



# Interspecific competition between *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa* on different phosphorus substrates

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## Abstract

Phosphorus (P) is responsible for algal growth and the structural changes in algal communities. Therefore, it is essential to know whether the different phosphorus availability to different algae can change the community structure. In this study, the interspecific competition was investigated at two bloom-forming cyanobacterium, *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa*, when both were treated with five different phosphate compounds, including  $K_2HPO_4$ ,  $\beta$ -glycerol phosphate, (2-aminoethyl)-phosphinic acid, glyphosate, and P-free. The results of mono-culture experiments showed that the two species could utilize the dissolved organic phosphorus (DOP) and  $K_2HPO_4$  (DIP) as the sole P resource. Moreover, the specific growth rates and the endogenous alkaline phosphatase activity in *M. aeruginosa* cells were much lower than those in *C. raciborskii* under DOP and DIP treatments. In the co-cultured experiments, however, a significant biomass increase in *C. raciborskii* was observed in all experimental P treatments, except for glyphosate, regardless of its initial cell density proportion. A 31.8–63.4% increase in cell number of *C. raciborskii* was found after incubated into  $K_2HPO_4$ , while the highest biomass of mixed samples,  $17.72 \times 10^6$  cell  $mL^{-1}$ , was observed in the (2-aminoethyl)-phosphinic acid treatment (50C50M). Additionally, higher specific growth rate was also found in *C. raciborskii* when compared with *M. aeruginosa* under P-free; the increasing proportion of *C. raciborskii* were 29.1% (50C50M), 16.4% (75C25M), and 36.7% (25C75M), respectively. When the mixed samples were co-cultivated under glyphosate, *C. raciborskii* cells appeared to be depressed, whereas the cell density of *M. aeruginosa* increased rapidly. The findings indicated that an excellent P competition might give some advantages for *C. raciborskii* dominance in natural waters with DIP limitation or DOP abundance.

**Keywords** *Cylindrospermopsis raciborskii* · Dissolved organic phosphorus · Interspecific competition · *Microcystis aeruginosa* · Phosphorus

## Abbreviations

|       |                                |
|-------|--------------------------------|
| APA   | Alkaline phosphatase activity  |
| C–P   | Carbon–phosphorus bond         |
| C–O–P | Carbon–oxygen–phosphorus bond  |
| DIP   | Dissolved inorganic phosphorus |

|     |                               |
|-----|-------------------------------|
| DOP | Dissolved organic phosphorus  |
| DRP | Dissolved reactive phosphorus |

## Introduction

With the **aggravation** of eutrophication and global climatic change, cyanobacteria harmful algae bloom (cyanoHAB) has considered to be one of the most serious problems in aquatic ecosystems (Paerl and Huisman 2009). As the two of major cyanoHAB species in the field waters, filamentous *Cylindrospermopsis* and colonial *Microcystis* have gained increasing attention from both the scientific community and managers of water facilities (Padisák and Reynolds 1998; Nixdorf et al. 2003; Tomioka et al. 2011).

Previous reports have shown *Cylindrospermopsis raciborskii* is well regarded as an invasive cyanobacterial

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species since it is now becoming prevalent in more temperate regions (Padisák 1997; Saker and Griffiths 2001; Hamilton et al. 2005; Stüken et al. 2006; Moreira et al. 2015). Furthermore, *C. raciborskii* can produce toxic substances, such as cylindrospermopsin (CYN) and paralytic shellfish-poisoning toxin (PSP) that may cause serious health risks to both humans and wildlife (Byth 1980; Bourke et al. 1983; Saker and Griffiths 2001; Lagos et al. 1999). Therefore, *C. raciborskii* has become one of the most notorious and conspicuous harmful algae. Numerous factors might have facilitated the fast expansion and invasion of *C. raciborskii*, such as its high phenotypic plasticity and high level of flexibility to critical environmental factors (light and nutrients) (Wu et al. 2009; Isvánovics et al. 2000; Bonilla et al. 2011; Bai et al. 2014; Burford et al. 2016; Braind et al. 2004; Coles and Jones 2000).

*C. raciborskii* is replacing other bloom formers, such as *Microcystis aeruginosa* and *Anabaena* spp., or is becoming co-dominant in some tropical reservoirs (Marinho and Huszar 2000; Molica et al. 2005; Soares et al. 2009; Moisan et al. 2012; Yatigamma and Perera 2017). Long-term monitoring of Lake Julius in Australia has also shown a gradual shift within the cyanobacterial composition, from dominance by *M. aeruginosa* (Finlayson et al. 1985; Boland 1993) to *C. raciborskii* dominance after 1991 (Saker and Griffiths, 2001). Shifts in phytoplankton community are often thought to be a result of changes in nutrient factors (Fujimoto and Sudo 1997; Pehler et al. 2004; Hlaili et al. 2006) and physical factors (Gomes and Miranda, 2001; Moisan et al. 2002). For instance, Karadžić et al. (2013) found that on account of the heterocyst and the low optimal light requirements for growth, *Cylindrospermopsis raciborskii* occupied the dominance for collapse of *Aphanizomenon flos-aquae* in the Ponjavica River in Serbia, a eutrophic river with the higher biochemical oxygen demand and turbidity and lower  $\text{NO}_3\text{-N}$  concentrations.

Simultaneously, nutrient composition and concentration of water indeed influence the formation of different algae blooms. As the most essential of nutrients in most ecosystems, the quantity and forms of phosphorus in the environment mediate phytoplankton biomass and influence phytoplankton species composition (Dyhrman et al. 2007). In natural waters, dissolved phosphorus comprises orthophosphate (DIP) (Diaz et al. 2008) and dissolved organic phosphorus (DOP) derived from the degradation of glycolipids, glycoproteins, and antibiotics (Clark et al. 1998; Kolowith et al. 2001; Dyhrman et al. 2006; White and Metcalf 2007). Although DIP is considered the most important P source for microalgae, the DIP is often low near the detection limit using standard analytical methods in many aquatic environments (Anagnostou and Sherrell 2008). However, organic phosphorus species have accounted for a substantial amount of the dissolved P found in many aquatic environments, such as nucleic acids (DNA and RNA), nucleotides

(e.g., ATP, GTP, AND, AMP), inositol phosphates, pyrophosphate, phospholipids, and phosphonates (Baldwin 2013). Numerous studies showed that DOP is available for the production of both phytoplankton and bacteria in some freshwater systems, when the DIP concentration is insufficient (Rinker and Powell 2006; Wang et al. 2011; Bai et al. 2014; Li et al. 2015).

