

## Inorganic Carbon Utilization of the Freshwater Red Alga *Compsopogon coeruleus* (Balbis) Montagne (Compsopogonaceae, Rhodophyta) Evaluated by *in situ* Measurement of Chlorophyll Fluorescence

Shao-Lun Liu<sup>(1)</sup>, Liang-Chi Wang<sup>(1)</sup> and Wei-Lung Wang<sup>(1,2)</sup>

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**ABSTRACT:** To explore the inorganic carbon utilization of the freshwater red alga *Compsopogon coeruleus*, photosynthetic rates in response to increasing of bicarbonate concentration, the addition of alkaline HEPES buffer (pH 8.8), acid HEPES buffer (pH 4.0) and the extracellular carbonic anhydrase inhibitor (acetazolamide, AZ), respectively, were examined *in situ* by using a submersible pulse amplitude modulated (PAM) fluorometer. Among the treatments, adding acid HEPES buffer significantly reduced photosynthetic rates of the alga, while others showed no effect. Accordingly, we concluded that *C. coeruleus* had less or no inorganic carbon ( $C_i$ ) limitation in its natural habitat. The alga might have higher affinity for bicarbonate and directly uptake bicarbonate as main  $C_i$  source without the aid of extracellular carbonic anhydrase.

**KEY WORDS:** Acetazolamide, Carbonic anhydrase, Fluorometer, Inorganic carbon, HEPES, PAM, Photosynthesis.

### INTRODUCTION

*Compsopogon coeruleus* (Balbis) Montagne (as *Compsopogon chalybeus* Kützing), a branched and filamentous macroscopic red alga habitat freshwater, has been recently found in Taiwan (Liu and Wang, 2004). The alga is widely distributed in clear and alkaline streams from lowland subtropical to tropical regions worldwide (Kumano, 2002). Most studies of *Compsopogon* have focused on its taxonomy and ecology (Necchi *et al.*, 1990; Vis *et al.*, 1992; Necchi and Dip, 1992; Necchi and Pascoaloto, 1995; Necchi *et al.*, 1999), and ignored its photosynthetic physiology, in contrast to other members of the freshwater red algae that have been more extensively examined (Raven and Beardall, 1981; Raven *et al.*, 1982; Kremer, 1983; Sheath, 1984; Sheath and Hambrook, 1990; Karsten *et al.*, 1993; Leukart and Hanelt, 1995; Necchi and Zucchi, 2001). Hence, it would be worthwhile to characterize the photosynthetic utilization of inorganic carbon in *C. coeruleus*.

Though there are three types of inorganic carbon ( $C_i$ , including  $CO_2$ ,  $HCO_3^-$  and  $CO_3^{2-}$ ) in aquatic systems, the major form of inorganic carbon available to *C. coeruleus* has been found to be bicarbonate (Necchi and Zucchi, 2001). Two processes of bicarbonate utilization in aquatic plants have been proposed (Zou *et al.*, 2003). Some plants convert  $HCO_3^-$  to  $CO_2$  prior to uptake with the aid of extracellular carbonic anhydrase (CA), or they may directly uptake bicarbonate without the aid of extracellular carbonic anhydrase. The enzyme CA is inhibited by the membrane-impermeable CA inhibitor, acetazolamide (AZ). Hence, AZ is generally used to detect whether plants can directly uptake bicarbonate.

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1. Department of Biology, National Changhua University of Education, Changhua 500, Taiwan.

2. Corresponding author. Tel: 886-4-7232105 ext. 3436; Email: wlwang@cc.ncue.edu.tw

Photosynthetic performance of aquatic plants is traditionally assayed by O<sub>2</sub> evolution and carbon isotope techniques in the lab (Raven and Beardall, 1981; Raven *et al.*, 1982; Kremer, 1983; Sheath, 1984; Sheath and Hambrook, 1990; Karsten *et al.*, 1993; Leukart and Hanelt, 1995; Gao and Zou, 2001; Necchi and Zucchi, 2001; Zou and Gao, 2002; Zou *et al.*, 2003). Recently, a new method of chlorophyll fluorescence, the pulse amplitude modulated technique (PAM), has been utilized as a non-intrusive indicator to study photosynthesis in algae and plants (Maxwell and Johnson, 2000). Schwarz *et al.* (2000) designed a special Perspex chamber, along with an underwater PAM fluorometer, permitting *in situ* measurements of photosynthetic rates of aquatic plants under the treatments of various reagents. According to their results, the authors argued that artificial physiological experiments in the lab would probably result in misleading information.

