

Changes in C uptake in populations of *Chlamydomonas reinhardtii* selected at high CO₂

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ABSTRACT

Estimates of the effect of increased global atmospheric CO₂ levels on oceanic primary productivity depend on the physiological responses of contemporary phytoplankton populations. However, microalgal populations will possibly adapt to rising CO₂ levels in such a way that they become genetically different from contemporary populations. The unknown properties of these future populations introduce an undefined error into predictions of C pool dynamics, especially the presence and size of the biological C pump. To address the bias in predictions introduced by evolution, we measured the kinetics of CO₂ uptake in populations of *Chlamydomonas reinhardtii* that had been selected for growth at high CO₂ for 1000 generations. Following selection at high CO₂, the populations were unable to induce high-affinity CO₂ uptake, and one line had a lower rate of net CO₂ uptake. We attribute this to conditionally neutral mutations in genes affecting the C concentrating mechanism (CCM). Lower affinity CO₂ uptake, in addition to smaller population sizes, results in a significant reduction in net CO₂ uptake of about 38% relative to contemporary populations under the same conditions. This shows how predictions about the properties of communities in the future can be influenced by the effect of natural selection.

Key-words: carbon concentrating mechanism; experimental evolution; natural selection.

INTRODUCTION

Plant growth depends on CO₂ concentration (reviewed by Urban 2003), which is expected to increase from current levels of about 400 ppm to between 700 and 1000 ppm during the next century (Watson *et al.* 2001). Oceanic primary production constitutes about 46% of the total primary production on earth (Field *et al.* 1998), and experiments examining how C uptake by microalgae responds to rising CO₂ are needed to understand how oceanic primary production will change in the future. The ocean–atmosphere flux is

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partially controlled by a biological pump, by which dying phytoplankton sink C into deep ocean sediments. Mathematical simulations have estimated that pre-industrial levels of CO₂ would have been as high as 460 ppm without the operation of such a pump (Sarmiento & Toggweiler 1984), whereas pre-industrial atmospheric CO₂ levels were around 280 ppm (Etheridge *et al.* 1996). The discrepancy between a model ocean lacking biologically mediated C fixation and an ocean with biological C fixation suggests that biotic sequestration of C plays an important role in regulating atmospheric CO₂ levels. It has been suggested that increases in CO₂ may lead to an increase in algal biomass, which would in turn lead to more CO₂ being removed from the atmosphere by these algae. In addition to these ecological responses to rising CO₂, microalgal communities may also adapt to the increased supply of CO₂, resulting in unknown long-term changes to C uptake and cycling in oceans.

Many microalgal species respond to CO₂ limitation by the induction of a C concentrating mechanism (CCM) (Badger *et al.* 1998; Sültemeyer 1998; Badger and Spalding 2000). The CCM is an inducible system that enables microalgae to respond to extracellular changes in inorganic C, and occurs in most microalgal species studied to date (Colman *et al.* 2002). The CCM elevates CO₂ concentration in the vicinity of ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco), the main carboxylating enzyme in C fixation, when C is scarce (Moroney & Somanchi 1999). Conversely, CCM expression decreases at higher levels of CO₂, resulting in lower affinity C uptake when inorganic C is abundant (Bozzo & Colman 2000). This presumably allows algae to avoid paying the cost of unnecessary enzyme production and active transport.

Over the long term, changes in CO₂ levels have the potential to affect two different processes connected to phytoplankton growth: C uptake and C fixation. Most predictions of phytoplankton responses to CO₂ enrichment make the tacit assumption that a functional CCM will continue to exist in phytoplankton and that their basic physiology will remain more or less unchanged as CO₂ rises. If prolonged growth at elevated CO₂ can result in drastic changes to the CCM or to C fixation, considerable uncertainty would be introduced into predictions about the outcome of competition between populations, as well as estimates of global C pool dynamics.

The presence of a CCM in microalgae buffers the C fixation machinery from changes in extracellular inorganic C concentrations. In some cases, growth and photosynthesis rates are nonetheless stimulated by elevated concentrations of CO₂ within the range of expected global increases (Hein & Sand-Jensen 1997). In other cases, primary productivity of species or species assemblages of phytoplankton are more or less insensitive to extracellular increases in inorganic C (Tortell & Morel 2002). Even in replicate lines descending from isogenic ancestors, both increases in growth and insensitivity to CO₂ enrichment have been reported (Collins & Bell 2004). This variability in responses over all time scales makes it difficult to make general predictions about how phytoplankton populations may respond to elevated CO₂, even when other complicating factors are ignored, such as changes in other nutrients or competition between species.