Our previous study has revealed that *C. raciborskii* could utilize organic phosphorus effectively (Bai et al. 2014). Otherwise, high storage capacity and high affinity for phosphorus might play essential roles in the dominance of *C. raciborskii* (Isvánovics et al. 2000; Wu et al. 2009). Furthermore, Wu et al. (2009) and Posselt et al. (2009) have shown that effective uptake and utilization to phosphate might play important roles in its occurrence and invasive behavior. Padisák (1997) revealed that the replacement of several cyanobacteria species by *C. raciborskii* in tropical aquatic ecosystems might be related to competition for resources. Therefore, we can suppose that the flexibility of the utilization for phosphorus might be a competitive strategy in *C. raciborskii*. Nevertheless, whether this factor of *C. raciborskii* might contribute to its success in interspecific competition is not yet well determined.

As the prevalent bloom-forming species of cyanoHAB, the growing properties of *M. aeruginosa* have been studied extensively under different environmental conditions. Ren et al. (2017) revealed that *M. aeruginosa* could utilize DOP under low P conditions and also exhibited a strong potential to scavenge DOP, which led to better compete with other species in the co-cultures. Moreover, *M. aeruginosa* could accumulate and store cellular P to ensure that its growth rates did not immediately decline (Li et al. 2015).

Base on the similarity of P utilization of the two species, we questioned (1) whether the phosphorus availability of the two strains was different or not and (2) which strain will be a winner in interspecific competition when the two strains co-existed under varying P sources. Therefore, to clarify the questions, the growth and competition between two bloom-forming cyanobacteria, *C. raciborskii* and *M. aeruginosa*, were investigated after the two species were mono-cultured and co-cultured at various phosphate treatments.

## Materials and methods

### Culture conditions

The *Cylindrospermopsis raciborskii* (Woloszýnska) strain FACHB 1096 we used in our experiments was obtained from the Culture Collections of the freshwater algae section of the Institute of Hydrobiology, Chinese Academy of Sciences (FACHB-Collection; Wuhan, China). A previous study had demonstrated the strain, *C. raciborskii*, was non-CYN (Bai

et al. 2014). The unicellular strain *Microcystis aeruginosa* (Kützing) strain 205 was separated from the Three Gorges Reservoir and was non-toxic determined by toxin detection using HPLC method. The axenic strains were cultivated in MA medium (Ichimura 1979) at  $25 \pm 1$  °C, and the light illumination was controlled at  $25 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , with a light and dark cycle of 12 h:12 h. The culture condition of the two strains was kept consistent. To guarantee the bacterial contamination remained less than 1% of the culture biomass, regular fluorescence microscopic inspection after acridine orange and 4', 6-diamidino-2-phenylindole staining was analyzed in our whole experiments.

## Experimental design

Before treatment with different phosphorus source, exponentially growing cells at MA medium were collected by centrifugation at 4000 rpm for 8 min, washed three times with a P-free medium, and then inoculated in a P-free medium for 3 days to remove excess phosphate of the cell.

After the P starvation, five phosphorus treatments, including  $\beta$ -glycerol phosphate, (2-aminoethyl)-phosphinic acid, glyphosate, dipotassium phosphate ( $\text{K}_2\text{HPO}_4$ , there for name after DIP), and P-free, were carried out in this study. The P element concentration in all media was about  $10.13 \text{ mg L}^{-1}$ . The different organophosphorus was used to test in this study, because it has been reported to be important and representative for P loading in eutrophic freshwater lakes in China (Bai et al. 2009). An adequate concentration of nitrogen,  $15.84 \text{ mg L}^{-1}$ , was supplied in all media in order to avoid the influence of nitrogen on both *C. raciborskii* and *M. aeruginosa*. For the mono-cultures, the algae were separately inoculated in 250-mL standard medium with different forms of phosphorus. To the co-culture experiments, the two species cells exposed to the P starvation were mixed and co-cultured in 500-mL culture flasks with different phosphorus treatments. The initial cell density proportions are as follows: (1) 25C75M: 25% *C. raciborskii*–75% *M. aeruginosa*, (2) 50C50M: 50% *C. raciborskii*–50% *M. aeruginosa*, and (3) 75C25M: 75% *C. raciborskii*–25% *M. aeruginosa*, respectively. The initial cell density of all the samples was  $1 \times 10^6 \text{ cells mL}^{-1}$ . All experiments were performed in triplicate.

Mono-culture and co-cultures experiments were carried out for 16 days under the same culture conditions as described above. Each flask was shaken twice a day manually. The algal cells were sampled at 0, 2, 4, 6, 8, 10, 13, and 16 days of inoculation.

## Growth analyses

The cell density was determined using a hemocytometer chamber under a Nikon CE-I microscope (Nikon, Tokyo,

Japan). The cell number of *M. aeruginosa* was obtained by direct counting, whereas the cell number of *C. raciborskii* was obtained according to methods provided by Bai et al. (2014). The specific growth rate ( $\mu$ ) in the experiment was calculated based on the cell density according to the following equation:  $\mu = (\ln C_{t_2} - \ln C_{t_1}) / (t_2 - t_1)$ , in which  $C_{t_2}$  and  $C_{t_1}$  reflect the cell density at times day 4 and day 13, respectively.

## Orthophosphate detection and extracellular alkaline phosphatase activity

Algae were filtered out, and the phosphorus molybdenum blue method was used for the measurement of orthophosphate concentration (Martin and Doty 1949). The extracellular alkaline phosphatase activity (APA) was assayed according to the protocol by Shen and Song (2007).

## Statistical analyses

One-way ANOVA was performed using the Microcal Origin Version 8.5 (OriginLab Corporation, Northampton, MA, USA), to determine differences in growth rates between treatments, species, and culture methods. In all the analyses, the  $p$  value  $< 0.05$  was considered as statistically significant. The statistical analyses were performed using the SPSS 17.0 Version (IBM, Armonk, New York City, USA).