In this study, we used an underwater PAM fluorometer with a specially designed Perspex chamber to examine *in situ* photosynthetic rates of *C. coeruleus* in response to increased bicarbonate concentration, addition of alkaline HEPES buffer (pH 8.8), addition of acid HEPES buffer (pH 4.0) and addition of acetazolamide (AZ). The purpose of this study was to obtain a better understanding of inorganic carbon utilization in this aquatic organism.

## MATERIALS AND METHODS

### Study site and the measurement of environmental variables

*Compsopogon coeruleus* (Balbis) Montagne was studied in April 2004, in a clear, small pool of water (ca. 4 m<sup>2</sup> in area, 20-50 cm in depth) in Bagu Mountain, Changhua City, Taiwan (N 24°04.948', E 120°33.451'). Water temperature, pH, conductivity, dissolved oxygen (DO) and turbidity of the water were measured by thermometer, pH meter, conductometer, DO meter and turbidity meter *in situ*, respectively. A water sample (approximately 1,000 ml) was collected, and then measured for dissolved inorganic nitrogen (DIN, including NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup> + NH<sub>3</sub>) and dissolved inorganic phosphate (DIP, PO<sub>4</sub><sup>3-</sup>) using a SMART<sub>Spectro</sub> spectrophotometer (LaMotte, Maryland, USA).

### Measurements of chlorophyll fluorescence

*In situ* photosynthetic performance of *C. coeruleus* was analyzed using an underwater fluorometer (Diving PAM, Heinz Waltz, Germany). A thallus 2-3 mm wide was used to determine the photosynthetic rates. The light response curve of samples was assessed for effective quantum yield measurements. The effective quantum yield of PSII ( $Y = (F_m' - F) / F_m'$ , where  $F_m'$  = light-adapted maximal fluorescence,  $F$  = fluorescence yield for a given light state before a saturating pulse) was measured in light-adapted samples at darkness and eight irradiance levels ranging from non-saturating to saturating intensities (139, 213, 299, 450, 612, 943, 1376, and 2097  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ ); light was provided by an internal halogen lamp and controlled by the Diving PAM's RLC (rapid light curve) function at 10s-interval. Because this study compared only the relative difference before and after treatment, only relative electron transport rate (RETR) was calculated, using the following equation according to the manufacture's manual:  $\text{RETR} = Y \cdot \text{PPFD} \cdot 0.5 \cdot \text{AF}$ , where  $Y$  is the effective quantum yield of PSII, PPFD is photosynthetically available irradiance reaching the leaf ( $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ ), 0.5 assumes that half of the PPFD was absorbed by PSII, and AF is the fraction of PPFD absorbed by the leaf (0.84 in the instrument default value). The light-saturated maximal

RETR (RETR<sub>max</sub>) and the slope of light-limited RETR ( $\alpha^{\text{RETR}}$ ) was calculated using the hyperbolic tangent equation:  $\text{RETR} = \text{RETR}_{\text{max}} \tanh(\alpha^{\text{RETR}} I / \text{RETR}_{\text{max}})$ , where  $I$  is the irradiance (Jassby and Platt, 1976). The light intensity at the onset of saturation ( $E_k$ ) was then calculated as:  $E_k = \text{RETR}_{\text{max}} / \alpha^{\text{RETR}}$ . The relationship between light intensity and photochemical quenching (qP) and nonphotochemical quenching (qN) was also measured via the RLC function of the Diving-PAM. Quenching analysis provides insight into the mechanisms controlling the overall photosynthetic activity. Due to the lack of measurement of dark-adapted chlorophyll fluorescence parameters, only relative values of quenching parameters, qN and qP, were determined from RLCs (Schreiber *et al.*, 1994; McMinn *et al.*, 2004).

