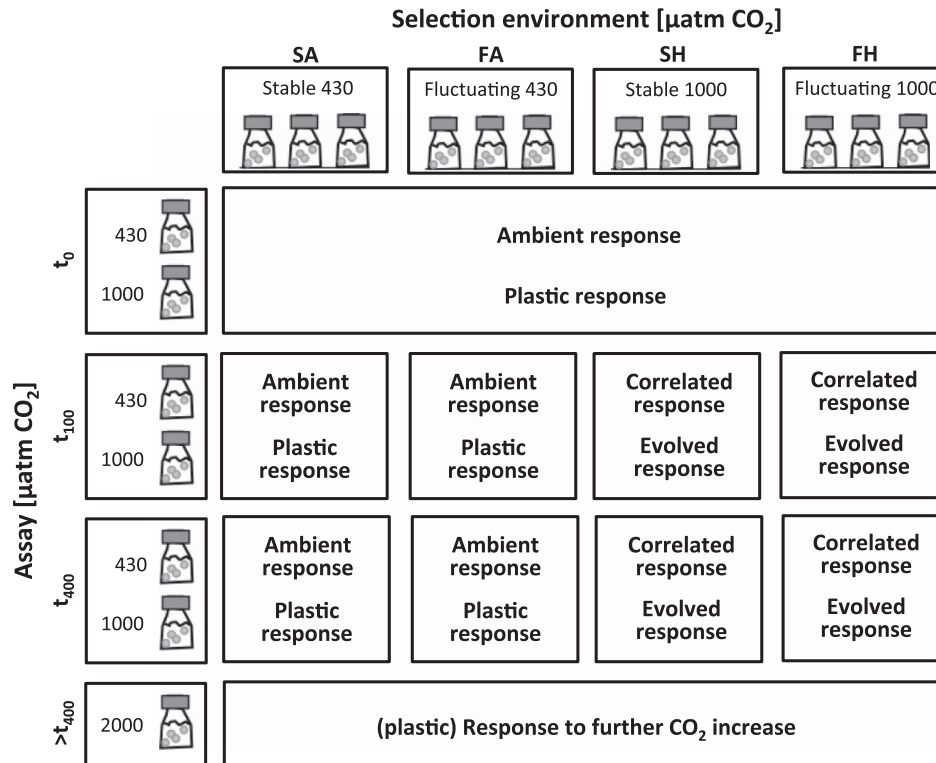


Figure 1 Experimental setup: 3 biological replicates of 16 lineages of the species complex *Ostreococcus* were evolved in 4 selection environments. These were SA ($444 \pm 43 \mu\text{atm CO}_2$), FA ($490 \pm 97 \mu\text{atm CO}_2$), SH ($1031 \pm 87 \mu\text{atm CO}_2$) and FH ($1012 \pm 244 \mu\text{atm CO}_2$). At the beginning of the experiment (t_0), as well as at 100 (t_{100}) and 400 (t_{400}) generations into the experiment, phenotypes of all samples were assayed at both 430 and 1000 $\mu\text{atm CO}_2$. We refer to SA- and FA-evolved phenotypes measured at 430 and 1000 $\mu\text{atm CO}_2$ as ambient and plastic responses, respectively. We refer to phenotypes of FH- and SH-evolved lineages measured in the 1000 $\mu\text{atm CO}_2$ assay as evolved responses. FH- and SH-evolved lineages assayed at 430 $\mu\text{atm CO}_2$ are referred to as correlated responses (see Supplementary Figure S1). Shortly after t_{400} , we also assayed all samples at 2000 $\mu\text{atm CO}_2$ and call this a response to further CO_2 increase. We discuss all but the correlated responses in detail in the main text. For correlated responses, see Supplementary Information.