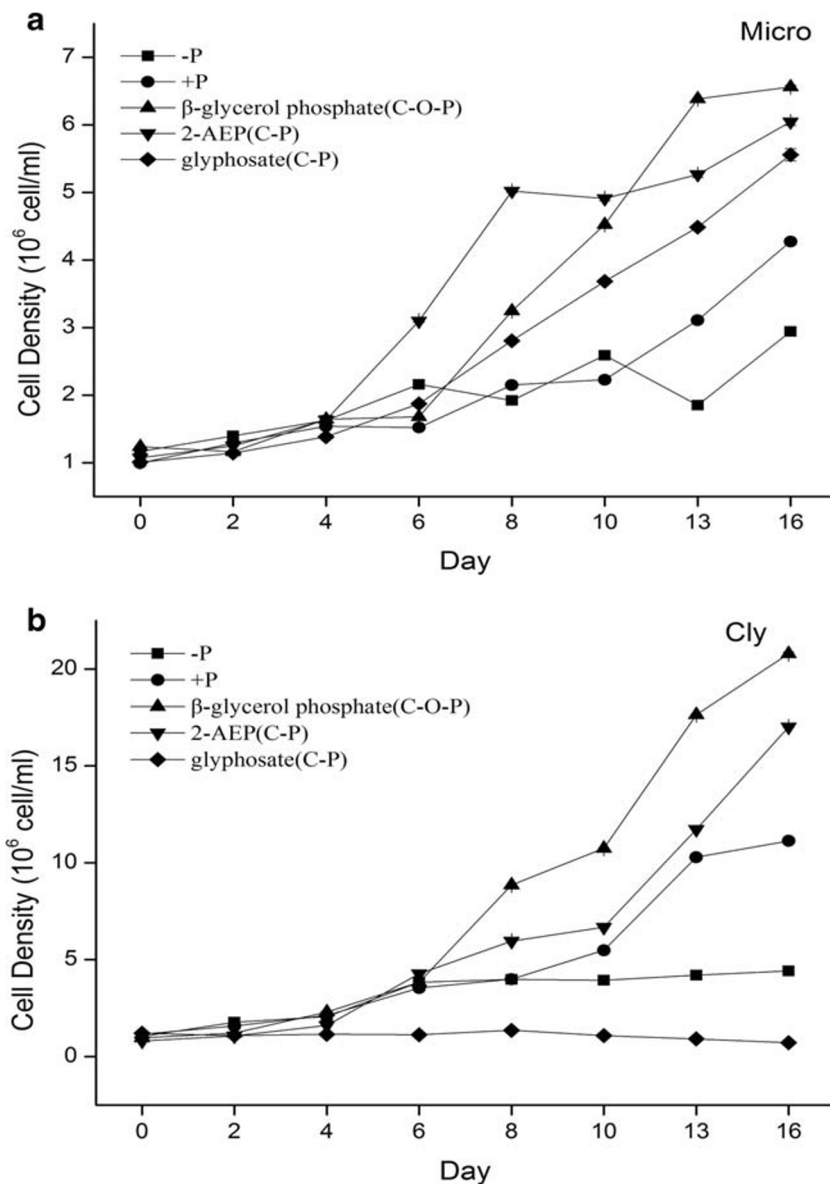
## Results

### Mono-culture experiments

#### Growth

The relationship between the growth curve and various phosphorus resources for *M. aeruginosa* and *C. raciborskii* was displayed in Fig. 1. In the P-free cultivation, the growth rate was kept a slightly increase in *M. aeruginosa* and *C. raciborskii*, and the cell number increased to  $2.94 \pm 0.21 \times 10^6 \text{ cells mL}^{-1}$  and  $4.42 \pm 0.14 \times 10^6 \text{ cells mL}^{-1}$  after 16-d culture, respectively. Both cells could keep the growth when the two strains were exposed to treatments of DIP, (2-aminoethyl)-phosphinic acid, and  $\beta$ -glycerol phosphate, respectively. After 16-d culture at treatments of DIP, (2-aminoethyl)-phosphinic acid, and  $\beta$ -glycerol phosphate, the final cell densities of *C. raciborskii* were  $11.13 \pm 0.25 \times 10^6$ ,  $17.00 \pm 0.23 \times 10^6$ , and  $20.78 \pm 0.14 \times 10^6 \text{ cells mL}^{-1}$ , which was 2.60, 2.81, and 3.17 times higher than those of *M. aeruginosa*, respectively (ANOVA,  $P < 0.05$ ). The growth rates ( $\mu$ ) of the two strains cultivated with (2-aminoethyl)-phosphinic acid and  $\beta$ -glycerol phosphate were significantly

**Fig. 1** Growth curve of *M. aeruginosa* (a) and *C. raciborskii* (b) with different phosphorous culture. Values shown are the means of three replicates ± SD



**Table 1** The specific growth rate of *C. raciborskii* and *M. aeruginosa* for mono-cultures and co-cultures under varying phosphorus substrate

| Treatment                       | Mono-cultures |               | Co-cultures |               |              |               |             |               |
|---------------------------------|---------------|---------------|-------------|---------------|--------------|---------------|-------------|---------------|
|                                 |               |               | 25C75M      |               | 50C50M       |               | 75C25M      |               |
|                                 | Micro         | Cyl           | Micro       | Cyl           | Micro        | Cyl           | Micro       | Cyl           |
| P-free                          | 0.06 ± 0.02   | 0.08 ± 0.02   | 0.05 ± 0.01 | 0.14 ± 0.02   | 0.04 ± 0.02  | 0.14 ± 0.03   | 0.03 ± 0.02 | 0.13 ± 0.02   |
| K <sub>2</sub> HPO <sub>4</sub> | 0.09 ± 0.01   | 0.14 ± 0.00*  | 0.05 ± 0.01 | 0.16 ± 0.02   | 0.04 ± 0.02* | 0.16 ± 0.04   | 0.05 ± 0.02 | 0.16 ± 0.03   |
| β-glycerol phosphate            | 0.11 ± 0.03*  | 0.19 ± 0.02*  | 0.13 ± 0.03 | 0.20 ± 0.05   | 0.10 ± 0.03  | 0.17 ± 0.05   | 0.10 ± 0.05 | 0.17 ± 0.02   |
| (2-aminoethyl)-phosphinic acid  | 0.12 ± 0.02*  | 0.19 ± 0.01*  | 0.14 ± 0.03 | 0.17 ± 0.02   | 0.13 ± 0.03  | 0.21 ± 0.07   | 0.11 ± 0.02 | 0.19 ± 0.03   |
| Glyphosate                      | 0.11 ± 0.01*  | -0.03 ± 0.01* | 0.10 ± 0.03 | 0.02 ± 0.01** | 0.09 ± 0.02  | 0.04 ± 0.02** | 0.09 ± 0.03 | 0.03 ± 0.02** |

\*P < 0.05

higher than those under P-free condition (Table 1, ANOVA,  $P < 0.05$ ). However, growth inhibition was found in *C. raciborskii* cultured at glyphosate treatment, while *M. aeruginosa* showed an increasing growth; the specific growth rate was  $0.11 \pm 0.01 \text{ d}^{-1}$  (ANOVA,  $P < 0.05$ ).

