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Effect of CO_2 concentrations on the activity of photosynthetic CO_2 fixation and extracelluar carbonic anhydrase in the marine diatom *Skeletonema costatum*

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Abstract **The growth and activity of photosynthetic CO_2 uptake and extracellular carbonic anhydrase (CA_{ext}) of the marine diatom *Skeletonema costatum* were investigated while cultured at different levels of CO_2 in order to see its physiological response to different CO_2 concentrations under either a low (30 mol·m^{-2}·s^{-1}) or high (210 mol·m^{-2}·s^{-1}) irradiance. The changes in CO_2 concentrations (4—31 mol/L) affected the growth and net photosynthesis to a greater extent under the low than under the high light regime. CA_{ext} was detected in the cells grown at 4 mol/L CO_2 but not at 31 and 12 mol/L CO_2, with its activity being about 2.5-fold higher at the high than at the low irradiance. Photosynthetic CO_2 affinity (1/ $K_{1/2}(CO_2)$) of the cells decreased with increased CO_2 concentrations in culture. The cells cultured under the high-light show significantly higher photosynthetic CO_2 affinity than those grown at the low-light level. It is concluded that the regulations of CA_{ext} activity and photosynthetic CO_2 affinity are dependent not only on CO_2 concentration but also on light availability, and that the development of higher CA_{ext} activity and CO_2 affinity under higher light level could sufficiently support the photosynthetic demand for CO_2 even at low level of CO_2.**

Keywords: acetazolamide (AZ), carbonic anhydrase, CO_2 affinity, dissolved inorganic carbon (DIC), light, photosynthesis.

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The primary production in the ocean is considered not to be limited by dissolved inorganic carbon (DIC) but by nutrients, light or zooplankton grazing[1,2]. However, recent studies with marine diatoms cultured in the laboratory[3] and with phytoplankton assembly in natural environments[4] have shown that the supply of CO_2 in seawater could restrict the photosynthesis and growth, suggesting that the atmospheric CO_2 rise associated with industrial combustion of fossil fuels would enhance the oceanic primary productivity. Nevertheless, the large-scale significance of these results remains to be assessed.

In nature, light fluctuates both temporally and spatially, and light availability has been shown to affect phytoplankton biomass in the ocean[5]. However, little is known about the interactive effects of light and CO_2 on marine phytoplankton, which usually concur and play important roles in controlling the physiological behavior of phytoplankton[6].

Marine diatoms, as dominant primary producers in marine ecosystems, contribute greatly to the marine new production in the oceans[6]. The carboxylation by ribulose-1, 5-bisphosposphate carboxylase and oxygenase (Rubisco) in marine diatoms has been shown to require much higher CO_2 concentration (30—60 mol/L CO_2) than that in seawater (about 10 mol/L CO_2) to become half-saturated[7], and their growth in general seawater has been proved to be CO_2-limited[3]. However, some kinetics and growth studies showed that marine diatoms could avoid CO_2-limitation[8—10], suggesting the existence of CO_2 concentrating mechanism (CCM)[6]. The CCM has been studied extensively in cyanobacteria and green microalgae[11—15], exhibiting two key components: a mechanism for active uptake of DIC and internal carbonic anhydrase (CA) that catalyzes the inter-conversion of HCO_3^- and CO_2[12]. Less knowledge on the regulation of the CCM has been documented in marine diatoms compared with green microalgae. Matsuda et al.[16] reported that dissolved CO_2 in culture medium was the critical signal for the regulation of photosynthetic affinity to dissolved inorganic carbon (DIC) in the marine diatom *Phaeodactylum tricornutum.* In addition to bulk CO_2 levels, light, temperature or nutrient levels may also play important roles in regulating the components of CCM. However, there is a paucity of information on this aspect in the marine diatoms. In the present study, we investigated the effects of varied CO_2 and light levels on the growth, photosynthesis and activity of CA_{ext} in *Skeletonema costatum,* in an effort to clarify their interactive effects, and to explain the nature of CCM in this marine diatom.