### ***In situ* experimental procedure**

The experimental procedure followed that of Schwarz *et al.* (2000) and was conducted using a special designed Perspex chamber (20 ml). Experiments were conducted to detect the preferred  $C_i$  source and the mechanism of  $C_i$  transport for *C. coeruleus*. First, to investigate the preferred  $C_i$  source for *C. coeruleus*, photosynthesis of the sample inside the chamber was measured using RLC function of the Diving PAM before and after the addition of 1 ml 1 M alkaline and acid HEPES buffer solution (pH 8.8 and pH 4.0, final concentration 50 mM), at pH 4.0  $\text{CO}_2/\text{H}_2\text{CO}_3$  constituted *ca.* 100% of inorganic carbon while at pH 8.8  $\text{HCO}_3^-$  constituted *ca.* 100% of inorganic carbon (Necchi and Zucchi, 2001). Secondly, to test for external CA-mediated  $\text{HCO}_3^-$  uptake, the photosynthetic light response curve of a separate sample inside the chamber was measured before and after addition of 0.2 ml 0.001 mM acetazolamide (AZ) solution (a membrane-impermeable CA inhibitor, final concentration 100  $\mu\text{M}$ ). All measurements were done in three replicates. In every treatment, the incubation time was 5 min, and then the light response curve was recorded. Finally to learn whether the alga had  $C_i$  limited in its natural habitat, we first measured the initial light response curve without additional treatment. 0.4 ml of 100 mM  $\text{NaHCO}_3$  solution was injected from a 1 ml micropipette to increase the concentration of  $C_i$  by about 2 mM in the chamber, and then the response curve was measured again. Prior to the third RLC measurement, a further 0.4 ml of 100 mM  $\text{NaHCO}_3$  was injected to the increased concentration of  $C_i$  in the chamber by another 2 mM.

### **Statistical analysis**

The data were expressed as means  $\pm$  standard error (SE). The *t*-test and analysis of variance (ANOVA) were used to test the statistical significance of the data with the significance level set at 0.05.

## **RESULTS AND DISCUSSION**

Results from measurements of environmental variables (Table 1) revealed that *C. coeruleus* in this study inhabits a clear, nearly neutral, warm and nutrient moderate aquatic environment. These results concur with previous reports on the algae in the genus *Compsopogon* prefers warmer, nearly neutral or more alkaline and slight by pollutant habitat (Sheath and Hambrook, 1990; Wehr and Sheath, 2003; Liu and Wang, 2004).

The Rapid light curve (RLC) of *C. coeruleus* is shown in Fig. 1. The light-saturated maximum relative electron transport rate (RETR<sub>max</sub>), the initial slope of light-limited RETR

Table 1. Environmental variables in the habitat of *Compsopogon coeruleus* in this study are listed.

Parameters	Values
Water temperature (°C)	20.3
pH	7.18
Conductivity ( $\mu\text{S cm}^{-1}$ )	469
Dissolved oxygen ( $\text{mg L}^{-1}$ )	5.01
Turbidity (NTU*)	10.0
Dissolved inorganic nitrogen (ppm)	0.367
Dissolved inorganic phosphate (ppm)	0.447

\*NTU, nephelometric turbidity unit.

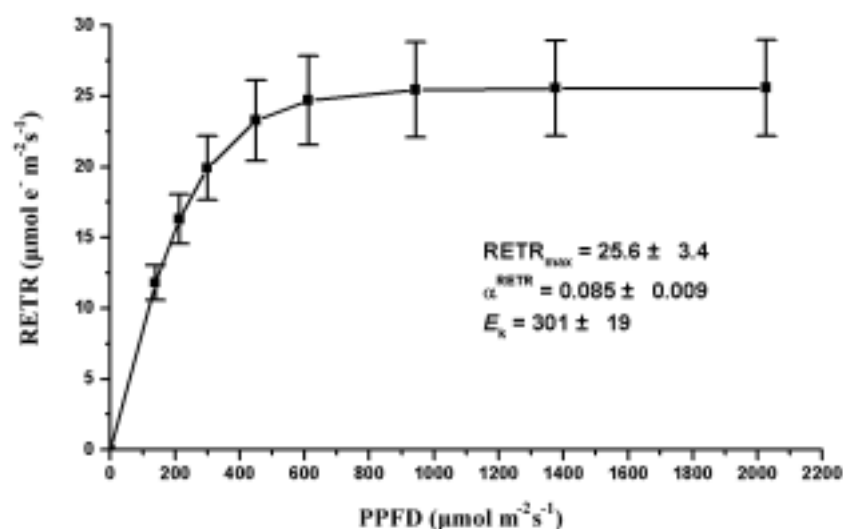


Fig. 1. The relative electron transport rate (RETR) of *Compsopogon coeruleus* at different photosynthetically photon flux densities (PPFD) measured by an underwater PAM fluorometer. Bars are standard error (SE) ( $n = 12$ ).

