PHYSIOLOGICAL AND ULTRASTRUCTURAL RESPONSES OF MICROCYSTIS AERUGINOSA TO DIFFERENT PHOSPHORUS CONCENTRATIONS

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ABSTRACT

Microcystis has become increasingly dominant during periods of algae bloom in freshwater environments where phosphorus (P) is usually the primary element limiting its propagation. In order to understand the mechanism of Microcystis dominance, the present study investigates the physiological, biochemical and ultrastructural responses of Microcystis aeruginosa (FACHB 469) to eight different inorganic phosphorus (Pi) concentrations under batch culture condition. The results showed that growth of M. aeruginosa was significantly restrained when Pi concentration was below 0.20 mg/L. Its alkaline phosphatase activity (APA) was induced under P deficiency stress and there was also a negative correlation between APA and external Pi concentration. Nevertheless, polyphosphate bodies (PP) were observed after M. aeruginosa, pre-cultured under Pi starvation, was exposed to higher Pi concentration. These results suggest that APA induction uptake and storage strategies of P might have ensured the dominance of M. aeruginosa in the freshwater environment with varying Pi concentrations.

KEYWORDS: Alkaline phosphatase activity; Microcystis aeruginosa; Inorganic phosphorus; Oxygen evolution activity; Polyphosphate bodies

1. INTRODUCTION

Eutrophication has become a world-wide phenomenon leading to vast proliferation of cyanobacteria particularly in highly eutrophic lakes. Frequently, Microcystis is the primary species in this type of environment and has received increasing attention due to its toxicity and massive propagation, therefore greatly threatening the quality of drinking water and leisure activities [1]. The increase in a number of nutrients including carbon (C), nitrogen (N), and phosphorus (P) is associated with eutrophication in natural freshwater. Although P is widely distributed in earth’s crust (about 0.1% by weight), its availability for organisms is restricted. Previous studies have shown that P and phytoplankton biomass are significantly correlated [2, 3], and P is often the limiting nutrient not only in oligotrophic freshwater, but also in meso-eutrophic and eutrophic waterbodies [4]. In addition, it has been shown that Microcystis sp. growth is inhibited under P-limited conditions [5, 6].

Inorganic phosphorus can be immediately utilized by phytoplankton. However, organic phosphates usually have to be hydrolyzed by alkaline phosphatases (AP) before they can be assimilated [7]. The alkaline phosphatase activity (APA) can be induced when inorganic phosphorus (Pi) is low [7]. As a consequence, APA is considered an indicator of P availability [8, 9]. On the other hand, the P uptake system is highly activated under phosphate limitation, and the cyanobacteria cells can store P in the form of polyphosphate bodies (PP) when the external P rapidly increases [10]. In addition, PP are used by cyanobacteria as intracellular P reserves and can help them to overcome short periods of P starvation [11].

Previous studies have described changes in cyanobacterial species composition and distribution in several water bodies [12, 13], in response to spatio-temporal variability of Pi concentration [11]. Field observations have been conducted to investigate the succession of cyanobacterial species during bloom formation [14, 15]. However, experiments under controlled conditions may be helpful to quantify the relationships between nutrients and cyanobacteria abundance.

The present investigation examines the lowest threshold Pi concentration at which M. aeruginosa can grow exponentially in batch culture conditions, and its physiological response to different Pi concentrations. In addition, the P uptake and storage strategies of M. aeruginosa under controlled laboratory conditions were determined. The objectives are to understand how M. aeruginosa
overcomes periods of P deprivation, and how it becomes the dominant species in a P enriched environment. This research should assist a better understanding of the dominance of *Microcystis* in natural water bodies with varying Pi concentrations.

### 2. MATERIALS AND METHODS

#### 2.1. Organisms and culture conditions

The algal strain, *Microcystis aeruginosa* 469, was provided by the FACHB (Freshwater Algae Collections, Institute of Hydrobiology of the Chinese Academy of Sciences). It was cultivated at 28°C with an irradiance of 25 µE·m⁻²·s⁻¹ under 12h/12h light-dark cycles in the HGZ medium [16]. By changing the amount of K₂HPO₄ (the reduced amount of K₂HPO₄ was replaced by equimolar KCl) in the HGZ medium, we prepared the experiment medium at 8 different Pi concentrations (P equivalent): 0.00 mg/L, 0.02 mg/L, 0.20 mg/L, 0.50 mg/L, 1.00 mg/L, 2.00 mg/L, and 6.95 mg/L, equivalent to Pi concentration in the basic concentration to trigger the cyanobacterium bloom in lakes [17]).

#### 2.2. Treatment of P starvation of algal strain

Algal cells in the exponential growth phase were collected by centrifugation at 4000 rpm for 7min, washed 3 times with distilled water to remove excess of P on cell surfaces, and re-suspended in a P-free HGZ medium (the original medium without K₂HPO₄). To validate that Pi concentration in the cells was almost zero, the optical density at 680nm (OD₆₈₀) was determined every 48 h until its variability was within the range of ± 5% and the orthophosphate concentration below 0.01mM [18]. After starvation, the cells were inoculated into 250 ml Erlenmeyer flasks containing 150 ml fresh HGZ medium at three Pi concentrations (0.00 mg/L, 6.95 mg/L, and 20.0 mg/L) and cultured for 14 days before TEM observation. The TEM procedure was performed following the method described by Venter et al. [21]. The deposition of PP involves the appearance of electron transparent areas in the nucleoplasm and the deposition of polyphosphate on and near this structure [22].

