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Comparative studies on photosynthesis and phosphate metabolism of *Cylindrospermopsis raciborskii* with *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*

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ARTICLE INFO

Article history: Received 3 March 2009 Received in revised form 10 April 2009 Accepted 7 May 2009

Keywords: Aphanizomenon flos-aquae Cylindrospermopsis raciborskii Extracellular phosphatase Microcystis aeruginosa Photosynthetic activity

ABSTRACT

The physiological differences for three bloom-forming cyanobacteria (*Cylindrospermopsis raciborskii*, *Microcystis aeruginosa*, and *Aphanizomenon flos-aquae*) were investigated. In comparison with *M. aeruginosa* and *A. flos-aquae*, *C. raciborskii* exhibited a significantly higher concentration of carotenoids, higher values in maximum photosynthesis rate (P_m), apparent photosynthetic efficieny (a), and maximum electron transport rate (ETR_{max}) during the growth period. In addition, higher extracellular alkaline phosphatase activities and lower light compensation point (I_c) were also detected in *C. raciborskii* (p < 0.05, ANOVA). Therefore, it is suggested that the higher photosynthetic activities, more effective uptake and utilization to phosphate, and low light requirements might play important roles in the occurrence and invasive behavior of *C. raciborskii*.

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1. Introduction

Cylindrospermopsis raciborskii (Woloszyńska) Seenayya and Subba-Raju (1972) is a planktonic filamentous cyanobacterium that has been becoming increasingly prevalent in water bodies worldwide, including ponds, lakes, reservoirs and other drinking and recreational water supplies (Dyble et al., 2006). Some strains have been found to produce hepatotoxic cylindrospermopsin (CYL) or paralytic shellfish-poisoning toxin (PSP), implying their threat to human and animal health (Byth, 1980; Bourke et al., 1983; Saker et al., 1999; Lagos et al., 1999; Li et al., 2001). Thus, *C. raciborskii* has attracted the attention of scientists and managers of water facilities.

Due to its growth requirement for high temperature and its inability to adapt to temperature fluctuation, *C. raciborskii* was thought to evolve in tropical lakes (Padisák, 1997). However, *C. raciborskii* is well regarded as an invasive cyanobacterial species since it is now becoming prevalent in more temperate regions (Padisák, 1997; Saker and Griffiths, 2001). *C. raciborskii* has been frequently reported to occur in Africa (Lagos et al., 1999), America (Chapman and Schelske, 1997; Vidal and Kruk, 2008), Australia (Byth, 1980; Bourke et al., 1983; Hawkins et al., 1985; Saker and Neilan, 2001), Asia (Li et al., 2001; Chonudomkul et al., 2004), and Europe (Briand et al., 2002; Shafik et al., 2003), and the northernmost margin was recorded as in Germany at the latitude of $53-54^{\circ}N$ (Krienitz and Hegewald, 1996).

Padisák (1997) summarized that the reasons for *C. raciborskii* succeeding in the world's lakes might be attributed to multiple factors including good floating ability, superior shade tolerance, high affinity ammonia uptake, N₂-fixation, resistance to grazing, and P-uptake and storage capacity. Briand et al. (2004) indicated that two main factors, global warming phenomenon and ability of *C. raciborskii* to tolerate a rather wide range of climatic conditions, were responsible for the colonization of this species in midlatitudes. However, Figueredo et al. (2007) suggested that a potential allelopathic advantage could explain the geographic expansion of *C. raciborskii*. Other reasons about *C. raciborskii* succession and proliferation in lakes worldwide have also been documented (Présing et al., 1996; Isvánovics et al., 2000; Shafik et al., 2001; Kovács et al., 2003; Padisák, 2003).

Nevertheless, knowledge about *C. raciborskii* and its physiological properties, such as photosynthetic characters and the mechanism dealing with phosphate deficiency, have not been sufficiently documented. Recently, *C. raciborskii* has also been found in freshwater reservoirs and ponds from several regions of China, indicating its expansion in Asian areas. In this paper, the photosynthetic activities and the capacities of phosphate uptake and utilization in *C. raciborskii* were studied. Simultaneously, *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*, reported two main bloom-forming species in China, were also studied to compare with *C. raciborskii*.