1 Materials and methods

(ⅰ) Cultures. *Skeletonema costatum* (Greville) Cleve was obtained from the Institute of Oceanography, the Chinese Academy of Sciences. All cultures were carried out in a CO_2 plant chamber (Canviron EF7, Canada). Cells at mid-exponential phase were inoculated at low density (less than 1×10^5 cells·L^{-1}) and grown in sealed flasks with nutrient-enriched (3.0 mmol/L KNO_3, 0.1 mmol/L Na_2HPO_3, 70 μmol/L $NaSiO_3$, 1.0 μmol/L $FeSO_4$ and 25 μmol/L EDTANa), filtered natural seawater at 20℃ and 210 or 30 μmol·m^{-2}·s^{-1} (12∶12 LD cycle), which were respectively representative of the average light levels at the surface and deep layers in the oceanic euphotic zones[4,17,18]. The seawater was collected from

Nan' ao Island, Shantou, China. Illumination was provided with white fluorescent lamps. CO_2 concentrations of 31, 12 and 4 μmol/L in the culture were obtained by adjusting pH of the medium with 0.1 mol·L^{-1} HCl or NaOH[3] to pH 7.8, pH 8.2 and pH 8.6, respectively. The CO_2 concentrations of 4, 12 and 31 μmol/L correspond roughly to the atmospheric CO_2 levels ranged from 100 to 1000 μL/L, with the low CO_2 level equivalent to the CO_2 concentration occurred in the last glaciation or during phytoplankton bloom formation[3] and the high CO_2 level corresponding to the CO_2 concentration predicted in the future oceanic surface water[19]. The dissolved inorganic carbon (DIC) in the medium was 2.0 mmol/L. Variation of CO_2 concentrations during each culture was less than 5%. The CO_2 concentrations were determined on the known levels of DIC, pH, salinity and temperature according to ref. [20]. DIC was measured by using a total organic carbon analyzer (TOC-5000A Shimadzu, Japan). The cells had been pre-cultured for at least six cell divisions (3—4 d, mid-exponential) for them to acclimate to the levels of CO_2 and irradiance before being used for experiments. The cells used for experiment were harvested within 3—6 h after the start of light period.

(ii) Determination of the growth rate. Specific growth rate (m) was determined by the following formula:

$$m = (\ln x_2 - \ln x_1)/(t_2 - t_1),$$

where x_2 and x_1 were the number of cells at t_2 and t_1 number of days ($t_2 - t_1$ was 3 or 4 d), respectively. Cells were counted microscopically by using a haemocytometer.

(iii) Measurement of extracellular carbonic anhydrase activity. The harvested cells were washed and re-suspended in the seawater buffered with 20 mmol/L veronal at pH 8.2. Activity of carbonic anhydrase (CA) was assayed by an electrometric method as described by Wilbur and Anderson[21]. Extracellular CA activity was measured with intact cells. 0.5 mL cell suspension was added to 4 mL cold veronal buffered seawater and mixed, the time required for pH drift from pH 8.2 to 7.2 was recorded after the addition of 2 mL ice-cold CO_2-saturated pure water (which was prepared by bubbling pure CO_2 into ice-cold pure water for at least 60 min). The temperature during the reaction was controlled at 4℃. Enzyme units were calculated from the equation:

$$EU = 10*(T_0 / T_{-1}),$$

where T_0 and T represent respectively the periods of time required for the pH drift in the presence and absence of 100 μmol/L acetazolamide (AZ) (Sigma), which inhibits the activity of extracellular CA and unpenetrates into cell.

(iv) Photosynthesis measurement. Photosynthetic oxygen evolution was measured with a Clark-type oxygen electrode (YSI-5300, USA). The harvested cells were washed and re-suspended in the fresh medium buffered with 20 mmol/L Tris-HCl (pH 7.8, 8.2 or 8.6). Temperature during the measurement was controlled at 20℃ by using a cooling circulator (Cole Parmer Instrument Co. USA). Illumination was supplied by a halogen lamp (220/240 V, 150 W, Phoenix Electric Co., Japan). Light intensity was measured with a quantum sensor (SKP 200, ELE international). 5 mL cell suspension (1×10^6—2×10^6 cell·mL^{-1}) was transferred to the electrode chamber. O_2 levels in the reaction chamber were reduced to less than 30% by bubbling N_2 prior to the measurements.

