Impacts of Elevated CO\textsubscript{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\textsubscript{2} concentrations, *Chlorella pyrenoidosa* Chick and *Chlamydomonas reinhardtii* Dang cells were cultured at different CO\textsubscript{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, carbohydrate, and protein contents were enhanced to varying extents with increasing CO\textsubscript{2} concentration from 3–186 µmol/L. The CO\textsubscript{2} enrichment significantly increased the Chl $a$/Chl $b$ ratio in *Chlorella pyrenoidosa*, but not in *Chlamydomonas reinhardtii*. The CO\textsubscript{2} concentration had significant effects on CA and nitrate reductase activity. Elevating CO\textsubscript{2} concentration to 186 µmol/L caused a decline in intracellular and extracellular CA activity. Nitrate reductase activity, under either light or dark conditions, in *C. reinhardtii* and *C. pyrenoidosa* was also significantly decreased with CO\textsubscript{2} enrichment. From this study, it can be concluded that CO\textsubscript{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\textsubscript{2}; nitrate reductase.

It is generally predicted that the atmospheric CO\textsubscript{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\textsubscript{2} enrichment would also result in a reduction of the pH and an increase in the CO\textsubscript{2} concentration of surface waters (Stumm and Morgan 1996). It is important to assess the impact of increasing atmospheric CO\textsubscript{2} on aquatic plants (Bowes 1993). Riebesell et al. (1993) showed that CO\textsubscript{2} availability could potentially influence the growth of marine diatoms. Hein and Sand-Jensen (1997) demonstrated that an increase in atmospheric CO\textsubscript{2} would stimulate phytoplankton primary production in open ocean. An elevated CO\textsubscript{2} concentration enhanced photosynthesis and the growth of macroalgae (Gao et al. 1991, 1993). The enriched CO\textsubscript{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\textsubscript{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\textsubscript{3} under CO\textsubscript{2}-limited conditions. Carbonic anhydrase (CA) was demonstrated to be essential for photosynthetic utilization of inorganic carbon at low
external CO₂ concentrations and alkaline pH (Moroney et al. 1985), which played an important role in CO₂ transport either at the cell surface or in the cell. Extracellular CA (CAₑₓₜ) activity increased substantially within a couple of hours when cells were allowed to acclimate to air levels of CO₂ (Badger and Price 1994). Similarly, Williams and Colman (1996) found that CAₑₓₜ activity increased with a decrease in CO₂ availability. Intracellular CA (CAᵢₘ) activity in *C. reinhardtii* was reported to be considerably higher in low CO₂-grown cells than in those grown under conditions of high CO₂ (Amoroso et al. 1996). The impact of enhanced CO₂ on growth and photosynthesis also depends on the availability of nitrogen in the plant (Stitt and Krapp 1999).

The availability of CO₂ 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 CO₂ concentrations to 5% resulted in an increase of NR activity in *Ulva rigida* (Magnusson et al. 1996). These findings regarding CA and NR activity in the presence of high concentrations of CO₂ (1%–5% CO₂) are fundamental in understanding the biochemical responses of microalgae, but have little ecological implication in view of increases in atmospheric CO₂. Conversely, how CA and NR activity in freshwater phytoplankton responds to elevated atmospheric CO₂ remains unknown. At the same time, CO₂ 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 CO₂ 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 NaHCO₃. The range of CO₂ 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 10⁴ cells/mL by renewing the culture medium before the beginning of illumination every day. Temperature and illumination were controlled at 25 °C and 200 µmol·m⁻²·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 CO₂ 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). Carbohydrate 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 CO₂-saturated water (Willbur and Anderson 1948). Cells were broken with an ultrasonic homogenizer at 4 °C. Intact and broken cells were assayed for CAₑₓₜ and total CA activity, respectively. Carbonic anhydrase activity was calculated according to the following formula:

\[ EU = 10 \left( \frac{t_b}{t_c} - 1 \right) \]

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 CAᵢₘ activity was determined by subtracting CAₑₓₜ 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 KNO₃ in a final volume of 10 mL. Cells, at a density of 10⁶ 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 µg NO₂⁻·cell⁻¹·h⁻¹.

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

2 Results

The CO₂ enrichment had a significant effect on CA_ext activity (P < 0.05; Fig. 1). The CA_ext activity in Chlamydomonas reinhardtii decreased with increased CO₂ concentrations (21 and 186 µmol/L CO₂) to 18.7% and 45.5% of that in cells grown in 3 µmol/L CO₂, respectively (both P < 0.05), whereas CA_int decreased by 32.6% only in the presence of 186 µmol/L CO₂ (Fig. 2). Compared with CA_ext activity in Chlorella pyrenoidosa and Chlamydomonas reinhardtii grown at 3 µmol/L CO₂, CA_ext activity did not change in the presence of 21 µmol/L CO₂ (P > 0.05), whereas CA_ext activity was significantly reduced at a concentration of 186 µmol/L CO₂ (P < 0.05; Fig. 1). The activity of CA_int activity declined significantly with an increase in CO₂ concentrations to 21 and 186 µmol/L CO₂, being 73.9% and 84.9% of that in the presence of 3 µmol/L CO₂, respectively (P < 0.05; Fig. 2).