Variability in the responses of phytoplankton to CO₂ enrichment has led to considerable debate over the role that C uptake by phytoplankton plays in the sequestration of anthropogenic CO₂. This has inspired several experiments designed to evaluate the presence and magnitude of a biological C pump (Honda 2003; Buesseler *et al.* 2004; Coale *et al.* 2004). For anthropogenic CO₂ to be 'sunk' by phytoplankton, it must stimulate increases in net primary productivity, either by increasing rates of net C uptake or by increasing population sizes. Although the response of phytoplankton to changes in CO₂ remains controversial, estimates of increases in primary production of 15–19% in response to reasonable increases in CO₂ have been observed in natural populations in unenriched seawater from the central Atlantic Ocean (Hein & Sand-Jensen 1997).

We previously used a microalgal model system, *Chlamydomonas reinhardtii*, to evaluate changes in fitness caused by the spread of novel mutations over 1000 generations in response to increasing CO₂ (Collins & Bell 2004). We found that there was no direct response to selection at high CO₂, whereas many of the high selected lines had lowered fitness at ambient CO₂. The lines also had lower maximum population densities at high CO₂, despite normal or increased rates of photosynthesis. From these characters, we suggested that this negative correlated response was caused by the accumulation of conditionally deleterious mutations in the CCM, though this hypothesis was not directly tested. Here, we measure rates and affinities of CO₂ uptake by mass spectrometry to evaluate the hypothesis that prolonged growth at elevated CO₂ can result in the degradation of high-affinity CO₂ uptake. A mass spectrometer was used to distinguish between the following hypotheses: that high selected lines were unable to take up C, that high selected lines were leaking C or that high selected lines were unable to fix C at ambient levels of CO₂, even though it was actively being taken up. Because changes in CO₂ uptake have the potential to affect larger-scale ecological processes, we then use these results to explore the possible effect of evolutionary change on net C uptake by algal populations.

MATERIALS AND METHODS

Selection experiment

We founded 10 replicate lines from a single clone of M566B (laboratory isolate), and 10 replicate lines were founded from a single clone of CC-2344 (*Chlamydomonas* Genetics Center, Duke University, Durham, NC, USA). Five replicates from each clone were grown in high and ambient CO₂ environments, respectively. The ambient CO₂ environment consisted of flasks being bubbled with air containing 430 ppm CO₂ for the entire experiment. Lines in the high CO₂ treatment were initially grown in flasks being bubbled with air containing 430 ppm CO₂, and CO₂ levels were raised steadily to 1050 ppm over the first 600 generations of the experiment. These lines were then grown at 1050 ppm CO₂ for a further 400 generations. Lines were propagated by batch culture grown in bubbled flasks containing 300 mL of Suoka high salt medium (HSM) (Harris 1989) (pH 7) in a chamber in the McGill Phytotron under constant light at 25 °C. We transferred 1 mL of culture (about 10⁵ cells) every 3–4 d for approximately 1000 generations for each replicate line.

Maximum population densities

Maximum population densities were measured in 384-well plates containing 90 µL HSM per well. The cultures were first acclimated (3–6 d), then diluted and transferred to assay plates. For two evolved lines that often failed to grow at ambient CO₂, several extra acclimation cultures were inoculated, and the surviving cultures were used for growth assays. This may have allowed some backselection of the high selection lines, so that the results presented here are conservative. The plates were grown in the same phytotron chamber as previously mentioned at either 430 or 1050 ppm CO₂. Absorbance of each culture was measured every 24 h. Relative maximum densities were calculated from the maximum absorbance maintained by a culture. The average maximum density obtained by the control cultures growing under ambient CO₂ was arbitrarily given a value of 1. Three independent triplicate measurements were made.

