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Impacts of Elevated CO_2 Concentration on Biochemical Composition, Carbonic Anhydrase, and Nitrate Reductase Activity of Freshwater Green Algae

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Abstract: To investigate the biochemical response of freshwater green algae to elevated CO_2 concentrations, *Chlorella pyrenoidosa* Chick and *Chlamydomonas reinhardtii* Dang cells were cultured at different CO_2 concentrations within the range 3–186 μmol/L and the biochemical composition, carbonic anhydrase (CA), and nitrate reductase activities of the cells were investigated. Chlorophylls (Chl), carotenoids, carbonhydrate, and protein contents were enhanced to varying extents with increasing CO_2 concentration from 3–186 μmol/L. The CO_2 enrichment significantly increased the Chl *a*/Chl *b* ratio in *Chlorella pyrenoidosa*, but not in *Chlamydomonas reinhardtii*. The CO_2 concentration had significant effects on CA and nitrate reductase activity. Elevating CO_2 concentration to 186 μmol/L caused a decline in intracellular and extracellullar CA activity. Nitrate reductase activity, under either light or dark conditions, in *C. reinhardtii* and *C. pyrenoidosa* was also significantly decreased with CO_2 enrichment. From this study, it can be concluded that CO_2 enrichment can affect biochemical composition, CA, and nitrate reductase activity, and that the biochemical response was species dependent.

Key words: carbonic anhydrase; *Chlamydomonas reinhardtii*; *Chlorella pyrenoidosa*; CO_2; nitrate reductase.

It is generally predicted that the atmospheric CO_2 concentration will be doubled to 700 μL/L at the end of this century because of the increasing industrial combustion of fossil fuels (King *et al*. 1992). Atmospheric CO_2 enrichment would also result in a reduction of the pH and an increase in the CO_2 concentration of surface waters (Stumm and Morgan 1996). It is important to assess the impact of increasing atmospheric CO_2 on aquatic plants (Bowes 1993). Riebesell *et al*. (1993) showed that CO_2 availability could potentially influence the growth of marine diatoms. Hein and Sand-Jensen (1997) demonstrated that an increase in atmospheric CO_2 would stimulate phytoplankton primary production in open ocean. An elevated CO_2 concentration enhanced photosynthesis and the growth of macroalgae (Gao *et al*. 1991, 1993). The enriched CO_2 concentration also affected growth of the freshwater green algae *Chlamydomonas reinhardtii* Dang, *Chlorella pyrenoidosa* Chick, and *Scenedesmus obliquus* Kütz when these were cultured at various concentrations of inorganic N and P (Yang and Gao 2003).

The effects of CO_2 enrichment on the growth and photosynthesis of freshwater green algae are usually related to the mechanisms of inorganic carbon acquisition. Some green algae have been reported to utilize HCO_3^- under CO_2-limited conditions. Carbonic anhydrase (CA) was demonstrated to be essential for photosynthetic utilization of inorganic carbon at low

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external CO_2 concentrations and alkaline pH (Moroney *et al*. 1985), which played an important role in CO_2 transport either at the cell surface or in the cell. Extracellular CA (CA_{ext}) activity increased substantially within a couple of hours when cells were allowed to acclimate to air levels of CO_2 (Badger and Price 1994). Similarly, Williams and Colman (1996) found that CA_{ext} activity increased with a decrease in CO_2 availability. Intracellular CA (CA_{int}) activity in *C. reinhardtii* was reported to be considerably higher in low CO_2-grown cells than in those grown under conditions of high CO_2 (Amoroso *et al*. 1996). The impact of enhanced CO_2 on growth and photosynthesis also depends on the availability of nitrogen in the plant (Stitt and Krapp 1999). The availability of CO_2 may also impact on the efficiency of nutrient use and alter the biogeochemistry of aquatic habitats (Giordano and Bowes 1997; Giordano 2001). Nitrate reductase (NR) plays an important role in N utilization. Elevating CO_2 concentrations to 5% resulted in an increase of NR activity in *Ulva ridiga* (Magnusson *et al*. 1996). These findings regarding CA and NR activity in the presence of high concentrations of CO_2 (1%–5% CO_2) are fundamental in understanding the biochemical responses of microalgae, but have little ecological implication in view of increases in atmospheric CO_2. Conversely, how CA and NR activity in freshwater phytoplankton responds to elevated atmospheric CO_2 remains unknown. At the same time, CO_2 enrichment could result in changes in cell composition by affecting C and N metabolism. However, there is little information published regarding these aspects, particularly in the case of freshwater green algae.