NR activity in Chlamydomonas reinhardtii and Chlorella pyrenoidosa was significantly (P < 0.05) affected by CO₂ concentration (Fig. 3). When cells were grown at 21 and 186 µmol/L CO₂, 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 CO₂. Nitrate reductase activity decreased by 67.9% and 76.4% in the dark in
Jian-Rong XIA and Kun-Shan GAO: Impacts of Elevated CO₂ Concentration on Biochemical Composition, Carbonic Anhydrase, and Nitrate Reductase Activity of Freshwater Green Algae

The CO₂ 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 CO₂ 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 CO₂. The Chl \(a/Chl b\) value increased significantly with CO₂ 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 CO₂ 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 CO₂. Increasing the CO₂ 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 CO₂, by 234.5% and 225.3%.

![Fig. 3](image) Nitrate reductase (NR) activity in *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* grown under various CO₂ concentrations (3, 21, and 186 µmol/L CO₂) at the end of photoperiod and darkness. Data are the mean ± SD \((n = 3)\).

**Table 1** Chlorophylls, carotenoids, carbohydrate, and protein content in *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* grown in the presence of different concentrations of CO₂

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CO₂ concentration (µmol/L)</th>
<th>3</th>
<th>21</th>
<th>186</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella pyrenoidosa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl ((10^6 \mu g/cell))</td>
<td></td>
<td>1.44 ± 0.09a</td>
<td>2.29 ± 0.22b</td>
<td>2.86 ± 0.21c</td>
</tr>
<tr>
<td>Carotenoids ((10^6 \mu g/cell))</td>
<td></td>
<td>0.28 ± 0.03a</td>
<td>0.38 ± 0.04b</td>
<td>0.43 ± 0.02b</td>
</tr>
<tr>
<td>Chl (a/Chl b)</td>
<td></td>
<td>2.82 ± 0.12a</td>
<td>3.03 ± 0.38a</td>
<td>3.88 ± 0.20b</td>
</tr>
<tr>
<td>Carbohydrate (%, w/w)</td>
<td></td>
<td>9.30 ± 0.62a</td>
<td>11.66 ± 0.26b</td>
<td>21.01 ± 0.96c</td>
</tr>
<tr>
<td>Protein (%, w/w)</td>
<td></td>
<td>15.90 ± 0.29a</td>
<td>53.18 ± 1.00b</td>
<td>51.73 ± 0.58b</td>
</tr>
<tr>
<td><em>Chlamydomonas reinhardtii</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl ((10^6 \mu g/cell))</td>
<td></td>
<td>5.56 ± 0.15a</td>
<td>7.22 ± 0.35b</td>
<td>6.82 ± 0.27b</td>
</tr>
<tr>
<td>Carotenoids ((10^6 \mu g/cell))</td>
<td></td>
<td>1.31 ± 0.03a</td>
<td>1.77 ± 0.08b</td>
<td>1.65 ± 0.08b</td>
</tr>
<tr>
<td>Chl (a/Chl b)</td>
<td></td>
<td>2.21 ± 0.02a</td>
<td>2.24 ± 0.03a</td>
<td>2.20 ± 0.04a</td>
</tr>
<tr>
<td>Carbohydrate (%, w/w)</td>
<td></td>
<td>3.19 ± 0.10a</td>
<td>7.40 ± 0.81b</td>
<td>6.41 ± 0.64b</td>
</tr>
<tr>
<td>Protein (%, w/w)</td>
<td></td>
<td>23.94 ± 0.23a</td>
<td>29.14 ± 0.19b</td>
<td>30.86 ± 1.06c</td>
</tr>
</tbody>
</table>

Data are the mean ± SD \((n = 3)\). Values with different letters in the same row are significantly different \((P < 0.05, \text{Duncan's multiple comparison})\). 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$_2$CO$_3$, 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 ($^1$O$_2$), which is produced by the excited triplet state of Chl. In the meantime, it can directly deactivate $^1$O$_2$ and can also quench the excited triplet state of Chl, thus reducing the formation of $^1$O$_2$ 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 Larsson 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 CO2 concentrations in *C. reinhardtii* (Sültemeyer 1998). Therefore, an increase in the CO2 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 CO2 enrichment (Besford and Hand 1989; Hocking and Meyer 1991) but, in another study, no effect of CO2 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 CO2, 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 CO2 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 CO2 concentrations. The species-dependent response to elevated CO2 concentrations in the freshwater ecosystem could alter species composition, decrease diversity, and alter food webs as a whole.

**References**


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