



Response of extracellular and intracellular alkaline phosphatase in *Microcystis aeruginosa* to organic phosphorus

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Received: 9 October 2019 / Accepted: 15 June 2020 / Published online: 23 June 2020
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Abstract

Cyanobacterial blooms caused by *Microcystis* have become a menace to public health and water quality in the global freshwater ecosystem. Alkaline phosphatases (APases) produced by microorganisms play an important role in the mineralization of dissolved organic phosphorus (DOP) into orthophosphate (Pi) to promote cyanobacterial blooms. However, the response of extracellular and intracellular alkaline phosphatase activity (APA) of *Microcystis* to different DOP sources is poorly understood. In this study, we compared the growth of *M. aeruginosa* on two DOP substrates (β -glycerol-phosphate (β -GP) and lecithin (LEC)) and monitored the changes of P fractions and the extra- and intracellular APA under different P sources and concentrations. *M. aeruginosa* can utilize both β -GP and LEC to sustain its growth, and the bioavailability of LEC was greater than β -GP. For the β -GP treatment, there was no significant difference in the algal growth at different concentrations ($P > 0.05$), while the algal growth in the LEC treatment groups was significantly affected by concentrations ($P < 0.05$). The results showed that intracellular APA of *M. aeruginosa* could be detected in all DOP treatment groups and generally higher than extracellular APA. In addition, the intracellular APA per cell increased first and then decreased in all DOP treatment groups. Compared with the β -GP treatment, *M. aeruginosa* in the LEC groups could secrete more extracellular APA.

Keywords *M. aeruginosa* · alkaline phosphatase activity · extracellular and intracellular · organic phosphorus · β -glycerol-phosphate · lecithin · mineralization

Introduction

Eutrophication in aquatic ecosystems is a major environmental issue, threatening water security and biodiversity.

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Responsible Editor: Vitor Manuel Oliveira Vasconcelos

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Cyanobacterial blooms mainly caused by *Microcystis* in freshwater lakes have become more frequent (Ho et al. 2019; Sato et al. 2017; Schindler et al. 2016; Yang and Kong 2013). Phosphorus (P) is an important requirement for microbial metabolism (Geider and La Roche 2002; Markou et al. 2014) and is a limiting nutrient of lakes primary productivity (Carpenter 2008; McMahon and Read 2013; Sondergaard et al. 2003). Long-term studies of lake ecosystems indicated that cyanobacterial blooms could be controlled by reducing the input of P (Schindler et al. 2016).

Orthophosphate (Pi) is the preferred P form for microorganisms (Paerl et al. 2011) and is generally present at low concentrations in freshwater ecosystems, accounting for just 5 to 10% of total P (Feng et al. 2008; Halemejkó and Chrost 1984). Dissolved organic P (DOP) accounts for a larger proportion of the total dissolved P in lake environments (Cao et al. 2005). It was estimated that most DOP exists in the form of P-esters (McMahon and Read 2013; Read et al. 2014), which are potential substrates for phosphatase and nucleotidases. Therefore, the release of Pi by enzymatic mineralization

of DOP is an important compensation process for Pi in eutrophic lakes.

Free alkaline phosphatases (APases) are believed to be responsible for the release of large amounts of Pi in freshwater and marine ecosystems (Reichardt et al. 1967). APases not only play a role in catalyzing the hydrolysis of phosphate monoesters (excluding phytic acid) (Labry et al. 2005; Martinez et al. 1996) but also can use diesters as hydrolysis substrates (Martinez et al. 1996). Microorganisms (bacteria and phytoplankton) are reported to produce APases when facing Pi starvation (Wetzel 1999; Apel et al. 2007; Vershinina and Znamenskaya 2002; Antelmann et al. 2000; Suzuki et al. 2004; Tiwari et al. 2015). Three bacterial APases families (PhoA, PhoD, and PhoX) have been identified (Dai et al. 2016; Luo et al. 2009). Subcellular localization of marine bacteria indicates that APases exist both extracellularly and intracellularly (Luo et al. 2009). The wide distribution of intracellular APases provides a possibility for microorganisms to directly uptake P-esters to support growth. The presence of a large number of P-ester transporter genes in the Global Ocean Sampling (GOS) metagenome indicates that direct transport of dissolved P-esters by bacteria could be an important mechanism of P acquisition in oceans (Luo et al. 2009); however, the knowledge of direct uptake of DOP by freshwater and marine bacteria is still missing.

