

LETTER

Evolution of natural algal populations at elevated CO₂

Sinéad Collins* and Graham Bell
Department of Biology, McGill
University, Montreal, QC,
Canada H3A 1B1
*Correspondence: E-mail:
sinead.collins@mail.mcgill.ca

Abstract

Over the next century, it is expected that the concentration of CO₂ in the atmosphere will roughly double (Watson *et al.*, 2001, *Climate Change 2001: the Scientific Basis*, Intergovernmental Panel on Climate Change, Geneva). Microbial populations, which have large population sizes and short generation times, may respond to CO₂ enrichment through genetic change. Here we describe microalgae isolated from the soil of natural CO₂ springs and compare these strains with lines of *Chlamydomonas* that were selected at elevated CO₂ in the laboratory. Both the laboratory and natural populations failed to evolve specific adaptations to elevated CO₂, and contain populations that grow poorly at ambient levels of CO₂. Laboratory and CO₂ spring populations also include lines whose growth rates are insensitive to CO₂. This demonstrates that, although laboratory selection experiments use simplified environments, the evolutionary responses that are seen following long-term CO₂ enrichment correspond to those found in natural populations that have experienced similar conditions.

Keywords

Adaptation, carbon dioxide spring, *Chlamydomonas*, CO₂, microalgae, model system.

Ecology Letters (2006) 9: 129–135

INTRODUCTION

Laboratory selection experiments are often used to understand the processes that underlie adaptation better. In contrast, selection experiments can also be used to obtain or describe a particular phenotype that is important for commercial, medical or ecological reasons. This is the case with experimental evolution studies examining the degradation of pollutants (Swenson *et al.* 2000) or antibiotic resistance (Chopra *et al.* 2003; Dobrindt *et al.* 2004) in bacteria or responses to rising CO₂ in algae (Collins & Bell 2004). In cases such as these, it is important to establish that the model system used mimics the outcome of selection in natural populations closely enough to produce realistic end outcomes.

Experimental approaches are powerful because they allow the experimenter to control the environment, and thus to attribute adaptive outcomes unambiguously to a defined environmental change. However, by defining and controlling the environment that adaptation occurs in, experimental systems run the risk of oversimplifying important aspects of the natural environment. This oversimplification may alter adaptive outcomes, which need not be a weakness in experiments meant to study a general process, but is of

concern when specific adaptive outcomes are being studied. We previously used an experimental model system so as to study the evolutionary effects of elevated CO₂ on microalgae (Collins & Bell 2004). The main simplifications used in the experimental design were that the algae populations were grown at a pH of 7 that did not change as CO₂ increased and were grown in a nutrient-rich media. Furthermore, they existed in a microcosm where no competition with other species could occur, although this has been shown to be important in multicellular plants as well as in mixed cultures of microalgae acclimating to changes in CO₂ levels (Bazzaz *et al.* 1995; Tortell & Morel 2002). The conditions of our previous experiment were not intended to mirror the natural growth conditions of microalgae accurately, but to detect any evolutionary change attributable solely to elevated CO₂. A more fundamental limitation of experimental evolution as a whole is that it may provide a range of possible outcomes, but cannot predict which outcome(s) will occur in nature (with respect to plant responses to elevated CO₂, e.g. see Klus *et al.* 2001; Collins & Bell 2004).

CO₂ springs offer a naturally occurring selection experiment, where populations have existed at elevated CO₂ for 100s of years or more (Raschi *et al.* 1997), which