underlying change in genotype (West-Eberhard, 2003). Plastic responses here are the short-term responses to elevated CO_2 levels. To measure the phenotype of the plastic response, lineages evolved at SA or FA are measured at 1000 $\mu\text{atm CO}_2$. These lineages have not evolved in high- CO_2 environments; hence, their phenotypes at high CO_2 are a measure of the phenotypic change that occurs in the absence of genetic evolution in response to long-term growth in a high- CO_2 environment. It is noteworthy that this is sometimes also called an acclimation response in other studies. Here we reserve the word ‘acclimation’ for the period where we allowed populations to acclimate to the assay environment before measuring traits. As our lineages were founded from clonal populations, we argue that any change in average population phenotype observed within 14 generations is unlikely to have arisen through *de novo* mutation or genotype sorting. We also show that the response is reversible by transferring the samples back to 430 μatm after exposure to elevated CO_2 (Supplementary Figure S1A). Likewise, the response of SA-, FA-, SH- and FH-evolved lineages to 2000 $\mu\text{atm CO}_2$ is a plastic response. Evolved responses are changes in phenotype that are due to changes in

the genotypic makeup of the population over time. Evolved traits are usually described as changes in phenotype of the evolved populations growing in their selective environment (here, FH or SH growing in high CO_2) relative to the phenotype of an ancestral or control population grown in the same environment (here, SA and FA evolved in ambient CO_2 environments but were measured in high CO_2). We refer to phenotypic traits measured in SH- and FH-evolved populations at 1000 $\mu\text{atm CO}_2$ as evolved responses and describe the correlated responses (SH and FH at 430 $\mu\text{atm CO}_2$) in the Supplementary Information (Supplementary Figure S1B).

The SA environment controlled for any changes in phenotype that might occur due to evolution in a lab environment. The FA environment selected for the maintenance and/or evolution of plasticity (that is, the ability to produce a plastic response) to fluctuations in pCO_2 alone. The SH environment selected for growth at high CO_2 alone, whereas the FH environment selected for the maintenance and/or evolution of plastic responses due to high as well as fluctuating CO_2 environment. For detailed carbonate chemistry tables for the different selection environments, please refer to Supplementary Table S2.

Physiological responses

Samples were acclimated to the appropriate CO₂ level as described above before the physiological assays. CO₂ levels of 430, 1000 or 2000 μ atm CO₂ were established through bubbling artificial seawater in the respective CO₂ incubator for at least 24 h before any cultures were added. Seawater DIC (dissolved inorganic carbon), alkalinity and pH were measured regularly as described in the Supplementary Information.

Growth rates, oxygen evolution and consumption rates. Growth rates were calculated from the intact, chlorophyll a-positive event counts within an *Ostreococcus*-specific gate of forward and side scatter on a FACS CANTO (BD Biosciences, Oxford, UK; see Supplementary Figure S2). Oxygen evolution and consumption rates were measured using a Clark-type oxygen electrode under conditions resembling those in the respective incubators (see Supplementary Methods for details).

Cellular stoichiometry. Particular organic carbon and nitrogen samples were taken for eight lineages per selection environment, using three technical replicates of each of three biological replicates. Samples were filtered onto pre-combusted (12 h, 500 °C) GF/F filters (1.2 mm; Whatman, GE Healthcare Life Sciences, Cardiff, UK) and stored in pre-combusted (500 °C) petri dishes. Filters were wrapped in tin foil and particular organic carbon and nitrogen were then measured using an Elementar Vario EL mass spectrometer (Elementar Analysensysteme GmbH, Hanau, Germany).

Cell size, chlorophyll a content and Nile Red fluorescence. Chlorophyll a content, lipid content and relative cell size were determined using a FACS CANTO (for general protocols, see (Marie *et al.*, 2005; Petersen *et al.*, 2012)). The red fluorescence channel was used as a proxy for total chlorophyll a content and was cross-calibrated using chlorophyll a calibration beads as standards (Bangs Laboratories, Inc., Fishers, IN, USA), as well as chlorophyll a extraction (Holm-Hansen and Riemann, 1978). The same channel was used to determine polar lipids after a Nile Red stain (la Jara de *et al.*, 2003; Guzmán *et al.*, 2009). Cell size was inferred from the FSC (forward scatter) and comparison with calibration beads and Coulter counter measurements. Owing to the nature of flow cytometry measurements, trait values retrieved from FACS CANTO measurements are approximate (Marie *et al.*, 2005; Petersen *et al.*, 2012), but relative changes between samples are highly informative for comparisons of strains and selection lines.