( $\alpha^{\text{RETR}}$ ) and the light intensity at the onset of RETR<sub>max</sub> ( $E_k$ ) were about 26  $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$ , 0.085 and 301  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ , respectively. The relationship between photochemical quenching and nonphotochemical quenching is shown in Fig. 2. The qP drops with increasing light, while the qN rises with increasing light (data not presented). The maximum value of qN is about half (67%) that of qP. Different relationships between qP and qN have been reported in plants at low light-adapted condition and high light-adapted condition (Ralph *et al.*, 1998; McMinn *et al.*, 2004). In plants adapted to low light conditions, qP drops with increasing light but qN remains low, or less than to half the value of qP. In plants adapted to high light conditions, qP sustains at high light levels and qN increases. The relationship between qP and qN of *C. coeruleus* resembles that of plants at low-light-adapted conditions. The onset of RETR<sub>max</sub> of *C. coeruleus* was observed at a low light level about 301  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ , which is consistent with the suggestion that the alga is adapted to low light. These results agree with those of previous studies showing that freshwater red algae prefer low light conditions (Kremer, 1983; Sheath, 1984; Sheath and Hambrook, 1990; Karsten *et al.*, 1993; Leukart and Hanelt, 1995; Necchi and Zucchi, 2001).

A 33-37% increase of photosynthetic rates was observed after the increase of 2-4 mM bicarbonate (Fig. 3). However, the difference between treatment and control is not statistically significant ( $p > 0.05$ ). These results indicate that *C. coeruleus* do not suffer C<sub>i</sub> limitations in their natural habitat. In addition, no significant effects on photosynthetic rates were observed

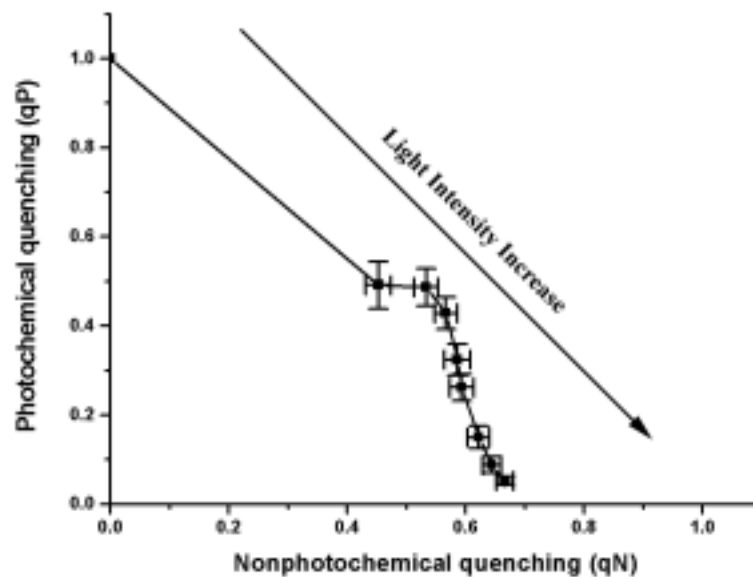


Fig. 2. Relationship between photochemical quenching (qP) and nonphotochemical quenching (qN) of *Compsopogon coeruleus* measured from the RLC function PAM fluorometer. These measurements are only relative values as the RLCs were conducted in a non-dark-adapted state. Bars are standard error (SE) ( $n = 12$ ).