#### 2.3. Measurement of algal growth

Chlorophyll a (Chl a) of algal cells was extracted with acetone and estimated every 48 h according to Arnon in 1949 [19].

#### 2.4. Determination of alkaline phosphatase activity (APA)

Phosphatase activity was determined using para-nitrophenyl phosphate (p-NPP) as substrate for phosphomonoesterase as previously described by Berman [20]. One unit of enzyme activity (U) was defined as the amount expressed in µg of para-nitrophenyl (p-NP) released by 1mg of Chl a per hour. APA was estimated every 2 days in triplicate. The means of APA in the exponential growth stage from day 12 to 28 after inoculation were calculated.

#### 2.5. Determination P-I equation and oxygen evolution activity

The photosynthesis–irradiance (P-I) equation was obtained by measuring oxygen evolution at different irradiance intensities through the following equation:

\[
P = P_m \times \tan h \left( \frac{\alpha \cdot I}{P_m} \right) + R_d [14].
\]

where P represents photosynthetic rate at a given irradiance; I, irradiance; Pₘ, the light-saturated photosynthetic rate; α, photosynthetic efficiency; Rₙ, the dark respiration rate. The light saturation point \(I_s\) is given as:

\[
I_s = \frac{(P_m - R_d)}{\alpha}.
\]

At the light saturation point, O₂ evolution activity was measured using a Clark oxygen electrode (Hansatech, UK) kept at 28°C by a recycled water bath thermostat (Polystat refrigerated bath, Cole Parmer Inc.).

#### 2.6. Preparations for the transmission electron microscope (TEM)

The cells after P starvation were inoculated into 250 ml Erlenmeyer flasks containing 150 ml fresh HGZ medium at three Pi concentrations (0.00 mg/L, 6.95 mg/L, and 20.0 mg/L) and cultured for 14 days before TEM observation. All statistical analysis and plotting were completed by SPSS 13.0 and Origin Pro 8.

### 3. RESULTS

The growth of *M. aeruginosa* at eight different P concentrations is shown in Fig. 1. The cells in the medium with the initial Pi concentration of less than 0.02 mg/L were unable to reach exponential growth. Though there was a period of exponential growth at Pi concentration of 0.2 mg/L, but the growth rate declined approximately 15 days later. When Pi concentration was higher than 0.50 mg/L, the cells rapidly entered into and remained in the exponential growth phase during the incubation period.

The \(I_s\) of *M. aeruginosa* was derived from the P-I equation (Fig. 2) and it was approximately 255 µ Em⁻²s⁻¹ PAR. The O₂ evolution activity increased with the increase of Pi concentration and reached a maximum at the Pi concentration of 6.95 mg/L. After incubation for 14 days, the O₂ evolution activities in the medium with Pi concentration ≤ 0.20 mg/L were significantly lower than those with Pi concentrations ≥0.5 mg/L (P<0.05) (Fig. 3).

APA reached a peak after 16 days under Pi concentration below 0.02 mg/L, but 28 days for those at Pi concentrations of 0.2 - 0.5 mg/L (Fig. 4). It was also estimated that the mean APA in the P free treatment reached almost 700 U/mg Chl a, and that at higher Pi concentrations such as 6.95 mg/L, Pi treatment was just 50 U/mg Chl a in the APA exponential growth stage, which showed...
FIGURE 1 - Growth of *M. aeruginosa* at different Pi concentrations (The error bars indicate standard deviation).

FIGURE 2 - Photosynthetic-light responses (P-I) of *M. aeruginosa* (The error bars indicate standard deviation).

FIGURE 3 - Photosynthetic O₂ evolution rate of *M. aeruginosa* strains grown for 14 days at different Pi concentrations (The initial O₂ evolution rate of *M. aeruginosa* before inoculation is 250.23 µmol O₂·mg Chl a⁻¹·h⁻¹. The error bars indicate standard deviation and different letters indicate significant difference at P<0.05 level).

FIGURE 4 - Changes in alkaline phosphatase activity (APA) over time at different Pi concentrations (The error bars indicate standard deviation).

that the APA value was negatively associated to the initial Pi concentrations to some extent. The APA from *M. aeruginosa* at Pi concentrations of <0.2 mg/L and 0.2 mg/L were significantly higher than those in Pi concentration >0.2 mg/L (P<0.01) (Fig. 5).

Polyhedral bodies (PH) now recognized as carboxosomes, were observed in cells grown in P-free medium, while a large amount of polyphosphate bodies (PP) were observed in cells at higher Pi concentrations (*i.e.*, 6.95 mg/L and 20.0 mg/L) (Fig. 6). The size of the detected PP in *M. aeruginosa* using TEM was about 0.4 µm (Fig. 6).