### Alkaline phosphatase activity (APA)

The extracellular alkaline phosphatase activity was displayed a similar tendency in both strains (Fig. 2); APA was rapidly increased and then decreased to a relatively constant level. Nevertheless, APA in *M. aeruginosa* was much lower than that in *C. raciborskii*. At treatments of P-free, DIP, (2-aminoethyl)-phosphinic acid,  $\beta$ -glycerol phosphate, and glyphosate, APA in *C. raciborskii* was 6.51, 1.94, 2.97, 3.78, and 4.02 times higher in contrast with *M. aeruginosa* at the 2 days (ANOVA,  $P < 0.05$ ).

### Phosphorus fraction

The change of phosphorus concentration in culture medium accompanying the growth of *M. aeruginosa* and *C. raciborskii* was showed in Fig. 3. The DIP concentration showed a similar trend between *M. aeruginosa* and *C. raciborskii*, representing a markedly increase at the early phase of their growth (day 0–6) under the treatments of  $\beta$ -glycerol phosphate and (2-aminoethyl)-phosphinic acid and then began to decline at after 6 days. In treatments of P-free and glyphosate, DIP concentration kept a relatively low level in both of the two species during the whole phase.

## Co-culture experiments

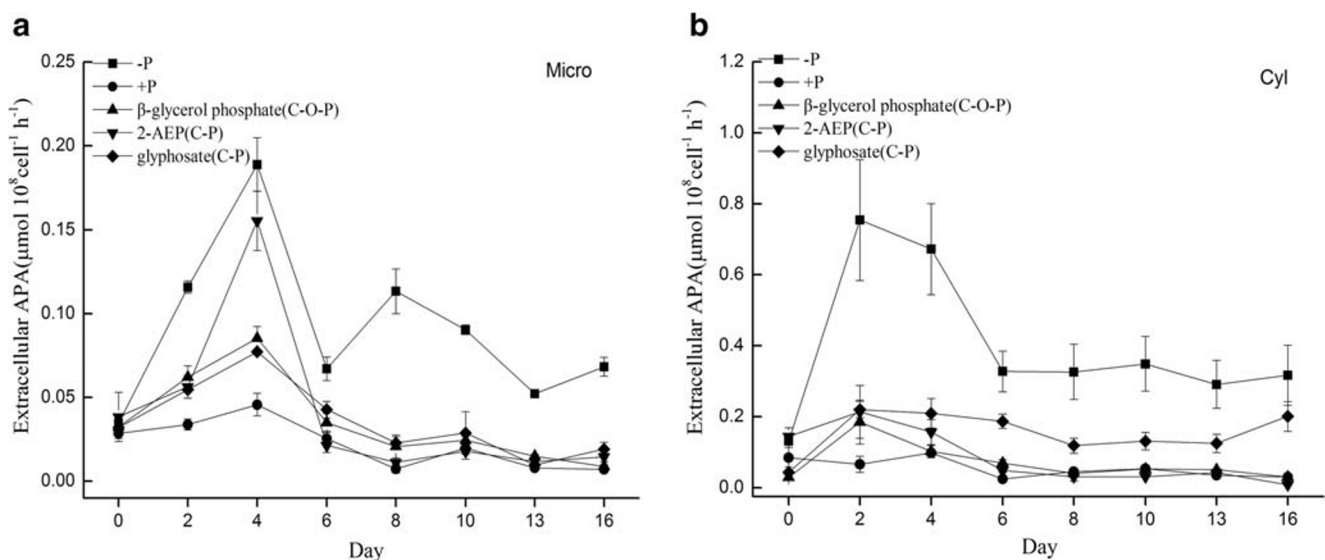
### Co-culture under P-free conditions

As the results showed in Fig. 4, the proportions of *C. raciborskii* revealed a mean 27.3% increase in the culture regardless of its initial cell density under P-free conditions. The lower the initial proportion of *C. raciborskii*, the higher an increase was observed in its proportion in the co-culture. The greatest change (36.7%) was found in 25C75M. Additionally, the specific growth rate ( $\mu$ ) of *C. raciborskii* was markedly higher than that of *M. aeruginosa* under three of the initial ratios (Table 1, ANOVA,  $P < 0.05$ ). Moreover, compared with the  $\mu$  values in mono-culture, the  $\mu$  values of *M. aeruginosa* decreased by a mean of 34.3%, while the values of *C. raciborskii* increased by a mean of 63.5% (Table 1, ANOVA,  $P < 0.05$ ).

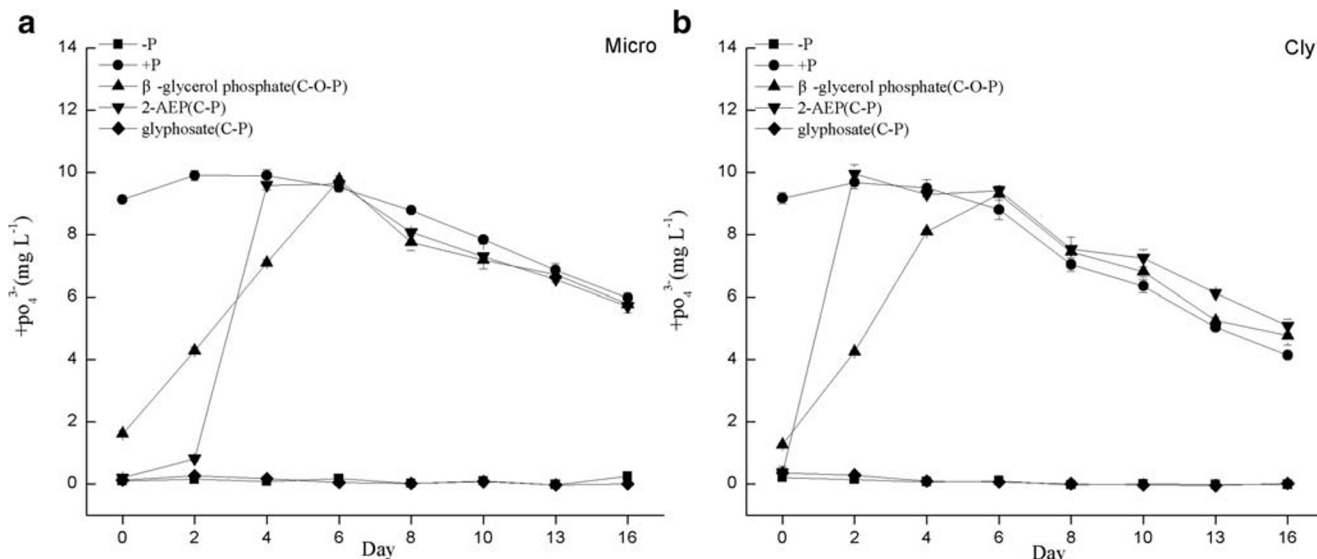
During the entire experiment and in all investigated samples, a relatively high and rapid increase of extracellular APA was observed at the beginning of the experiment. The most significant up-regulation was observed in the 75C25M sample, in which APA was increased to a constant value of  $0.65 \pm 0.09 \mu\text{mol} (10^8 \text{ cells})^{-1} \text{ h}^{-1}$  (Fig. 5).