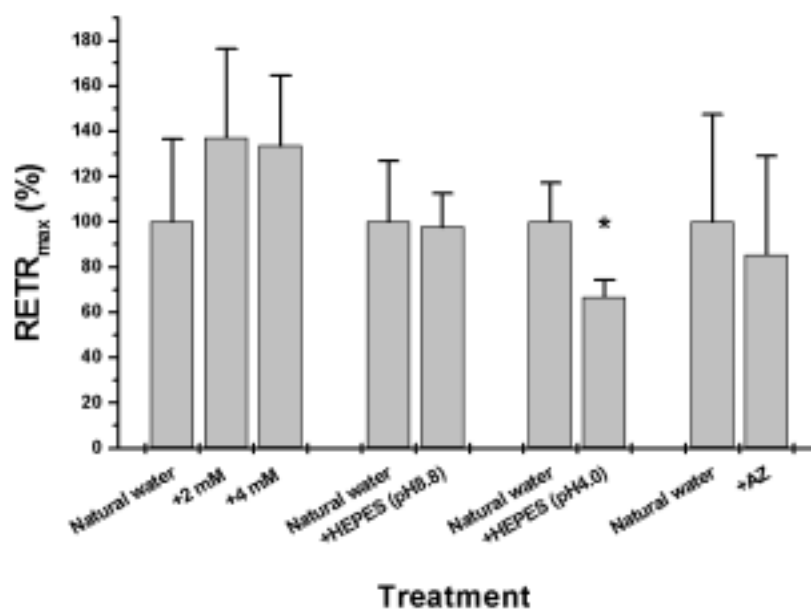


Fig. 3.  $RETR_{max}$  of *Compsopogon coeruleus* under different bicarbonate concentration, the addition of alkaline HEPES buffer (pH 8.8), the acid HEPES buffer (pH 4.0) and acetazolamide (AZ). Before treatment, the values of RETR at the highest irradiance are set to 100%. Star indicates statistical significance ( $p < 0.05$ ). Bars are standard error (SE) ( $n = 3$ ).

( $p > 0.05$ ) (Fig. 3) when an alkaline HEPES buffer and AZ were added, while a significant decrease in the photosynthetic rate was observed after adding acid HEPES buffer ( $p < 0.05$ ). These results indicate that *C. coeruleus* has a higher affinity for an inorganic carbon source as  $HCO_3^-$ , and directly uptakes  $HCO_3^-$  without the aid of external CA-mediated conversion of  $HCO_3^-$  to  $CO_2$ . Similarly most marine macroalgae are not  $C_i$  limited under natural conditions

and using uptake  $\text{HCO}_3^-$  as their main  $\text{C}_i$  source without the aid of external CA (Beer, 1994). However, very few studies have examined inorganic carbon utilization in freshwater macroalgae, particularly freshwater red algae (Raven and Beardall, 1981; Raven *et al.*, 1982; Necchi and Zucchi, 2001). Raven and Beardall (1981) and Raven *et al.* (1982) analyzed the inorganic carbon utilization of *Batrachospermum* sp. and *Lemanea mamilliosa* Kützinger, which use only free carbon oxide, not bicarbonate, as the main  $\text{C}_i$  source. On the other hand, Necchi and Zucchi (2001) investigated at the  $\text{C}_i$  utilization of eight freshwater red algal species (*Compsopogon coeruleus*, *Audouinella hemannii* (Roth) Duby, *A. pygmaea* (Kützinger) Weber-van Bosse, *Batrachospermum ambiguum* Montagne, *B. delicatum* (Skuja) Necchi *et* Entwisle, *B. vogesiacum* Skuja, and *Thorea hispida* (Thore) Desvaux) and found that they varied in the form of inorganic carbon used. According to these data, three types of  $\text{C}_i$  utilization can be found in the freshwater red algae: (1) higher photosynthetic rates, under pH 8.5, suggesting higher affinity for inorganic carbon in the form of bicarbonate, found in algae such as *C. coeruleus*, *A. hermannii*, *A. pygmaea*, *B. ambiguum*; (2) rates that do not differ when pH falls between 4.0 and 8.5, suggesting affinity for both carbon dioxide and bicarbonate, found in algae such as *B. delicatum* and *T. hispida*; and (3) higher rates under pH 4.0, suggesting higher affinity for inorganic carbon such as carbon dioxide, found in algae such as *Batrachospermum* sp., *B. vogesiacum* and *L. mamilliosa*. *C. coeruleus* as studied here and by Necchi and Zucchi (2001). Schwarz *et al.* (2000) studied the  $\text{C}_i$  utilization of seagrasses *in situ* using the Diving-PAM and argued that artificial physiological experiments in the lab would give misleading result. In contrast, we found consistent results between *in situ* measurements in the field (this study) and artificial measurements in the lab (Necchi and Zucchi, 2001). In addition, the present study was the first to demonstrate that *C. coeruleus* can directly uptake bicarbonate without the aid of external CA-mediated conversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$ . This more efficient means of  $\text{HCO}_3^-$  utilization may be an adaptation for highly-unstable aquatic environments (Axelsson *et al.*, 1995). After excluding the possibility of CA-mediated transport of  $\text{HCO}_3^-$ , we examined other possible transport pathways of  $\text{HCO}_3^-$  in *C. coeruleus*. Transport of  $\text{HCO}_3^-$  across the plasma membrane may be either associated with  $\text{Na}^+/\text{HCO}_3^-$  symport,  $\text{Na}^+/\text{H}^+$  antiport,  $\text{HCO}_3^-/\text{Cl}^-$  exchange, or  $\text{OH}^-/\text{HCO}_3^-$  exchange (Gao and Zou, 2001). In one experiment we found that the net photosynthesis of *C. coeruleus* was not affected under  $\text{Cl}^-$ -free and  $\text{Na}^+$ -free medium (data not shown). These results indicate that  $\text{HCO}_3^-$  utilization by *C. coeruleus* involves neither a  $\text{Na}^+/\text{HCO}_3^-$  symport nor a  $\text{Cl}^-/\text{HCO}_3^-$  exchange system. Whether the transport pathway of  $\text{HCO}_3^-$  across the plasma membrane involves the other two possible systems in *C. coeruleus* needs further investigation.