4. DISCUSSION

Previous studies have shown that P deficiency limits algal growth by comparing algal activities at different Pi concentrations [23, 24]. Our results also reveal that P deficiency restrains the growth of *M. aeruginosa* and further
FIGURE 6 - P storage with inoculation of *M. aeruginosa* strains for 14 days at three Pi concentrations (A, B, P free; C, 6.95 mg/L P treatment; D, E, 20.0 mg/L P treatment). A and B, observed polyhedral bodies (PH) and their magnified view in P deficient condition; C, D, and E, observed polyphosphate bodies (PP) and their magnified view in P enriched environment

demonstrate the threshold value of initial Pi concentration, *i.e.*, 0.20 mg/L, which can ensure the exponential growth phase in batch cultures (Fig. 1). Additionally, the O₂ evolution activity also indicates the threshold value of Pi concentration at 0.20 mg/L (Fig. 3). Compared to the growth of *M. aeruginosa* in treatments with Pi concentrations ≥ 0.5 mg/L, *M. aeruginosa* at 0.20 mg/L Pi concentration showed no difference in growth before 16 days of incubation (Fig. 1). However, a significant decrease in O₂ evolution activity (P<0.05) was observed after 14 days (Fig. 3). This result suggests that algal cells may firstly reduce photosynthesis under P deficient conditions.

A variety of microorganisms including bacteria can produce alkaline phosphatase (AP) [25, 26], and more than 50 species of cyanobacteria can synthesize the extracellular phosphatase to utilize organic P when it is the only available P source [27]. Despite previous studies reporting that AP excreted from cyanobacteria could not be detected or observed in natural aquatic environments, the present study confirms that *M. aeruginosa* can synthesize AP. The reason explaining the results from this previous study might be that cyanobacteria in natural water bodies have luxury uptake ability and can store a large amount of P in P enrichment, and therefore they are more
tolerant to P deficiency stress and it is unnecessary for them in natural environment to activate the procedure to synthesize AP.

Fitzgerald and Nelson [28] found that AP excreted by algae in P deficient conditions was 25 times higher than that in a P-enriched environment. Previous studies have shown that phytoplankton can be synthesized and excrete AP when P availability was low and that APA was inhibited when P bioavailability increased, corroborating that APA can be used as an indicator of P deficiency. In accordance with results from previous studies [29, 30], we found that APA was 9 times higher than that in P sufficient conditions and we also found a significant negative correlation between APA and initial concentrations of Pi in the medium (P<0.01).

Algal responses to P limitation are species-specific [24]. For M. aeruginosa, its mechanism of P luxury uptake could assist it to overcome P deficient conditions. M. aeruginosa did not reach exponential growth phase until day 6 (Fig. 1) when the algae finished absorbing external P to saturation, indicated by the non-decrease of residual Pi concentrations [6]. This is probably related to the time needed for a P starved cell to build up a new metabolic mechanism for its growth and reproduction [31]. In addition, residual Pi in the medium did not decrease after M. aeruginosa entered into the exponential growth phase [6]. Thus, M. aeruginosa can grow exponentially with the P stored in the cells. This luxury uptake of P in M. aeruginosa was also demonstrated by the observation of PP in higher Pi concentrations (Fig. 6).

The most common P storage products of cyanobacteria are polyphosphate bodies [22, 32]. These polyphosphate bodies can function as a metabolic sink of P (Fig. 6) [32]. Some algal species can absorb phosphate and store it in the form of PP at higher external Pi conditions [22, 11], and the stored P could be utilized to maintain proliferation for a short period [11]. Besides phosphorus, polyphosphate bodies normally contain other elements, mostly potassium, calcium and magnesium, which are available during phosphate uptake [22, 32]. One or more PP can form in one cell, and the diameter of PP in our study was about 0.4 µm, which was much less than the maximum diameter of 1.5 µm previously observed in other studies [22]. Other factors such as sulphur starvation and nitrogen limitation can affect PP formation. It has been reported that deposition of polyphosphate bodies (or granules) occurs in other cyanobacteria (Calothrix elenkinii, Nostoc punctiforme and Rivularia biaesolettiana) [11, 33], but there is no clear evidence of their formation in Microcystis aeruginosa. Therefore, the structure of PP needs further research including using techniques such as spectrum of energy dispersion and nuclear magnetic resonance spectroscopy.

5. CONCLUSION

M. aeruginosa can store P in the form of polyphosphate bodies (PP) in P enriched environment, and its growth is significantly restrained when Pi concentration is below 0.20 mg/L as shown by the results of Chl a, O2 evolution, APA in cells. Furthermore, its APA was induced under P deficiency stress, and- was also a negative correlation between APA and external Pi concentration. Specifically, M. aeruginosa restrains unnecessary P consumption by reducing photosynthesis, and produces alkaline phosphatase (AP) to utilize organic P in P deficient conditions. These series of strategies may give M. aeruginosa an advantage in competition over other algae in natural environments. Nevertheless, why M. aeruginosa can survive and become dominant in worldwide natural water systems with significant variability in Pi concentration is worthy of further investigation.

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REFERENCES


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