The aim of the present study was to gather basic information on changes in the biochemical composition and CA and NR activity of freshwater microalgae *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* under conditions of CO_2 enrichment.

1 Materials and Methods

The freshwater green algae *Chlamydomonas reinhardtii* Dang and *Chlorella pyrenoidosa* Chick were obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, the Chinese Academy of Sciences (Wuhan, China). All experiments were performed in 1 000-mL batch cultures with modified Bristol's medium (Fujita 1972) containing 20 mmol/L Tris and 20 mg/L $NaHCO_3$. The range of CO_2 concentrations from 3 to 186 μmol/L in the media were obtained by adjusting the pH to 7.2, 8.2, and 9.0 according to the methods of Xia and Gao (2002). Cells were cultured in a plant growth chamber (EF7; Conviron) with a 12 h/12 h light-dark cycle and were maintained at original densities of 10^4 cells/mL by renewing the culture medium before the beginning of illumination every day. Temperature and illumination were controlled at 25 °C and 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, respectively. Dissolved inorganic carbon (DIC) was measured with a Total Organic Carbon Analyser (TOC-5000; Shimadzu, Kyoto, Japan); pH was also monitored and maintained at constant levels, as above. The CO_2 concentrations in the media were calculated from pH and DIC values according to Stumm and Morgan (1996). After 7 d of culture, cells were harvested for analysis.

Chlorophylls (Chl) and carotenoids were extracted in 80% acetone and determined according to the methods of Lichtenthaler and Wellburn (1983). Protein was estimated according to the Bradford method, with bovine serum albumin as the standard (Kochert 1978a). Carbonhydrate was determined according to Kochert (1978b) using sugar as the standard.

CA activity assay was determined at 2 °C by measuring the time for the pH to drop from 8.3 to 7.3 in 20 mmol/L Veronal buffer after the addition of ice-cold CO_2-saturated water (Willbur and Anderson 1948). Cells were broken with an ultrasonic homogenizer at 4 °C. Intact and broken cells were assayed for CA_{ext} and total CA activity, respectively. Carbonic anhydrase activity was calculated according to the following formula:

$$EU = 10\ (t_b/t_c - 1)$$

where t_b and t_c are the time taken for the pH to drop from 8.3 to 7.3 in intact or broken cells and boiled cells, respectively. The CA_{int} activity was determined by subtracting CA_{ext} activity from total CA activity.

NR activity was determined according to the

methods of Radin (1973). The reaction mixture contained 0.1 mol/L sodium phosphate buffer and 0.2 mol/L KNO_3 in a final volume of 10 mL. Cells, at a density of 10^6 cells/mL, were incubated in this mixture at 30 °C for 30 min in darkness under anaerobic conditions. The nitrite concentration was determined by the addition of 2 mL sulfanilamide (1 g sulfanilamide in 3 mol/L HCl) and 2 mL ethylene-diamine (0.2 g ethylene-diamine in 0.3 mol/L HCl) to the filtered mixture and recording the absorbance at 520 nm. Nitrate reductase activity was expressed as $\mu g\ NO_2^- \cdot cell^{-1} \cdot h^{-1}$.

Treatments were compared using ANOVA followed by Duncan's test. The confidence level was set at 5%.