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2. Materials and methods

2.1. Strains and culture conditions

C. raciborskii HAB 151 was isolated from a fish pond near to Dianchi Lake, Kunming, China, in 2006. Dianchi Lake (24.9°N, 102.7°E) is a hypertrophic water body with perennial water blooms, however, the shift of dominant cyanobacterial species occurred from *Microcystis* spp. in warm seasons to *Aphanozomenon flos-aquae* in cool seasons and seasonal *Aphanizomenon* bloom. *A. flos-aquae* HAB 176 was isolated from a water bloom of Dianchi Lake in March, 2006. *M. aeruginosa* PCC 7806 was employed in this study as well. The strains were grown in 250 ml Erlenmeyer flasks with 100 ml MA medium (Ichimura, 1979) under a constant white light intensity of 30 µmol photons m⁻² s⁻¹, on 12:12 L:D cycle, and at a temperature of 25 ± 1°C.

2.2. Pigments, growth rate and dry weight measurements

Under the culture conditions described above, the strains were sampled every other day for measurements of photosynthetic pigments, growth rate and dry weight. Chlorophyll a was extracted in 90% acetone and measured spectrophotometrically as described by Nusch (1980). Pelleted cells were extracted with 90% acetone, and carotenoids concentration was estimated from the optical density at 480 nm (Davies, 1976). The phycobilins were extracted with repeated freezing in liquid N_2 and thawing at 4°C in the presence of 0.05 mol l⁻¹ phosphate buffer (pH 6.7). The concentrations of phycocyanin were calculated according to Abelson and Simon (1988). The growth was shown with the increase of optimal density at 680 nm. Specific growth rate was calculated according to: $\mu_{\text{max}} = (\ln t_1 - \ln t_0)/(t_1 - t_0)$. The samples were centrifuged at 4000 rpm for 15 min, and pelleted cells were dried at 105 °C in an oven until the weight did not change. The increasing dry weight (W) was calculated as $W = W_t - W_i$, where W_t , the dry weigh after t days; W_i , the dry weigh after i days.

2.3. Photosynthetic parameters measurements

The photosynthetic oxygen evolution of the studied cyanobacterial strains in the exponential growth phase was measured with a Clark-type oxygen electrode at 25 °C. Illumination was provided by a halogen lamp ranging from 17 to 955 μ mol photons m⁻² s⁻¹, which was measured with a quantum sensor (LI-185B, Li-Cor Inc. USA). Oxygen evolution was measured for at least 5 min at each irradiance value. Parameters for the photosynthetic responses to irradiance curves (*P–I* curves) was analyzed according to the equation (Henley, 1993):

$$P = P_{\rm m} tanh(\alpha I/P_{\rm m}) + R_{\rm d}, I_{\rm k} = P_{\rm m}/\alpha, I_{\rm c} = -R_{\rm d}/\alpha$$

where *P* represents photosynthetic rate at irradiance *I*; *P*_m, maximum photosynthesis rate; α , slope of light-limited part of *P*–*I* curve; *I*, irradiance; *R*_d, dark respiration rate; *I*_k, saturating irradiance for photosynthesis, and *I*_c, light compensation point. The non-linear curve fitting of the data was performed with Microcal Origin (Version 6.1, Microcal Software Inc.).

Photosynthetic oxygen evolution responses to dissolved inorganic carbon (DIC) concentration were measured at 25 °C and 600 μ mol photons m⁻² s⁻¹. The method was minorly modified according to Qiu and Gao (2002). Briefly, fresh samples were washed with reaction medium four times for each measurement. Different concentrations of DIC (0–200 μ mol l⁻¹) were obtained by adding known concentration of NaHCO₃ to the DIC-free medium. Parameters of photosynthetic responses to DIC were obtained by fitting net photosynthetic rates at various DIC concentrations with the Michaelis–Menten formula: $v = V_{max}[S]/$ $(K_{0.5} (\text{DIC}) + [S])$. Where v, photosynthetic rate; V_{max} , DIC saturated photosynthetic rate; [S], concentration of DIC; and $K_{0.5}$ (DIC), DIC concentration where photosynthetic activity is the half of the maximum value.

Light response curve and electrons transport rate of the three cyanobacterial strains in the exponential growth phase were measured with a pulse-amplitude-modulated fluorescence monitoring system (PAM, Walz, Effeltrich, Germany). The method was conducted according to the description by Wu et al. (2008). The electron transport rate of PS II (ETR) was calculated as: relative ETR = ((($F_m' - F_t$)/ F_m') × 0.5 × PAR ($m^{-2} s^{-1}$)) (Figueredo et al., 2007).