Statistics and graphical representation

Data were analysed in the R environment, using mixed models within the 'nlme' and 'lme4' packages

and models built based on (Pinheiro and Bates, 2000), to control for temporal effects of CO₂ in assay and selection environments, and to account for the same clonal lineages being used across treatments. Owing to the multiple comparisons performed in the omnibus analysis of variance (ANOVA) detecting changes between fluctuating and stable environments across all traits, selection regimes and lineages, *P*-values were adjusted through a Holm–Bonferroni approach before Tukey's honest significant difference *post-hoc* tests performed only on *P*-values < 0.05, that is, all *post-hoc* results presented throughout are already corrected for multiple comparisons. Smaller ANOVA analyses were performed to gain more detailed information, for example, on the effect of environmental stability on specific traits or groups of lineages. The number of biological replicates per lineages was three. For graphic representation, means and s.e. per lineage, as well as per CO₂ treatment, were calculated within the 'ggplot2' and 'plotrix' package. See Supplementary Information for details.

Results and Discussion

Fluctuating and stable CO₂ select for drastically different phenotypes

The plastic responses of the marine picoplankton *Ostreococcus* to elevated CO₂ before the evolution experiment have been described in Schaum *et al.* (2013), and in line with this we find elevated oxygen evolution and respiration rates, as well as an increase in cell size and C:N ratio in lineages exposed to 1000 μ atm CO₂ at t0 (before evolution) and in SA- and FA-evolved populations (at t400) assayed at 1000 μ atm CO₂. Notably, phenotypes characteristic of the plastic response to elevated CO₂ are not maintained when populations are allowed enough time to evolve in high-CO₂ environments (Figure 2, ANOVA all traits, selection environment \times assay environment $F_{3,124} = 9.69$, $P < 0.05$) and begin to reverse after 75–100 generations in the high-CO₂ environments (Supplementary Figure S3). This partial reversal of the plastic response in lineages evolving in high-CO₂ environments is extremely likely to be due to evolution. This is because first, it occurs while populations are still growing in the high-CO₂ environment and, second, it is considerably slower than the reversal of the plastic response seen when we transfer SA or FA populations from 1000 μ atm CO₂ back to 430 μ atm CO₂. There, the SA- or FA-evolved phenotype is re-established with 7–14 generations (Supplementary Figure S1A).

In the long-term, elevated CO₂ and fluctuations in CO₂ both drive evolution of phenotypes in marine phytoplankton and explain ~30% and ~60% of the variation in the evolved phenotypes, respectively (Figure 3). Lineages evolved in FH are different from those evolved in SH (ANOVA all traits, selection CO₂