In the measurements of net photosynthesis (P_n) and dark respiration (R_d), the buffered seawater contained 2.0 mmol/L DIC. The net photosynthetic rates of cells grown at 210 or 30 μmol·m^{-2}·s^{-1} under 31, 12 and 4 μmol/L CO_2 were respectively measured under the corresponding conditions. Their respiratory rates were respectively measured at the corresponding CO_2 concentrations in the dark.

In measurement of DIC-dependent oxygen evolution, "CO_2"-free seawater (pH 8.2) was prepared according to Gao et al.[22]. The cells were allowed to photosynthesize to deplete possible intracellular pool of "CO_2" until no net O_2 evolution was observed before the measurement. Following the addition of different amounts of $NaHCO_3$, rate of oxygen evolution was measured at 400 μmol·m^{-2}·s^{-1}. The $K_{1/2}$ (DIC) value for photosynthesis was determined by fitting the net photosynthetic rates at various DIC concentrations with the Michaelis-Menten formula:

$$V = V_{\max}[S]/(K_{1/2}(\mathrm{DIC}) + [S]),$$

where [S] is for DIC concentration; V, net photosynthetic rate at a given DIC concentration; V_{max}, the DIC-saturated rate of photosynthesis; $K_{1/2}$(DIC), the DIC concentration required to give half-maximal photosynthetic rate, representing the photosynthetic affinity to DIC. $K_{1/2}(CO_2)$ was estimated from $K_{1/2}$(DIC).

(v) Measurement of chlorophyll a concentration. Chlorophyll a concentration was determined by the spectrophotometric method according to Jeffrey and Humphrey[23].

2 Results

Skeletonema costatum grew much faster at 210 μmol·m^{-2}·s^{-1} than at 30 μmol·m^{-2}·s^{-1} regardless of CO_2 concentrations (Fig. 1). In the high irradiance, when CO_2 concentration was raised from 4 to 31 μmol/L, the growth was not enhanced (ANOVA, $P > 0.5$). In the low irradiance, it showed no significant difference in the specific growth rate between 31 and 12 μmol/L CO_2 (t-test, $P > 0.5$). However, the growth rate at 4.0 μmol/L CO_2 significantly declined (t-test, $P < 0.01$) (Fig. 1). Dark respiration was not affected by the variations in CO_2 concentration under both the high and low light regimes (ANOVA, $P > 0.2$) (Table 1). In the high irradiance, net photosynthesis was not enhanced (ANOVA, $P > 0.5$) when CO_2 concentration was raised from 4 to 31 μmol/L. However, it significantly declined at 4 μmol/L CO_2 under the low irradi-

ance (*t*-test, $P < 0.05$) (Table 1). It appeared that the growth and photosynthesis were limited at the low CO_2 concentration under the low irradiance.

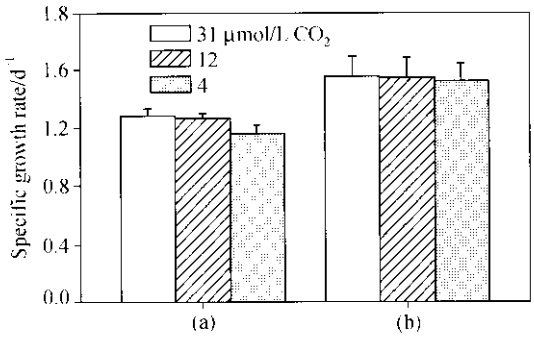

Fig. 1. Growth of *Skeletonema costatum* as a function of CO_2 concentrations at (a) 30 and (b) 210 μmol·m^{-2}·s^{-1}. Data are the means ± SE (n=7).

Table 1 Net photosynthesis (P_n) and dark respiration (R_d) of *Skeletonema costatum* cells grown under varied levels of CO_2 and light

Rates of / mol O_2 · mg chla^{-1} · h^{-1}	210 μ mol · m^{-2} · s^{-1}			30 μ mol · m^{-2} · s^{-1}		
	31	12	4	31	12	4
	μ mol/L CO_2			μ mol/L CO_2		
P_n	256±17	257±62	259±20	76±4	76±3	65±7
R_d	57±9	52±5	54±6	28±4	28±3	27±2

The data are the means ± SE (n = 4—6).