M. aeruginosa, a kind of cyanobacteria, is the common dominant species in eutrophic freshwater ecosystems. The dominant APase produced by cyanobacteria is PhoX (Harke et al. 2012; Luo et al. 2009; Zhao 2015), which is critical for the bioavailability of DOP in *M. aeruginosa* (Harke et al. 2012). Most research on DOP utilization by *M. aeruginosa* has focused on different organic P sources and extracellular alkaline phosphatase activity (APA) (Li and Brett 2013; Li et al. 2015; Pang et al. 2016; Ren et al. 2017; Yi et al. 2004; Zhang et al. 2015; Zhu et al. 2015). Even though the mineralization mechanism of DOP in eutrophic lakes has been a research hotspot in recent years (McMahon and Read 2013), the changes of extracellular and intracellular alkaline phosphatase in *M. aeruginosa* to different DOP sources are still unclear.

In this study, we investigated the growth of *M. aeruginosa* on dissolved inorganic phosphorus (DIP) and two DOP substrates (β -glycerol-phosphate (β -GP) and lecithin (LEC)) while monitoring the changes of P fractions and the extra- and intracellular APAs and comparing the differences in DOP utilization under different P sources and concentrations. This study aims to (i) identify and characterize the role of extra- and intracellular APases in the utilization of DOP by *M. aeruginosa* and (ii) examine the effect of DOP with different molecular weight on the growth of *M. aeruginosa*.

Materials and methods

M. aeruginosa culture

M. aeruginosa PCC 7806 used in the experiment was obtained from the Microcystis Research Laboratory of the College of Life Sciences of Nanjing Normal University. Cultures were grown in BG11 medium at 25 ± 1 °C and illuminated by cool-white fluorescent (3000 lx; 12 h light/12 h dark). Cultures were gently shaken by hand three times daily.

Experimental setup

M. aeruginosa cells from logarithmic growth period were inoculated into regular BG11 medium and P-free (-Pi) medium in triplicate. KCl was used to keep the -Pi medium at the same K^+ concentrations as regular BG11 medium. Subsamples were collected every 2 days for cell density, until there were no significant increases in cell density.

Prior to DOP experiments, cells were harvested in the logarithmic growth period by centrifugation (8000 rpm, 5 min), rinsed 3 times with -Pi medium, and then resuspended in medium for 48 h to exhaust the stored P in cells. After P starvation, cells were separately re-inoculated in 250-mL flasks, each containing 100 mL BG11 medium, which contained two different phosphorus compounds: β -glycerophosphate (β -GP) and lecithin from soybean (LEC) with a gradient P concentration at 0.1, 0.35, 3.5, and 7.5 μ g/mL (C1, C2, C3, and C4). Subsamples were periodically examined for cell density, P fractions, and extra- and intracellular APAs.

All experiments were performed in triplicate and carried out in batches, and BG11 medium without *M. aeruginosa* cells was used as a control. The culture flasks and media were sterilized by autoclaving at 121 °C and 150 kPa for 20 min before inoculation. Organic P sources were added to media cooled to room temperature to avoid the influence of high temperature.

Determination of algal growth

Algal samples (5 mL) were collected every 2 days during the experiment. The absorbance (A) of the algal samples was measured at 440 nm, and the corresponding cell densities were calculated according to a standard curve (Zhou et al. 2016). The cell specific growth rate (μ) was calculated with the following equation: $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_1 and N_2 are the cell densities on days t_1 and t_2 , respectively (Li et al. 2015).

Estimation of extracellular P fractions

Total P (TP) and inorganic P (IP) concentrations in cultures were measured with the P molybdenum blue method (Jin and Tu 1990). Extracellular P concentrations were determined from culture supernatants. Since the LEC-treated cultures were turbid, filtrated supernatants were used for the extracellular P determination.

Estimation of extra- and intracellular APAs

APA was estimated within 2 h after the collection of algal samples using *p*-nitrophenyl phosphate (*p*NPP) as the substrate (Hadas and Pinkas 1997). The extraction methods for extra-APases were consistent with that of P fractions. Intracellular APA was extracted through repeated freeze-thaw and ultrasound. Algal samples (40 mL) were centrifuged, rinsed 3 times with -Pi medium, and then resuspended in deionized water to freeze at $-20\text{ }^{\circ}\text{C}$. After cells swell, sonication (100 W for 1 min, stop for 1 min, 5 times in succession) was used to release intracellular APA. Centrifuged supernatants (4500 rpm, 10 min) were used to determine intracellular APA.

APA reaction mixture, including 1.3 mL of sample solution, 0.7 mL of buffer (pH 8.4, Tris-HCl buffer for extracellular APA assay, pH 5.0 sodium acetate buffer for intracellular APA assay), and $1\text{ mL} \times 10^{-3}\text{ mol/L}$ *p*NPP solution, was incubated at $37\text{ }^{\circ}\text{C}$ for 90 min in the dark. Subsequently, 0.3 mL of 1 mol/L NaOH solution was added to terminate the reaction. After mixing, *p*-nitrophenol (*p*NP), the product of the enzymatic hydrolysis of *p*NPP, was determined at 410 nm using a spectrophotometer. APA units (U) are expressed in milligrams of *p*NP produced per milliliter of sample per hour. Single-cell APA was estimated by dividing the measured APA by cell density.

