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Alkaline Phosphatase Activity in Four *Microcystis aeruginosa* Species and Their Responses to Nonylphenol Stress

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In recent years, algal blooms occur frequently in freshwater bodies all over the world. This phenomenon is necessarily related with industrial pollution, which contains a vast amount of organic pollutants. A bloom that occurred in the Modder River of South Africa originated from organic pollution (Koning and Roos 1999). The relative activity of alkaline phosphatase (APase) has been suggested as a potential measure of microbial biomass and general trophic conditions of aquatic environments (Jones 1972). Sabil et al. (1994) found that immobilized enzymes, including phosphatase in the sediment phase of the Venice lagoon within an area of shallow water, showed marked heat stability and an increased resistance to environmental changes. These enzymes could be useful as diagnostic factors of the ecosystem, since their presence is related to waste products and their life span is prolonged by their insolubilization (Zhou et al. 2002). Therefore, it is essential to study the relationships between organic pollutants and alkaline phosphatase activity (APA) to clarify the reasons for APA variation caused by complex pollution stress. There are limited reports available on chemical stress on APA variation and no information concerning the effect of persistent organic pollution on APA variation.

Cyanobacteria are the dominant phytoplankton group in eutrophic freshwater bodies worldwide, causing harmful impacts on recreation, ecosystem integrity, and human and animal health. Cyanobacterial poisoning of livestock, pets and humans has been widely documented (Sivonen 1996). Nonylphenol (NP) is a degradation product of the alkylphenol polyethoxylates, an important class of nonionic surfactants employed in many detergent formulations for industrial and household use. Considerable amounts of these persistent compounds have been discharged into natural waters via industrial and municipal wastewater effluents (Ahel et al. 1994). Concerns about the toxicity and endocrine potential of NP have led to extensive studies on its fate in the environment and toxic effects on aquatic animals and plants over the last decade (Servos 1999). Therefore, there is ecological importance of studying the interaction between NP and cyanobacteria in water systems. To our knowledge, studies on effects of NP on APase activity and kinetics in cyanobacteria are still lacking.

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Laboratory culture experiments were conducted using four *Microcystis areuginosa* strains under different concentrations of NP. The main objective of the present study was to investigate the APase activities in different *Microcystis* strains and evaluate the impact of different nonylphenol concentrations on activity and kinetics of dissolved alkaline phosphatase (as expressed by the Michaelis constant, K_m and the maximum enzyme reaction velocity, V_{max}) of the four *M. areuginosa* strains.

MATERIALS AND METHODS

Four axenic *M. aeruginosa* strains, the toxic PCC7820 and 562 and the nontoxic PCC7820N and 315, provided by Prof. Lirong Song, the Culture Collection of Algae at the Institute of Hydrobiology, were used for this study. Both PCC7820 and 562 produce mainly microcystin-LR as the major toxin. The nontoxic strain of PCC7820N was obtained from the toxic one under laboratory conditions.

The strains were grown in CT medium (Jang et al. 2004) as batch cultures in an incubation chamber set up with a starting cell number of 6×10^6. The laboratory conditions were controlled at 25±1 °C using cool white fluorescent lights (2400 lux) with a light-dark regime of 14:10 h. The strains were inoculated in 250-mL Erlenmeyer flasks with 150 mL growth medium. The flasks were incubated with continuous shaking (100 rev/min). Technical nonylphenol was kindly donated by Dr. Schramm (GSF-National Research Center of Environment and Health, Germany). The NP was dissolved in dimethyl sulfoxide (DMSO) as a carrier solvent, which was diluted to give the desired concentrations of NP in growth medium with DMSO <0.02%. Eight different NP concentrations (0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2 mg L^{-1}) were chosen for algal exposure experiments. The experiments were repeated three times and each treatment had at least two replicates.

The algal cells were harvested after incubation for 12 days by centrifugation at 12000 g at 20 °C prior to APA analysis. The supernatants were decanted and filtered for dissolved APA analysis. The harvested cells were ground to a slurry with a mortar and pestle which was kept cool on liquid nitrogen, and then 4 mL phosphate buffer (pH 7.8) containing 1% (w/v) of insoluble polyvinylpyrrolidone (PVPP) was used to extract enzymes. Protein was assayed according to Bradford (1976). Bovine serum albumin was used as the standard.