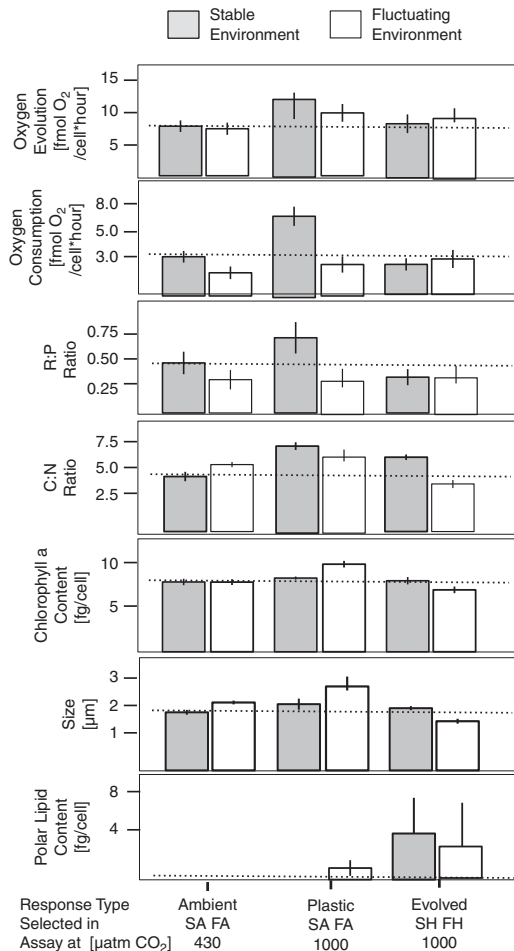


Figure 2 Ambient (left), plastic (middle) and evolved responses (right) to stable (grey) and fluctuating (white) pCO₂ of phenotypic traits in *Ostreococcus*. Selection in SH and FH environments yields different phenotypes, which also differ from plastic response. As a guideline, the dashed line represents mean values of each trait for SA lineages assayed at 430 μatm CO₂. Bars are means ± 1 s.e. *Post-hoc* results are summarized in Supplementary Table S3. In the stable environment (grey), high CO₂-evolved phenotypes differ from the ambient response with regards to oxygen evolution, oxygen consumption, R:P ratio, C:N ratio and lipid content. The plastic response of SA-evolved lineages differs from the evolved response (SH-evolved lineages) for oxygen evolution, oxygen consumption, R:P ratio, C:N ratio and lipid content. For lineages evolved in a fluctuating environment, there are significant differences between FA- and FH-evolved lineages in oxygen evolution, C:N ratio, chlorophyll a content, size and polar lipid content. The plastic response of FA lineages is different from the evolved response (that is, FH lineages) for C:N ratio, chlorophyll a content, cell size and polar lipid content. Phenotypes of SH lineages are different from those measured in FH lineages for oxygen consumption, C:N ratio chlorophyll a content and size. After evolution in SH or FH, lineages from the stable environment are characterized by lower oxygen consumption rates, higher C:N ratios, higher chlorophyll content per cell volume and larger cells than lineages that had evolved in a fluctuating environment.

(sco) × environmental fluctuation (ef), $F_{1,118} = 11.34$, $P < 0.01$, Figure 2 and also Supplementary Table S3). For example, FH-evolved lineages respire more than SH-evolved lineages do (*post-hoc* $P < 0.05$), have lower C:N ratios (*post-hoc* $P < 0.01$) and are smaller

with less chlorophyll a per cell than SH-evolved lineages (both *post-hoc* $P < 0.01$). Oxygen evolution rates, respiration to photosynthesis ratio (R:P ratio) and lipid content are not significantly different overall, but there are significant differences on the level of the individual lineages for these traits (ANOVA, $sco \times ef$, $F_{15,109} = 77.43$, $P < 0.05$, Supplementary Figure S4). Overall, we cannot use SH-evolved phenotypes to predict FH-evolved ones (see Supplementary Figure S5). Below, we describe the SH- and FH-evolved phenotypes in detail and discuss the potential implications on marine food webs and carbon cycling.

Trait evolution and their implications for productivity and higher trophic levels. Oxygen evolution rates are higher than they are at ambient CO₂ levels in both SH-evolved and FH-evolved lineages (Figure 2), with rates of 9.07 ± 0.14 and 9.6 ± 1.3 fmol O₂ per cell per hour, respectively. The average oxygen consumption rate after selection at SH is 3.2 ± 0.2 fmol O₂ per cell and 3.6 ± 0.2 fmol O₂ per cell per hour in FH. Mean R:P ratios are 0.35 ± 0.2 and 0.38 ± 0.2 for SH and FH, respectively. Even though there is a general decrease in R:P ratios in both SH- and FH-evolved lineages compared with the ancestral phenotype, there are large differences depending on the lineages' sampling origin (Figure 3 and Supplementary Figure S6, ANOVA RP ratio, $sco \times ef$, $F_{1,122} = 59.6$, $P < 0.01$).