2.4. Phosphate uptake and extracellular phosphatase detection

After cultivation of the three strains in phosphate-free MA medium for 10 days, the initial uptake rate of phosphate and extracellular phosphatase were measured. After 4 h, initial uptake was estimated by phosphate loss from five phosphate concentrations: 1, 2, 5, 10, and 20 μ mol l⁻¹. The concentration of phosphate in medium was determined using acid hydrolysis to convert acidhydrolysable phosphorus to orthophosphate which can be determined by the ascorbic acid/molybdate method (APHA, 1995) for filtered samples through a membrane filter (0.45 μ m pore size; Millipore). Cells were counted with a haemocytometer chamber under Olympus CX41 microscopy (Olympus, Japan). The uptake rate (V_p) was calculated from the difference between the initial concentration and the final concentration for each phosphate treatment. The results were fitted to the Michaelis-Menten equation: $V_p = V_{pmax}[S]/(K_{0.5} [Pi] + [S])$, where V_{pmax} is the maximum uptake rate (pg cell⁻¹ h⁻¹), $K_{0.5}$ [Pi] is the halfsaturation constant (μ mol 1⁻¹) and S is the ambient phosphate concentration.

ELF[®]97 phosphate dye (ELFP, Molecular Probes) was utilized for the microscopic detection of extracellular phosphatases in the strains. In the presence of phosphatases, a fluorogenic substrate, enzyme-labeled fluorescence phosphate (ELFP), is cleaved and forms an intensive green fluorescent insoluble product, ELF alcohol (ELFA), which tags individual cyanobacterial cells at or near the sites of enzymatic activity (Gozález-Gil et al., 1998). The method for extracellular phosphatase in the three strains was measured according to the protocol of Štrojsová et al. (2003) with a minor modification by which the fresh samples was incubated into the ELFP solution (final concentration 27 mmol l⁻¹) after phosphorus starvation. After 4 h incubation at darkness and 25 °C, the samples were filtered by 0.22 µm pore size filters, and washed 2-3 times to exclude heterotrophic bacteria to be dyed and eliminate the background noise. The samples were observed using a fluorescence microscope with UV-excitation (Nikon Universal condenser C-CU, Japan).

2.5. Statistical analysis

All experiments were performed in three replicates. Data shown in this study are presented in means \pm standard deviation (SD). Analysis for significance was carried out with Microcal Origin (version 6.1, Microcal Software Inc.). Significant differences among three strains were determined by ANOVA, and differences were considered to be significant at p < 0.05.

3. Results

3.1. Photosynthetic pigments and specific growth rate

Pigment concentrations of *C. raciborskii* HAB151, *A. flos-aquae* HAB 176 and *M. aeruginosa* PCC7806 were shown in Fig. 1. The



Fig. 1. Chlorophyll a concentrations in Cylindrospermopsis raciborskii HAB 151, Microcystis aeruginosa PCC 7806, and Aphanizomenon flos-aquae HAB 176 during cultured for 20 d.

highest concentration of Chl *a* in *C. raciborskii* HAB 151 was found on 12 day, afterwards Chl *a* concentration was kept a relative constant value as aproximately 1.30 ± 0.05 mg l⁻¹. However, the Chl *a* concentrations of *Microcystis* PCC 7806 and *Aphanizomenon* HAB 176 increased along the cultivated time. After 4 day, the value of carotenoids/Chl *a* of *C. raciborskii* had a significant increase in comparison with those of *Microcystis* PCC 7806 and *Aphanizomenon* HAB 176 (p < 0.05, ANOVA) (Fig. 2A). In addition, higher relative concentrations of phycobilins in *Cylindrospermopsis* were also found as described in Fig. 2B. However, specific growth rates of the three



Fig. 2. The relative concentrations of carotenoids and phycobilins (CPC) in *Cylindrospermopsis raciborskii* HAB 151, *Microcystis aeruginosa* PCC 7806, and *Aphanizomenon flos-aquae* HAB 176 during cultured for 20 d. A, carotenoids/Chl *a*; B, phycobilins/Chl *a*.