is ample time for evolutionary change to occur in microorganisms or plants with short generation times. Material from CO₂ springs has been used in order to understand better long-term genetic changes that occur in response to increased CO₂ (Raschi *et al.* 1999). This work has focused primarily on multicellular plants, although a few studies of photosynthetic algae found in lichen exposed to CO₂-enriched air from CO₂ springs have been published (Balaguer & Barnes 1999; Balaguer *et al.* 1999). Responses observed in multicellular plants from CO₂ springs have been shown to be comparable with those observed in microevolutionary experiments spanning several generations (Marchi *et al.* 2004). One of the weaknesses in using CO₂ springs alone to look at evolutionary responses is that it is very difficult to attribute responses definitively to high CO₂ (e.g. see Andalo *et al.* 1999). Parameters that co-vary with CO₂ levels, such as pH or the presence of competing species can confound the cause of microevolutionary change and may also make it difficult to find an appropriate ambient CO₂ comparison population nearby. It is also difficult to be sure that heritable physiological differences translate into differences in fitness, rather than to neutral variation between populations (Fordham & Barnes 1999; Woodward 1999). In addition, unknown immigration rates from nearby populations adapted to ambient CO₂ levels may impede adaptation to high CO₂ in resident populations. Despite these ambiguities, CO₂ springs offer long-term mesocosm experiments that should be taken advantage of both as experimental systems on their own and in combination with laboratory studies. Here, we have isolated soil microalgae from two well-studied CO₂ springs so as to characterize their growth and compare it with laboratory selection lines.

In contrast to studies using multicellular plants, almost all work to date on microalgal responses to rising CO₂ has been performed using short-term studies in natural and laboratory populations (reviewed recently by Beardall *et al.* 1998; Colman *et al.* 2002; Riebesell, 2004) or by comparisons between different taxa of extant phytoplankton (Raven 2003). These studies have resulted in detailed descriptions of the physiological responses of contemporary populations to changes in CO₂. The strength of this approach is that it has established that the changes in carbon uptake in standard microalgal model systems behave like natural populations over short time scales. In this study, we show that the same holds true for using the model alga *Chlamydomonas* to experimentally test long-term evolutionary responses to increasing CO₂ in microalgae. In demonstrating that the model system we have used to date behaves like comparable natural populations, we show that CO₂ springs and laboratory experiments can be used together in order to obtain both a realistic description of a high CO₂ evolved

phenotype, as well as explain how natural selection can lead to this phenotype.

MATERIALS AND METHODS

Collection and isolation of natural populations

Soil samples were collected at the Bossoleto CO₂ spring near Sienna, Italy (latitude 43°17', longitude 11°35') and at the Strmec spring near Radenci, Slovenia (latitude 46°39', longitude 16°00'). The Bossoleto and Strmec springs are well-characterized CO₂ springs with undetectable levels of methane and sulphur in their emissions. Average daytime CO₂ levels at the springs range from 600 to 1200 p.p.m. in air at the Bossoleto spring and 2600–4200 p.p.m. in soil at the Strmec spring (Bettarini *et al.* 1999; Kaligarić 2001; Marchi *et al.* 2004), although CO₂ levels in soil very near the springs can exceed 5%. Sulphur levels were undetectable near both springs. Soil samples were collected in CO₂-enriched areas around the springs, and comparison soil samples were collected in nearby areas that were not enriched. Samples were not taken from the mud near or in the spring water. Soil samples were mixed with 1/4 strength HSM (High Salt Medium) (Harris, 1989) and illuminated under either very high (5%) or ambient levels of CO₂ and sampled after 48 and 72 h of growth. This level of CO₂ was within the range of CO₂ levels measured at the Bossoleto spring during sampling. Microalgae were isolated from soil by phototaxis (Sack *et al.* 1994). Algae were allowed up to 24 h to swim through agarose-filled pipettes; cells that reached the illuminated end of the pipettes were then grown on HSM agar plates at either very high or ambient CO₂. This procedure would have failed to isolate any types that had an absolute (short term) requirement for high CO₂ or which could not grow in HSM. Populations were identified by visual inspection by Thomas Pröschold (texts used: Ettl & Gärtner 1988, 1995; Gärtner & Ettl 1988). Isolates were nearly monospecific, made up of one of the following: *Tetracystis* sp., *Chloranomala* sp. or *Chlorococcum* sp., all of which are common soil algae (Bold & Wynne 1985). Individual isolate identifications are listed in Appendix S1.

Maximum population densities and growth rates

Maximum population densities and growth rates were measured in 384-well plates containing 90 µL HSM per well. All cultures were first acclimated for a single culture cycle (3–6 days), then diluted and transferred to assay plates. For spring 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 back-selection of the spring lines, so that the results presented here are conservative. The plates were

grown in the same phytotron chamber as above at either 430 or 2000 p.p.m. CO₂. Absorbance of each culture was measured every 24 h. Maximum densities were calculated from the maximum absorbance attained by a culture in the same assay as was used to measure growth rates. Growth rates and maximum densities are relative, such that the average value of the control cultures growing at ambient CO₂ is 1.0. Three independent triplicate measurements were made for each culture. The physiological response to changes in CO₂ was also calculated from this data.