Statistical analysis

One-way analysis of variance (ANOVA) was used to assess the statistical significance of differences among different treatment groups using SPSS 19.0, with significance determined at $P < 0.05$.

Results and discussion

Changes in algal growth and extracellular TP

The growth of *M. aeruginosa* treated with DIP (dipotassium phosphate (K_2HPO_4)) source and P-free (-Pi) for 10 days is shown in Fig. 1. The cell density of *M. aeruginosa* with the K_2HPO_4 treatment increased gradually with incubation time and reached the maximum value of $1681.73 (\pm 15.32) \times 10^7$

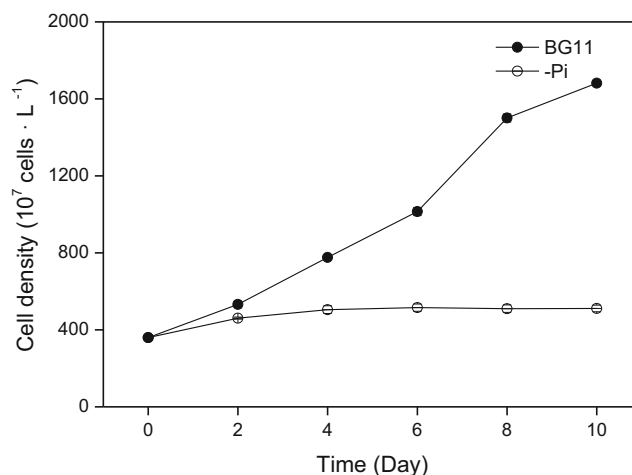


Fig. 1 Cell density of *M. aeruginosa* in different treatments (BG11: K_2HPO_4 treatment group, -Pi: P-free treatment group)

cell/L on the day 10, and the *M. aeruginosa* cells entered the logarithmic growth period after 2 days. However, the cell density remained at a constant level about $510.00 \times 10^7\text{ cell/L}$ under P-free treatment. Huang et al. (2005) reported that DIP was negatively correlated with algal APA. However, in media with high concentrations of DIP, the expression of extracellular APA increased with the growth of *M. aeruginosa* (Li et al. 2015). There are few studies on the relationship between DIP and APA (extracellular and intracellular APAs). The extracellular and intracellular APA per cell of *M. aeruginosa* cultured under K_2HPO_4 (BG11) and P-free (-Pi) treatments are shown in Fig. 2. Our results revealed that the extracellular and intracellular APAs per cell were still expressed in DIP sufficient medium. This result is consistent with the results of previous study (Li et al. 2015).

The growth of *M. aeruginosa* in DOP experimental treatment under different P concentrations is shown in Figs. 3 and 4. Cyanobacteria have been reported to maintain growth by hydrolyzing organic P under P limitation (Fernando 2015; Tiwari et al. 2015). Our results showed that *M. aeruginosa* cultured in β -GP and LEC grew well than the control treatment (-Pi) (Fig. 4). In both β -GP and LEC treatment groups, the cell density of *M. aeruginosa* increased most significantly on the day 2 (Fig. 3). *M. aeruginosa* might temporarily accumulate P facing Pi starvation and slowly utilize it during the following incubation (Ren et al. 2017; Van Moorleghe et al. 2013).

The total P (TP) concentrations in the culture medium were monitored to determine the bioavailability of DOP to *M. aeruginosa*. The variations of TP in the solutions with β -GP and LEC treatment groups are shown in Fig. 5. A general decrease of TP concentration was observed in both β -GP- and LEC-treated cultures. TP concentrations in two DOP source treatment groups decreased significantly in the initial two days. In addition, TP concentration in the medium treated with LEC decreased faster due to the rapid growth of *M. aeruginosa*. This