### Co-culture experiment under dissolved inorganic phosphorus

A proportion of *C. raciborskii* was increased under the treatment of  $\text{K}_2\text{HPO}_4$ , with a mean increasing rate of 31.8% for the three initial proportions (Fig. 6). Here, the highest increasing rate of *C. raciborskii* also was found (42.4%) among all of the co-culture experiments when the initial proportion was 75C25M. When the co-culture included  $\text{K}_2\text{HPO}_4$  treatment, the growth curve of *C. raciborskii* showed a rapid increase,



**Fig. 2** Extracellular APA for the responses of *M. aeruginosa* (a) and *C. raciborskii* (b) to different phosphorous compounds. Values shown are the means of three replicates  $\pm$  SD



**Fig. 3** Dissolved inorganic phosphate concentration for the responses of *M. aeruginosa* (a) and *C. raciborskii* (b) to different phosphorous compounds. Values shown are the means of three replicates  $\pm$  SD

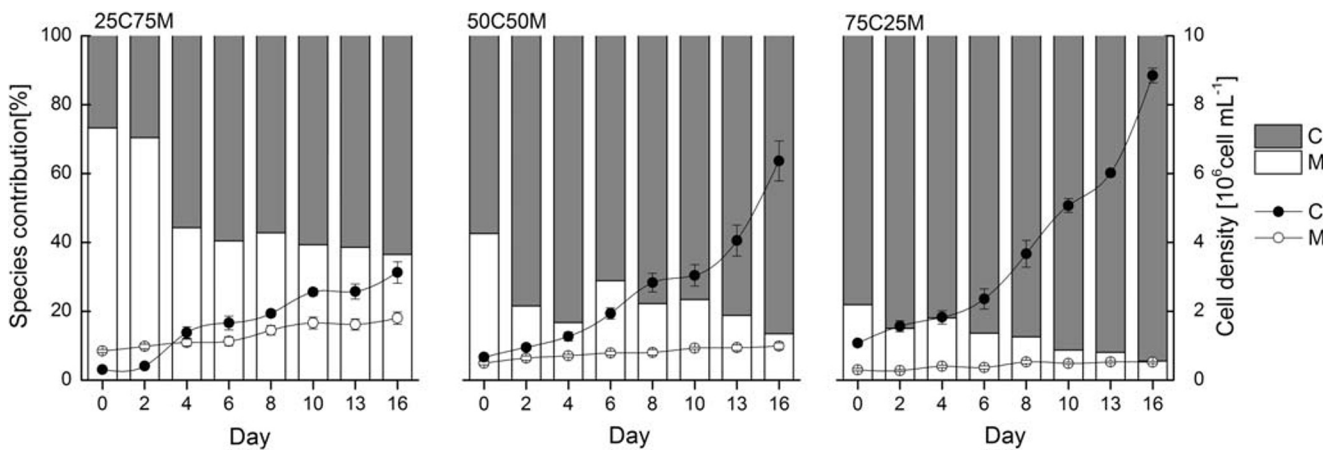
and the ratio of *C. raciborskii* increased to 63.4% when the initial proportion was 25C75M. Also, the growth rates of *M. aeruginosa* showed a lower value when compared with the mono cultivation (Table 1, ANOVA,  $P < 0.05$ ).

**Co-culture experiment using dissolved organic P sources**

The results of the co-culture experiment using dissolved organic P treatments were shown in Fig. 7. After 16 days in culture, the cell density of *C. raciborskii* increased rapidly, when the co-culture cells were cultured in treatments of  $\beta$ -glycerol phosphate and (2-aminoethyl)-phosphinic acid and an increasing proportion of *C. raciborskii* was revealed in these two treatments regardless of the initial ratio. The mean increasing rate was 22.8% and 20.87%, respectively (Fig. 7a

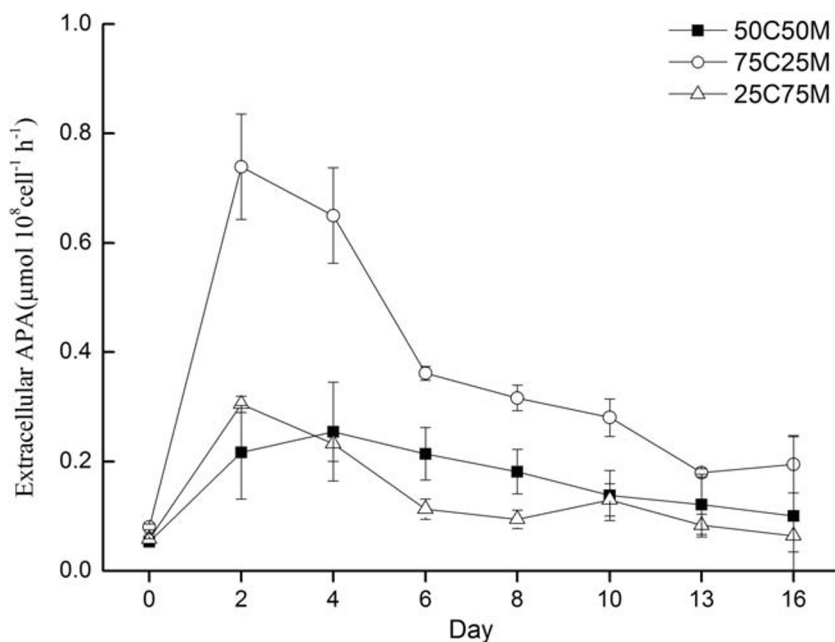
and b). The highest biomass of co-culture cells was observed in the 50C50M samples when the co-culture was incubated with (2-aminoethyl)-phosphinic acid, in which the cell density was increased to  $17.72 \pm 0.52 \times 10^6\ cell\ mL^{-1}$ . In the meantime, a decreasing proportion was found in *M. aeruginosa* co-cultured at  $\beta$ -glycerol phosphate and (2-aminoethyl)-phosphinic acid (Fig. 7a and b). However, the specific growth rates in the two strains did not show a difference between the mono-culture and the co-culture (Table 1, ANOVA,  $P > 0.05$ ).