RESULTS

Growth rates and maximum population densities

Figure 1 shows the growth rates and maximum population densities of CO₂ spring and comparison isolates. Both comparison and CO₂ spring populations were successfully

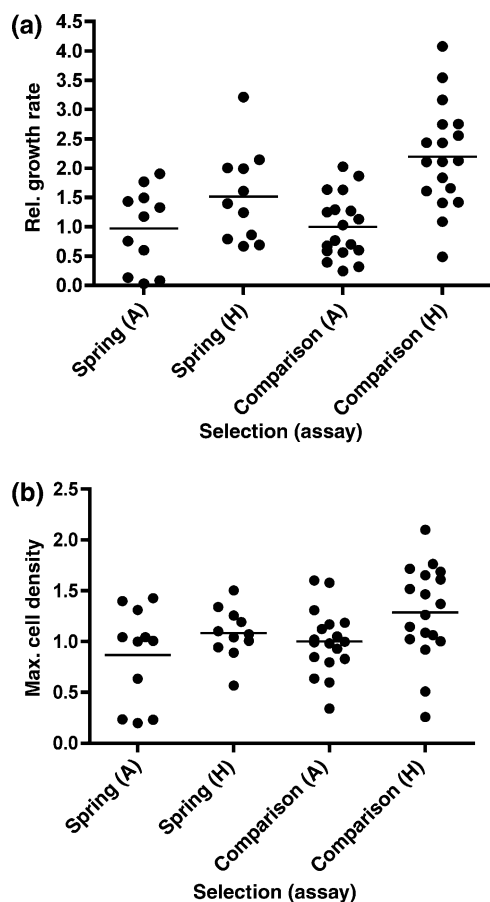


Figure 1 (a) Relative growth rates of CO₂ spring and comparison algae at high (2000 p.p.m.) and ambient levels of CO₂. (b) Relative maximum cell density attained by cultures of CO₂ spring and comparison algae at high and ambient levels of CO₂. In both cases, each point represents the mean value from three independent replicate measurements for a single population.

isolated from Strmec soil samples, but no comparison populations were isolated from the Bossoleto spring. Results from the Strmec isolates only are analysed below.

There was no detectable effect of genus on either growth rates ($F_{5,5} = 1.83$, $P = 0.19$) or maximum population densities ($F_{5,5} = 1.24$, $P = 0.35$). Because of this, the isolates are only divided into CO₂ spring and comparison populations below, regardless of identity. This may be partially because the isolation procedure used would have excluded cells that grew very slowly or were not motile, and any populations that could not grow detectably in the media used. Thus, the isolation procedure itself might select for relatively homogenous growth rates. In addition, there are relatively few isolates of each genus, making it unlikely that small differences in growth rate or maximum population density would be detected here.

Figure 1a shows the relative growth rates of the spring and comparison isolates. The expected physiological response to an increase in CO₂ is an increase in growth rate. The effect of the assay environment (high or ambient CO₂) is significant ($F_{1,1} = 17.95$, $P < 0.0001$) whereas the main effect of habitat (spring vs. comparison) is ambiguous ($F_{1,1} = 3.48$, $P = 0.068$) and the assay \times habitat interaction is non-significant. A direct evolutionary response to long-term growth at high CO₂ would result in the CO₂ spring isolates having a higher growth rate or maximum population density than the comparison cultures at high CO₂. However, populations from the CO₂ springs on average have growth rate 31% lower than the comparison populations under high CO₂ conditions, although the range of growth rates does not extend below that of the controls. This effect is only marginally significant (see above), mostly because there is no systematic difference in growth rates between high and comparison isolates at ambient CO₂. There is no significant difference in maximum population densities achieved by the CO₂ spring isolates and the comparison isolates when grown at high CO₂.