Fig. 3. The changes of the optimal light density at 680 nm in *Cylindrospermopsis* raciborskii HAB 151, *Microcystis aeruginosa* PCC 7806, and *Aphanizomenon flos-aquae* HAB 176 during cultured for 20 d.

strains did not show a significant difference (p > 0.05, ANOVA) (Fig. 3). Specific growth rates of *Cylindrospermopsis*, *Microcystis*, and *Aphanizomenon* strains were 0.36 ± 0.09 , 0.26 ± 0.064 , and $0.33 \pm 0.04 \text{ d}^{-1}$, respectively. An alternative calculation method by using dry weights also showed that specific growth rates of the three strains were not significantly different (data not shown).

3.2. Photosynthetic activities

Photosynthetic responses to light intensity presented that *Cylindrospermopsis* HAB 151 has higher values of maximal net photosynthesis (P_m), apparent photosynthetic efficiency (α), and dark respiration (R_d) than *Microcystis* PCC 7806 and *Aphanizomenon* HAB176 (p < 0.05, ANOVA). However, comparison among the three strains showed that the lowest values for saturation light intensity (I_k) and light compensation point (I_c) were found in *Cylindrospermopsis* (p < 0.05, ANOVA) (Fig. 4, Table 1).

Photosynthetic responses to dissolved inorganic carbon (DIC) concentrations in these three strains were shown in Fig. 5 and Table 2, indicating that V_{max} and $K_{0.5}$ (DIC) differed significantly in the three strains (p < 0.05, ANOVA). The values of V_{max} in *Cylindrospermopsis*, *Microcystis*, and *Aphanizomenon* were 109.06 ± 17.35, 70.70 ± 15.15, and 51.97 ± 12.03 µmol O₂ g⁻¹ DW min⁻¹, respectively. However, the values of $K_{0.5}$ (DIC) showed a



Fig. 4. Photosynthetic O₂ evolution as a function of irradiance for *Cylindrospermopsis* raciborskii HAB 151, *Microcystis aeruginosa* PCC 7806, and *Aphanizomenon flos-aquae* HAB 176.

Table 1

Parameters of photosynthesis-irradiance curves for Cylindrospermopsis raciborskii HAB 151, Microcystis aeruginosa PCC 7806, and Aphanizomenon flos-aquae HAB 176.

Strains	$P_{\rm m}~(\mu~{ m mol}~{ m O}_2~{ m g}^{-1}~{ m DW}~{ m min}^{-1})$	α (μ mol O ₂ g ⁻¹ DW min ⁻¹)	$R_{\rm d} \ (\mu \ { m mol} \ { m O}_2 \ { m g}^{-1} \ { m DW} \ { m min}^{-1})$	$I_{\rm k}$ (µ mol m ⁻² s ⁻¹)	$I_{\rm c} (\mu { m mol} { m O}_2 { m g}^{-1} { m DW} { m min}^{-1})$
HAB 151	82.50 ± 4.27	0.46 ± 0.06	-2.45 ± 0.21	179.35 ± 12.30	5.33 ± 0.66
PCC 7806	31.85 ± 4.17	0.16 ± 0.06	-5.09 ± 0.74	199.06 ± 39.77	31.81 ± 6.54
HAB 176	46.71 ± 4.08	0.21 ± 0.05	-3.17 ± 0.41	222.43 ± 26.94	15.10 ± 2.76

Table 2

Parameters of photosynthesis-dissolved inorganic carbon (DIC) curves for Cylindrospermopsis raciborskii HAB 151, Microcystis aeruginosa PCC 7806, and Aphanizomenon flos-aquae HAB 176.

Strains	$V_{\rm max} (\mu \ { m mol} \ { m O}_2 { m g}^{-1} { m DW} { m min}^{-1})$	<i>K</i> _{0.5} (DIC) (μM)
HAB 151 PCC 7806 HAB 176	$\begin{array}{c} 109.06 \pm 17.35 \\ 70.70 \pm 15.15 \\ 51.97 \pm 12.03 \end{array}$	$\begin{array}{c} 51.27 \pm 12.09 \\ 42.24 \pm 4.13 \\ 28.71 \pm 2.86 \end{array}$



Fig. 5. Photosynthetic O_2 evolution as a function of dissolved inorganic carbon (DIC) for *Cylindrospermopsis raciborskii* HAB 151, *Microcystis aeruginosa* PCC 7806, and *Aphanizomenon flos-aquae* HAB 176.

significant decrease in Cylindrospermopsis, Microcystis, and Aphanizomenon, the values were 51.27 \pm 12.09, 42.24 \pm 4.13, and 28.71 \pm 2.86 μ mol l^{-1} , respectively.