C flux and photosynthesis measurements

We chose three high selection lines which differed in photosynthesis rates, competitive fitness and maximum population densities. CO₂ exchange rates and photosynthetic O₂ evolution in whole cells were measured by mass spectrometry as described by Amoroso *et al.* (1998), with the following modifications. Cells were acclimated to either 1050 ppm CO₂ (high CO₂) or to air (ambient CO₂) for 24 h. Washed cells were resuspended in an HSM buffered with 50 mM bistrisphosphate (BTP) at pH 7.0 at 25 °C. Because it is necessary to inhibit the extracellular carbonic anhydrase (CA) to use this method, 20 µM acetazolamide (AZA) was added to the cells. AZA inhibits cell surface CA, but cannot enter the cell. All selection lines (high and control) as well as the wild-type line had external CA activity. We

determined that this concentration of AZA had no effect on intracellular CA, but inhibited external CA in the high and wild-type lines. All our control lines had evolved insensitivity to AZA and autolysin, presumably because of changes in cell wall composition. Because the previous method could not be used on them, the well-characterized wild-type strain [11–32b from culture collection of algae, University of Göttingen, Göttingen, Germany; described in Amoroso *et al.* (1998)] was used for comparison in CO₂ uptake. Control cultures were used for photosynthetic O₂ evolution, as this does not require inhibition by AZA. CO₂ fluxes were calculated using previously published formulas (Badger, Palmqvist & Yu 1994). In short, gross CO₂ uptake and CO₂ leakage were measured, and net CO₂ uptake was calculated by difference. Bicarbonate uptake was measured. Net total inorganic C uptake was calculated as bicarbonate uptake + net CO₂ uptake. $K_{0.5}$ (half-saturation constant) and V_{max} (maximum rate of uptake) were calculated by least squares non-linear regression using Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). We made two or three independent measurements for each estimate.

Net CO₂ uptake calculations

Expected steady-state CO₂ uptake was calculated as maximum population density (relative maximum cell number/population) × [rate of net CO₂ uptake/unit chlorophyll (Chl)]. The rate of CO₂ uptake at a given CO₂ concentration was calculated using the standard Michaelis-Menten formula for a single-substrate reaction from the estimated values of $K_{0.5}$ and V_{max} for cells acclimated to either high or ambient CO₂. CO₂ concentrations used in the calculations were the concentration of dissolved CO₂ present in a buffered solution at pH 7 and 25 °C at equilibrium, given atmospheric concentrations of 1050 ppm (high CO₂) or 430 ppm (ambient CO₂ during selection experiment). The calculations are given in the Appendix.

RESULTS

Changes in kinetic parameters

All data discussed are for populations. Populations can no longer be considered clonal after 1000 generations of growth and mass transfer, so the error bars represent error + genetic variance in all cases.

Figure 1a and b shows the maximum rates of CO₂ uptake for wild-type and high selection lines. The maximum rate of gross CO₂ uptake at a given level of CO₂ is slightly lower in high selected lines than in wild-type lines ($F_{1,20} = 4.83$, $P = 0.04$), indicating that the high selected lines do not take up CO₂ as quickly as wild type. The response of wild-type and high selected lines to changes in CO₂ is the same.

The V_{max} values for net CO₂ uptake vary considerably among high selection lines, but they have a somewhat lower net V_{max} than wild type at a given level of CO₂ ($F_{1,16} = 4.83$, $P = 0.04$). One high selected line (line 10) has a lower maximum rate of net CO₂ uptake than the other high selection