No extracellular carbonic anhydrase (CA_{ext}) activity was detected in the cells grown at 31 and 12 μ mol/L CO_2 at 30 or 210 μmol·m^{-2}·s^{-1}, but it was significantly recognized in those grown at 4 μ mol/L CO_2 under both the low and high light levels (Fig. 2(a)). The CA_{ext} activity of the cells grown at 4 μ mol/L CO_2 was about 2.5-fold higher at the high irradiance than at the low irradiance (Fig. 2(a)). In the presence of AZ, net photosynthesis was not affected in the cells grown at 31 and 12 μ mol/L CO_2 under 30 or 210 μ mol·m^{-2}·s^{-1}, but was reduced in the cells grown at 4 μ mol/L CO_2 by 11% and 24% under the low or the high light regimes, respectively (Fig. 2(b)), indicating that there was greater photosynthetic contribution of CA_{ext} under the high light.

Response of photosynthesis to DIC in *S. costatum* is shown in Fig. 3. In the high-light cultures, CO_2 affinity for photosynthesis ($K_{1/2}(CO_2)$) for the cells grown at 31, 12 and 4 μ mol/L CO_2 was 1.4, 1.2 and 0.5 μ mol/L CO_2, respectively (Fig. 3, Table 2). The cells grown at the lowest CO_2 level showed 2- to 3-fold increase in the CO_2 affinity for photosynthesis. In the low-light cultures, $K_{1/2}(CO_2)$ values were 1.6 and 1.3 and 1.0 μ mol/L for the cells grown at 31, 12 or 4 μ mol/L CO_2, respectively. The photosynthetic affinity for CO_2 was about 23%—38% higher at the lowest CO_2 compared with the higher levels (Fig. 3, Table 2). In comparison of $K_{1/2}(CO_2)$ values between the high and low light cultures, it appeared that in the low-light cultures the CO_2 affinity of the alga was about 10% at 31 and 12 μ mol/L CO_2 and about 50% at 4 mol/L CO_2 lower than the corresponding values in the high-light cultures (Fig. 3, Table 2).

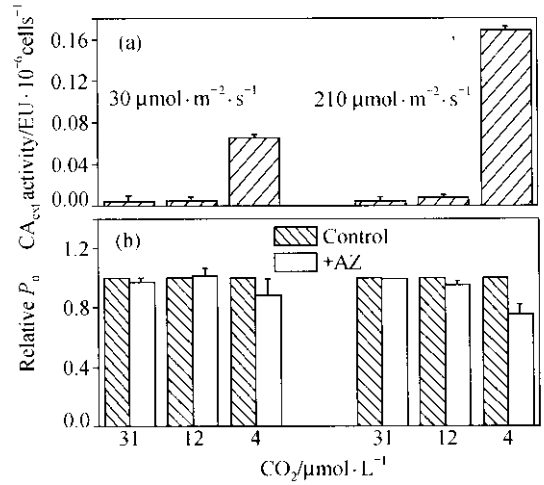

Fig. 2. Activity of extracellular carbonic anhydrase (CA_{ext}) (a) and relative net photosynthesis (P_n) (b) of *Skeletonema costatum* grown at varied levels of CO_2 and light. P_n was measured at 400 μ mol·m^{-2}·s^{-1} in the absence (control) and presence (+AZ) of 100 μ mol/L AZ. Data are the means ± SE (n=3—5).

