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Physiological and biochemical responses of *Microcystis aeruginosa* to phosphite

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ABSTRACT

Phosphorus (P) is a key biological element and limiting nutrient in aquatic environments. Phosphate (+5) is traditionally associated with the P nutrient supply. However, phosphite (+3) has recently generated a great deal of interest, because of the possibility that it is a P source based on recognition of its vital role in the original life of the early earth. This study investigated whether phosphite can be an alternative P source for *Microcystis aeruginosa* PCC 7806, one of the predominant bloom species in freshwater systems. The results indicated that *M. aeruginosa* could not utilize phosphite as a sole P-nutrient directly for cell growth at any concentration, but that phosphite could boost cell numbers and chlorophyll a (Chl-a) content as long as phosphate was provided simultaneously. Specifically, Chl-a production increased sharply when 5.44 mg P L^{-1} phosphite was added to 0.54 mg P L^{-1} phosphate medium. Analysis of the maximum yield of PSII indicated that phosphite may stimulate the photosynthesis process of cells in phosphate–phosphite medium. In addition, phosphite failed to support cell growth, even though it more readily permeated the cells in P-deficient medium than in P-sufficient medium. Alkaline phosphatase activity (APA) analysis indicated that, unlike organic P, phosphite inhibits the response of cells to deficient P status, especially under P-deprived conditions.

1. Introduction

Phosphorus (P) is a necessary nutrient for life on earth. Phosphate minerals, which comprise the majority of inorganic P, have low solubility and mobility, which limits their availability (Benitez-Nelson, 2000). The concentration of P can be used to predict the total biomass of phytoplankton (Zhang et al., 2007). It has been assumed that P occurs exclusively as phosphate (Geng et al., 2005a; Han et al., 2011). However, recent evidence suggests that other less oxidized forms of P (namely reduced P, with an oxidation state lower than +5) that are more soluble and active play a critical role in P bioavailability (Metcalf and Wolfe, 1998; Morton et al., 2003). It has even been suggested that the P geochemistry in the early earth was controlled by reduced forms of P such as phosphite ($H_2PO_3^-$ and/or HPO_3^{2-}, +3) (Pasek, 2008). Many microorganisms can use phosphite and hypophosphite ($H_2PO_2^-$, +1) as alternative P sources, and there is genetic evidence that this capability is ancient (White and Metcalf, 2007). Moreover, application of phosphite fertilizer in agriculture is increasing worldwide, despite the dispute over whether it can be used as a P source (Rickard, 2000; McDonald et al., 2001a; Thao and Yamakawa, 2009). Phosphite has been sold as commercial products known as biostimulants or nutri-phosphite in the American and European market since 1998, and claims that these products boost plant growth have been made; however no studies have confirmed these reports or that phosphite was actually involved in P metabolism. In addition to nutrient function, phosphite also serves as a fungicide in agricultural field. Phosphite compounds have been recognized as excellent fungicides for the control of many important plant diseases caused by Oomycetes, particularly *Phytophthora* sp. (Thao and Yamakawa, 2009). Nevertheless, few studies have investigated the existence and role of reduced P in the biogeochemical P cycle in lake ecosystems.

P is an important nutrient for algal growth, and its deficiency can greatly influence the production of Chl-a and the rate of photosynthesis (Shen and Song, 2007; Wu et al., 2009). The role of P in eutrophication has been at the forefront of hydro-biological research during the last few decades (Correll, 1998; Geng et al., 2005b). However, the effects of reduced P on eutrophication have not been considered in past studies, in part because these previous efforts have neglected the existence of reduced P in the environment. Reduced forms of P are often misclassified as organic P by traditional analysis methods (Morton et al., 2003), even though they are actually potential contributors to measured dissolved reactive P concentrations (Hanrahan et al., 2005). To date, ion chromatography methods have been widely employed to identify phosphite and hypophosphite in the environment (McDowell et al., 2004). Pech et al. (2009) confirmed the presence of 0.06 ± 0.02 μM phosphite and 0.05 ± 0.01 μM phosphate in a geothermal pool. We recently measured 0–0.05 mg P L^{-1} of phosphite in bottom water collected from Lake Taihu, China (unpublished data). Reduced P may be introduced to the environment from many different sources, including

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phosphate ore, corroding metals such as iron, and industrial products. The primary product of schreibersite oxidation by water is phosphite, with >50% of the total aqueous P occurring in this form (Pasek, 2008). Moreover, reduced P products are routinely used in environmental applications, including fertilizers, fungicides, insecticides, herbicides, rodenticides, fumigants, flame retardants, and chemical intermediates (Morton et al., 2005).

Approximately 1% of bacterial species are capable of using reduced P compounds such as phosphite and hypophosphite as the sole P sources (Pasek, 2008). Additionally, some bacteria can oxidize hypophosphite to phosphite. Phosphine gas (PH_3, −3) is detectable globally in the atmosphere and as part of the atmospheric link of the P cycle on earth (Han et al., 2011); accordingly, PH_3 has been attributed to anaerobic metabolism and can ultimately be converted to phosphate after complex oxidation through hypophosphite and phosphite. PH_3 had a positive relationship with the biomass of algae and Chl-a in Lake Taihu (Geng et al., 2005b; Niu et al., 2003). However, no studies have been conducted to investigate the effects of phosphite on algal growth and photosynthesis to date. Therefore, it is necessary to study the algal bioavailability of phosphite in lakes and its possible link to eutrophication.

Given the importance of P cycling in lake ecosystems, additional studies are needed to clarify the relationship between reduced P and lake eutrophication. In this study, the bioavailability of phosphite by *M. aeruginosa*, one of the predominant species involved in algal blooms in freshwater lakes of China (Cembella et al., 1984; Fujimoto et al., 1997; Wu et al., 2009), was investigated. The purpose of this study was to determine if phosphite will boost the cell growth and photosynthesis of *M. aeruginosa*.