Figure 1b shows the maximum cell density attainable by a culture. There is no significant difference in mean growth rates ($F_{1,1} = 0.07$, $P = 0.79$) or maximum population densities ($F_{1,1} = 1.15$, $P = 0.29$) between the CO₂ spring and comparison populations when grown at ambient CO₂. However, three of 10 CO₂ spring populations have near-zero growth rates at ambient CO₂, and periodically fail to grow at all ambient CO₂. These three isolates do not have unusually low growth rates when grown at high CO₂.

Changes in sensitivity to CO₂

Figure 2 shows the physiological response to changes in CO₂ in populations collected from either CO₂ springs or from surrounding areas where CO₂ was not elevated (comparison). The response is shown as either change in

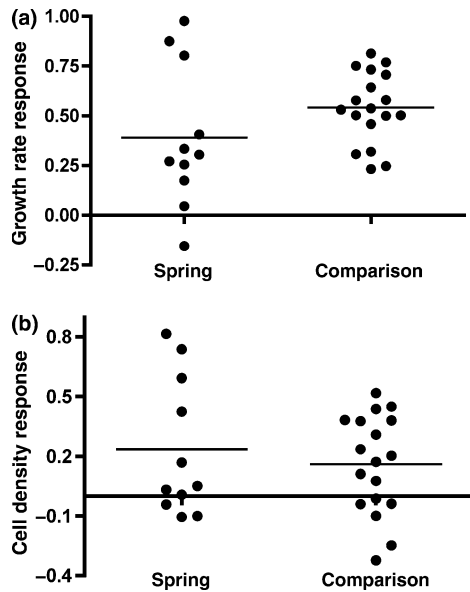


Figure 2 (a) Response of growth rate to an increase in CO₂ concentration from ambient to high (2000 p.p.m.) in CO₂ spring and comparison cultures. (b) Response of maximum cell density to an increase in CO₂ concentration from ambient to high in CO₂ spring and comparison cultures. In all cases, a value of 0 denotes no change between ambient and high CO₂, a value of 1 denotes no growth at ambient CO₂. Each point represents the mean value from three independent replicate measurements for a single population.

growth rate (Fig. 2a) or change in maximum population size (Fig. 2b). The physiological response is calculated as proportional increase in growth rate or maximum population size at high CO₂, calculated as $(Y_{\text{high}} - Y_{\text{amb}}/Y_{\text{high}})$ for each isolate such that a value of 0 indicates that growth rate or maximum population size is the same at high and ambient CO₂.

There is no significant effect of genus on the physiological response to increases in CO₂ (growth rate: $F_{5,5} = 2.29$, $P = 0.12$; maximum population density: $F_{5,5} = 1.05$, $P = 0.44$). Because of this, the isolates are pooled into either spring or comparison treatments below, regardless of identity. We found no effect of long-term growth at high

CO₂ on the mean physiological response to CO₂ levels in terms of growth rate ($t_{0.05(2)13} = 1.3$, $P = 0.21$) or maximum population densities ($t_{0.05(2)27} = 0.51$, $P = 0.67$). The populations isolated from the CO₂ spring did, however, have a larger variance in the response of growth rate to CO₂ than did the comparison cultures ($F = 6.64$, $P = 0.016$), as the sample from the CO₂ spring contained both isolates with very reduced growth at ambient CO₂ (values at or near 1) and CO₂-insensitive isolates (values at or near 0), which the comparison sample did not.

The Bossoleto samples were not analysed quantitatively because of the lack of comparison populations. Qualitatively, we see the presence of a high CO₂-requiring isolate of *Chlorella minutissima* (response = 1), which does not grow at all at ambient CO₂, although standard laboratory populations of *Chlorella* are usually cultured in air. *Chlorella* sp. was not isolated in any of the comparison samples from either spring.

DISCUSSION

In this study, natural isolates from populations representing three genera of microalgae did not show any evidence of adaptive evolutionary change that could be attributed to elevated CO₂. This is in good agreement with the results obtained from a laboratory selection experiment carried out at elevated CO₂ over 1000 generations in *Chlamydomonas*, a widely used model system for photosynthesis and carbon uptake (Collins & Bell 2004). The phenotypes of the laboratory lines and natural isolates are summarized in Table 1. Neither laboratory selection lines nor CO₂ spring isolates show a direct response to selection at high CO₂ in terms of either growth rate or maximum population density. Selection at high CO₂ resulted in an apparently maladaptive response in some cases. Both laboratory and CO₂ spring isolates had populations that failed to grow reliably at ambient CO₂, present at a frequency of 2/10 in the laboratory lines and 3/10 in the natural isolates from the Strmec spring, as well as one isolate from the Bossoleto spring.