C:N ratios in SH-evolved lineages are at 7.5 ± 0.15 and at 5.3 ± 0.3 in FH-evolved lineages (Figure 2). Strikingly, this means that SH-evolved lineages evolve a 1.34-fold higher C:N ratio than SA-evolved lineages (*post-hoc* $P < 0.01$), whereas FH-evolved lineages have lower C:N ratios than FA-evolved lineages (*post-hoc* $P < 0.01$). C:N ratios in FA are 1.2-fold higher than in FH). The decrease in C:N is particularly marked for *Ostreococcus* isolated in 'deep-sea' locations (Supplementary Figure S6, *post-hoc* $P < 0.05$).

The average size and chlorophyll content of *Ostreococcus* cells also changes in response to SH and FH. In SH-evolved lineages, *Ostreococcus* from the surface and mid-depth locations are slightly larger in size with higher chlorophyll a content per cell than 'deep sea' ones (*post-hoc* $P < 0.05$, Figure 3 and Supplementary Figure S6). On average, SH-evolved cells are 2.19 ± 0.14 μm in size and contain 10.9 ± 1.93 fg chlorophyll a (Figure 2). FH-evolved cells are smaller and contain less chlorophyll a than FA-evolved cells (both *post-hoc* $P < 0.01$, Figure 2), measuring 1.73 ± 0.03 μm and containing 9.49 ± 0.2 fg per cell. After evolution at SH and FH, intracellular polar lipid content measured as Nile Red fluorescence increases in 6 out of 16 lineages, but no change in polar lipid content was found in the other lineages (Figure 2 and Supplementary Figures S4).

Differences in C:N ratio, cell size and lipid content between lineages evolved in stable and FH environments may affect trophic interactions

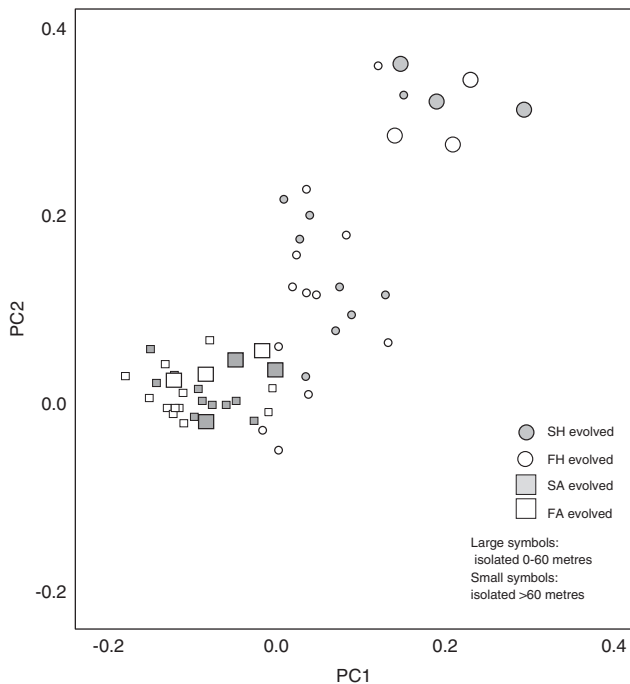


Figure 3 PCA showing that both environmental stability (PC1) and CO₂ elevation (PC2) drive phenotypic evolution of lineages. Large symbols, lineages sampled from above 60-m depth; small symbols, lineages sampled from below 60-m depth; circles, evolved in high-CO₂ environment; squares, evolved in ambient CO₂ environment; grey, evolved in fluctuating environment; white, evolved in stable environment. See Supplementary Information for a color-coded version where each lineage is represented by a unique symbol (Supplementary Figure S7). Together, fluctuation and elevation of pCO₂ explain ~90% of the variance in evolved phenotypes and lineages (within CO₂ treatments) cluster largely according to sampling depth.

and biogeochemical cycles. This is particularly the case when we also consider that frequencies of lineages within phytoplankton communities are expected to change. We have previously shown that lineages with larger plastic response to elevated CO₂ will increase in frequency in the short term and also have a larger evolutionary response in the long term (Schaum and Collins, 2014). Although between-lineage variance in growth rate is larger before evolution (0.36, see Schaum *et al.*, 2013) than after evolution in SH (0.12) and FH (0.13), our findings show that SH- and FH-evolved lineages have evolved differently.