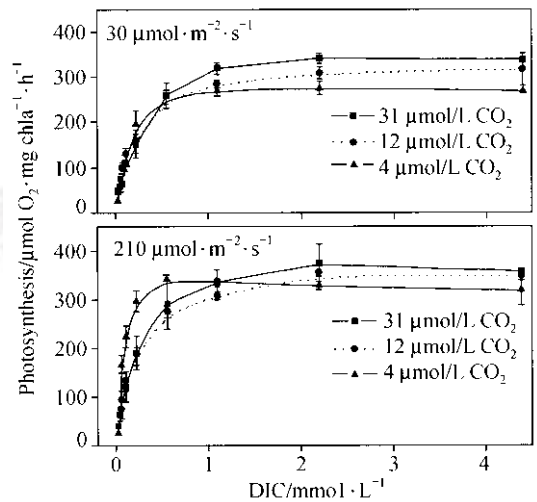

Fig. 3. Photosynthetic response of *Skeletonema costatum* grown under varied levels of CO_2 and light to varied concentrations of dissolved inorganic carbon (DIC). Data are the means ± SE (n=3).

In the presence of AZ, $K_{1/2}(CO_2)$ values of the cells grown at 210 or 30 μ mol·m^{-2}·s^{-1} were 1.8 and 1.5 μ mol/L CO_2, respectively, being increased by 3.5- or 1.5-fold compared with the controls (Fig. 4, Table 3). It appeared that the effect of CA_{ext} on the photosynthetic affinity for CO_2 was greater under the high light than the low light.

Table 2 $K_{1/2}$ values of DIC and CO_2 in *Skeletonema costatum* grown under varied levels of CO_2 and light

$K_{1/2}$ values[a)] / μmol·L^{-1}	210 μmol·m^{-2}·s^{-1}			30 μmol·m^{-2}·s^{-1}		
	31	12	4	31	12	4
	μmol/L CO_2			μmol/L CO_2		
DIC	241±23	203±13	84±22	277±37	210±20	161±26
CO_2	1.4±0.1	1.2±0.1	0.5±0.1	1.6±0.2	1.2±0.1	1.0±0.2

a) $K_{1/2}$ (DIC) was estimated from Fig. 3.

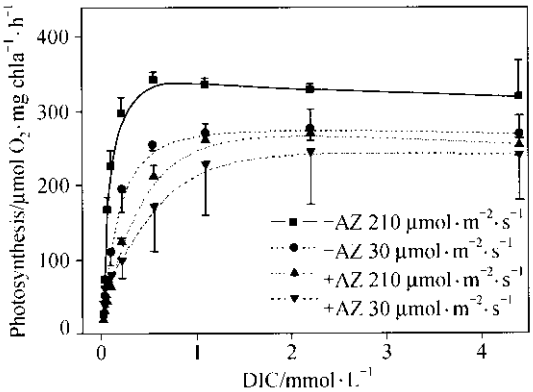

Fig. 4. Effect of extracellular CA inhibitor AZ (100 μmol/L) on the relationship of photosynthesis and DIC in *Skeletonema costatum* grown at 4 μmol/L CO_2 and 210 or 30 μmol·m^{-2}·s^{-1}. Data are the means ± SE (n=3).

Table 3 $K_{1/2}$ values of DIC and CO_2 for *Skeletonema costatum* grown at 4 μmol/L CO_2 and 210 or 30 μmol·m^{-2}·s^{-1} in the absence and presence of 100 μmol/L AZ

$K_{1/2}$ values[a)] /μmol·L^{-1}	4 μmol/L CO_2 210 μmol·m^{-2}·s^{-1}		4 μmol/L CO_2 30 μmol·m^{-2}·s^{-1}	
	−AZ	+AZ	−AZ	+AZ
DIC	84±22	300±57	161±26	247±47
CO_2	0.5±0.1	1.8±0.3	1.0±0.2	1.5±0.3

a) $K_{1/2}$ (DIC) was estimated from Fig. 4.