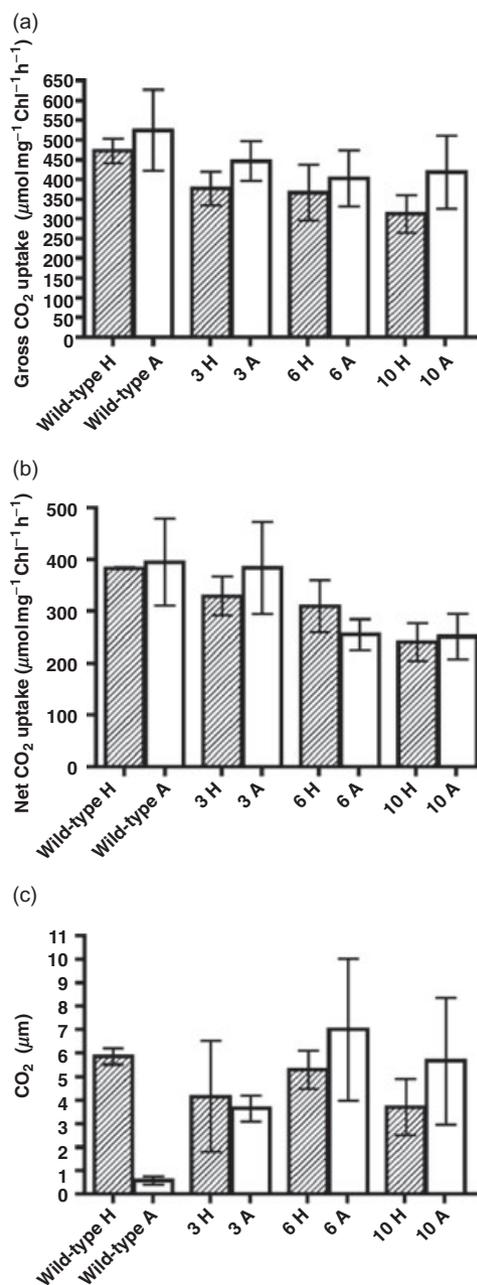


Figure 1. (a) Maximum rate of gross CO₂ uptake in wild-type and high selection (numbered) lines acclimated to 1050 ppm CO₂ (filled bars, H) and air (white bars, A). (b) Maximum rate of net CO₂ uptake in wild-type and high selection (numbered) lines acclimated to 1050 ppm CO₂ (filled bars, H) and air (white bars, A). (c) $K_{0.5}$ of net CO₂ uptake in wild-type and high selected (numbered) lines acclimated to 1050 ppm CO₂ (filled bars, H) and air (white bars, A). In all cases, each bar represents averages ± standard error of the mean (SEM) from independent duplicate or triplicate measurements. $K_{0.5}$, half-saturation constant; Chl, chlorophyll.

lines. This line appears unable to prevent CO₂ from leaking out of the cells. The decrease in net CO₂ uptake in the high selection lines is not a consequence of parallel changes in net C fixation rates.

Figure 1c shows $K_{0.5}$ for net CO_2 uptake in the wild-type and high selection lines. When grown at high CO_2 , wild-type and high selection lines have similar $K_{0.5}$ values, showing that at high CO_2 , substrate affinity has not been changed by selection. Wild-type lines show the expected response in $K_{0.5}$ to a decrease in CO_2 . There is a selection–assay interaction ($F_{1,14} = 3.57$, $P = 0.08$), indicating that the reaction norm has been changed by selection, such that the high selection lines are unable to induce high-affinity CO_2 uptake at ambient CO_2 .

In the affinity for CO_2 , the high selection lines do not differ from the wild-type line when grown at high CO_2 , which is consistent with our previous findings that there is no direct response to selection after 1000 generations of selection at high CO_2 . However, the high selection lines have lower affinity for CO_2 when grown at ambient CO_2 . This is consistent with both the lower maximum population densities and competitive fitnesses of these lines that have been previously reported (Collins & Bell 2004).

Although the high selection lines were unable to induce high-affinity CO_2 uptake, the extent of impairment varies among lines. One of the lines (line 3) has a normal maximum rate of net CO_2 uptake, and is able to prevent CO_2 from leaking out of the cell as effectively as the control and wild-type lines. This line also appears to have a $K_{0.5}$ for net CO_2 uptake that is intermediate between the wild-type and other high selection lines. The other two high selection lines, however, have lower V_{\max} for net CO_2 uptake, indicating that the cells are leaking CO_2 , and also appear to have much higher $K_{0.5}$ for CO_2 uptake. Interestingly, line 10, which has the lowest V_{\max} and also has a high $K_{0.5}$, was often unable to grow at ambient CO_2 , while the other two selection lines always grew.

Representative data for bicarbonate uptake is shown in Fig. 2a. The affinity for total inorganic C (bicarbonate + CO_2) is shown in Fig. 2b. All lines are capable of taking up bicarbonate. Bicarbonate uptake is important when inorganic C is very scarce, and lowers the $K_{0.5}$ values of total inorganic C uptake relative to net CO_2 uptake. Bicarbonate uptake is undetectable when CO_2 is abundant, so the V_{\max} values are unaffected. Microalgae have been shown to shift from bicarbonate to CO_2 uptake as the amount of available CO_2 increases (Tortell & Morel 2002), which can occur either as a result of an increase in total C or a decrease in pH. Evidence of this can be seen in all lines when the affinity of CO_2 uptake is compared with the affinity of total inorganic C uptake. $K_{0.5}$ values for total inorganic C uptake are lower than for net CO_2 uptake in all cases [$t = 4.38$, degrees of freedom (d.f.) = 7, $P = 0.002$]. The wild-type line shows an induction of higher affinity inorganic C uptake when CO_2 is lowered, but only one of the three high selection lines can induce higher affinity total C uptake. The wild-type shows a 12.5-fold increase in affinity for inorganic C, while the high selection line shows a 3.8-fold increase. In this case, the increase in affinity is attributable to increased bicarbonate uptake at CO_2 levels well below ambient. This would not contribute to higher affinity C uptake when CO_2 is lowered from high to ambient levels,