Ostreococcus evolved in high-CO₂ environments show changes in growth rate, size and C:N ratio, indicating that future changes to productivity and carbon export by picoplankton are likely. Our data suggest that the magnitude of this change will vary locally, as there was substantial variation among lineages in some evolved traits (measured in this experiment and in Schaum and Collins, 2014), local variation in both CO₂ levels and fluctuations in CO₂ levels (Eggleston *et al.*, 2010), which will affect how natural selection acts.

Our study was done using many lineages of a single genus. However, differences in evolutionary

responses to environmental change between species is also likely to drive changes in primary production and carbon export. For example, mesocosm studies suggest that non-calcifying picoplankton, such as *Ostreococcus*, may become more frequent in future oceans, as they are less adversely affected by elevated CO₂ levels than calcifying phytoplankton (Riebesell *et al.*, 2008; Scheinin *et al.*, 2015). Calcifying phytoplankton (for example, coccolithophores) and non-calcifying phytoplankton (for example, diatoms and green algae) belong to different functional groups (Falkowski *et al.*, 1998) and have different roles in ecosystem functions and services, which means that the relationship between within-group evolution and ecosystem function is likely complex. For example, picoplankton communities of future oceans may be more productive than picoplankton communities in today's oceans, but the overall productivity and export of the mixed functional group assemblages might still be lower under CO₂-enrichment scenarios that favor an increase in frequency in picoplankton. This is because picoplankton are smaller and sink more slowly than larger plankton. However, even small picoplankton do contribute to carbon export after ingestion by planktonic grazers, or when they become heavier through aggregation (Riebesell and Wolf-Gladrow, 1992).

Size, lipid content and C:N ratio are important aspects of food quality (Thomsen *et al.*, 2013; Rossoll *et al.*, 2012). Organisms that graze on *Ostreococcus*, mainly heterotrophic nanoflagellates (Christaki *et al.*, 1999; Cuevas, 2006), are size selective and may not be physically able to ingest a food source if it doubles in size, or eat enough of a food source if it decreases substantially in size. Here, FH-evolved lineages were smaller than lineages evolved in the SH environment, with little variation between lineages. Several lineages also evolved phenotypes that stored more lipids at high CO₂ than at ambient CO₂, which may be part of a general response to elevated CO₂, causing a shift from oxidative toward reductive pathways (Rokitta *et al.*, 2012). Overall changes in fatty acid composition of marine plankton can lead to lowered reproductive success in zooplankton grazers (Rossoll *et al.*, 2012). If selection imposed in the FH CO₂ environment informs us about selection imposed by global change in oceans, grazers will have to feed on smaller, nitrogen-deplete, lipid-rich cells, and thus will have to ingest more cells than under the present day conditions. If the overall phytoplankton biomass in some lineages decreases, grazers and filter feeders may not be able to fulfil their nutritional requirements, or, alternatively, changes in phytoplankton may drive a co-evolutionary response in grazers. As the plastic response of *Ostreococcus* to elevated CO₂ is different from the evolutionary response, grazers, with longer generation times than phytoplankton, may have to cope with an increasingly variable food source. There are a number of evolutionary responses to changes in their food source that could occur in grazers. For example, grazers may use conservative or diversified bet-hedging strategies to