Both laboratory and natural populations showed an apparently maladaptive response when grown at high CO₂: a

Table 1 Qualitative descriptions of populations grown for prolonged periods of time, either in the laboratory, or in the soil near CO₂ springs

	Characters at high CO ₂	Characters at ambient CO ₂	Response to changes in CO ₂
Laboratory selection	No adaptive response to selection Lower average limiting density Higher variance in limiting density	Presence of high CO ₂ requiring lines	Presence of CO ₂ insensitive lines
CO ₂ spring	No adaptive response to selection Lower average growth rate No difference in variances	Presence of high CO ₂ requiring lines	Presence of CO ₂ insensitive lines

reduction in mean maximum population density in the laboratory populations and a reduction in mean growth rate in the natural isolates. The nature of this apparently maladaptive response differs between the laboratory lines and the spring isolates in that it is seen as significantly lowered population density in the laboratory cultures and as lowered growth rates in the CO₂ spring isolates. This suggests that some loss of function in carbon uptake or metabolism is a general feature of long-term exposure to elevated CO₂ in microalgal populations. However, the nature and extent of this loss appears to vary between the laboratory selection lines and the spring isolates, as well as between replicate laboratory lines (Collins & Bell 2004). This is consistent with the usual divergence seen between replicate lines in selection experiments. It has been suggested that contemporary populations of plants are adapted to lower (pre-industrial or Pleistocene) CO₂ levels, and that this may constrain or preclude adaptive evolution to elevated CO₂ (Sage & Coleman 2001). While this study does not address the reason that both natural and laboratory populations of microalgae failed to adapt to elevated CO₂, it does empirically show that adaptation is unlikely to occur over 1000s of generations. This is in agreement with studies spanning a comparable number of generations which were carried out using banked seeds from the mid-19th century (Balaguer & Barnes 1999), and in multicellular plants found at CO₂ springs (Andalo *et al.* 1999; Augusti *et al.* 1999; Balaguer & Barnes 1999), although failure to adapt to elevated CO₂ is not always the case (Tousignant & Potvin 1996; Polle *et al.* 2001; Vodnik *et al.* 2002).

Although there is no adaptive response to growth at elevated CO₂, a range of phenotypes can be seen in populations propagated at high CO₂, such as isolates that are unable to grow at ambient CO₂. All the control isolates are able to increase their growth rate in response to an increase in CO₂, whereas two of the CO₂ spring isolates are unable to do so. These growth–CO₂ relationships do not systematically correspond to growth rate or maximum population density at high CO₂. A similar range evolved among replicate experimental lines of *Chlamydomonas* (Collins & Bell 2004), demonstrating that long-term responses to elevated CO₂ vary among natural populations in the same way that they do between experimental lines in the laboratory.

Because there is a range of neutral phenotypes, long-term growth at elevated CO₂ has the potential to cause non-adaptive evolutionary change and phenotypic divergence in natural populations of microalgae as well as in a laboratory model system. The repercussions of this could be especially important in isolated populations of the same species, which have the potential to diverge as CO₂ levels rise. The range of neutral phenotypes found in high CO₂ populations introduces a source of uncertainty and the possibility of

multiple simultaneous outcomes into predictions of how microalgal populations are likely to change as CO₂ rises. This uncertainty should not be mistaken for a lack of understanding or be used as an excuse either to discount models based on current growth–CO₂ relationships or to fail to take prudent measures that could manage anthropogenic impacts on natural systems. However, findings such as the ones in this study highlight the inherent unpredictability of biological systems, even when they have been greatly simplified for laboratory experiments.