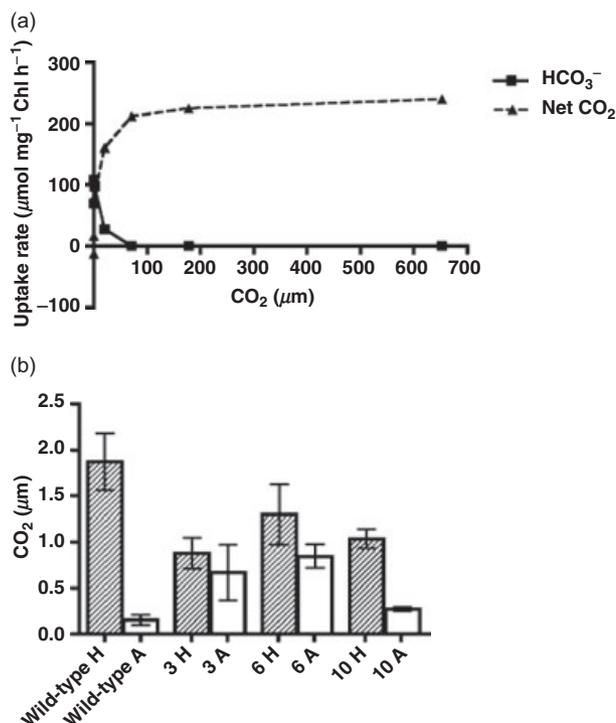


Figure 2. (a) Representative bicarbonate uptake data (squares) from a high selection line. Control and wild-type lines show a similar pattern. Net CO_2 uptake (triangles) is shown to illustrate the relative contributions of CO_2 and HCO_3^- as the concentration of external CO_2 increases. (b) $K_{0.5}$ of net total inorganic C uptake in wild-type and high selection (numbered) lines acclimated to 1050 ppm CO_2 (filled bars, H) and air (white bars, A). Net total inorganic C uptake was calculated as (HCO_3^- uptake + net CO_2 uptake). Each bar represents averages \pm standard error of the mean (SEM) from independent duplicate or triplicate measurements. $K_{0.5}$, half-saturation constant; Chl, chlorophyll.

because bicarbonate uptake does not contribute to total inorganic C uptake at either high or ambient CO_2 . As such, the kinetics of bicarbonate uptake at very low external concentrations of C does not appreciably change the conclusions reached using the CO_2 uptake data alone.

Figure 3a and b shows V_{\max} and $K_{0.5}$ for photosynthetic O_2 evolution. The wild-type and control lines show the same affinity photosynthesis at high CO_2 . However, the control line shows higher affinity photosynthesis at ambient CO_2 than the wild type, which may be a result of selection for growth at ambient CO_2 .

There is a significant effect of selection on V_{\max} ($F_{1,1} = 16.96$, $P < 0.001$), such that the high lines show a reduced maximum rate of photosynthesis. There is no significant effects of selection on $K_{0.5}$ ($F_{1,1} = 3.60$, $P = 0.72$), but there is a significant selection–assay interaction ($F_{1,1} = 9.75$, $P = 0.005$), indicating that the reaction norm has been changed by selection. In this case, the high selected lines fail to change the affinity of photosynthesis when CO_2 levels change. The lower rate of photosynthesis could be due to changes in levels, regulation or activity of Rubisco or

