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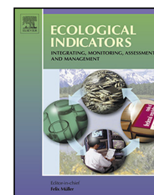

Effects of phosphorus stress on the photosynthetic and physiological characteristics of *Chlorella vulgaris* based on chlorophyll fluorescence and flow cytometric analysis

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ABSTRACT

This paper discusses the effects of phosphorus stress on the photosynthetic and physiological characteristics of *Chlorella vulgaris*. The results indicate that the growth and photosynthesis of *C. vulgaris* were affected under non-phosphorus control and non-orthophosphate treatments (pyrophosphate, tripolyphosphate and glycerophosphate). Orthophosphate promoted the growth of *C. vulgaris* most significantly, while the growth of *C. vulgaris* under non-phosphorus control was slow and soon entered a stable period. The growth of *C. vulgaris* was also promoted under non-orthophosphate treatments, but slower than orthophosphate treatment, with no significant differences among the three. A reversible inactivation occurred in the PSII system of *C. vulgaris* under non-phosphorus control after 24 h, while the same phenomenon occurred after exposure for 7 days under non-orthophosphate treatments. The decrease in actual photochemical efficiency was related to the fall in the ratios of the opening reaction centers in the PSII system. For non-orthophosphate treatments, it was also related to other factors, as not all opening reaction centers transferred electrons. Phosphorus stress did not affect the integrity of the cell membranes, demonstrating that phosphorus stress could result in the inhibition of cell yield, rather than cell death. Phosphorus stress could also decrease the esterase activities and increase the mean size of algal cells.

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1. Introduction

Algae are not only the primary producers of aquatic ecosystems but also the foundation of the food chain. The relative stability of the algal yield plays an important role in maintaining the normal operation of aquatic ecosystems. Algae are also often used for monitoring environmental changes and assessing ecological risks.

Phosphorus is one of the essential nutrients for algae and is the basis of energy transfer and material synthesis. A lack of phosphorus could affect the physiological characteristics of *Chlorella kessleri*, resulting in increased algal biomass (dry weight yield), reduced algal optical density values, and so on (Elsheek and Rady, 1995). Phosphorus could also affect the growth and photosynthesis of *Nitzschia* sp., *Sphaerocystis schroeteri* and *Phormidium luridum* (Litchman et al., 2003).

The main uptake and utilization form of phosphorus for most algae is orthophosphate ($H_2PO_4^-$ and HPO_4^{2-}) (Cembella et al., 1984), and some algae can use hydrolysates of organic matter as a source of nutrients (e.g., Oh et al., 2002; Zhang et al., 2002; Qian et al., 2010). When lacking orthophosphate, algae can be induced to produce alkaline phosphatase, which is used to transform non-orthophosphate into orthophosphate (Wynne et al., 1991). Under natural conditions, only 5–8% of dissolved phosphorus is orthophosphate, which can be used directly; most phosphorus is non-orthophosphate dissolved or suspended in water (Jansson, 1988; Wetzel, 2001). As a consequence, using only dissolved orthophosphate cannot effectively predict the growth potential of algae in water, and it is important to study the effects of non-orthophosphate on algae.

Previous results showed that *Alexandrium tamarense* grew poorly under fructose-6-phosphate, glucose-1-phosphate, glycerophosphate, and ribose-5-phosphate treatments but grew well under treatment of adenosine-5-diphosphate (ADP), adenosine triphosphate (ATP), and dissolved inorganic phosphorus (Oh et al., 2002). *Gymnodinium catenatum* could utilize various forms of

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organic and inorganic phosphorus efficiently (Oh et al., 2002). According to other studies, exogenous organic phosphorus (sodium glycerophosphate) was more conducive to the growth of *Microcystis aeruginosa* than inorganic phosphorus (KH_2PO_4) (Zhang et al., 2002).

The forms of phosphorus in natural water body are very rich, and, at the same time, the emissions of human activities have a great influence on the forms of phosphorus in natural water. For example, tripolyphosphates are important components of modern synthetic detergents and common chemicals in waste water. As a consequence, relevant studies on the effects of different phosphorus forms on algae are still needed. In addition, traditional research methods in algal toxicology emphasize mortality, not physiological responses such as photosynthesis or changes on a cellular level. This focus is not conducive to a comprehensive understanding of the action mechanism of external stress. Chlorophyll fluorescence and flow cytometric analysis have been used in recent years to study the photosynthetic toxicity and cytotoxicity, which could detect the changes of cells on a cellular level and make up for the deficiencies of traditional algal toxicology.

Chlorophyll fluorescence analysis is based on the theory of photosynthesis, using chlorophyll in vivo as a probe to detect the photosynthetic physiological status and the effects of external factors on photosynthesis quickly, sensitively and without any damage (e.g., Govindjee, 1995; Lazár, 1999; Maxwell and Johnson, 2000). FCM (flow cytometry) could quantitatively detect changes in individual cells (fluorescence, light scattering and so on) (e.g., Szakács et al., 1998; Brown and Wittwer, 2000; Iannone et al., 2000) and quickly measure the physiological characteristics of cells (sizes and activities of cells, number of nucleic acids, and so on) (Song and Li, 1992; Du and Feng, 2008).