In the natural isolates described here, microalgal populations are unable to improve their performance significantly at elevated CO₂, but this does not exclude the possibility that elevated CO₂ could indirectly cause adaptive change. A negative correlated response, where some CO₂ spring isolates show reduced growth at ambient CO₂, shows that some degree of normal function can be lost when CO₂ is constantly abundant. Similarly, it was found that *Chlamydomonas* selected at high CO₂ lost the ability to induce high-affinity CO₂ uptake as effectively as wild-type cells (S. Collins, D. Sültemeyer and G. Bell, unpublished data). This might allow energy diverted from CO₂ uptake or regulation to be devoted to the acquisition of limiting resources, such as iron (Falkowski 1994; Riebesell 2004). This hypothesis has yet to be directly tested in microalgae, although there is some support for it in land plants grown in competition under high CO₂ for several generations (Bazzaz *et al.* 1995).

The aim of this study was to compare the evolutionary responses of natural and laboratory populations of microalgae to elevated CO₂. For the laboratory selection experiment, an algal model system, *Chlamydomonas reinhardtii*, was used. *Chlamydomonas* is a common soil algae that possesses the ability to actively concentrate inorganic carbon inside its cell (Badger *et al.* 1980). Two of the three genera of soil algae isolated from the Strmec spring, *Chlorococcum* and *Tetracystis*, are part of the Order Chlamydomonales along with *Chlamydomonas* (Lewis & McCourt 2004). *Chlorococcum* sp. is known to induce active concentration of carbon inside its cell under low CO₂ (Miyachi *et al.* 2003), as does *Chlamydomonas*. To the best of our knowledge, the carbon uptake physiology of *Tetracystis* or *Chloranomala* has not been reported, although the ability to concentrate inorganic carbon is present in most microalgae studied to date (Colman *et al.* 2002). In multicellular plants, responses to CO₂ measured over several generations vary idiosyncratically between species (reviewed by Ward & Strain 1999; Urban 2003). In contrast with this, our present study failed to detect any systematic between-genus differences in evolutionary responses to elevated CO₂, although small differences may be detectable with more intensive sampling. This suggests that the range of results obtained using replicate laboratory populations approximates the range of

results found in the same size sample of natural populations with similar biology.

Experimental studies designed to examine the effects of specific environmental changes must simplify both the biotic and physical environment in order to draw clear conclusions about the nature and cause of adaptation. While this simplification is necessary, it is important to establish to what extent model systems behave like the natural populations that they are meant to represent. Our findings validate the use of a model system for studying the evolutionary response of algae to elevated CO₂ and verifies that the phenotypes found in the laboratory correspond with the outcome of selection in natural communities.

ACKNOWLEDGEMENTS

We thank Antonio Raschi and Mitja Kaligarić for help with sample collection, and Thomas Pröschold for algae identification.