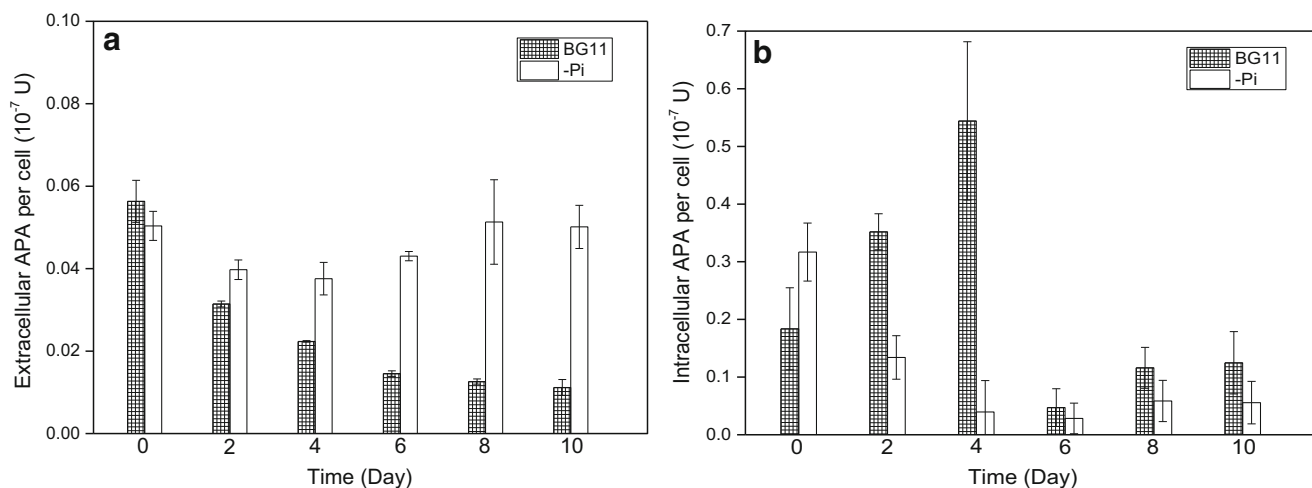


Fig. 2 Extracellular APA per cell (10^{-7} U) (a) and intracellular APA per cell (10^{-7} U) (b) of *M. aeruginosa* cultured under K_2HPO_4 (BG11) and P-free (-Pi) treatment

indicates that *M. aeruginosa* may have higher LEC uptake and utilization capacity. LEC is a kind of natural nutrient, which is generally used as a DOP source in the selective medium for DOP-dissolving bacteria (Wang et al. 2015; Yang 2014). Therefore, compared with β -GP, LEC may have a higher bio-availability. The μ value of cells in the LEC treatment groups increased with the increase of LEC concentration, and the maximum μ value was 0.146 ± 0.008 at the $7.50 \mu\text{g/mL}$ group (Fig. 4), suggesting that the concentration of phosphate diester LEC is an important factor affecting algal growth, and high concentration has a positive effect on algal growth. However, although our results showed that the growth trend of *M. aeruginosa* cultured in the β -GP at different concentrations was similar (Fig. 3a), there was no significant difference in the μ value among these groups ($P > 0.05$, ANOVA; Fig. 4). The growth of *M. aeruginosa* in culture medium was not affected

by the concentration of β -GP. It is suggested that *M. aeruginosa* may have different utilization strategies for LEC and β -GP.

Utilization of phosphomonoester β -GP by *M. aeruginosa*

As shown in Fig. 6, when DOP concentration was 3.50 and $7.50 \mu\text{g/mL}$, the extracellular IP concentrations in β -GP-treated culture increased with the prolongation of incubation time, while at lower DOP concentrations (0.10 and $0.35 \mu\text{g/mL}$), the extracellular IP concentrations tended to a constant level about $0.085 \pm 0.016 \mu\text{g/mL}$. Research showed that due to high bioavailability, phosphomonoesters were consumed rapidly and their hydrolysis exceeded the algal Pi uptake, leading to a gradually increasing DIP in the culture medium

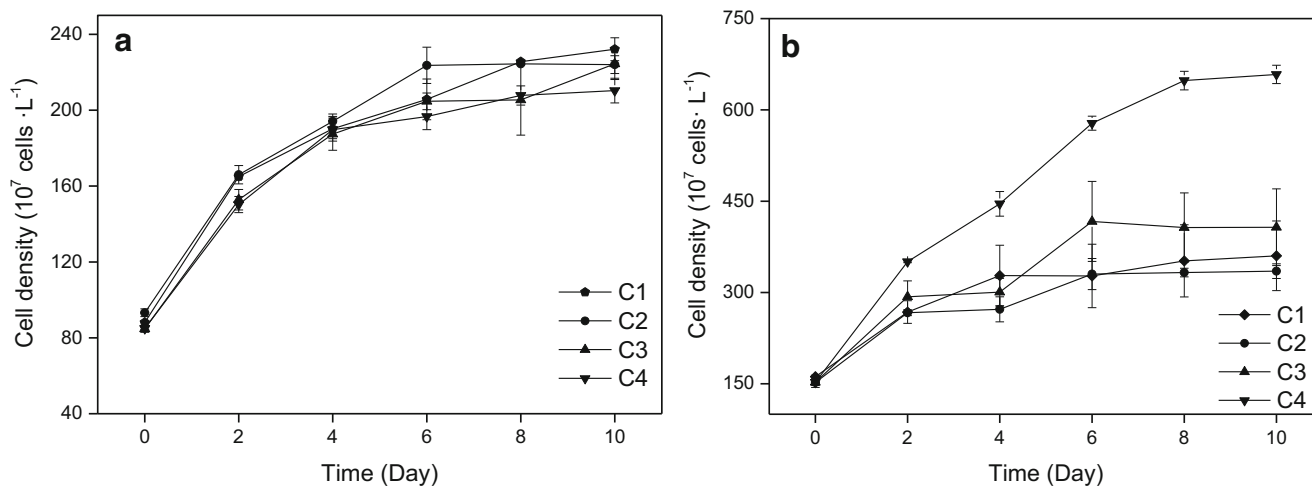


Fig. 3 Cell density of *M. aeruginosa* in the β -GP treatment group (a); LEC treatment group (b) with different concentrations (C1 0.10 , C2 0.35 , C3 3.50 , C4 $7.50 \mu\text{g/mL}$)