In this paper, four common forms of phosphorus (K_2HPO_4, $C_3H_7Na_2O_6P{\cdot}5H_2O$, $Na_4P_2O_7{\cdot}10H_2O$, and $Na_5P_3O_{10}$) were selected as phosphorus source, and traditional research methods in algal toxicology, as well as chlorophyll fluorescence and flow cytometric analysis, were used to study the uptake and utilization of different forms of phosphorus and their effects on the photosynthetic and physiological characteristics of *C. vulgaris*. Studies on *C. vulgaris* based on chlorophyll fluorescence and flow cytometric analysis can help understand the mechanism of the effects of different phosphorus forms on algae, which provides theoretical bases for relevant ecological studies and has significance for both further studies and practical applications.

2. Materials and methods

2.1. Experimental materials

2.1.1. Algal strain

In this paper, *C. vulgaris* (purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China) was selected as the algal material. Green algae are the most common algal species in some areas. In many previous domestic and overseas studies, *C. vulgaris* has also been selected as the algal material, which makes comparing findings convenient (Fergola et al., 2007).

2.1.2. Chemical materials

BG11 medium was used for algal culture. The main components of the medium and partial exposure reagents (K_2HPO_4, $C_3H_7Na_2O_6P{\cdot}5H_2O$, and $Na_4P_2O_7{\cdot}10H_2O$) were purchased from Sinopharm Chemical Reagent Co., Ltd., Beijing, China. $Na_5P_3O_{10}$ and dyes used in flow cytometric analysis (fluorescein diacetate, FDA; propidium iodide, PI) were purchased from Sigma-Aldrich Corp., St. Louis, U.S. Acetone was purchased from Beijing Chemical Works, Beijing, China, used for solubilization of FDA.

2.2. Experimental methods

2.2.1. Algal culture conditions

C. vulgaris was cultured statically in BG11 medium at $24 \pm 1\,^{\circ}C$ with a cycle of light (14 h, 4000 lx) and dark (10 h, 0 lx) in a GXZ-280B illumination cultivation cabinet (Ningbo Jiangnan Instrument Factory, Ningbo, China). The cultures were shaken two to three times daily, and their positions were changed randomly.

2.2.2. Exposure experiments

Before the exposure experiments, *C. vulgaris* was cultured in BG11 medium without phosphorus for 3 days to remove the phosphorus from the original medium. At the beginning of the exposure experiments, the initial cell density of *C. vulgaris* was approximate 10^6 cells/mL. No phosphorus was added in the medium for non-phosphorus control. K_2HPO_4, $Na_5P_3O_{10}$, $Na_4P_2O_7{\cdot}10H_2O$, and $C_3H_7Na_2O_6P{\cdot}5H_2O$, four common forms of phosphorus in natural water body, were selected as exposure reagents and separately added in four treatments (the final concentration of total phosphorus in each medium was 1.75×10^{-4} mol/L). The non-phosphorus control and each treatment were all set up in triplicate and cultured for 7 days. Samples were taken for analysis every 24 h.

2.2.3. Measurements of the indicators

2.2.3.1. Biomass analysis. In this paper, biomass was used as the indicator of algal growth. Based on the results of the preliminary experiments, $OD_{450\,nm}$ (optical density of algal samples at 450 nm) correlated with algal cell density (cell density (10^6 cells/mL) = $27.6 \times OD_{450\,nm}$, $R^2 = 0.9992$). $OD_{450\,nm}$ was measured using a microplate reader (Model-680, Bio-Rad Laboratories, Inc., California, U.S.). The growth rates of *C. vulgaris* were calculated by the following formula:

$$\text{Growth rates}\,(t) = \text{In}N_t - \text{In}N_{t-24} \quad (1)$$

N_t and N_{t-24} were the biomass of algal cells under non-phosphorus control and phosphorus treatments at time t (h) and $t-24$ (h).

2.2.3.2. Chlorophyll fluorescence analysis. Chlorophyll fluorescence analysis was selected to detect the photosynthetic changes of *C. vulgaris*. After dark adaptation for 25 min, light-induced curves were measured by MAXI-Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany). From the curves, many photosynthetic parameter values could be measured or calculated, such as *F0* (initial fluorescence), *Fm* (maximum fluorescence after dark adaptation), *Fm′* (maximum fluorescence after light adaptation), *Fv*/*Fm* (maximum photochemical efficiency), Φ_{PSII} (actual photochemical efficiency), ETR (photosynthetic electron transport rate), qL (photochemical quenching coefficient), NPQ/4 (non-photochemical quenching coefficient) and so on.

2.2.3.3. Flow cytometric analysis. To detect the responses of algal cells at a cell level, flow cytometric analysis was selected to measure esterase activities, cell membrane integrity, chlorophyll *a* fluorescence and cell size.

FDA could be used to evaluate the esterase activities of cells (Franklin et al., 2001a; Stauber et al., 2002; Yu et al., 2007). In this study, after stained by FDA (dissolved in acetone, final concentration 25 μmol/L) for 8 min in the dark (Yu et al., 2007), the fluorescence in the FL1 channel (515–545 nm) was measured. Based on the results of the preliminary experiments, acetone exhibited no significant effects on the esterase activities of *C. vulgaris*.

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