By contrast, under glyphosate treatment, the ratio of *C. raciborskii* showed a distinctly decrease compared with the initial rate (Fig. 7c), with a depressed rate of 25.3% (25C75M), 40.8% (50C50M), and 43.2% (75C25M), respectively. However, the growth rate for *C. raciborskii* displayed an increase with the value of  $0.02 \pm 0.01$ ,  $0.04 \pm 0.02$ , and



**Fig. 4** Variation of percentages of cell numbers of *M. aeruginosa* and *C. raciborskii* without phosphorous added (-P) in co-existence incubation experiments

**Fig. 5** Extracellular APA of *M. aeruginosa* and *C. raciborskii* without phosphorous added (-P) in co-existence incubation experiments. Values shown are the means of three replicates  $\pm$  SD



$0.03 \pm 0.02 \text{ d}^{-1}$  for each initial proportion, which was much higher than those in the mono-culture. There was no difference of the growth rate between the mono-culture and the co-culture for *M. aeruginosa* under the glyphosate condition.

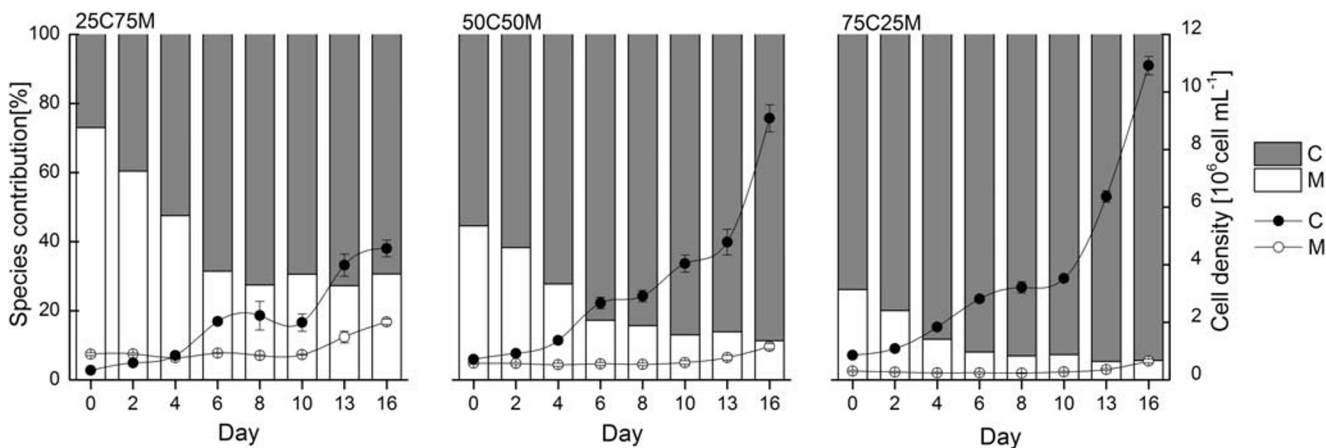
## Discussion

Numerous researches have shown that phosphorus availability in aquatic ecosystems is crucial to control harmful algae blooms (Laws et al. 2011; Wang et al. 2011; Abell and Hamilton 2013). Some algae that pose a capacity to use various phosphorus forms as sole phosphorus source have been widely investigated (Stihl et al. 2001; Dyhrman et al. 2002; Dyhrman et al. 2006; Shi et al. 2011; Li et al. 2015). In this study, the specific growth rate in *C. raciborskii* and

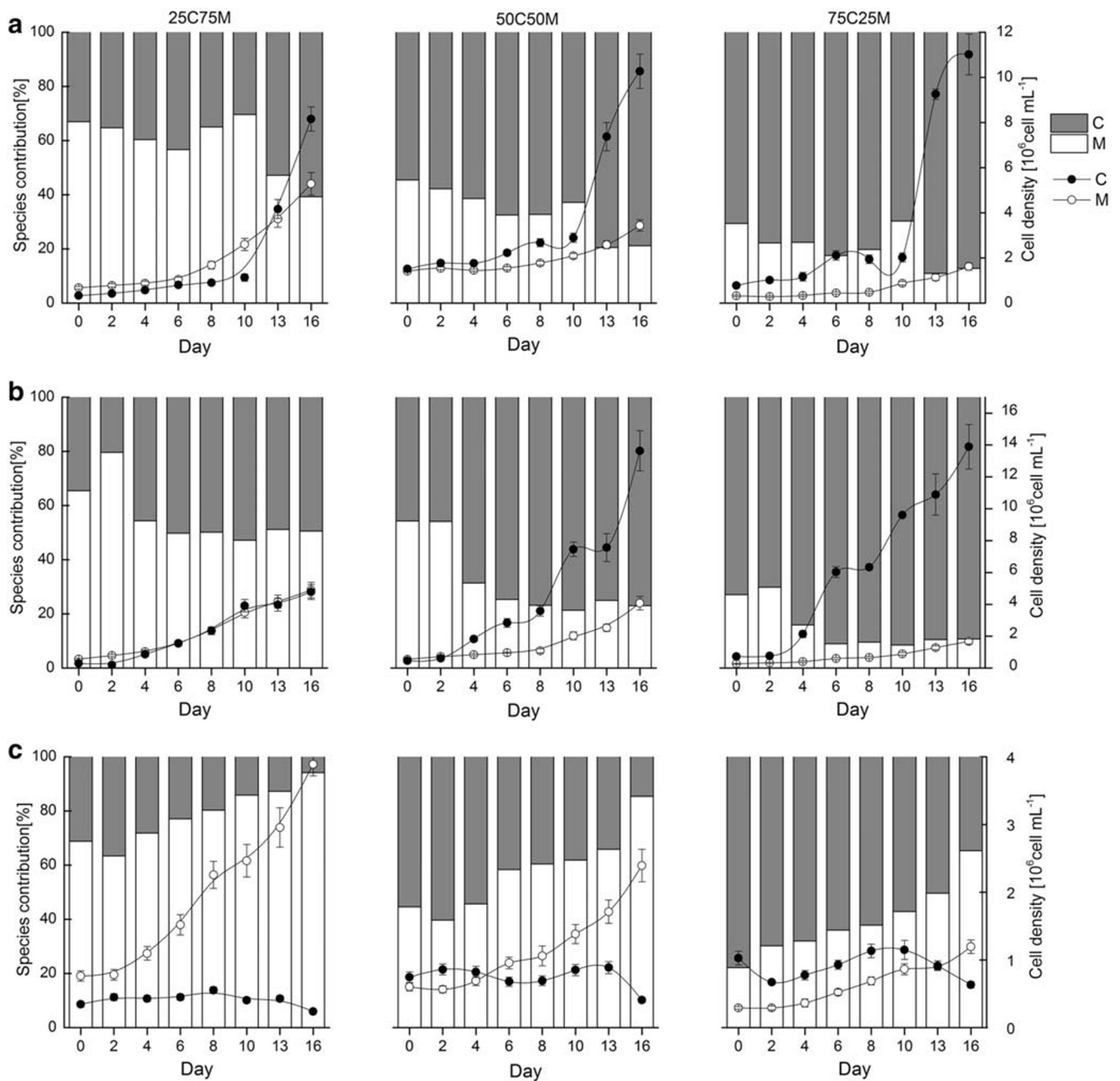
*M. aeruginosa* was  $0.09\text{--}0.20 \text{ d}^{-1}$  and  $0.06\text{--}0.11 \text{ d}^{-1}$ , respectively (Fig. 1 and Table 1) in the mono-cultures, which supported the results of Wu et al. (2009). A similar result was also proposed in the study of Xiao et al. (2017a, b) that the specific growth rate in *C. raciborskii* and *M. aeruginosa* was  $0.15\text{--}0.70 \text{ d}^{-1}$  and  $0.16\text{--}0.55 \text{ d}^{-1}$  when cultured at different light and temperature, suggesting that the utilization ability for phosphorus in *C. raciborskii* might be much stronger than that in *M. aeruginosa* and the two species showed apparent physiological plasticity (ecotype) (Willis et al. 2015; Burford et al. 2016; Robarts and Zohary 1992; Briand et al. 2004).