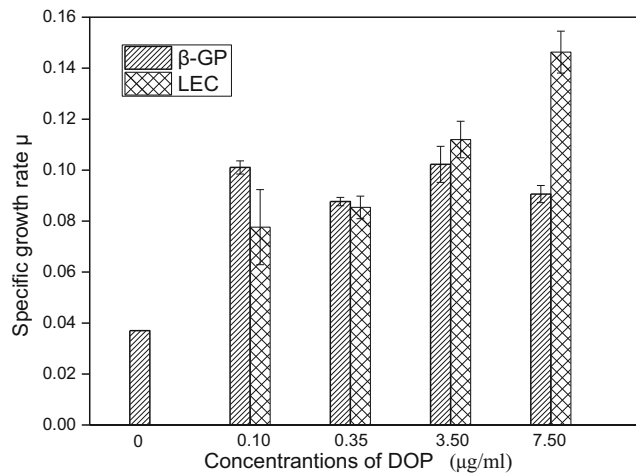


Fig. 4 Specific growth rate (μ) of *M. aeruginosa* in the β -GP and LEC treatment groups with different concentrations (C1 0.10, C2 0.35, C3 3.50, C4 7.50 $\mu\text{g/mL}$) in 10 days of incubation

(Ren et al. 2017). However, it seemed that the algal cells under low β -GP concentration groups could gain a balance between the production and the uptake of Pi. When facing Pi starvation, *M. aeruginosa* rapidly produces APases which can hydrolyze DOP to release IP (Labry et al. 2005; Tiwari et al. 2015). This is consistent with our results that extracellular APA secretion increases with the decreasing TP concentration as the cultures progress (Figs. 5a and 7a).

Interestingly, our results showed that intracellular APA (mean = $0.026 (\pm 0.003) \times 10^{-7}$ U) was generally higher than extracellular APA (mean = $0.006 (\pm 0.001) \times 10^{-7}$ U) in the β -GP treatment groups (Fig. 7). This phenomenon increases the possibility of intracellular hydrolysis of DOP by *M. aeruginosa*. It is generally believed that APases is a kind of extracellular enzyme, but recent studies (Weiss et al. 1991; White 2009) suggested that glycerophosphoric acid, a small

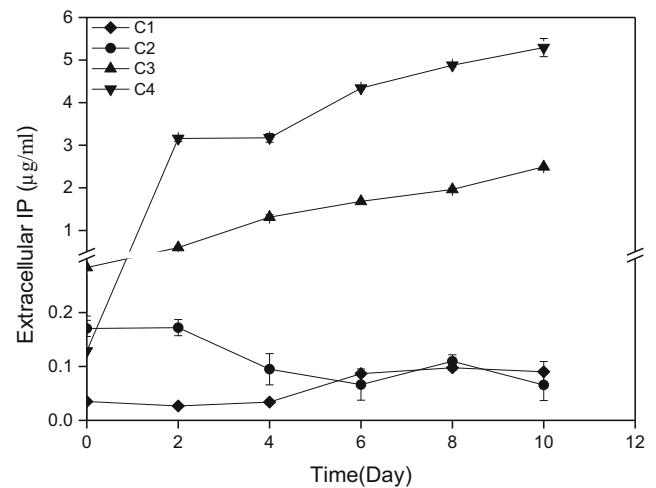


Fig. 6 Extracellular IP concentrations of *M. aeruginosa* in the β -GP treatment group with different concentrations (C1 0.10, C2 0.35, C3 3.50, C4 7.50 $\mu\text{g/mL}$)

hydrophilic molecules compounds, can be assimilated intact by certain bacteria and hydrolyzed in the cytoplasm by specific bacterial phosphatases (APases, predominately PhoA and PhoD proteins). Studies also found that the intracellular hydrolysis of the exogenous DOP might be significant (Luo et al. 2009). Our results showed that the estimated intracellular APA per single cell first increased to $0.033 (\pm 0.001) \times 10^{-7}$ (C1), $0.053 (\pm 0.002) \times 10^{-7}$ (C2), $0.038 (\pm 0.003) \times 10^{-7}$ (C3), $0.047 (\pm 0.005) \times 10^{-7}$ (C4) U, and then decreased (Fig. 7b). Compared with the higher β -GP concentrations treatment groups, the intracellular APA per cell peaked earlier in cultures treated with lower concentrations (Fig. 7b). This may be an algal survival strategy to accumulate and store P in phosphate-rich environments (Watanabe et al. 1987).