The maximal electron transport rates (ETR_{max}) in *Cylindrospermopsis*, *Aphanizomenon*, and *Microcystis* were 123.77 \pm 1.64, 95.93 \pm 0.75, and 84.77 \pm 2.20 μ mol electrons m⁻² s⁻¹, respectively (Fig. 6), reflecting that *Cylindrospermopsis* had a significant higher value of ETR_{max} than either *Microcystis* or *Aphanizomenon* (p < 0.05, ANOVA).

3.3. Phosphate uptake and extracellular phosphatase

The uptake rate (V_p) and half-saturation constant $(K_{0.5}$ [Pi]) of phosphate depended on the strains. The results based on uptake kinetics were shown in Table 3. The maximum uptake rate (V_{pmax}) of *Cylindrospermopsis* was about 0.61 ± 0.03 pg cell⁻¹ h⁻¹, which was significant higher than those of *Microcystis* and *Aphanizomenon*.

Table 3Parameters of inorganic phosphate (Pi) uptake for Cylindrospermopsis raciborskiiHAB 151, Microcystis aeruginosa PCC 7806, and Aphanizomenon flos-aquae HAB 176.





Fig. 6. The electron transport rates (ETR) of Cylindrospermopsis raciborskii HAB 151, Microcystis aeruginosa PCC 7806, and Aphanizomenon flos-aquae HAB 176 at log phase.

However, $K_{0.5}$ [Pi] did not show a significant difference among three strains.

Extracellular alkaline phosphatase in the three cyanobacteria was shown in Fig. 7. The results indicated that the activities of extracellular phosphatase also depended on the strains. After inoculation of phosphate starvation for 10 days, extracellular alkaline phosphatase in *Cylindrospermopsis* was induced. Compared with *Aphanizomenon*, occurrence of the green fluorescent insoluble products were observed in most individual algal cells of *Cylindrospermopsis*. However, the cells of *Microcystis* were not observed any fluorescent products.

4. Discussion

The widespread occurrence of cyanobacteria has been explained due to their ecological and physiological advantages over other algae (Oliver and Ganf, 2000). However, except for a few species, biological features of a large portion of bloom-forming cyanobacteria have been still unexplored (Yamamoto and Nakahara, 2005). In this study, the physiological parameters of C. raciborskii were measured, in parallel with those of Microcystis and Aphanizomenon strains together. The results showed that the concentrations and ratios of pigments in three strains varied (Figs. 1 and 2). Carotenoid concentration in the Cylindrospermopsis strain was significantly higher than those of Microcystis and Aphanizomenon strains (p < 0.05, ANOVA). However, Chl a concentration of Cylindrospermopsis was lowest among the three strains. Briand et al. (2002) described that low transparency of the water during blooms in shallow ponds is one of main environmental factors involved in proliferation of Cylindrospermopsis since providing an indirect competitive advantage over other heterocystic cyanobacteria. It suggested that antenna pigments such as carotenoids and/or phycobilins played an important role in light absorption and photosynthesis (Sathyendranathe et al., 1987), and the result showing higher concentrations of carotenoids and phycobilins in Cylindrospermopsis in this study, is in agreement with that of Briand et al. (2002) from a physiological perspective.



Fig. 7. Extracellurar phosphatase activities for *Cylindrospermopsis raciborskii* HAB 151, *Microcystis aeruginosa* PCC 7806, and *Aphanizomenon flos-aquae* HAB 176 by ELFA labeling after 10 d of P starve culture. Red colors indicate that autofluorescence of chlorophyll *a* in algal cells; green colors are ELFA fluorescence of extracellular alkaline phosphatase precipitates. (A) *C. raciborskii* HAB 151; (B) *Aphanizomenon flos-aquae* HAB 176; (C) *M. aeruginosa* PCC 7806. Arrow, extracellural phosphatase.