REFERENCES

- Andalo, C., Godelle, B. & Mousseau, M. (1999). Are *Arabidopsis thaliana* from a natural CO₂ spring adapted to elevated CO₂? In: *Ecosystem Response to CO₂: the MAPLE Project Results* (eds Raschi, A., Vaccari, F.P. and Miglietta, F.). European Commission Directorate-General for Research. Unit D.1.1-Preserving the ecosystem, Brussels, pp. 158–167.
- Augusti, A., Lauteri, M., Spaccino, L. & Brugnoli, E. (1999). Short- and long-term responses of carbon isotope discrimination and photosynthetic energy dissipation to elevated CO₂ concentration. In: *Ecosystem Response to CO₂: the MAPLE Project Results* (eds Raschi, A., Vaccari, F.P. and Miglietta, F.). European Commission Directorate-General for Research. Unit D.1.1-Preserving the ecosystem, Brussels, pp. 117–132.
- Badger, M.R., Kaplan, A. & Berry, J.A. (1980). Internal inorganic carbon pool of *Chlamydomonas reinhardtii*: evidence for a carbon dioxide-concentrating mechanism. *Plant Physiol.*, 66, 407–413.
- Balaguer, L. & Barnes, J.D. (1999). Potential impacts of long-term CO₂ enrichment on green-algal lichens. In: *Ecosystem Response to CO₂: the MAPLE Project Results* (eds Raschi, A., Vaccari, F.P. and Miglietta, F.). European Commission Directorate-General for Research. Unit D.1.1-Preserving the ecosystem, Brussels, pp. 106–116.
- Balaguer, L. *et al.* (1999). Long-term responses of the green-algal lichen *Parmelia caperata* to natural CO₂ enrichment. *Oecologia*, 119, 166–174.
- Bazzaz, F.A., Jasienski, M., Thomas, S.C. & Wayne, P. (1995). Microevolutionary responses in experimental populations of plants to CO₂-enriched environments: parallel results from two model systems. *PNAS*, 92, 8161–8165.
- Beardall, J., Johnston, A. & Raven, J. (1998). Environmental regulation of CO₂-concentrating mechanisms in microalgae. *Can. J. Bot.*, 76, 1010–1017.
- Bettarini, I., Grifoni, D., Miglietta, F. & Raschi, A. (1999). Local greenhouse effect in a CO₂ spring in central Italy. In: *Ecosystem Response to CO₂: the MAPLE Project Results* (eds Raschi, A., Vaccari, F.P. and Miglietta, F.). European Commission Directorate-General for Research. Unit D.1.1-Preserving the ecosystem, Brussels, pp. 39–52.
- Bold, H.C. & Wynne, M.J. (1985). *Introduction to the Algae: Structure and Reproduction*, 2nd edn. Prentice-Hall, Inc., Prentice, NJ.
- Chopra, I., O'Neill, A.J. & Miller, K. (2003). The role of mutators in the emergence of antibiotic-resistant bacteria. *Drug Resist. Updat.*, 6, 137–145.
- Collins, S. & Bell, G. (2004). Phenotypic consequences of 1000 generations of selection at elevated CO₂ in a green alga. *Nature*, 431, 566–569.
- Colman, B., Huertas, I.E., Bhatti, S. & Dason, J.S. (2002). The diversity of inorganic carbon acquisition mechanisms in eukaryotic microalgae. *Funct. Plant Biol.*, 29, 261–270.
- Dobrindt, U., Hochhut, B., Hentschel, U. & Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. *Nat. Rev. Microbiol.*, 2, 414–424.
- Ettl, H. & Gärtner, G. (1988). Chlorophyta II – Tetrasporales, Chlorococcales, Gloenodendrales. In: *Süßwasserflora von Mitteleuropa* (eds Ettl, H., Gerloff, G., Heynig, H. and Mollenhauer, D.). Gustav Fischer, Stuttgart-New York, pp. ??.
- Ettl, H. & Gärtner, G. (1995). *Syllabus der Boden-, Luft- und Flechtalgen*. Gustav-Fischer, Stuttgart-Jena-New York.
- Falkowski, P.G. (1994). The role of phytoplankton photosynthesis in global biogeochemical cycles. *Photosyn. Res.*, 39, 235–258.
- Fordham, M. & Barnes, J. (1999). Growth and photosynthetic capacity in *Agrostis canina* and *Plantago major* adapted to contrasting long-term atmospheric CO₂ concentrations. In: *Ecosystem Response to CO₂: the MAPLE Project Results* (eds Raschi, A., Vaccari, F.P. and Miglietta, F.). European Commission Directorate-General for Research. Unit D.1.1-Preserving the ecosystem, Brussels, pp. 143–157.
- Gärtner, G. & Ettl, H. (1988). Neugliederung der Gattung *Chlorococcum* Meneghini (Chlorophyta, Chlamydomyces, Chlorococcales). *Nova Hedwegia*, 47, 271–278.
- Harris, E. (1989). *The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use*. Academic Press, Inc., San Diego, CA, USA.
- Kaligarić, M. (2001). Vegetation patterns and responses to elevated CO₂ from natural CO₂ springs at Strmec (Radenci, Slovenia). *Acta Biol. Slovenica*, 44, 31–38.
- Klus, D., Kalisz, S., Curtis, P.S., Teeri, J.A. & Tonsor, S.J. (2001). Family- and population-level responses to atmospheric CO₂ concentration: gas exchange and the allocation of C, N, and biomass in *Plantago lanceolata* (Plantaginaceae). *Am. J. Bot.*, 88, 1080–1087.
- Lewis, L.A. & McCourt, R.M. (2004). Green algae and the origin of land plants. *Am. J. Bot.*, 91, 1535–1556.
- Marchi, S. *et al.* (2004). Physiological and morphological responses of grassland species to elevated CO₂ concentrations in FACE-systems and natural CO₂ springs. *Funct. Plant Biol.*, 31, 181–194.
- Miyachi, S., Iwasaki, I. & Shiraiwa, Y. (2003). Historical perspective on microalgal and cyanobacterial acclimation to low- and extremely high-CO₂ conditions. *Photosyn. Res.*, 77, 139–153.
- Polle, A., McKee, I. & Blaschke, L. (2001). Altered physiological and growth responses to elevated [CO₂] in offspring from holm oak (*Quercus ilex* L.) mother trees with lifetime exposure to naturally elevated [CO₂]. *Plant Cell Environ.*, 24, 1075–1083.