Alkaline phosphatase activity in cultured solutions and algal cells was assayed at 37 °C by a method involving the hydrolysis of *p*-nitrophenylphosphate (*p*NPP) to *p*-nitrophenol (*p*NP), by reading absorbance at 410 nm. The reaction mixture was made of 0.1 M KCl/H_3BO_4, pH 10.2, 0.1 M NaOH and 2 mM 4-nitrophenyl-phosphate (Qin et al. 2001).

Kinetic measurements were conducted at eight different concentrations of *p*NPP from 0.01 to 1.8 mmol L^{-1} in the absence or the presence of NP exposed cultured solutions. The enzyme solution was prepared by dissolving the enzyme

preparation in 100 mM Tris/HCl, pH 7.4, containing 100 mM NaCl, 0.1 mM $MgCl_2$, 0.02 mM $ZnCl_2$, 10% glycerol and 0.02% NaN_3. The actual procedure followed that described by Sayler et al. (1979) and Zhou et al. (2002). 0.1 M NaOH was used to stop the reaction. Phosphatase activity was converted to absolute units using a standard curve based on enzymatically hydrolyzed *p*-nitrophenol. The Lineweaver-Burke transformation (1/V vs. 1/s) of the Michaelis-Menten equation was used to calculate the Michaelis constant (K_m) and maximum velocity of the enzyme (V_{max}). K_m and V_{max} were computed by linear regression analysis of the values obtained in the assay.

Data presented were expressed as mean ± standard deviation. Statistical significance was established at $P < 0.05$. Student Newman-Keuls test was used to compare the means of observations at the $p = 0.05$ level. All statistical analyses were carried out by the STATISTICA 6.0 (Statsoft, Inc. Tulsa Oklahoma, USA).

RESULTS AND DISCUSSION

Alkaline phosphatase activities in *M. aeruginosa* were determined in laboratory cultures of four strains in logarithmic phase of growth. Toxic strain PCC7820 showed the highest cell-bound APA activity of 327.1 ± 41.9 μg mg^{-1} protein h^{-1}, followed by the nontoxic PCC7820N and toxic 562 with 193.0 ± 7.3 and 83.7 ± 7.0 μg mg^{-1} protein h^{-1} respectively, and the nontoxic 315 had the lowest activity of 23.0 ± 13.8 μg mg^{-1} protein h^{-1}. Dissolved APase activities in culture media after 12 days' incubation were 6.64 ± 0.99, 7.40 ± 0.58, 7.28 ± 0.12 and 3.81 ± 0.67 μg mL^{-1} h^{-1} for toxic PCC7820, nontoxic PCC7820N, toxic562 and nontoxic 315, respectively. Since the first report of extracellular APase by multicellular marine algae, APA has been measured in a variety of algal groups such as red algae and brown algae (Hernández et al. 2002). However, limited information is available on APase in freshwater cyanobacteria. In the present study, APase activities in four species of *M. aeruginosa* were detected and these results indicated that cell-bound APA and APA dissolved in media differed markedly between species. Both the studies of Rengefors et al. (2001) that single cells could express APA at different levels and Spijkerman and Coesel (1998) that *S. chaetoceras* is liable to release APase into water, obtained results that are consistent with our conclusion. Field investigations show that variability of APase activities is directly related to the heterogeneity of the samples within a given site (Sayler et al. 1979). Song et al. (2004) also found the distribution and kinetics of APA at the bottom of the ponds with *M. aeruginosa* bloom are diversified. These phenomena encountered in the field may be explained by previous studies (Rengefors et al. 2001; Spijkerman and Coesel 1998), and by ours, that cell-bound APA and APA release are species specific.

There were obviously different patterns of cell-bound APA variation in response to NP stress between toxic strains and nontoxic strains (Fig.1). No obvious change of APA was observed in the nontoxic strains PCC7820N and 315, whereas APA in the toxic strains PCC7820 and 562 increased greatly upon exposure to 2 mg L^{-1} NP. APA decreased slightly when exposed to 0.2-1 mg L^{-1} NP and 0.05- 0.5 mg