Raps et al. (1983) indicated that *Microcystis* colonization and formation of blooms in water bodies worldwide may result from a light intensity adaptation. Briand et al. (2004) also showed that *Cylindrospermopsis* displayed a positive net growth in wide range of

ligh intensities, which may result in the colonization of midlatitudes by this organism. In the present work, specific growth rates in the three strains did not display a significant difference (Fig. 3), suggesting that *C. raciborskii* had a similar growth characterization to *Microcystis* and *Aphanizomenon*.

The results of photosynthetic activities and ETR indicated that Cylindrospermopsis had higher photosynthetic activities than Microcystis and Aphanizomenon (Figs. 4 and 6, Table 1). Furthermore, the lower values of I_c and I_{l_r} in *Cylindrospermopsis* reflected that it could tolerate lower light irradiances. This is in agreement with the results by Padisák and Reynolds (1998) and Briand et al. (2004) who indicated that C. raciborskii was a good low lightadapted species. Therefore, it implied that Cylindrospermopsis had higher competitive advantages in photosynthetic characterizations over Microcystis and Aphanizomenon. Fabbro and Duivenvoorden (1996) described that C. raciborskii could form blooms at very high incident irradiance intensities (up to 2500 µmol photon $m^{-2} s^{-1}$) in a tropical water body, the Fitzroy River, Australia. Briand et al. (2004) also showed that C. raciborskii is a species with the low light requirements, but good tolerance to high light intensities up to 500 μ mol photon m⁻² s⁻¹. The present study, based on a subtropical strain of *C. raciborskii* from China, showed that higher growth at lower light irradiance and higher tolerance to high light intensity up to 900 μ mol photon m⁻² s⁻¹, consistent with the previous findings described above.

Advantageous DIC uptake abilities seem to be responsible for the competitive dominance of cyanobacteria in eutrophic water bodies (King, 1970; Yamamoto and Nakahara, 2005). Thus, DIC might be a limiting factor to other algae when low concentration of DIC occurred in water bodies. In the present study, *Cylindrospermopsis* had a significantly higher value of DIC uptake rate (Fig. 5, Table 2) in comparison with *Microcystis* and *Aphanizomenon*. However, surprisingly, higher thresholds for DIC (high $K_{0.5}$ (DIC)) was found in *Cylindrospermopsis*, indicating that *Cylindrospermopsis* had relatively low affinity to DIC. It is therefore speculated that *Cylindrospermopsis* might develop an effective DIC uptake mechanism to avoid the DIC limitation at low concentration of DIC condition.

Previous studies had shown that high affinity phosphate uptake and high P-storage capacity played an important role in the success of *C. raciborskii* (Padisák, 1997; Isvánovics et al., 2000). Similar results were also obtained in the present study (Table 3) to show that *Cylindrospermopsis* had a higher phosphate uptake rate in contrast to *Microcystis* and *Aphanizomenon*, which indicated that *Cylindrospermopsis* had more advantage with the competitive ability to Pi than *Microcystis* and *Aphanizomenon*.

The production of extracellular phosphatases, which can hydrolyse dissolved organic P compounds, is one possible mechanism allowing cyanobacteria to overcome P limitation (Cembella et al., 1984; Jansson et al., 1988). The bulks of extracellular alkaline phosphatases were observed in *Cylindrospermopsis* (Fig. 7), implying that *Cylindrospermopsis* might also display an important feature for invasion by developing an effective utilization to Pi.

5. Conclusions

Conclusively, in comparison with *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii* exhibited several advantageous features such as higher effective photosynthetic activities, higher DIC uptake, and higher effective uptake and transformation to Pi, which suggested that these physiological characteristics may be responsible for mass occurrence or invasion of *C. raciborskii* in waters from tropical to temperate climates.

Acknowledgements

The author thanks Gongliang Yu for the collection and isolation of *Cylindrospermopsis raciborskii*, Hong Shen for help in the EFLA labeling, and Lin Chen for help in the use of Clark-type oxygen electrode in oxygen evolution. We are also grateful to two anonymous reviewers for helpful comments and suggestions on the manuscript.

The study was supported by the National Natural Science Foundation of China (30800123), the Knowledge Innovation Program of the Chinese Academy of Sciences (KZCX1-YW-14-1), the State Key Basic Research and Development Plan of China (2008CB418002), and the Frontier Research Project of the Chinese Academy of Science (055102-1-501).[SS]

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