2 Results

The CO_2 enrichment had a significant effect on CA_{ext} activity ($P < 0.05$; Fig. 1). The CA_{ext} activity in *Chlamydomonas reinhardtii* decreased with increased CO_2 concentrations (21 and 186 μmol/L CO_2) to 18.7% and 45.5% of that in cells grown in 3 μmol/L CO_2, respectively (both $P < 0.05$), whereas CA_{int} decreased by

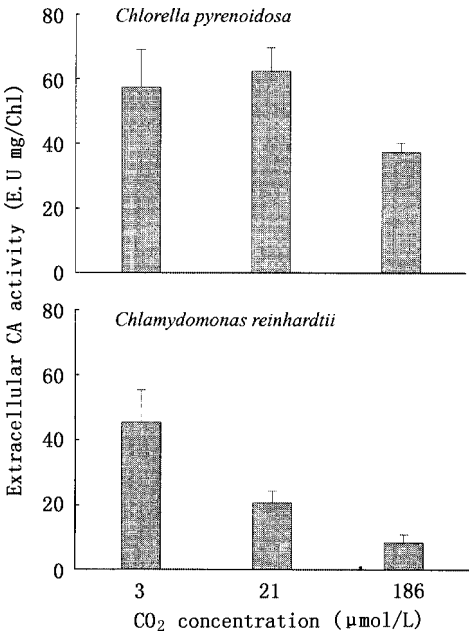

Fig. 1. Extracellular carbonic anhydrase (CA) activity in *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* grown under various CO_2 concentrations (3, 21, and 186 μmol/L CO_2). Data are the mean ± *SD* (n = 3). Chl, chlorophyll.

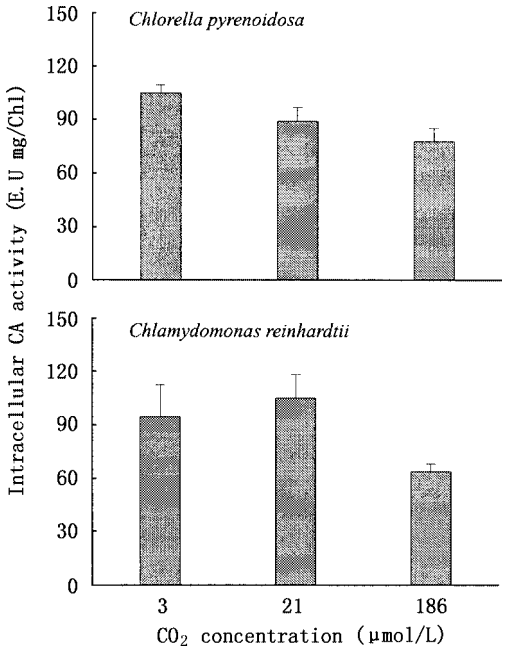

Fig. 2. Intracellular carbonic anhydrase (CA) activity in *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* grown under various CO_2 concentrations (3, 21, and 186 μmol/L CO_2). Data are the mean ± *SD* (n = 3). Chl, chlorophyll.

32.6% only in the presence of 186 μmol/L CO_2 (Fig. 2). Compared with CA_{ext} activity in *Chlorella pyrenoidosa* at 3 μmol/L CO_2, CA_{ext} activity did not change in the presence of 21 μmol/L CO_2 ($P > 0.05$), whereas CA_{ext} activity was significantly reduced at a concentration of 186 μmol/L CO_2 ($P < 0.05$; Fig. 1). The activity of CA_{int} activity declined significantly with an increase in CO_2 concentrations to 21 and 186 μmol/L CO_2, being 73.9% and 84.9% of that in the presence of 3 μmol/L CO_2, respectively ($P < 0.05$; Fig. 2).

NR activity in *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa* was significantly ($P < 0.05$) affected by CO_2 concentration (Fig. 3). When cells were grown at 21 and 186 μmol/L CO_2, NR activity was reduced by 15.9% and 59.5%, respectively, in *Chlorella pyrenoidosa* ($P < 0.05$) and by 72.8% and 83.2%, respectively, in *Chlamydomonas reinhardtii* ($P < 0.05$) at the end of the photoperiod compared with cells grown in the presence of 3 μmol/L CO_2. Nitrate reductase activity decreased by 67.9% and 76.4% in the dark in