The extracellular APA (mean = $0.002 (\pm 0.001) \times 10^{-7}$ U) was lower during the early period of *M. aeruginosa* culture, so

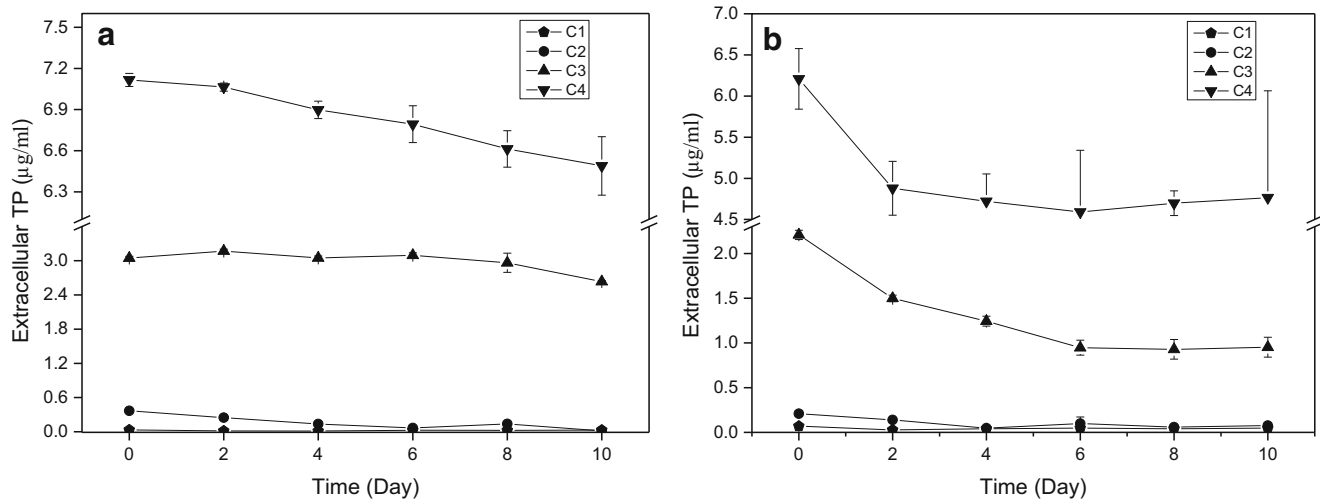


Fig. 5 TP concentrations in media under different treatments: (a) β -GP and (b) LEC with different concentrations (C1 0.10, C2 0.35, C3 3.50, C4 7.50 $\mu\text{g/mL}$)

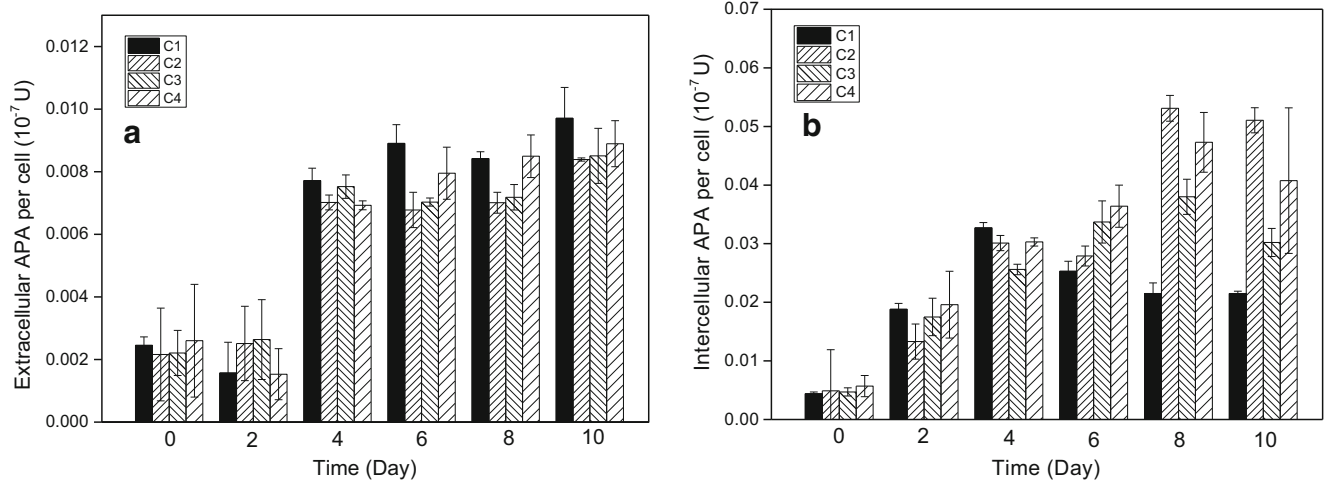


Fig. 7 Extracellular APA per cell (10^{-7} U) (a) and intracellular APA per cell (10^{-7} U) (b) of *M. aeruginosa* in the β -GP treatment group with different concentrations (C1 0.10, C2 0.35, C3 3.50, C4 7.50 $\mu\text{g/mL}$)