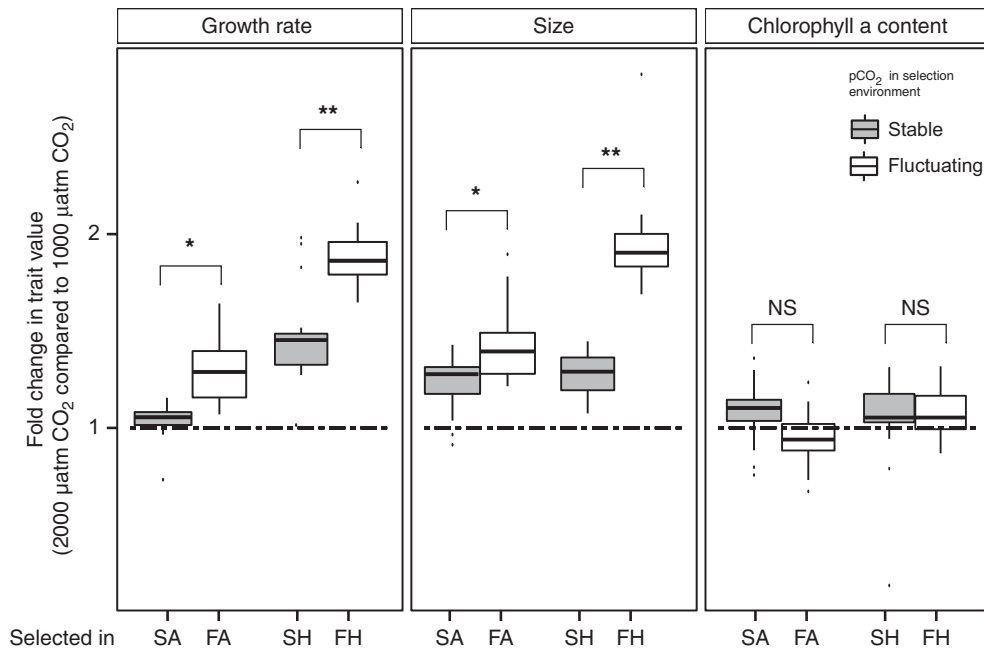


Figure 4 Selection history affects responses to different levels of elevated pCO₂. Here, grey boxes represent pooled lineages from stable environments (SA and SH) and white boxes represent pooled lineages from fluctuating environments (FA and FH). Boxplots are displayed with whiskers extending to the highest and the lowest values that are within 1 interquartile range (IQR) of lower/upper quartile. $n = 3$ per lineage. NS, difference between lineages from stable and fluctuating selection regimes not significant; * $P < 0.05$ and ** $P < 0.01$, significant difference between lineages from stable or fluctuating environment, respectively. All values are displayed as fold change compared with the response to 1000 µatm at t400. This is the evolved response for FH and SH, and the plastic response for SA and FA. Any values larger than 1 indicate that the response to pCO₂ 2000 µatm is stronger than the plastic or evolved (for SH and FH) response to pCO₂ 1000 µatm.

minimize fitness variance in the long term. Here the organisms either use the same, low-risk strategy (conservative) or invest in several strategies at once (diversified) (Beaumont *et al.*, 2009; Ripa *et al.*, 2010). In addition, grazers themselves may respond plastically to changes in their food source by switching the feeding mechanisms depending on the food source present in their habitat (Mariani *et al.*, 2013). Finally, it is also possible that grazers will adjust to more variable food sources through evolution and adaptive tracking (that is, mutants arise and fix to fit the food source when it changes), or use a combination of the responses above.

Exposure to elevated fluctuating CO₂ levels broadens the scope to react to further CO₂ enrichment

A striking difference between lineages evolved in stable versus fluctuating environments in our experiment is how they respond to further increases in pCO₂. Most CO₂-enrichment experiments for marine phytoplankton use partial pressures of CO₂ of around 700–1000 µatm as their high-CO₂ environment. As CO₂ levels are expected to rise further and can also transiently rise above these levels today (IPCC report, 2007), it is important to investigate how evolution in moderately high-CO₂ environments affects the response to short-term exposure to even higher CO₂ levels.