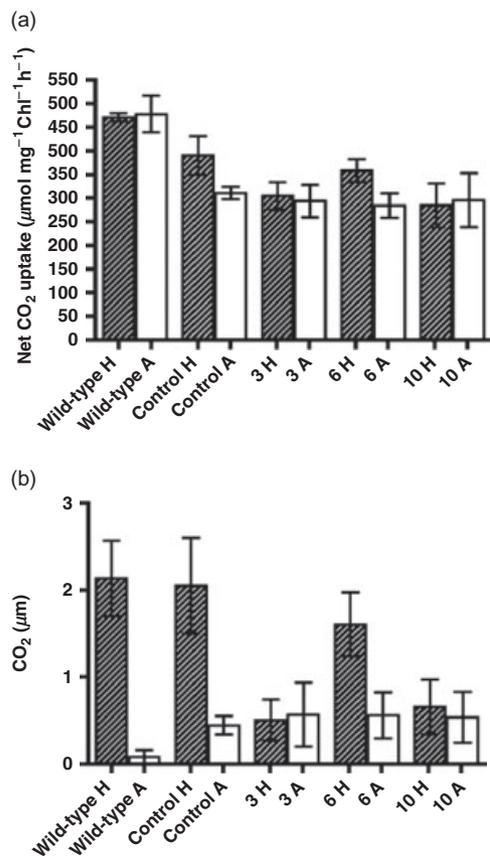


Figure 3. (a) Maximum rate of photosynthetic O₂ evolution in wild-type, control and high selection (numbered) lines acclimated to 1050 ppm CO₂ (filled bars, H) and air (white bars, A). (b) K_{0.5} of photosynthetic O₂ evolution in wild-type, control and high selected (numbered) lines acclimated to 1050 ppm CO₂ (filled bars, H) and air (white bars, A). In all cases, each bar represents averages \pm standard error of the mean (SEM), from independent duplicate or triplicate measurements. K_{0.5}, half-saturation constant; Chl, chlorophyll.

CA, but this was not measured in this study. The high selected lines have higher affinity photosynthesis at high CO₂ than the wild-type and control lines ($F_{1,1} = 9.51$, $P = 0.01$). This may partially compensate for the reduced affinity of CO₂ uptake into the cells, as we have previously shown that the high lines do not show a decrease in the actual rate of photosynthetic oxygen evolution at 1050 ppm CO₂ relative to the control lines (Collins & Bell 2004). At ambient CO₂, the high lines are not significantly different than the control line either in affinity or maximum rate of photosynthesis.

The control line has the same response to decreased CO₂ as does the wild-type line (assay–line interaction $F_{1,1} = 0.32$, $P = 0.60$), showing higher affinity photosynthetic O₂ evolution at ambient CO₂ than at high CO₂ ($F_{1,1} = 20.61$, $P = 0.006$). One of the high selected lines also shows this response but the other two fail to induce higher affinity photosynthesis when CO₂ is lowered. Most lines show slightly higher maximum photosynthesis rates than would

be expected from CO₂ uptake rates alone; this is because of some bicarbonate uptake at low external concentrations of inorganic C.

Net C uptake calculations

Primary production will respond to changes in population size or in the kinetic parameters of C uptake in the individuals making up the populations. We combined changes in population density and kinetic parameters to describe how C pool dynamics might change as a result of selection. Net C uptake was calculated for each population at a given level of CO₂ using the maximum population size and the kinetic parameters estimated for that population. Table 1 shows the expected relative net CO₂ uptake by contemporary (wild type) and high selection lines at current and projected levels of CO₂. Both high selection and wild-type lines show a significant increase in net CO₂ uptake when CO₂ is increased ($F_{1,4} = 125.8$, $P < 0.001$). In all cases, however, the high selected lines have lower net CO₂ uptake than do wild-type lines at a given level of CO₂ ($F_{1,4} = 50.0$, $P = 0.002$). On average, the estimates of net CO₂ uptake per population at high CO₂ is approximately 38% less in the high selection lines than in the wild-type lines at high CO₂. In the wild-type populations, net CO₂ uptake increases by 2.4-fold when atmospheric CO₂ increases. In contrast, comparing a wild-type population with a high selection line shows only a 1.5-fold increase between current and projected rates of net CO₂ uptake.

DISCUSSION

The behaviour of future phytoplankton populations is of great ecological interest, in part because they have the potential to affect global CO₂ levels. Over the short term, increasing the external concentration of CO₂ can cause the phytoplankton to shift from one species of inorganic C (bicarbonate) to another (CO₂), accompanied by a decrease in CCM activity (Burkhardt *et al.* 2001; Tortell & Morel 2002; Rost *et al.* 2003). The longer term outcome of growth at elevated CO₂ is seen in our high selection lines, where CCM activity became degraded to the point where

Table 1. Relative rates of net CO₂ uptake per population in wild-type and high selected lines at current and high levels (1050 ppm) of CO₂

	Ambient (current) CO ₂	High (projected) CO ₂
Wild type	250	607
High selected lines	57.6 \pm 16	375 \pm 26