the intracellular APases is required to participate in the mineralization of DOP for algae growth. The concentration of extracellular TP decreased with the rapid growth of *M. aeruginosa*, and the algal growth was slowed down due to the P consumption after 8 days of cultivation (Figs. 3a and 5a). Thus, we infer that *M. aeruginosa* may cope with complex environmental changes by regulating the intracellular and extracellular APAs.

Utilization of phosphodiester LEC by *M. aeruginosa*

The extracellular IP concentrations in LEC-treated culture with different P concentrations are shown in Fig. 8, and the extracellular and intracellular APAs of *M. aeruginosa* under LEC culture of incubation are shown in Fig. 9. Overall, the change trend of extracellular APA per cell treated with LEC and β -GP was similar, except that the extracellular APA increased significantly in the initial 2 and 4 days, respectively (Figs. 7a and 9a). In this study, the extracellular APA per cell

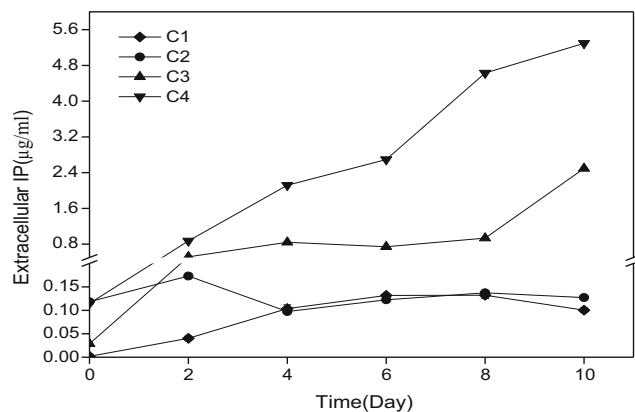


Fig. 8 Extracellular IP concentrations in the LEC treatment group with different concentrations (C1 0.10, C2 0.35, C3 3.50, C4 7.50 $\mu\text{g/mL}$)

(mean = $0.004 (\pm 0.001) \times 10^{-7}$ U) was lower in the LEC treatment group on the day 1 of cultivation (Fig. 9a). Subsequently, the extracellular APA per cell increased significantly to average $0.01 (\pm 0.001) \times 10^{-7}$ U on day 2, which promoted DOP mineralization by *M. aeruginosa*. The above results are similar to the previous research (Li et al. 2015), indicating that *M. aeruginosa* could utilize LEC due to the gradual hydrolysis of LEC by APases.

The extracellular APA and IP in the two DOP source treatment groups increased gradually with incubation time (Figs. 6; 7a; 8; and 9a). However, during the incubation, the extracellular APA in the LEC treatment group (mean = $0.008 (\pm 0.001) \times 10^{-7}$ U) was higher than that in the β -GP group (mean = $0.006 (\pm 0.001) \times 10^{-7}$ U), while the extracellular IP in the LEC group (mean = $0.885 \pm 0.190 \mu\text{g/mL}$) was lower than that in the β -GP group (mean = $1.397 \pm 0.096 \mu\text{g/mL}$) (Figs. 6; 7a; 8; and 9a). This may be as the result of the fact that LEC is more difficult to hydrolyze than β -GP, and more secreted APases are needed for DOP mineralization by *M. aeruginosa*. Moreover, the increase of extracellular IP concentration in the cultures might due to the fact that the greater was the degree of hydrolysis of DOP by APases than the absorption of DIP by *M. aeruginosa*. Interestingly, although the increase of extracellular IP in the LEC treatment group was lower than that in the β -GP group before day 6 (Figs. 6 and 8), the extracellular APA per cell in the LEC treatment group was always higher than that in the β -GP group during the culture period, leading to a similar concentration of extracellular IP on the day 10 (Figs. 6; 7a; 8; and 9a). However, the total reduction of TP in the LEC treatment group (mean = $0.446 \pm 0.328 \mu\text{g/mL}$) was greater than that in the β -GP group (mean = $0.350 \pm 0.040 \mu\text{g/mL}$) (Fig. 5). Thus, for *M. aeruginosa*, although β -GP was easily hydrolyzed by APases, the bioavailability of LEC is greater than β -GP, which may be related to the composition and structure of DOP.