## Mono-culture in *C. raciborskii* and *M. aeruginosa*

Reynolds (1997) indicated that aquatic microbial organisms could only directly assimilate DIP. Ren et al. (2017) also



**Fig. 6** Variation of percentages of cell numbers of *M. aeruginosa* and *C. raciborskii* to dissolved inorganic phosphorus in co-existence incubation experiments



**Fig. 7** Variation of percentages of cell numbers of *M. aeruginosa* and *C. raciborskii* to different phosphorous culture in co-existence incubation experiments **a**  $\beta$ -glycerol-phosphate, **b** (2-aminoethyl) phosphinic acid, and **c** glyphosate

showed that DIP was the preferred phosphorus substrate in both culture types for *M. aeruginosa*, *Chlorella pyrenoidosa*, and *Pseudokirchneriella subcapitata*. However, in this study, higher growth rates were found in the two strains exposed to  $\beta$ -glycerol phosphate and (2-aminoethyl)-phosphinic acid in contrast to DIP treatment (Table 1), indicating that both *Cylindrospermopsis* and *Microcystis* could be efficient to use DOP. It was consistent with previous findings that the two strains could utilize the DOP as a solo P source in the mono-culture (Shi et al. 2011; Bai et al. 2014; Li et al. 2015; Ren et al. 2017). A similar result was also reported by Wang

et al. (2011), who revealed that *Prorocentrum micans* and *Chattonella marina* used phosphomonoesters ( $\beta$ -glycerol phosphate and glyphosate) more efficiently than inorganic phosphorus. Dyhrman et al. (2007) showed that  $\beta$ -glycerol phosphate and (2-aminoethyl)-phosphinic acid were efficient to support algal growth when DIP was insufficient in natural waters. These suggested that the utilization of DOP by cyanobacteria might play essential roles in the frequent occurrence of cyanoHABs.

Wong (2000) and Forlani et al. (2008) showed that different appropriate concentration glyphosate might stimulate the



growth of different bloom algae, such as *Scenedesmus quadricauda* at  $0.0037 \text{ mg L}^{-1}$ , *Leptolyngbya boryana* at  $6.2 \text{ mg L}^{-1}$ , and *Nostoc punctiforme* at  $3.1 \text{ mg L}^{-1}$ . Qiu et al. (2013) had shown that *M. aeruginosa* could utilize glyphosate (a typical exogenous DOP caused by anthropogenic activity) at a low concentration. In the present study, an increasing growth was found in *M. aeruginosa* exposed to glyphosate, whereas *C. raciborskii* showed an inhibiting growth, even more cell death at the treatment of glyphosate (Fig. 1 and Table 1), supporting our previous result that *C. raciborskii* could not utilize the glyphosate (Bai et al. 2014). The result suggested that glyphosate might be toxic to *C. raciborskii* (Ren et al. 2017). Tsui and Chu (2008) stated that glyphosate could influence the microbial communities in freshwater lakes.

Alkaline phosphatase (AP) activity is often recommended as an indicator of P status, since it was induced to hydrolyze organic phosphorus to compensate for the deficiency in ambient DIP (Gillor et al. 2002; Dyhrman and Ruttenberg 2006), indicating that *C. raciborskii* could produce AP as a spontaneous response to P limitations (Gillor et al. 2002; Li and Brett 2013; Prentice et al. 2019). Reynolds (1997) demonstrated that cyanobacteria could first hydrolyze DOP through AP to assimilate the DOP. In the present study, alkaline phosphatase activity in both *C. raciborskii* and *M. aeruginosa* was significantly increased at mono-cultures of P-free,  $\beta$ -glycerol phosphate, (2-aminoethyl)-phosphinic acid, and glyphosate (Fig. 2). Tan et al. (2007) found that a lower capacity to excrete AP of *M. aeruginosa* was determined than other cyanobacteria, *A. flos-aquae* and *O. planctonica*, under P-stress. A similar result was also observed in the present study, in that APA in *M. aeruginosa* was much lower than that in *C. raciborskii* (Fig. 2), reflecting that high bioavailability of DOP in *C. raciborskii* at treatments of DOP and P-free.