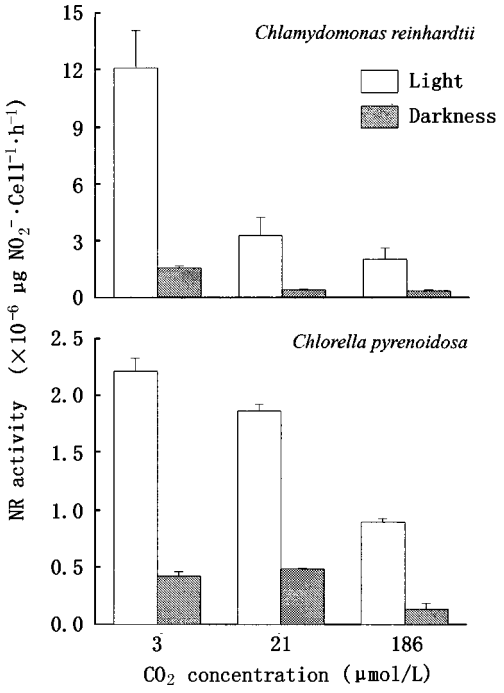

Fig. 3. Nitrate reductase (NR) activity in *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* grown under various CO_2 concentrations (3, 21, and 186 μmol/L CO_2) at the end of photoperiod and darkness. Data are the mean ± *SD* (n = 3).

cells from *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii*, respectively, that were exposed to 186 μmol/L CO_2. Darkness also induced a significant decrease in NR activity in contrast with illumination.

The CO_2 concentration had significant effects on the content of total Chl, carotenoids, carbohydrate, and protein in *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa* ($P < 0.05$) (Table 1). Total Chl content in cells cultured at a concentration of 21 and 186 μmol/L CO_2 increased by 59.1% and 98.6% ($P < 0.05$) in *Chlorella pyrenoidosa* cells, respectively, and by 30.1% and 28.2% ($P < 0.05$) in *Chlamydomonas reinhardtii* cells, respectively, compared with cells cultured in the presence of 3 μmol/L CO_2. The Chl *a*/Chl *b* value increased significantly with CO_2 enrichment in *Chlorella pyrenoidosa* ($P < 0.05$), but not in *Chlamydomonas reinhardtii* ($P > 0.05$).

Carbohydrate in cells grown in the presence of 21 and 186 μmol/L CO_2 increased by 131.3% and 93.1%, respectively, in *Chlorella pyrenoidosa*, and by 25.4% and 126.1%, respectively, in *Chlamydomonas reinhardtii* compared with cells grown at 3 μmol/L CO_2. Increasing the CO_2 concentration to 21 and 186 μmol/L resulted in a significant increase in protein content, compared with the protein content of cells grown in the presence of 3 μmol/L CO_2, by 234.5% and 225.3%,

Table 1 Chlorophylls, carotenoids, carbohydrate, and protein content in *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* grown in the presence of different concentrations of CO_2

Parameters	CO_2 concentration (μmol/L)		
	3	21	186
Chlorella pyrenoidosa			
Chl (10^{-6} μg/cell)	1.44 ± 0.09a	2.29 ± 0.22b	2.86 ± 0.21c
Carotenoids (10^{-6} μg/cell)	0.28 ± 0.03a	0.38 ± 0.04b	0.43 ± 0.02b
Chl *a*/Chl *b*	2.82 ± 0.12a	3.03 ± 0.38a	3.88 ± 0.20b
Carbohydrate (%, w/w)	9.30 ± 0.62a	11.66 ± 0.26b	21.01 ± 0.96c
Protein (%, w/w)	15.90 ± 0.29a	53.18 ± 1.00b	51.73 ± 0.58b
Chlamydomonas reinhardtii			
Chl (10^{-6} μg/cell)	5.56 ± 0.15a	7.22 ± 0.35b	6.82 ± 0.27b
Carotenoids (10^{-6} μg/cell)	1.31 ± 0.03a	1.77 ± 0.08b	1.65 ± 0.08b
Chl *a*/Chl *b*	2.21 ± 0.02a	2.24 ± 0.03a	2.20 ± 0.04a
Carbohydrate (%, w/w)	3.19 ± 0.10a	7.40 ± 0.81b	6.41 ± 0.64b
Protein (%, w/w)	23.94 ± 0.23a	29.14 ± 0.19b	30.86 ± 1.06c