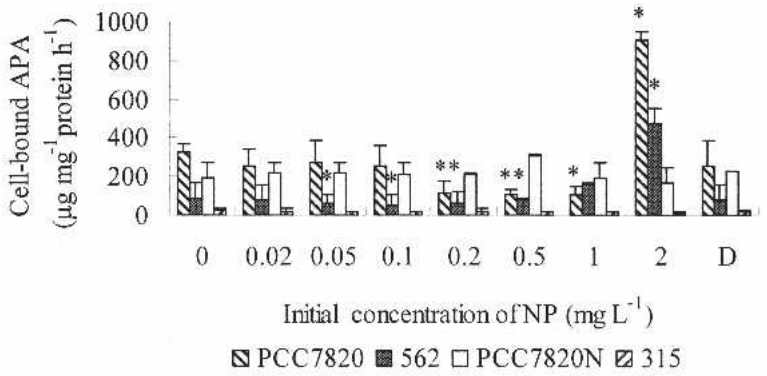

Figure 1. Cell-bound APase activities in *M. areuginosa* strains after 12 days' incubation with different concentrations of NP. Vertical bars show standard deviation. D represents solvent control. * indicates a significant difference from control ($p < 0.05$).

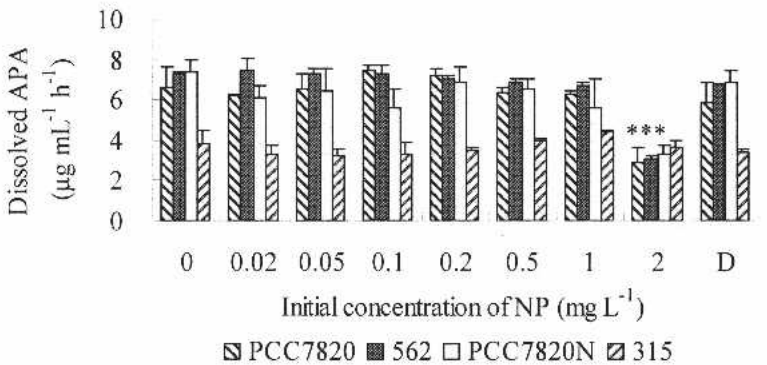

Figure 2. Dissolved APase activities in *M. areuginosa* strains after 12 days' incubation with different concentrations of NP. Vertical bars show standard deviation. D represents solvent control. * indicates a significant difference from control ($p < 0.05$).

L^{-1} NP for the toxic PCC7820 and 562, respectively. The APA dissolved in culture media was only inhibited when exposed to 2 mg L^{-1} NP for the toxic strains PCC7820 and 562 and nontoxic PCC7820N (Fig. 2). Serrano and Boon (1991) found that polyphenol extracted from *Eucalyptus camaldulensis* leaves were extremely inhibitory to purified APase and inhibited APA of various waters in Australian rivers. The threshold for the inhibition ranged from < 2 to 10 mg L^{-1}, which was within the range of polyphenol concentrations found in freshwater environments. Freeman et al. (1990) found that after removing organic matter with a molecular weight of >1000 daltons, the activities of β-glucosidase,

phosphatase and esterase were increased, suggesting that phenolic materials were responsible for the inhibition they observed. Our study showed that APAs dissolved in culture water released by *M. aeruginosa*, both toxic and nontoxic strains, were inhibited when the concentration of nonylphenol was as high as 2 mg L^{-1}, which was within the range of toxic concentrations of polyphenols found by Serrano and Boon (1991).

There are only limited studies on chemical stress on APA. Kim et al. (1998) reported that animal wastes stimulated phosphatase activity. Zhou et al. (2001) postulated that fish feces enhanced APA in sediments. The present study is the first report of the effects of nonylphenol, a persistent organic pollutant and endocrine disrupter on APA in *M.aeruginosa* strains. Our study showed that NP of 1-2 mg L^{-1} evidently enhanced APA in algal cells of the toxic *M. aeruginosa* strains PCC7820 and 562, but NP had no obvious influence on APA of the nontoxic strains PCC7820N and 315. In addition, APA in toxic strains PCC7820 and 562 decreased slightly in response to 0.2-1 and 0.05-0.5 mg L^{-1} NP, whereas this tendency did not occur in nontoxic strains. Apart from the hydrolysis function, APA also plays a role in many aspects of cell metabolism, such as material transport across the cell membrane, organic phosphorus synthesis in the body, etc. (Muda et al. 1992). Therefore, we speculated that APA might have some relationship with microcystin production in the algal cell and further studies are needed to clarify the reasons.