Under higher pH conditions, the resulting  $\text{CO}_2$  concentration is very low. The ability of direct  $\text{HCO}_3^-$  utilization without the aid of external CA is very important in maintaining photosynthetic efficiency (Axelsson *et al.*, 1995). This ability to sustain photosynthetic performance under higher pH conditions may explain the reason that most species of *Compsopogon* are able to endure higher pH environments (Raven and Beardall, 1981; Raven *et al.*, 1982; Sheath and Hambrook, 1990; Wu, 1999; Necchi and Zucchi, 2001; Wu, 2001; Wehr and Sheath, 2003; Liu and Wang, 2004). Dreschsler *et al.* (1994) found that in the green macroalgae *Ulva* sp. the probable active sites of direct  $\text{HCO}_3^-$  transport protein contain high-pK amino acid arginine and lysine residues. These residues maintain a positive charge and hence attract  $\text{HCO}_3^-$  even at very high pH values. Although no similar research has been done on *Compsopogon*, it is possible that a similar amino acid structure may be found in  $\text{HCO}_3^-$  transport proteins in *C. coeruleus*.

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利用葉綠素螢光分析法實地研究淡水紅藻  
*Compsopogon coeruleus* (Balbis) Montagne 無機碳使用情形

劉少倫<sup>(1)</sup>、汪良奇<sup>(1)</sup>、王瑋龍<sup>(1,2)</sup>

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摘 要

本研究使用水中葉綠素螢光分析儀，實地測定在增加無機碳酸鹽濃度、添加鹼性 HEPES 緩衝液 (pH 8.8)、添加酸性 HEPES 緩衝液 (pH 4.0) 和胞外碳酸酐酶抑制劑 acetazolamide (AZ) 前後對淡水紅藻 *Compsopogon coeruleus* 光合作用速率的改變狀況，以期了解 *C. coeruleus* 對外在環境無機碳使用情形。研究結果顯示，添加酸性 HEPES 緩衝液 (pH 4.0) 後，*C. coeruleus* 光合作用速率有顯著下降 ( $p < 0.05$ )，其他處理則無顯著影響。因此，我們認為 *C. coeruleus* 在其生長棲地並未遭受到碳源不足的限制；在無機碳源的利用中，對重碳酸鹽具有較高的親合力；而重碳酸鹽的吸收方式是採用不需要胞外碳酸酐酶幫助的直接吸收方式。

關鍵詞：Acetazolamide、碳酸酐酶、螢光分析儀、無機碳、HEPES、飽和性光脈衝、光合作用。

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1. 國立彰化師範大學生物系，彰化市 500 進德路 1 號，台灣。

2. 通訊作者。Tel: 886-4-7232105 ext. 3436; Email: wlwang@cc.ncue.edu.tw