Data are the mean ± *SD* (n = 3). Values with different letters in the same row are significantly different ($P < 0.05$, Duncan's multiple comparison). Chl, chlorophyll.

respectively, in *Chlorella pyrenoidosa* and by 21.7% and 28.9%, respectively, in *Chlamydomonas reinhardtii* ($P < 0.05$).

3 Discussion

Inorganic carbon in water exists in the forms of CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}, and the concentration of dissolved CO_2 in freshwater is approximately 15 μmol/L when the CO_2 partial pressure is balanced between air and water. An increase in CO_2 concentration is usually associated with a decline in pH. Doubling the atmospheric CO_2 level to 700 μL/L would result in a pH drop of approximately 0.28. In view of the ecological implications of increased atmospheric CO_2, it is irrelevant to differentiate between the effects of changing levels of CO_2 and pH. The CO_2 enrichment associated with a pH drop has been used extensively to assess the CO_2 response of algae (Riebesell *et al*. 1993; Burkhardt and Riebesell 1997; Burkhardt *et al*. 1999; Xia and Gao 2002; Yang and Gao 2003). pH has an indirect effect on the growth and carbon uptake of algae by affecting the distribution of inorganic carbon species (Chen and Durbin 1994). A limitation of inorganic carbon can severely inhibit the photosynthesis of phytoplankton in lakes with algal blooms, especially eutrophic alkaline lakes dominated by green algae (Hein 1997).

In the present study, the increased Chl and carotenoids content that results following CO_2 enrichment would be available for trapping more light energy for photosynthesis. Yang and Gao (2003) have shown that a high CO_2 concentration (186 μmol/L) leads to enhanced photosynthesis in the freshwater green algae *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* in contrast with photosynthesis at 3 μmol/L CO_2. However, in the present study, a significant increase was observed in the Chl *a*/Chl *b* ratio for *Chlorella pyrenoidosa*, whereas the Chl *a*/Chl *b* ratio in *Chlamydomonas reinhardtii* cells remained constant regardless of the CO_2 concentration, which implies that the Chl *b* pool in *Chlorella pyrenoidosa* was more sensitive to elevated CO_2 than that in *Chlamydomonas reinhardtii*. Furthermore, the increased carotenoids content may also indicate that CO_2 enrichment could improve resistance to stress because carotenoids are involved in the protection of the photosynthetic apparatus against photoinhibitory damage by singlet oxygen ($^1O_2$), which is produced by the excited triplet state of Chl. In the meantime, it can directly deactivate $^1O_2$ and can also quench the excited triplet state of Chl, thus reducing the formation of $^1O_2$ species (Siefermann-Harms 1987). It has been routinely reported that enhancement of carbohydrate occurs in response to elevated CO_2 concentrations (Gordillo *et al*. 1999; Giordano 2001). This may be why CO_2 enrichment usually enhances photosynthesis, leading to an increase in soluble carbohydrate. In the present study, the protein content was markedly increased with increasing CO_2 concentration, which in consistent with the report of Larrsson *et al*. (1985), who also suggested that CO_2 led to increased internal N content in the unicellular Chlorophyta *Scenedesmus obtusiusculus* because a higher N content in cells was usually associated with a higher protein content. Stimulation of growth by CO_2 was correlated with higher nitrate uptake rates and enhanced protein synthesis.