Rates are in arbitrary units, and are only intended to show relative values. Rates for high selected lines represent the average of the three selected lines studied \pm standard error (SE). Rate for wild-type line is estimated from a single wild-type line (11–32b). For calculation procedure, see Appendix.

high-affinity uptake could no longer be induced at ambient CO₂, though it would normally be expressed at ambient CO₂ concentrations in this species (Bozzo & Colman 2000). In this case, the high selection lines are not being directly compared to an ancestor; however, the control line could induce higher affinity photosynthesis, suggesting that our control line is not qualitatively different from wild type. In addition, the control lines originate from standard unmutagenized laboratory cultures and grow normally in air, which strongly suggests that they have a functional CCM. The complete disappearance of high-affinity CO₂ uptake is a fundamental difference in C uptake between wild-type lines of *Chlamydomonas* and the high selected lines characterized here. The induction of higher affinity CO₂ uptake at ambient CO₂ is a well-documented response in laboratory and natural populations of most microalgae and cyanobacteria, including *Chlamydomonas* (Miyachi, Iwasaki & Shiraiwa 2003). In this study, we evaluate three possible causes for the phenotypes of algae selected at elevated CO₂: changes in CO₂ uptake, changes in CO₂ leakage and changes in CO₂ fixation. Our data show that long-term increases in atmospheric CO₂ can degrade CCM induction, leading to greater dependence on CO₂ entry into cells by diffusion, and in some cases, more CO₂ leakage from the cell. An increased dependence on diffusion at elevated CO₂ may be partially compensated for by higher affinity photosynthesis in some of the high selected lines.

The loss of high-affinity CO₂ uptake during long-term growth at elevated CO₂ calls into question the tacit assumption that CCM function will remain unchanged as CO₂ rises. This assumption is often the basis for predictions about the behaviour of future phytoplankton populations. For example, the presence of a CCM has been used to suggest that elevated CO₂ alone is unlikely to affect some species (Casar, Laws & Bidigare 2004), as well as to explain interactions between species that may occur at different levels of CO₂ (Tortell 2000; Riebesell 2000). Our demonstration that this very basic physiological feature can change in response to elevated CO₂ has major implications for estimations of future population sizes and CO₂ uptake affinities, which directly affect the amount of CO₂ that is fixed by marine populations. Changes in CCM induction also introduce uncertainty into predictions of relative fitnesses of future populations, which determine the outcome of competition between types.

Previous studies have described CCM knockout phenotypes that resemble our high selection phenotypes (Suzuki & Spalding 1988; Spalding *et al.* 2002; Thyssen, Hermes & Sültemeyer 2003; Soupene, Inwood & Kustu 2004). The results obtained in this study show that the kinetics of CO₂ uptake in the high selection lines has not changed at high CO₂, whereas CO₂ uptake is impaired at ambient CO₂. Consequently, we suggest that the high selection phenotypes can be attributed tentatively to conditionally neutral mutations in genes encoding the CCM or related systems during selection at high CO₂. Because the great majority of reported selection experiments show a direct response to selection, it is often difficult to attribute a negative

correlated response unambiguously to mutation accumulation rather than to antagonistic pleiotropy (Turner & Elena 2000; Maclean & Bell 2002), though studying the dynamics of responses can indirectly demonstrate the presence of mutation accumulation (Funchain *et al.* 2000). In this case, there was no increase in competitive fitness, growth rate or maximum sustainable population density in the high selection lines (Collins & Bell 2004), which strongly suggests that the correlated response is due to mutation accumulation and not to antagonistic pleiotropy. Clear examples of mutation accumulation in large (non-bottlenecked) populations during a selection experiment are rare (Goho & Bell 2000; Giraud *et al.* 2001), and are often associated with elevated mutation rates. Our results show that it is possible for mutation accumulation to play an important role in adaptive outcomes, even in large populations and in the presumed absence of mutators. The degeneration of an unused metabolic pathway has been studied in yeast, in which it was found that most or all genes in an unused pathway were inactivated or lost in independent lineages, suggesting that gene inactivation or loss may be common following ecological change (Hittinger, Rokas & Carroll 2004). In addition, some natural populations of microalgae without CCMs have been found, often in environments where it is thought that a CCM would not be needed for growth, though a straightforward link between CCM characters and algal biology is not always possible (Raven 2003). Given that CO₂ levels have varied over evolutionary time, it is reasonable to suspect that some conditionally deleterious mutations affecting C uptake are fixed in contemporary phytoplankton populations. Further genetic work was not pursued in this study because the underlying genetics have little bearing on characters that are interesting for practical purposes, such as the ability to sink CO₂ into the deep ocean or the ability to compete with other species. In cases such as the one presented in this paper, where the phenotype is of ecological interest, phenotypic convergence between replicate lines can be used to estimate the range of future phenotypes possible. Our finding that a non-adaptive process such as mutation accumulation is likely to affect CO₂ uptake allows a more realistic view of how phytoplankton populations may respond to elevated CO₂.