Both of the kinetic parameters K_m and V_{max} presented species specific characters and NP showed different effects on K_m and V_{max} (Fig. 3-4). K_m of APase dissolved in the culture media measured on exposure to different concentrations of NP did not show an obvious difference. V_{max} of APase in media was induced under 2 mg L^{-1} of NP exposure for the toxic PCC7820 strain and 1-2 mg L^{-1} NP for strain 562, while there was no obvious change in V_{max} values for the nontoxic strains PCC7820N and 315. The Michaelis constant K_m of an enzyme may be regarded at a specific set of environmental conditions as a suitable measure of the affinity of that enzyme for substrate: the higher the K_m value, the lower the affinity for that particular substrate. Marxsen and Schmidt (1993) showed that V_{max} and K_m for phosphatase in stream sediments were influenced by the discharge rate, the water quality, the substrate (phosphomonoesters), and the concentration of dissolved orthophosphate (o-P). Zhou et al. (2001) reported that V_{max} values of APase in surface sediments increased markedly at sites immediately under and adjacent to the cage that would be subjected to the deposition of fish feces, but the K_m values showed much more variability indicating the composts contained different phosphomonoesterases. Our study showed that K_m was not influenced by nonylphenol, i.e. NP did not affect the affinity of APA for the particular substrate but different *Microcystis* strains showed different K_m values. Whereas V_{max} was different among different *Microcystis* strains and NP obviously affected APA in toxic *Microcystis* strains. The patterns of V_{max} and K_m in our experiment were similar to the previous field study conducted by Zhou et al. (2001). Phytoplankton can enhance the efficiency of organic phosphorus utilization by increasing the reaction velocity of the enzyme and improving the affinity of the enzyme to the

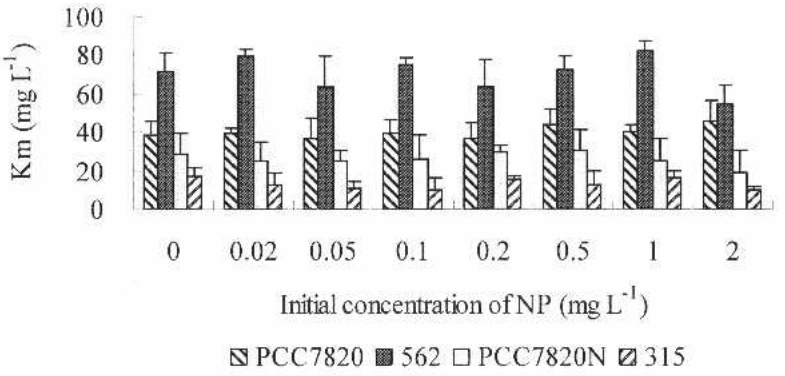

Figure 3. Variation of APase kinetic parameter (K_m) in culture media of *M. areuginosa* strains after 12 days' incubation with different concentrations of NP. Vertical bars show standard deviation. No significant difference from control.

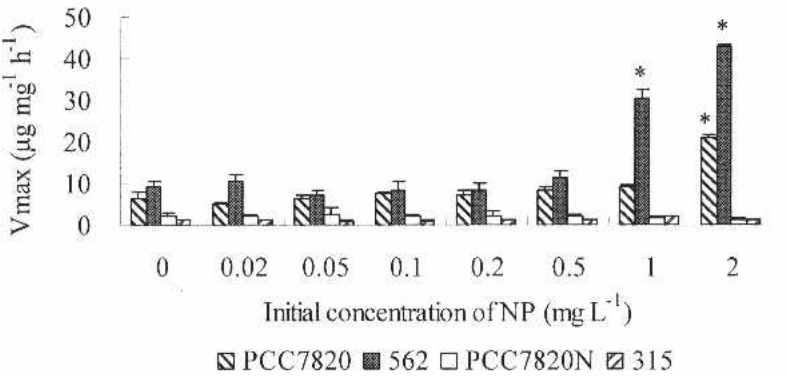

Figure 4. Variation of APase kinetic parameter (V_{max}) in culture media of *M. areuginosa* strains after 12 days' incubation with different concentrations of NP. Vertical bars show standard deviation. * indicates a significant difference from control ($p < 0.05$).

substrate (i.e. deceasing K_m value) (Francko and Heath 1979). Therefore, the toxic strains may also increase the efficiency of organic phosphorus utilization by increasing the reaction velocity of the enzyme in response to the toxic effect of NP.

In conclusion, this study discovered that APAs produced by *M. aeruginosa* and the APase kinetics were species specific. Nonylphenol had obvious effects on APA and APase kinetics in *M. aeruginosa*. These findings are helpful in explaining some phenomena encountered in field experiments. Therefore, it is essential to

further study the relationships among organic pollutants and APA and cyanobacteria.

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