The activity of CA_{ext} can be induced by a change in environmental CO_2 concentration and CA_{ext} activity is presumed to play an important role in photosynthesis by maintaining the Ci species equilibrium at the site of transport or diffusion into cells. It is well known for eukaryotic microalgae that CA_{ext} activity increases when cells are grown under air levels of CO_2 and decreases or disappears when cells are grown under air enriched with 1%–5% CO_2 (Moroney and Somanchi 1999). The activity of CA_{ext} in *Chlamydomonas reinhardtii* starts to increase when the dissolved CO_2 concentration in the external medium is lower than 100 μmol/L (Bozzo and Colman 2000). In the present study, with the CO_2 concentration ranging from 3 to 186 μmol/L, both CA_{ext} and CA_{int} activity exhibited a significant decline, but to varying extents, which implies that the susceptibility of CA activity to CO_2 enrichment was species dependent. One possibility is that the inorganic carbon

utilization mechanism differed among the species. Intracellular CA activity in *Chlorella* species and *Chlamydomonas reinhardtii* has been shown to be of at least two types: (i) a soluble CA, located in the cytosol; and (ii) an insoluble membrane-bound CA, which is associated with the chloroplast (Miyachi *et al*. 1983; Pronina and Semenenko 1984; Sültemeyer *et al*. 1990, 1995). In a recent paper, intracellular CA was also found in mitochondria during acclimation to low CO_2 concentrations in *C. reinhardtii* (Sültemeyer 1998). Therefore, an increase in the CO_2 concentration from 3 to 186 μmol/L resulted in a decrease of CA_{int} activity in *C. pyrenoidosa* and *C. reinhardtii* to different extents, which may also be associated with CA distribution in the cell.

A decrease in NR activity has been observed in a variety of species following CO_2 enrichment (Besford and Hand 1989; Hocking and Meyer 1991) but, in another study, no effect of CO_2 enrichment on NR activity was found (Riviere-Rolland 1996). The present results demonstrated that NR activity in the light was higher than that in the dark for both *C. reinhardtii* and *C. pyrenoidosa*. This must be because NR gene expression is under the control of light (Deng *et al*. 1990). In *Chlamydomonas*, nitrate appears to be essential for the increase in active NR protein (Navarro *et al*. 1996). However, in the present study, nitrate was not a limiting factor because the culture medium was renewed each day. Thus, nitrate was not responsible for the decrease in NR activity. Activation of NR may be related to a redox interconversion process (Franco *et al*. 1987). In the presence of high CO_2, the decrease in NR activity may be attributed to the covalent modification of the NR protein via protein phosphorylation (Huber *et al*. 1992) because the phosphorylation level was higher in cells incubated in the presence of air enriched with CO_2 compared with air (Lee *et al*. 1999). It was apparent from this study that different species of freshwater green algae exhibited varied biochemical responses to elevated CO_2 concentrations. The species-dependent response to elevated CO_2 concentrations in the freshwater ecosystem could alter species composition, decrease diversity, and alter food webs as a whole.

References

Amoroso G, Weber C, Sültemeyer D, Fock HP (1996). Intracellular carbonic anhydrase activities in *Dunaliella tertiolecta* (Butcher) and *Chlamydomonas reinhardtii* (Dangeard) in relation to inorganic carbon concentration during growth: Further evidence for the existence of two distinct carbonic anhydrase associated with the chloroplasts. *Planta* **199**, 177–184.

Badger MR, Price GD (1994). The role of carbonic anhydrase in photosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* **45**, 369–392.

Besford RT, Hand DW (1989). The effects of carbon dioxide enrichment and nitrogen oxides on some Calvin cycle enzymes and nitrite reductase in glasshouse lettuce. *J Exp Bot* **40**, 329–336.

Bowes G (1993). Facing the inevitable: Plants and increasing atmospheric CO_2. *Annu Rev Plant Physiol Plant Mol Biol* **44,** 309–332.

Bozzo GG, Colman B (2000). The induction of inorganic carbon transport and external carbonic anhydrase in *Chlamydomonas reinhardtii* is regulated by external CO_2 concentration. *Plant Cell Environ* **23**, 1137–1144.

Burkhardt S, Riebesell U (1997). CO_2 availability affects elemental composition (C:N:P) of the marine diatom *Skeletonema costatum*. *Mar Ecol Prog Ser* **155**, 67–76.