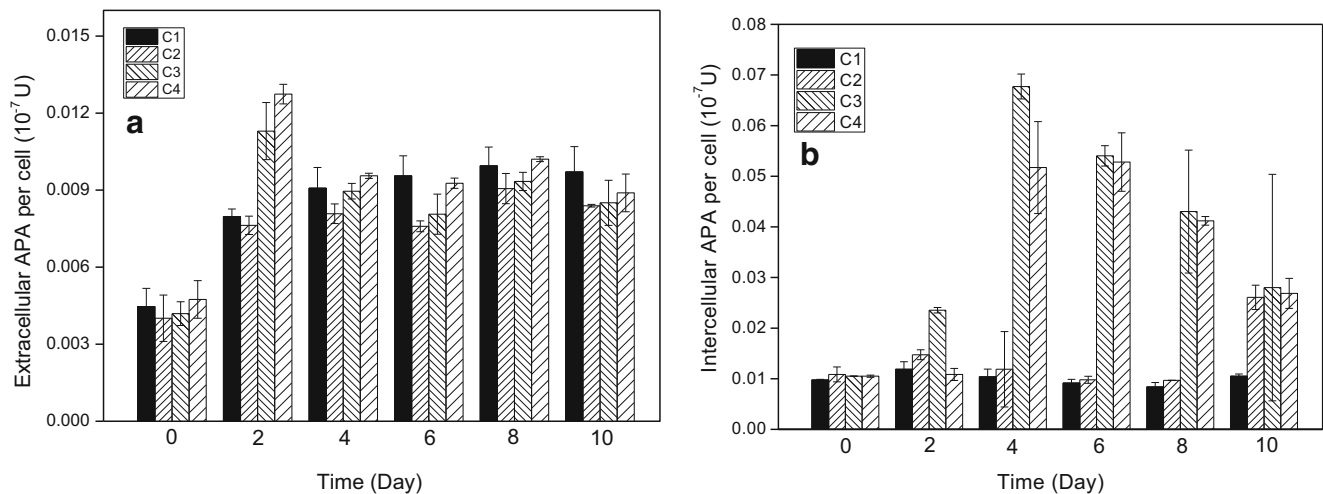


Fig. 9 Extracellular APA per cell (10^{-7} U) (a) and intracellular APA per cell (10^{-7} U) (b) of *M. aeruginosa* in the LEC treatment group with different concentrations (C1 0.10, C2 0.35, C3 3.50, C4 7.50 $\mu\text{g/mL}$)

The trend of the estimated intracellular APA per cell in the LEC treatment group was similar to that in the β -GP group: it first increased and then decreased (Figs. 7b and 9b). However, the intracellular APA per cell in the LEC treatment group peaked earlier in cultures treated with higher concentrations, for example, the peak of the intracellular APA per cell in the 3.50 $\mu\text{g/mL}$ group was $0.068 (\pm 0.002) \times 10^{-7}$ U on the day 4 (Fig. 9b). This further suggests that *M. aeruginosa* has different strategies for utilizing LEC and β -GP. A previous study (Luo et al. 2009) showed that the intracellular hydrolysis of exogenous DOP may play an important role in marine bacteria P acquisition due to the presence of abundant cytoplasmic phosphatase and P-ester transporter genes. Our results also found that the intracellular APA in *M. aeruginosa* was generally higher than the extracellular APA in LEC culture during the whole incubation (Fig. 9). Though no research has proved that LEC, which is a macromolecular DOP, can be directly transport into the cell, our results provide the possibility for the direct uptake of LEC by *M. aeruginosa*. However, whether there are other mechanisms, such as endocytosis (Zhang et al. 2019), the ability to absorb phosphate diesters into the cell requires further study at the molecular level.

Conclusion

M. aeruginosa can utilize β -GP and LEC to sustain its growth. The bioavailability of LEC was greater than β -GP, perhaps as a result of the different compositions and structures of DOP. The growth of *M. aeruginosa* under different DOP treatment groups was significantly affected by LEC concentration but not by β -GP concentration, indicating different DOP utilization strategies. The presence of generally

higher intracellular APA of *M. aeruginosa* than extracellular APA in β -GP and LEC treatment groups suggests the possibility to directly uptake DOP by *M. aeruginosa*. The intracellular APAs per cell were first increased and then decreased in all DOP treatment groups with different concentrations. These results improve our understanding of the response of extracellular and intracellular APases to DOP in *M. aeruginosa*. Future research will need to be done to focus on the quantitative study of the direct uptake and utilization of DOP by *M. aeruginosa*.

Acknowledgments We greatly thank Prof. Li J.H. from Nanjing Normal University for guidance on experimental protocols and methods, as well as students Liu L.W. and Zu Y. for help with algae inoculation. We greatly thank Dr. Malott T.M. from Mascoma LLC for the paper revise.

Funding information This work was funded by National Natural Science Foundation of China (Nos. 41303058, 31971561).

Compliance with ethical standards

Competing interests The authors declare that there are no competing interests.

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