Studies also declared that DOP might be associated with surface enzymes, which was then hydrolyzed to DIP by algae for direct utilization or transient accumulation in culture medium (Jacobson and Halmann 1982; Van Moorleghem et al. 2013). Generally, AP played a vital role in the utilization of phosphomonoesters and phosphonate (Reynolds 1997; Wan et al. 2019). In this study, DIP release showed a significant increase at treatments of  $\beta$ -glycerol phosphate and (2-aminoethyl)-phosphinic acid (Fig. 3), indicating that cyanobacteria could assimilate P from complex organic P sources by AP to recalcitrant and non-bioavailable (Ilikhyan et al. 2009). However, DIP release was not found in treatments of P-free and glyphosate (Fig. 3), implying that high bulk APA did not necessarily lead to DIP release in glyphosate as solo P source (Ren et al. 2017).

### Co-culture between *C. raciborskii* and *M. aeruginosa*

Studies have indicated that *M. aeruginosa* and *C. raciborskii* could co-occur in some places (Soares et al. 2009; Dantas

et al. 2011). It has already been shown that *C. raciborskii* is capable of dominating to *M. aeruginosa* in a lake or lowland river (Soares et al. 2009; Karadžić et al. 2013). Marinho et al. (2013) found that *C. raciborskii* can either dominate or be displaced by *M. aeruginosa* when exposed to different conditions of light or phosphate limitation. In the present study, when the co-culture was implemented in P-free medium, the proportion of *C. raciborskii* showed an increase under different treatments of the initial cell density (Fig. 4), suggesting that competition for resources and the regulatory mechanisms may have developed between these two species. In this study, a relatively high and rapid increase of extracellular APA was observed at the beginning of the experiment (Fig. 5). Moreover, the higher the initial proportion of *C. raciborskii*, the more APase was synthesized (Fig. 5), which is consistent with the result of mono-culture experiment. Tan et al. (2007) indicated that the tolerance to P-stress in *M. aeruginosa* was weaker than that observed in two other bloom-forming cyanobacteria species, *Aphanizomenon flos-aquae* and *Oscillatoria planctonica*. Additionally, the capacity to excrete AP was determined for three cyanobacteria under P-stress: *A. flos-aquae* > *O. planctonica* > *M. aeruginosa* (Tan et al. 2007). Shen and Song (2007) determined that the unicellular *M. aeruginosa* consumed more P to maintain the growth, resulting in a weaker tolerance to P-free conditions. These suggested that *C. raciborskii* could synthesize more APase to acclimatize and tolerance to P-starvation (Wu et al. 2012; Bai et al. 2014; Rzymyski et al. 2014).

Studies have shown that the stronger competitive capacity to P might lead to the dominance of algae (De Nobel et al., 1997). When co-cultured under the treatment of  $\text{K}_2\text{HPO}_4$ , increasing proportions of *C. raciborskii* were the highest (Fig. 6). The growth rates of *C. raciborskii* were significantly higher than of *M. aeruginosa* in three initial proportions (Table 1). Wu et al. (2009) found that in comparison with *M. aeruginosa* and *A. flos-aquae*, *C. raciborskii* exhibited a more effective uptake and utilization of phosphate. The result further supported that high uptake to DIP of *C. raciborskii* provides an advantage for its dominance (Isvánovics et al. 2000). Interestingly, the growth curve of *C. raciborskii* occurred at a great fluctuation in the 25C75M group. One explanation could be that chemical substances were secreted by a large number of *M. aeruginosa* cells and then induced a suppression of *C. raciborskii* (Mello et al. 2012).

In this study, the co-culture in media treated with  $\beta$ -glycerol phosphate revealed a mean 22.8% increase of *C. raciborskii* proportion at the expense of *M. aeruginosa* growth, independent of its initial cell density. Similarly, an 20.87% increase of *C. raciborskii* proportion in media treated with (2-aminoethyl)-phosphinic acid was observed in all samples (Table 1, Fig. 7a and b). Shi et al. (2011) showed that the growth rates and cell abundances decreased when *M. aeruginosa* was inoculated into DOP, such as glucose-1-

phosphate, adenosine triphosphate, and cyclic-adenosine monophosphate, in contrast with DIP group. Smith and Kalff (1982) found that the interspecific variation of phosphate uptake kinetics is mainly dependent on phytoplankton cell size, nutrition quota in the cell, and environmental factors. In this study, the size of *C. raciborskii* filament was 20  $\mu\text{m}$ ; however, the cell diameter of *M. aeruginosa* is mainly less than 3.5  $\mu\text{m}$ , suggesting that large cell size might be responsible for the competition of *C. raciborskii* by phosphate uptake, storage, and capacity.

Previous results have shown that the cell density of *M. aeruginosa* increased rapidly when the strain was cultured at different appropriate concentration glyphosate (Wong, 2000; Forlani et al. 2008; Qiu et al. 2013). Bai et al. (2014) found that *C. raciborskii* did not survive at the treatment of glyphosate. Similar result was also found at co-culture between *C. raciborskii* and *M. aeruginosa* exposed to glyphosate. When the co-culture was incubated into the media with glyphosate, the decreased contribution of *C. raciborskii* was found in the present study (Table 1, Fig. 7c), reflecting that a difference in the competition and the regulatory for glyphosate between *C. raciborskii* and *M. aeruginosa* was developed.

In conclusion, the study has shown that both *C. raciborskii* and *M. aeruginosa* could utilize the DOP as a useful P source in the mono-culture. However, a significant increase of *C. raciborskii* proportion was observed independent of its initial contribution in the co-culture, except for glyphosate treatment, revealing that *C. raciborskii* was a strategist with high bioavailable P and a good competitor (Shen and Song 2007; Willis et al. 2015, 2017). Moreover, *Cylindrospermopsis* is known to dominate in water bodies with very low DIP ( $< 0.06 \mu\text{m}$ ) (Padisák 1997; Burford and O'Donohue 2006; Marinho et al. 2013). Therefore, studies on Pi deficiency and DOP utilization capability by *Cylindrospermopsis* are important to control *Cylindrospermopsis* bloom. However, Xiao et al. (2017b) indicated cyanobacterial competition was highly variable due to be depended on strains present and environmental conditions, such as light and temperature, etc., suggesting that there was no absolute “winner” under all conditions as there were always strains predicted to co-exist with the dominant strains. Therefore, in order to clarify P storage and utilization mechanisms of phytoplankton further, studies about the different strains and different cultivation environment are needed in the future.

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