Burkhardt S, Zondervan I, Riebesell U (1999). Effect of CO_2 concentration on C:N:P ratio in marine phytoplankton: A species comparison. *Limnol Oceanogr* **44**, 683–690.

Chen CY, Durbin EG (1994). Effects of pH on the growth and carbon uptake of marine phytoplankton. *Mar Ecol Prog Ser* **109**, 83–94.

Deng MD, Moureaux T, Leydecker MT, Caboche M (1990). Nitrate reductase expression is under control of the circadian rhythm and is light inducible in *Nicotiana tabacum* leaves. *Planta* **180**, 257–261.

Franco AR, Cardenas J, Fernandez E (1987). Involvement of reversible inactivation of nitrate reductase in the regulation of enzyme levels in *Chlamydomonas reinhardtii*. *Plant Physiol* **84**, 665–669.

Fujita Y (1972). Composition of culture media. In: Tammiya H,

Watanable T, eds. *Experimental Methods of Algae*. Nankoto, Tokyo. pp. 68–104 (in Japanese).

Gao KS, Aruga Y, Asada K, Ishihara, Akano T, Kiyohara M (1991). Enhanced growth of the red algae *Porphyra yezoensis* Ueda in high CO_2 concentration. *J Appl Phycol* **3**, 355–362.

Gao KS, Aruga Y, Asada K, Kiyohara M (1993). Influence of enhanced CO_2 on growth and photosynthesis of the red algae *Gracilaria* sp. and *G. chilensis*. *J Appl Phycol* **5**, 563–571.

Giordano M (2001). Interactions between C and N metabolism in *Dunaliella salina* cells cultured at elevated CO_2 and high N concentrations. *J Plant Physiol* **158**, 577–581.

Giordano M, Bowes G (1997). Gas exchanges, metabolism, and morphology of *Dunaliella salina* in response to the CO_2 concentration and nitrogen source used for growth. *Plant Physiol* **115**, 1049–1056.

Gordillo FJL, Jiménez C, Figueroa FL, Niell FX (1999). Effects of increased atmospheric CO_2 and N supply on photosynthesis, growth and cell composition of the cyanobacterium *Spirulina platensis* (Arthrospira). *J Appl Phycol* **10**, 461–469.

Hein M (1997). Inorganic carbon limitation of photosynthesis in lake phytoplankton. *Freshwater Biol* **37**, 545–552.

Hein M, Sand-Jensen K (1997). CO_2 increases oceanic primary production. *Nature* **388**, 526–527.

Hocking PJ, Meyer CP (1991). Effects of CO_2 enrichment and nitrogen stress on growth and partitioning of dry matter and nitrogen in wheat and maize. *Aust J Plant Physiol* **18**, 339–356.

Huber JL, Huber SC, Campbell WH, Redinbaugh MG (1992). Reversible light/dark modulation of spinach leaf nitrate reductase activity involves protein phosphorylation. *Arch Biochem Biophys* **296**, 58–65.

King AW, Emanuel WR, Post WM (1992). Projecting future concentrations of atmospheric CO_2 with global carbon cycle models: importance of simulating historical changes. *Environ Manage* **16**, 91–108.

Kochert G (1978a). Carbohydrate determination by phenolsulfuric acid methods. In: Hellebust JA, Craigie JS, eds. *Handbook of Physiological Methods*: *Physiological and Biochemical Methods*. Cambridge University Press, London. pp. 95–97.

Kochert G (1978b). Protein determination by dye binding. In: Hellebust JA, Craigie JS, eds. *Handbook of Physiological Methods*: *Physiological and Biochemical Methods*. Cambridge University Press, London. pp. 90–93.

Larsson M, Larsson CM, Guerrero MG (1985). Photosynthetic nitrogen metabolism in high CO_2 and low-CO_2 adapted *Scenedesmus*. *J Exp Bot* **36**, 1373–1386.

Lee HM, Vasquez-Bermudez MF, Tandeau de Marsac N (1999). The global nitrogen regulator NtcA regulates transcription of the signal transducer P_{II} (GlnB) and influences its phosphorylation level in response to nitrogen and carbon supplies in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J Bacteriol* **181**, 2697–2702.