Both 1000 and 2000 µatm CO₂ are considered to be ‘high-CO₂ conditions’, but the ability of lineages to modulate their growth rates depends on the high-CO₂ environment they experienced in

their previous selection environment (ANOVA growth, response 2000 µatm × selection environment, $F_{3,105} = 138.72$, $P < 0.0001$). Specifically, only lineages evolved in elevated or fluctuating CO₂ environments grow differently at 1000 and 2000 µatm CO₂, whereas SA-evolved lineages have the same growth rate in both 1000 and 2000 µatm CO₂ (*post-hoc* $P = 0.67$ for SA-evolved lineages, Figure 4). In FA-evolved lineages, which were transiently exposed to higher CO₂ levels during evolution, growth rates are on average 1.22-fold higher at 2000 than at 1000 µatm CO₂ (*post-hoc* $P < 0.001$, Figure 4). In SH- and FH-evolved lineages, growth rates at 2000 µatm CO₂ were 1.5- and 1.9-fold higher than at 1000 µatm CO₂, respectively (*post-hoc* difference μ SH and FH at 2000 µatm < 0.01). Changes in cell size in response to 2000 µatm CO₂ were also larger in lineages from the FH environments than in lineages from the SH environment (ANOVA trait values, response 2000 µatm × selection environment, $F_{3,78} = 2.89$, $P < 0.05$), whereas there was no significant difference in chlorophyll levels.

The differences in lineages evolved in stable and fluctuating environments show that evolving or maintaining the ability to distinguish between different high CO₂ levels, even those outside the range of CO₂ levels encountered in the past few hundred generations of evolution, only requires occasional exposure to high CO₂. In our experiment, even the highest CO₂ levels in the FA environment were lower than those in either SH or FH, yet

responses of FA to 2000 μatm were comparable to those of lineages evolved in the stable and FH CO_2 selection regimes. Although our experiment did not test this directly, we suggest that evolving in an environment where fluctuations are random has a role in lineages evolving the ability to specifically modulate their responses to further changes to the environment. Our reasoning is that an environment switching between two set levels of CO_2 could select for generalists displaying the same phenotype across environments or a population composed of specialist subpopulations with distinct phenotypes in high- and low- CO_2 environments, but no specific evolution of the ability to deal with or distinguish between different high levels of CO_2 (Kassen, 2002; Ketola *et al.*, 2013). One possible reason that lineages evolved in fluctuating environments are able to respond by increasing growth at 2000 μatm CO_2 is that they are not operating near limits set by oxidative damage (for example, see Dowling and Simmons, 2009) and are thus metabolically better equipped to deal with further perturbations to their environment. This is consistent with previous work showing the eventual evolution of reduced population growth rates but higher heat shock survival and better competitive abilities against novel conspecific competitors in *Ostreococcus* lineages evolving in high- CO_2 environments (Schaum and Collins, 2014).

Regardless of the underlying cause, we show that evolving in fluctuating environments broadens the scope for using plastic responses in the face of continued environmental change (Figure 4). This is not limited to further increases in $p\text{CO}_2$, but also extends to reduced CO_2 levels: the correlated responses show that FH-evolved lineages grew more rapidly in their ancestral environment (430 μatm CO_2) than did the SH-evolved lineages (Supplementary Figure S1A and B and details in Schaum and Collins, 2014).

These findings indicate that both the average environment and the stability of the environment during evolution affect how populations respond to subsequent environmental fluctuations, and that changes in the stability of marine environments will likely have a key role in driving evolutionary outcomes. Taken together, the differences between lineages evolved in stable and fluctuating environments show that selection experiments carried out under stable conditions will fail to detect key changes in phenotype associated with evolving in fluctuating environments, in particular as we cannot use SH-evolved phenotypes to predict FH-evolved ones.

Conclusions

Phenotypic evolution in high- CO_2 environments depends on both deviations from the mean CO_2 level and the absolute CO_2 levels, and this is consistent over several lineages of a species complex. This study shows that fluctuations in CO_2 , in

addition to differences in average CO_2 , are likely to be important drivers of evolutionary change in green algae as CO_2 levels rise. Although the evolutionary response to long-term CO_2 enrichment is to partially revert the plastic (short term) response, persistent changes in cellular size and composition remain. This indicates that evolutionary responses to high and fluctuating CO_2 can change the role of marine phytoplankton in food webs and nutrient cycles.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

ES designed and performed the experiments, analysed data and wrote the manuscript. SC designed the experiments, analysed data, wrote the manuscript and supervised the laboratory work. BR supervised the laboratory work at the Alfred Wegener institute and contributed to the writing of the manuscript.

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