These results have important implications for projected changes in primary production and C sequestration in response to anthropogenic increases in atmospheric CO₂. For increased CO₂ to have an effect on C sequestration to the deep ocean, it must affect primary production, that is, natural populations must be limited by CO₂ (Riebesell, Wolf-Gladrow & Smetacek 1993). Though CO₂ limitation of natural populations remains controversial, it has been shown to exist in some laboratory (Riebesell *et al.* 1993) and natural populations (Chen & Dubin 1994; Hein & Sand-Jensen 1997). Our results show that high CO₂ selection lines have a compromised CCM and attain smaller population sizes. As a result of this, the stimulation of net C uptake in evolved populations could be significantly less than that projected from the responses of contemporary

populations if elevated CO₂ can affect populations through non-adaptive change. Doubts have been voiced over the future possibility of algae acting as a large C sink for anthropogenic CO₂ (Hein & Sand-Jensen 1997; Buesseler *et al.* 2004; Cassar *et al.* 2004), even without any sort of evolutionary change in phytoplankton.

The outcome of our experiments suggests that over the next century, phytoplankton may evolve a decreased ability to induce high-affinity C uptake because of the accumulation of conditionally deleterious mutations in the CCM. The goal of this experiment was not to create a realistic scenario to quantify changes in primary productivity and the biological C pump. Rather, by changing CO₂ alone in a controlled environment with initially isogenic populations, we have demonstrated the magnitude of bias that evolutionary change can introduce into estimates of future CO₂ uptake by phytoplankton populations. To do this, we used a freshwater algae that could grow very rapidly such that mutations could occur, fix and be detected, and at a pH where the results were attributable to growth at elevated CO₂. We do not intend that these results be applied directly to marine microalgae, which experience a different abiotic and biotic environment. However, experiments of this type show that changes in CO₂ alone can change fundamental physiological properties of populations over evolutionary time, in this case disabling the induction of high-affinity C uptake. This suggests that projections based on the physiological properties of current populations and the assumption that these properties will not change on the scale of hundreds of years may be unrealistic. In addition to evolutionary change within populations, ecological processes such as changes in species composition are likely to make it difficult to predict biotic responses to global change. Selection experiments in more realistic systems with natural populations are needed to evaluate evolutionary responses to global change.

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APPENDIX

Net C uptake calculations

- 1 Rate of net C uptake ($V_{\text{net}} = V_{\text{max}}[\text{CO}_2]/(K_{0.5} + [\text{CO}_2])$), where V_{max} and $K_{0.5}$ values are those estimated for a particular line at a particular $[\text{CO}_2]$. $[\text{CO}_2]$ values used correspond to dissolved CO₂ at 1050 ppm for high and 430 ppm for ambient, respectively. Values used can be found in Table 2.
- 2 Net C uptake per population = V_{net} (relative maximum population size), at a given $[\text{CO}_2]$. Maximum population size is relative, with the average of ambient CO₂ selected lines grown at ambient having an arbitrary value of 1 as described in Collins & Bell (2004). Values used can be found in Table 3.

Table 2. V_{net} values used

	V_{net} at ambient (current) CO ₂ ($\mu\text{mol mg}^{-1} \text{Chl}^{-1} \text{h}^{-1}$)	V_{net} at high (projected) CO ₂ ($\mu\text{mol mg}^{-1} \text{Chl}^{-1} \text{h}^{-1}$)
Wild type	250.6	371.9
High selected lines	50.7	286.7

Chl, chlorophyll.

Table 3. Maximum population size values used

	Relative population size at ambient (current) CO ₂	Relative population size at high (projected) CO ₂
Wild type	1.0	1.63
High selected lines	1.12 (average of high lines)	1.31 (average of high lines)