Lichtenthaler HK, Wellburn AR (1983). Determinations of total carotenoids and chlorophyll *a* and *b* of leaf extracts in different solvents. *Biochem Soc Trans* **63**, 591–592.

Magnusson G, Larsson C, Axelsson L (1996). Effects of high CO_2 treatment on nitrate and ammonium uptake by *Ulva lactuca* grown in different nutrient regimes. *Sci Mar* **60** (Suppl. 1), 179–189.

Miyachi S, Tsuzuki M, Avramova ST (1983). Utilization modes of inorganic carbon for photosynthesis in various species of *Chlorella*. *Plant Cell Physiol* **24**, 64–68.

Moroney JV, Somanchi A (1999). How do algae concentrate CO_2 to increase the efficiency of photosynthetic carbon fixation. *Plant Physiol* **119**, 9–16.

Moroney JV, Husic HD, Tolbert NE (1985). Effects of carbonic anhydrase inhibitors on inorganic carbon accumulation by *Chlamydomonas reinhardtii*. *Plant Physiol* **79**, 177–183.

Navarro MT, Prieto R, Fernandez E, Galvan A (1996). Constitutive expression of nitrate reductase changes the regulation of nitrate and nitrate transporters in *Chlamydomonas reinhardtii*. *Plant J* **9**, 819–827.

Pronina NA, Semenenko VE (1984). Localization of membrane bound and soluble carbonic anhydrase in the *Chlorella* cells. *Fiziol Rast* **31**, 241–251.

Radin JW (1973). *In vivo* assay of nitrate reductase in cotton leaf discs. *Plant Physiol* **51**, 332–336.

Riebesell U, Wolf-Gladrow DA, Smetacek T (1993). Carbon dioxide limitation of marine phytoplankton growth rates. *Nature* **361**, 249–251.

Riviere-Rolland, Contard P, Betsche T (1996). Adaptation of pea to elevated atmospheric CO_2: Rubisco phosphoenolpyru-

vate carboxylase and chloroplast phosphate translocator at different levels of nitrogen and phosphorus nutrition. *Plant Cell Environ* **19**, 109–117.

Siefermann-Harms D (1987). The light-harvesting and protective function of carotenoids in photosynthetic membranes. *Physiol Plant* **69**, 561–568.

Stitt M, Krapp A (1999). The interaction between elevated carbon dioxide and nitrogen nutrition: The physiological and molecular background. *Plant Cell Environ* **22**, 583–621.

Stumm W, Morgan JJ (1996). *Aquatic Chemistry*, 3rd edn. Wiley-Interscience, New York.

Sültemeyer D (1998). Carbonic anhydrase in eukaryotic algae: Characterization, regulation, and possible function during photosynthesis. *Can J Bot* **76**, 962–972.

Sültemeyer D, Fock HP, Canvin DT (1990). Mass spectrometric measurements of intracellular carbonic anhydrase activity in high and low C_i cells of *Chlamydomonas*. *Plant Physiol* **88**, 179–190.

Sültemeyer D, Amoroso G, Fock HP (1995). Induction of intracellular carbonic anhydrase during the adaptation to low inorganic carbon concentrations in wild-type and *ca*-1 mutant cells of *Chlamydomonas reinhardtii*. *Planta* **196**, 217–224.

Willbur KM, Anderson NG (1948). Electronric and colorimetric determination of carbon anhydrase. *J Biol Chem* **176**, 147–154.

Williams TG, Colman B (1996). The effect of pH and dissolved inorganic carbon on external carbonic anhydrase activity in *Chlorella saccharophila*. *Plant Cell Environ* **19**, 485–489.

Xia JR, Gao KS (2002). Effects of CO_2 enrichment on microstructure and ultrastructure of two species of freshwater green algae. *Acta Bot Sin* **44**, 527–531.

Yang Y, Gao KS (2003). Effects of CO_2 concentrations on the freshwater microalgae, *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (Chlorophyta). *J Appl Phycol* **15**, 379–389.

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