2. Materials and methods

2.1. Algae and culture conditions

M. aeruginosa (PCC 7806) obtained from the Institute of Hydrobiology of Chinese Academy was investigated in modified-P BG-11 medium at 25 ± 1 °C under illumination (2400 lx) using cool white fluorescent lights with a 12-h dark:12-h light cycle. The basal P-free BG-11 medium consisted of 150 g L^{-1} $NaNO_3$, 20 g L^{-1} Na_2CO_3, 75 g L^{-1} $MgSO_4{\cdot}7H_2O$, 36 g L^{-1} $CaCl_2{\cdot}2H_2O$, 1 g L^{-1}EDTA (disodium salt), 6 g L^{-1} citric acid, 6 g L^{-1} $(NH_4)_3Fe(C_6H_5O_7)_2$, 35 g L^{-1} of KNO_3, 2.86 g L^{-1} H_3BO_3, 1.81 g L^{-1} $MnCl_2{\cdot}4H_2O$, 0.22 g L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.39 g L^{-1} $NaMoO_4{\cdot}2H_2O$, 0.079 g L^{-1} $CuSO_4{\cdot}5H_2O$, and 0.049 g L^{-1} $Co(NO_3)_2{\cdot}6H_2O$ in distilled water. All components were autoclaved separately and mixed upon cooling. All P substrates were prepared based on P-free BG-11 medium using a single filter-sterilized (0.22 μm) P source (Na_2 (HPO_3) $\cdot 5H_2O$ and $NaH_2PO_4{\cdot}H_2O$) to form different P media.

2.2. Sample collection and analysis

Before the experiment, cells were cultured in phosphate medium until they reached their exponential growth stage, after which they were transferred to P-free BG-11 medium for about 1 week to exhaust the accumulated P in the cells. The P-starved cells were then re-inoculated into different P media for 2 or 3 weeks (depending on the growth status).

All the experiments were conducted in triplicate. At regular intervals, samples were taken and cell numbers were counted on a compound microscope equipped with a hemocytometer. Each sample was measured at least three times, with a maximum deviation of about 20%. Chl-a was extracted with 90% acetone extraction and measured as described by the standard methods (State Environmental Protection Administration of China, 2002).

A chlorophyll fluorescence monitoring system (PAM 210; Walz, Efeltrich, Germany) was applied to monitor the photosynthetic performance in algae as described by Schreiber and Bilger (1987). Dark-adapted minimal fluorescence (F_o) with all PSII reaction centers open was determined by measuring the modulated light (0.15 μmol m^{-2} s^{-1} PAR), which was sufficiently low not to induce any significant variable fluorescence. The dark adapted maximal fluorescence (F_m) with all PSII reaction centers closed was determined by 0.8 s saturating pulse 8000 μmol m^{-2} s^{-1} PAR. The variable fluorescence, F_v, was calculated as the difference between F_o and F_m. After being dark-adapted for 15 min, the optimal quantum yield of PS II photochemistry was measured and calculated from the F_v/F_m ratio based on the method described by Ting and Owens (1992). The detection limit of the fluorometer was approximately 10^6 cells of *M. aeruginosa* per mL.

Phosphate was measured using the molybdenum-antimony spectrophotometric method (State Environmental Protection Administration of China, 2002). Because phosphite is unable to react with molybdenum-antimony regent, its levels can be determined in vitro after oxidation into phosphate with persulfate solution (Morton et al., 2003). Cells in the P medium were centrifuged at 12,000 *g* for 10 min at 4 °C, washed three times with P-free medium and then digested with 5% persulfate for 30 min.

The alkaline phosphatase activity (APA) was assayed according to the method described by Berman (1970). Briefly, a 2 mL cell sample (unfiltered) was mixed with 1 mL of freshly prepared p-nitrophenyl phosphate, disodium hexahydrate (p-NPP) at 10^{-3} mol L^{-1} and 2 mL of Tris-Cl (pH = 8.6), after which it was incubated under dark culture conditions for 24 h and then measured at 410 nm by UV-1100. Reactions were monitored by continuously following the production of p-nitrophenol.

3. Results and discussion

3.1. Phosphite stability in medium

Before the experiment, the stability of phosphite in the medium was investigated. The percentage of oxidation of phosphite in BG-11 medium was examined in freshly prepared and after 3 weeks of storage medium under the culture conditions. As shown in Table 1, phosphite was stable in medium during the 3 weeks of storage under the cultured conditions. In phosphite medium with phosphite concentrations ranging from 0 to 500 mg P L^{-1}, the oxidation percentage of the phosphite ranged from 0% to 1.48%.

If the phosphite is unstable and easily oxidized to phosphate, the generated phosphate rather than the phosphite itself will serve as the P source. The results indicated that the phosphite was stable in BG-11 medium, at least during the experimental period. Other studies have also shown that phosphite solution is stable, even under aerobic conditions, and that it will not be readily oxidized without strong oxidants (McDowell et al., 2004). ^{31}P nuclear magnetic resonance (NMR) revealed that phosphite in morpholinepropanesulfonic acid (MOPs) medium had no detectable oxidation products after 2 weeks under aerobic conditions when the P concentration ranged from 250 to 1000 μM (Metcalf and Wolfe, 1998).

3.2. Phosphite as the sole P source for growth of M. aeruginosa

P-starved *M. aeruginosa* were inoculated into medium containing four levels of phosphite ranging from 0.5 to 500 mg P L^{-1}. The results revealed that 500 mg P L^{-1} phosphite caused a decrease in the cell number and even death after 16 d, whereas the cell number of cultures grown on medium containing 0–50 mg P L^{-1}