- Raschi, A., Miglietta, F., van Gardingen, P. & Tognetti, R. (1997). *Plant Responses to Elevated CO₂: Evidence from Natural Springs*. (eds Raschi, A., Vaccari, F.P. and Miglietta, F.) Cambridge University Press, Cambridge.
- Raschi, A., Vaccari, F.P. & Miglietta, F. (eds) (1999). *Ecosystem Response to CO₂: the MAPLE Project Results*. European Commission Directorate-General for Research. Unit D.1.1-Preserving the ecosystem, Brussels.
- Raven, J.A. (2003) Inorganic carbon concentrating mechanisms in relation to the biology of algae. *Photosyn. Res.*, 77, 155–171.
- Riebesell, U. (2004). Effects of CO₂ enrichment on marine phytoplankton. *J. Oceanogr.*, 60, 719–729.
- Sack, L. *et al.* (1994). Isolation of 4 new strains of *Chlamydomonas reinhardtii* (Chlorophyta) from soil samples. *J. Phycol.*, 30, 770–773.
- Sage, R.F. & Coleman, J.R. (2001). Effects of low atmospheric CO₂ on plants: more than a thing of the past. *Trends Plant Sci.*, 6, 18–24.
- Swenson, W., Wilson, D.S. & Elias, R. (2000). Artificial ecosystem selection. *PNAS*, 97, 9110–9114.
- Tortell, P.D. & Morel, F.M.M. (2002). Sources of inorganic carbon for phytoplankton in the eastern subtropical and equatorial Pacific Ocean. *Limnol. Oceanogr.*, 47, 1012–1022.
- Tousignant, D. & Potvin, C. (1996). Selective responses to global change: experimental results on *Brassica juncea* (L.) Czern. In: *Carbon dioxide, Populations, and Communities* (eds Bazzaz, F.A. and Korner, K.). Academic Press, San Diego, CA, pp. 23–30.
- Urban, O. (2003). Physiological impacts of elevated CO₂ concentration ranging from molecular to whole plant responses. *Photosynthetica*, 41, 9–20.
- Vodnik, D., Pfanz, H., Macek, I., Kastelec, D., Lojen, S. & Batic, F. (2002). Photosynthesis of cockspar [*Echinochloa crus-galli* (L.) Beauv.] at sites of naturally elevated CO₂ concentration. *Photosynthetica*, 40, 575–579.
- Ward, J.K. & Strain, B.R. (1999). Elevated CO₂ studies: past, present and future. *Tree Physiol.*, 19, 211–220.
- Watson, R., Houghton, J. & Yihui, D. (eds) (2001). *Climate Change 2001: The Scientific Basis*. Intergovernmental Panel on Climate Change, Geneva.
- Woodward, F.I. (1999). Adaptation by *Plantago lanceolata* and *Tussilago farfara* to CO₂ enrichment. In: *Ecosystem Response to CO₂: the MAPLE Project Results* (eds Raschi, A., Vaccari, F.P. and Miglietta, F.). European Commission Directorate-General for Research. Unit D.1.1-Preserving the ecosystem, Brussels, pp. 133–142.

SUPPLEMENTARY MATERIAL

The following supplementary material is available online for this article from <http://www.Blackwell-Synergy.com>:

Appendix S1 Identification of soil algae.

Editor, Jim Elser

Manuscript received 9 August 2005

First decision made 14 September 2005

Manuscript accepted 6 October 2005