3 Discussion

In natural seawaters and culture tanks using *S. costatum* as a food organism, CO_2 concentration usually fluctuates during a day. Atmospheric CO_2 rise to the double level of the present would increase the dissolved CO_2 and decrease the pH of seawater. In the present study, CO_2 level raised to 31 μmol/L with pH lowered to 7.8 did not enhance the growth at either 30 or 210 μmol·m^{-2}·s^{-1}. However, when the CO_2 level was reduced to 4 μmol/L from 12 μmol/L which is equivalent to the common CO_2 concentration in general seawater, specific growth rate decreased significantly only under the low light regime, implying that growth of the alga could be limited in the water where both CO_2 and light were reduced by dense biomass or during specific periods of a day. *Skeletonema costatum* constitutes a major component of most marine phytoplankton blooms. During the development of *S. costatum* bloom, light and CO_2 availability in seawater was reduced by dense biomass and seawater pH rise from 8.2 to 8.5—8.7[24], this indicated that its growth could be CO_2-limited during its bloom. Culture experiments under optimal growth conditions showed that atmospheric CO_2 rise might not affect photosynthesis and growth of marine phytoplankton[6]. The present study demonstrated that growth and photosynthesis of *S. costatum* were CO_2-limited under 30 μmol·m^{-2}·s^{-1} but unaffected under 210 μmol·m^{-2}·s^{-1} when the dissolved CO_2 was lowered to 4 μmol/L by raising pH to 8.6, implying that influences of CO_2 concentrations on phytoplankton might have been underestimated under low light conditions.

The response to carbon limitation in *S. costatum* was associated with the development of CA_{ext}[25,26], which catalyzes the interconversion of HCO_3^- and CO_2 in the periplasm space. In the present study, induction of CA_{ext} activity was recognized at 4 μmol/L CO_2 in both the high- and low–light cultures. However, the CA_{ext} activities were lower under the low light compared with the high light, suggesting that light plays an important role in regulating CA_{ext} activity of this diatom. Nimer et al.[25] have reported that redox activity external to the plasma membrane was required for the development of CA_{ext} activity in *S. costatum* under the condition of CO_2-limitation. Plasma membrane redox activity in plants was light-dependent[27]. Thus low redox activity under the low light could be accounted for the lower CA_{ext} activity in *S. costatum.*

In the present study, the long-term (3—4 d, at least six generations) acclimation of *S. costatum* to the higher CO_2 level (31 μmol/L) led to the declined CO_2 affinity for photosynthesis (Fig. 3, Table 2). Marine phytoplankton in the long-term growth commonly showed a lower inorganic carbon affinity when CO_2 was enriched[6,28]. Such a down-regulation of CO_2 affinity for photosynthesis was generally explained as the function of CCM adjusting to increased CO_2 concentrations[13,14]. *Skeletonema costatum* grown at 4 μmol/L CO_2, in the present study, showed higher CO_2 affinity under the high light compared with the low light. This can be accounted for the lower CA_{ext} activity in the low light (Fig. 4, Table 3). The efficiency of CO_2 uptaken by *S. costatum* appeared to be dependent on the availability of light in addition to CO_2.

The development of higher CA_{ext} activity at 4 mol/L CO_2 in the high-light-grown cells could lead to faster conversion of HCO_3^- to CO_2, resulting in faster CO_2 uptake that offsets the limitation of CO_2 diffusion at the low CO_2 level. The compensated CO_2 uptake efficiency by the enhanced CA_{ext} activity gave rise to the specific growth rates at 4 μmol/L CO_2 equivalent to those at 12 and 31 μmol/L CO_2. Under the low-light condition, CA_{ext} activity was enhanced at 4 mol/L CO_2 compared with 12 and 31 μmol/L CO_2, but to a less extent compared to that under the high light. The lower CA_{ext} could not efficiently supply enough CO_2 to meet uptake of CO_2 into the cells

and led to the reduced growth and photosynthesis rates, so the low-light-suppressed CA_{ext} activity could be accountable for the reduced growth and photosynthetic rates.

In the oceanic system, many marine diatoms could develop CA_{ext} under conditions of carbon limitation[26]; their growth and photosynthesis therefore were considered not to be affected in nature by the rise in atmospheric CO_2 concentration[13]. However, the light-dependency of CA_{ext} in *S. costatum* demonstrated in the present study reflects that growth and photosynthesis of marine diatoms grown in waters of low CO_2 concentrations would be CO_2-limited at lower levels of irradiance. Subsequently, it can be deduced that marine diatoms, whose growth in nature commonly proceeds under the condition of light limitation[6,29], could have increased oceanic primary productivity when the atmospheric CO_2 level rose from the last glaciation to the present.

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